AhR-Mediated Effects of Dioxin on Neuronal Acetylcholinesterase Expression in Vitro

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BACKGROUND: Deficits in cognitive functioning have been reported in humans exposed to dioxins and dioxin-like compounds. Evidence suggests that dioxins induce cholinergic dysfunction mediated by hypothyroidism. However, little is known about direct effects of dioxins on the cholinergic system.

OBJECTIVES: We investigated the action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on acetylcholinesterase (AChE), a key enzyme in cholinergic neurotransmission.

METHODS: We used SK-N-SH human-derived neuronal cells to evaluate the effect of dioxin exposure on AChE.

RESULTS: We consistently found a significant decrease in enzymatic activity of AChE in cultured neurons treated with TCDD. We also found that, unlike organophosphate pesticides that directly act on the catalytic center of AChE, the suppressive effect of dioxin was through transcriptional regulation. The addition of CH223191, an inhibitor of the aryl hydrocarbon receptor (AhR)-dependent pathway, counteracted the TCDD-induced suppression of AChE, suggesting involvement of the AhR-dependent pathway. The existence of putative dioxin-responsive element (DRE) consensus sequences in the human AChE promoter region further supported this hypothesis. Consistent with the absence of DRE elements in mouse or rat AChE promoter regions, suppression of AChE by TCDD did not occur in rat neuronal cells, indicating a species-specific effect.

CONCLUSIONS: In SK-N-SH cells, dioxin suppressed the activity of neuronal AChE via AhR-mediated transcriptional down-regulation. This is the first study to report direct interference by dioxin with the cholinergic neurotransmission system.

KEY WORDS: acetylcholinesterase (AChE), aryl hydrocarbon receptor (AhR), dioxin-responsive element (DRE), neuron, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), transcriptional regulation.


Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and related dioxin-like compounds (DLCs) represent a diverse group of contaminants, many of which are highly toxic, and both environmentally and biologically persistent (reviewed by Mandal 2005). Dioxins and DLCs cause multiple toxic effects, including increased risk of cancer and interference with the function and development of the nervous, immune, and reproductive systems (Boucher et al. 2009; reviewed by Marinković et al. 2010, and by White and Birnbaum 2009).

Cholinergic neurotransmission and acetylcholinesterase (AChE; a vital functional enzyme in cholinergic neurotransmission) play important roles in multiple advanced brain functions, such as memory, learning, and attention (Hasselmo and Sarter 2011; reviewed by Soreq and Seidman 2001 and by Woolf and Butcher 2011). Emerging evidence suggests that maternal or perinatal exposure to dioxins or DLCs can interfere with the development of the central cholinergic system, including the development of AChE in the cerebellum (Ahmed 2011) and expression of muscarinic acetylcholine receptors in the cerebrum and cerebellum of rats (Coccini et al. 2007). Ahmed (2011) suggested that the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on brain AChE were related to alterations in thyroid development. It is also plausible that dioxins could directly affect cholinergic neurotransmission, a possibility that we explored in the present study.

In general, activity of AChE can be affected in two ways: direct inhibition of enzymatic activity or suppression of transcription. Inhibition of AChE activity has been used as an indicator of organophosphorus insecticide (OP) exposure because OPs irreversibly inhibit the activity of AChE by binding to its catalytic residue (reviewed by Chen et al. 1999; Farahat et al. 2011). Therefore, we investigated the possibility that dioxin affects the enzymatic activity of AChE in cultured neurons, and further explored the possibility that this occurs by a transcriptional mechanism.

Dioxin is thought to exert its biological and toxicological effects primarily by binding to the aryl hydrocarbon receptor (AhR, dioxin receptor) followed by nuclear translocation and binding to dioxin-responsive elements (DREs) in gene promoters (reviewed by Beislag et al. 2008). Putative DREs were observed in the promoter of the human AChE gene but not in the mouse or rat AChE genes (Sun et al. 2004), suggesting the possibility of species-specific effects of dioxins on AChE. Therefore, we also studied the role of the AhR-dependent pathway in dioxin-induced alterations of AChE and the species specificity of the effects.

Materials and Methods

Cell culture. SK-N-SH cells (a cell line derived from human neuroblastoma cells) were purchased from the cell resource center of Chinese Academy of Medical Sciences (Beijing, China). SK-N-SH cells express both AChE and muscarinic acetylcholine receptor (Ezotiuk et al. 2008; Pizzi et al. 2002; Popova and Rasenick 2004). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), and incubated at 37°C in a water-saturated 5% CO2 incubator. PC12 cells [a cell line derived from a pheochromocytoma of the rat adrenal medulla; a gift from K.W. Taim (The Hong Kong University of Science and Technology)] were maintained in DMEM, supplemented with 6% FBS and 6% heat-inactivated horse serum, and incubated at 37°C in a water-saturated 5% CO2 incubator. All reagents for cell culture were obtained from Invitrogen (Carlsbad, CA, USA).

Chemical treatment. The cells were seeded in 6-well-plates at 500,000 cells/well 24 hr before exposure to dioxin or other chemical treatment for determination of AChE activity. TCDD, the most potent congener of dioxins, was purchased from Wellington Laboratories Inc. (Ontario, Canada) and employed at low concentrations of 10⁻¹¹ to 10⁻⁹ M. We also examined 2,3,7,8-tetrachlorodibenzoofuran (TCDF; 10⁻⁸ M) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF; 3 × 10⁻⁹ M) (both purchased from Wellington Laboratories Inc. (Ontario, Canada)).

Conclusive statements.

The authors declare they have no actual or potential competing financial interests.

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from Wellington, Ontario, Canada), forskolin (5 × 10⁻⁵ M; Sigma, St. Louis, MO, USA), and nerve growth factor (50 ng/mL; Alomone Labs, Jerusalem, Israel). CH223191, an inhibitor of the AhR-dependent pathway (Zhao et al. 2010), was obtained from Sigma and used at a concentration of 10⁻⁵ M. To examine the role of AhR, we pretreated cells with CH223191 3 hr before incubation with TCDD. The solvent dimethyl sulfoxide (DMSO) was present in all treatments at 0.1%. In the in vitro assay, SK-N-SH cell lysate was incubated with TCDD (10⁻¹¹ to 10⁻⁹ M), BW284c51 (a specific inhibitor of AChE; Sigma), at 2 × 10⁻⁵ M, or 0.1% DMSO alone (control). After 1 hr incubation at room temperature, enzymatic activity of AChE was determined by the Ellman assay (Ellman et al. 1961). BW284c51 served as an assay control.

**Reporter gene constructs and transfections.** pAChE-Luc and pAChEm-Luc consist of the human AChE and mouse promoter sequences upstream of a firefly luciferase gene in pGL4.10 and pGL3-Basic vectors (Promega, Madison, WI, USA), respectively. The truncated construct, pAChE-T-Luc, derived from pAChE-Luc, was constructed using sense primer 5’-TTG GAC AGA GAA TTC GCA GAC GGA C-3´, in which the original sequence of the AChE promoter was kept. The fragments were ligated with EcoRI restriction site (5´-GAA TTC-3´) and a HindIII restriction site, to produce pAChE-T-Luc. AChE T subunit (AChET) reporter construct (NM_0008065), 5’-CTG GGC TGC GGA GGA CAG-3´, and 5’-TG AGC AGC GAT CCT GCT-3´ for human AChET, was obtained from Sigma (GenBank accession numbers; GenBank: http://www.ncbi.nlm.nih.gov/GenBank/). As for the other constructs, 5’-GGG GCC TGC AGA TTT AAC CAG GTC TGA GCA GCG TTC CTG-3´ and 5’-AGG GCC TGC AGA TTT AAC CAG GTC TGA GCA GCG TTC CTG-3´ for human PRiMA (NM_178013), and 5’-GAC TGT CTT AAC AGT CCA CCC TCT GAG TCT TAT CTA CCA C-3´ for human β-galactosidase gene (AY750146; Sudhakar et al. 2003), were used for promoter truncation and mutation studies. We used the Bonferroni test to determine statistical significance. The expression of AChE was normalized to total protein and β-galactosidase activity.

**Luciferase assay.** Cells were transfected with promoter–reporter constructs together with cDNA encoding the β-galactosidase gene at 10:1 weight ratio. Twenty-four hours later, cells were treated with chemicals as described above. For luciferase measurement, sample wells were washed twice with phosphate-buffered saline (PBS), followed by the addition of cell lysis buffer (Promega) and shaking of the plates for 10 min at room temperature to allow cell lysis. Insoluble material was removed by centrifugation, and the resulting lysates were transferred to white 96-well microplates with a Tecan Infinite F200 Pro luminometer with automatic injection of Promega stabilized luciferase reagent. Luciferase activity in each well was normalized to total protein and to transfection efficiency as determined by β-galactosidase activity.

**Real-time quantitative PCR.** We isolated total RNA (5 μg) from SK-N-SH cultures using TRIzol reagent (Invitrogen); cDNA was prepared using 5 μg of RNA and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time PCR of AChE T subunit (AChET), AChE R subunit (AChER), PRiMA (proline-rich membrane anchor), and 18S rDNA transcripts was performed on equal amounts of cDNA using SYBR Green Master mix and Rox reference dye, according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). The primers (with GenBank accession numbers; GenBank: http://www.ncbi.nlm.nih.gov/GenBank/) were as follows: 5’-GGG GCC TGC AGA TTT AAC CAG GTC TGA GCA GCG TTC CTG-3´ and 5’-TG AGC AGC GAT CCT GCT-3´ for human AChET, 5’-CTG GGC TGC GGA GGA CAG-3´ and 5’-TG AGC AGC GAT CCT GCT-3´ for human PRiMA (NM_178013), and 5’-GAC TGT CTT AAC AGT CCA CCC TCT GAG TCT TAT CTA CCA C-3´ for human β-galactosidase (AY750146; Sudhakar et al. 2003).

**MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.** For cell viability tests, cultured SK-N-SH cells in 96-well plates were treated with TCDD for 48 hr, followed by the addition of MTT in PBS at final concentration of 0.5 mg/mL for 2 hr. The medium was aspirated, and the cultures were resuspended in 150 μL DMSO to determine cell viability by absorbance at 570 nm.

**Other assays.** To determine β-galactosidase enzymatic activity, 20 μL of cell lysate was mixed with 80 μL of sodium phosphate buffer (pH 7.5) containing 0.8 mg/mL o-nitrophenyl-β-D-galactopyranoside. After incubation at 37°C for 1 hr, absorbance was measured at 410 nm. In this period of time, OD derived from the cell lysate varied linearly with time.

**Statistics.** We performed statistical tests using Origin Pro software (version 8; OriginLab, Northampton, MA, USA). One-way analysis of variance (ANOVA) was used for most analyses, but two-way ANOVA was used for promoter truncation and mutation studies. We used the Bonferroni test to determine statistical significance.
bution to the nucleus and forms a heterodimer with its partner ARNT (aryl hydrocarbon receptor nuclear translocator). The heterodimer binds to the DRE in the promoter region upstream of target genes and thus regulates transcription. The role of AhR in the dioxin-induced decrease of AChE activity was first investigated using CH223191, a ligand-selective antagonist of the AhR, which can preferentially inhibit effects of certain classes of AhR agonists, including TCDD (Zhao et al. 2010). We found that, compared with DMSO (mean ± SE, 3.8 ± 0.18 mOD/min/μg), 10–9 M TCDD significantly decreased the activity (Figure 2B), consistent with the result in Figure 1B. In contrast, pretreatment with CH223191 blocked this decrease, indicating that AhR was involved in the dioxin-induced effect.

**Dioxin causes transcriptional down-regulation of AChE.** Using a human AChE promoter–driven luciferase reporter construct (pAChE–Luc) with approximately 2.2 kb of the regulatory region upstream of the human AChE gene, we evaluated TCDD’s effects on the promoter activity of human AChE. This construct has been well characterized and extensively used to study the regulation of the AChE gene (Getman et al. 1995; Siow et al. 2002). Quiescent SK-N-SH cells were transiently transfected with pAChE–Luc 1 day before the application of TCDD (10–11 to 10–9 M). Promoter activity was determined by luciferase assay after 24 hr of TCDD treatment. Consistent with AChE

![Figure 2](https://dx.doi.org/10.1289/ehp.1206066)

**Figure 2.** Effects of TCDD on AChE activity via direct inhibition of the enzyme (A) and via the AhR-dependent pathway (B). (A) AChE activity in cell lysate of SK-N-SH incubated with TCDD (10–11 to 10–9 M), 2 × 10–6 M BW284c51 (AChE inhibitor used as the positive control), or with 0.1% DMSO (control) for 1 hr. (B) AChE activity in cultured SK-N-SH cells incubated with 10–6 M CH223191 (AhR inhibitor) or 0.1% DMSO for 3 hr and then incubated with 10–9 M TCDD or 0.01% DMSO for 24 hr. See “Materials and Methods” for additional details. Values were calculated as a percentage of control and are expressed as mean ± SE (n = 4); each independent sample was tested in triplicate.

*p < 0.05 compared with control by one-way ANOVA with Bonferroni test. †p < 0.05 compared with TCDD alone by one-way ANOVA with Bonferroni test.
activity, we observed a significant decrease (-30%) in human AChE promoter activity after TCDD exposure (10^{-10} to 10^{-8} M) compared with the DMSO control (Figure 3A). Similar to the effects on AChE activity, pretreatment with CH223191 (10^{-8} M) significantly reversed the suppressive effect of TCDD (10^{-9} M) on the promoter activity of human AChE, consistent with our assumption that an AhR-dependent pathway directs dioxin-induced transcriptional suppression of AChE (Figure 3B).

The transcriptional regulation of AChE by dioxin was further confirmed by real-time PCR analyses to determine expression levels of AChE_T mRNA (the major AChE transcript in neurons), AChE_R mRNA (the minor AChE transcript in the brain), and PRIMA mRNA (a structural subunit of the active form of neuronal AChE) (reviewed by Massoulie 2002). Results showed an approximately 25% decrease in AChE_T mRNA in response to 10^{-9} M TCDD, with no significant changes in AChE_R and PRIMA mRNA levels (Figure 3C). Similar to the change in AChE mRNA, the protein level of AChE was obviously reduced after TCDD exposure [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1206066)]. Therefore, we conclude that exposure to dioxin leads to a decrease in the mRNA expression of AChE catalytic subunit, resulting in decreased expression of the active form of AChE.

**Putative DRE(s) in the human AChE promoter.** The presence of DREs in the regulatory region upstream of dioxin-responsive genes is a key component of the AhR-dependent signaling pathway in response to dioxin. We found four putative consensus core sequences of DRE (5'-TNGCCGTG-3' or 5'-CAGCGCA-3') (Nukaya et al. 2009) within the approximately 2.2-kb region upstream of the human AChE gene (GenBank NM_000665). All of the putative DREs are exact matches or reverse complements of 5'-GCCGTG-3' [see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ehp.1206066)]. Considering that the 5' DRE is the only one located upstream of the transcription start sites among all four putative DREs (Getman et al. 1995; Figure 4A), we concentrated our investigation on the role of this putative DRE. In cultures transfected with truncated human AChE promoter without the 5'-DRE site (pAChE-T) or with a promoter containing a specific mutation of the DRE site (pAChE-M), we found that TCDD no longer suppressed promoter activity (Figure 4B). Furthermore, the effect of 10^{-10} M and 10^{-9} M TCDD on pAChE-M was significantly different from that on the wild type (pAChE). These results suggest that the 5' DRE may play a critical role in mediating suppression.

**Differential responses of rodent AChE to dioxin.** Because rodent AChE genes lack obvious DREs [Sun et al. 2004; see Supplemental Material, Figure S2 (http://dx.doi.org/10.1289/ehp.1206066)], we tested the effects of dioxin on AChE in PC12 cells, a rat neuronal cell line widely used in toxicology studies. After 24 hr exposure, none of the TCDD treatment groups (10^{-10}, 10^{-9}, or 10^{-8} M) showed suppression of the AChE activity (Figure 5A). In a similar manner, TCDD exposure had no effect on promoter activity of mouse AChE when we used a construct (pAChE_Luc) consisting of an approximately 2.1-kb regulatory region upstream of the mouse AChE gene driving luciferase reporter gene expression (Figure 5B) (Jiang et al. 2003). The absence of consensus DRE sequences in the 2.1-kb regulatory region upstream of the mouse AChE gene may help to explain the different responses of rodent and human AChE genes (Sun et al. 2004). PC12 cells exhibited normal responses to treatment with forskolin, which activates the cAMP-dependent pathway, and nerve growth factor. We observed neurite outgrowth and an increment in AChE activity in cells treated with forskolin and nerve growth factor, respectively (see Supplemental Material, Figure S3). These data further support the explanation that the unresponsiveness to TCDD may be due to the absence of DRE on the promoter of rodent AChE.

**Discussion.** Emerging evidence has shown effects of dioxin in the central cholinergic system. Ahmed (2011) reported that daily administration of TCDD (0.2 or 0.4 µg/kg body weight) to pregnant rats from gestation day 1 to lactation day 30 interfered with the development of AChE expression in cerebella of the offspring, although there was no obvious sign of developmental toxicity. Ahmed (2011) suggested that the effects of TCDD on brain AChE were related to alterations in thyroid development. Ahmed’s in vitro study suggested that TCDD is able to affect AChE in the brain. In the present in vitro study, we observed that TCDD suppressed AChE activity in cultured human neuronal cells. However, AChE may not be the only target of dioxin in the cholinergic system, because putative DRE sites are also present in the promoter region of acetylcholine receptor (Sun et al. 2004). Thus, our finding on AChE is a starting point for the exploration for other abnormalities in cholinergic function directly caused by dioxin.

AChE may have functions besides the classical function in cholinergic neurotransmission; evidence has suggested functions in, for example, synapse transmission (reviewed by Zimmerman and Soreq 2006), neurite outgrowth (reviewed by Paraonau and Layer 2013).
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2008), apoptosis (reviewed by Jiang and Zhang 2008), and bone formation (Vogel-Hopker et al. 2012). Thus, the interference of dioxin with AChE might bring new insight into the biological or toxicological effects mediated by AhR not only in the nervous system but also elsewhere in the human body.

Monitoring of AChE inhibition has been used as an indicator of OP exposure (reviewed by Chen et al. 1999; Farahat et al. 2011). Emerging evidence suggests that other types of xenobiotics, such as heavy metals (Richetti et al. 2011) and nanoparticles (Wang et al. 2009), can also affect AChE activity. Here we provide evidence of neuronal AChE regulation by another type of xenobiotic, dioxins. Our results show that AhR mediated the transcriptional regulation of AChE. Considering the ability of AhR to bind diverse structures of chemicals (Zhao et al. 2010), the spectrum of xenobiotics affecting AChE transcription could extend beyond dioxin-like chemicals.

These results suggest that in the future, in addition to assaying for AChE activity, it may be possible to monitor exposures by demonstrating down-regulation of AChE transcripts.

In our experiments, the alterations in neuronal AChE expression were induced by low concentrations of TCDD, close to environmental levels. In several accidental exposures to dioxins, such as in Vietnam (Tai et al. 2011), Seveso, Italy (Needham et al. 1997), and Taiwan (Guo et al. 2004), exposed individuals had median serum levels of approximately 1,000, 450, and 180 pg/g fat, respectively. Based on the estimated average serum fat content of 6.9 g/L (Phillips et al. 1989), the average serum concentration of dioxin (TCDD) in these individuals would be 10\(^{-10}\) to 10\(^{-11}\) M. Although concentrations of dioxins in brain tissue of exposed individuals are unknown, we based our experimental concentrations on these serum concentrations and on concentrations used in other studies (Jin et al. 2004; Sánchez-Martín et al. 2010).

In the present study using relatively low concentrations of TCDD, cultured neuronal cells exhibited no significant change in viability, which made it feasible to study the functional alterations induced by dioxin.

On the basis of the present findings and the literature (reviewed by Beischlag et al. 2008), we assume that:

- When dioxin enters the neuronal cells, it will bind to AhR in the cytosol, resulting in the transformation and translocation of the receptor.
- The active AhR then goes into the nucleus and binds with ARNT to form a heterodimer.
- The heterodimer will then bind to the putative DRE site(s) on the promoter region of the AChE gene, suppressing the expression of AChET transcripts.
- This transcriptional suppression of the major neuronal AChE transcript leads to a decrease in the production of the AChE catalytic subunits and finally causes the decrease in enzymatic activity.

However, further investigations are needed to clarify the role of the putative DRE(s) and how transcriptional suppression occurs. Apart from this transcriptional mechanism, we found no evidence showing that dioxin could inhibit AChE activity by direct interaction with the catalytic subunit.

Toxic effects of environmental chemicals can be species specific. For example, the binding affinity of dioxin to AhR is higher in mice than in humans (reviewed by Denison et al. 2011), and endocrine-disrupting chemicals, such as bisphenol A and its analogs, are potent agonists for human pregnane X receptor (hPXR) but do not affect mouse PXR activity (Sui et al. 2012). Our findings show that dioxin has a suppressive effect on AChE expression in human, but not rat, neuronal cells (Figure 5). The species specificity found in the present study is unlikely to have been caused by differences in affinities of dioxin–AhR binding, but rather resulted from the presence and absence of DREs in the regulatory regions of human and rodent AChE genes, respectively. This finding highlights the advantage of in vitro toxicity testing using human cell lines instead of animal-derived cell lines in assessment of effects of human exposure to xenobiotics, as proposed by Tox21 (Bhattacharya et al. 2011; Hartung 2009; reviewed by Krewski et al. 2010).

**Conclusion**

We found a novel mechanism whereby dioxin may produce its biological or toxicological effects by decreasing neuronal AChE activity through a transcriptional down-regulation mechanism via the AhR-dependent pathway. To our knowledge, this is the first study to report direct interference by dioxin with the cholinergic neurotransmission system.

**Figure 4.** Putative DREs in the human AChE promoter and their response to TCDD exposure. (A) Putative DRE consensus sequences in the pAChE-Luc construct (full-length; top), pAChE-T-Luc (truncated; middle), and pAChE-M-Luc (mutant; bottom). (B) The three constructs were transiently transfected into cultured SK-N-SH cells 1 day before 24 hr incubation with TCDD (10\(^{-10}\) to 10\(^{-8}\) M) or 0.1% DMSO (control); luciferase assays were then performed to determine the promoter activity. See “Materials and Methods” for additional details. Values were calculated as a percentage of control and are expressed as mean ± SE (n = 3); each independent sample was tested in triplicate.

\(p < 0.05\) compared with control by one-way ANOVA with Bonferroni test. \(p < 0.05\) compared with pAChE-Luc transfected cells by two-way ANOVA with Bonferroni test.

**Figure 5.** Effect of TCDD (10\(^{-10}\) to 10\(^{-8}\) M) on AChE enzymatic activity (A) and promoter activity (B) in PC12 rat neuronal cells. (A) AChE enzymatic activity in cells incubated with TCDD or 0.1% DMSO (control) for 24 hr. (B) The mouse AChE promoter–reporter construct (pAChE-Luc) was transiently transfected into PC12 cells 1 day before treatment; after 24 hr of treatment with TCDD or DMSO, promoter activity was determined by luciferase assays. Values were calculated as a percentage of control and are expressed as mean ± SE (n = 3); each independent sample was tested in triplicate.
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