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Aryl Hydrocarbon Receptor Regulation of Cytochrome P4501B1 in Rat Mammary Fibroblasts: Evidence for Transcriptional Repression by Glucocorticoids

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ABSTRACT

Cytochrome P450 1B1 (CYP1B1), which actively metabolizes polycyclic aromatic hydrocarbons, is regulated by the aryl hydrocarbon receptor (AhR) in primary cultures of rat mammary fibroblasts (RMF) and rat embryo fibroblasts (REF). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induced the 5.2-kilobase CYP1B1 mRNA in RMF (12-fold) and REF (14-fold) after a 6-hr treatment, with comparable increases in the microsomal protein. The synthetic glucocorticoid dexamethasone (DEX) suppresses TCDD-induced expression of CYP1B1 in RMF and REF. Suppression of CYP1B1 mRNA in RMF (maximal suppression, 70%) was observed when DEX was added 2 hr before TCDD, but was not observed with co-administration. The concentration dependence ($EC_{50} \approx 10$ nM) and reversal by the antagonist, RU486, implicates the glucocorticoid receptor. DEX inhibition of TCDD-induced CYP1B1 protein needed more extensive preincubation (>6 hr). TCDD induction of CYP1B1-luciferase constructs in RMF was mediated by a 265-base-pair upstream region (−810 to −1075), which was similarly suppressed (50–70%) by a 2-hr preincubation with 10^{-7} M DEX via this enhancer region. Expression of the AhR is suppressed by DEX (70% after 12 hr), but not after the 2-hr period that was sufficient for suppression of transcription. The AhR nuclear translocator is not affected by this treatment. We conclude that glucocorticoid receptor rapidly suppresses activity of the AhR/AhR nuclear translocator complex in the CYP1B1 enhancer region, even though lacking glucocorticoid responsive element(s). DEX inhibits proliferation of RMF in this same concentration range (35%, $EC_{50} \approx 5$ nM), indicating additional effects on intracellular activity that may link to this suppression.

This laboratory has established expression of CYP1B1 in the rat mammary gland. In culture, rat mammary epithelial cells express CYP1A1 but very little CYP1B1 when induced by AhR agonists, whereas RMF express both constitutive and AhR-inducible CYP1B1 (Christou *et al*., 1995). In RMF, CYP1B1 expression is suppressed by a complete hormonal mixture that contains, in part, progesterone and GCs.

Systemic hormones and locally acting growth factors combine to control the proliferation and differentiation of the mammary gland (Imagawa, 1990). Lactogenic hormones, such as GCs and prolactin, are examples of systemically derived molecules that play a role in maintenance of mammary gland development (Dembinski *et al*. and Shiu, 1987; Haslam, 1987). For example, the stage-specific regulation of milk protein gene expression is controlled by GC and prolactin. Multiple GR-binding sites are present in the promoters of the β-casein and whey acidic protein genes and seem to work in synergy with prolactin-stimulated signal transduction pathways to activate transcription (Lechner *et al*., 1997) or to maintain the lactogenic state by down-regulating remodeling proteases (Andreasen *et al*., 1990; Lund *et al*., 1996).

We have reported previously that a complete hormonal mixture, including GCs, suppressed constitutive and PAH-induced levels of CYP1B1 in isolated RMF (Christou *et al*.,

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ABBREVIATIONS: CYP, cytochrome P450; RMF, rat mammary fibroblasts; REF, rat embryo fibroblasts; PAH, polycyclic aromatic hydrocarbon; TCDD, 3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon nuclear translocator; GC, glucocorticoid; GR, glucocorticoid receptor; DEX, dexamethasone; XRE, xenobiotic response element; bp, base pair(s); kb, kilobase pair(s); GRE, glucocorticoid responsive element; DME/F12, Dulbecco's modified Eagle's/Ham's F12 medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TBST, Tris-buffered saline/Tween 20; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

1995). GCs have been shown to regulate a number of other drug metabolizing genes (Prough *et al.*, 1996). In cultured adult rat hepatocytes, GCs potentiated the PAH-induction of CYP1A1, as well as a number of other phase II enzymes (glutathione *S*-transferase-Ya and UDP-glucuronyltransferase), and suppressed PAH-induction of NADPH:quinone oxidoreductase (Xiao *et al.*, 1995). The *CYP1A1* gene contains a functional GC response element in the first intron that mediates this potentiation (Mathis *et al.*, 1989). GC suppression of CYP1A1 is developmentally specific; neonatal rats are responsive to exogenously applied DEX, wheras adolescent rats are relatively unaffected, possibly because of the much higher endogenous levels of GCs in adult rats (Linder, 1993).

TCDD activates transcription of CYP1A1 by stimulating AhR complex formation with the Arnt protein, which then targets a cluster of XREs approximately 1 kb upstream of the transcriptional start site (Denison *et al.*, 1988; Denison *et al.*, 1989; Whitlock *et al.*, 1996). CYP1B1 is also regulated by the AhR in stromal fibroblasts from a number of sources, including the mammary gland, uterus, and embryo (Christou *et al.*, 1995; Pottenger *et al.* and Jefcoate, 1990; Savas *et al.*, 1993). Basal levels of the 5.2 kilobase (kb) CYP1B1 mRNA are elevated several fold following TCDD treatment of these cells. Recently, a 265-bp enhancer region of the CYP1B1 gene promoter, containing 5 XREs, has been shown to be essential for both basal and TCDD-induced expression (Zhang *et al.*, 1998).

The AhR also controls a number of genes whose products may be involved in a number of cellular proliferation and differentiation processes (Okey *et al.*, 1994). Recently, AhR-deficient mice were generated, and their phenotypes suggest a role for this receptor in hepatic growth and development (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). AhR-deficient cells exhibit a decreased rate of cell proliferation because of a prolongation of cells in G_1 (Ma and Whitlock, 1996; Weiss *et al.*, 1996), and TCDD has been demonstrated to exert a delay in G_1-S progression in hepatoma cells (Wiebel and Cikryt, 1991).

The GR regulates transcription in multiple ways that may affect AhR activity. GR homodimers bind to cognate DNA sequences known as GREs then interact with the initiation complex on the promoter and enhance transcription (Bamberger *et al.*, 1996, and references therein). In some cases, activated GR binds to so-called negative GREs, which causes inhibition rather than enhancement of transcription. In other cases, genes regulated by activating protein-1, involving dimers of the Jun and Fos family of proteins, are negatively regulated when activated GR interacts directly with c-jun. GCs may also act indirectly and more slowly to inhibit transcriptional regulation of certain genes. For example, GCs may suppress a number of genes involved in the inflammatory response, such as cyclooxygenase-2, inducible nitric oxide synthase, and cytosolic phospholipase A, through induction of the transcriptional repressor, adenovirus E4 promoter binding protein (Wallace *et al.*, 1997).

In this article, we establish a similar regulation of CYP1B1 in RMF and in primary REF. We present a first analysis of promoter regulation by the AhR in these primary cells and link this to suppression of CYP1B1 expression by GCs (Christou *et al.*, 1995). Notably, this study addresses whether steroid regulation is mediated through the TCDD enhancer region in the CYP1B1 gene through use of CYP1B1 promoter constructs. We will also address whether GC affects TCDD induction of CYP1B1 by an indirect mechanism, such as through changes in AhR expression.

Materials and Methods

Chemicals. Dexamethasone (DEX), DNase II, trypsin, and dimethylsulfoxide were purchased from Sigma Chemical (St. Louis, MO). TCDD was purchased from Chemsyn Science Laboratories (Lenexa, KS). RU486 was a kind gift from Dr. Terence Berry (University of Wisconsin, Madison). Proteinase K was purchased from Boehringer Mannheim (Indianapolis, IN). Collagenase (type III) for rat mammary fibroblast preparations was purchased from Worthington Biochemical (Freehold, NJ). Dulbecco's modified Eagle's /Ham's F12 medium [DME/F12, 1:1 (v/v)] for cell culture work was purchased from Gibco (Grand Island, NY). FBS was purchased from Gemini Bioproducts (Calabasas, CA). Tissue culture plates (Falcon) were purchased from Fisher Scientific (Itasca, IL). Oligo(dT)-cellulose was purchased from Collaborative Biomedical Products. (Bedford, MA). All other chemicals were purchased from Sigma.

Animals and tissues. All animals and animal tissues used in these studies were purchased from Harlan Bioproducts for Science (Madison, WI). Fresh mammary glands from virgin female Sprague-Dawley rats, 50–55 days old, were used to isolate RMF. REF were isolated from 15-day-old fetuses isolated from timed-pregnant Sprague-Dawley rats.

Preparation of rat mammary fibroblasts and cell culture. RMF were isolated as follows. Briefly, the lower four to six abdominal/anogenital mammary glands were excised from virgin female Sprague-Dawley rats and placed in PBS buffer on ice. In a sterile hood, the glands were finely minced with a scalpel and resuspended in a digestion solution [DME/F12 (1:1, v/v), pH 7.2, supplemented with 0.2% (w/v) collagenase (type III), 0.2% (w/v) dispase (grade II), 5% FBS, 50 μg/ml gentamycin]. This mixture was incubated in a shaking incubator (200 rpm) at 37° for 3 hr. At the end of this incubation, 100 μg/ml DNase (type III) was added and the mixture incubated another 10 min. Undigested tissue was allowed to settle for 2–3 min and the cells aspirated off, removed to another sterile 50-ml tube, and centrifuged at 500 $\times$ *g* for 5 min to pellet the cells. Fat was aspirated off and discarded and the cell/organoid pellet was resuspended in fresh medium (DME/F12, pH 7.2, supplemented with 5% FBS and 50 μg/ml gentamycin) and centrifuged at 500 $\times$ *g* for 5 min. The pellet was resuspended in 10 ml of fresh medium and cells were filtered through a sterile nylon mesh (0.22 μm). The flow-through, which is composed of single cells and small cell clumps, was collected and centrifuged at 500 $\times$ *g* for 5 min, and the resulting cell pellet was resuspended in fresh fibroblast medium [DME/F12 (1:1, v/v), pH 7.2, supplemented with 10% FBS] and plated in 175 cm^2 flasks. RMF were grown in a humidified atmosphere of 5% CO_2/95% air at 37°, and reseeded three or four times to remove any contaminating epithelial cells before experiments were begun on these cells.

Preparation of rat embryo fibroblasts and cell culture. REF were prepared as follows. Fifteen-day-old rat embryos were removed from a freshly killed pregnant Sprague-Dawley rat, decapitated, and eviscerated. The resulting tissue was minced and placed in a sterile 50-ml polypropylene tube containing trypsin solution [0.05% trypsin, 5 mM EDTA] at room temperature for 1 hr with constant mixing. The resulting tissue fragments were gently pipetted to loosen adherent cells and the cell solution centrifuged at 500 $\times$ *g* for 5 min to pellet the cells. The cells were washed twice by resuspension in fresh cell medium (DME/F12, pH 7.2, supplemented with 10% FBS) and pelleting at 500 $\times$ *g* for 5 min. The final suspension of cells were plated onto 175-cm^2 dishes and allowed to attach and grow overnight in a humidified atmosphere of 5% CO_2/95% air at 37°. At confluency, rat embryo fibroblasts were collected by trypsinization and split 1:3 for reseeding. All experiments on REF were performed on passages 3 to 5.

Preparation of microsomal protein from cells. For microsomal preparations from monolayers of cultured cells, cells were

washed once with PBS buffer and collected by scraping. Cells were resuspended in 2 volumes of hypotonic buffer and swelled on ice for 10 min then 2 volumes of homogenization buffer (0.1 M $KHPO_4$, pH 7.25, 150 mM KCl, 10 mM EDTA, 0.25 mM PMSF, 0.1 mM dithiothreitol) were added and the cells lysed by sonication using a sonicator cell disruptor (model W185F; Heat Systems-Ultrasonics, Plainview, NY) at 10-sec pulses. The lysate was centrifuged at 15,000 × *g* for 20 min to remove the mitochondrial fraction. The postmitochondrial fraction was then centrifuged at 105,000 × *g* for 90 min to pellet the microsomal fraction. The resulting cytosolic fraction was collected and the microsomal pellet was resuspended in 2 volumes of dilution buffer (0.1 M $KHPO_4$, pH 7.25, 10 mM EDTA, 0.25 mM PMSF, 0.1 mM dithiothreitol, 20% glycerol) and kept at −70° until further use. Both cytosolic and microsomal protein concentrations determined by the bicinchoninic protocol (Pierce, Rockford, IL).

Preparation of cytosolic and nuclear protein for AhR and Arnt studies. To determine the effects of GCs on expression of AhR and Arnt, cytosolic and nuclear protein were isolated as follows. Briefly, cells were preincubated with DEX, diluted in DMSO, for various times before addition of the AhR ligand, TCDD. Cells were collected by scraping at the time of TCDD addition (0 hr) and 1 hr later, pelleted, and resuspended (100 μl/1 × 10^6 cells) in lysis buffer (25 mM MOPS, pH 7.4, 5 mM EGTA, 1 mM EDTA, 10% glycerol, 0.02% sodium azide) containing protease inhibitors (5 μg/ml leupeptin, 100 units/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 50 μg/ml PMSF) and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM sodium fluoride, 20 mM sodium molybdate). The cells were incubated for 30 min at 4° to facilitate lysis, and centrifuged at 100 × *g* for 5 min to isolate the nuclear fraction. The cytosolic fraction was collected and kept at −20° until further use, and the nuclear pellet was carefully washed three times in lysis buffer to remove contaminating cytosolic proteins. After the final wash, the nuclear pellet was resuspended in lysis buffer and disrupted two or three times by sonication using a sonicator cell disruptor at 10-sec pulses. This nuclear lysate was centrifuged at 15,000 × *g* for 2 min to remove debris and the nuclear protein collected and kept at −20° until further use.

Western immunoblot analysis. Proteins were prepared for immunoblot analysis by suspension in sample loading buffer, heated at 100° for 5 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% acrylamide). After separation, the proteins were transferred to nitrocellulose membranes (Shleicher & Schuell) and blocked in 1 × TBST containing 5% milk overnight at 4° (or for 1 hr at room temperature). The membranes were washed in 1 × TBST for 20 min before addition of the primary antibodies. Primary antibodies used in these studies include affinity-purified polyclonal antibodies to recombinant mouse CYP1B1 (Savas *et al*., 1997), mouse AhR, and mouse Arnt (gifts from Dr. Richard Pollenz, Medical College of South Carolina, Charleston, SC). Following incubation with primary antibodies, the membranes were washed with 1 × TBST for 20 min, then incubated with secondary antibody, anti-rabbit horseradish peroxidase (Promega, Madison, WI). After washing the membranes, immunoreactive proteins were visualized by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL) according to manufacturer's instructions.

Poly(A)$^+$ RNA isolation. Isolation of poly(A)$^+$ RNA from cultured cells was carried out as described previously (Badley *et al*., 1988) with modifications. After treatment of the cells, the media was removed and the cells were washed once with sterile PBS (0.01 M phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl) containing 25 μM aurin tricarboxylic acid, an RNase inhibitor. Cell lysis buffer (0.2 M Tris·HCl, pH 7.5, 0.2 M NaCl, 0.15 mM $MgCl_2$, 2% sodium dodecyl sulfate, 200 μg/ml proteinase K, and 20 μM aurin tricarboxylic acid) was added and the cell lysate was collected and placed in sterile polypropylene tubes. DNA was sheared by passing the lysate through a sterile plastic syringe fitted with a 23-gauge needle four or five times, and the lysate incubated at 45° for 2 hr in a shaker waterbath to digest proteins, including RNase. At the end of the incubation period, the salt content of the lysate was adjusted to a final concentration of 0.5 M NaCl to facilitate binding of the poly(A)$^+$ RNA to oligo(dT)-cellulose. Binding, washing, and elution of mRNA was carried out in sterile Eppendorf tubes according to standard protocols (Sambrook *et al*., 1989).

Northern hybridization analysis. Poly(A)$^+$ RNA for each sample was separated on a 1% agarose-formaldehyde-formamide denaturing gel as described previously (Sambrook *et al*., 1989) and transferred by capillary action to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH) in 20 × standard saline citrate (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 18 hr. RNA was immobilized by UV-induced covalent linkage to the membrane using a UV Stratalinker 1800 (Fisher Scientific) (1900 joule × 100 for 30 sec). Hybridization was carried out with an *Eco*RI-*Hin*dIII fragment of the carboxyl terminus of rat CYP1B1 cDNA (700 bp) (Bhattacharyya, 1995). A β-actin probe was used to quantify the levels of RNA in each lane. Each probe was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by the

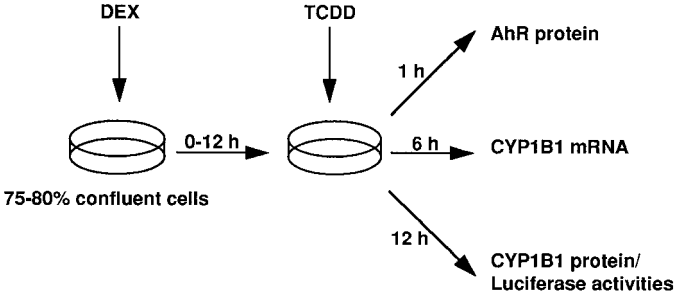

Fig. 1. Protocol for treatment of rat fibroblasts with DEX and TCDD. Cells at 75–80% confluency were preincubated with 10^{-7} M DEX for 0 to 12 hr (*h*) (as indicated) before addition of 10^{-9} M TCDD for varying times depending on the particular endpoint: 1 hr (for AhR expression), 6 hr (for CYP1B1 mRNA expression), and 12 hr (for CYP1B1 protein expression and luciferase activities). Treatments with DEX were staggered to isolate the cells at the same time after TCDD treatment.

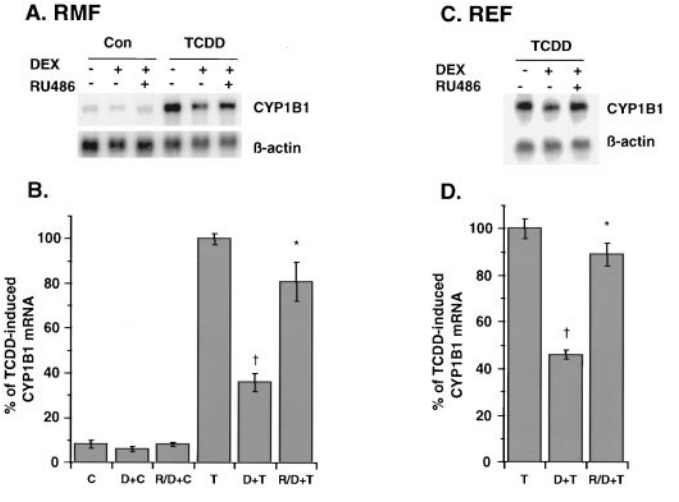

Fig. 2. Effect of DEX preincubation on constitutive and TCDD-induced CYP1B1 mRNA. Primary cultures of RMF were isolated and cultured until 75–80% confluent, then pretreated for 2 hr with 10^{-7} M DEX (*D*) alone or in combination with 10^{-6} M RU486 (*R*), before addition of 0.1% DMSO (*Con, C*) or 10^{-9} M TCDD (*T*) for 6 hr (A). The effect of a 2-hr preincubation with DEX on TCDD-induction of CYP1B1 was duplicated in REF (C). Poly(A)$^+$ RNA (5 μg/lane) was isolated and separated in 1% agarose/formaldehyde gels, transferred to nylon membranes, and probed by Northern hybridization analysis of CYP1B1, as described in Materials and Methods. Messenger RNA levels were quantified, normalized to TCDD-induced levels, and presented in graphical form below each blot for RMF (B) and REF (D). Values represent the mean ± standard error from three separate experiments. ∗, Significantly different from DEX+TCDD treatment; †, significantly different from TCDD treatment.

random-primed labeling method of Stratagene (San Diego, CA) according to manufacturer's instructions. RNA signals were visualized either by autoradiography or by PhosphorImager analysis.

Transient transfection of primary cells. Primary cultures of RMF were transiently transfected with various CYP1B1 promoter-luciferase reporter gene constructs (Zhang *et al*., 1998) using a modified calcium phosphate method (Ausubel, 1996). Briefly, reseeded cells were grown to 50% confluency, at which time the medium was changed to low serum (DME/F12, pH 7.2, supplemented with 4% FBS, without antibiotics) for 2–3 hr. Meanwhile, the transfection buffer was prepared (0.125 M $CaCl_2$, 25 mM BES, 140 mM NaCl, 0.75 mM Na_2HPO_4, pH 6.95) containing the DNA construct of interest (6.5 μg of luciferase + 1.5 μg of β-galactosidase DNA/30 cm^2 well). The transfection buffer containing the DNA constructs of interest were added directly to the cells (100 μl/30 cm^2 well) and the DNA was allowed to precipitate and attach to the cells for 5–6 hr in a humidified atmosphere of 5% CO_2/95% air at 37°. The medium was removed and the cells shocked at room temperature for 5 min with warmed medium [DME/F12, pH 7.2, no serum] containing 10% glycerol to facilitate uptake of the precipitated DNA by the cells. The cells were then rinsed with fresh warmed medium (no glycerol) once, placed in regular culture medium (DME/F12, pH 7.2, supplemented with 10% FBS and antibiotics), and incubated overnight in a humidified atmosphere of 5% CO_2/95% air at 37°. The next day, the cells were treated as described in the figure legends. For the luciferase assay, the cells were collected in lysis buffer (Promega) by scraping and lysed according to manufacturer's instructions. Protein concentrations were determined and the luciferase assay carried out according to manufacturer's instructions and determined in a luminometer. Transfection efficiency and normalization of activities of different constructs was carried out with the use of a co-transfection with a β-galactosidase-expressing vector (gift from Dr. John Fagan, Maharishi University) and measurement of β-galactosidase activity.

Cell proliferation assay. The effect of GCs on proliferation of RMF was carried out with a CellTiter 96 Aqueous nonradioactive cell proliferation assay (Promega, Madison, WI). The assay is based on a novel tetrazolium compound that is bioreduced by metabolically active cells to a water-soluble formazan product that can be determined by absorbance at 490 nm in a 96-well assay plate. The quantity of formazan product is directly proportional to the number of actively growing cells in culture. Briefly, cells were plated into 96-well plates at 20,000 cells/well and allowed to attach for 2 hr. At this time the cells were treated with various concentrations of DEX, 0.1% DMSO, or 10^{-9} M TCDD for 24 hr and the assay performed according to manufacturer's instructions.

Analytical methods. Quantitation and densitometry of the immunoblot and Northern blots was performed using a Zeineh soft laser scanning densitometer (model SL-504-XL; Biomed Instruments, Fullerton, CA) and by analysis of electronically scanned images on a Power Macintosh 6100/60 using the public domain NIH Image (ver. 1.56; written by Wayne Rasband, National Institutes of Health and available from the Internet by anonymous FTP from *zippy.nimh.nih.gov*). Quantitation of phosphorimages and electronically scanned images (saved as TIFF files) was also performed with the software Imagequant (ver. 1.0; Molecular Dynamics, Sunnyvale, CA).

Statistics. For comparison among several groups, statistical analysis of results was carried out using one-way analysis of variance, followed by a two-tailed Student's *t* test. Significance was set at $p < 0.05$.

Results

Effect of preincubation with DEX on constitutive and induced CYP1B1 mRNA. For the experiments presented herein, subconfluent cultures of rat fibroblasts were treated with DEX and TCDD and analyzed for either CYP1B1 or AhR expression according to the protocol schematized in Fig. 1. Treatments with DEX were staggered to isolate the cells at the same time after TCDD treatment. This ensures that any effects we observe are caused by DEX and TCDD and are not attributable to cell culture phenomenon. RMF and REF were treated with 10^{-9} M TCDD for 6 hr after a pretreatment with 10^{-7} M DEX for 2 hr. The 5.2-kb CYP1B1 mRNA was constitutively expressed in primary cultures of RMF and levels were elevated 12-fold after 6 hr of TCDD treatment (Fig. 2A). Constitutive CYP1B1 mRNA and TCDD induction were similarly seen for REF (not shown). RMF were pretreated with 10^{-7} M DEX for 2 hr before addition of either 0.1% DMSO (Control) or 10^{-9} M TCDD for 6 hr. DEX preincubated for 2 hr lowered constitutive levels of CYP1B1 only marginally (20%) but significantly suppressed TCDD induction of CYP1B1 mRNA (65%) ($p < 0.05$) (Fig. 2B). This suppression was almost fully relieved with 1 μM of the strong GR antagonist RU486, establishing a role for the GR in mediating this suppression. Co-administration of DEX with TCDD had no measurable effect on transcriptional stimulation of CYP1B1 by TCDD (not shown). DEX suppression of TCDD-induced CYP1B1 mRNA was duplicated in REF (Fig. 2, C and D). For both cell types, inhibition of TCDD-induced CYP1B1 by DEX exhibited a concentration-dependence typical of GR binding ($EC_{50} \approx 10$ nM, $p < 0.05$) (Fig. 3, A and B). Interestingly, the suppressive effects of GCs on CYP1B1 were not confined to fibroblasts or AhR regulation,

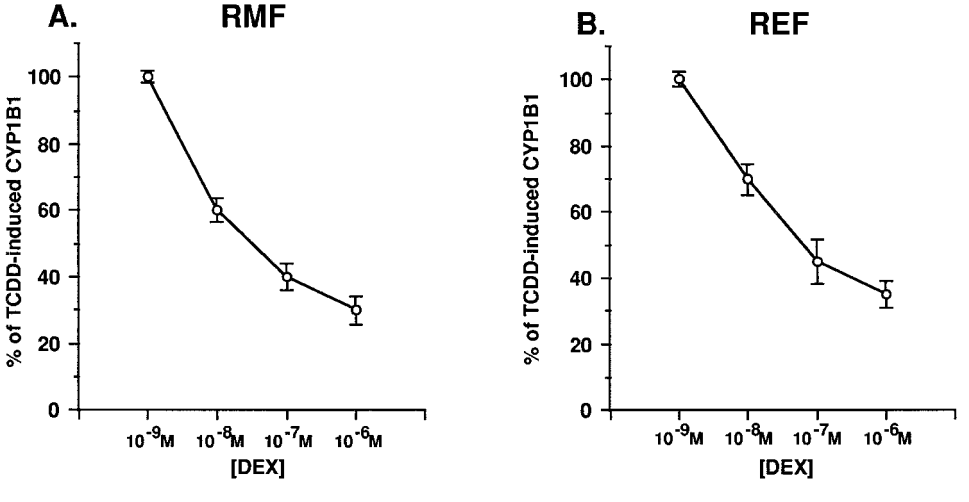

Fig. 3. Dose-response relationship of DEX suppression of TCDD induction of CYP1B1 mRNA. Primary cultures of RMF (A) and REF (B) were isolated and cultured until 75–80% confluent, then pretreated for 2 hr with various concentrations of DEX as indicated, before addition of 10^{-9} M TCDD for 6 hr. Poly(A)$^+$ RNA (5 μg/lane) was isolated and separated in 1% agarose/formaldehyde gels, transferred to nylon membranes, and probed by Northern hybridization analysis of CYP1B1, as described in Materials and Methods. Expression of CYP1B1 was normalized to β-actin, quantified, and displayed in graphical form as percent of TCDD-induced CYP1B1 mRNA (normalized as 100%) versus concentration of DEX. Values presented represent the mean and range of results from two experiments.

because in primary rat adrenocortical cells, GCs suppress cAMP stimulation of CYP1B1 by 70% (not shown).

Effect of DEX on TCDD induction of CYP1B1 protein. The level of TCDD-induced CYP1B1 protein in RMF was also quantified. Primary cultures of RMF were pretreated with 10^{-7} M DEX for various times to assess the time-dependency of GC suppression on TCDD-induced CYP1B1 protein. This represents a longer induction period than for the mRNA measurements above (Fig. 1; 12 hr versus 6 hr). Again a preincubation with DEX was required for inhibition, because co-administration with TCDD did not result in inhibition of CYP1B1 protein expression (Fig. 4A). RMF that were not treated with DEX before incubation with TCDD exhibited levels of CYP1B1 protein similar to those of cells that were co-administered DEX and TCDD; we conclude, therefore, that the effects of DEX on CYP1B1 expression are caused by DEX itself and not by cell culturing conditions. However, in contrast to the rapid effects on CYP1B1 mRNA, a 6-hr preincubation produced only a marginal suppression, whereas this effect increased rapidly between 6 and 12 hr of preincubation (Fig. 4, A and B). The levels of TCDD-induced CYP1B1 protein were inhibited 65% after 12 and 24 hr of DEX pretreatment.

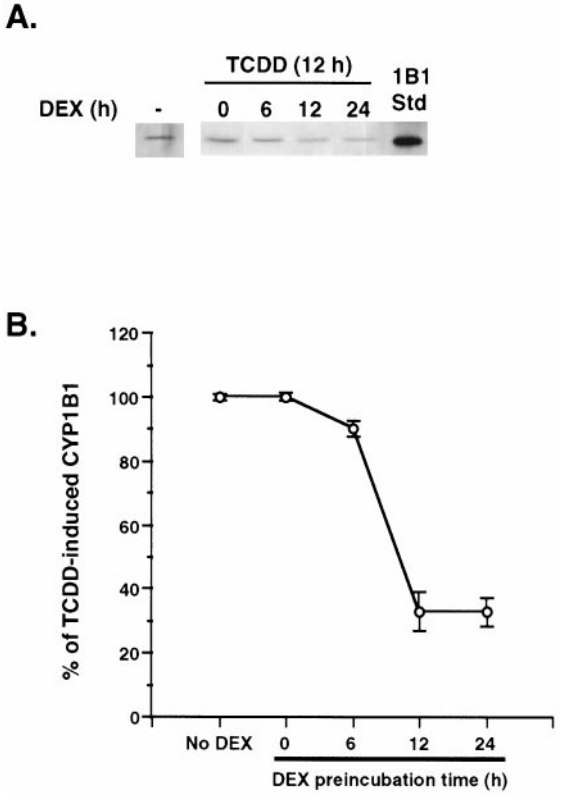

Fig. 4. Effect of DEX on TCDD induction of CYP1B1 protein. RMF were isolated and cultured until 75–80% confluent, as described in Materials and Methods, then preincubated with 10^{-7} M DEX for times indicated (*h*, hour), or left untreated (-), before addition of 10^{-9} M TCDD for 12 hr to assess the effects of DEX pretreatment time on TCDD induction of CYP1B1 protein (A). After 12 hr of TCDD treatment, the cells were harvested and microsomal protein was isolated and probed for immunodetectable CYP1B1. Protein (5 μg) were loaded in each lane for both A and B. Shown are representative blots of repeated experiments. CYP1B1 protein levels were quantified as described in Materials and Methods, and relative levels were determined from two separate experiments and presented in graphical form (B), as mean and range, for percent inhibition of TCDD-induced CYP1B1 protein versus preincubation times for DEX.

Analysis of CYP1B1 promoter-luciferase activities. To further define the mechanism of GC/GR activity on the regulation of *CYP1B1*, we examined the effects of GCs on *CYP1B1* promoter activity. Several 5′-flanking sequences have been isolated from mouse genomic CYP1B1 clones and have been characterized for responsiveness to TCDD in mouse embryo fibroblasts (Zhang *et al*., 1998). These studies identified a 265-bp enhancer region located about 1 kb upstream (−810 to −1075) that is essential for TCDD induction,. A minimal promoter of 210 bp was also characterized immediately upstream of the transcriptional start site that is active in the absence of TCDD. An additional sequence in

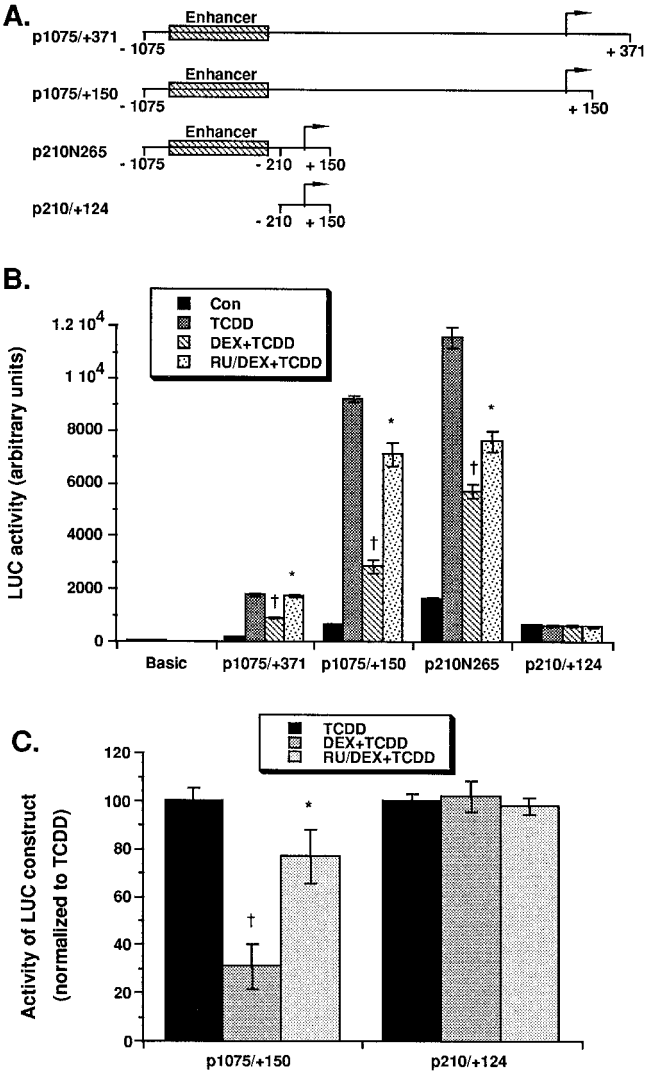

Fig. 5. Effect of DEX on *CYP1B1* promoter-luciferase activity. RMF were transiently transfected with a number of *CYP1B1* promoter-luciferase constructs (as described in Fig. 4) and assessed for effects of DEX on TCDD-induced luciferase activities (A), as described in Materials and Methods. Luciferase activities (*LUC*, arbitrary units) were determined in response to 12 hr of 0.1% DMSO (*Con*) or 10^{-9} M TCDD treatment following a 2-hr preincubation with 10^{-7} M DEX alone, or in combination with 10^{-6} M RU486 (*RU*). B, A comparison of the effects of DEX on TCDD induction of two CYP1B1 constructs (p1075/+150 and p210/+124) that were repeated in another experiment in RMF. Values for A represent mean ± standard error for triplicate samples; values for B represent the mean ± standard error of triplicate samples from two experiments (n = 6). *, Significantly different from TCDD treatment ($p < 0.05$); †, significantly different from DEX treatment ($p < 0.05$).

exon1 was also inhibitory. These constructs were used here to determine the effect of GCs on CYP1B1 promoter activities and regulatory effects of *exon1* (Fig. 5A).

A 1.4-kb construct (p1075/+371) that contains the TCDD enhancer region and a complete *exon1* was transiently transfected into RMF and assessed for responsiveness to DEX. We used a 2-hr preincubation period that was optimized for DEX effects on CYP1B1 transcription. It should be recognized that the 12-hr response time here for luciferase measurements is much longer than that used for CYP1B1 transcription, but the same as for CYP1B1 protein measurement (Fig. 1). This construct expressed constitutive activity and responded to TCDD with a 10-fold increase in activity (Fig. 5B). A second construct, p1075/+150, in which a previously identified inhibitory sequence in *exon1* was deleted, exhibited a much higher basal activity consistent with observations seen in mouse embryo fibroblasts, and was highly responsive to TCDD (17-fold increase) (Fig. 5B). A third construct, p210N265, which contains the TCDD enhancer region fused directly to the proximal promoter and which lacked a second identified suppressive region, was highly responsive to TCDD (Fig. 5B). Preincubation with 10^{-7} M DEX for 2 hr suppressed activity of each of these TCDD-induced constructs (50–70%, $p < 0.05$), with suppression partially relieved ($p < 0.05$) by co-treatment with the GC antagonist RU486 [10^{-6} M] (Fig. 5C). A fourth construct, p210/+124, which contains only the proximal promoter of CYP1B1, was unresponsive to both TCDD and to DEX (Fig. 5, B and C). Therefore, we conclude that GCs directly inhibit *CYP1B1* at the transcriptional level and that this inhibition is mediated through the enhancer region.

Effect of DEX on AhR and Arnt expression. We reasoned that DEX could be inhibiting CYP1B1 induction by decreasing AhR expression. When TCDD binds to the cytosolic AhR protein, this complex translocates to the nucleus, briefly accumulates, along with its heterodimerization partner, Arnt, and then is down-regulated (Pendurthi *et al.*, 1993; Pollenz *et al.*, 1994). In RMF, TCDD translocates about 60% of the receptor to the nucleus in a 1-hr treatment (Fig. 6A), consistent with previous observations in mouse cells (Pollenz *et al.*, 1994). The AhR antibody used in these studies consistently recognizes two bands in rat fibroblasts with mobilities concordant with that of the rat liver AhR (Denison *et al.*, 1986). Under these conditions in mouse embryo fibroblasts a single band is recognized (Alexander *et al.*, 1997), which suggests that the two variants are indeed expressed in these cells. The less mobile form predominates in the nucleus and has therefore been used for quantification. The lower band is equally down-regulated by TCDD without concomitant appearance in the nucleus suggesting a more rapid nuclear degradation. In parallel with the other endpoints, we have examined the consequences of varying the length of DEX pretreatment on the cellular distribution and total expression of AhR and Arnt. Co-administration of DEX with TCDD had no effect on AhR levels or accumulation of the receptor in the nucleus. RMF that were pretreated with DEX for 2 hr exhibited marginal effects on initial levels of cytosolic AhR or TCDD-induced translocation to the nucleus (Fig. 6, A and B). When RMF were preincubated with DEX for 12 hr before TCDD stimulation, expression of the AhR was significantly ($p < 0.05$) suppressed 70% (Fig. 6, A and B). The same proportion of AhR was translocated to the nucleus after 1 hr TCDD treatment. Levels of Arnt protein were not affected by DEX treatment (Fig. 6, C and D). Some Arnt is found in the cytosol and decreases with TCDD treatment. This probably

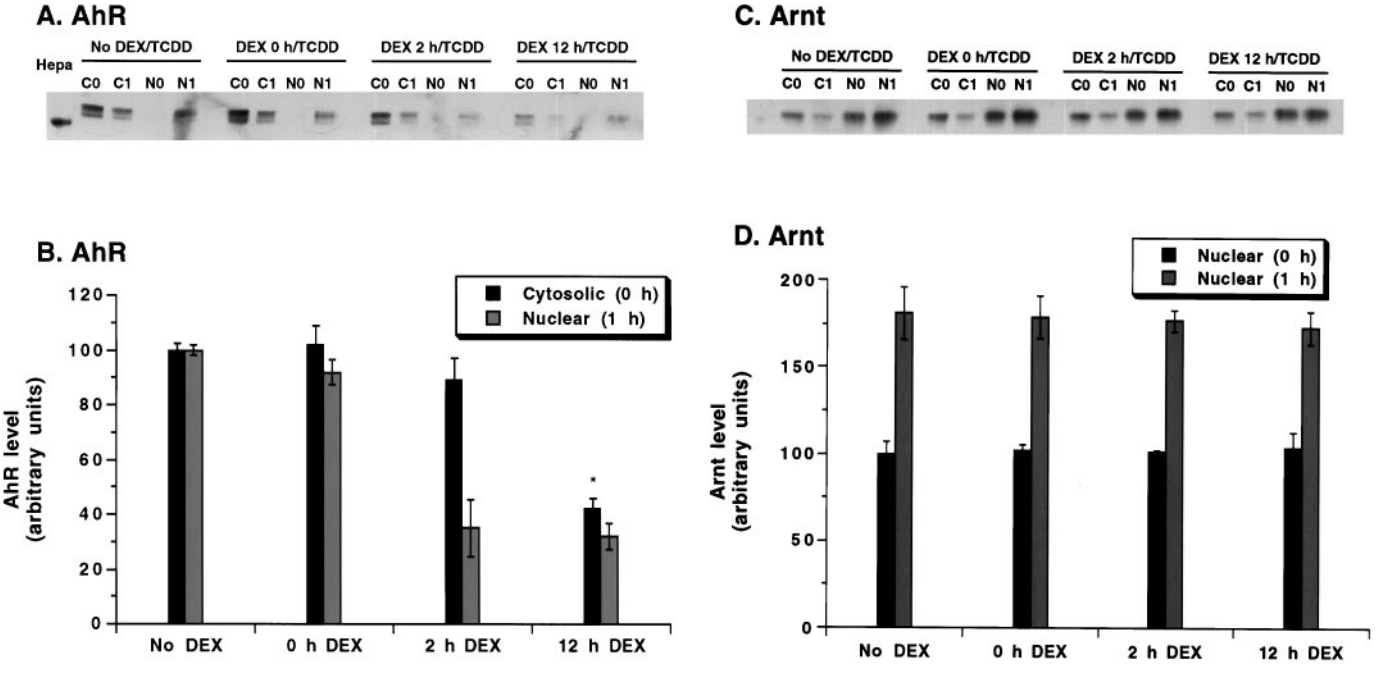

Fig. 6. Effect of DEX on AhR and Arnt expression. RMF were isolated and cultured until 50% confluent, then pretreated with 10^{-7} M DEX for the times indicated (*h*, hour), and treated with 10^{-9} M TCDD for 0 and 1 hr. Cells were then harvested and cytosolic (*C*) and nuclear (*N*) proteins were isolated and probed for immunodetectable AhR (A, B) or Arnt (C, D), as described in Materials and Methods. Cytosolic and nuclear levels were quantified from multiple experiments, normalized to untreated (0 hr TCDD) samples, and presented in graphical form for the AhR (B). These samples were also probed for immunodetectable Arnt (C) and similarly quantified and presented in graphical form (D). Values presented represent the mean ± standard error for three experiments (B, D). *, significantly different from No DEX treatment ($p < 0.05$).

reflects leakage from the nucleus during preparation of the cytosol and nuclear extracts.

Effect of DEX on cellular proliferation of RMF. GCs can give rise to multiple effects that may impact on AhR activity, including inhibition of fibroblast growth (Durant, 1986). We treated actively proliferating RMF with various concentrations of DEX to quantify this effect of GCs. A 24-hr treatment of RMF with DEX resulted in a dose-dependent ($EC_{50} \approx 5$ nM) inhibition of cell proliferation, with maximal suppression of 35% at 10^{-6} M DEX (Fig. 7). This level of growth inhibition is consistent with DEX's effects in other cells, including rat hepatoma and mammary tumor cell lines (Cook, 1988; Webster, 1990). TCDD alone had no effect on cell growth.

Discussion

The data presented here document the expression of CYP1B1 in primary cultures of RMF and REF. We show that basal and TCDD-induced expression of the 5.2-kb CYP1B1 mRNA is comparable in both cell types, paralleling previous findings in C3H10T1/2 cells (Pottenger *et al*., 1991) and mouse embryo fibroblasts (Alexander *et al*., 1997). We have established suppression of this AhR regulation by GCs; this suppression is similar in RMF and REF. We have determined that transcription of the *CYP1B1* gene is enhanced by TCDD through a 265-bp enhancer region that is located 1 kb upstream of the transcriptional start site, and we have provided evidence that the transcriptional control by GCs acts through the GR. The suppression by GCs occurs in this same region, even though there is no GRE. These effects of GCs on TCDD-induced transcription require addition of DEX 2 hr before TCDD. DEX suppresses AhR expression in RMF, but this effect is not seen at preincubation times that suppress CYP1B1 mRNA.

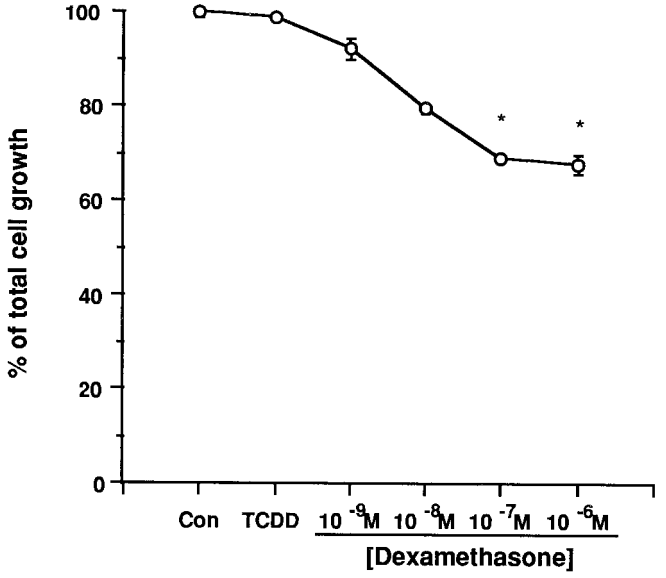

Fig. 7. Effect of DEX on cellular proliferation of RMF. Actively proliferating RMF (passage 3) were plated into a 96-well plate and allowed to attach for 3 hr. At this time the cells were treated with either 0.1% DMSO (*Con*), 10^{-9} M TCDD, or various concentrations of DEX for 24 hr, at which time actively growing cells were determined using a nonradioactive cellular proliferation assay kit based on reduction of a tetrazolium compound to formazan that can be measured by absorbance at 490 nm (Promega Corp., Madison, WI). The graphical representation compares proliferating activity (absorbance at 490 nm, arbitrary units) versus various concentrations of DEX. Values represent the mean ± standard error for triplicate samples ($n = 3$). *, Significantly different from control ($p < 0.05$).

These results suggest a pattern of regulation different than what was reported in previous investigations where PAH induction of CYP1A1 was potentiated by GCs in cultured hepatocytes (Mathis *et al*., 1986; Xiao *et al*., 1995). These effects of GCs were seen at the transcriptional level and produced comparable effects at the activity, protein, and mRNA levels. Work presented here demonstrates that TCDD enhances CYP1B1 expression at the transcriptional level to an extent comparable with the increases in promoter activity as measured with CYP1B1-luciferase reporter constructs. DEX also suppressed CYP1B1 mRNA levels and promoter activity to comparable extents (70%). Both TCDD induction and DEX suppression were mediated via the 265-bp enhancer region. A functional GRE that binds GR has been identified in the first intron of the rat *CYP1A1* gene and is linked to the effects of GR (Mathis *et al*., 1989). No consensus GRE has been identified in the first 1.08 kb of the 5′-flanking sequences or the first intron of mouse *CYP1B1* gene structures, notably in the active 265-bp enhancer (Zhang *et al*., 1998). CYP1A1 is not expressed in rat fibroblasts (Christou *et al*., 1995); therefore, it could not be compared in these studies with CYP1B1 in response to DEX.

We have shown that DEX inhibits CYP1B1 up-regulation after TCDD treatment with an apparent $EC_{50} \approx 10$ nM in both RMF and REF. Thus, the effect of DEX on this induction is not specific to mammary cells. This EC_{50} is comparable with those seen for other DEX-mediated up- or down-regulation of gene transcription (Guller, 1994; Schoffelmeer *et al*., 1995; Yang *et al*., 1994). Involvement of the GR was evident by effects of the antagonist, RU486 (1 μM), which almost fully relieved suppression. Another group has reported that 10 μM of RU486 fully reverses DEX's effect on potentiation of PAH-induction of CYP1A1 (Xiao *et al*., 1995). DEX inhibits CYP1B1 expression at transcriptional and protein levels to a comparable extent (70%). However, there was no effect of DEX on protein expression with preincubations (2 hr) that effectively lowered RNA levels, indicating that translation or protein stability may control CYP1B1 protein levels. Substantially longer DEX pretreatment was needed for a comparable CYP1B1 protein suppression (12 hr versus 2 hr). These pretreatment times correspond to those needed for AhR suppression, which suggests that this plays an additional role in determining CYP1B1 protein levels. We have provided previous evidence that CYP1B1 protein in mouse embryo fibroblasts is relatively labile and is regulated separately from mRNA (Savas and Jefcoate, 1994).

The 2-hr preincubation with GCs is crucial, even for a subsequent 6-hr incubation of DEX and TCDD, as co-administration of DEX and TCDD together had no effect on transcription of CYP1B1. This suggests one of two mechanisms for the suppression. First, there is an ordered process in which DEX changes initiating events immediately after translocation of AhR to the nucleus. AhR activity is then less critical for the subsequent maintenance of transcription. This is consistent with the idea that AhR activity at the enhancer opens up proximal promoter sequences by relaxing nucleosomes (Whitlock *et al*., 1996) (for example, by an increase in histone acetylation). A second possibility is that once present, TCDD activation of the AhR overrides the activity of GR.

Evidence presented here suggests that GCs suppress expression of the AhR, the major regulatory factor for constitutive and inducible CYP1B1 in fibroblasts (Zhang *et al*., 1998). However, because short preincubations (2 hr) with DEX that suppress CYP1B1 mRNA do not affect AhR levels, we conclude that the primary effect of GCs on transcription of CYP1B1 does not involve expression or translocation of the AhR. Levels of the heterodimer partner Arnt were not affected by DEX. At this point, down-regulation of AhR expression has only been demonstrated in response to exogenous ligand binding (Prokipcak and Okey *et al*., 1991; Swanson and Perdew, 1993). Sadek and Hoffmann (1994a; 1994b) have reported up-regulation of AhR activity in the absence of exogenous ligand, by suspension of human keratinocytes. This suspension prohibits adhesion and promotes differentiation. Other researchers have reported similar up-regulation of the AhR during the differentiation of cells (Hayashi *et al*., 1995; Wanner *et al*., 1995), whereas the GC, hydrocortisone, completely antagonized TCDD-mediated growth inhibition of a human keratinocyte cell line (Rice and Cline, 1984). Here, GCs effects on CYP1B1 protein expression occur several hours after the effects on transcription and in parallel to changes in AhR levels.

In summary, it is presented here that DEX inhibits CYP1B1 regulation by TCDD, effects that are mediated through the enhancer region of the *CYP1B1* gene in a dose-dependent manner. These effects are mediated through the GR as evidenced by the potency of the GR antagonist RU486 at relieving this suppression. The inhibition of CYP1B1 transcription by GCs could be a direct effect mediated by competition for *trans*-acting factors or effects on histone acetylation, or could be secondary to changes in early response genes that affect AhR activity. This may reflect the slower synthesis or degradation of CYP1B1 protein and possibly also the longer observation period for protein expression (24 hr versus 6 hr). In addition, the AhR may possibly play a role in determining the translation of CYP1B1 protein.

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