POSSIBLE INTRACELLULAR TARGETS OF TCDD IN CEREBELLAR GRANULE CELLS

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Introduction

Both human and animal studies showed neurotoxic effects of TCDD such as cognitive impairment and motor dysfunctions as neurodevelopmental outcomes [1,2]. While TCDD may lead to neurodevelopmental and neurobehavioral deficit[3], it is not known which molecular substances are intracelluler targets for TCDD-induced neurotoxicity. 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.

One of the most pivotal second messenger molecules involved in neuronal function and development is PKC. PKC signaling pathways have been implicated as an important factor in learning and memory processes[4]. Alteration of PKC in cerebellum is suggested to be associated with impaired motor dysfunction[5]. Since PKC isozymes are differentially distributed in the brain cells and their roles are isozyme-specific and species-specific[6], it is important to identify the individual isoforms involved in the neurotoxic effects to understand the mechanism of action. A recent *in vitro* studies using cerebellar granule cells demonstrated a translocation of PKC-α and ε following the TCDD or PCB exposure [7,8]. RACK (receptor for activated C-kinase) proteins play a key role in PKC activation and in membrane anchoring [9]. Activation of certain isoforms (PKC-β, δ, and ε) is preferentially associated with RACK-1 which plays a significant role in PKC signaling pathway [10]. However, it is not known whether RACK is a possible intracellular target for TCDD-mediated signaling pathway.

To identify the intracellular target for TCDD and understand a signaling pathway in the developing brain, the present study attempted to analyze the PKC isoforms and RACK in the cerebellar granule cells

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 ì M) 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, and 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (>99% purity; KOR, Boston) for 60 min. 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. For the inhibition study, cells were treated with 10 ì M dequalinium chloride (Sigma, MO) for 1 hr and UV-irradiation for 5min 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 ì g/ml leupeptin, and 10 ì g/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 [12]. Proteins (10 ìg) from cytosolic and membrane fractions were separated by 7.5 %(PKC isoforms) 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 isoforms and RACK 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, 50 ìg 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). The data was represented as % controls.

Results and Discussion

TCDD is known to be sensitive to the developing brain and to affect the central nerve system[13,14]. The TCDD-induced neurodevelopmental deficits include the cognitive disability and motor dysfunction. PKC is implicated in learning and memory as well as in LTP. PKCs are abundant in neuronal tissue and are involved in neuronal survival and functions of neuronal trophic factors, suggesting a crucial role for PKC in the signal transduction between neurons and the etiology of the neuronal diseases [4,15]. Since functional roles and subcelluar distributions of individual PKC isoforms are isoform-specific and species-specific, identification of specific isoforms targeted for TCDD is required to understand the mechanism of the TCDD-induced neurotoxicity.

Ca^{2+}-independent PKCs have different substrate specificity or phospholipid dependency as compared to Ca^{2+}-dependent isoforms. Ca^{2+}-independent forms are suggested to be involved in the different cellular functions than Ca^{2+}-dependent forms[16]. Although the physiological roles of Ca^{2+}-independent forms have not been fully clarified, it is known that PKC-å, one of the Ca^{2+}-independent forms, is most abundant in the brain.

In the present study, the translocational effects of PKC-åand δ were observed after a high dose exposure of TCDD for 60 min (Fig. 1&2). Because regulation of PKC-åmRNA showed a similar time course to GAP-43 mRNA and both PKC- åand GAP-43 are located in the presynaptic terminals[17,18], it is speculated that PKC-åmay play a role in expression of GAP-43 in neuronal cells. In addition, PKC-åhas been suggested to be a candidate isoform associated with a mechanism of LTP. Thus, it is suggested that alteration of this particular isoform may perturb the normal maintenance of LTP and lead to the impairment of learning and memory. A subsequent abnormal expression of GAP-43 by the altered PKC-åmay disturb structural formation of neuronal cells. Regulation of neurotransmitter release may be interfered via altered cytoskeleton networks.

PKC-δ is known to play an important role in regulating cell cycle of cerebellar granule cells [19]. Inhibition of PKC-δ led to the apoptosis of cerebellar granule cells derived from 8-day old rat cerebellum. Thus, the altered activation of PKC-δ may induce the dysregulation of neuronal cell proliferation, which may result in the neurological diseases. Since PKC-å and δ have been associated with a variety of pivotal biological events in neuronal cells, it is feasible that altered subcellular distribution of these isoforms may play important roles in the TCDD-induced neurotoxicity.

RACKs are 30- and 36-kDa proteins located in cytoskeletal compartment. RACK is one of proteins that anchor the activated PKC at the site of translocation [9]. RACKs bind PKC only in the presence of PKC activators [20]. Since different PKC isoforms translocate to distinct subcellular sites on activation [21], it is suggested that isoform-specific RACK may be present. Whereas isoform specificity of RACK is not known, PKC-β, δ, and ε tend to bind RACK more preferentially than other isoforms [10]. Immunoblot analysis of RACK in the present study showed that level of RACK protein was increased with exposure to TCDD (Fig.3), suggesting that RACK may be an intracellular target molecule for TCDD. Dequalinium is known to PKC activity by blocking RACK-1 binding domain [22]. Pretreatment of dequalinium for 1 hr blocked the increase of RACK protein induced by TCDD (Fig 3). This result further suggests that TCDD be involved in the alteration of PKC signaling pathway in this neuronal cell system. Since RACK is a homolog of the G-protein beta subunit [23], altered level of RACK with TCDD exposure may disrupt normal signal pathway in brain, which ultimately may lead to neurobehavioral and cognitive deficit.

Translocational effects of PKC-δ and ε with exposure to TCDD was slightly dampened by dequalinium chloride (Fig. 1 and 2), indicating that TCDD targets PKC signaling system by altering a binding potential between RACK and activated PKCs. The results suggest that certain PKC isoforms and their anchoring protein are possible intracellular targets of TCDD and alteration of these proteins may be associated with a mechanism of TCDD-induced neurotoxicity.

While RACKs are known to bind activated PKC in the presence of phosphatidylserine(PS), diacylglycerol(DAG) and calcium [20], PKC isoforms associated with these protein seems not to be limited to classical PKC isoforms, which require

both calcium and DAG. The present study showed that RACK might be, at least, in part, involved in activation of novel PKC class, which does not require calcium for its activation.

The study provided the evidence that TCDD altered selective PKC isoforms and their anchoring protein in the developing neuron. Identification of target molecules as shown in the present study may contribute to understanding TCDD-induced neurotoxic mechanism of action in neuronal cells, thereby improving the health risk assessment in humans.

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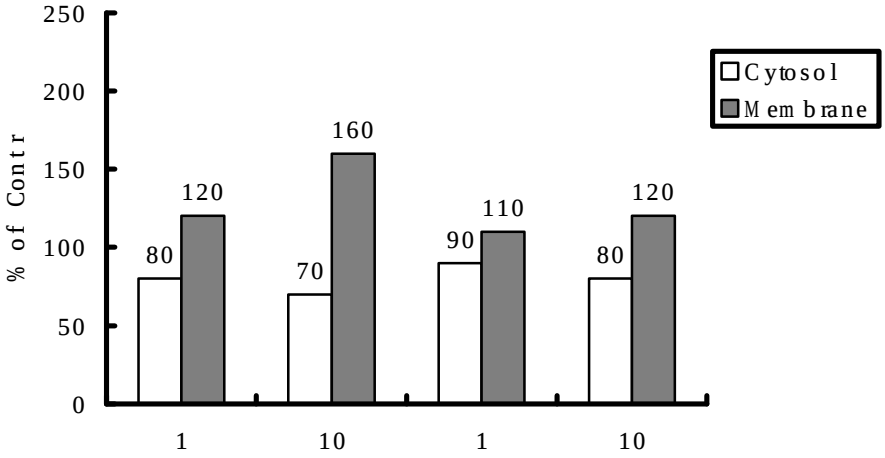

Figure 1. Effects of TCDD (nM) on subcellular distribution of PKC-ε in SD rat cerebellar granule cells in presence or absence of dequalinium (10uM).

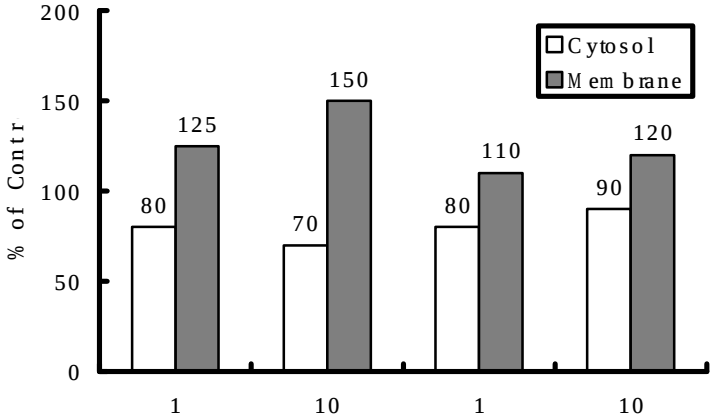

Figure 2. Effects of TCDD on subcellular distribution of PKC-δ in SD rat cerebellar granule cells
in presence or absence of dequalinium (10uM).

Figure 3. Effects of RACK following TCDD exposure in presence or absence of dequalinium. Whole cell lysates (50ug) were separated by 10% SDS-PAGE. 1)DMSO (0.1%)only; 2) Dequalinium (10uM) only; 3) TCDD (1nM) only; 4) TCDD (10nM) + Dequalinium(10UM); 5) TCDD(10uM) only.