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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces alterations in myogenic differentiation of C2C12 cells☆

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ABSTRACT

Dioxin-induced toxicities that affect the development of the motor system have been proposed since many years. However, cellular evidence and the molecular basis for the effects are limited. In this study, a cultured mouse myoblast cell line, C2C12, was utilized to examine the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on myogenic differentiation and expression of acetylcholinesterase (AChE), a neuromuscular transmission-related gene. The results showed that TCDD exposure at 10^{-10} M repressed the myotube formation of C2C12 cells by disturbing the fusion process and suppressing the expression of myosin heavy chain, a myobute structural protein, and not by induction of cytotoxicity. Furthermore, TCDD dose dependently suppressed the transcriptional expression and enzymatic activity of AChE during the myogenic differentiation, particularly in the middle stage. However, the administration of aryl hydrocarbon receptor antagonists, CH223191 and alpha-naphthoflavone, did not completely reverse the TCDD-induced downregulation of muscular AChE during myogenic differentiation. These findings suggest that low dose exposure to dioxin may result in disturbances of muscle differentiation and neuromuscular transmission.

1. Introduction

Dioxins have been linked to multiple intoxications including chloracne, immunotoxicity, neurotoxicity, hepatoxicity, reproductive toxicity, and tumor development (Denison et al., 2011; Pohjanvirta and Tuomisto, 1994; van Leeuwen et al., 2000). In recent years, people have paid more attention to dioxins' neurotoxicities, especially the developmental toxicities in the nervous system. Significant negative associations between the mental developmental index and the dioxin level in maternal blood have been found among 6-month-old male infants in Sapporo cohort study (Kishi et al., 2013; Nakajima et al., 2006). Additionally, animal studies have also revealed that maternal exposures to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could disrupt the memory function of the offspring and alter the differentiation pattern of the neural progenitor cells in mice (Haijima et al., 2010; Mitsuhashi et al., 2010). Dioxins not only cause alterations in advanced brain functions (Michalek et al., 2001; Powers et al., 2005; Schantz and Bowman, 1989) but also in the development and function of the skeletal muscles. A recent epidemiological study showed that altered functions in motor domains of the nervous system occurred in 4-month-old infants living in dioxin-contaminated areas in Vietnam, whose function of object manipulation and movement of the limbs and torso were interfered by perinatal dioxin exposure (Tai et al., 2013). Furthermore, defects in the myogenesis of the palate has been proposed as one of the mechanisms for the formation of the cleft palate in prenatal TCDD-exposed mice (Yamada et al., 2014). Coletti et al. also reported that the differentiation of cells in both the myogenic cell line and the primary myogenic cell cultures was specifically impaired by exposure to commercial mixtures of polychlorobiphenyl (PCBs) congeners, in which dioxin-

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like PCBs were included (Coletti et al., 2001). Thus, dioxin exposure might have effects on the myogenic differentiation of the skeletal muscle cells, which deserves extensive investigations on the direct cellular evidence and underlining molecular basis.

Apart from muscle development, dioxin could affect various aspects of functions associated with locomotion, such as functions of the motor neurons, conduction of the motor nerves, and functions of the neuromuscular junction (NMJ) and muscle innervation. Decrease in motor nerve conduction velocity has been reported to occur in the peroneal nerve of 156 dioxin-exposed workers from a pesticide plant (Thomke et al., 1999). Consistent with this finding in human, electrophysiological studies showed a dose-dependent slowing down in the conduction velocity of motor and sensory parts of the sciatic nerve in adult male Han/Wistar rats exposed to TCDD (Grahmann et al., 1993). Apart from peripheral nerves, dioxins may affect innervation of the muscles. Myalgia and myasthenia were major complaints among dioxin-exposed chemists (Schecter and Ryan, 1992). Amyotrophy could be observed in muscular tissues of dioxin-exposed rats (Max and Silbergeld, 1987). However, whether the neuromuscular transmission can be affected by dioxin exposure is still devoid of solid evidence.

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine into acetic acid and choline and plays a vital role in terminating the nervous impulse in the peripheral and central cholinergic nervous systems. In the peripheral nervous system, AChE mainly exerts its function at NMJs, and the major functional asymmetric form of AChE is present at the basal lamina of the NMJs (Soreq and Seidman, 2001). Abnormal expression levels or subcellular locations of AChE at NMJs may lead to abnormalities in neuromuscular transmission, which controls muscle contractions to maintain normal movement function including breathing (Massoulie and Millard, 2009). The expression of AChE at NMJs is tightly controlled during development and after maturation, in which both the presynaptic neurons and postsynaptic myotubes make contributions (Tsim et al., 2010). The regulations of AChE during the process of myogenesis and NMJ formation have been extensively studied (Gaspersic et al., 1999; Siow et al., 2002). The C2C12 mouse myoblast cell line is widely used as an *in vitro* model for the study of myogenesis (Katase et al., 2016; Nozaki et al., 2016; Siow et al., 2002). The differentiation profile of AChE during myogenesis has revealed that AChE expression is markedly increased from the myoblast to myotube stages of cultured C2C12 cells and getting prepared for the innervation process (Fuentes and Taylor, 1993; Siow et al., 2002). Recently, alterations in neuronal AChE expression have been studied in dioxin-treated neuroblastoma cells, in which dioxin exposure significantly suppressed the AChE activity through aryl hydrocarbon receptor (AhR)-mediated transcriptional down-regulation in the SK-N-SH cells (Xie et al., 2013). However, whether muscular AChE expression could be altered by dioxins remains unclear.

Given the aforementioned evidence, we hypothesized that dioxin may interfere the process of myogenesis and the expression of AChE during myogenic differentiation. Therefore, in this study, we sought to define alterations in myogenic differentiation of C2C12 cells and in the differentiation profile of AChE expression upon dioxin exposure during the time. Finally, the role of AhR in gene alterations was explored.

2. Materials and methods

2.1. Cell culture and differentiation

C2C12 murine cell line, obtained from the American Type Culture Collection (Manassas, VA, USA), was maintained in a growth medium (GM), including Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 20% fetal bovine serum (FBS) (Corning, New York, USA) and 1% penicillin–streptomycin (P/S) (Gibco), and incubated at 37 °C in a water-saturated 5% CO_2 incubator. To induce fusion of cultured myoblasts, cells were first allowed to grow in DMEM with 10% FBS until confluent. Then the medium was changed to a differentiation medium (DM) including DMEM with 2% heat-inactivated horse serum (HS) (Gibco) to induce the differentiation. The medium was changed every 24 h over the following 6 days.

2.2. Chemical treatment

C2C12 cells were seeded onto 6-well plates and cultured in the GM or DM. TCDD, the most potent congener of dioxins, was purchased from Wellington Laboratories, Inc. (Ontario, Canada). Two antagonists of the AhR-dependent pathway, CH223191 or alpha-naphthoflavone (ANF) (Zhao et al., 2010; Ramadass et al., 2003), were obtained from Sigma (St. Louis, MO, USA).

C2C12 cells were continuously treated with TCDD during the myogenic differentiation. The dosing of C2C12 cells was conducted every day in which the DM containing TCDD or solvent control was replenished every 24 h from the first day of induction (day 0). To reveal the role of AhR, C2C12 cells were pretreated with CH223191 (10^{-6} M) or ANF (10^{-5} M) for 3 h before the treatment with TCDD (Zhao et al., 2010; Ramadass et al., 2003). The solvent dimethyl sulfoxide (DMSO) was present in all treatments at less than 0.1%.

2.3. Morphological analysis

The morphological change of the cells during myogenic differentiation was analyzed by hematoxylin and eosin (H&E) staining. C2C12 cells were seeded onto 6-well plates. Five different images were randomly captured per well under an inverted light microscope (CKX41, Olympus, Japan) with a digital camera (DS126311; Canon Inc., Taiwan). The myotube and nuclei numbers were counted by using Image-Pro Plus 6.0 from which the average numbers from the five images randomly captured per well were obtained. The fusion index was calculated as the ratio of nuclei incorporated into myotubes to the total number of nuclei in all images captured. The number of nuclei per myotube was determined as the average number of the nuclei in the myotubes from the images captured per well. In this study, the myotubes with two or more nuclei were counted (Ge et al., 2014; Shafey et al., 2005).

2.4. Cell viability analysis

The cells were cultured in 6-well plates to induce differentiation and carry out TCDD treatment. Cell viability was determined using CellTiter-Glo® luminescent cell viability assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The absorbance was measured with a GLOMAX™ Multi detection system (Promega).

2.5. Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted using GeneJET RNA purification kit (Thermo Waltham, MA, USA). cDNA was prepared using 2 μg of total RNA and the RevertAid first strand cDNA synthesis kit (Thermo) according to the manufacturer's instructions. Real-time PCR was performed on equal amounts of cDNA using GoTaq® qPCR master mix kit (Promega) according to the manufacturer's instructions. The SYBR green signal was detected by a Roche 480 multiplex quantitative PCR system (Roche, Basel, Switzerland) and the ΔΔCT method was used to quantify the relative mRNA