DIOXIN-INDUCED APOPTOSIS OF NEURONAL CELLS: A POSSIBLE INVOLVEMENT OF ARYL HYDROCARBON RECEPTOR AND ESTROGEN RECEPTOR BETA SIGNALING

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Abstract

This study demonstrated that TCDD (10-500 nM) activated caspase-3 in the mouse primary neuronal cell cultures. TCDD-mediated activation of caspase-3 was most prominent at early culture stage. A time-dependent activation of caspase-3 was found within several hours after the treatment and followed by moderate increase in LDH-release. Ac-DEVD-CHO inhibited both dioxin-induced caspase-3 and dioxin-induced LDH-release. Hippocampal neurons appeared more vulnerable to TCDD than the neocortical or cerebellar cells. The main achievement of the study is demonstration that TCDD-initiated apoptotic processes were accompanied by increased expression of AHR and ERβ. In hippocampal cultures exposed to TCDD on 1 DIV, a time-dependent increase in AHR and ERβ protein levels has been found. Immunoblotting analysis revealed also the constitutive expression of AHR and ERβ?in mouse hippocampal neurons at early stages in vitro. Summing up, the present data demonstrated that pro-apoptotic effects of TCDD in neuronal cells are age- and tissue-dependent. This study has also provided evidence for increased expression of AHR and ERβ in response to TCDD, which may have implication for treatment or prevention of dioxin intoxication during brain development.

Introduction

Dioxin intoxication results in severe clinical problems, such as behavioural and cognitive impairments and an increased number of newborns without properly formed brains. Knowledge about dioxin effects on nervous system is related mainly to necrosis. Little is known, however, about apoptotic effects of dioxins. It is particularly important because apoptosis occurs at each stage of neurodevelopment being also attributed to neurodegenerative diseases. There are only several data pointing to dioxins as regulators of brain apoptosis. Tillitt and Papoulias (2002) indicated TCDD-induced apoptosis in the dorsal midbrain of zebrafish, since they noticed chromatin condensation, apoptotic DNA fragmentation, and apoptotically altered cell morphology.[1] Dioxin apoptotic effects were inhibited by AHR antagonist, α-naphtoflavone, and DNA anti-sense to AHR 2 gene as well as by caspase inhibitor, Z-VAD-FMK.[2, 3]

Majority of toxic and anti-estrogenic effects of dioxins are mediated by aryl hydrocarbon receptors (AHRs). AHRs are present in many tissues including brain, where their pattern of distribution in preoptic area closely overlaps that of GABAergic neurons.[4] In the absence of ligand, AHRs are bound to heat shock protein Hsp90. Upon ligand binding, the AHR translocates into the nucleus whereupon it heterodimerizes with the ARNT protein (hydrocarbon receptor nuclear translocator) and binds to AHR DNA recognition sites, known as XRE (xenobiotic response element). XRE is located close to ERE (estrogen response element), thus allowing dioxin- and estrogen-mediated transcription processes to be reciprocally affected.[5]

No effective therapy against dioxin intoxication has been established yet. Some studies suggest that vitamin E and dihydroascorbic acid protect human epithelial cells from TCDD toxicity.[6] Some others impose protection to resveratrol, which has antagonist activity on the aryl hydrocarbon receptor.[7] Protective properties could also posses estrogens, which stimulate neurotrophin expression and regulate cell survival and apoptosis signalling, especially that mediated by the mitochondrial pathway.[8]

Defining molecular mechanisms of dioxin action in neuronal cells is the necessary step preceding the adaptation of new therapeutic strategies against dioxin neurotoxicity. Our previous study indicated that toxic and apoptotic effects of NMDA (N-methyl-d-aspartic acid) in neuronal cells are age- and tissue-dependent.[9, 10] Since dioxins are able to affect NMDA receptors, we made an attempt to evaluate developmental and tissue-specific regulation of dioxin-induced apoptotic processes and their interactions with estrogen receptor β (ERβ) in primary neuronal cell cultures. We investigated action of TCDD (tetrachlorodibenzo-*p*-dioxin; 10-500 nM) on caspase-3 and lactate dehydrogenase (LDH) activities in mouse hippocampal, neocortical, and cerebellar

neurons. Specificity of TCDD-induced activation of caspase-3 was confirmed by Ac-DEVD-CHO, a selective caspase-3-like protease inhibitor. An involvement of AHR in TCDD action was checked with the receptor antagonist, α-naphtoflavone. The protein levels of AHR and ERβ were evaluated with immunoblotting.

Materials and Methods

Primary neuronal cell cultures, assessment of caspase-3 activity, measurement of lactate dehydrogenase activity, data analysis and immunoblotting for AHR and ERβ were generally performed as previously described. [11, 12] In order to evoke toxic effects, including possibly apoptotic cell death, mouse primary neuronal cell cultures were exposed either to TCDD (tetrachlorodibenzo-*p*-dioxin; 10-500 nM; Sigma) or staurosporine (0.5 μM; Sigma) for 1 h, 3 h, and 6 h. In case of immunoblottings, hippocampal cells were treated with 100 nM TCDD for 1 h to 48 h. An involvement of aryl hydrocarbon receptor (AHR) in TCDD action was checked with the receptor antagonist, α-naphtoflavone (1 μM; Sigma). Specificity of TCDD-induced activation of caspase-3 was confirmed by Ac-DEVD-CHO (40 μM; aldehyde caspase substrate; Molecular Probes). All compounds were originally dissolved in dimethyl sulfoxide (DMSO), and then further diluted in culture medium to result in DMSO concentrations below 0.1%.

Results and Discussion

Central issue of the study was to demonstrate that TCDD activated caspase-3 in the mouse hippocampal, neocortical and cerebellar neurons, thus initiating there the apoptotic processes. We indicated that TCDD-mediated activation of caspase-3 was most prominent at early culture stage, i.e. on 1 DIV. A time-dependent activation of caspase-3 was found within several hours after the treatment in all the tissues examined (Fig. 1). In hippocampal cultures exposed to TCDD, the activity of caspase-3 reached the maximal value of about 200% of control already at 1 h. In neocortical and cerebellar cultures, the maximal activity of TCDD-induced caspase-3 was below 200% of control value and appeared at 3 h of post-treatment. Therefore, hippocampal neurons seem to be more vulnerable to TCDD than the other tissues. Similar observation was noted

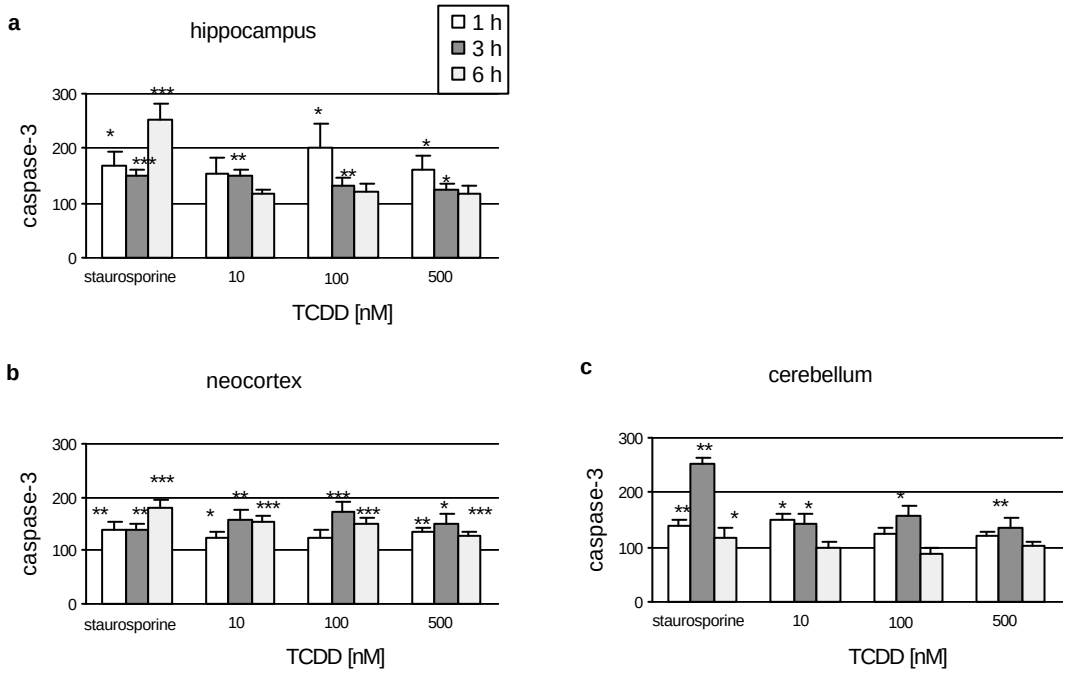

Fig. 1. Time-course effects of TCDD (10-500 nM) and staurosporine (0.5 μM) on caspase-3 activity in primary cultures of hippocampal (a), neocortical (b), and cerebellar cells (c). Cells were treated with these compounds for 1 h, 3 h, and 6 h. The results are presented as a percentage of control. Each bar represents the mean of three to four independent experiments ± SEM. A number of replicates in each experiment ranged from 5 to 8. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ (versus control cultures).

by Howard et al. (2003), who demonstrated that hippocampal, but not neocortical cells, were sensitive to Aroclor 1254 (a mixture of polychlorinated biphenyls acting in dioxin- or non dioxin-like way) in terms of caspase-dependent DNA-fragmentation.[13]

In our study, a time-dependent activation of caspase-3 was followed by moderate (7-13% over control), but significant increase in LDH-release, as demonstrated at 6 h after the treatment with 10 nM TCDD (Fig. 2). Likewise in our study, Williamson et al. (2005) noticed moderate TCDD toxicity indicating 5-20% loss of cell viability in TCDD-treated cerebellar neuroblasts.[14] Since the neuroblasts were exposed to 0.1-10 nM TCDD for 24 h, the authors might have observed late apoptotic effects of the dioxin. Indeed, in contrast to caspase activation, which is directly associated with apoptosis and observed already within a few hours after an injury, late apoptotic features, including LDH-release and cell death, need even days to reveal themselves.[15] Thus, our findings are not in conflict with these of Carpenter et al. (1997), who did not notice significant cell death in dissociated rat cerebellar neurons treated acutely with 100 nM TCDD.[16] There is a strong linkage between the activation of caspase-3 and apoptotic degradation of genomic DNA. A specific caspase-3-activated DNase (DFF40 or CAD) has been identified and characterized as an enzyme which is involved in the internucleosomal fragmentation of DNA and, finally, in apoptotic cell death.[17] Since in our study paradigm, 1-day-old neurons were sensitive to TCDD, responding by the caspase-3 activation, we suggest that these neurons underwent apoptotic death. Our assumption has been confirmed by Ac-DEVD-CHO, a selective caspase-3-like protease inhibitor, which inhibited not only dioxin-induced caspase-3, but also dioxin-induced LDH-release, as indicated at 3 h and 6 h of experiment. Toxic effects of TCDD were inhibited also by α-naphtoflavone, which points to AHR-mediated response of neuronal cells to this dioxin (data not shown). However, non-genomic effects of dioxin such as a rise of neuronal calcium level, a decrease in mitochondrial membrane potential or accelerated production of reactive oxygen species can not be excluded.[18]

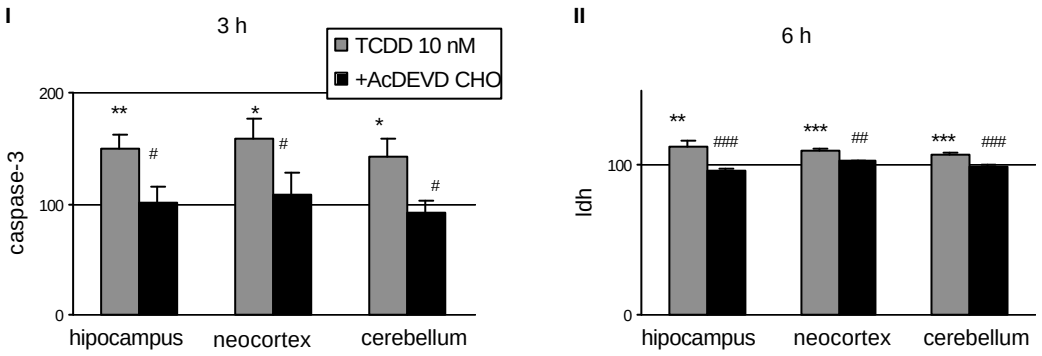

Fig. 2. Effects of Ac-DEVD-CHO (40 μM) on TCDD (10 nM)-induced caspase-3 activity (Ⅰ) and LDH-release (Ⅱ) in primary neuronal cell cultures. Cells were treated with the compounds for 3 h or 6 h. The results are presented as a percentage of control. Each bar represents the mean of three to four independent experiments ± SEM. A number of replicates in each experiment ranged from 5 to 8. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ (versus control cultures), and $^{\#}p< 0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ (versus TCDD-treated cultures).

The main achievement of this study is demonstration that TCDD-initiated apoptotic processes were accompanied by increased expression of AHR and ERβ, thus pointing to the complex mechanism involving estrogen and aryl hydrocarbon receptor cross-talk in neuronal tissue. In hippocampal cultures exposed to TCDD on 1 DIV, a time-dependent increase in AHR and ERβ protein levels has been found (Fig. 3A). In case of AHR, TCDD-mediated effect was delayed, since it was evident at 48 h. In case of ERβ, the stimulation was maximal already at 6 h, suggesting that ERβ is an important target for TCDD during neurodevelopment. Recently, it has become evident that apart from response to environmental pollutants, including dioxins, AHR is involved in neural development and apoptosis.[14, 19] However, toxicological and developmental functions of AHR may not

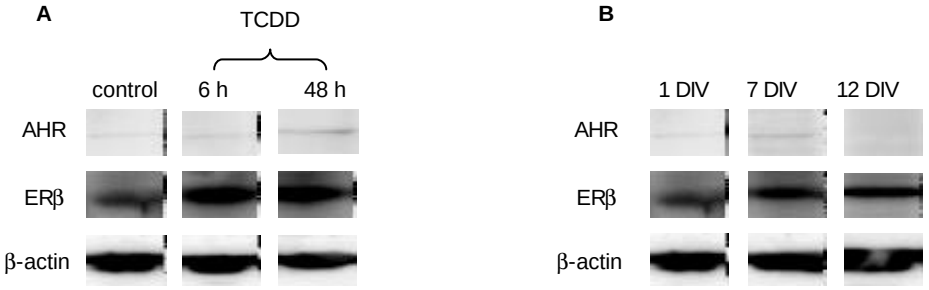

Fig. 3. The protein levels of ERβ, AHR, and β-actin used as a loading control, in primary hippocampal cell cultures. A - cell cultures treated with TCDD (100 nM) for 6 h and 48 h, on 1 DIV. B - control cell cultures on 1, 7, and 12 days in vitro (DIV). Tissue lysates (20 µg) were separated by SDS-PAGE (7.5% acrylamide gels) and transferred onto nitrocellulose membranes. The membranes were processed using standard procedure and incubated with polyclonal antibody anti-AHR (goat; Santa Cruz Biotechnology Inc.; 1:100), anti-ER-β (rabbit; Santa Cruz Biotechnology Inc.; 1:100), and anti-β-actin (Dako; 1-2000) followed by peroxidase-labelled secondary antibody. Immunoblots were visualized with chemiluminescence detection kit (Roche). Semiquantitative analysis of band intensity was performed using FujiLas 1000 and FujiGauge softwares.

always be related. Similarly, ERβ has been attributed to neurodevelopment and apoptosis mediated, possibly, via death receptor pathway.[20] Our immunoblotting analysis revealed that the constitutive expression of AHR in mouse hippocampal neurons was overlapped by the expression of ERβ at early stages in vitro, i.e. on 1 and 7 DIVs (Fig. 3B). These in vitro stages correspond with intensive programmed cell death in developing brain, thus supporting developmental and apoptosis-related functions of the receptors.

In summary, the present data demonstrated that pro-apoptotic effects of TCDD in neuronal cells are age- and tissue-dependent. This study has also provided evidence for increased expression of AHR and ERβ in response to TCDD, which may have implication for treatment or prevention of dioxin intoxication during brain development.

Acknowledgements
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