FEBS Letters 462 (1999) 430–434 FEBS 23005

Expression of E16/CD98LC/hLAT1 is responsive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Received 20 September 1999; received in revised form 27 October 1999

Edited by Masayuki Miyasaka

Abstract We employed cDNA representational difference analysis to identify new genes that are upregulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in a human hepatoblastoma cell line HepG2. We isolated several TCDD-responsive cDNAs. Sequence analysis revealed that one of them encodes E16/CD98LC/hLAT1, an integral membrane protein involved in multiple cellular functions including cellular transport of L-type amino acids. Northern blot analysis confirmed the TCDD-dependent upregulation of the mRNA. Induction of E16/CD98LC/hLAT1 mRNA by TCDD did not require de novo protein synthesis as revealed by the experiment using cycloheximide. Consistent with the changes at mRNA level, the transport of ^{3}H-leucine into HepG2 cells was significantly increased by TCDD treatment. These findings provide a novel aspect of biological effects of TCDD on human hepatocytes.

Key words: cDNA representational difference analysis; Dioxin; Amino acid transporter E16; Gene regulation; Hepatocyte

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons are potential environmental contaminants that have a pleiotropic adverse biological effect on living animals. Exposure to TCDD leads to tumor promotion, teratogenesis, immunosuppression, hepatotoxicity, epithelial dysplasia, reproductive toxicity and thymic atrophy [1,2]. Most of the effects of TCDD are believed to be mediated by aryl hydrocarbon receptor (AhR) that is a ligand-activated transcription factor belonging to basic helix-loop-helix (bHLH)/PAS protein family [3]. In unstimulated cells, AhR is present in the cytoplasm in association with Hsp90. Upon TCDD binding, liganded AhR translocates into nucleus and heterodimerizes with another bHLH/PAS protein, aryl hydrocarbon nuclear translocator (ARNT). Subsequently, AhR/ARNT complex binds to the specific DNA sequence (xenobiotic response element (XRE)) in the enhancer region of target genes, and regulates their transcription. Thus, initial AhR-mediated gene regulation does not require de novo protein synthesis.

Most of the TCDD-responsive genes identified to date code for enzymes associated with various metabolic pathways within the liver, for instance, cytochrome P450 CYP1A1, CYP1B1, aldehyde dehydrogenase, NADPH-quinone-oxidoreductase, glutathione-*S*-transferase (GST) Ya, UDP-glucuronosyltransferase 1A1 and ecto-ATPase [4–8]. However, considering the divergent biological effects of TCDD like teratogenesis and tumor promotion, genes associated with cell division, cell cycle regulation or cell adhesion are likely to be regulated by TCDD. We, therefore, endeavored to discover other TCDD-responsive genes.

We employed cDNA representational difference analysis (cDNA RDA) to isolate TCDD-responsive genes in human hepatoblastoma cell line HepG2. cDNA RDA is a polymerase chain reaction (PCR)-coupled subtractive enrichment procedure for the isolation of differentially expressed cDNAs among two different cDNA populations (called Tester and Driver) [9,10]. Usually, Driver cDNA population is subtracted from Tester cDNA population by hybridization. Thus, cDNAs present only in the Tester are enriched and amplified by PCR. In order to identify genes that are directly upregulated by TCDD, the Tester cDNA was prepared from the cells that were pretreated with protein synthesis inhibitor cycloheximide (CHX), followed by TCDD, and the Driver cDNA was prepared from the cells treated with CHX alone. We cloned several TCDD-responsive cDNAs. Sequence analysis revealed that one of them encodes E16/CD98LC/hLAT1 [11–13].

2. Materials and methods

2.1. Cell culture

Human hepatoblastoma cell line HepG2 (ATCC HB-8065) was cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin G and 50 μg/ml of streptomycin at 37°C in 5% CO_2 and 100% relative humidity. For cDNA RDA, the experimental cells were treated with 10 μg/ml CHX (Wako, Osaka, Japan) for 30 min followed by 1 nM TCDD (dissolved in toluene; SUPELCO, Supelco Park, Bellefonte, PA, USA) for 4 h and the control cells were treated with CHX and vehicle.

2.2. Extraction of RNA and cDNA synthesis

Total RNA was extracted according to the method of Chomczynski and Sacchi [14]. Total RNA (600 μg) was used to purify poly (A^+) mRNA by Oligotex mRNA Isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Double-stranded cDNA was synthesized from 2.3 μg of poly (A^+) mRNA using a cDNA synthesis system (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. cDNA was finally eluted in 20 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.3. cDNA representational difference analysis (cDNA RDA)

cDNA RDA was carried out essentially according to the protocol described by Hubank and Schatz [10]. Control and TCDD-treated

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PII: S0014-5793(99)01574-4

groups were regarded as Driver and Tester, respectively. The sequences of the adapter oligonucleotides are shown in Table 1. Briefly, cDNAs (1 μg each) were digested with *Dpn*II. R-24 and R-12-mer adapters were ligated to the digested cDNAs. Representative amplicons were generated by melting away R-12-mer, filling in the ends and then amplifying by PCR using the adapter-ligated cDNAs as templates and R-24-mer as primer. Representative amplicon (100 μg each) was digested with *Dpn*II and J-24 and J-12-mers were ligated only to the Tester. Subtractive hybridization was carried out using 20 μg of digested Driver amplicon and 0.2 μg J-ligated Tester amplicon at 67°C for 20 h. J-12-mer was melted away from the hybridization mix and the hybridized product was amplified by PCR using J-24-mer as primer. The PCR product was digested with mung bean nuclease (MBN; New England Biolabs, Beverly, MA, USA) to remove single-stranded DNA and linear amplified products and the MBN digested product was amplified with J-24-mer to generate the first difference product (DP1). The product of DP1 was digested with *Dpn*II and N-24 and N-12-mers were ligated to it. Hybridization was carried out with 20 μg of digested Driver amplicon and 25 ng of N-ligated DP1. The second difference product (DP2) was generated using N-24-mer as primer. The product of DP2 was digested with *Dpn*II and J-24 and J-12-mers were ligated to it. Hybridization was carried out with 20 μg of digested Driver amplicon and 0.5 ng of J-ligated DP2. The third difference product (DP3) was generated using J-24-mer as primer and was cloned into pGEM-T vector (Promega, Madison, WI, USA). The cloned fragments were sequenced by ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

2.4. Northern blot analysis

Fifteen micrograms of total RNA from HepG2 cells, cultured in the presence or absence of TCDD, was subjected to Northern blot analysis as described previously [15]. The membrane was hybridized with human E16/CD98LC/hLAT1 cDNA, one of the cDNA fragments cloned by cDNA RDA, labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA, USA). The same membrane was rehybridized with human CYP1A1 and rat glyceryldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. A 288-bp fragment of human CYP1A1 cDNA was cloned by RT-PCR from HepG2 mRNA using primers, sense: 5′-ACT GCT ATC TCC TGG AGC CTC ATG-3′ and antisense: 5′-CTT CTG GTC ATG GTT GAT CTG CCA-3′. The cloning of rat GAPDH was reported previously [15]. Densitometric analysis was carried out by BAS 2000 bioimage analyzing system (Fuji film, Tokyo, Japan). E16/CD98LC/hLAT1 mRNA levels were normalized by GAPDH mRNA levels.

2.5. Analysis of amino acid transport

HepG2 cells (1×10^5) were plated into each well of a 12-well plate and were cultured for 24 h. The cells were treated with or without 1 nM TCDD for 6 h and then incubated in the transport buffer (1×Hanks' balanced salt solution (Life Technologies) with 10 mM HEPES-KOH (pH 7.5)) containing 5 μCi (31.45 nmol)/ml ^{3}H-leucine for 15 or 30 min at 37°C or on ice (the latter for measuring background). The cells were washed with ice-cold transport buffer twice and lysed with 400 μl of 0.5% SDS in 0.2 N NaOH. The lysates were centrifuged at 12 000 rpm for 10 min to precipitate DNA and aliquots of 10 μl of each supernatant were analyzed for radioactivity by a liquid scintillation counter. Duplicate measurements were performed and the background was subtracted for each sample. The experiments were carried out in triplicate for three times.

2.6. Statistical analysis

Statistical analysis was carried out using one-way ANOVA followed by Fisher's protected least significant difference (PLSD) analysis.

3. Results

HepG2 cells were treated with 10 μg/ml CHX for 30 min followed by 1 nM TCDD (Tester) or not (Driver) for 4 h. After synthesis of cDNAs and *Dpn*II digestion, series of three subtractions of Driver cDNA population from the Tester cDNA population and PCR amplifications were carried out.

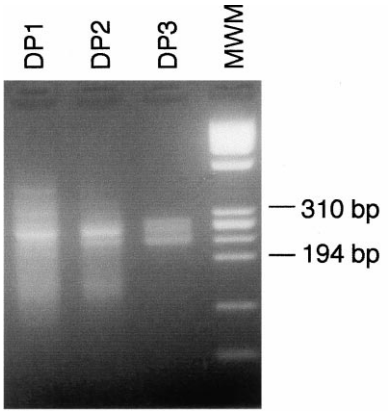

Fig. 1. Representation of difference products of cDNA RDA. PCR products at different steps of cDNA RDA were electrophoresed in a 4% agarose gel. DP1, DP2 and DP3 represent first, second and third difference products, respectively. MWM represents molecular weight marker (φX174/*Hae*III digest). The numbers on the right side indicate the size of the DNA bands in the MWM.

Fig. 1 shows the profile on the agarose gel electrophoresis of the amplified difference products (DP) after each subtraction. There was a gradual decrease in the number of the products from DP1 to DP3. Cloning of cDNAs in DP3 and subsequent sequence analysis showed that most of the clones were independent. BLAST search revealed that the nucleotide sequence of one clone of 178 bp, harboring *Dpn*II sites at both ends, was completely identical to the region from 853 to 1030 bp of E16/CD98LC/hLAT1 cDNA (EMBL/DDBJ accession number AF077866).

To verify the upregulation of E16/CD98LC/hLAT1 mRNA by TCDD, HepG2 cells were treated with 1 nM TCDD for 0, 1, 3, 6 and 12 h and Northern blot analysis was performed using the cloned cDNA fragment as a probe. As shown in Fig. 2A, a single band of approximately 3.8 kb was detected in the absence of TCDD. This size of the band was consistent with the previous report [16], indicating that E16/CD98LC/hLAT1 mRNA is constitutively expressed in HepG2 cells. TCDD treatment resulted in significant increases in the mRNA level, and the level reached its maximum at 6 h after TCDD treatment and returned to the basal level at 12 h. Similar pattern of induction was observed in the expression of the CYP1A1 gene, a well characterized TCDD-responsive gene [4]. CYP1A1 mRNA expression increased maximally at 6 h and returned to the basal level at 12 h. In contrast, GAPDH mRNA levels were not altered by TCDD treatment. Next we studied the dose-effect of TCDD on E16/CD98LC/hLAT1 mRNA level. The cells were exposed to various con-

Table 1
Sequences of the oligonucleotides

R-24-mer:	5'- AGCACTCTCCAGCCTCTCACCGCA - 3'
R-12-mer:	3'- AGTGGCGTCTAG - 5'
J-24-mer:	5'- ACCGACGTCGACTATCCATGAACA - 3'
J-12-mer:	3'- GTACTTGTCTAG - 5'
N-24-mer:	5'- AGGCAACTGTGCTATCCGAGGGAA - 3'
N-12-mer:	3'- GCTCCCTTCTAG - 5'

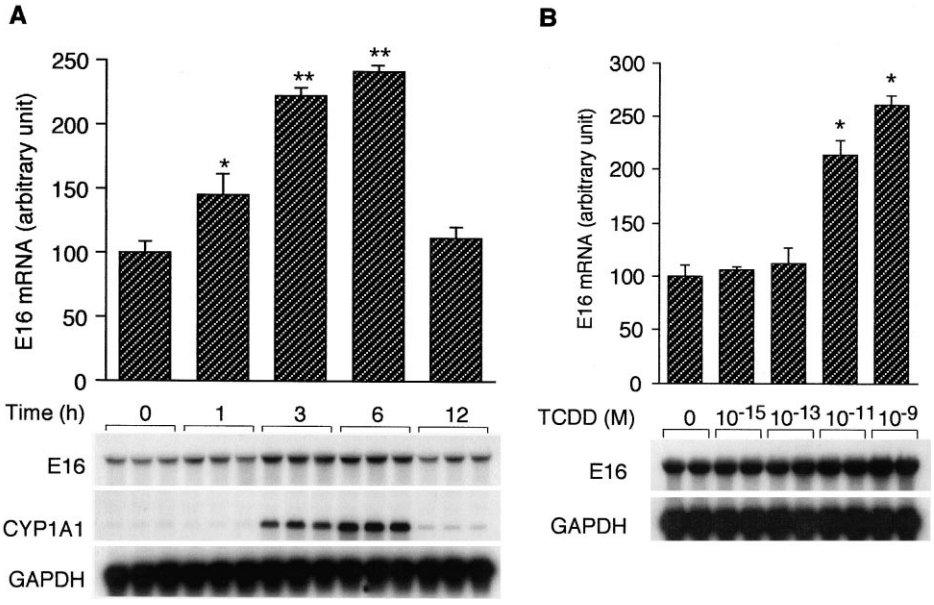

Fig. 2. TCDD upregulates E16/CD98LC/hLAT1 mRNA expression. A: HepG2 cells were treated with 1 nM TCDD for 0, 1, 3, 6 and 12 h. Total RNA (15 μg) from these cells was subjected to Northern blot analysis using E16/CD98LC/hLAT1, CYP1A1 and GAPDH cDNAs as probes. The asterisks represent significant differences from 0 h (*: $P<0.01$; **: $P<0.001$). B: HepG2 cells were treated with indicated concentrations of TCDD for 6 h, and 15 μg of total RNA from these cells was subjected to Northern blot analysis. The asterisk represents significant difference from 0 M TCDD ($P<0.01$). E16 stands for E16/CD98LC/hLAT1. The experiments were carried out twice. The data represent mean ± S.D.

centrations of TCDD for 6 h. As shown in Fig. 2B, treatment with 10^{-15} and 10^{-13} M TCDD did not alter the mRNA level. Treatment with 10^{-11} M (10 pM) and 10^{-9} M (1 nM) TCDD induced a significant increase in the mRNA level. Taken together, these results demonstrated that the E16/CD98LC/hLAT1 mRNA level was positively regulated by TCDD.

The next experiment was performed to study the effect of CHX on TCDD-dependent increase in E16/CD98LC/hLAT1 mRNA. The cells were pretreated with CHX for 30 min and then treated with TCDD for 0, 1, 3, 6 and 12 h. As shown in Fig. 3, Northern blot analysis revealed a greater extent of increase in E16/CD98LC/hLAT1 mRNA levels by TCDD in the presence of CHX, when compared to its absence (Fig. 2A). It was noted that, in the absence of CHX, the mRNA level returned to the basal level at 12 h (Fig. 2A), while in its presence, the mRNA level increased further by six-fold above the basal level. These observations indicated that the TCDD-dependent increase in E16/CD98LC/hLAT1 did not require de novo protein synthesis, and that the presence of protein synthesis inhibitor resulted in superinduction of the mRNA level.

Finally, we examined the functional significance of the upregulation of E16/CD98LC/hLAT1 mRNA by TCDD. One of the known functions of E16/CD98LC/hLAT1 is transport of large neutral amino acids into the cells. We, therefore, tested

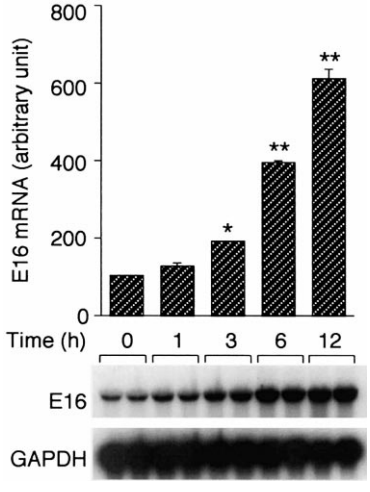

Fig. 3. Cycloheximide superinduces TCDD-mediated upregulation of E16/CD98LC/hLAT1 mRNA. HepG2 cells were pretreated with cycloheximide followed by 1 nM TCDD for 0, 1, 3, 6 and 12 h, and 15 μg of total RNA from these cells was subjected to Northern blot analysis. E16 stands for E16/CD98LC/hLAT1. The experiment was carried out twice. The data represent mean ± S.D. The asterisks represent significant differences from 0 h (*: $P<0.01$; **: $P<0.001$).

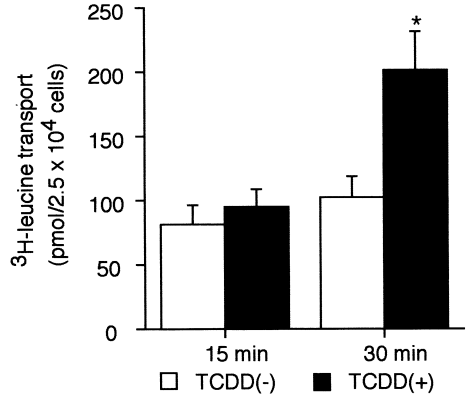

Fig. 4. TCDD increases transport of ^{3}H-leucine in HepG2 cells. HepG2 cells were treated with 1 nM TCDD for 6 h followed by incubation in transport buffer containing 50 μCi/ml of ^{3}H-leucine for 15 or 30 min. Aliquots of cell lysates were analyzed for radioactivity using a liquid scintillation counter. Data represent mean ± S.D. The asterisk represents significant difference ($P<0.01$) between TCDD (−) and TCDD (+) cells at 30 min.

whether TCDD treatment results in increased transport of ^{3}H-leucine into the cells or not. The cells were treated with 1 nM TCDD for 6 h and then incubated for 15 or 30 min in a transport buffer containing ^{3}H-leucine. As shown in Fig. 4, a significant increase in the uptake of ^{3}H-leucine was observed at 30 min.

4. Discussion

In the present study, we succeeded in identifying a novel TCDD-responsive gene, E16/CD98LC/hLAT1, in human hepatocytes by utilizing cDNA RDA. E16/CD98LC/hLAT1 was originally cloned as E16, a transiently expressed protein upregulated by the tumor promoter phorbol myristate acetate (PMA) in Jurkat T-cells, which contained only the 3′-half of the cDNA [11]. Cloning of the full cDNA revealed that E16 is identical to the light chain of CD98/4F2 (CD98LC) and L-type amino acid transporter-1 (hLAT1) [12,13].

Our Northern blot analysis demonstrated that TCDD transiently increased E16/CD98LC/hLAT1 mRNA level without de novo protein synthesis, suggesting that TCDD may directly increase the transcription of the gene via activation of AhR/ARNT. We also observed that the mRNA level can be superinduced in the presence of protein synthesis inhibitor, CHX. Two mechanisms may be involved in this phenomenon. Inhibition of synthesis of new proteins, such as 'AUUUA' binding protein, critical for mRNA degradation might be one of the mechanisms [17–19]. Indeed, the mRNA contains this conserved sequence 'AUUUA' in the 3′-untranslated region [11]. Another mechanism might be the inhibition of AhR repressor (AhRR) [20]. AhRR represses the transcriptional activity of AhR by competing with AhR in forming a heterodimer with ARNT and by binding with XRE sequence. The expression of AhRR gene is shown to be induced by AhR/ARNT which binds to the XRE present in the promoter of the AhRR gene. These dual effects of CHX on the synthesis of mRNA binding proteins and AhRR may account for the superinduction of E16/CD98LC/hLAT1 mRNA.

Human E16/CD98LC/hLAT1 consists of 507 amino acids with 12 transmembrane domains, indicating that it is an integral membrane protein [12]. It was demonstrated that E16/CD98LC/hLAT1 heterodimerizes with the heavy chain of CD98 (CD98HC) and constitutes the Na^{+}-independent amino acid transport system L which is the major route for the transport of large, neutral amino acids with branched or aromatic side chains [16,21,22]. CD98HC is constitutively expressed in almost all tissues and tumor cell lines and functions as a 'guidance molecule' for the expression of CD98LC in the plasma membrane [23]. This might explain the finding that TCDD-mediated E16/CD98LC/hLAT1 mRNA upregulation is coupled with increased amino acid transport as demonstrated by the present study.

It is intriguing to speculate the functional significance of TCDD-mediated upregulation of E16/CD98LC/hLAT1 mRNA and increased amino acid transport for the cells. The mRNA was found to be highly expressed in human colon cancers, while it was almost undetectable in normal intestine [24]. It was also reported that E16/CD98LC/hLAT1 was hardly detectable in adult human tissues such as brain, lung and liver, but it was detected in various, immortalized cell lines [11]. In addition, TA1, the rat homologue, was identified to be an oncofetal gene which was highly expressed in fetal liver and in hepatocellular carcinoma [25]. TA1 expression was shown to be induced by acute and chronic liver injury mediated by carbon tetrachloride which acts as a tumor promoter in rat models of chronic liver injury [26]. These broad expression patterns of E16/CD98LC/hLAT1 in rapidly dividing cells and its rapid induction upon treatment with tumor promoters suggest that it might have a direct functional involvement in the process of cell division or tumor progression. Recently, it has been documented that overexpression of CD98 results in malignant transformation of NIH3T3 cells [27]. TCDD has been shown to act as a potent tumor promoter [28]. These findings suggest that the TCDD-mediated increase in E16/CD98LC/hLAT1 expression and the resultant increase in uptake of amino acid may be coupled with cell proliferation and tumor progression.

CD98 is involved not only in the cellular uptake of amino acids, but also in the activation of integrin signaling pathway, calcium homeostasis, cellular activation of lymphocytes and cell fusion [29]. It remains to be studied whether these pleiotropic functions of CD98 are related to the biological effects of TCDD or not.

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