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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-mediated membrane translocation of c-Src protein kinase in liver WB-F344 cells

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Abstract 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a widespread environmental contaminant and the most potent agonist of the aryl hydrocarbon receptor (AhR). Persistent activation of the AhR has been shown to be responsible for most TCDD-mediated toxic responses, including liver tumour promotion. However, the mechanisms responsible for these complex toxic reactions are still unknown. TCDD (1 nM) has previously been shown to reduce DNA synthesis of primary hepatocyte cultures and cell contact inhibition of confluent WB-F344 cells. The latter model was used to study early effects of TCDD on protein tyrosine kinase c-Src in confluent WB-F344 cells. It was found that TCDD decreased cytosolic c-Src (protein and tyrosine kinase activity) after 20–60 min, and increased c-Src in the membrane fraction. Membrane translocation of c-Src occurred in the presence of 100 μM cycloheximide and was observed after treatment with 1 nM TCDD or 50 nM 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin. Under these conditions epidermal growth factor (EGF) receptor tyrosine phosphorylation was also studied. As expected, its phosphorylation was low in confluent cells but was significantly enhanced by TCDD treatment. Pretreatment of WB-F344 cells for 1 h with 1 μM geldanamycin, which disrupts cytosolic heat shock protein Hsp90 complexes with AhR and Src, abolished TCDD-mediated Src translocation and TCDD-mediated reduction of cell contact inhibition. The WB-F344 cell model appears to be very useful to study TCDD effects on protein tyrosine kinases and of signaling pathways responsible for modulation of the cell cycle by TCDD.

Key words  Aromatic hydrocarbon receptor · Dioxins · c-Src protein kinase · EGF receptor · WB-F344 cells

Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and related compounds are widespread environmental contaminants and potent inducers of xenobiotic metabolizing enzymes. These compounds in a species- and cell-specific manner trigger various toxicities, which led to public concern, such as wasting syndrome, immunosuppression, reproductive toxicity and liver tumour promotion (Poland and Knutson 1982; Safe 1986; Bock 1993; Okey et al. 1994. Hankinson 1995). Studies with aryl hydrocarbon receptor (AhR) knock-out mice indicate that most toxic responses are mediated via the AhR (Fernandez-Salguero et al. 1996; Mimura et al. 1997). However, mechanisms responsible for these toxicities are still unknown. The AhR has been characterized as a ligand-activated transcription factor and member of the bHLH/PAS (basic helix-loop-helix/Arnt-sim) family (Burbach et al. 1992; Ema et al. 1992). AhR is normally present in the cytosol in a multiprotein complex with the heat shock protein Hsp90 (Chen and Perdew 1994). Hsp90 acts as a chaperone, and prevents transcriptional activation of the AhR but keeps the receptor in a conformation recognizing its ligands. After ligand binding Hsp90 is released, the AhR is translocated to the nucleus and binds to its partner Ah receptor nuclear translocator (Arnt). The heterodimer binds to GCGTG DNA sequences, the core binding motif of the xenobiotic responsive element (XRE; Whitlock et al. 1996). Binding of AhR/Arnt leads to changes of the chromatin structure allowing transactivation of AhR-controlled genes, such as CYP1A1. In addition to this AhR/Arnt transcriptional pathway, other signalling modes have also been suggested. For example, accumulating evidence suggests that the liganded AhR can affect cellular functions by activating cellular kinases, such as the protein kinase c-Src (Matsumura 1994).
TCDD-mediated c-Src activation appears to be AhR-dependent but Arnt-independent (Blankenship and Matsumura 1997a). c-Src has been shown in vivo and in vitro to be responsible for some toxicities of TCDD (Bombick et al. 1988; Matsumura et al. 1997). In the present study the following aspects were investigated. (1) Previous findings (Münzel et al. 1996) on TCDD-mediated reduction of contact inhibition in WB-F344 cells were substantiated. (2) The WB-F344 cell model was selected to study effects of TCDD on c-Src protein kinase and on its substrate, the EGF receptor, as early regulators of many cellular functions including the cell cycle.

Materials and methods

Chemicals

Reagents were obtained from the following sources: TCDD and 1,2,3,4,6,7,8 heptachlorodibenzo-p-dioxin (HpCDD) from Ökometric (Bayreuth, Germany); [3H]thymidine (dT) and [γ-32P]ATP from Amersham Buchler (Braunschweig, Germany). Antibodies against c-Src and against EGF receptor for immunoblotting, protein A/G plus agarose and the anti-phosphotyrosine antibodies PY20 were obtained from Santa Cruz, Inc. (Calif., USA). Non-inhibiting c-Src antibodies were obtained from Calbiochem-Novabiochem (Cambridge, Mass., USA). c-Src substrate peptide, RR-Src (Conventional one-letter code, RRLIEDAEYAARG) for the c-Src-type tyrosine kinase assay, was purchased from Sigma (St. Louis, Mo., USA). Alkaline phosphatase-conjugated antibodies and CDP-Star™ were obtained from Tropix (Perkin Elmer Applied Biosystems; Bedford, Mass., USA).

Cell culture and treatment

WB-F344 cells (passages 15–25), kindly provided by Dr J. Grisham (Coleman et al. 1997; Tsao et al. 1984), were seeded at a density of 250 000 cells on 90-mm tissue culture dishes (Falcon) in Richer’s IMEMZO medium supplemented with 20 mM HEPES, 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and insulin (4 μg/ml). Cells were incubated in a humidified atmosphere containing 5% (v/v) CO2 in air at 37 °C and the medium was renewed every other day. When the cells reached confluency at a cell density of ca. 1.5 × 106 cells per plate, the medium was changed and 24 h later cells were treated with TCDD or HpCDD, dissolved in dimethylsulphoxide (DMSO). Controls were treated with DMSO alone.

c-Src immune complex kinase assay

Membrane and cytosolic fractions were prepared as described (Oude Weernink et al. 1994). Briefly, confluent cells were washed once with ice-cold phosphate-buffered saline (PBS) and harvested with 700 μl homogenization buffer-1 [50 mM HEPES, pH 7.5, 1.5 mM magnesium acetate, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(aminomethyl) ether) tetraacetatic acid, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), aprotinin (5 μg/ml) and 100 μM vanadate]. Samples were lysed by ultrasonication (1 × 5 sec). After centrifugation at 800 g for 10 min, supernatants were again centrifuged at 48 000 g for 60 min to collect the cytosolic fraction. Pellets were extracted for 60 min with 700 μl homogenization buffer-1 supplemented with 1% (v/v) Triton X-100. Supernatants after centrifugation at 48 000 g for 60 min were used as solubilized membrane fraction.

c-Src kinase activity was determined as described by Steward (1993). Briefly, cytosolic fractions (0.5 mg of protein) and Triton X-100-solubilized membranes (0.5 mg protein) were incubated with 4 μg anti-Src antibodies and 20 μl protein A/G plus agarose for 16–24 h at 4 °C under constant shaking. Precipitates were washed twice with homogenization buffer-1 and twice with Src kinase buffer [50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.3 mg/ml bovine serum albumin (BSA) 3 mM DTT, 0.1% (v/v) Triton X-100, 100 μM vanadate]. Src phosphorylation of RR-Src was determined in 30 μl Src kinase buffer supplemented with 10 mM MgCl2 and substrate (0.25 mg/ml). The reaction was started by the addition of 1.2 mM [γ-32P]ATP (5000 Ci/mmol). After 30 min at 30 °C reactions were stopped with 80 μl 10% (v/v) phosphoric acid. Samples were spotted onto phosphocellulose (P81 Whatman, UK), and after washing four times with 0.5% (v/v) phosphoric acid and once with acetone radioactivity was counted.

c-Src immunoblotting

Immunoprecipitation was carried out as described above for the Src-immune complex kinase assay, but with 20 μg anti-Src antibody and 20 μl protein A/G plus agarose. After incubation for 16–24 h at 4 °C under constant shaking, precipitates were washed three times with homogenization buffer and denatured in Laemmli buffer at 95 °C for 5 min (Laemmli 1970). Proteins were separated by 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrothermally to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Mass., USA). The membrane was blocked overnight with a solution of 0.66% (w/v) I-Block™ in PBS and incubated with 0.2 μg/ml anti-c-Src antibody for 1 h in PBS. Immunocomplexes were detected by incubation with alkaline phosphatase-conjugated immunoglobulins for 45 min at room temperature and CDP-Star as chemoluminescence substrate. Imaging was performed with a CCD camera system (Raytest, Straubenhardt, Germany).

Immunoblotting of EGF receptor tyrosine phosphorylation

Logarithmically growing cultures contained ca. 1.5 × 106 cells/90 mm plate and confluent cultures ca. 1.5 × 107 cells/90 mm plate. Prior to TCDD treatment the medium was changed to serum-free Richter’s IMEMZO medium supplemented with insulin (4 μg/ml). After 4 h cells were treated with 10–9 M TCDD for 1 h at 37 °C. EGF stimulation was carried out according to Sörry and Östman (1996). The medium was changed to ice-cold serum-free medium containing EGF (50 ng/ml) or EGF + 10−7 M TCDD and incubated at 37 °C for 1 h. Subsequently the cells were incubated for 5 min at 37 °C.

After EGF stimulation, cells were immediately washed twice with ice-cold PBS and scraped off into homogenization buffer 2 [Bohmer et al. 1995; 0.15 M NaCl, 50 mM HEPES, pH 7.4, 10% (v/v) glycerol, 1.5 mM MgCl2, 2 mM EGTA, 40 mM NaF, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 50 μM phenylarsine oxide, 0.5 mM benzamidine, 0.25 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 2 μg/ml pepstatin]. The cell suspension was centrifuged for 10 min at 1000 g and the pellet lysed in homogenization buffer 2 containing 1% (v/v) Triton X-100 for 30 min at 4 °C. After centrifugation for 60 min at 48 000 g the supernatant was removed and stored at −70 °C. Protein was determined according to Bradford (1976). Lysate proteins were separated by discontinuous SDS-PAGE (2 μg protein per lane; Laemmli 1970) and EGF receptor protein was detected by immunoblotting. Dilution of the primary polyclonal rabbit anti-EGF receptor antibody 1005 was 1:10000.

Equal amounts of EGF receptor protein were separated by a second SDS-PAGE and blotted as described above. The membrane was incubated in a solution of 3% (w/v) bovine serum albumin in TRIS-buffered saline, 0.1% (v/v) Tween 20 to block nonspecific protein-binding sites. Immunodetection was achieved by incubation of the membrane with anti-phosphotyrosine antibody PY20.