TCDD-inducible plasminogen activator inhibitor type 2 (PAI-2) in human hepatocytes, HepG2 and monocytic U937 cells

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Induction of PAI-2 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been studied in human primary hepatocytes, hepatoma HepG2 cells and monocytic U937 cells, extending recent findings in human keratinocytes. PAI-2 represents a serpine-type protease inhibitor with wide-ranging implications in fibrinolysis, extracellular matrix proteolysis, growth factor activation and carcinogenesis. PAI-2 was induced by >10−9 M TCDD in hepatocytes and HepG2 cells and by >10−10 M TCDD in U937 cells. In the latter cell line, PAI-2 induction by TCDD and by 12-O-tetradecanoyl phorbol-13-acetate (TPA) has been compared. TCDD appeared to be less efficient than TPA as an inducer of PAI-2. In contrast to induction by TPA, PAI-2 induction by TCDD was found to be biphasic, with an early peak of mRNA at 1–3 h and a late peak at 12–24 h. A biphasic response was also seen at the protein level although production of PAI-2 protein lagged behind the corresponding mRNA. PAI-2 is known to contain AP-1 sites, i.e. Jun/Fos protein-binding sites, in its promoter region. Hence, PAI-2 induction by TCDD has originally been conceived to be due to an indirect response, secondary to the induction of Jun/Fos proteins. Therefore, expression of Jun/fos genes and their AP-1 activity were studied at the early phase of PAI-2 induction by TCDD. TCDD did not increase mRNA of c-fos, c-jun, junB or junD (in contrast to TPA which markedly increased the expression of c-fos and junB), nor did TCDD increase AP-1 activity. In conclusion, the findings suggest that PAI-2 induction by TCDD is not restricted to human keratinocytes but includes liver cells and monocytic U937 cells. The induction mechanism is complex but the early phase does not appear to involve Jun/Fos proteins.

Introduction

Plasminogen activator inhibitor type 2 (PAI-2*), a fast-acting inhibitor of tissue- and urokinase-type plasminogen activators, has recently been added to the list of dioxin-inducible proteins (1,2). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a prototype of ubiquitous environmental contaminants which exerts a variety of biologic and toxic responses in experimental animals, wildlife and humans (3–5). These responses are believed to be mediated through the Ah or dioxin receptor. The Ah receptor acts as a dimeric complex together with its partner protein aryl hydrocarbon nuclear translocator (ARNT). It has recently been characterized as a basic region–helix–loop–helix transcription factor (6–9). PAI-2 belongs to the family of serine protease inhibitors (serpins; 10) which play important roles in fibrinolysis, proteolysis of extracellular matrix, activation of growth factors (TGFA, TGFB, etc.) and carcinogenesis (10–13). Extending recent findings of Sutter et al. in keratinocytes (1), we investigated whether PAI-2 induction is detectable in other possible human target cells of TCDD toxicity. Primary hepatocytes and hepatoma cells (HepG2) were studied because of the known tumour-promoting actions of TCDD in rodent liver (14) and possibly also in humans at high doses (15).

Regulation of PAI-2 has already been studied in human monocytes and monocytic U937 cells (16,17). For example, PAI-2 is known to be inducible in U937 cells by phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (16), an agonist of protein kinase C signalling. TPA treatment leads to increased activity of Jun/Fos proteins, which are components of the activator protein-1 (AP-1) transcription factor (18). AP-1 activity is mainly regulated by (i) transcriptional activation of jun/fos genes and (ii) by post-translational phosphorylation/dephosphorylation of Jun/Fos proteins (18–20). Interestingly, PAI-2 contains several AP-1 binding sites in its promoter region (21,22). Furthermore, a consensus dioxin receptor responsive element (DRE) is present in the regulatory region of PAI-2 (1). It was therefore of interest to investigate whether PAI-2 induction by TCDD occurs as a primary event, e.g. by the Ah receptor/ARNT mechanism, or as a secondary event via activation of Jun/Fos proteins. The present study describes PAI-2 induction by TCDD in a variety of human tissues. Comparative studies of TCDD and TPA in U937 cells suggest that TCDD induction of PAI-2 occurs independently of the activation of Jun/Fos proteins.

Materials and methods

Materials

Eagle’s minimal essential medium (Eagle’s MEM) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Biochrom (Berlin, Germany) and RPMI 1640 medium, Waymouth’s MD 7051 medium, fetal calf serum and calf serum from Gibco, now Life Technologies (Eggenstein, Germany). The random primed DNA labelling kit and l-glutamine were from Boehringer (Mannheim, Germany). TPA, dexamethasone and oligo(dT)-cellulose were purchased from Sigma (St Louis, MO) and TCDD from Okometric GmbH (Bayreuth, Germany).

Cell culture and treatment

All cells were cultured in a humidified atmosphere of 5% CO2 in air at 37°C. TCDD and TPA were added in DMSO (0.1% final concentration) and cells were harvested at the time points indicated.

HepG2 cells

HepG2 cells were grown on 100×20 mm Falcon tissue culture dishes (Becton Dickinson, Heidelberg, Germany) in Eagle’s MEM supplemented with 10% fetal calf serum, 10% calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), dexamethasone (100 nM) and l-glutamine (2 mM).
U937 cells

U937 cells were grown in suspension culture on 94×16 mm tissue culture dishes (Greiner, Nürtingen, Germany) in RPMI 1640 containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM).

Primary hepatocytes

Primary hepatocytes were isolated from four human individuals who were either transplant donors or underwent hepatic surgery because of benign or malignant liver tumours. In the case of partial liver resection, tissue surrounding the tumour was used exclusively for preparation of hepatocytes. For cell preparation, tissue was perfused sequentially using the EDTA-collagenase procedure as described (23). Hepatocytes were plated on 94×16 mm Petri dishes (Greiner, Nürtingen, Germany) covered with rat tail collagen. Cells were seeded at a density of 100,000 cells/cm², and after 12-24 h medium was replaced.

Northern blot analysis

The isolation of total RNA was performed by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (24). For isolation of poly(A)+ RNA, total RNA was prepared by the method of Chirgwin et al. (25) and poly(A)+ RNA was selected on oligo(dT)-cellulose (26). Total RNA samples (10 or 20 µg) or poly(A)+ RNA samples (10 µg) were denatured with 2.2 M formaldehyde, 50% formamide and 1×MOPS buffer, pH 7.0 (20 mM 3-N-morpholino propane sulfuric acid, 5 mM sodium acetate, 1 mM EDTA) by heating for 3 min. An RNA ladder of fragment size of 0.24–9.5 kb (Gibco) was used as mol wt standard. After electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde, RNA was transferred to a nylon membrane (Hybond N, Amersham Buchler, Braunschweig, Germany) by capillary blotting in 20×SSC (3 M sodium chloride, 0.3 M sodium citrate buffer, pH 7.0), RNA was covalently bound to the membrane by UV radiation and then prehybridized in 5×SSC (0.5 M sodium thiosulfate, 50 mM sodium citrate, 0.5% SDS) for 1 h at 65°C. Hybridization was carried out in 5×SSC, 5% dextran sulfate, 5% SDS and 0.4 µg/ml denaturated herring sperm DNA at 44°C for 24 h. Hybridization was performed at 44°C for 48 h using selective cDNA probes.

Human PAI-2 cDNA was obtained from the American Type Culture Collection. Full-length cDNA of mouse CYP1A2 was provided by Dr F. Gonzalez (National Cancer Institute, Bethesda, MD; 27). Plasmids with cDNAs for junB (28), c-junD (30) and c-junB (31) were provided by Dr P. Angel (Kernforschungszentrum Karlsruhe, Germany). A full-length Ah receptor clone was provided by Dr L. Poellinger (Karolinska Institute, Huddinge, Sweden; 32). Loading of the gels was controlled using a cDNA probe for the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (32). The probes were labelled by the random primer method using (α-32P)dCTP (34). Washing of the membrane was carried out twice in 2×SSC, 0.1% SDS at 50°C for 20 min. The membrane was exposed for 1–7 days at -70°C to Kodak XAR-5 film with intensifying screens. For densitometry the system and Hyper-CAM software of Cybertech (Berlin, Germany) were usually used. Recently (Table I), PAI-2 mRNA was quantified by direct phosphor image analysis using the Fuji Bio-Image Analyser BAS 1000 (Fuji Film, Tokyo, Japan).

Western blot analysis

U937 cells were washed with 0.9% sodium chloride solution and homogenized in PBS with 0.5% (w/v) Triton X-100. To remove cell debris, the sample was centrifuged at 5,000 × g for 5 min, and the supernatant was stored at -20°C until used. SDS-PAGE was performed according to the method of Laemmli (35) using slab gels with 10% polyacrylamide separating gel and 6% stacking gel. Molecular weight markers were purchased from Biorad (Richmond, CA) and were used. SDS-PAGE was usually used. Recently (Table I), PAI-2 mRNA was quantified by direct phosphor image analysis using the Fuji Bio-Image Analyser BAS 1000 (Fuji Film, Tokyo, Japan).

Results

HepG2 hepatoma cells

Induction of PAI-2 mRNA by TCDD was clearly detectable in human HepG2 hepatoma cells. However, PAI-2 mRNA was not detectable until 24 h, whereas CYP1A1 mRNA was already induced at 3 h (Figure 1). Both PAI-2 and CYP1A1 mRNA were increased ~30-fold. TCDD appeared to be more potent as an inducer of CYP1A1 than of PAI-2 (Figure 2).
Primary cultures of human hepatocytes

Dose dependence of PAI-2 induction by TCDD was investigated in human primary hepatocytes from four different donors (Figure 3). PAI-2 mRNA was clearly detectable in all four preparations. A 2- to 3-fold induction of PAI-2 mRNA was observed in three livers (HL2, HL4 and HL10). HL5 showed no inducibility; instead, high PAI-2 expression was already observed in the absence of TCDD treatment. CYP1A1 was clearly inducible in hepatocytes obtained from the four livers investigated.

Human monocytic U937 cells

Ah receptor mRNA at 6.6 kb was constitutively expressed throughout the experiment (Figure 4A). It is noteworthy that a 1.7 kb fragment was also consistently recognized by the Ah receptor probe. Furthermore, the Ah receptor (105 kDa; 37) and ARNT protein (87 kDa; 38) were present in the cytosol and in nuclear extracts (Figure 4B). TCDD-mediated transfer of the Ah receptor to the nucleus was also seen, similar to findings of Pollenz et al. (39). Using a rat CYP1A1 probe (40) increased CYP1A1 mRNA by TCDD was detectable in our U937 cells (not shown). These findings indicate the functionality of the Ah receptor–ARNT complex in these cells, confirming recent observations (41).

Time courses of PAI-2 induction were compared after treatment with TCDD and TPA (Figure 5). After TPA treatment PAI-2 mRNA increased within 2 h, reaching a maximum (~5-fold) at 4 h and declining to baseline levels after 48 h. After TCDD treatment, induction of PAI-2 mRNA appeared to be lower (2- to 3-fold induction) and biphasic. An early maximum of mRNA was observed at 2–3 h and a late maximum at 12 h. As expected, appearance of PAI-2 protein lagged behind, with an early maximum at ~6 h. A second increase occurred up to 72 h (Figure 6). The dose dependence of PAI-2 induction by TCDD and TPA was studied after 2 h of treatment (Figure 7). Maximum induction of PAI-2 mRNA by TPA was observed at a concentration of 40 nM, whereas induction of PAI-2 mRNA by TCDD increased up to 100 nM. When treated with both inducers, PAI-2 induction appeared to be further increased (Table I).

Experiments with actinomycin D were conducted to exclude the possibility that induction was due to stabilization of its mRNA. U937 cells were treated with 10 nM TCDD for 2 h prior to the addition of actinomycin D (10 µg/ml). Controls received DMSO instead of TCDD. RNA was isolated at 0, 1, 2, 4 and 6 h and PAI-2 mRNA was analysed by direct phosphor image analysis. PAI-2 mRNA decreased linearly with time with a half-life of ~4 h. However, there was no significant difference between TCDD-treated cultures and solvent-treated controls (not shown). The half-life found in U937 cells was similar to that observed in human monocytes (2 h; 17).

To investigate whether PAI-2 induction by TCDD occurs secondary to the activation of Jun/Fos proteins, mRNA levels of jun/fos genes were studied and compared to those after TPA treatment (Figure 8). TPA strongly induced c-fos and junB...
Fig. 5. Time course of PAI-2 gene expression in human U-937 cells treated with DMSO (-), 40 nM TPA (+) and 10 nM TCDD (+). Total RNA (10 μg) was isolated at the indicated time points after treatment and subjected to Northern blot analysis using a cDNA probe for human PAI-2. Data are representative of three experiments.

Fig. 6. Western blot analysis of PAI-2 protein of human U-937 cells treated with 40 nM TPA or 10 nM TCDD. Each lane contained 60 μg of cellular protein isolated at the indicated time points after treatment. Mouse anti-PAI-2 IgG was used for immunoblotting. Data are representative of two experiments.

mRNA, whereas TCDD did not markedly induce jun/fos genes. Furthermore, gel mobility shift analysis utilizing an oligonucleotide encompassing AP-1 sites in the promotor region of PAI-2 did not show increased AP-1 activity after 4 h treatment with TCDD (Figure 9), whereas AP-1 activity was clearly increased after TPA treatment. The reaction was specific since it was abolished by competition with the unlabelled oligonucleotide (compare lines 2 and 3). Similar results were obtained after 2 h treatment (not shown).

Discussion
In this report two questions have been addressed. (i) Is PAI-2 induction by TCDD detectable in human target tissues of TCDD toxicity other than keratinocytes (1)? (ii) Is PAI-2 directly induced by the Ah receptor/ARNT mechanism or secondarily via Jun/Fos proteins? The latter study was carried out with monocytic U937 cells.
Table I. Influence of TCDD and TPA on PAI-2 mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAI-2 mRNA (fold increase over controls)</th>
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<tbody>
<tr>
<td>Controls</td>
<td>1.0 ± 0.4 (^b)</td>
</tr>
<tr>
<td>TCDD (10 nM)</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>TPA (40 nM)</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>TCDD (10 nM) + TPA (40 nM)</td>
<td>5.3 ± 0.3</td>
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Radioactivity of the Northern blots was detected by direct phosphor image analysis.
\(^a\)Figure 7 lists a representative Northern blot.
\(^b\)Data represent means ± SD of 4 experiments.

## Induction of PAI-2 in HepG2 cells and primary cultures of hepatocytes

PAI-2 was shown to be inducible in human HepG2 and primary cultures of hepatocytes, although in primary cultures of hepatocytes, interindividual differences were also noted. For example, in one liver (HL5) high PAI-2 mRNA was present without TCDD treatment. In this liver, transcriptional activation of PAI-2 may have occurred prior to the isolation of hepatocytes. Interestingly, induction of PAI-2 in HepG2 cells does not occur until 24 h (Figure 1). It appears therefore that in HepG2 cells only the late phase response of PAI-2 induction by TCDD occurs (see below, studies with U937 cells).

PAI-2 expression has also been studied in rat liver cells. Since proteolysis by the plasminogen activator/plasmin cascade appears to be involved in the activation of TGFα, TGFβ (10–13) and in the activation of the EGF receptor (42), TCDD-altered PAI-2 expression would have provided a link to liver tumour promotion by TCDD (43). A 701 bp DNA fragment in the region of highest homology between rat and human PAI-2 was obtained by PCR using human PAI-2 and primers of the rat PAI-2 which were selected from EMBL Data Bank, accession no. X64563 (forward primer: nucleotides 501–523; reverse primer: nucleotides 1202–1181). Although constitutive expression of PAI-2 could be detected in a mixed Kupffer and endothelial cell fraction (prepared according to 44) TCDD induction of PAI-2 was not found in rat parenchymal and non-parenchymal cells (not shown), confirming the findings of others (45). Hence, PAI-2 induction by TCDD appears to be remarkably species specific. Different regulation of PAI-2 in rat and human cells adds to the marked species specificity of Ah receptor responses (3–5,8,9).

## Comparison of PAI-2 induction by TCDD and TPA in U937 cells

The Ah receptor, ARNT, and TCDD induction of CYP1A1 activity were detectable in U937 cells, the latter indicating the functionality of the Ah receptor–ARNT complex. Regulation of PAI-2 expression by TPA and bacterial lipopolysaccharides has already been studied in U937 cells and human monocytes (16,17). As shown in Figures 5 and 6, PAI-2 is also induced by TCDD in these cells. In contrast to observations after TPA treatment, the time course of PAI-2 induction by TCDD was found to be biphasic. An early phase of PAI-2 induction occurred between 1 and 3 h, and a late phase between 6 and 24 h. PAI-2 protein accumulation lagged behind, with an early maximum between 3 and 6 h and a late increase from 48 h up to at least 72 h. Interestingly, biphasic induction was also
observed after treatment with bacterial lipopolysaccharides (17). In the latter studies PAI-2 induction could be attributed to increased transcription and in part to stabilization of its mRNA. Stabilization of mRNA has been described as the mechanism responsible for TGFα induction after TCDD treatment (46).

From these observations it was conceivable that enhanced stability of PAI-2 transcripts may have been responsible for the increased PAI-2 mRNA. However, experiments with actinomycin D did not suggest altered stability of PAI-2 mRNA as the mechanism responsible for PAI-2 induction by TCDD. PAI-2 contains multiple AP-1 sites in its promoter region (22). It was therefore conceivable that TCDD induction of PAI-2 occurred secondary to the activation of Jun/Fos proteins. However, neither increased expression of jun/fos genes nor increased AP-1 activity could be detected after TCDD treatment. These findings, as well as further induction by TCDD after maximal induction by TPA, suggest that TCD and TPA act as independent inducers of PAI-2.

The present findings suggest that the mechanism of PAI-2 induction appears to be complex, including early and late phases, similar to the responses to the addition of bacterial lipopolysaccharides (17). At the beginning of our experiments it seemed conceivable that PAI-2 induction may be an indirect response secondary to the induction of Jun/Fos proteins. Induction of jun/fos genes by TCDD has been described (47). However, in the present study no evidence for the involvement of Jun/Fos proteins has been obtained. There was also no evidence for post-transcriptional stabilization of PAI-2 mRNA. Although indirect mechanisms cannot be excluded, the hypothesis has been strengthened that PAI-2 induction is due to direct transcriptional activation. One DRE-like motif has been observed in the regulatory region of PAI-2 (1). The hypothesis of direct transcriptional activation is currently being tested by nuclear run-off and by gel-shift analysis using the DRE-like motif of PAI-2 as the labelled oligonucleotide.

Our findings suggest that PAI-2 induction by TCDD can be demonstrated in a variety of human cells, in addition to keratinocytes (1), including liver and monocytic cells. The absence of TCDD-induced PAI-2 induction in rat liver cells represents a remarkable example of species differences in the actions of TCDD. PAI-2 probably fulfils important roles in the activation of growth factors such as TGFα and TGFβ (10-13,42,43). TCDD may act as a liver tumour promoter in highly exposed human populations (15). Excretion of PAI-2 by transformed cells suggests additional functions of this protease inhibitor at the progression stage of carcinogenesis (48,49). However, the role of TCDD-inducibility of PAI-2 in these processes still remains elusive.

Acknowledgements

The authors wish to thank Mrs G.Eisenhardt and S.Vetter for expert technical assistance, Dr L.Poellinger (Karolinska Institute, Huddinge, Sweden) for generously providing the plasmid containing the Ah receptor and antibodies to the Ah receptor and ARNT. They also acknowledge financial support by the Deutsche Forschungsgemeinschaft.

References


Received on June 30, 1995; revised on November 3, 1995; accepted on November 17, 1995.