Toxicology 309 (2013) 107–116

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

TCDD induces the expression of insulin-like growth factor binding protein 4 in 5L rat hepatoma cells: A cautionary tale of the use of this cell line in studies on dioxin toxicity

Stefanie Brandner[a], Carola Eberhagen[a], Josef Lichtmannegger[a], Ludwig Hieber[b], Ulrich Andrae[a,*]

[a] Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Molecular Toxicology and Pharmacology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

[b] Helmholtz Zentrum München, German Research Center for Environmental Health, Department of Radiation Sciences, Research Unit of Radiation Cytogenetics, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Received 8 April 2013
Received in revised form 18 April 2013
Accepted 19 April 2013
Available online 29 April 2013

Keywords:
Dioxin
5L hepatoma cells
IGF-I
IGFBP-4
AKT

ABSTRACT

Previous quantitative proteomic studies on the actions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in 5L rat hepatoma cells, a cell model frequently used for investigating the mechanisms of TCDD toxicity, had indicated that dioxin exposure reduced the abundance of numerous proteins which are regulated at the level of protein synthesis initiation. In the present study, we have analysed the mechanism mediating this inhibition. TCDD treatment of the cells largely prevented the activation of eukaryotic translation initiation factor 4E-binding protein 1, a regulator of translation initiation and substrate of the mammalian target of rapamycin (mTOR). By "working upwards" from mTOR, we observed that TCDD inhibited endogenous and IGF-I-induced AKT and ERK activation by interfering with tyrosine phosphorylation of insulin receptor substrate 1. This inhibition was mediated by a TCDD-induced secreted factor which was identified as insulin-like growth factor binding protein 4 (IGFBP-4). The induction of IGFBP-4 protein was dependent on a functional aryl hydrocarbon receptor and was preceded by a rapid increase in the level of IGFBP-4 mRNA indicating that IGFBP-4 is a previously unknown transcriptional target of TCDD in 5L cells. IGFBP-4 was not induced by TCDD in the parental cell line of 5L cells, Fao, and in various closely related rat hepatoma cell lines as well as in other unrelated cell types. Analysis of 5L cell chromosomes by multicolour spectral karyotyping (SKY) revealed that the cells carry several hitherto uncharacterised chromosomal translocations. The observations suggest that in 5L cells the *Igfbp-4* gene may have got under the control of a promoter containing dioxin responsive element(s) leading to the induction of IGFBP-4 by TCDD. These findings emphasise a particular caution when interpreting and extrapolating results on the action mechanisms of TCDD obtained in studies using 5L cells as a model system.

1. Introduction

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic chemicals identified to date. In experimental animals, TCDD causes a plethora of adverse effects, such as cardiovascular disease, developmental defects, porphyria, diabetes, hormonal disturbances, and cancer. Studies on humans have mostly focused on the induction of cancer, endocrine and reproductive toxicity and cardiovascular diseases (White and Birnbaum, 2009). In spite of decades of painstaking research, most of the molecular mechanisms mediating dioxin toxicity are still poorly understood. Clarification of the action mechanisms of TCDD is an important task as it is deemed essential for assessing the risk associated with dioxin exposure of humans.

We have recently characterised the proteome changes elicited by TCDD in 5L rat hepatoma cells, a cell model frequently used for studying the mechanistic aspects of dioxin toxicity (Sarioglu et al.,

Abbreviations: AhR, aryl hydrocarbon receptor; AKT1S1, proline-rich AKT1 substrate 1; CYP1A1, cytochrome P450 1A1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ERK, extracellular signal-regulated protein kinase; GSK3, glycogen synthase kinase 3; IGF-I, insulin-like growth factor I; IGFBP, insulin-like growth factor binding protein; IGF1R, insulin-like growth factor receptor type 1; IRS-1, insulin receptor substrate 1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; RPS6, 40S ribosomal protein S6; TSC, tuberous sclerosis complex; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element.

* Corresponding author. Tel.: +49 089 3187 2221; fax: +49 089 3187 3449.
E-mail addresses: brandner@helmholtz-muenchen.de (S. Brandner), carola.eberhagen@helmholtz-muenchen.de (C. Eberhagen), lichti@helmholtz-muenchen.de (J. Lichtmannegger), ludwig.hieber@helmholtz-muenchen.de (L. Hieber), andrae@helmholtz-muenchen.de (U. Andrae).

0300-483X/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2013.04.014

2006, 2008). In these analyses, a large number of protein species were identified whose abundance was changed due to TCDD exposure. The dioxin not only increased the expression of known members of the aryl hydrocarbon receptor (AhR) gene battery but, at the same time, caused a striking down-regulation of the amounts of many other proteins. Down-regulation of protein levels can be caused by several mechanisms, such as inhibition of transcription, inhibition of translation or accelerated protein degradation. With respect to these possibilities, an intriguing outcome of our studies was the observation of a coordinate down-regulation of many proteins whose expression is known to be regulated at the level of translation initiation. This regulation involves growth factor-mediated control of polysome association of ribosomal protein encoding mRNAs, a process mediated by terminal oligopyrimidine (TOP) tracts in the 5′ untranslated region of these constitutively expressed mRNAs (Meyuhas, 2000). The TOP mRNA-encoded proteins observed as down-regulated after TCDD exposure included ~20 ribosomal proteins and several other proteins involved in either ribosome biogenesis or translation control.

Since initiation of translation is controlled by several signalling pathways regulated by, *e.g.*, the supply of amino acids, glucose or oxygen as well as the availability of growth factors (Ma and Blenis, 2009) it appeared likely that in 5L cells TCDD interfered with a pathway involved in the stimulation of protein synthesis. In the present study, we set out to clarify the mechanism underlying the inhibition effected by TCDD in 5L cells. The identification of the alterations in signal transduction by TCDD was expected to yield new insight into the cross talk of AhR signalling with other signalling pathways and potential mechanisms of dioxin toxicity.

2. Materials and methods

2.1. Reagents

TCDD (purity >99%) in DMSO was obtained from Ökometric (Bayreuth, Germany). IGF-I (human, recombinant), LONG® R^3 IGF-I (human, recombinant) and IGF-II (human, recombinant) were from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Cells

The 5L cell line is a variant of the Fao line (Moore and Weiss, 1982), a descendant of the cell line H-4-II-E-C3 established by Pitot et al. (1964) from the Reuber H35 rat hepatoma (Reuber, 1961). BP8 cells represent a clone of 5L cells which lacks the aryl hydrocarbon receptor (AhR) (Göttlicher et al., 1990; Weiss et al., 1996). Cells were grown as monolayers in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin ("complete RPMI 1640 medium") at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Primary cultures of rat hepatocytes derived from male Sprague Dawley rats (~350 g body weight) were purchased from PRIMACYT Cell Culture Technology, Schwerin, Germany (6-well plates containing ~1.2×10^6 cells/well) and cultured in Hepatocyte Growth Medium (Primacyt, 2.5 ml/well) for maximally 32 h after receiving the cells, *i.e.* ~50 h after hepatocyte isolation.

2.3. Treatment of the cells with TCDD for Western blot analyses

Cell lines were seeded at a density of 1.5×10^6 cells/10-cm dish in complete RPMI medium. One day later, the medium was replaced by 5 ml of fresh complete RPMI medium, and two days after seeding, 1 nM TCCD dissolved in DMSO or solvent alone was added for the times indicated. The DMSO concentration in the medium was 0.1%. When indicated, IGF-I (30 or 50 ng/ml) was added to the cells 30 min before the end of the incubation.

2.4. Western blot analysis

For Western blot analysis of protein abundance and phosphorylation, cells were lysed in ice-cold Cell Extraction (CE) Buffer (Invitrogen, Darmstadt, Germany) supplemented with Complete Protease Inhibitor Cocktail Tablets and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Twenty micrograms of total protein were resolved on SDS polyacrylamide gels of appropriate density (XCell SureLock Mini-Cell, Invitrogen) and blotted onto nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Bands were visualised using specific primary antibodies, horseradish peroxidase-labelled secondary antibodies and an ECL detection kit (LumiGLO, Cell Signaling Technology, Beverly, USA). Actin served as a loading control. Band intensities were quantitated using the ImageMaster 1D software (GE Healthcare, Freiburg, Germany). A list of the primary antibodies used is provided as Supplemental Table 1.

2.5. Ligand blot analysis

For the detection of IGF-I binding activity within the cells or secreted into the medium by the cells, ligand blots were prepared. Cells were grown and treated with DMSO or TCCD (1 nM) for 24 h in RPMI 1640 medium as described above except that serum was omitted from the medium. Subsequently, samples corresponding to 2.5 ml of culture medium were removed, supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche), centrifuged (5 min, 5450 *g* at room temperature) and concentrated using Amicon® Ultra-4 Centrifugal Filter Units (Millipore). The concentrate (~100 μl) was diluted with 1/3 volume of 4× Laemmli buffer containing 0.5% SDS but no mercaptoethanol. The cell monolayers were thoroughly washed with ice-cold PBS, lysed in ice-cold CE buffer and diluted with Laemmli buffer. Samples were electrophoresed on 12% SDS PAGE gels using Dual Color molecular weight standards (BioRad) and electroblotted as described above. The blots were blocked with 3% BSA and incubated with 45×10^3 Bq of human recombinant ^{125}I-IGF-I (PerkinElmer, Rodgau, Germany) in 5 ml Tris-bufferd saline (TBS) under continuous agitation at room temperature for 3 h. Subsequently, the blots were washed 3× for 10 min each with TBS containing 0.1 % Tween 20 and twice with TBS. The bands of the molecular weight standards were traced with fluorescent ink and the blots exposed to Amersham Hyperfilm MP (GE Healthcare) at −80 °C for 3 days. Protein bands with bound ^{125}I-IGF-I were visualised using ECL detection.

2.6. Assay of total and tyrosine-phosphorylated IRS-1

The effects of TCDD and IGF-I on the levels of total insulin receptor substrate 1 (IRS-1) and phospho-IRS-1 (panTyr) in 5L cells were determined using PathScan® Sandwich ELISA Kits (Cell Signaling Technology) according to the manufacturer's protocol.

2.7. Real-time quantitative PCR

The expression of IGFBP-4 at the mRNA level was quantitated by real-time quantitative PCR with a LightCycler instrument (Roche Applied Science) using the following primers which were selected using the Primer3Plus programme: forward, 5′-TTGGAGTGGGCTATACTCAGACT-3′; reverse, 5′-GCATTAAAGCTCTTCCCGTCT-3′. Expression levels were normalised to that of the mRNA transcribed from the house-keeping gene encoding porphobilinogen deaminase (Bohle et al., 2000). Quantitation was done using the mathematical model described by Pfaffl (2001).

2.8. siRNA-mediated knockdown of IGFBP-4

A set of three different siRNAs specifically targeting different regions of the rat IGFBP-4 mRNA and contained in the Stealth RNAi™ siRNA Select RNAi (Invitrogen) was initially used in experiments aimed at identifying the most effective of the set. 1.5×10^6 5L cells in 1.84 ml RPMI 1640 medium without antibiotics were added to a mixture of 30 μl Lipofectamine™ RNAiMAX reagent (Invitrogen) and 30 μl (600 pmol) of the respective siRNA in 600 μl Opti-MEM® medium (Invitrogen), preincubated for 20 min at room temperature in a 5-cm cell culture dish and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Nontargeting siRNAs with the corresponding GC content recommended by the manufacturer were used as negative controls. One day later, the cells were treated with DMSO or 1 nM TCDD for 24 h. Subsequently, the medium was removed, centrifuged, concentrated, diluted with complete Laemmli buffer, electrophoresed and analysed by Western blotting as described above for the ligand blot analysis. Lysates of the cell monolayers were analysed in parallel. The siRNA designated RSS337648 with the target sequence 5′-GCCAAAGTGAGAGATCGGAGCAAGA-3′ turned out to be particularly effective in inhibiting IGFBP-4 expression and was subsequently used in the knock-down experiments together with the corresponding negative control (Stealth RNAi™ siRNA Negative Control Hi GC, no. 12935-400).

2.9. Growth of 5L and other hepatoma cells

The growth behaviour of 5L cells and other hepatoma cell lines in the presence of TCDD and IGFs was determined by monitoring the DNA content of the cultures using the method of Labarca and Paigen (1980) with minor modifications. In brief, 10^4 cells/well were seeded in 96-well microtiter plates containing 100 μl complete RPMI 1640 medium/well and grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Subsequently, the medium was removed, 100 μl of fresh complete RPMI medium containing the test agents were added and the incubation was continued for up to 48 h. Following removal of the medium and cell lysis, bisbenzimide was added and the fluorescence read in a microplate reader.