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Effect of Transforming Growth Factor-β_1 on Expression of Aryl Hydrocarbon Receptor and Genes of *Ah* Gene Battery: Clues for Independent Down-Regulation in A549 Cells

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SUMMARY

An inhibitory effect on both constitutive and inducible expression of cytochrome P450 isoenzymes has been shown for different cytokines and growth factors. We previously described an inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1A1 mRNA and enzyme activity by transforming growth factor-β_1 (TGF-β_1) in human lung cancer A549 cells. In the present study, we report that not only TCDD-induced expression of *CYP1A1* but also basal mRNA expression of CYP1A1, CYP1B1, and aryl hydrocarbon receptor (AHR) was down-regulated by TGF-β_1 in cells not treated with TCDD. In contrast, mRNA expression of the AHR partner protein Arnt (aryl hydrocarbon receptor nuclear translocator) was not influenced. Furthermore, TCDD-induced expression of CYP1B1 and NMO-1 was inhibited, and the IC_{50} values of 5–10 pM TGF-β_1 were in the same range as observed for inhibition of CYP1A1 and AHR mRNA expression. Transfection studies with a plasmid containing a luciferase reporter gene under control of two dioxin-responsive elements indicate an effect on AHR protein expression. Results of time-course studies revealed a parallel inhibition of AHR and CYP1 mRNA expression, indicating that TGF-β_1 is a direct negative regulator of transcription of these genes. The treatment of cells with cycloheximide led to a superinduction of TCDD-induced CYP1A1 and CYP1B1 mRNA expression and abolished the inhibitory effect of TGF-β_1 on basal as well as TCDD-induced CYP1 and AHR mRNA expression. TGF-β_1 seems not to influence the stability of AHR mRNA. The results suggest that TGF-β_1 induces rapid transcription and translation of an as-yet-unknown negative regulatory factor or factors that may directly regulate expression of AHR and genes of *Ah* gene battery.

The cytochrome P450 enzyme family is a group of hemethiolate monooxygenases important in metabolism of many endogenous as well as exogenous compounds; so far, >481 different cytochrome P450 genes have been identified and classified into 74 gene families according to their amino acid sequences (1). The CYP1 family, which consists of at least three enzymes, CYP1A1, CYP1A2, and CYP1B1, has been shown to be important in the metabolism of several xenobiotics, such as PAH and heterocyclic amines, and expression of these enzymes is inducible by PAHs like TCDD. TCDD inducibility of CYP1 transcription is mediated by the cytosolic AHR, which belongs to a group of ligand-activated transcription factors. Activation of AHR involves ligand binding, dissociation of heat-shock protein-90, nuclear translocation, and dimerization with Arnt followed by binding to DRE enhancer elements in the 5′-noncoding region of the respective gene (2). In addition to CYP1 genes, transcription of several other genes is inducible by TCDD, including phase II enzymes like NMO-1 und UGT1A6 and different cytokines and growth factors like IL-1β, TGF-β, TNF-α, and TGF-α (2–4). Although involvement of AHR in transcriptional activation has been well proved for phase I and phase II genes of *Ah* gene battery (i.e., CYP1, NMO-1, UGT1A6, GST, and ADH) (3), the TCDD-induced expression of cytokines and growth factors may instead be attributable to secondary effects (4–6).

An inhibition of cytochrome P450-related enzyme activities and, therefore, altered drug metabolism has been shown during infection and inflammation in rodents and humans (7–9). This inhibition has been linked to an increased serum concentration of proinflammatory cytokines exerting a nega-

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; ActD, actinomycin D; AHR, aryl hydrocarbon receptor; AP, activator protein; Arnt, aryl hydrocarbon receptor nuclear translocator; BMS, basal medium supplement; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; DRE, dioxin responsive element; EROD, 7-ethoxyresorufin-*O*-deethylase; FCS, fetal calf serum; IL, interleukin; NMO, NADPH:quinone oxidoreductase; NRE, negative regulatory element i; RT, reverse transcription or transcriptase; PCR, polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transforming growth factor; TNF, tumor necrosis factor; UGT, UDP-glucuronosyltransferase.

tive regulatory effect on cytochrome P450 expression and therefore on drug metabolism. For example, male volunteers challenged with lipopolysaccharide exhibited a lower extension rate of antipyrine, hexobarbital, and theophylline, whereas serum concentrations of TNF-α, IL-1β, and IL-6 were enhanced (9). An influence of cytokines and growth factors on constitutive CYP expression has also been shown in cell culture models [e.g., IL-6 repressed CYP1A1, CYP1A2, and CYP3A mRNA in human hepatoma cells (10), and IL-1β, IL-6, and TNF-α inhibited CYP1A2, CYP2D, CYP2E1, and CYP3A mRNA and related enzyme activities in human primary hepatocytes (11)]. An inhibition of PAH-induced CYP1 expression by cytokines and growth factors has also been demonstrated in different cell systems *in vitro* [e.g., PAH-induced CYP1A mRNA expression was inhibited by IL-1β in rat and human primary hepatocytes, by IL-6 in human HepG2 cells and primary hepatocytes, and by TNF-α in human primary hepatocytes (12–15)]. We recently reported that TGF-β_1 inhibited TCDD-induced EROD activity and CYP1A1 mRNA expression in human lung cancer A549 cells (16); similar results were observed in human keratinocytes and human primary hepatocytes (14, 17).

TGF-β_1 belongs to a superfamily of paracrine-acting peptides known to elicit a variety of biological activities in many cell types, including effects on cell proliferation, cell differentiation, cell adhesion, cell migration, and regulation of extracellular matrix compositions. It inhibits the proliferation of many different cell lines, mainly epithelial cells. Although a stimulation of cell growth by TGF-β_1 has been shown, this mitogenic effect has been considered to be secondary in regard to other cellular responses (18). The effects of TGF-β_1 on gene expression are bipartite; both up- and down-regulation of TGF-β_1-sensitive genes were observed in many cell systems. For example, TGF-β_1 stimulated cell adhesion by modeling the expression of cell adhesion molecules and extracellular matrix proteins like plasminogen activator inhibitor type I, whereas it inhibited transcription of matrix-degrading metalloproteinases like transin/stromelysin (18–20). Similar distinct effects of TGF-β_1 on expression of cell cycle-regulating genes have been observed. TGF-β_1 increased transcription of p21/WAF1/Cip1 cyclin-dependent kinase inhibitor and immediate-early genes like c-*jun*, *jun* D, and c-*fos*, whereas it down-regulated mRNA expression of c-*myc* and G1-specific cyclin A (21, 22).

As outlined above, cytokines and growth factors interact with expression of drug-metabolizing enzymes. Because these peptides are increasingly used for therapeutic applications, studies on the mechanisms of these interactions are of considerable health concern in respect to possible side effects. The current study was performed to determine whether previously reported inhibition of TCDD-induced CYP1A1 expression by TGF-β_1 is due to an effect on AHR, the transcriptional activator of CYP1A1. The results show that AHR expression was down-regulated by TGF-β_1 at picomolar concentrations. In addition to CYP1A1, expression of two other members of *Ah* gene battery, CYP1B1 and NMO-1, was inhibited by TGF-β_1. Time-response experiments revealed that down-regulation of AHR is not required for inhibition of basal and TCDD-induced CYP1 mRNA expression, indicating that TGF-β_1 has direct negative regulation of expression of these genes.

Experimental Procedures

Materials. TCDD (purity, ≥ 99%) was obtained from Ökometric (Bayreuth, Germany). Recombinant human TGF-β_1, 7-ethoxyresorufin, resorufin, rhodamine B, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dicoumarol, NADPH, CHX, and ActD were supplied by Sigma (Taufkirchen, Germany). Moloney murine leukemia virus-RT and TRIzol total RNA preparation kit were from GIBCO-BRL (Eggenstein, Germany). Oligo(dT)$_{15}$ primer and DNase I were from Boehringer-Mannheim Biochemica (Mannheim, Germany). Deoxynucleotide triphosphates and RNase inhibitor were from Pharmacia (Freiburg, Germany). *Taq* DNA polymerase, transfectam, pSV-β-Gal, and luciferase assay system were from Promega (Heidelberg, Germany). [α-^{32}P]dCTP was from ICN (Costa Mesa, CA). Media for cell cultures were purchased from Sigma (Taufkirchen, Germany), and penicillin/streptomycin, BMS, FCS, and glutamine were from Seromed (Berlin, Germany).

Cell culture and treatment. The human lung cancer cell line A549 was a kind gift from Dr. Knabbe (UKE, Hamburg, Germany). Cells were cultured in DMEM, supplemented with 10% FCS (v/v), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM glutamine. Cells were maintained under standard conditions at 37° in 5% CO_2. Before treatment, nearly confluent monolayers were cultured overnight in low Ca^{2+} (50 μM) containing DMEM, supplemented with 5% BMS (v/v). Cells were then treated in high Ca^{2+} (1.8 mM) DMEM/5% BMS (v/v) with TCDD, TGF-β_1, CHX, or ActD as indicated. TCDD (1 μM stock solution) was dissolved in DMSO, TGF-β_1 (80 nM) was dissolved in 4 mM HCl/0.1% (w/v) bovine serum albumin, ActD (2.5 mg/ml) was dissolved in ethanol, and CHX (35 mM) was dissolved in sterile water. Control cells received the respective solvent vehicle, and the final concentration of DMSO in the medium was 0.1% (v/v).

EROD activity. For determination of EROD activity, TCDD-treated cells were harvested in ice-cold Tris/sucrose (10 mM/25 mM, pH 7.4), collected by centrifugation, and homogenized in 1 ml of Tris/sucrose. EROD activity was measured spectrofluorometrically as previously described using a Jobin Yvon spectrofluorometer (23). The spectrofluorometer was calibrated with a solution of rhodamine B in methanol, and amounts of resorufin were calculated from a standard curve.

RT-PCR. RT-PCR was performed as previously described (23). Total RNAs were prepared with TRIzol total RNA isolation kit according to the manufacturer's instructions followed by digestion with RNase-free DNase I. PCR amplifications were performed using a DNA thermal cycler (Hybaid-Omnigene, MWG-Biotech, Ebersberg, Germany) for the indicated cycles with the following profile: 4 min at 94° before the first cycle, 1 min for denaturation at 94°, 1 min for primer annealing, 1 min for primer extension at 72°, and 7 min at 72° after the last cycle. PCR primers were synthesized with an Applied Biosystems 391 DNA synthesizer (Weiterstadt, Germany), and primer sequences were taken from published sources. The following annealing temperatures and cycle numbers were used for gene-specific amplification: β-actin (23): 60°, 19 cycles; AHR (24): 61°, 25 cycles; Arnt (23): 65°, 26 cycles; c-*myc* (25): 61°, 25 cycles; CYP1A1 (23): 60°, 25 cycles; CYP1B1 (23): 63°, 22 cycles; NMO-1 (23): 68°, 20 cycles; TGF-β_1 (5): 60°, 22 cycles; and UGT1A6 (26): 65°, 32 cycles. Basal CYP1 mRNA expression was analyzed in cells not treated with TCDD by RT-PCR at higher cycle numbers using 29 and 25 cycles for CYP1A1 and CYP1B1, respectively. Linearity of amplification was controlled by three different cycle numbers for one cDNA concentration. PCR products were analyzed on 10% (w/v) polyacrylamide gels, and gels were dried and autoradiographed. For semiquantitative analyses, respective bands were quantified using a OmniMedia gel scanner (Millipore, Überlingen, Germany).

Transfection experiments. A549 cells (1×10^6 cells) were seeded onto 100-mm culture dishes and maintained for 7 hr in supplemented DMEM/10% FCS (v/v) under standard conditions. Cells were then transiently transfected with 4 μg of luciferase re-

porter plasmid (27) and 1 μg of pSV-β-Gal using 25 μg of the cationic lipopolyamine transfectam per culture dish in DMEM without FCS according to the manufacturer's instructions. The cells were incubated with the DNA/liposome mixture overnight and subsequently maintained in fresh DMEM supplemented with 5% (v/v) BMS for 24 hr. Cells were then treated in DMEM/5% (v/v) BMS for 40 hr with TCDD, TGF-β_1, or the respective vehicle control followed by cell lysis in 500 μl of reporter lysis buffer. Luciferase activities in cell lysates were determined using the luciferase assay system in a Berthold Multi-Bioluminat LB 9505C luminometer. Luciferase activity was corrected by β-Gal activity determined photometrically as previously described (28).

Results

Dose-dependent inhibition of TCDD-induced CYP1 expression by TGF-β_1. TCDD is a potent inducer of CYP1A1 and CYP1B1 mRNA expression in human lung cancer A549 cells with a maximum induction at a concentration of 1 nM TCDD (data not shown). Pretreatment of A549 cells for 2 hr with 0.5–250 pM TGF-β_1 led to a dose-dependent inhibition of TCDD-induced CYP1A1 and CYP1B1 mRNA expression with a complete inhibition at a concentration of ~100 pM TGF-β_1 in cells cotreated for 24 hr with TCDD (Fig. 1, *left*). The IC_{50} values for inhibition of both CYP1A1 and CYP1B1 were ~10 pM TGF-β_1. A dose-dependent inhibition of TCDD-induced CYP1A1-associated EROD enzyme activity was also shown in A549 cells. Pretreatment of cells for 2 hr with 10 or 50 pM TGF-β_1 repressed TCDD-induced EROD activity to 61% and 29%, respectively (Table 1).

Effect of TGF-β_1 on UGT1A6 and NMO-1 mRNA expression. To examine specificity of AHR-dependent gene transcription, the effect of TGF-β_1 on two other members of *Ah* gene battery was analyzed. Neither TCDD nor TGF-β_1 affected UGT1A6 mRNA expression (Fig. 1, *right*). In contrast, treatment of cells with 1 nM TCDD led to an ~3-fold induction of NMO-1 mRNA expression that was dose-dependently inhibited by TGF-β_1 (Fig. 1, *right*). A maximum response was found at a concentration of ~50 pM TGF-β_1 with an inhibition of TCDD-induced NMO-1 mRNA by 65%.

Effect of TGF-β_1 on AHR and Arnt mRNA expression. Because TCDD-induced gene expression is mediated by the heterodimeric AHR/Arnt complex, we examined the effect of TGF-β_1 on mRNA expression of both genes. As previously shown for human breast cancer MCF-7 and MDA-MB 231 cells (23), TCDD had no effect on mRNA expression of these genes in A549 cells. TGF-β_1 led to a dose-dependent repression of AHR mRNA expression, and a maximum inhibition was observed at a concentration of 100 pM TGF-β_1 (Fig. 2). The IC_{50} value (~8 pM TGF-β_1) calculated for down-regulation of AHR mRNA is in the same order of magnitude found for inhibition of TCDD-induced CYP1A1, CYP1B1, and NMO-1 mRNA expression (Fig. 1). In contrast, TGF-β_1 had no effect on mRNA expression of Arnt (Fig. 2).

Time course of down-regulation of basal AHR and CYP1 mRNA expression by TGF-β_1. Inhibition of AHR and TCDD-induced CYP1A1 mRNA expression by TGF-β_1 can be due to a direct effect on transcription of both genes as well as to the inhibition of AHR expression and, as a consequence, the lack of activation of AHR-dependent gene expression. To examine whether TGF-β_1-mediated down-regulation of CYP1 mRNA is an AHR-dependent response, the time course of inhibition of basal AHR as well as CYP1A1 and CYP1B1 mRNA expression was analyzed in cells treated for 2, 8, and 24 hr with 100 pM TGF-β_1 but not with TCDD. A significant decrease in AHR, CYP1A1, and CYP1B1 mRNA was observed after a 2-hr incubation, indicating that expression of these genes is rapidly down-regulated by TGF-β_1 (Fig.

TABLE 1

Inhibition of TCDD-induced EROD-activity in A549 cells by TGF-β_1

Cells were pretreated for 2 hr with 10 or 50 pM TGF-β_1 and cotreated for an additional 24 hr with 1 nM TCDD; control cells received 0.1% (v/v) DMSO. EROD activity was determined in cell homogenates as described in Materials and Methods. Mean ± standard deviation values of three independent experiments are given.

Cell treatment	Resorufin	% of Effect
	pmol/mg/min	
0.1% DMSO	N.D.[a]	
1 nM TCDD	1.35 ± 0.14	100
10 pM TGF-β_1/1 nM TCDD	0.83 ± 0.05	61
50 pM TGF-β_1/1 nM TCDD	0.39 ± 0.09	29

[a] N.D., not detectable.

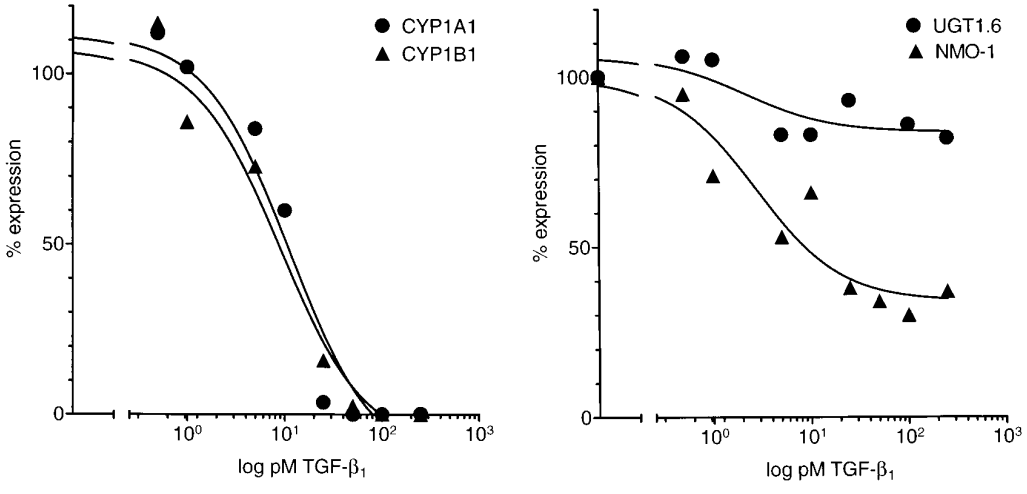

Fig. 1. Dose-dependent inhibition of TCDD-induced mRNA expression of TCDD-sensitive genes by TGF-β_1 in human lung cancer A549 cells. *Left*, CYP1A1 (●) and CYP1B1 (▲). *Right*, UGT1A6 (●) and NMO-1 (▲). Cells were pretreated for 2 hr with 0.5–250 pM TGF-β_1 and then cotreated for 24 hr with 1 nM TCDD. Control cells received only TCDD. mRNA expression was detected by RT-PCR, the respective bands were scanned, and mRNA levels are given as relative intensities to β-actin as described in Experimental Procedures.

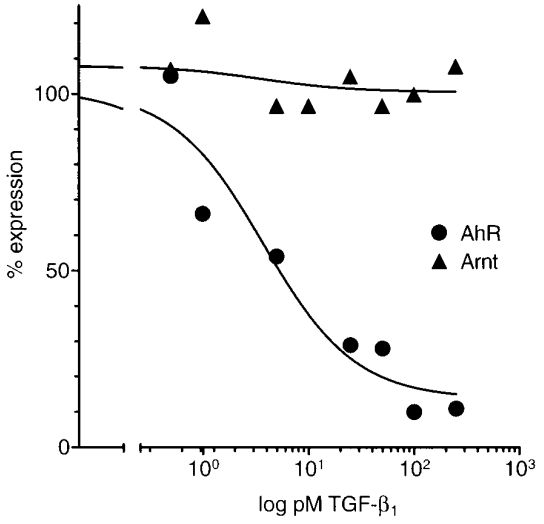

Fig. 2. Effect of TGF-β_1 on AHR (●) and Arnt (▲) mRNA expression in A549 cells. See legend to Fig. 1.

3). A maximum repression was observed for AHR and CYP1B1 in cells treated for 8 hr with TGF-β_1, with an inhibition to 80% of control levels, whereas CYP1A1 mRNA was nearly completely inhibited after 24 hr. Prolonged treatment with TGF-β_1 did not further reduce the mRNA content of AHR and CYP1B1. The results of time course studies shown in Fig. 3 reveal that down-regulation of AHR and CYP1 mRNA expression are parallel rapid processes that implicate the direct action of TGF-β_1 on expression of AHR and CYP1 genes. Furthermore, cotreatment of cells with TCDD seems to not be necessary for the inhibitory effect of TGF-β_1.

Inhibition of TCDD-induced luciferase activity by TGF-β_1 in A549 cells. To analyze the effect of TGF-β_1 on AHR expression, we performed transfection experiments with the minimal dioxin-responsive reporter construct pTX-.DIR, which contains two DREs. This plasmid has been shown to be inducible by AHR agonists in human cells and therefore is a useful tool for the study of AHR-dependent gene activation (27). Treatment of transiently transfected A549 cells with 10 nM TCDD for 40 hr led to a 5.6-fold induction of luciferase activity compared with untreated cells (Fig. 4). Cotreatment of these cells with 50 pM TGF-β_1 significantly antagonized TCDD-induced luciferase activity to ~60%. TCDD had no effect on luciferase activity in cells transiently transfected with parental pT81 lacking the two DREs. TGF-β_1 itself slightly induced luciferase activity ~2-fold compared with controls in both pT81- and pTX.DIR-transfected A549 cells.

Effect of protein synthesis inhibitor CHX on TGF-β_1-mediated mRNA decrease. We have previously shown that protein synthesis is necessary for TGF-β_1-mediated inhibition of TCDD-induced CYP1A1 expression (16); therefore, we tested whether protein synthesis is also required for down-regulation of AHR mRNA expression. The cells were treated for 24 hr with 100 pM TGF-β_1 in the presence or absence of 35 μM CHX (Fig. 5). CHX abolished TGF-β_1-induced effect on AHR mRNA expression. Furthermore, AHR mRNA was overexpressed in cells treated with TGF-β_1 and CHX compared with untreated cells. Similar results were observed for CYP1A1 and CYP1B1. CHX neutralized TGF-β_1-mediated inhibition of TCDD-induced CYP1 mRNA, and a superinduction of TCDD-induced CYP1A1 and CYP1B1 mRNA expression by CHX was observed. These results indicate that in addition to CYP1A1, the expression of CYP1B1 and AHR seems to be transcriptionally controlled by a negative regulatory protein or proteins.

Influence of TGF-β_1 on AHR mRNA stability in A549 cells. To examine whether TGF-β_1 influences AHR mRNA stability, experiments with transcription inhibitor ActD were performed. Cells were first treated simultaneously with 100 pM TGF-β_1 and 5 μg/ml ActD for 5 and 10 hr. The inhibitory effect of TGF-β_1 was abolished in these cells (Fig. 6, *left*, *lanes 1–5*), indicating that mRNA synthesis seems to be necessary for repression of AHR mRNA. Cells were then pretreated for

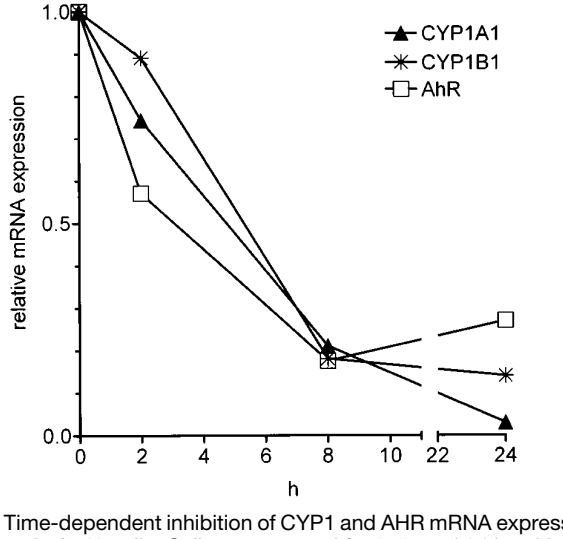

Fig. 3. Time-dependent inhibition of CYP1 and AHR mRNA expression by TGF-β_1 in A549 cells. Cells were treated for 2, 8, and 24 hr with 100 pM TGF-β_1, and relative mRNA expression is given to respective controls. Basal CYP1 mRNA expression in cells not treated with TCDD was detectable at higher cycle numbers with 29 and 25 cycles for CYP1A1 and CYP1B1, respectively, and RT-PCR was performed as described in Experimental Procedures. Typical results of three independent experiments are given.

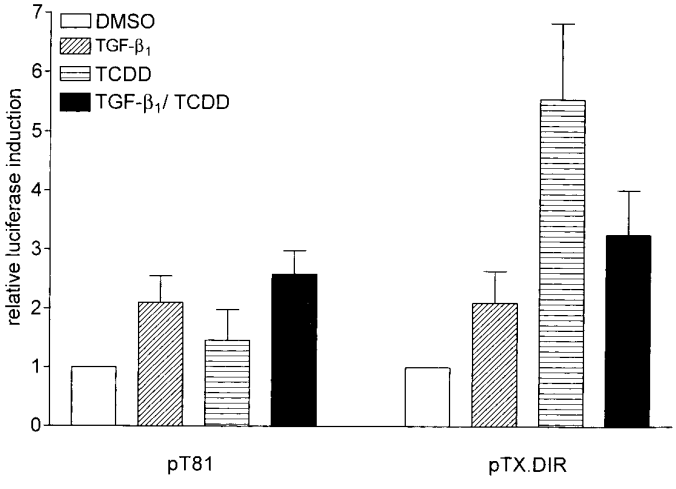

Fig. 4. Inhibition of TCDD-induced luciferase activity by TGF-β_1 in A549 cells. Cells transiently transfected with pTX.DIR or pT81 were treated for 40 hr with 50 pM TGF-β_1, 10 nM TCDD, or 50 pM TGF-β_1/10 nM TCDD. Luciferase activities were measured as described in Experimental Procedures and were corrected by activities of cotransfected pSV-β-Gal control plasmid. *Bars*, mean ± standard deviation of triplicate experiments.

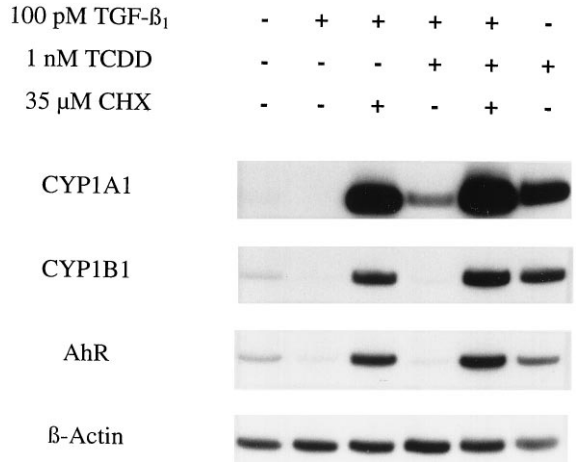

Fig. 5. Effect of protein synthesis inhibitor CHX on TGF-β_1-mediated down-regulation of TCDD-induced CYP1 and basal AHR mRNA expression in A549 cells. Cells were pretreated for 2 hr with 100 pM TGF-β_1 in the presence of 35 μM CHX and then cotreated for 24 hr with 0.1% (v/v) DMSO (*lanes 1–3*) or 1 nM TCDD (*lanes 4–6*). mRNA expression was analyzed by RT-PCR as described in Experimental Procedures.

3 hr with 100 pM TGF-β_1, followed by cotreatment with 5 μg/ml ActD for 5 and 10 hr (Fig. 6, *left*, lanes *6–11*). The results showed that pretreatment for 3 hr was sufficient to restore TGF-β_1-mediated down-regulation of AHR mRNA expression. The graph of band intensities shown in Fig. 6, *left* (*lanes 6–11*) revealed that TGF-β_1 does not influence AHR mRNA stability (Fig. 6, *right*). A half-life for AHR mRNA of ~8 hr was observed in both control and TGF-β_1-treated cells.

Effect of TGF-β_1 on expression of TGF-β-sensitive genes in A549 cells. Because TGF-β_1 elicits both negative and positive regulatory activities on transcription of several genes, the analysis of TGF-β-sensitive genes may be useful in the control of down-regulation of AHR and CYP1 mRNA by TGF-β_1 in A549 cells. Thus, we analyzed the effects of TGF-β_1 on its own gene, which is autoinduced (29), and c-*myc*, which is down-regulated by TGF-β_1 (30). The cells were treated for 2, 8, and 24 hr with 100 pM TGF-β_1 (Fig. 7). As expected, TGF-β_1 led to a rapid decrease in c-*myc* mRNA expression, and this down-regulation was parallel to AHR and CYP1 mRNA repression (Fig. 3). In contrast to c-*myc*, expression of TGF-β_1 mRNA was autoinduced in these cells (Fig. 7).

Discussion

The human lung carcinoma A549 cells are well characterized in their sensitivity toward TGF-β_1. Both anchorage-dependent and -independent growth of A549 cells is inhibited by TGF-β_1 at a picomolar range (31); TGF-β_1 also regulates transcription of immediate-early genes as well as its own gene in this cell line (21, 29). As shown in this study, A549 cells express AHR mRNA and are TCDD sensitive because mRNA expression and enzyme activity of genes of the *Ah* gene battery are inducible in these cells by TCDD. A549 cells therefore represent a useful cell model for the study of the influence of TGF-β_1 on the expression of genes of the *Ah* gene battery. TGF-β_1 inhibited dose- and time-dependently TCDD-induced CYP1A1 mRNA expression and EROD activity in A549 cells (16, this study). To determine whether TGF-β_1 in general affects expression of genes of the *Ah* gene battery, we further analyzed the influence of TGF-β_1 on expression of CYP1B1, NMO-1, and UGT1A6 as well as AHR and Arnt. TGF-β_1 inhibited TCDD-induced CYP1B1 and NMO-1 mRNA expression dose-dependently at similar concentrations as inhibition of CYP1A1 mRNA. Furthermore, CYP1A1 and CYP1B1 mRNA expression was down-regulated by TGF-β_1 in cells not treated with TCDD. According to previous studies in A549 cells, UGT1A6 mRNA expression was observed at a constitutive level but was not inducible by TCDD (26), and TGF-β_1 had no influence on UGT1A6 mRNA. TGF-β_1 also repressed AHR mRNA expression, whereas Arnt mRNA level was unaffected. Because TGF-β_1 alters numerous cellular functions and processes, the effect on CYP1 and AHR expression can be interpreted as a result of a general inhibition of cellular processes rather than of specific effects. Therefore, we analyzed the expression of two TGF-β-sensitive genes, c-*myc* and TGF-β_1, as positive controls. In agreement with reported data (29, 30), we observed an induction in TGF-β_1 and an inhibition of c-*myc* mRNA expression. Be-

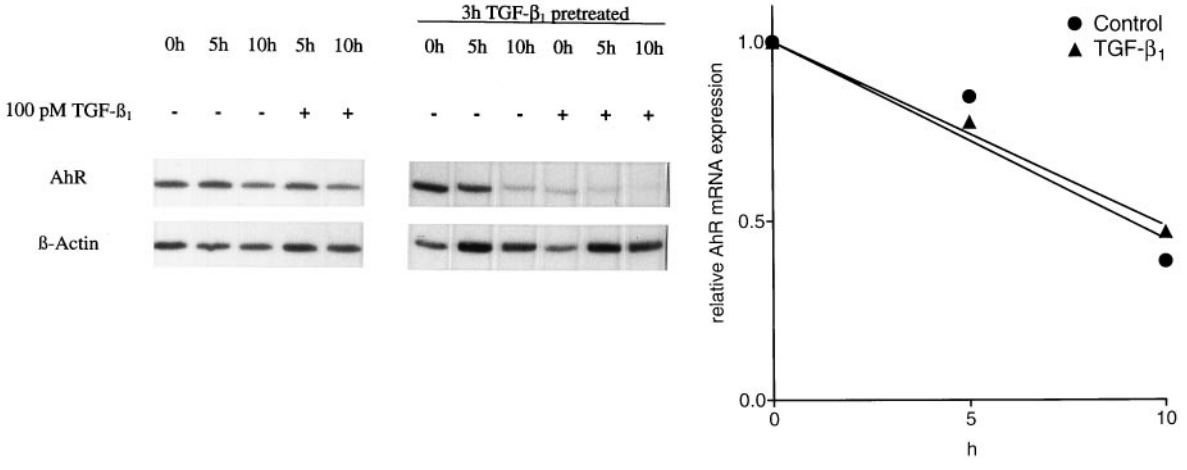

Fig. 6. Influence of TGF-β_1 on AHR mRNA stability in A549 cells. *Left*, cells were treated simultaneously with 100 pM TGF-β_1 and 5 μg/ml ActD for 5 or 10 hr (*lanes 1–5*) or pretreated with 100 pM TGF-β_1 for 3 hr and then cotreated with 5 μg/ml ActD for an additional 5 or 10 hr (*lanes 6–11*). Control cells received the respective vehicle solvent. AHR mRNA expression was analyzed by RT-PCR as described in Experimental Procedures. *Right*, band intensities shown on *left* (*lanes 6–11*) were normalized to respective controls and are given as relative expression to respective untreated cells (0 hr). Mean values of two independent experiments are given.

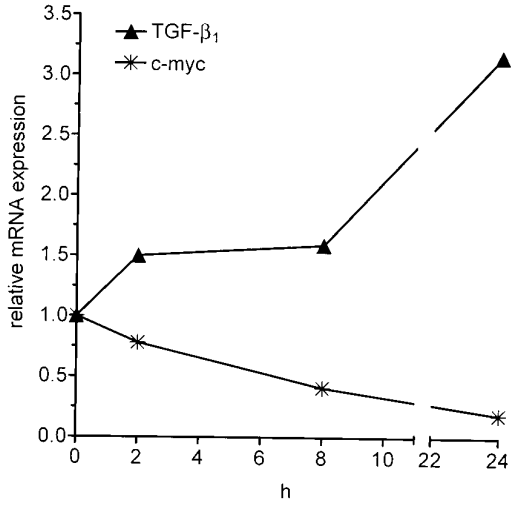

Fig. 7. Effect of TGF-β_1 on c-*myc* and TGF-β_1 gene expression. See legend to Fig. 3.

cause down-regulation of CYP1A1, CYP1B1, NMO-1, and AHR occurred at similar concentrations of TGF-β_1, our results favor a specific effect of TGF-β_1 on expression of genes of the *Ah* gene battery and AHR rather than an artificial one.

We also performed Western blot analyses to determine whether TGF-β_1 alters AHR expression, but we failed to detect the AHR in A549 cells. Proteins prepared from TCDD-sensitive MDA-MB 231 cells were used as positive controls, and a specific AHR band was identified in these cells (data not shown). Therefore, the most likely interpretation may be that the concentration of AHR in A549 cells was below the detection limit of our Western blot analyses. This assumption is supported by results of RT-PCR analyses revealing that the AHR mRNA level is significantly lower in A549 cells than in MDA-MB 231 cells (not shown). However, to demonstrate an effect of TGF-β_1 on AHR protein, transfection experiments were performed with the minimal dioxin responsive reporter construct pTX.DIR (27). The inhibition of TCDD-induced luciferase enzyme activity in cells cotreated for 40 hr with TGF-β_1 and TCDD indicates an effect on AHR protein expression and agrees with the observed inhibitory effect of TGF-β_1 on AHR mRNA expression.

Because AHR is the transcriptional regulator of TCDD-induced CYP1 mRNA expression, time-response studies were performed to examine whether down-regulation of AHR expression is required for inhibition of CYP1 mRNA expression. The results show that inhibition of TCDD-induced as well as basal CYP1 mRNA expression seems to be independent of down-regulation of AHR mRNA. A parallel decrease in these mRNAs was observed, leading to the hypothesis that TGF-β_1 is a direct negative regulator of expression of AHR and CYP1 genes. For further characterization of the mechanism of AHR down-regulation (e.g., whether TGF-β_1 elicits transcriptional or post-transcriptional effects), experiments with ActD and CHX were done. Simultaneous treatment with ActD or CHX abolished the inhibitory effect of TGF-β_1 on AHR mRNA expression, indicating the necessity of both transcription and translation of an as-yet-unknown negative regulator by TGF-β_1. Furthermore, experiments performed with ActD revealed that TGF-β_1 does not influence the stability of AHR mRNA, and a half-life of ~8 hr was found in control and TGF-β_1-treated cells. However, post-transcriptional effects of TGF-β_1 on AHR mRNA have to be verified in additional, appropriate experiments. Results obtained from experiments with CHX revealed that protein synthesis is also required for TGF-β_1-mediated repression of basal as well as TCDD-induced CYP1 mRNA expression and that CHX led to a superinduction of TCDD-induced CYP1A1 and CYP1B1 mRNA expression. Superinduction of CYP1A1 mRNA expression by CHX is a well-known effect (32) leading to the assumption of a constitutive negative regulator of CYP1A1 gene expression. Thus, both CYP1A1 and CYP1B1 genes seem to be negatively regulated by a similar mechanism.

Through computer research, we identified three different known NREs in the promoters of human CYP1A1 and AHR genes (33, 34). Responsiveness toward TGF-β has been shown for two of these NREs (Table 2). A Fos-binding sequence has been identified as TGF-β inhibitory element in the 5′-regulatory region of transin/stromelysin gene and other TGF-β-inhibited genes like urokinase and c-*myc* (19). The Fos protein is also necessary for positive regulation of transin/stromelysin expression induced by EGF. The specificity of Fos (e.g., positive or negative transcriptional regulation) seems to be mediated by heterodimerization with different members of the Jun family (20). Increased mRNA levels of the protooncogenes c-*fos*, c-*jun*, and *jun* B are early responses in TGF-β_1-treated A549 cells, and autoinduction of TGF-β_1 is mediated by the transcription factor AP-1 (21, 29). Therefore, TGF-β_1-induced down-regulation of AHR mRNA expression may be mediated by protein products of immediate early genes. Furthermore, TGF-β induction of c-*fos* was required for repression of transin/stromelysin expression

TABLE 2

Identification of putative NREs in promotor of AHR and CYP1A1 genes

AHR (33) and CYP1A1 promoter (34) were screened for known negative regulatory *cis*-acting elements.

NRE	CYP1A1	AHR	TGF-β^a
Rat Transin[b]	5′-GNNTTGGNGA nt −1447 to −1438 nt −127 to −118	5′-GNNTTGGNGA nt −1433 to −1424	+
Human IL-2[c]	5′-TGTcaAAa*ATGcAAA* nt −2632 to −2618	5′-TGatTcA*aATGTAcAt* nt −1151 to −1136	+
Human CYP1A1[d]	5′-GTGCTCTGCCAATCAAAGCAC nt −794 to −774	5′-GTaCTCTGaatAgCAAAGCAC nt −1046 to −1026	?

[a] TGF-β responsiveness: +, proven; ?, not shown.
[b] Consensus sequence for transin/stomelysin TGF-β inhibitory element: GNNTTGGNGA (20).
[c] Noncanonical AP-1/Oct-1 binding sites in human IL-2 gene: −87 5′-TGTGTAA*TATGTAAAA* (35). Putative Ap-1 [TGARTC/AA (36)] and Oct-1 binding site [5′-*ATGCAAAT* (36)] as described for IL-2 NRE are underlined or given in italics, respectively.
[d] Palindromic sequences identified in the NRE of human CYP1A1 (37) are double-underlined.
nt, nucleotides.

(20), which is consistent with the current results showing that transcription and translation of an as-yet-unidentified factor are necessary for down-regulation of AHR mRNA expression. IL-2 is another gene that is negatively regulated by TGF-β_1, and the NRE in human IL-2 promoter has been identified as noncanonical AP-1/Oct-1 binding site. Although consensus sequences of AP-1 and Oct-1 are degenerated, a binding of both factors to this sequence has been shown (35, 36). Similar degenerated noncanonical AP-1/Oct-1 binding sites lie within the CYP1A1 and AHR promoters (Table 2). The promoter of the human CYP1A1 gene contains another NRE capable of down-regulating a heterologous promoter, and specific as-yet-unidentified nuclear proteins have been shown to bind to a palindromic sequence identified in this NRE (37). The promoter of human AHR gene contains a similar palindromic sequence with an identity of 15 to 16 base pairs (Table 2), but TGF-β responsiveness of this element has yet to be shown. Taken together, different putative negative regulatory elements were found in the promoter of both CYP1A1 and AHR genes, which may give an explanation for observed parallel inhibition of mRNA expression by TGF-β_1. However, although the TGF-β inhibitory element of transin/stromelysin gene has been identified in the promoter of c-*myc* gene, this element seems to not be involved in negative regulation of c-*myc* by TGF-β_1 (20), indicating that negative regulation of AHR and CYP1 gene expression by TGF-β_1 may also be mediated by different mechanisms.

The physiological function of AHR is still unknown. An involvement of AHR in hepatic growth and development has been suggested on the basis of AHR-deficient mice that displayed reduced liver weights, transient microvesicular fatty metamorphosis, prolonged extramedullary hematopoiesis, and portal hypercellularity with thickening and fibrosis (38). An involvement of AHR in cell cycle progression has recently been proposed from *in vitro* studies. AHR-deficient mouse hepatoma cells exhibited a prolonged G1 phase. This effect was abolished in cells stably transfected with AHR, leading to the hypothesis that AHR is a modulator of cell cycle progression in Hepa 1c1c7 cells (39). TGF-β_1 is a potent inhibitor of cell cycle progression of many different cell types, and several cell cycle-regulating genes and proteins have been identified as targets for the growth inhibitory effect (22). In addition to other factors acting in early or late G1 phase, TGF-β_1 induces the rapid down-regulation of c-*myc* mRNA and protein levels thought to result in a cell cycle arrest in G1 phase. TGF-β_1 also inhibits c-*myc* mRNA expression in A549 cells; therefore, the growth-inhibitory effect of TGF-β_1 on these cells may be due to a common action of TGF-β_1 on expression of several cell cycle-regulating genes like c-*myc* and possibly AHR. The precise mechanism of TGF-β_1-induced inhibition of gene expression is more or less unknown. However, the current results indicate that TGF-β_1 seems to not influence stability of AHR mRNA, whereas it seems to induce rapid transcription and translation of a factor or factors with negative regulatory effects on expression of AHR and genes of *Ah* gene battery. This negative regulator or regulators remain to be identified in further experiments.

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