DIOXIN MODULATES EXPRESSION OF RECEPTOR FOR ACTIVATED C KINASE (RACK-1) IN DEVELOPING NEURONS

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Introduction

TCDD is sensitive to the central nerve system of the developing brain[1,2]. The TCDD-induced neurodevelopmental deficits include the cognitive disability and motor dysfunction. While TCDD may lead to neurodevelopmental and neurobehavioral deficit[3], it is not known which molecular substances are intracellular targets for TCDD. Since TCDD accumulates in brain and the brain contains the Ah receptor, it is possible that TCDD may act at the target site such as cerebellum, which is responsible for cognitive abilities and motor function. A recent *in vitro* studies using cerebellar granule cells demonstrated a translocation of PKC-α and ε following the TCDD or PCB exposure[4].

One of the most pivotal second messenger molecules involved in neuronal function and development is protein kinase C (PKC). PKC signaling pathways have been implicated as an important factor in learning and memory processes[5]. PKC signaling events are optimized by the adaptor proteins, which organize PKCs near their selective substrates and away from others. RACK-1(receptor for activated C-kinase) is one of adaptor proteins that anchor the activated PKC at the site of translocation [6]. RACKs bind PKC only in the presence of PKC activators[7]. RACKs are 30- and 36-kDa proteins located in cytoskeletal compartment and play a key role in PKC activation and in membrane amchoring. Since different PKC isoforms translocate to distinct subcellular sites on activation [8], it is suggested that isoform-specific RACK may be present. Activation of certain PKC isoforms (PKC-a and βII) is preferentially associated with RACK-1[9, 10]. While TCDD modulates PKC signaling pathway, role of RACK-1 on TCDD-mediated signaling pathway is not known. To identify the intracellular target for TCDD and understand a mechanism of signaling pathway in the developing brain, the present study attempted to analyze effects of RACK-1 in the cerebellar granule cells following TCDD exposure

Materials and Methods

Cerebellar granule cell culture. Cerebellar granule cell cultures were prepared from the cerebella of 7-day old Long Evans Hooded rat pups as described previously[11]. Cells were plated at 3 x 10^6 cells/well in 6-well plates. After plating, cells were incubated at 37 ^{0}C in a humidified incubator

with 5% CO_2 atmosphere. Cytosine arabinoside (5 µM) was added after 24 hr to prevent growth of non-neuronal cells. Cells were used for the experiments after 7 days in culture. Cultures typically contained >95% neurons.

Exposure. Cerebellar granule cells grown on 6-well culture plates were exposed to 0, 1, 10 and 100 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (>99% purity; KOR, Boston) for the duration of various time points, as described in Table 1. In order to get enough protein for immunoblots, 4 culture plates were used for each concentration. After the exposure, cultures were washed twice with lock's buffer and the cells were harvested in a final volume of 2 ml buffer A (20 mM Tris-HCl. pH7.5, containing 0.25 M sucrose, 2 mM EDTA, 2 mM EDTA and cocktail of protease inhibitors including 0.5 mM phenylmehylsulfonylfluoride (PMSF), 10 µg/ml leupeptin, and 10 µg/ml pepstatin). For the inhibition study, cells were treated with 10 µM a-naphthoflavone (a-NF) (Sigma, MO) for 1 hr prior to the exposure of 2,3,7,8-tetrachlorodibenzo-p-dioxin .

Cell fractionation. Cells were scraped off into buffer A (20 mM Tris-HCl. pH7.5, containing 0.25 M sucrose, 2 mM EDTA, 2 mM EDTA and cocktail of protease inhibitors including 0.5 mM phenylmehylsulfonylfluoride (PMSF), 10 µg/ml leupeptin, and 10 µg/ml pepstatin). The cells were briefly sonicated and centrifuged at 100,000g for 1 h. The supernatants were designated as cytosolic fraction. The membrane proteins in the pellets were extracted with buffer B (20 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA and protease inhibitors) on ice for 30 min followed by centrifugation at 15,000g, and the supernatants were saved as detergent-soluble-membrane fraction.

Immunoblotting. Immunoblot analysis was performed as described previously [11]. Proteins (10 µg) from cytosolic and membrane fractions were separated by 7.5 % SDS-PAGE and transferred to nitrocellulose membrane by Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The nitrocellulose sheet was blocked with 5% non-fat dry milk in Tris buffered saline. PKC isozymes and RACK-1 were detected with monoclonal antibodies (Transduction Lab, Lexington, KY). The blots were reacted with a peroxidase-conjugated anti-mouse IgG and detected by the Super Signal (Pierce, Rockford, IL). For the detection of RACK-1, 50 µg of whole cell lysate was analyzed by 10% SDS-PAGE. The density of respective bands was analyzed by the Fluor-S (Bio-Rad, Hercules, CA).

Results and Discussion

TCDD altered the levels of RACK-1 in a dose- and time-dependent manner (Fig. 1). When the cerebellar cells in culture were exposed to the various concentrations TCDD, maximum increase was observed at 10nM. The highest induction of RACK-1 was shown at the 30 min exposure and beyond a point of 30 min, the levels showed a trend of decrease (data not shown). Since RACK-1 binds to PKC and other growth regulatory proteins[12], it is suggested that altered expression of RACK-1 disrupt the signaling pathway, which may lead to TCDD-induced neurotoxicity. The induction of RACK-1 was blocked by a-Naphthoflavone (a-NF), Ah receptor inhibitor, indicating that the induction was mediated by the Ah receptor (Fig. 2A). Since neurotoxic responses of non AhR-mediated pathway are in dispute, evidence on AhR-dependent induction of RACK-1 will help understand mechanism of PKC signaling pathways in the neuronal cells.

TCDD induced the translocation of PKC-bII and PKC-e (data not shown), but the mechanisms involved might take different pathways. While the translocation of PKC-bII was blocked by the Ah

receptor blocker, that of PKC-e was not affected by the same blocker. Thus, activations of PKC-bII and PKC-e may be mediated via the AhR –dependent and independent pathway, respectively. AhR-independent action of PKC-e in the nervous system observed in this study is compatible with other previous reports[4]

Since RACK-1 binding is known to increase PKC-bII activity, a lack of RACK-1 induction by the AhR inhibitor may lead to the inhibition of translocation in PKC-bII. The results suggest that PKC-bII may be dependent of both RACK-1 and AhR for its activation upon TCDD exposure. In addition, each PKC isozyme, which is differentiated by the subcellular distribution and functional role, may have its unique pattern of mechanism in this *in vitro* system. Because, in this study, a specific blockage of RACK-1 or RACK-2, specific adaptor protein to PKC-e, was not performed, further studies are warranted to determine if RACK-2 is also mediated by the AhR and if a direct blockage of RACK-1 or RACK-2 leads to inactivation of the respective PKC isozymes.

Our study revealed that induction of RACK-1 was AhR-dependent. So, we investigated a structure-activity relationship among the structurally similar organohalogenated compounds. Treatment of cerebellar granule cells with PCBs resulted in a selectively higher induction of RACK-1 with coplanar PCB, as compared to non-coplanar structure (Fig. 2B). The result indicates that RACK-1 induction may be an important event for the AhR agonists of organohalogenated compounds. RACK-1 induction may be a useful biomarker to discrete the moieties between coplanar/non-coplanar structures among PCB mixtures.

RACK-1 is not only a shuttling or anchoring protein to PKCs, but a binding protein to other cellular growth regulatory factors. RACK-1 is also associated with the altered production of cytokines and other inflammatory factors[13]. Modulation of RACK-1 interaction caused by the altered expression may lead to the TCDD-induced neurotoxic effects including neurobehavioral and neurodevelopmental deficits. The study demonstrated that RACK-1 is a possible target molecules for TCDD and other structurally related compounds and plays an important role for understanding PKC-isozyme specific mechanism of action and signaling pathways. As TCDD induced the activation of PKC in isozyme-specific manners, identification of the specific isozymes and their adaptor proteins involved in the action will be a crucial step in the future to elucidate the mechanism of action and pinpoint the target molecules.

Acknowledgments
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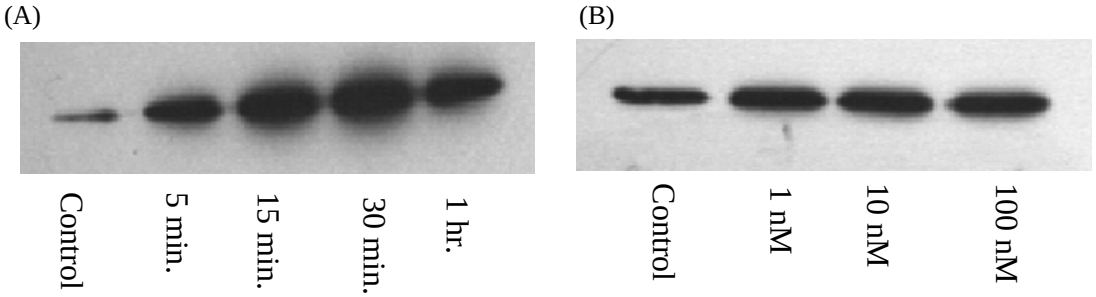

Figure. 1 Time-dependent increase of RACK-1 with exposure to 10nM TCDD (A), Dose-dependent increase of RACK-1 following 30 min. exposure to TCDD (B)

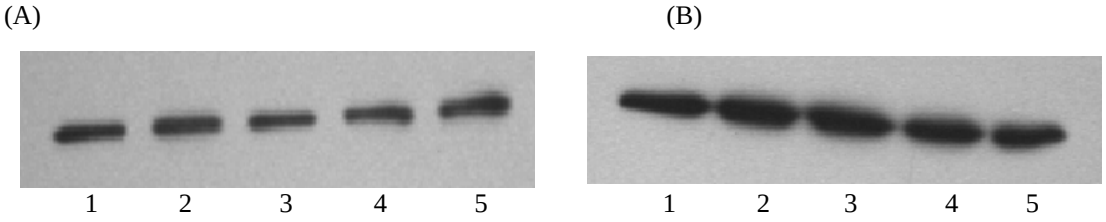

Figure 2. Levels of RACK-1 with exposure to TCDD in presence of 10μM α-naphthoflavone.; 1) control, 2) α-NF only , 3) 1nM TCDD + α-NF, 4) 10nM TCDD + α-NF, 5) 100nM TCDD + α-NF (A)
Levels of RACK-1 with exposure to 50μM PCBs. 1) control, 2) 4,4'-DCB, 3) 2,3,4,4',5-PCB, 4) Aroclor 1254, 5) 2,2'-DCB (B)