*J. Toxicol. Pub. Health*
Vol. 15, No. 1, pp. 89-93, 1999

# 2,3,7,8-Tetrachlorodibenzo-p-dioxin Induces Recruitment of Shc/Cbl/Grb2/Sos Complex in Early Signaling Pathway of CYP1A1 Induction in the Primary Culture of Rat Hepatocytes

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*(Received November 12, 1998)*
*(Accepted December 27, 1998)*

**ABSTRACT** : *2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is known to induce cytochrome P450 1A1 and to activate c-Src kinase and p21 Ras. This study examined the molecular interactions of adaptor proteins including Shc, Grb2, and Sos in rat primary hepatocytes and their relationship to the induction of CYP1A1 by TCDD. TCDD induced CYP1A1 level and EROD activity in a dose-dependent mode. Sos/Grb2 association is increased by TCDD in a dose dependent mode. Tyrosine phosphorylated Shc, mainly p52, onloads to Grb2/Sos complex upon TCDD stimulation. The electrophoretic mobility shift of Sos is showed by TCDD. These results indicate that TCDD modulates the molecular interaction features of adaptor complex proteins including Shc, Grb2, Sos and Cbl in early signaling pathway of TCDD-mediated CYP 1A1 induction of rat primary hepatocytes.*

**Key Words** : *TCDD, Hepatocytes, CYP1A1, Shc, Grb2, Sos*

## I. INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental contaminant formed as a by-product in the production of several herbicides and chlorine bleaching of paper pulp. TCDD activates the expression of several cytochrome P450 (CYP) enzymes, especially, CYP1A1 (Whitlock, 1993). TCDD enters cells and binds with cytosolic AhR. According to Blankenship and Matsumura (1997) TCDD binding dissociates hsp90 and c-Src kinase from AhR. The TCDD complex travels into the nucleus where it forms a heterodimer with Arnt and finally binds with dioxin or xenobiotics response element and thereby activates the expression of CYP1A1 gene.

c-Src kinase and small GTPase $p21^{Ras}$ which are activated by TCDD are implicated in AhR-mediated CYP1A1 induction (Enan and Matsumura, 1994; Enan and Matsumura, 1996; Reiners *et al.*, 1997). However, the role of adaptor complex proteins positioned between protein tyrosine kinase (PTK) and small GTPase $p21^{Ras}$ in TCDD-mediated CYP induction is remainded to be demonstrated. The adaptor complex proteins containing Grb2, Shc, Cbl, Sos, CrkL and PI3K link the signals of receptor or non-PTK from extracellular stimuli to small GTPase such as Ras, Rho, and Rap. The activation of Ras occurs predominantly via Grb2-Sos complex, either alone or with Shc via the formation of Shc/Grb2/Sos complex (Holt *et al.*, 1996; Buday *et al.*, 1996). Thus, Sos-bound Grb2 helps to translocate Sos to the cytoplasmic face of plasma membrane so that it can convert Ras from its inactive GDP-bound to its active GTP-bound state. $p120^{c\text{-}Cbl}$, a mammalian homologue of v-Cbl, is tyrosine phosphorylated following stimulation of growth factors receptors, cytokine receptors, and receptors lacking intrinsic enzymatic activity such as TCR, BCR, or Fc receptors (Holt *et al.*, 1996; Smit *et al.*, 1996). Cbl has already been shown to bind to the SH3 domains of a number of proteins, including Fyn, Grb2, Lck, Fgr Nck, Crk, PLC$\gamma$1, and p85 of PI 3K (Buday *et al.* 1996; Holt *et al.*, 1996; Anderson

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*et al.*, 1997).

In this report, we examined whether an adaptor molecular complex including Shc/Grb2/Sos is implicated in TCDD-mediated induction of CYP1A1. Herein, we demonstrated that treatment of primary rat hepatocytes with TCDD induced the modulation of molecular interactions of adaptor proteins and subsequent induction of CYP1A1.

## II. MATERIALS AND METHODS

### 1. Materials

TCDD was obtained from Cambridge Isotope Laboratories (Woburn, MA) and collagenase from Gibco (Grand Island, NY). Polyclonal antibody to CYP1A1 was obtained from Daiichi Pure Chemicals (Tokyo, Japan) and swine anti-goat IgG (H+L)-alkaline phosphatase conjugate was purchased from Vector Laboratories (Burlingame, CA). Anti-phosphotyrosine (anti-Tyr(p)) and anti-Shc were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Grb2, and anti-Sos antibodies were obtained from Santa Cruz Co. (Santa Cruz, CA).

### 2. Hepatocyte isolation, culture and TCDD treatment

Hepatocytes were isolated from adult male Sprague-Dawley rat (250~300 g) by collagenase perfusion. The dead hepatocytes and Kuffer cells were removed by density gradient centrifugation in Percoll (Kim *et al.*, 1997). Hepatocytes were seeded at a density of $1\times 10^5$ cells/$cm^2$ with William' E media containing 10 mM HEPES, pH 7.4, 1.25 mg/*ml* bovine serum albumin, 6.25 $\mu$g/*ml* insulin, 6.25 $\mu$g/*ml* transferrin, 6.25 ng/ml selenious acid, 1.0 $\mu$M dexamethasone, 100 units/*ml* penicillin G and 100 $\mu$g/*ml* streptomycin. Media was changed at 4 and 24 hr of incubation. Hepatocytes were treated with 0, 10, or 100 nM TCDD (dissolved in DMSO) for 15 min or 24 hr.

### 3. Preparation of microsomal fraction and measurement of microsomal CYP1A1 activity and its protein level

At the end of 24-hr incubation, hepatocytes were homogenized in 0.1 M potassium phosphate, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, and 0.1 mM DTT. After centrifugation at 10,000$\times$g for 30 min, the supernatant was centrifuged at 100,000$\times$g for 1 hr. The pellet was homogenized in 0.1 M phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA and 0.1 mM DTT. 7-Ethoxyresorufin-O-deethylase (EROD) activity was determined using a fluorimetric method (Xu *et al.*, 1997) with 10 mM 7-ethoxyresorufin. SDS-polyacrylamide gel electrophoresis was performed with 5 $\mu$g microsomes using a 12.5% acrylamide resolving gel and a 5% stacking gel. Electrophoretically separated proteins were transferred to nitrocellulose and the membranes were blocked overnight at 4°C in Tris-buffered-saline, pH 7.5, with 0.05% Tween containing 5% non-fat milk. Membranes were incubated with a goat polyclonal antibody against CYP1A1. The membrane was then exposed to swine anti-goat IgG (H+L)-alkaline phosphatase conjugate. Bands were visualized by an alkaline phosphatase reaction for 5 min.

### 4. Immunoprecipitation and immunoblotting

At the end of the 15 min incubation of hepatocytes with TCDD, the cells were lysated with Triton X-100 extraction buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 0.1% BSA, 1 mM PMSF, 1% aprotinin, 5 mM EDTA, 50 mM NaF, 0.1% 2-mercaptoethanol, 5 $\mu$M phenylarsine oxide and 100 $\mu$M sodium orthovanadate. To immunoprecipitate, 1 $\mu$g of anti-Grb2 antibody was added to the lysates. Immunoprecipitates or the lysates of primary hepatocytes were resolved on 12.5% acrylamide and 0.2% bisacrylamide gels by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane (11 mAh/cm2) using semi-dry blotting transfer system (Ellard Inc., Seattle, WA). The membrane was incubated with blocking solution for 1 h and then incubated with specific anti-Tyr(p), anti-Shc, anti-Grb2, and anti-Sos antibodies with continuous agitation. The membrane was incubated with anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase for enhanced chemiluminescence detection (Amersham Co., Arlington Heights, IL) or

conjugated with alkaline phosphatase for colorimetric development. For reprobing, the membrane was stripped with 0.1 M glycine, pH 2.5 at room temperature for 30 min and then reblotted with primary antibody (Park *et al.*, 1996).

## III. RESULTS

### 1. Induction of CYP1A1 level and EROD activity upon TCDD treatment

Cultured hepatocytes were treated with TCDD and the induction of CYP1A1 expression was examined. The treatment of TCDD increased CYP1A1 expression in a dose-response way (Fig. 1A). First lane was anti-CYP1A1 blot of 3-methylcholanthrene-treated rat microsomes. EROD activity has been used as a marker for CYP1A1. EROD activity in microsomes of untreated hepatocytes was 17±2 pmol/min/mg protein (Fig. 1B). EROD activity was significantly induced by TCDD treatment and correlated with CYP1A1 protein level. While EROD was induced 16- or 48-fold and CYP1A1 level was induced 45-or 114-fold at a concentration of 10 nM or 100 nM, respectively. Anyway, these data suggested TCDD treatment in the cultures of hepatocytes was effective.

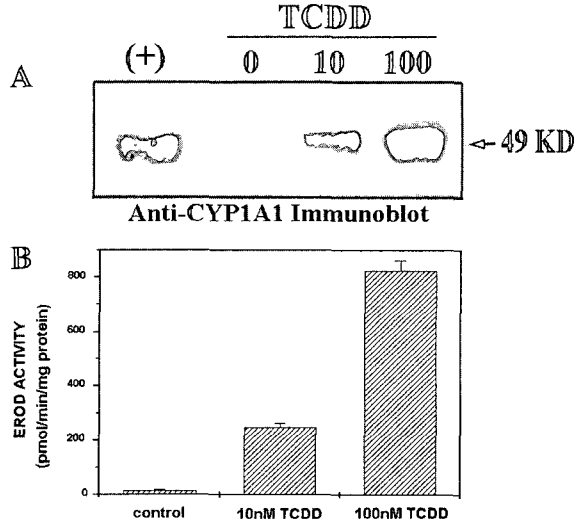


**Fig. 1.** Dose-dependent induction of CYP1A1 and EROD activity. Rat primary hepatocytes were treated with TCDD for 24 hr and prepared microsomes as described in the Materials and Methods. (A). CYP1A1 level was determined by Western blot analysis. Lane 1, standard microsomes for CYP1A1. Lane 2, DMSO. Lane 3, 10 nM TCDD. Lane 4, 100 nM. (B). EROD activity.

### 2. Interaction of Shc, Cbl, and Sos with Grb2 was inducible upon TCDD stimulation in rat primary hepatocytes

We examined the molecular interaction of adaptor molecules among Shc, Cbl, and Grb2 whose complex shuttled Sos to Ras at the plasma membrane (Byrne *et al.*, 1996). Tyrosine phosphorylation of Shc is critically required for the activation of Ras (Ponk *et al.*, 1994). To confirm the association of Shc with Grb2 in the signaling pathway of TCDD-mediated CYP1A1 induction, the TCDD-treated cell lysates were immunoprecipitated with anti-Grb2 antibodies and analysed by Western blot. Anti-Grb2 bolt confirmed that all lanes brought down equal amounts of Grb2 protein except pre-immune precipitates (Fig. 2A, lanes 2~4). There was a tyrosine phosphorylated protein, p52, in precipitated protein mixture with anti-Grb2 antibodies (Fig. 2A, lanes 2~4). The interaction of Shc with Grb2 presented in a resting state was increased a little following TCDD treatment (Fig. 2B, lanes 2~5). Cbl brought down Grb2 proteins in both resting rat primary hepatocytes and stimulated with TCDD except preimmune precipitates (Fig. 2C). Anti-Sos blot of Grb2 immunoprecipitates revealed that Sos was coprecipitated with Shc/Grb 2 complex in both resting and TCDD treatment of rat primary hepatocytes (Fig. 2D). TCDD increased the association of Sos with Grb2. The level of tyrosine phosphorylation of the p52 in a resting state was markedly increased following TCDD treatment in a dose-dependent mode (Fig. 2E, lanes 3). We confirmed the integrity of p52 phosphoprotein to Shc with rabbit anti-Shc antibodies in the same membrane after stripping (Fig. 2B). The presence of Sos protein in Shc/Grb2 complex was also tested. These results suggested that the tyrosine phosphorylation of Shc and its association with Grb2 were induced in the signaling pathway of TCDD-mediated CYP1A1 induction. Also, Sos was coprecipitated with Shc/Grb2 complex and showed mobility shift upon TCDD treatment of rat primary hepatocytes. The mobility shift of Sos was reversed by potato acid phosphatase treatment

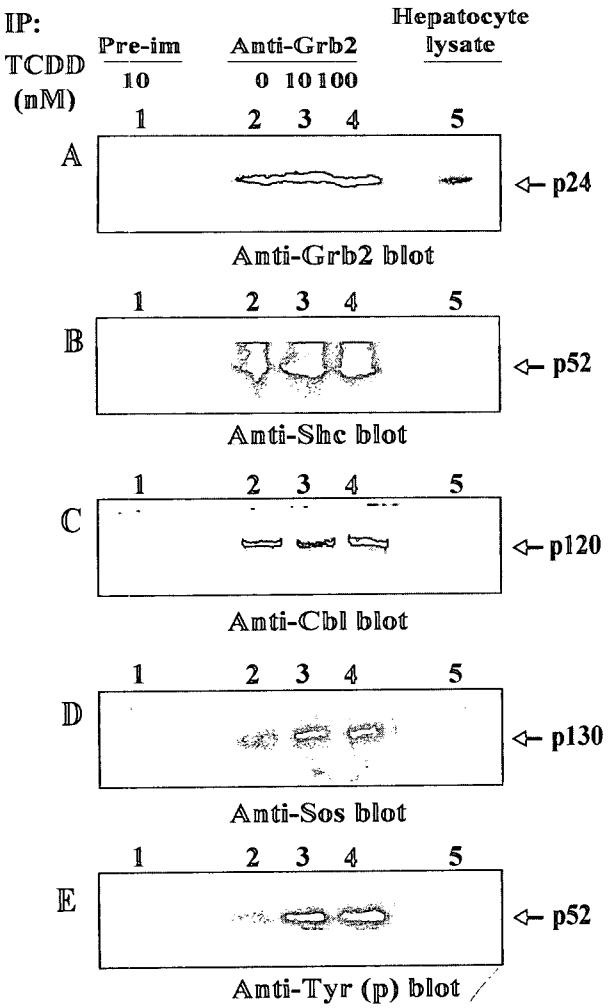


**Fig. 2.** TCDD induced the tyrosine phosphorylation of Shc and increased the interaction of Shc with Grb2. Rat primary hepatocytes were treated with TCDD for 15min and the cell lysates were immunoprecipitated with anti-Grb2 antibodies. The precipitated immune complexes were resolved on 12.5% SDS-PAGE and analyzed by Western blot. The membrane was blotted with anti-Grb2 (A), anti-Shc (B), anti-Cbl (C), anti-Sos (D), and anti-Tyr (p) (E). Lane 1 represents pre-immune immunoprecipitates of rat primary hepatocytes. Other lanes correspond to resting primary hepatocytes, lane 2; the cells stimulated with 10 nM TCDD lane 3; with 100 nM TCDD lane 4. Lane 5 was a whole lysate of rat primary hepatocytes.

(unpublished data).

## IV. DISCUSSION

In the present study, we designed the experiments to determine the molecular interaction features of adaptor complex proteins including Shc, Grb2, Cbl, and Sos in the signaling pathway of TCDD-mediated CYP1A1 induction. One possible mechanistic insight is that phosphorylation events are occurred in the signaling pathway of TCDD-mediated CYP1A1 induction. Src kinase was directly tyrosine phosphorylated by TCDD (Enan and Matsumura, 1996; Blankenship and Matsumura, 1997). Also, AhR was associated with Src kinase and its tyrosine phosphorylation sites were localized in the C-terminal half of the protein (Mahon and Gasiewicz, 1995). These findings indicate that AhR may be a substrate of protein tyrosine kinase, such as Src kinase, but upstream and downstream of the signaling molecule of Src kianse activation have yet to be proven. The signals from protein tyrosine kinase (PTK) including receptor or non-receptor type PTK are transduced to $p21^{Ras}$ small GTPase via adaptor complex proteins (Byrne *et al.*, 1996).

The implication of $p21^{Ras}$ GTPase was demonstrated in the process of CYP1A1 induction by TCDD (Reiners *et al.*, 1997). Induction of CYP1A1 by TCDD was suppressed in skin papillomas having highly expression of Ha-ras. This finding was supported by the data showing that epidermal growth factor (EGF), a strong activator of $p21^{Ras}$, suppressed the induction of CYP1A1 in cultured rat hepatocytes (Hohne *et al.*, 1990). However, the mechanism by which $p21^{Ras}$ modulates AhR function after TCDD treatment is not known even though AhR and ARNT proteins underwent phosphorylation. The notion that both Src kinase and $p21^{Ras}$ were involved in CYP1A1 induction by TCDD led us explore the role of adaptor complex proteins including Shc, Grb2, and Sos in the signaling pathway of TCDD-mediated CYP induction. We here suggested the evidence that treatment of TCDD modulated the molecular interaction pattern of adaptor complex proteins. However we could not prove the mechanism by which TCDD or AhR modulated the interaction features of adaptor proteins.

Shc and Grb2 functionally couple upstream protein tyrosine kinases to Ras in mammalian cells (Byrne *et al.*, 1996; Park *et al.*, 1996). In our studies, Shc was tyrosine phosphorylated and coprecipitated with Grb2 (Fig. 2). In rat primary hepatocytes Shc/Grb2 complex was already present in the resting state. Shc and Grb2

recruited each other after TCDD treatment in a phosphorylation dependent manner. Sos in anti-Grb2 (Fig. 2), anti-Shc, or anti-Cbl (unpublished data) precipitates showed a mobility shift at 10 and 100 nM TCDD but this was not distinctive because a 12.5% polyacrylamide gel was used to show p24 Grb2 and this high percentage of polyacrylamide decreased the separation of high molecular weight $p130^{Sos}$. These data indicate that TCDD induces the expression of CYP1A1 and the association of adaptor proteins which link the signals of receptor or non-receptor type PTK stimuli to Ras in a dose-response way. However, the relationship between two response should be investigated more.

## ACKNOWLEDGEMENT

The author wishes to acknowledge the financial support of the Korea Research Foundation made in the program year of 1997 (GE-97-126).

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