Identification of differentially expressed genes response to TCDD in rat brain after long-term low-dose exposure

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A B S T R A C T

Several cohort studies have reported that dioxin and dioxin-like polychlorinated biphenyls might impair the nervous system and lead to neurological or neurodegenerative diseases in the elder people, but there is limited research on the involved mechanism. By using microarray analysis, we figured out the differentially expressed genes between brain samples from SD rats after low-dose (0.1 μg/(kg▪bw)) dioxin exposure for six months and controls. To investigate the function changes in the course of dioxin exposure, Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on the differentially expressed genes. And the changes of several picked genes have been verified by real-time PCR. A total of 145 up-regulated and 64 down-regulated genes were identified. The metabolic processes, interleukin-1 secretion and production were significantly associated with the differentially expressed genes. And the genes regulated by dioxin also clustered to cholinergic synapse and long-term potentiation. Candidate biomarker genes such as egr1, gad2, gabrb3, abca1, ccr5 and pycard may be toxicological targets for dioxin. Furthermore, synaptic plasticity and neuro-immune system may be two principal affected areas by dioxin.

Introduction

Dioxin represents a group of persistent organic pollutants which exerts threats on human health. Recently, many studies focused on the neuronal effects of dioxin. The cohort studies focusing on the neurotoxicity of dioxin could be classified into two categories based on the age of people in the cohorts. One major category aiming at children after perinatal dioxin exposure, demonstrated that dioxin might disrupt regular neuronal development and lead to cognitive deficiency. The results of cohort studies have been verified by animal experiments (Kakeyama et al., 2014), and several researches also tried to demonstrate the mechanism involved in the neurotoxicity of dioxin on the neurodevelopment by both animal behavior tests and molecular experiments (Mitsuhashi et al., 2010; Nayyar et al., 2002; Nguyen et al., 2013; Williamson et al., 2005).

But to the other category, in which the cohorts concerned about the effects of dioxin on the nervous system of elder people, there is limited relevant research on the mechanisms. A study to evaluate the consequences of severe occupational intoxication with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) that occurred during production of the herbicide trichlorophenoxyacetic acid...
in the period 1965–1968, indicated that even forty years past, the blood level of TCDD was still 100 times in the intoxication people higher than in the general population, and a high percent of subjects suffered from neurological and vascular disorders (Pelclova et al., 2009). Another study carried out in 2014 also suggested that dioxin exposure several decades earlier might increase morbidity from Alzheimer disease and peripheral polyneuropathies (Yi et al., 2014). Generally, dietary exposure is the main way for dioxin to enter human body, especially for the majority population. It has been reported that the elder Michigan residents who consume amount of fish from the Great Lakes, get impairments in memory and learning due to the dioxin-like Polychlorinated biphenyls (PCB) intake from the fish (Schantz et al., 2000). Even in the developed countries like the U.S, older individuals suffer from high body burden, and their cognitive score was significantly affected by serum concentration of dioxin (Chen et al., 2007; Chen et al., 2009). After 24 weeks of exposure, brain tissue was dissected and stored in −80°C for further studies. All animal experiments were conducted in accordance with animal protocols approved by the Animal Care and Use Committee of Institute of Psychology (CAS).

1.2. Ribonucleic acid (RNA) isolation, quality control and microarray analysis

Rat brain tissue was thoroughly grinded in liquid nitrogen. Total RNA of rat brain tissue was isolated using the Thermo Scientific GeneJet RNA Purification Kit (Thermo) and genomic Deoxyribonucleic acid (DNA) was removal by RapidOut DNA Removal Kit following the manufacturer’s instructions. One microliter total RNA aliquots were used for quality control by agarose gel electrophoresis and quantified by Nanodrop 2000. All RNA samples used in this study showed no sign of degradation and the ratio of $A_{260}/A_{280}$ reached to 2.0.

Preparation of cDNA, complementary ribonucleic acid (cRNA), hybridization and scanning of microarrays was performed following manufacturer’s protocol. cDNA and biotinylated cRNAs were synthesized from 5 μg RNA samples with the Genechip expression 3′ amplification reagents (one-cycle cDNA synthesis, and IFT labeling) kits of Affymetrix, and biotinylated probes were hybridized to an Affymetrix Rat Genome 230 2.0 array. Microarrays were washed and scanned in an Affymetrix GeneChip Scanner 3000, and the comparison of gene expression profiles between the control and treated samples was performed by using a standard significance analysis of microarray software (Affymetrix Microarray Suite 5.0).

1.3. Gene Ontology (GO) and pathway enrichment analysis

Genes showing differential expression were functionally classified based on the GO database using the Database for Annotation, Visualization and Integrated Discovery rate (DAVID) tool (Huang et al., 2009). The significantly altered GO functions with a false discovery rate (FDR) < 0.05 were identified.

To identify the pathways significantly associated with the genes of interest, the potentially altered pathways with a p-value <0.05 were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System (KOBAS) based on the cumulative hypergeometric distribution algorithm (Wu et al., 2006).

1.4. Quantitative RT-PCR

Real-time quantitative real-time polymerase chain reaction (RT-PCR) was used to confirm the differential expression of several dioxin-responsive genes detected in the microarray analysis. First strand complementary cDNA synthesis was performed on 2 μg RNA (RevertAid First Strand cDNA Synthesis Kit, Thermo), previously treated with DNase (Rapidout DNA Removal Kit, Thermo). Two microliters of diluted cDNA (1:50) was subjected to PCR cycles by using the GoTaq qPCR Master Mix (Promega). The reaction was incubated at 95°C for 2 min, and then followed by 40 cycles of amplification.
Assays were performed by using QuantStudioTM 6 Flex Real-Time PCR System (instrument and software) from ThermoFisher. All samples were run in triplicate, and results were analyzed by the CT method. The following primers (forward and reverse) were designed by Primer Premier 6 from sequences obtained from Genbank, and synthesized by Sangon.

Results were normalized to the expression levels of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The primers were as follows:

**GAPDH:** 5′-GGTAGTTGGCCACTTCTCGT-3′

**GABRB3:** 5′-TCTGGGCTCTGAGGAGTGGAAACCTC-3′

**GAD2:** 5′-GGCAACGCACGCACTTGGCA-3′ (NM_017000.3; GI:4026917)

**CDKN1a:** 5′-AGTTCAACGGCACAGTCAAG-3′ (NM_017000.3; GI:4026917)

**CCR5:** 5′-CTGGCTCTGAGGAGTGGAAACCTC-3′ (NM_172212.2; GI:83992993)

**PYCARD:** 5′-ATCACGCTGTCTGTTCCGATCC-3′ (NM_012563.1; GI:69788)

**ERG1:** 5′-GGGAACTGCAGCAGTGTGCTC-3′ (NM_172212.2; GI:83992993)

**C4A:** 5′-TCTGGCCTGAGGAGTGGAAACCTC-3′ (NM_031504.3; GI:259013549)

**ABCa1:** 5′-ATCACGCTGTCTGTTCCGATCC-3′ (NM_012563.1; GI:69788)

**Plekk:** 5′-AGTTCAACGGCACAGTCAAG-3′ (NM_017000.3; GI:4026917)

**Cdkn1a:** 5′-ATCACGCTGTCTGTTCCGATCC-3′ (NM_017000.3; GI:4026917)

**Ccr5:** 5′-ATCACGCTGTCTGTTCCGATCC-3′ (NM_017000.3; GI:4026917)

Results demonstrated that the TCDD induced changes of the differentially expressed genes from the microarray results. Results indicated that the TCDD induced changes differed between the two assays (Fig. 2). Among these up-regulated genes, the change of ERG1 was most promising in the expression of ABCa1, C4A, CCR5, PYCARD, CDKN1a, GAD, GABRB3 and ERG1, closely correlated with the corresponding microarray data, although the exact fold change differed between the two assays (Fig. 2). Among these up-regulated genes, the change of ERG1 was most promising (2.40 fold compared with the control group), while CDKN1a and GAD2 were also up-regulated to more than 2 folds (2.15 and 2.10 folds, respectively). ABCa1, C4A, CCR5 and PYCARD are down-regulated more than 25%.

### 2. Results

#### 2.1. Identification of differentially expressed gene profiling in rat brain tissue exposed to TCDD

In present study, we investigate the differentially expressed genes from SD rat brain tissue after TCDD exposure, microarray analysis was performed and results selectively compared with gene expression of tissue exposed to DMSO. From the microarray results, a total of 209 genes were regulated by TCDD. Among them, 145 genes were up-regulated, whereas 64 genes were down-regulated (Table 1). The heat map for the hierarchical clustering of gene expression is shown in Fig. 1, which illustrated distinct gene expression profiles between TCDD exposure sample and DMSO controls.

#### 2.2. Verification of the differentially expressed genes

The real-time PCR experiment was performed the verify some of the differentially expressed genes from the microarray results. Results demonstrated that the TCDD induced changes in the expression of ABCa1, C4A, CCR5, PYCARD, CDKN1a, GAD, GABRB3 and ERG1, closely correlated with the corresponding microarray data, although the exact fold change differed between the two assays (Fig. 2). Among these up-regulated genes, the change of ERG1 was most promising (2.40 fold compared with the control group), while CDKN1a and GAD2 were also up-regulated to more than 2 folds (2.15 and 2.10 folds, respectively). ABCa1, C4A, CCR5 and PYCARD are down-regulated more than 25%.
Fig. 1 – A heat map for the hierarchical clustering of gene expression. The results of 3 parallel samples from TCDD (left panel) and Dimethyl Sulphoxide (DMSO) groups (right panel) are shown in the heat map. Red and green bars show up- and down-regulations, respectively. The names of the differently expressed genes are shown at the right side. TCDD: tetrachlorodibenzo-p-dioxin.
2.3. GO function and KEGG enrichment analysis

The GO functions closely related with genes of interest are shown in Table 2. A total of 10 biological functions were significantly enriched, such as regulation of primary metabolic process (GO: 0080090) and positive regulation of cellular metabolic process (GO: 0031323). The majority of the annotated genes were related to regulation of primary metabolic process. Pathway analysis is a functional analysis mapping genes to KEGG pathways. The Enrichment Score represents the significance of enrichment for each pathway. From this result, we found that 10 pathways were significantly up-regulated, while 3 pathways were significantly down-regulated by the differentially expressed genes in TCDD exposure group. Among the up-regulated pathways, Long-term potentiation (pathway ID: rno04720), Cholinergic synapse (rno04725) and Insulin secretion (rno04911) are most significant ones. Arachidonic acid metabolism (rno00590), Endocytosis (rno04144) and Serotonergic synapse (rno04726) were down-regulated by a lower significance (Fig. 3).

3. Discussion

Dioxins, as a kind of notorious persistent organic pollutant, exert their toxicity through a relatively long period and an accumulation process in human body, especially in nervous system. Several cohort studies implied potential adverse health effects such as cognitive deficit and high risk of neurological diseases, emerged after decades years of dioxin exposure (Yi et al., 2014; Schantz et al., 2001; Bouchard et al., 1999). Based on the plasma dioxin concentration from dioxin polluted area (Pelclova et al., 2009), we designed our exposure dose of dioxin for rat experiments. To our knowledge, this study is the first application of animals after prolonged exposure of dioxin at low concentration for the analysis of effects of dioxin on brain tissue, which could reflect more real and comprehensive effects of dioxin in brains.

Microarray data suggested that synaptic plasticity might be a vulnerable target of dioxin. The up-regulation expression of camkII and camk4 in the TCDD-treated group suggested the activated Ca^{2+} signal pathway by dioxin, and creb1 expression

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Fig. 2 – The messenger ribonucleic acid (mRNA) expression level of differently expressed genes in rat brain. (A) The values from microarray results are shown. (B) Total RNA was extracted from rat brain. Real-time polymerase chain reaction (PCR) was performed to measure the mRNA level of the genes ABCa1, C4A, CCR5, PYCARD, CDKN1a, GAD2, GABRB3 and EGR1. Values are expressed as the fold of change (X Basal against the control (DMSO treatment)). Statistical differences relative to the control (DMSO treatment) were analyzed by the student t-test: *p < 0.05 and **p < 0.01 vs. control.

Table 2 – The results of GO function analysis.

| GO.ID   | Term                                     | Fold Enrichment | p-Value       |
|---------|-------------------------------------------|-----------------|---------------|
| GO: 0080090 | Regulation of primary metabolic process | 1.984103585  | 4.19063E−11  |
| GO: 0031323 | Regulation of cellular metabolic process | 1.944189418  | 7.71575E−11  |
| GO: 0019222 | Regulation of metabolic process          | 1.774316373  | 2.53258E−09  |
| GO: 0006810 | Transport                                | 1.968416287  | 1.58244E−08  |
| GO: 0060255 | Regulation of macromolecule metabolic process | 1.836576337  | 3.27817E−08  |
| GO: 0051234 | Establishment of localization            | 1.922079371  | 3.99049E−08  |
| GO: 0048518 | Positive regulation of biological process | 1.788416287  | 1.33064E−07  |
| GO: 0048519 | Negative regulation of biological process  | 1.859544993  | 1.40202E−07  |
| GO: 0048518 | Regulation of apoptotic process          | 2.75768416    | 1.79676E−07  |
| GO: 0009893 | Positive regulation of metabolic process  | 2.174421257  | 1.86674E−07  |

GO: Gene Ontology.
suggesting time difference of gene expression. PSD-95 is a member of the synapse-associated protein family of scaffolding molecules that control the organization, composition and function of synapses, the abnormal expression of dlg4 gene shown synaptic plasticity defect and was detected in Alzheimer’s patients (Migaud et al., 1998; Leuba et al., 2008). gad2 encoding one isoform of glutamate decarboxylase, which is the rate-limiting enzyme in the synthesis of GABA, was also detected up-regulated by dioxin. gad2 knockout mice displayed impaired GABA synaptic release indicated its important role in the plasticity of central GABA synapses (Pan, 2012; Kash et al., 1997). Another GABAAergic system member, gabra3, together with gad2 has been regarded as candidate gene of autism spectrum disorders (Chen et al., 2014). Autistic traits have been observed in the children after perinatal dioxin exposure (Nishijo et al., 2014), but the underlying mechanism still needs to be elucidated.

The genes (abca1, pycard, ccr5) involved in the production and of Interleukin-1 (IL-1), were down-regulated by dioxin suggested immune system dysfunction in the brain caused by dioxin. IL-1, as A pro-inflammatory cytokine, plays an important role in immune response in central nervous system (Weiss et al., 1989), and have influence on the dopaminergic and serotonergic neurotransmission systems (Felger and Miller, 2012; Felger et al., 2013). In addition, IL-1 could also influence brain function through their effect on hippocampal neuroplasticity and neurogenesis (Yirmiya and Goshen, 2011) or via neuro-endocrine mechanisms involving the hypothalamic pituitary–adrenal axis (HPA) functioning (Goshen and Yirmiya, 2009). Dioxin has been reported to up-regulate the expression and secretion of pro-inflammatory cytokines in cultured HAPI microglial cells after acute exposure (Xu et al., 2013). Although our microarray results did not show any significant changes of the expression of genes coding pro-inflammatory cytokines in the TCDD-treated group, the down-regulation of ATP-binding membrane cassette transporter A1 (ABCA1) might also cause inflammatory response (Schmitz et al., 1999; Aiello et al., 2003). Moreover, the main role of ABCA1 is mediation of the transport of cholesterol, phospholipids and other lipophilic molecules across cellular membranes to lipid-poor High-density lipoproteins (HDL) apolipoproteins (Tang and Oram, 2009). Dysregulation of cholesterol transportation and metabolism has been proved to be associated with several neurodegenerative diseases, such as Alzheimer’s disease (AD) and Huntington’s disease (HD) (Di Paolo and Kim, 2011; Karasinska and Hayden, 2011). PYCARD (apoptosis-associated speck-like protein containing a CARD or ASC) mediates the assembly of large signaling complexes in the inflammatory and apoptotic signaling pathways via activation of caspase. PYCARD is a key factor in the caspase1 activation and IL-1β maturation (Kumar et al., 2013). Down-regulation of pycard might lead to suppression of secretion of IL-1β (Couturier et al., 2016). Deficiency of C-C chemokine receptor S(CCR5) activated astrocytes and induced Aβ accumulation, these processes might extend neuro-inflammation and contribute to the development and the progression of AD (Hwang et al., 2016; Simard et al., 2006; Stalder et al., 2005). Suppression messenger ribonucleic acid (mRNA) level of ccr5 caused by dioxin might give some support to associate dioxin exposure to immune system dysfunction.

was also induced by dioxin. These molecular physiological processes have been linked to the synaptic activity such as changes in synaptic strength and the regulation of neuronal survival and death (Curtis and Finkbeiner, 1999; Xi et al., 2016; Lee et al., 2004). And another up-regulated gene, Early Growth Response 1 (EGR1), has been documented as a critical integrator and mediator of synaptic plasticity and neuronal activity in both physiological and pathological conditions (Duclot and Kabbaj, 2017). It is noted that EGR1 could regulate multiple aspects of synaptic plasticity, such as neurotransmitters metabolism, actin cytoskeleton, Long-term potentiation (LTP) maintenance, and vesicular release endocytosis (Koldamova et al., 2014; Duclot and Kabbaj, 2015; Penke et al., 2014). In fact, the intracellular calcium increases in hippocampal neurons followed by up-regulation of EGR1 (Bading et al., 1995). Furthermore, the expression of creb1 and jund in the brain was elevated by dioxin, these two transcription factors could bind their respective response elements located in the erg1 promoter (Tur et al., 2010). It is known that TCDD increases the expression of transcription factor EGR1 in the lung epithelial cells, kidney cells and hematopoietic stem cells (Martinez et al., 2004; Aida-Yasuoka et al., 2014; Casado et al., 2011; Keshava et al., 2005), but this is the first time to report that TCDD regulates erg1 expression in nervous system. Although overexpression of EGR1 in primary cultured rat hippocampal neurons caused reduction in PSD-95 protein level (Qin et al., 2015), our data showed an increase in the expression of dlg4 (a gene encoding PSD-95), which was consistent with the expression profile in the rat after chronic electroconvulsive stimulation (ECS) (Dyrvig et al., 2014),
in brain. Furthermore, the activation of Ca\(^{2+}\) signal pathway and junD up-regulation caused by dioxin also predict the neuro-inflammation toxicity of dioxin (Xu et al., 2013; Sul et al., 2009; N'Diaye et al., 2006).

4. Conclusions

This is the first report about the microarray study to identify differentially expressed genes in rat brain after environmental related dose and long-term dioxin exposure. Several genes encoding key protein in maintaining the synaptic plasticity and participating in the neuro-inflammation have been identified to respond to dioxin.

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