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Alteration of the PKCθ–Vav1 complex and phosphorylation of Vav1 in TCDD-induced apoptosis in the lymphoblastic T cell line, L-MAT

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ABSTRACT

We have previously reported that protein kinase C (PKC) theta (θ) and protein tyrosine kinase are involved in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced apoptosis of L-MAT, a human lymphoblastic T cell line. In the current report, we show that Vav1, a GDP/GTP exchange factor for Rho-like small GTPases, could be detected by Western blotting in the membrane fraction of L-MAT cells after TCDD treatment and was precipitated by incubating with an antibody against PKCθ. Furthermore, the degree of phosphorylation of Vav1, which can be detected using the phosphotyrosine-specific antibody PY-20 or 4G10, is significantly increased after treatment with TCDD. In addition, pretreatment of the cells with genistein, a protein tyrosine kinase inhibitor, abolished the phosphorylation of Vav1 and inhibited the apoptosis. These results suggest that TCDD treatment may activate an unidentified protein tyrosine kinase. Accordingly we hypothesize that this kinase phosphorylates Vav1, following which phosphorylated Vav1 may translocate to the membrane with PKCθ. Finally, PKCθ may mediate the transfer of the apoptotic signal to downstream components.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental pollutant and triggers apoptosis in both thymocytes and T cells. The results of in vivo studies of immunotoxicity have suggested that TCDD-induced thymic atrophy is a consequence of thymocyte apoptosis (McConkey and Orrenius, 1989; Kamath et al., 1997). TCDD-induced T cell apoptosis occurs mainly in immature T cells such as double positive ($CD4^+/CD8^+$) T cells. Our group has previously reported that cells of the human lymphoblastic T cell leukemia line, L-MAT cells, which are sensitive to TCDD, is a good cell system to explore the mechanism of TCDD-induced T cell apoptosis (Hossain et al., 1998). In L-MAT cells, apoptosis does not depend upon stimulation of aryl hydrocarbon receptors (AhR), because they do not express the receptor. Furthermore, gene transcription and *de novo* protein synthesis are not required for TCDD-induced apoptosis (Ahmed et al., 2005). In a previous report, we showed that treatment of L-MAT cells with TCDD decreased amounts of Bcl-2 protein, which is involved in mitochondrial pathway of apoptosis, and that PKCθ activity was involved in the TCDD-induced apoptosis of L-MAT cells (Ahmed et al., 2005). However, the precise mechanism of the signal transduction pathway involved in TCDD-mediated apoptosis of L-MAT cells is not clear.

The novel protein kinase C (PKC) isoform, PKC-theta (θ), and the proto-oncogene Vav1 are involved in various signal transduction pathways in T cells (Tybulewicz, 2005; Barouch-Bentov and Altman, 2006). Vav1 is a GDP/GTP exchange factor (GEF) for Rho-like small GTPase. It is also a scaffold protein that is able to bind various other proteins by means of its calponin homology domain, SH2 and SH3 domains (Barouch-Bentov and Altman, 2006). Activation of Vav1 and PKCθ usually occurs during T cell receptor (TCR)-mediated T cell activation. Although two proteins have been studied extensively, the roles of Vav1 and PKCθ in the process of negative selection of thymocyte have not been described. A work of Kong et al. has suggested that the activation of Vav1 and its interaction with PKCθ are involved in peptide-specific apoptosis of thymocytes (Kong et al., 1998). Furthermore, actin reorganization, which is regulated by Vav1, has been found to be required for antigen receptor-mediated selection and peptide-specific apoptosis in thymocytes (Kong et al., 1998). It has also been reported that a Ca^{2+}-independent isoform of PKC, PKCθ, is involved in an early stage of CD3/CD28-mediated induction of thymocyte apoptosis (Asada et al., 2000). Therefore, it is possible that Vav1, together with PKCθ regulates negative and/or positive selection of thymocytes.

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In a recent study, it was shown that TCDD enhanced the negative selection of T cells in the thymus of the HY-TCR Tg male-mice (Fisher et al., 2005). Treatment with TCDD resulted in an increased level of phosphorylation of extracellularly regulated kinase and expression of lymphocyte-specific protein tyrosine kinase in their thymocytes (Fisher et al., 2005).

Tannheimer et al. have demonstrated that TCDD was found to significantly increase the amount of phosphoinositide-3 kinase (PI3K) activity, as assessed by γ-^{32}P phosphorylation of the substrate phosphatidylinositol, using human mammary epithelial cells (MCF10A) (Tannheimer et al., 1998). Products of PI3K are phosphatidylinositol (4,5)-diphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Vav1 possesses pleckstrin homology (PH) domain, which may regulate guanine exchange factor (GEF) activity following binding to the phospholipids, PIP2 and PIP3 (Tybulewicz, 2005).

These findings prompted us to investigate whether the phosphorylation status of Vav1, which mediates PKCθ translocation to the membrane, is a determinant of TCDD-induced apoptosis, using our L-MAT. To this end, we took advantage of the lack of the AhR in L-MAT cells to examine the AhR-independent signal transduction pathway in TCDD-induced apoptosis.

2. Materials and methods

2.1. Cell culture and reagents

L-MAT cells were grown in RPMI 1640 (MP Biomedicals Inc., Solon, OH) containing 5% FBS, 100 IU/ml penicillin and 0.1% (v/v) streptomycin at 37 °C, in 95% air, 5% CO_2. Antibodies against human PKCθ (goat polyclonal, sc-1875) and normal rabbit immunoglobulin G (IgG) (sc-3888) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Another Human PKCθ antibody (mouse IgG_{2a}, Cat. No. 610089) and antibody against phosphotyrosine (PY-20, mouse IgG2b, Cat No. 610000) were obtained from BD Transduction Laboratories (BD Biosciences, San Jose, CA).

Antibodies against human Vav1 and phosphotyrosine (4G10) were purchased from Upstate Inc. (Charlottesville, VA). The antibody against IgG was either a horseradish peroxidase-conjugated rabbit IgG against goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), or a mouse antibody against rabbit IgG (Santa Cruz Biotechnology). Tyrosine kinase inhibitors, genistein, herbimycin A and PI3K inhibitor, wortmannin were purchased from Waco Pure Chemical Industrial Ltd. (Osaka, Japan). The tyrphostin inhibitors (A1, A25, B44, B48, B56, B50, B42 and B46) were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA).

2.2. Cell lysis, immunoprecipitation and Western blot analysis

Cells were lysed as described previously (Ahmed et al., 2005). For the detection of the interaction between PKCθ and Vav1 by immunoprecipitation, L-MAT cells were incubated for 1–2 h at 37 °C in 95% air and 5% CO_2 at a density of 2.0×10^6 cells/ml in serum-free RPMI 1640 medium either in the presence of TCDD or in an equal volume of solvent DMSO. Upon completion of the incubation, the cells were lysed in Buffer S1 (10 mM HEPES/KOH, pH 7.4, 38 mM NaCl, 1 mM PMSF, 0.2 U/ml aprotinin, 50 μg/ml leupeptin, 25 mM NaF, 1 mM sodium orthovanadate), subjected to one freeze thaw cycle and then disrupted using a Dounce homogenizer (Wheaton Science Products, Millville, NJ). The cell nuclei were removed by centrifugation at $1190 \times g$ for 10 min at 4 °C. Approximately 1 mg of protein was incubated for 2 h at 4 °C with antibodies against human PKCθ or against human Vav1 and then immunoprecipitated overnight at 4 °C with Protein A Sepharose or Protein G Sepharose (Amersham Biosciences, London, UK). The trapped proteins were separated by SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membrane (Hybond-P, Amersham Biosciences). The membrane was blocked with 5% (v/v) skimmed milk in 1× TTBS (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% (w/v) Tween 20) for 1 h at room temperature on a shaker, and then incubated with an antibody against for PKCθ or Vav1 2 h at 4 °C. The membrane was washed five times (5 min each wash) with 1× TTBS at room temperature, and then was incubated with an antibody against IgG for 2 h at room temperature. The antibody against IgG was a horseradish peroxidase (HRP)-conjugated antibody against IgG. Finally, the signal was visualized by ECL™ (GE Healthcare).

2.3. Assay of apoptosis by determination of acetyl-Asp-Glu-Val-Asp/7-amino-4-methylcoumarin (AcDEVD-AMC) cleavage

We used caspase-3 activation to detect apoptosis in L-MAT cells as described previously (Kikuchi et al., 2001). L-MAT cells growing exponentially in RPMI 1640 medium containing 5% (v/v) FBS were collected and washed twice with phosphate-buffered saline (PBS). The cells were then incubated at a density of 6×10^6 cells/well in serum-free RPMI 1640 medium for 1–2 h at 37 °C in 95% air and 5% CO_2 for sensitization before treatment with various inhibitors for PTK or PI3K. The cells were incubated in serum-free RPMI 1640 medium either in the presence of PTK inhibitor, herbimycin A (100 nM–5 μM), genistein (1–50 μM), tyrphostin (50 μM) or PI3K inhibitor, wortmannin (1–50 nM), LY249002 (1–50 μM) or an equal volume of DMSO. The cells were treated with 20 nM TCDD for 3 h. Following this, 75 μl of the medium was removed and frozen at −80 °C for 30 min, then thawed on ice for 30 min. Next, 50 μl of 100 mM Hepes (pH 7.25), 20% (w/v) sucrose, 5 mM dithiothreitol, 0.1% (v/v) CHAPS, and 10^{-6}% Nonidet P-40 (NP-40) containing 100 mM acetyl-asp-Glu-Val-Asp/7-amino-4-methylcoumarin (AcDEVD-AMC) (Calbiochem, San Diego, CA) was added to each well containing the lysed cells. Substrate cleavage was monitored against time at 37 °C using a Fluoroscan Ascent microplate reader (Labsystems, Helsinki, Finland). The amount of 7-amino-4-methylcoumarin (AMC) in the reaction mixture was calculated from the emission at 460 nm (excitation at 355 nm), using a standard curve for AMC. Fluorescence units were converted to pmoles of AMC with the aid of a standard curve that had been generated using free AMC as described (Kikuchi et al., 2001).

2.4. Gel filtration

Analysis of the cellular multi-protein complexes was performed on a Superose 6 column (1 × 30 cm) (Villalba et al., 2002). L-MAT cells were sensitized as described above and were incubated for 1 h at 37 °C in 95% air and 5% CO_2 with 20 nM TCDD or left untreated. Total cell extracts from 3×10^8 L-MAT cells in 100 μl of octylglucoside lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 5 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1% [v/v] octylglucoside and 10% glycerol) were analyzed at a flow rate of 0.5 ml/min. Fractions of 500 μl were collected and aliquots from the respective fractions were analyzed by Western blot analysis as described above for the presence of Vav1 and PKCθ.

2.5. Subcellular fractionation

L-MAT cells (2×10^7 cells) were treated with 20 nM TCDD or left untreated. The cells were collected by centrifugation and washed in PBS. The cell pellet was resuspended in 1 ml of buffer S1 and disrupted with a Dounce homogenizer. Nuclei were removed by centrifugation and membrane/cytoskeletal fractions (particulate fractions) were obtained after centrifugation of cytosolic extracts for 30 min at $10{,}000 \times g$ at 4 °C. The supernatant of this step was the cytosolic fraction. The particulate pellets were washed twice with buffer S1 and resuspended in NP-40 buffer containing 10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 5 mM PMSF, 2 mM EDTA, 200 μM sodium orthovanadate, 10 mg/ml aprotinin and leupeptin. Solubilization was performed by pipetting and vigorous vortexing for 1 min. The protein concentrations of samples were then estimated by a standard procedure using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.6. Image analysis

Image analysis was performed on a Macintosh computer using the public domain NIH Image program developed at the US National Institutes of Health (http://rsb.info.nih.gov/nih-image/).

3. Results

3.1. TCDD-induced translocation of Vav1 and PKCθ to the membrane fraction in L-MAT cells

L-MAT cells that had been treated with 20 nM TCDD or with vehicle (DMSO) were separated into particulate and cytosol fractions. The protein samples from the particulate fraction were subjected to SDS-PAGE and detected using antibody against PKCθ or Vav1 (Fig. 1). The signal intensity of PKCθ and Vav1 in particulate fraction increased after 1 min of treatment with TCDD (Fig. 1A and B). The increase of Vav1 in particulate fraction (membrane fraction) was significant at 20 min after TCDD treatment as shown in Fig. 1B. L-MAT is a lymphoblastic T cell line, so that CD3 was used as an internal standard of membrane protein to normalize the amount of Vav1 in this experiment. The increase pattern of PKCθ in the particulate fraction [Figs. 1A and 7 in a previous report (Ahmed et al., 2005)] was almost the same as that of Vav1. In contrast, the amounts of PKCθ and Vav1 in the cytosol fraction decreased. These results show that TCDD treatment induced the translocation of PKCθ and Vav1 from the cytosol to the cell membrane.