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Transforming Growth Factor-β1 Down-regulates Basal and Polycyclic Aromatic Hydrocarbon-Induced Cytochromes P-450 1A1 and 1A2 in Adult Human Hepatocytes in Primary Culture

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SUMMARY

The effects of interleukin (IL)-1β, IL-4, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-γ, and transforming growth factor (TGF)-β1 on cytochrome P-450 (CYP)1A expression and polycyclic aromatic hydrocarbon (PAH)-mediated induction in primary human hepatocyte cultures were determined. Most cytokines that were previously found to decrease basal CYP expression could counteract PAH induction of CYP1A mRNA and its associated ethoxyresorufin-*O*-deethylation (EROD) activity. IL-1β and TNF-α blocked 3-methylcholanthrene (3-MC)-induced EROD activity by up to 25 and 44%, respectively. IFN-α and IFN-γ antagonized EROD induction by up to 61 and 70%, respectively. TGF-β1 proved to be the most effective cytokine, because 72 hr of treatment with 2 ng/ml TGF-β1 produced nearly 100% inhibition of 3-MC- and benzo(*a*)pyrene-induced CYP1A1 and CYP1A2 mRNAs and EROD activity. Treatment with cycloheximide in combination with 3-MC led to superinduction of CYP1A mRNA, under which conditions TGF-β1 did not block induction, suggesting the requirement for protein synthesis for the suppressive effect of the cytokine. In addition, TGF-β1 augmented AP-1-binding activity, suggesting that *fos* and/or *jun* protooncogene products could be implicated in the response. Our results demonstrate that IL-1β, TNF-α, and IFNs antagonized PAH-mediated induction of *CYP1A* gene expression in human hepatocytes. In addition, we report the finding of a novel effect of TGF-β1, which was able to prevent CYP1A1 and -1A2 induction by two different PAHs.

CYP monooxygenases play an important role in the metabolism of drugs and other chemicals, including the activation of procarcinogens and the synthesis of endogenous compounds such as steroid hormones, fatty acids, and prostaglandins (1). *CYP* genes are under complex regulatory control exerted both by xenobiotics, such as environmental pollutants, and by physio-pathological status, including diet, age, and health status (2). CYPs belong to a supergene family, of which four gene families are primarily involved in xenobiotic biotransformation in mammals (1).

CYPs are known to be increased by xenobiotic inducers, such as PAHs, ethanol, phenobarbital, and rifampicin (3). The mechanisms by which CYP induction occurs are diverse, such that PAHs, phenobarbital, and glucocorticoids mainly induce transcriptional activation, whereas ethanol is responsible for increased stability of CYP2E1 apoprotein (3). The mechanism of PAH-mediated induction of CYPs from family 1A has been studied extensively. Dioxin, 3-MC, and BaP are able to increase transcription of *CYP1A1* and *-1A2* in rodents and humans, both in the liver and in cultured cells of hepatic origin (4, 5). This transcriptional activation occurs via a cytosolic receptor that, upon inducer binding, can translocate into the nucleus and interact with DNA response elements, termed XREs, located in the promoter regions of *CYP1A* genes (6). The increased transcription results in increases in both CYP1A mRNA accumulation and its associated EROD activity. XRE sequences have also been shown to interact with nonreceptor molecules involved in induction and/or basal activity of the *CYP1A1* gene (7, 8). In addition, other promoter sequences have been shown to mediate down-regulation of *CYP1A1* in rodent and human hepatocytes and/or in hepatoma cells (9–11).

CYP activities are altered during inflammation, both in laboratory animals and in humans (12). *In vivo* and *in vitro* experiments have provided evidence that several cytokines can produce decreased CYP levels. Recently, we reported inhibition

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ABBREVIATIONS: CYP, cytochrome P-450; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor; EROD, ethoxyresorufin-*O*-deethylase; PAH, polycyclic aromatic hydrocarbon; 3-MC, 3-methylcholanthrene; BaP, benzo(*a*)pyrene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; XRE, xenobiotic-responsive element; Ah, aryl hydrocarbon; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; AP-1, Activator Protein-1; C/EBP, CABT Enhancer Binding Protein.

of *CYP1A2*, *CYP2C*, *CYP2E1*, and *CYP3A* gene expression by IL-1β, IL-6, and TNF-α, the three major inflammatory cytokines, in human hepatocytes in primary culture (13). Other cytokines can influence CYP expression in cultured human hepatocytes. IFNs down-regulated human *CYP1A2* and *-2E1*, whereas IL-4, while having no effect on *CYP1A2*, was able to up-regulate *CYP2E1* (13). Another cytokine, TGF-β1, which has been reported to down-regulate albumin and haptoglobin expression in adult human hepatocytes and hepatoma cells (14), has not yet been tested for its ability to influence expression of human CYPs.

Because the levels of CYPs in organisms may determine the potential for either metabolic activation of drugs or decreased efficacy of therapeutic molecules, it is of importance to analyze the effect of both positive and negative regulators of *CYP* gene expression. However, the interaction between PAH inducers and cytokines remains poorly understood. The possibility of direct analysis of human hepatocytes in primary culture provides an appropriate *in vitro* system in which to test the combined effects of PAHs and cytokines. A recent report showed that induction of *CYP1A1* and *CYP1A2* by dioxin could be inhibited by IL-1β at the transcriptional level in primary rat hepatocyte cultures (15). IFN-α and IFN-γ could prevent induction of *CYP1A* by 3-MC in cultured human hepatocytes (16). In the present study, we have investigated the effects of seven cytokines (IL-1β, IL-4, IL-6, TNF-α, IFN-α, IFN-γ, and TGF-β1) on the induction by PAHs of CYP1A1 and -1A2 mRNAs and EROD activity in human hepatocytes in primary culture. We demonstrate that, among the tested cytokines, TGF-β1 is the strongest antagonist of human *CYP1A1* and *-1A2* induction by two distinct PAHs, i.e., 3-MC and BaP.

Materials and Methods

Recombinant cytokines and chemicals. Human recombinant IL-1β, IL-6, TNF-α, and IFN-α were purchased from Genzyme (Cambridge, UK). IL-4 was from Immugenex (Los Angeles, CA). Human recombinant IFN-γ was kindly provided by Roussel-Uclaf (Romainville, France). Platelet-derived human TGF-β1 was purchased from British Biotech (Oxon, UK). 7-Ethoxyresorufin, 3-MC, BaP, α-amanitin, and cycloheximide were from Sigma Chemical Co. (St. Louis, MO).

Cell isolation and culture. Seventeen adult human liver fragments (referred to as HL14 to HL30) were resected from healthy tissue surrounding primary or secondary tumors and were used for isolation of hepatocytes by the two-step collagenase perfusion method (17). All studies were done according to French laws and regulations and were approved by the National Ethics Committee. Cell viability, estimated by trypan blue exclusion, ranged between 75 and 85%. Hepatocytes were seeded on plastic dishes at a density of $63 \times 10^3/cm^2$, in a standard medium consisting of 75% (v/v) minimum essential medium/25% (v/v) medium 199 buffered with 0.22% (w/v) sodium bicarbonate and supplemented with 10 μg/ml bovine insulin, 1 mg/ml bovine serum albumin, and 10% (v/v) fetal calf serum. Treatment with cytokines began 15–24 hr after seeding, at the first medium renewal. The medium was deprived of serum and supplemented with 1 μM hydrocortisone hemisuccinate and was then changed every day. Cytokines were added at the following concentrations: IL-1β, 100 units/ml; IL-4, 150 units/ml; IL-6, 50 units/ml; TNF-α, 50 units/ml; IFN-α, 50 units/ml; IFN-γ, 50 units/ml; TGF-β1, 0.04–5 ng/ml. These concentrations were previously found to regulate basal CYP expression in primary cultured human hepatocytes (13) and/or acute-phase protein expression in both rat and human hepatocytes in primary culture (14, 18). At these concentrations, none of the cytokines induced morphological alterations. 3-MC and BaP were dissolved in dimethylsulfoxide and were added at concentrations of 5 μM and 1 μM, respectively. All culture conditions included 0.2% dimethylsulfoxide, a concentration having no influence on CYP expression. α-Amanitin was added at the concentration of 1 μg/ml for 17 hr. Cycloheximide (60 μg/ml) was added concomitantly with inducers and/or TGF-β1 for 17 hr.

Isolation of RNA and blot analysis. Hepatocyte monolayers were scraped in 0.1 M phosphate-buffered saline. Total RNA was prepared as described previously (19), dissolved in sterile water, and stored at −80°. For Northern blotting, 10 μg of each RNA sample were subjected to electrophoresis in a denaturing 6% (v/v) formaldehyde/1.2% (w/v) agarose gel and transferred onto Hybond-N^+ nylon filters (Amersham, Arlington Heights, IL). For dot blot analyses, dilutions of each RNA sample in a denaturing buffer (6%, v/v, formaldehyde, 450 mM sodium chloride, 44 mM sodium citrate) were adsorbed onto Hybond-N^+ nylon membranes by suction and were washed with 20× standard saline citrate (3 M sodium chloride, 0.3 M sodium citrate, pH 7). Equal RNA sample loading and integrity were routinely confirmed by ethidium bromide staining of minigels and filters after transfer or dot blotting. Prehybridization, hybridization, and washes were performed according to the method of Church and Gilbert (20). cDNA probes were ^{32}P-labeled by random priming. Filters were autoradiographed at −80°. Hybridization signals were quantified by densitometry and integration of unsaturated signals. CYP1A1 and CYP1A2 coding sequences were cloned after amplification of total liver cDNA by the polymerase chain reaction (21). The specificity of the cDNA probes was determined using cDNA either as a target or as a probe in hybridization experiments. Under our conditions, cross-reactivity of the probes with one another could be estimated as <4%. In addition, probing the same filters with both probes gave very dissimilar hybridization patterns for RNA from freshly isolated hepatocytes; CYP1A1 mRNA was detectable in only two of eight samples, whereas CYP1A2 was expressed in eight of eight samples.

EROD assay. EROD activity was measured essentially according to the method of Burke and co-workers (22, 23), in living hepatocyte monolayers, after 24, 48, and 72 hr of treatment.

Nuclear protein extracts and gel retardation assays. Nuclear extracts from hepatocytes were prepared, protein concentration was determined, and gel shift assays were performed as described by Cereghini *et al.* (24), except that the nuclear extracts were not dialyzed. Binding reactions were carried out in a 15-μl volume containing 1 mM sodium phosphate, pH 7.5, 0.1 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol, 10% (v/v) glycerol, 0.5 μg of poly(dI·dC), 0.5 μg of sonicated salmon sperm DNA, 1 mM $MgCl_2$, 10 mM spermidine, and 0.1–0.2 ng of ^{32}P-labeled, 5′-end, double-stranded oligonucleotide. The AP-1 binding site had the following sequence: 5′-CTAGTGATGAGTCAGCCGGATC-3′ (25). Double-stranded C/EBPα oligonucleotide (5′-GATCAAGCTGCAGATTGCGCAATCTGCAGCTTA-3′) (26) was used as a nonspecific competitor. Four micrograms of proteins were added to the reaction mixture, which was then incubated for 10 min on ice. The DNA-protein complexes were resolved on 6% acrylamide gels in 0.5× TBE (45 mM Tris-borate, 1.25 mM EDTA) at 22 mA for 2 hr. The gel was then fixed, dried, and subjected to autoradiography.

Statistical analysis. The Wilcoxon, matched-pairs, signed-rank test was used when experiments were repeated with more than six different cell populations, i.e., only for analysis of the effect of TGF-β1 on 3-MC induction of both EROD activity and CYP1A1/1A2 mRNA levels. This test allows comparisons to be performed throughout a set of cell populations, by comparing the average values obtained for each experimental condition (eight repeated determinations for EROD activity and single determinations for CYP1A1 and CYP1A2 mRNA hybridization signals).

Student's *t* test was used when fewer than six different cell populations were analyzed. In the latter case, each experiment was analyzed separately by comparing, for each condition, eight repeated determinations of EROD activity in the same cell population. Significance was set at a limit of <5% for the two tests. The specific sets of comparisons are described in the legends to the tables and figures.

Results

Effect of Cytokines on 3-MC-Induced EROD Activity

To analyze the effects of various cytokines on 3-MC-induced EROD activity, we incubated human hepatocytes with both cytokines and 3-MC for 72 hr. Results were obtained for five cell populations (HL14 to HL18) (Table 1). In all hepatocyte cultures, basal EROD activity was detectable throughout the experiment, ranging between 0.3 and 4.6 pmol/mg of protein/min. The effect of cytokines on basal EROD activity showed a tendency towards decreased expression, which was statistically significant ($p < 0.05$) in the majority of cases; the exception was IL-4, which had no effect. These observations are in agreement with our previous studies (13). EROD activity was induced by an average of 12.8-fold (five experiments, $p < 0.05$) by 3-MC treatment. IL-4 and IL-6 had no significant effect on EROD induction. The effects of IL-1β (HL15, 25% reduction) and TNF-α (HL14, 33% reduction) were significant in only one of three cases. IFN-α also decreased induced EROD activity (HL14, 61% reduction; HL17, 56% reduction; HL18, 59% reduction; $p < 0.05$). IFN-γ significantly antagonized EROD induction (HL14, 61% reduction; HL15, 70% reduction; HL17, 48% reduction; $p < 0.05$), and TGF-β1 had the strongest inhibitory effect (77, 85, 91, and 94% decreases). In view of its greatest efficacy, only this cytokine was studied further.

Dose-Response and Time Course Analyses of the Effect of TGF-β1 on 3-MC-Induced EROD Activity

To determine whether TGF-β1 acted in a dose-dependent manner, we conducted a dose-response analysis for one concentration of 3-MC (5 μM) with cells from two samples (HL19 and HL20). Similar results were obtained for both cell populations. Data from HL19 are presented. The dose-response analysis was performed for 72 hr of TGF-β1 treatment, with both 3-MC-treated and untreated cells. At that time point, basal and induced EROD activities were diminished in a dose-dependent manner (Fig. 1). TGF-β1 had a significant effect on EROD induction ($p < 0.01$) at a low dose (0.2 ng/ml), but its effect on basal EROD activity was not significant. A concentration of 2 ng/ml TGF-β1 completely prevented 3-MC induction ($p < 0.01$) and, in fact, decreased basal EROD levels ($p < 0.01$). TGF-β1 also decreased both basal and 3-MC-induced EROD activity after 48 hr of treatment, but its strongest effect was observed after 72 hr (Fig. 2). Time dependence of TGF-β1 effects on basal and/or induced EROD activity was observed with different cell populations (HL16 and HL19–HL24) (data not shown). Results for HL21 are illustrated in Fig. 2. Decreases in basal EROD activity were 59, 68, and 66% (Student's t test, $p < 0.0005$) after 24, 48, and 72 hr of treatment, respectively. 3-MC-induced EROD activity was reduced by 58 (Student's t test, $p < 0.005$), 70 (Student's t test, $p < 0.0005$), and 100% (Student's t test, $p < 0.0005$) at 24, 48, and 72 hr, respectively. The ability of TGF-β1 to antagonize induction by 3-MC was further analyzed with several additional cell populations (Table 2). 3-MC induction factors ranged between 6 and 42 for nine of 10 cell populations, with one population (HL22) being unresponsive. TGF-β1 (2 ng/ml) was able to block this induction by 75–100%.

Statistical analysis (Wilcoxon's test) showed significant decreases in EROD activity levels in TGF-β1-treated cells, compared with control untreated cells ($p < 0.01$). EROD activity differed significantly in TGF-β1-treated- and 3-MC/TGF-β1-treated cells ($p < 0.05$). In addition, EROD activity in cells treated with 3-MC plus TGF-β1 was significantly lower than that in 3-MC-treated cells ($p < 0.01$) but was not lower than that measured in control cells ($p > 0.05$). Differences between untreated and 3-MC-treated cells were also statistically significant ($p < 0.01$).

Effect of TGF-β1 on BaP-Induced EROD Activity

To determine whether the effects of TGF-β1 on EROD induction were restricted to 3-MC only, we made use of a

TABLE 1

Effects of cytokine treatment on basal and 3-MC-induced EROD activity in primary human hepatocytes

Five different samples were used. Cytokines were added for 72 hr at the following concentrations: IL-1β, 100 units/ml; IL-4, 150 units/ml; IL-6, 50 units/ml; TNF-α, 50 units/ml; TGF-β1, 2 ng/ml; IFN-α, 50 units/ml; IFN-γ, 50 units/ml. 3-MC was used at a concentration of 5 μM. Cytokines and 3-MC were added concomitantly, for 72 hr, beginning 24 hr after seeding. Each EROD activity value represents the mean ± standard error of eight cultures from the same cell population. Control refers to cell maintained in culture for the same time as treated cells.

Condition	EROD activity				
	HL14	HL15	HL16	HL17	HL18
			pmol/mg of protein/min		
Control	4.56 ± 0.56	3.38 ± 0.66	0.32 ± 0.05	0.33 ± 0.16	2.04 ± 0.37
3-MC	28.02 ± 3.90[b]	20.43 ± 4.87[b]	10.37 ± 0.76[b]	4.12 ± 0.67[b]	14.46 ± 4.11[b]
3-MC + IL-1β	24.00 ± 4.13	15.27 ± 4.59[c]	9.75 ± 0.70	2.5 ± 1.53	ND[a]
3-MC + IL-4	25.69 ± 6.05	30.08 ± 8.22	ND	4.03 ± 0.34	ND
3-MC + IL-6	25.92 ± 4.69	21.08 ± 2.87	ND	3.71 ± 1.87	ND
3-MC + TNF-α	18.77 ± 3.15[c]	16.40 ± 3.74	ND	2.32 ± 1.59	ND
3-MG + TGF-β1	6.56 ± 2.56	ND	1.53 ± 0.20	0.24 ± 0.10	1.23 ± 0.44
3-MG + IFN-α	10.80 ± 3.27[c]	18.20 ± 7.65	ND	1.79 ± 0.48[c]	5.93 ± 1.13[c]
3-MC + IFN-γ	10.89 ± 2.33[c]	6.04 ± 5.17[c]	10.36 ± 0.43	2.13 ± 0.44[c]	ND
IL-1β	1.63 ± 0.24[b]	2.56 ± 0.44	0.21 ± 0.05[b]	0.26 ± 0.05	ND
IL-4	5.20 ± 0.47	4.90 ± 0.50	ND	0.31 ± 0.06	ND
IL-6	3.86 ± 0.12[b]	4.80 ± 0.50	ND	0.14 ± 0.10[b]	ND
TNF-α	1.82 ± 0.24[b]	2.26 ± 0.16[b]	ND	0.22 ± 0.06[b]	ND
TGF-β1	2.72 ± 0.13	ND	0.24 ± 0.15	0.25 ± 0.06	0.32 ± 0.12[b]
IFN-α	2.32 ± 0.22[b]	4.76 ± 0.62	ND	0.33 ± 0.15	1.41 ± 0.45[b]
IFN-γ	2.84 ± 0.13[b]	2.08 ± 0.53[b]	0.25 ± 0.07	0.19 ± 0.03[b]	ND

[a] ND, not determined.

[b] Statistically different from control untreated cells (Student's t test, $p < 0.05$).

[c] Statistically different from 3-MC-treated cells (Student's t test, $p < 0.05$). The significance of the TGF-β1 effect is analyzed in the legend to Table 2.

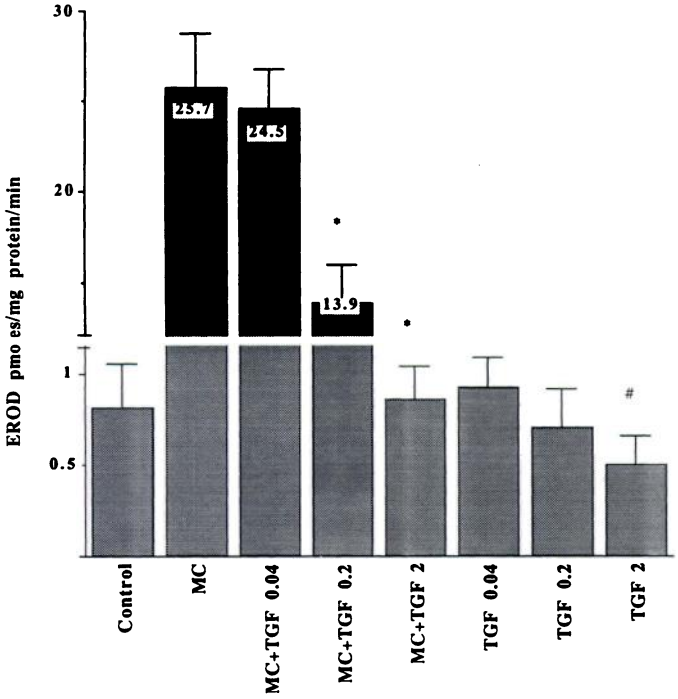

Fig. 1. Dose-response analysis of TGF-β1 effects on 3-MC-induced EROD activity. Primary human hepatocytes from HL19 were treated for 72 hr with various concentrations (indicated in ng/ml) of TGF-β1 combined with 3-MC (5 μM), beginning 24 hr after seeding. *Control*, cells maintained in culture for the same time as treated cells. Each value represents the mean ± standard error of eight samples. *, Statistically different from 3-MC-treated cells (Student's *t* test, $p < 0.01$). #, Statistically different from control untreated cells (Student's *t* test, $p < 0.01$).

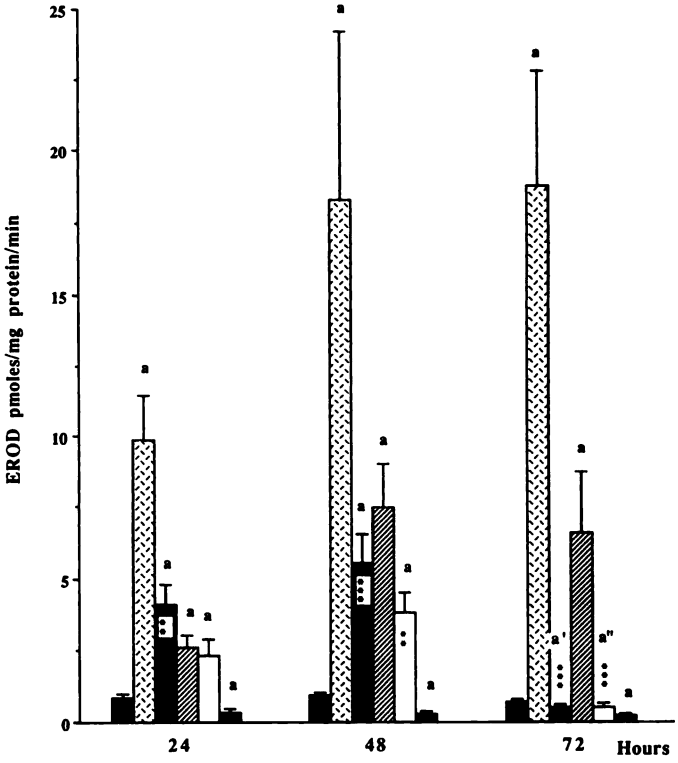

Fig. 2. Time course analysis of TGF-β1 effects on 3-MC- and BaP-induced EROD activity. Primary human hepatocytes from HL21 were treated with TGF-β1 (2 ng/ml), in combination with 3-MC (5 μM) or BaP (1 μM), for 24, 48, and 72 hr, beginning 24 hr after seeding. ■, Control cells kept in culture for the same time as treated cells; ⊠, 3-MC; ■, 3-MC plus TGF-β1; ▨, BaP; □, BaP plus TGF-β1; ■, TGF-β1. Each value represents the mean ± standard error of eight samples. *a*, *a'*, and *a"*, Statistically different from the corresponding control untreated cells (Student's *t* test; *a*, $p < 0.0005$; *a'*, $p < 0.005$; *a"*, $p < 0.05$). ***, **, Statistically different from the level in inducer-treated cells (Student's *t* test; ***, $p < 0.0005$; **, $p < 0.005$).

different PAH compound, BaP, another known ligand of the Ah receptor. BaP induction was statistically significant for HL21, HL23, and HL24 (average of 6.6-fold, $p < 0.01$). BaP-induced EROD activity was reduced by 10% (Student's *t* test, not significant), 48% (Student's *t* test, $p < 0.005$), and 100% (Student's *t* test, $p < 0.0005$) at 24, 48, and 72 hr, respectively, of exposure to TGF-β1 (Fig. 2). At the latter time point, EROD induction ranged between 1.8- and 9-fold, depending on the cell population ($p < 0.01$) (Table 2). TGF-β1 fully inhibited the BaP induction ($p < 0.01$). It is worth noting that, despite large variations in the BaP induction factors, TGF-β1 was always able to strongly antagonize induction. In addition, the fact that BaP induction factors were lower than those for 3-MC may be attributable to the fact that BaP, but not 3-MC, is metabolized, to some extent, by CYPs in hepatocytes.

Depression by TGF-β1 of PAH-Induced Levels of CYP1A mRNAs

The effect of TGF-β1 on 3-MC-induced CYP1A mRNAs at various time points was investigated with eight cell populations (HL16, HL19, HL20, HL24–HL27, and HL30) (Table 3). The effect of TGF-β1 on 3-MC induction was observed as early as 17 hr after the start of treatment. After 48 hr, 3-MC induced CYP1A1 mRNA by a factor ranging between 7-fold and >30-fold. TGF-β1 blocked 3-MC induction by at least 64% and up to 100%. TGF-β1 also produced strong decreases in 3-MC-induced CYP1A2 mRNA levels (57–98% reduction). Similar results were observed after 24 and 72 hr of treatment. Statistical analyses (Wilcoxon's test) showed significant differences for both CYP1A1 and CYP1A2 mRNA levels when comparisons were performed between cells treated with either 3-MC ($p < 0.05$) or 3-MC plus TGF-β1 ($p < 0.05$) and control untreated cells. In addition, after 3-MC plus TGF-β1 treatment CYP1A mRNA levels were significantly different from those found in 3-MC-treated cells ($p < 0.05$). Moreover, the effect of TGF-β1 treatment was statistically different from that of 3-MC plus TGF-β1 ($p < 0.05$). Although TGF-β1 was shown to decrease basal EROD activity ($p < 0.01$), no conclusion could be drawn concerning its effect on basal CYP1A1 and CYP1A2 mRNA levels, which were undetectable, both in control untreated cells and in TGF-β1-treated cells, in five of eight cell populations. Hence, the statistical analysis for TGF-β1-treated cells versus control untreated cells did not show a significant decrease in CYP1A mRNA levels.

The effect of BaP was investigated after 24, 48, and 72 hr. BaP was barely active on cells from HL25 and HL27 but was as strong an inducer as 3-MC in cells from HL26. It is possible that BaP was metabolized more quickly in cells from HL25 and HL27. In any event, TGF-β1 strongly antagonized induction of CYP1A1 (85% reduction) and CYP1A2 (98% reduction) mRNAs in HL26 cells. Therefore, similarly to its effect on EROD activity, TGF-β1 could block 3-MC and BaP induction of CYP1A mRNAs.

TABLE 2

Effects of TGF-β1 on basal, 3-MC-induced, and BaP-induced EROD activity in primary human hepatocytes

TGF-β1 (2 ng/ml) was added concomitantly with 3-MC (5 μM) or BaP (1 μM) for 72 hr, beginning 24 hr after seeding. Control refers to cells kept in culture for the same time as treated cells. Each value represents the mean ± standard error of eight cultures from the same cell population.

Condition	EROD activity									
	HL14	HL16	HL17	HL18	HL19	HL20	HL21	HL22	HL23	HL24
	pmol/mg of protein/min									
Control	4.56 ± 0.56	0.32 ± 0.05	0.33 ± 0.16	2.04 ± 0.37	0.93 ± 0.21	1.21 ± 0.27	0.72 ± 0.10	0.45 ± 0.30	1.39 ± 0.18	0.90 ± 0.09
3-MC[b]	28.02 ± 3.90	10.37 ± 0.76	4.12 ± 0.67	14.46 ± 4.11	26.23 ± 2.45	50.31 ± 6.69	18.75 ± 4.03	0.53 ± 0.08	9.27 ± 2.40	6.85 ± 2.82
3-MC + TGF-β1[c]	6.56 ± 2.56	1.53 ± 0.20	0.24 ± 0.10	1.23 ± 0.44	1.11 ± 0.21	0.73 ± 0.19	0.52 ± 0.09	0.35 ± 0.20	2.27 ± 1.00	0.33 ± 0.10
BaP	ND[a]	ND	ND	ND	ND	ND	6.56 ± 2.19[e]	0.70 ± 0.31	2.46 ± 0.98[e]	7.80 ± 0.75[e]
BaP + TGF-β1	ND	ND	ND	ND	ND	ND	0.51 ± 0.15[ef]	0.36 ± 0.18[f]	1.08 ± 0.05[ef]	0.18 ± 0.10[ef]
TGF-β1[bcd]	2.72 ± 0.13	0.24 ± 0.15	0.25 ± 0.06	0.32 ± 0.12	0.85 ± 0.17	0.08 ± 0.07	0.24 ± 0.05	0.30 ± 0.28	0.37 ± 0.14	0.50 ± 0.13

[a] ND, not determined.
[b] Statistically different from control untreated cells (Wilcoxon's test, $p < 0.01$).
[c] Statistically different from 3-MC-treated cells (Wilcoxon's test, $p < 0.01$).
[d] Statistically different from 3-MC/TGF-β1-treated cells (Wilcoxon's test, $p < 0.01$).
[e] Statistically different from control untreated cells (Student's t test, $p < 0.01$).
[f] Statistically different from BaP-treated cells (Student's t test, $p < 0.01$).

TABLE 3

Effects of TGF-β1 on CYP1A1 and CYP1A2 mRNA induction by 3-MC and BaP

Total RNA was prepared from cultures that had been treated with TGF-β1 and 3-MC or BaP for different lengths of time (17, 24, 48, or 72 hr, as described in the legend to Table 2). RNAs were analyzed by Northern blots and/or dot blots. Filters were hybridized with ^{32}P-labeled cDNA probes and autoradiographed. Relative RNA amounts were determined by densitometry.

Condition	Relative mRNA level															
	HL16			HL19		HL20, 72 hr	HL24, 17 hr	HL25			HL26		HL27			HL30, 72 hr
	24 hr	48 hr	72 hr	24 hr	48 hr			24 hr	48 hr	72 hr	24 hr	48 hr	24 hr	48 hr	72 hr	
CYP1A1																
Control	–[a]	–	–	–	–	0.1	–	0.4	0.3	0.7	0.1	0.24	–	–	–	–
3-MC[c]	2.9	2.0	2.2	2.2	3.0	6.6	4.0	9.3	9.1	12.5	8.4	5.0	0.7	0.7	1.0	5.0
3-MC + TGF-β1[cd]	1.9	0.7	0.6	1.2	0.6	3.7	2.1	4.0	2.9	3.2	3.0	1.8	0.6	–	–	2.2
BaP	ND[b]	ND	ND	ND	ND	ND	ND	0.4	0.8	0.6	0.8	2.4	–	–	–	ND
BaP + TGF-β1	ND	ND	ND	ND	ND	ND	ND	0.4	0.4	0.4	0.16	0.36	–	–	–	ND
TGF-β1[de]	–	–	–	–	–	–	–	0.3	0.2	0.2	–	–	–	–	–	–
CYP1A2																
Control	–	–	–	–	–	0.2	–	0.3	0.2	0.3	0.24	0.5	–	–	–	–
3-MC[c]	3.0	2.8	2.3	10	11.5	7.4	3.4	9.3	10.2	13.4	9.1	8.95	0.2	0.7	1.0	10
3-MC + TGF-β1[cd]	1.6	0.5	0.4	3.1	2.4	1.8	2.5	0.3	0.2	0.3	2.1	2.2	0.05	0.3	0.1	4.0
BaP	ND	ND	ND	ND	ND	ND	ND	0.3	0.35	0.3	0.8	9.2	–	0.2	0.4	ND
BaP + TGF-β1	ND	ND	ND	ND	ND	ND	ND	0.3	0.2	0.2	–	0.2	–	–	0.1	ND
TGF-β1[de]	–	–	–	–	–	0.1	–	0.2	0.1	0.15	–	0.1	–	–	–	–

[a] –, Undetectable.
[b] ND, not determined.
[c] Statistically different from control untreated cells (Wilcoxon's test, $p < 0.05$).
[d] Statistically different from 3-MC-treated cells (Wilcoxon's test, $p < 0.05$).
[e] Statistically different from 3-MC/TGF-β1-treated cells (Wilcoxon's test, $p < 0.05$).

Effects of Sequential Additions of TGF-β1 and PAH on CYP1A Expression

EROD activity. To determine the effects of sequential addition of TGF-β1 and PAH to the cells, we added TGF-β1, 24 or 48 hr before 3-MC or BaP addition, to cells from HL19. EROD activity was measured after 48 hr (Fig. 3A) or 72 hr (Fig. 3B) of treatment, corresponding to 72 and 96 hr of culture. We also performed the reverse experiment by adding TGF-β1 24 or 48 hr after 3-MC or BaP addition. When TGF-β1 was added to the cells 24 hr before 3-MC addition, 3-MC induction was only partially blocked (condition 8 is statistically different from condition 9, Student's t test, $p < 0.005$) (Fig. 3A). However, a 48-hr pretreatment of the cells with TGF-β1 fully prevented any induction (condition 8 is not statistically different from condition 9, Student's t test) (Fig. 3B). When TGF-β1 was added to cells that had been pretreated with 3-MC for 24 or 48 hr, induced EROD activity decreased more quickly (conditions 4 and 5, Student's t test, $p < 0.0005$) (Fig. 3), and to a lower level for identical time points, than when 3-MC was withdrawn in the absence of TGF-β1 (condition 5 is statistically different from condition 6, Student's t test, $p < 0.005$ in Fig. 3A and $p < 0.0005$ in Fig. 3B). Removal of TGF-β1 after 24 hr of treatment with both TGF-β1 and 3-MC still allowed a slight induction of EROD activity in the continued presence of 3-MC (23% of the induced level observed with 3-MC alone), but a very modest induction was observed when TGF-β1 was removed after 48 hr (condition 9 is statistically different from condition 10, Student's t test, $p < 0.0005$ in Fig. 3A and $p < 0.05$ in Fig. 3B), corroborating the results described above. Similar results were obtained with cells from HL20 that were treated with either 3-MC or BaP (data not shown); however, when TGF-β1 was added to cells that had been pretreated with BaP for 48 or 72 hr, induced EROD activity decreased more slowly, and to a higher level for identical time points, than when BaP was withdrawn in the absence of TGF-β1. The origin of the difference with 3-MC is unknown. Taken together, our results demonstrate that longer treatments (48 hr) of the cells with

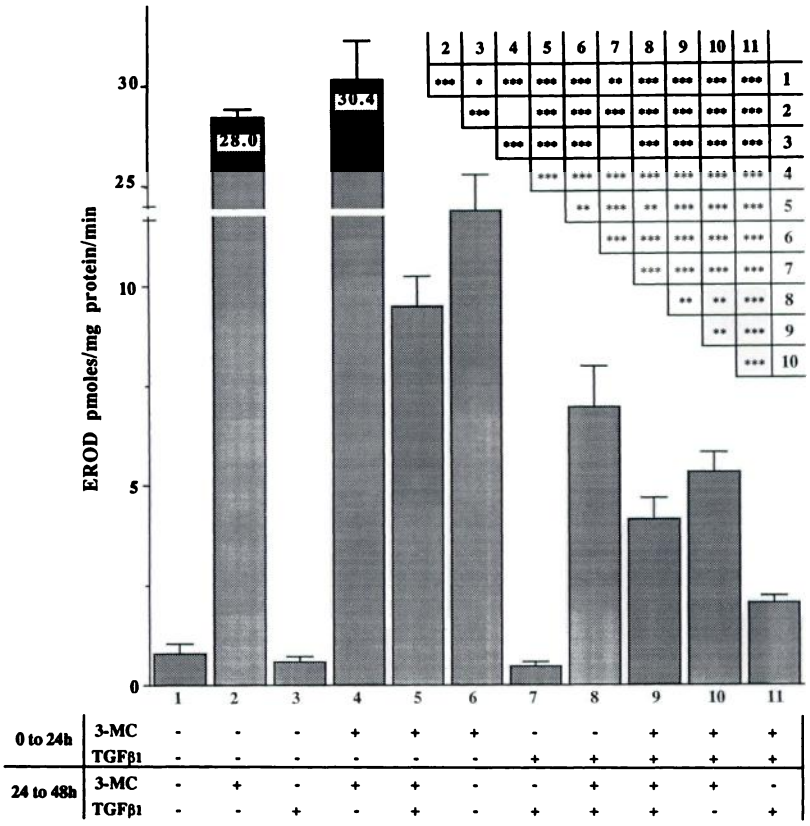

Fig. 3A. Effect of sequential addition of TGF-β1 and 3-MC on 3-MC-induced EROD activity. Primary human hepatocytes from HL19 were treated with TGF-β1 (2 ng/ml) and 3-MC (5 μM). TGF-β1 was added either 24 hr before or 24 hr after 3-MC (A) or 48 hr before or 48 hr after 3-MC (B). EROD activity was determined 48 hr (A) or 72 hr (B) after the beginning of treatment (which was started 24 hr after seeding). Each value represents the mean ± standard error of eight samples. +, Presence of TGF-β1 or 3-MC; −, absence of TGF-β1 or 3-MC. *Inset tables*, statistical analysis (Student's *t* test) performed by comparison of each condition with all of the other conditions. ***, **, and *, Significance (***, $p < 0.0005$; **, $p < 0.005$; *, $p < 0.05$). *Empty squares*, absence of significance.

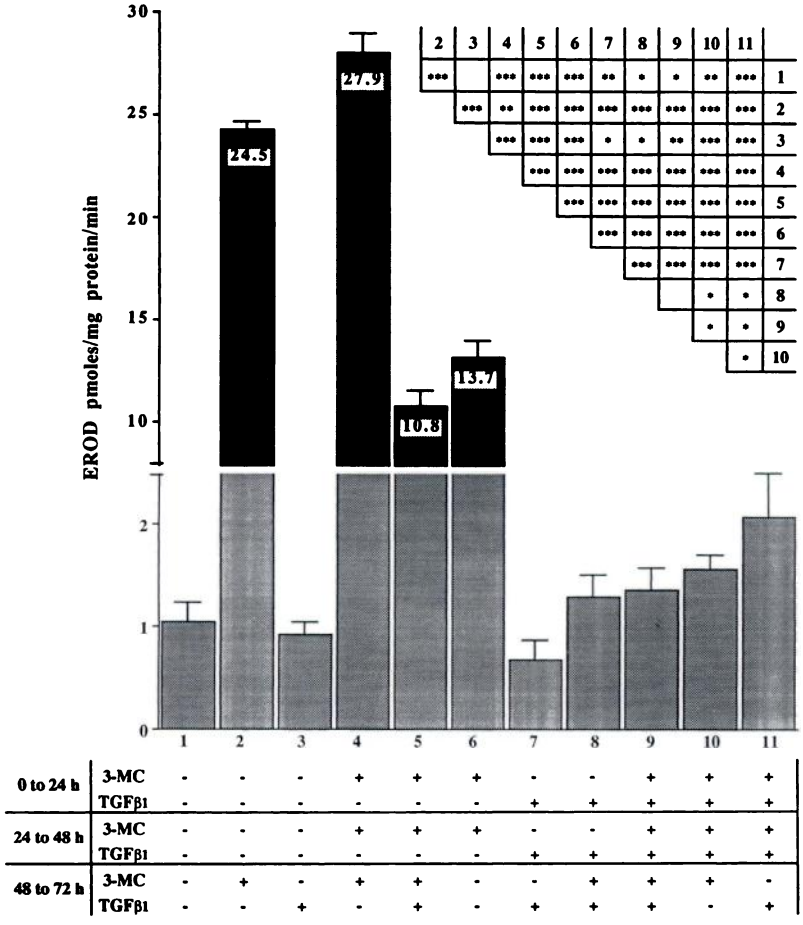

Fig. 3B. Continued

TGF-β1 lead to more severe blockades in induction by PAH compounds than do shorter treatments (24 hr), regardless of TGF-β1 addition time.

RNA levels. CYP1A1 and CYP1A2 mRNAs were analyzed after addition of TGF-β1, either before or after the inducer, in hepatocytes from the same sample (HL19) as used for analysis of EROD activity (Fig. 4). Although this hybridization experiment was performed only once, precluding the possibility of conducting a statistical analysis of the results, it should be noted that the statistical analysis performed in the case of EROD activity (Fig. 3) in cells from the same sample demonstrated highly significant results. Moreover, there was a good agreement between EROD activity data and RNA levels. Nevertheless, the densitometric analysis should be seen only as indicative.

The 3-MC induction of mRNA levels was partially blocked (69 and 32% of maximal levels with 3-MC alone for CYP1A1 and CYP1A2, respectively) when TGF-β1 was added to the cells 24 hr before 3-MC addition. When TGF-β1 was added to cells that had been pretreated with 3-MC for 24 hr, the induced CYP1A mRNA level was also lower than with 3-MC alone. Removal of TGF-β1 after 24 hr of treatment with both TGF-β1 and 3-MC, in the continued presence of 3-MC, still allowed full induction of CYP1A1 mRNA and 66% of full induction for CYP1A2 mRNA. Similarly to the results obtained for EROD activity, partial inhibition of 3-MC induction occurred at the mRNA level in cells treated with TGF-β1 either before or after exposure to 3-MC. However, removal of TGF-β1 from cells treated for only 24 hr allowed almost full induction by 3-MC.

Effects of Transcription and Protein Synthesis Inhibitors on the Down-Regulation by TGF-β1 of CYP1A Induction

To determine whether inhibition of transcription by RNA polymerase II could affect induction, we incubated human liver parenchymal cells (HL28) with α-amanitin or actinomycin D either 2 hr before or concomitantly with 3-MC or BaP, and EROD activity was measured 17 or 24 hr later. Under these conditions, BaP induction was abolished (0.8- versus 9.3-fold (α-amanatin) and 1.5- versus 9.8-fold induction actinomycin D), whereas 3-MC induction was strongly reduced (2.3- versus 22-fold (α-amanatin) and 2.5- versus 26-fold induction actinomycin D) (data not shown), suggesting a strong pretranslational effect of PAHs on hepatocytes, as reported previously (15).

Cycloheximide has been shown to induce CYP1A mRNA levels in the absence of inducers and, in addition, it was able to superinduce CYP1A mRNA accumulation in the presence of PAH inducers (27). When added to human hepatocytes for 17 hr, cycloheximide alone was able to induce both CYP1A1 and CYP1A2 mRNAs above control levels (HL24) (Fig. 5) and led to a superinduction (2.1 and 2.7 times the induced levels ob-

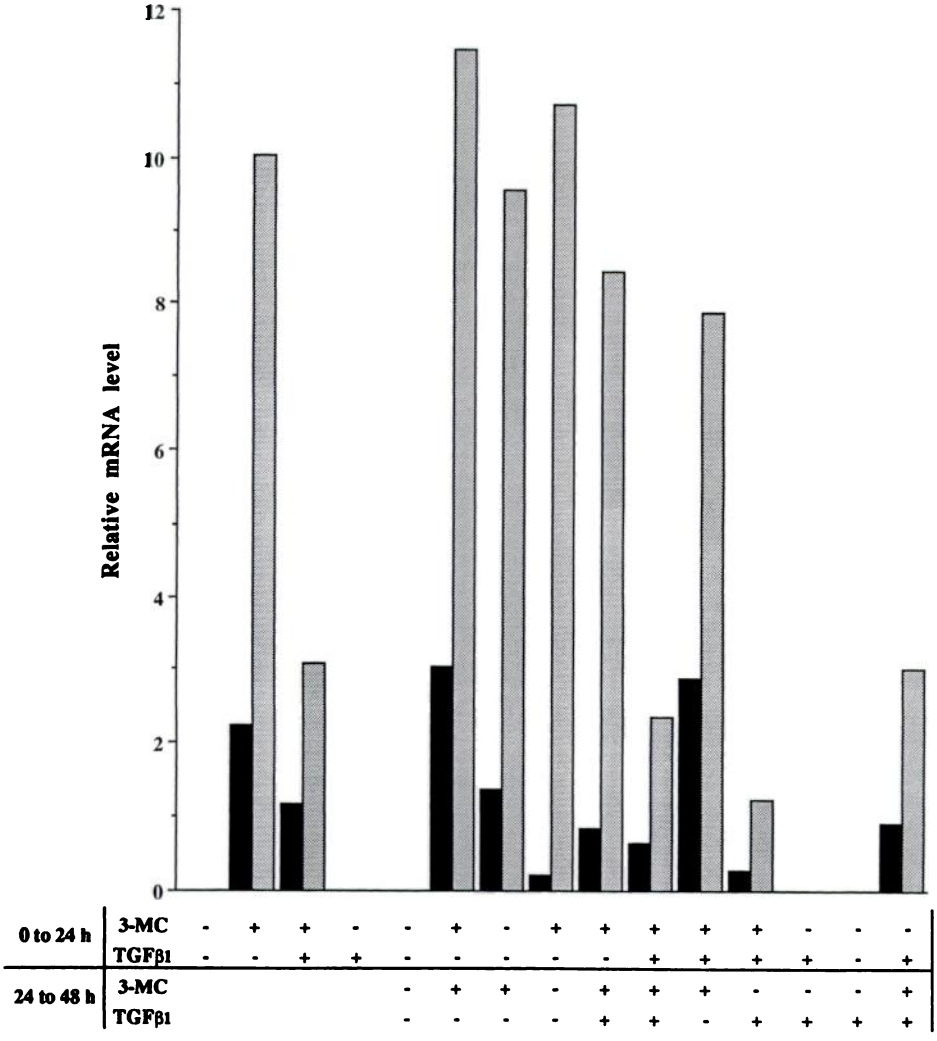

Fig. 4. Effect of sequential addition of TGF-β1 and 3-MC on CYP1A1 (■) and CYP1A2 (□) mRNA induction. Primary human hepatocytes from HL19 were treated with TGF-β1 (2 ng/ml) and 3-MC (5 μM). TGF-β1 was added either 24 hr before or 24 hr after 3-MC addition. Total RNA was prepared after 24 or 48 hr of treatment, and CYP1A mRNA expression was analyzed by Northern blots and/or dot blots. Filters were hybridized with ^{32}P-labeled cDNA probes and autoradiographed. Relative RNA amounts were determined by densitometry. +, Presence of TGF-β1 or 3-MC; −, absence of TGF-β1 or 3-MC.

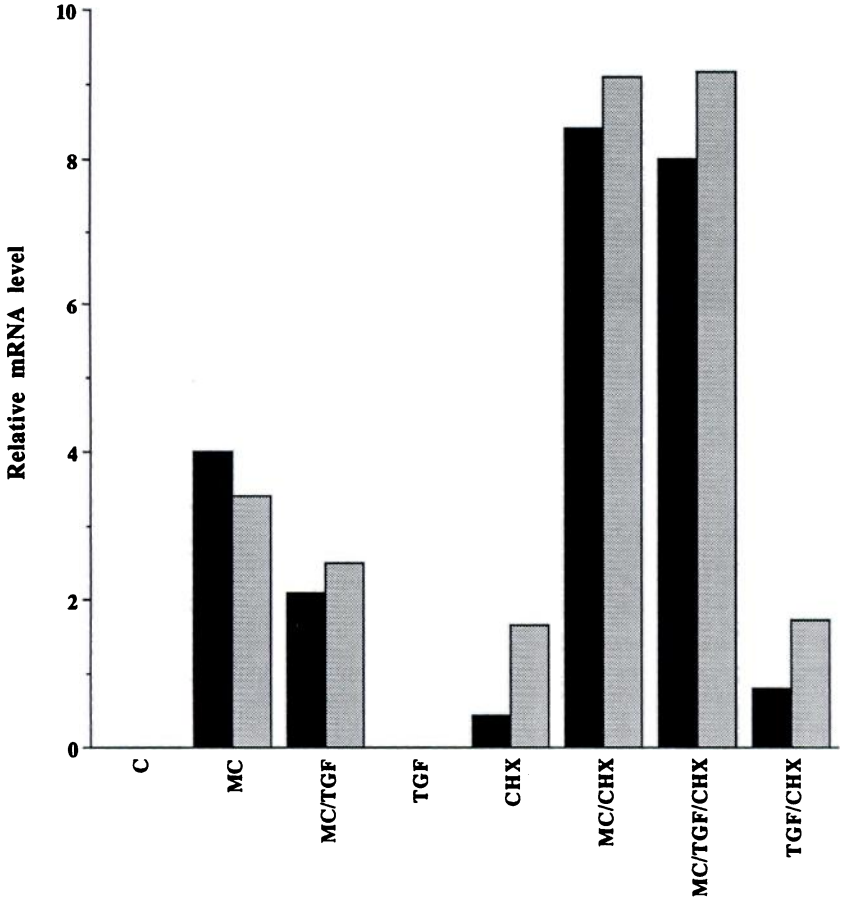

Fig. 5. Effect of protein synthesis inhibitors on the down-regulation of 3-MC-induced CYP1A1 (■) and CYP1A2 (▩) mRNAs by TGF-β1. Primary human hepatocytes from HL24 were treated concomitantly with TGF-β1 (2 ng/ml), 3-MC (5 μM), and cycloheximide (*CHX*) (60 μg/ml). Total RNA was prepared after 17 hr of treatment, and CYP1A mRNA expression was analyzed by Northern blots and/or dot blots. Filters were hybridized with ^{32}P-labeled cDNA probes and autoradiographed. Relative RNA amounts were determined by densitometry. *C*, control.

tained with 3-MC alone for CYP1A1 and CYP1A2, respectively) of the mRNAs in cells treated concomitantly with 3-MC. TGF-β1 did not influence the effect of cycloheximide, regardless of the presence of 3-MC, suggesting a requirement for protein synthesis for the effect of TGF-β1 to take place.

Influence of TGF-β1 on Nuclear Protein Binding to the AP-1 Site

It was shown that TGF-β1 could induce Fos and Jun proteins that were able to bind to a consensus AP-1 binding site (28). Therefore, some of the effects of the cytokine could be mediated through increased expression of AP-1-binding protein species. To determine whether the antagonistic effect of TGF-β1 on 3-MC induction could be correlated with increased AP-1 binding, we analyzed binding to a canonical AP-1 site (derived from the PAH-inducible quinone reductase gene) (25) by gel retardation with nuclear extracts from human hepatocytes cultivated for 72 hr in the presence or absence of TGF-β1 and/or 3-MC. Fig. 6, *HL17* and *HL29*, shows that the basal level of DNA-binding activity was very low, whereas it was induced in control cultures. 3-MC had no effect on AP-1 binding. In contrast, TGF-β1 induced a strong increase in the specifically retarded fraction, either alone or in combination with 3-MC. In addition, increased AP-1 binding in response to TGF-β1 was observed with nuclear extracts from one additional sample (HL18) (data not shown). Fig. 6, *HL30*, shows a low basal AP-1 activity and, again, a spontaneous increase in culture that was not influenced by 3-MC. No increase in the DNA-binding activity was observed as a consequence of TGF-β1 treatment, either alone or in combination with 3-MC. However, in the latter case, compared with freshly isolated cells, the level of AP-1-binding activity attained spontaneously in culture was much higher than that for samples HL17, HL18, and HL29. Therefore, the apparent lack of effect of TGF-β1 could be due to a high control AP-1-binding level.

Discussion

In a previous work, we showed that the major inflammatory cytokines, i.e., IL-1β, IL-6, and TNF-α, depressed, albeit to various extents, several CYPs (including CYP1A2) in primary human hepatocyte cultures (13). In the present study, we have demonstrated that most of the cytokines known to decrease basal expression of several human CYPs in primary hepatocyte cultures can also antagonize induction of *CYP1A* genes by PAHs. In addition, we provide the first demonstration that TGF-β1 can both decrease basal *CYP1A* gene expression and strongly inhibit its induction by PAHs. This cytokine was used at a concentration previously reported to mediate inhibition of albumin secretion (14), and we found it to be similarly active in the present study (data not shown). No morphological changes were observed throughout the treatment, indicating that decreased *CYP1A* expression did not reflect cell damage.

The antagonistic activities of the tested cytokines on PAH induction did not correlate with their abilities to negatively influence basal *CYP1A* expression. Regardless of the mechanistic interpretation, this observation suggests uncoupling between basal mRNA accumulation and PAH-induced mRNA levels. This may not be surprising, because XRE sequences

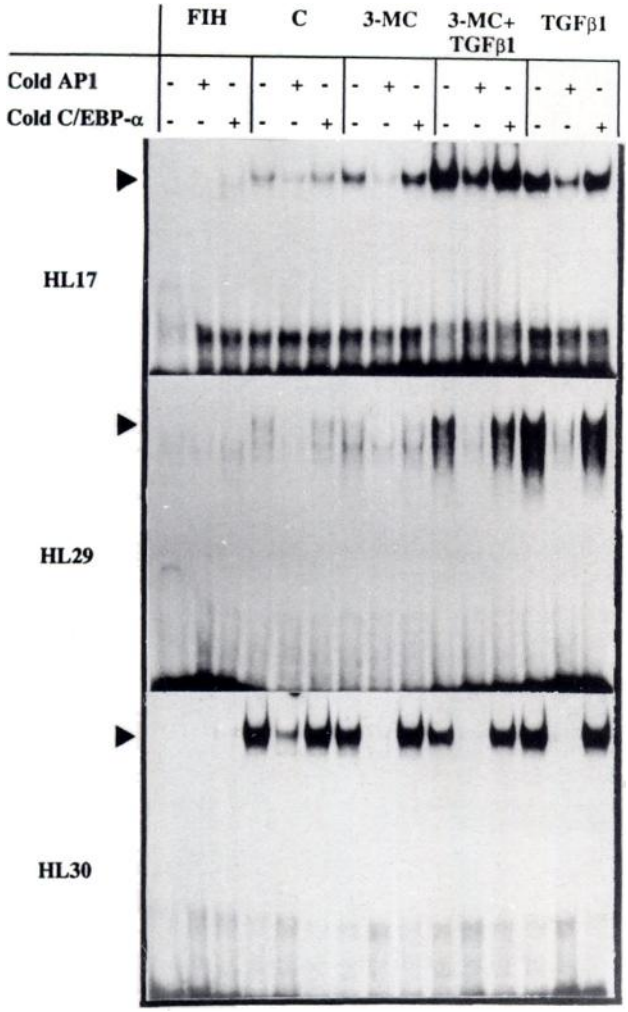

Fig. 6. Influence of TGF-β1 on nuclear protein binding to the AP-1 site. Nuclear protein extracts from three cell populations (HL17, HL29, and HL30) were prepared and resolved on acrylamide gels, as described in Materials and Methods, in the absence (−) or presence (+) of a 25-fold excess of unlabeled specific (AP-1) or nonspecific (C/EBPα) double-stranded oligonucleotide competitor. *Arrowheads*, position of the specifically retarded protein-DNA complex. *FIH*, freshly isolated hepatocytes; *C*, control untreated hepatocytes.

have been shown to direct dioxin-mediated induction in a context distinct from that of the promoter region of the *CYP1A1* gene, i.e., linked to a thymidine kinase promoter (29). In addition, it is possible that the level of mRNA accumulation attained upon induction partly reflects message stabilization. In this regard, PAH induction of the *CYP1A2* gene in primary rat hepatocytes was initially shown to occur through a post-transcriptional mechanism, at variance with induction of the *CYP1A1* gene in the same cells (30, 31). However, it was more recently shown that some transcriptional activation occurred in response to PAHs, despite the fact that XRE sequences have not been identified in the proximal promoter regions of the *CYP1A2* gene (15). Therefore, it is entirely possible that the negative effects of cytokines on CYP1A1 and CYP1A2 induction occur via distinct mechanisms. Indeed, if PAH-mediated transcriptional activation were to require XRE sequences only in the case of the *CYP1A1* gene, it is likely that antagonism by cytokines would require other sequences, presumably conserved in both the *CYP1A1* and *CYP1A2* genes. However, in the absence of the measurement of transcription rates, we cannot conclude that the antagonistic activities of the cytokines on either *CYP1A* gene occur at the level of transcription, despite the finding that PAH-mediated induction was very likely transcriptional, because it was blocked by α-amanitin treatment of the cells (data not shown). Nevertheless, by analogy with their effects on other cell types, it is likely that cytokines, upon binding to hepatocyte surface receptors, activate multiple signaling pathways (32), thereby leading to specific effets on *CYP1A* gene regulation.

The fact that IL-1β, TNF-α, IFN-α, IFN-γ, and TGF-β1 were able to negatively influence *CYP1A* expression stresses the importance of the effect of inflammatory mediators on detoxication enzymes. The fact that the tested cytokines were not equally effective suggests that they do not act through identical pathways. IL-1β, which was previously shown to strongly inhibit 3-MC induction of *CYP1A1* and *CYP1A2* transcription and mRNA accumulation in rat hepatocytes (15), had only a limited effect on 3-MC induction of *CYP1A* in human hepatocytes. IL-6 had no effect on rat *CYP1A* induction by PAHs (15) and had at best a minor inhibitory effect on basal EROD activity (HL14 and HL17 samples gave significant results). In addition, our initial survey of the effects of IL-6 on basal EROD activity in seven human cell populations did not show any significant inhibitory effect on basal EROD activity, although the effect on basal EROD activity might have been significant in some of the individual cases (13). TNF-α, which was a strong inhibitor of basal CYP expression (13), was weakly effective at blocking induction by PAHs. IFNs strongly affected basal *CYP1A* expression and had a strong antagonistic effect on induction. Similar observations have been made by others (15). Remarkably, IL-4 had no effect on basal or induced EROD activity. In addition, it was the only cytokine found to induce expression of one CYP mRNA, namely that encoding human CYP2E1 (13).

The most effective cytokine, TGF-β1, could antagonize induction in all of the analyzed cell populations. The inhibitory effect was observed whatever the magnitude of induction, both on EROD activity and on mRNA levels, and occurred in a dose-dependent (EROD activity) and time-dependent (EROD activity and mRNA level) manner. Thus, induction was completely blocked after 72 hr of treatment with either inducer (3-MC or BaP) and 2 ng/ml TGF-β1, which also decreased basal EROD activity. This effect was specific, inasmuch as TGF-β1 did not seem to influence basal expression of CYP2E1 and CYP3A mRNAs, which were not inducible by PAHs (data not shown). Interestingly, TGF-β1 was also shown to antagonize both basal and dibutyryl-cAMP-induced expression of the aromatase gene in human fetal hepatocytes at doses ranging between 2 and 5 ng/ml, indicating that fetal hepatocytes are also competent for cytokine-mediated antagonism of CYP activities (33). However, TGF-β1 was found to be ineffective on rat hepatocytes in primary culture (15). In our hands, it proved to be toxic to rat hepatocytes (at doses of 0.2 and 2.0 ng/ml) and, therefore, we could not examine potential effects on rat *CYP1A* genes.[1] The differences in sensitivity to TGF-β1 may be due to differences in the activity/origin of the cytokine (porcine versus human TGF-β1 in our study).

To obtain some insight into the mechanism(s) by which TGF-β1 was able to antagonize *CYP1A* gene induction by

[1] Z. Abdel-Razzak and L. Corcos, unpublished observations.

PAHs in human hepatocytes, we performed sequential additions of TGF-β1 and 3-MC. The cytokine produced an effect within 17 hr of addition to the cells but required 72 hr to be maximally effective. Therefore, it seems that, rather than a strict dominance over the effect of inducers, TGF-β1 may modify the steady state level of endogenous factors required for the induction (and/or basal expression) to take place. Alternatively, TGF-β1 could influence the cellular level of the inducer. However, this would require a mechanism distinct from that leading to increased metabolism of the PAH compound, because 3-MC is known to have a very long half-life in hepatic cells. The observation that blockade of 3-MC induction occurs in cells either pretreated or post-treated with TGF-β1 is in agreement with the notion that TGF-β1 could modify either the level or the activity of a factor required for induction. In this regard, binding of the Ah receptor to its target XRE sequences could be modulated by TGF-β1, resulting in impaired transcriptional activation of *CYP1A* genes in response to PAHs. However, our preliminary investigations failed to demonstrate any effect of TGF-β1 on Ah receptor binding to XRE sequences in human hepatocytes.[2] Conceivably, other XRE-binding proteins, such as the Ah receptor nuclear translocator protein (34) or C/EBPα (7), required for basal and/or induced CYP1A expression could be affected by TGF-β1 treatment. Alternatively, factor binding to the recently described, negative regulatory elements located in the promoter region of the human *CYP1A1* gene (11) could be activated as a consequence of TGF-β1 treatment.

Another type of negative regulation of PAH-mediated induction was recently described, showing that TPA, which can induce AP-1 activity, could block dioxin-mediated induction of murine CYP1A1 enzyme activity and mRNA accumulation (35, 36). Additional experiments led those authors to propose the involvement of protein kinase C in increasing the DNA binding affinity of the Ah receptor upon phosphorylation (35, 37). The possibility of other effects, mediated by AP-1, might have been overlooked in explaining the block of CYP1A induction by PAH compounds. Possibly, these could involve negative regulation via AP-1 binding to target response elements. These sequences need not be in the promoter regions of *CYP1A* genes but could belong to control regions of genes whose products are involved in the expression of *CYP1A* genes. Induction of AP-1-binding activity after 72 hr of treatment with TGF-β1 is correlated with increased mRNA levels for c-*jun*, *jun*-B, and *jun*-D, suggesting the occurrence of *de novo* synthesis of the proteins, rather than only activation of the DNA binding activity of preexisting molecules (data not shown). The possibility of a correlation between the effect of TGF-β1 on 3-MC induction of CYP1A and increased AP-1-binding activity, although observed in only three of four samples, supports the idea that increased AP-1-binding activity could be one of the primary determinants of TGF-β1 action. Indeed, AP-1 is able to confer both TPA and TGF-β1 responsiveness upon binding to TPA-responsive element-like elements, some of which are located in the promoter region of the TGF-β1 gene itself (38). Moreover, negative regulation mediated by TGF-β1 on expression of the stromelysin gene in rat fibroblasts was clearly shown to involve increased expression of the c-*fos* and *jun*-B genes, the products of which were able to bind two unrelated sequences, namely the TPA-responsive element (AP-1) site and a TGF-β1 inhibitory element with the consensus sequence GNNTTGGNGA (39). Furthermore, we have identified such a sequence in the promoter region of the human *CYP1A1* gene and are currently involved in determining its functional role in the antagonism of 3-MC induction by TGF-β1.

Clearly, the mechanisms by which TGF-β1 regulates expression of human *CYP* genes warrant further investigations. The possibility of a positive autoregulatory loop for the TGF-β1 gene itself may add an extra level of complexity to the regulation of detoxication enzymes, as well as other differentiated hepatic functions (38). In addition, TGF-β1 could also regulate expression of other cytokine-encoding genes whose products could participate, in concert with the effect of TGF-β1, in *CYP1A* gene regulation. Targeted disruption of the murine TGF-β1 gene resulted in a strong increase in expression of the TNF-α and IFN-γ genes and a slight increase in expression of the IL-1β gene in the liver, suggesting repression of these cytokine-encoding genes by TGF-β1 (40). Such observations, if transposable to human hepatocytes, would seem to suggest distinct pathways for TGF-β1 versus TNF-α or IFN-γ inhibition of PAH-mediated induction of *CYP1A* genes in these cells, because TNF-α and IFN-γ would not need to be expressed for the repressive effect of TGF-β1 to occur, although they are able to antagonize induction by themselves. Analysis of the possible cross-talk between cytokines, as well as determination of their effects on expression and induction of other members of the so-called "Ah gene battery," should greatly improve our understanding of how detoxication enzymes are regulated by both xenobiotics and inflammatory mediators.

[2] L. Corcos and Z. Abdel-Razzak, unpublished observations.

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