Arсенит Ингибирование CYP1A1 Индукции 2,3,7,8-Тетрахлордibenzo-p-dioxин есть независимым от клеточного цикла задержки

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АБСТРЭКТ
Мы показываем здесь, что арсенит (As³⁺) идет на мультифакторное действие на генетический материал, так как прерывание цикла клеточного деления может быть инициировано G₂/M задержкой а также торможением aryl hydrocarbon ген контроля, включая прерывание клеточного цикла контроля. As³⁺ стимулировал концентрационно-зависимое увеличение задержки G₂/M фазы задержки, что было обнаружено в 12.5 μM As³⁺. Однако, дозирование HepG2 с TCDD и концентраций As³⁺ как низких, как 0.5 μM стимулировало дозированное прерывание в индукции CYP1A1-зависимой ethoxyresorufin-O-deethylase активности и протеин, указывая на то, что индукция CYP1A1 индукции As³⁺ была значительно более сильной, чем As³⁺-инициированная клеточная задержка. Малые концентрации As³⁺ также инициируют дозо-зависимое снижение TCDD-индуцированного муска Cyp1a1, а также как гуман CYP1A1 в основном печених культурах из транс-генецин CYP1A1N⁺/⁻ мышей. Так как основной печень в культурах являются quiescent, эти результаты указывают на то, что действия As³⁺ на TCDD-индуцированной индукции CYP1A1 независимы от клеточного цикла задержки. As³⁺ не влияет на Ah receptor функцию, которая оценивается на уровне транспортной и связывания с xenobiotic responsive element последовательностей, но это может понижать TCDD-индуцированную CYP1A1 mRNA, что является конкордантным с RNA полимераза II ассоциацией с геном и уменьшением в транскрипционной реакционной части. Мы выводим из этих результатов, что прерывание CYP1A1-индуцированной транскрипции As³⁺-инициированной, а также независимо от клеточного цикла задержки.

Inorganic arsenic (arsenite, As³⁺) is an environmental contaminant released from a number of anthropogenic sources and results in As³⁺ sequestration in groundwater, which is often consumed as drinking water. Epidemiological studies indicate that chronic exposure is linked to vascular diseases associated with the cardiovascular and cerebrovascular systems, as well as the peripheral vasculature that leads to Blackfoot disease (Chiou et al., 1997; Lee et al., 2003; Tchounwou et al., 2003). In humans, As³⁺ exposure has also been thought to be a human carcinogen because epidemiological studies have linked As³⁺ exposure to skin, lung, liver, bladder, prostate, and kidney cancers (Smith et al., 1992; Tchounwou et al., 2003). However, exposure of laboratory animals to As³⁺ has failed to produce organ-specific cancers. This would indicate that cancers in humans linked to As³⁺ exposure may be associated with additional waterborne contaminants that work in concert to predispose humans to a carcinogenic episode. Although the relationship between As³⁺ exposure and other environmental toxicants associated with a carcinogenic episode is not known, it would seem that the actions of As³⁺ on cellular functions influence the biological actions of other environmental toxicants. As³⁺ has been shown to alter cell cycle control, causing G₁ and/or G₂/M phase arrest with subsequent programmed cell death (Park et al., 2000; Yih and Lee, 2000). Evidence suggests that the phase arrest is induced by DNA damage. Telomere shortening and chromosome end-to-end fusions (Liu et al., 2003), oxidative DNA base modifications (Schwerdtle et al., 2003), and DNA strand breaks (Yih and Lee, 2000) indicate that small amounts of direct DNA damage can be caused by low concentrations of As³⁺. If DNA damage is
not repaired, p53 induces cell arrest, which eventually leads to apoptosis. It has been shown that p53 expression and ataxia telangiectasia-mutated-dependent activation associated with G$_1$ and G$_2$/M arrest and apoptosis is up-regulated upon As$_3^{\text{3+}}$ treatment (Park et al., 2000; Yih and Lee, 2000; Filippova and Duerksen-Hughes, 2003). Thus, modulations in cell cycle control by As$_3^{\text{3+}}$ exposure may impact on the expression of other cellular components.

The expression of CYP1A1 is dependent on the induction and activation of the aryl hydrocarbon (Ah) receptor. Cell cycle control has been shown to influence CYP1A1 expression through mechanisms involving the Ah receptor and other independent pathways. When murine hepatic $1c1c$ cells were treated with microtubule disrupters known to cause G$_2$/M phase arrest, induction patterns of Cyp1a1 after exposure to TCDD were dramatically reduced (Santini et al., 2001). In addition, this arrest seemed to not disrupt Ah receptor functionality. Analysis of Ah receptor and Arnt proteins revealed no decrease in protein levels, nor was nuclear translocation of the activated Ah receptor impaired in G$_2$/M-arrested cells. Further implication that cell cycle control has an effect on CYP1A1 expression comes from data demonstrating that pRb binds to the Ah receptor and is necessary for maximal CYP1A1 induction by TCDD in G$_1$ phase (Elferink et al., 2001).

Although As$_3^{\text{3+}}$ can initiate cell cycle arrest, the levels of polycyclic aromatic hydrocarbon (PAH)-induced CYP1A1 are also inhibited when cells are exposed to As$_3^{\text{3+}}$ (Jacobs et al., 1999; Vernhet et al., 2003). Because the Ah receptor is activated in response to PAHs leading to transcriptional activation of CYP1A1, it has been suggested that As$_3^{\text{3+}}$ mediates the down-regulation of CYP1A1 through modulation of Ah receptor function. Transcriptional assays using reporter genes containing repeats of the xenobiotic responsive element (XRE) have demonstrated slight inhibition of transcriptional activity, suggesting that As$_3^{\text{3+}}$ inhibits CYP1A1 induction through a transcriptional-based mechanism (Vernhet et al., 2003). However, the cellular mechanism underlying inhibition of CYP1A1 induction by As$_3^{\text{3+}}$ remains largely unknown.

In this study, we have examined the effects of As$_3^{\text{3+}}$ on Ah receptor control and the impact on TCDD-induced CYP1A1 expression. Given the known effects of As$_3^{\text{3+}}$ on cell cycle control and apoptosis, the role of As$_3^{\text{3+}}$-induced G$_2$/M arrest on TCDD-initiated induction of CYP1A1 in HepG2 cells was investigated. Using a range of As$_3^{\text{3+}}$ concentrations from sub-cytotoxic to levels that cause cellular arrest and apoptosis, we show that inhibition of CYP1A1 induction occurs at concentrations of As$_3^{\text{3+}}$ well below those that initiate cell cycle arrest and apoptosis. The effects of As$_3^{\text{3+}}$ on human CYP1A1 gene expression in primary hepatocytes from transgenic mice (Galijatovic et al., 2004) coupled with analysis of polymerase II recruitment lead us to conclude that As$_3^{\text{3+}}$ inhibits CYP1A1 expression by modifying transcription independent of cell cycle control.

Materials and Methods

Materials. TCDD was purchased from Wellington Laboratories Inc. (Guelph, ON, Canada) and dissolved in DMSO. Sodium arsenite and the horseradish peroxidase-conjugated secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO). A mouse anti-human PARP-1 antibody was purchased from BD Biosciences PharMingen (San Diego, CA), and the mouse anti-human β-actin and anti-Pol II (sc-5943) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human CYP1A1 was a generous gift from Dr. Fred Guengerich (Vanderbilt University, Nashville, TN). The anti-human Ah receptor and anti-human Arnt antibodies were a generous gift from Dr. Christopher Bradfield (Mc Ardle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) and the anti-human UGT antibody was a generous gift from Dr. Wilbert H. Peters (St. Radbound University Hospital, Nijmegen, The Netherlands). All other chemicals and reagents were obtained through standard suppliers.

Cell Culture. The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, Manassas, VA) and mouse liver hepatoma hepali$1c1c$ cells (a generous gift of Dr. James Whitlock, Stanford University, Stanford, CA) were cultured in DMEM supplemented with 10% fetal bovine serum. TV101L cells were derived from HepG2 cells that stably express a CYP1A1-luciferase reporter gene (Postlind et al., 1993). TV101L cells were cultured under the same conditions as described above, except G418 (Geneticin; Invitrogen, Carlsbad, CA) was added to 0.8 mg/ml.

Primary hepatocytes were isolated from 8- to 12-week-old CYP1A1$^{+/+}$ mice (Galijatovic et al., 2004). Mice were anesthetized by isoflurane inhalation. The portal vein was cannulated, and the anterior vena cava was sectioned to allow flow-through from the liver. Perfusion of the liver was started with Hank's balanced salt solution (no Ca$^{2+}$ or Mg$^{2+}$) containing 0.5 mM EDTA and 10 mM HEPES, pH 7.4, at a rate of 4 ml/min and continued for 4 min. The perfusate was then changed to Hank's balanced salt solution (with Ca$^{2+}$ and Mg$^{2+}$) containing 10 mM HEPES, pH 7.4, and 0.2 mg/ml collagenase. The liver was gently teased apart while in a solution of DMEM containing 10% fetal bovine serum supplemented with penicillin/streptomycin. The cells were filtered through a 70-μm cell strainer and washed twice by centrifugation at 50g for 5 min. The hepatocytes were cultured into six-well collagen-coated tissue culture plates (BD BioCoat; BD Biosciences Discovery Labware, Bedford, MA). Four hours after plating, the medium was replaced. Primary hepatocytes were then treated 48 h after seeding and collected at the appropriate times after treatment.

Cell Viability Assay (MTT Assay). Cell viability was measured by MTT as described previously (Mosmann, 1983). After treatment with various chemicals for 18 h, culture medium was replaced with serum-free medium containing 0.5 mg/ml MTT, and cultures were incubated for an additional 3 h. Assay medium was removed, and 1 ml of isopropanol with 0.04% HCl was added. Absorbance values were determined at 570 and 630 nm. Results are displayed as percentage of viable cells compared to TCDD-treated cells.

Cell Cycle Analysis. Approximately 1 × 10$^6$ HepG2 cells were exposed to TCDD and As$_3^{\text{3+}}$ for 18 h, and the cells were collected by trypsinization and pelleted at 1000g for 5 min. Cells were washed twice with 1× DPBS and resuspended in 50 μl of 1× DPBS. Cells were fixed by the slow addition of 70% ethanol with constant vortexing to a volume of 5 ml. The cells were pelleted and resuspended in 800 μl of 1× DPBS containing 3% fetal bovine serum. Then, 100 μl of PI solution (final concentration 50 μg/ml PI) and 100 μl of boiled RNase A (final concentration 1 mg/ml) were added and incubated at 37°C for 30 min. Approximately 1 × 10$^5$ cells were acquired on a FACScan (Becton, Dickinson and Co., San Diego, CA) and analyzed using the CELLQuest software.

Preparation of Cellular and Microsomal Protein. Total cellular protein was obtained by lysing cells directly on the tissue culture plates in 25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM α-glycerophosphate, 0.5 mM DTT, 1 mM sodium orthovanadate, 0.1 μM okadaic acid, and 1 mM PMSF. The solubilized cell lysate was collected and centrifuged at 10,000g, and the supernatant was collected.

Microsomal protein was obtained by scraping cells from the tissue culture plates in a suspension of 10 mM KH$_2$PO$_4$, 0.15 M KCl, 2 mM PMSF, 2 mg/ml aprotenin, 0.2 mg/ml benzamidine, 0.5 mg/ml leu-

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peptin, and 1 μg/ml pepstatin. The cells were disrupted on ice by ultrasonic disruption using five repetitive 5-s bursts, followed by centrifugation at 10,000g. Supernatants were collected and centrifuged at 105,000g for 1 h in a Beckman TL100 tabletop ultracentrifuge. Microsomal pellet was resuspended in 100 μl of the phosphate buffer and stored at −70°C. All protein concentrations were determined by Bio-Rad analysis according to the manufacturer’s instructions.

Western Blot Analysis. Western blots for detection of PARP-1 were performed using NuPAGE Bis-Tris gel electrophoresis units as outlined by the manufacturer (Invitrogen). A 20-μg aliquot of total cellular protein was heated for 10 min in loading buffer and resolved on a 10% Bis-Tris gel under reducing conditions, and protein was transferred to a nitrocellulose membrane using a semi-dry transfer system (Novex; Invitrogen). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (0.01 M Tris, pH 8.0, 0.150 M NaCl, and 0.05% Tween 20) overnight at 4°C. This was followed by incubation with an anti-human/mouse PARP-1 primary antibody in Tris-buffered saline for 1 h at room temperature. Membranes were then washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at room temperature. The conjugated horseradish peroxidase was detected using ECL Plus Western blotting detection system (Amersham Biosciences Inc., Piscataway, NJ), and blots were exposed to X-ray film.

For detection of microsomal CYP1A1, 10 μg of microsomal protein was boiled in nonreducing loading buffer and added to Novex 10% Tris-glycine gels (Invitrogen), and electrophoresis carried out according to manufacturer’s instructions. Membranes were then prepared as described above with the exception of the use of rabbit anti-human CYP1A1 primary antibody. For detection of CYP1A1 from primary hepatocytes, 20 μg of total cellular protein was used, and blots performed as described for microsomal CYP1A1.

Detection of Ethoxyresorufin O-deethylase Activity. EROD measurement was performed as described previously (Cidino et al., 1998). Approximately 2.5 × 10⁵ cells/well were plated in six-well plates. Cells were exposed to TCDD and As³⁺ for 18 h, and the media were removed and replaced with DMEM containing 10% fetal bovine serum, 1.5 mM salicylamide, and 2.5 μM 7-ethoxyresorufin. After incubation for 30 min at 37°C, the media were removed, and fluorescence was measured with 530-nm excitation and 590-nm emission on a FluoroMax-2 (HORIBA Jobin Yvon SPEX Instruments, Inc., Edison, NJ). Resorufin standard curves were used to convert fluorescence to picomoles of resorufin formed. Results were normalized to reaction time and cellular protein.

For determination of microsomal EROD activity, microsomes were collected and resuspended in 50 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), and 1 mM DTT. Reactions contained 20 μg of protein and 8 μM 7-ethoxyresorufin and were initiated by the addition of 1 mM NADPH. After incubation for 30 min at 37°C, each reaction was stopped with the addition of chilled methanol. Fluorescence was detected as described above.

RNA Analysis for CYP1A1 Expression. Total RNA was extracted from cells using TRIzol (Invitrogen) according to manufacturer’s protocol and resuspended in diethyl pyrocarbonate-treated water. Each preparation of RNA was treated with DNase I (Invitrogen) and was reverse transcribed using Omniscript RT (QIAGEN, Valencia, CA). The CDNA was amplified with forward (5’-TAG-ACA-CTC-ATG-TGG-CAG-3’) and reverse (5’-GGG-AAG-GCT-CCA-TCA-GCA-TC-3’) human CYP1A1 mRNA primers. Primers for human CYP1A1 mRNA were published previously (Hestermann and Brown, 2003). For quantitation of human GAPDH, the forward (5’-GCT-GAG-ACA-CCA-TGG-GGA-AAG-3’, bases 93–113) and reverse (5’-CTT-CCC-GTT-CTC-AGC-GTC-3’, bases 282–301) primers were identified from the CDNA sequence (GenBank accession no. AF261085). Each RNA was amplified in a 50-μl PCR reaction that contained 25 μl of QuantiTect SYBR Green PCR Master Mix (QIAGEN), 100 nM each of forward and reverse primers, and 2 μl of cDNA. The initial activation was proceeded at 95°C for 10 min followed by 40 cycles of amplification: 95°C for 30 s, 60°C for 1 min, and 72°C for 45 s. Amplification was followed by DNA melt at 95°C for 1 min and a 41-cycle dissociation curve starting at 55°C and ramping 1°C every 30 s. The MX4000 Multiplex QPCR (Stratagene, La Jolla, CA) was programmed to take three fluorescence data points at the endpoint of each annealing plateau. All PCR reactions were performed in triplicates. CYP1A1 Ct(t) values were normalized to GAPDH Ct(t) values [ΔCt(t)]. CYP1A1 cDNA and hnRNA was expressed as induction fold of vehicle-treated cells using the equation ratio = 2−(ΔCtVehicle−ΔCtSample). TCDD-treated samples were set to maximal induction, and all other treatments are expressed as a percentage of TCDD induction of CYP1A1 mRNA.

Nuclear and Cytosolic Protein Preparation. Nuclear protein was prepared as described previously (Chen and Tukey, 1996), and all procedures were performed at 4°C. After treatment, the tissue culture plates were washed twice with ice-cold 10 mM HEPES. Cells were collected by scraping into MDH buffer (3 mM MgCl₂, 25 mM HEPES, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 2500g for 5 min, and the resulting nuclear pellet was resuspended and washed three times with MDHK buffer (3 mM MgCl₂, 25 mM HEPES, 0.1 M KCl, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The nuclear fraction was resuspended in 100 μl of HDK buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, and 20% glycerol). The homogenate was centrifuged at 105,000g for 1 h, and the supernatant was collected. Cytosolic Ah receptor was activated by incubation of cytosol with 20 μM TCDD for 24 h at 4°C.

Electrophoretic Mobility Shift Assay (EMSA). As described previously (Yueh et al., 2003), nuclear or cytosolic extracts were incubated on ice for 15 min with 2.2 μg of poly(dI-dC) and 1 μg of salmon sperm DNA in HED2G buffer (25 mM HEPES, pH 7.4, 1.5 mM EDTA, 1 mM DTT). The cells were homogenized with a Dounce homogenizer and then diluted 1:1 with HED2G buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, and 20% glycerol). The homogenate was centrifuged at 105,000g for 1 h, and the supernatant was collected. Cytosolic Ah receptor was activated by incubation of cytosol with 20 μM TCDD for 24 h at 4°C.
et al., 2004). HepG2 cells were grown to confluence on 150-cm plates and then treated for 1 h with DMSO, TCDD, 0.5 μM As³⁺ + TCDD, or 5 μM As⁵⁺ + TCDD. Cells were cross-linked by addition of formaldehyde to 1% directly to culture media for 10 min. Cross-linking was stopped by the addition of 125 mM glycine, and cells were centrifuged at room temperature for 10 min with gentle rocking. Plates were washed with phosphate-buffered saline, and then cells were collected by scraping in ice-cold phosphate-buffered saline. Cellular pellet was lysed (1% SDS, 2 mM EDTA, 20 mM Tris, pH 8, and protease inhibitors) for 10 min on ice and then sonicated for 15 s at 20 W in 1-min intervals. The sample was cleared of cellular debris by centrifugation at 16,000g for 10 min at 4°C. One hundred-microliter aliquots were diluted to 1 ml in dilution buffer (1% Triton, 2 mM EDTA, 20 mM Tris, pH 8, 150 mM NaCl, and protease inhibitors) and preincubated for 1 h at 4°C with 50 μl of protein agarose A/G (Santa Cruz Biotechnology, Inc.) with 1 μg/μl salmon sperm DNA. Aliquots were removed at this time for use as input control and processed along with pull-down DNA at reversal of cross-linking step. Precleared supernatants were then incubated overnight at 4°C on a rotating platform with 1 μg of α-Pol II. Fifty microliters of protein agarose A/G plus salmon sperm DNA was added and incubated on rocking platform for 1 h at 4°C. Beads were pelleted and washed for 10 min each in the following buffers (buffer 1, 0.1% SDS, 2 mM EDTA, 20 mM Tris, and 150 mM NaCl; buffer 2, 0.1% SDS, 2 mM EDTA, 20 mM Tris, and 500 mM NaCl; and buffer 3, 1% LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris). Pellets were washed twice in Tris/EDTA buffer and then eluted in 100 μl of 1% SDS, 0.1 M sodium bicarbonate, and 0.2 M NaCl at 65°C overnight. Eluates were digested with proteinase K at 45°C for 1 h and then purified using Qiagen spin columns. DNA was quantified using a spectrophotometer. Equal amounts of pulled-down DNA as well as input controls were used for each quantitative real-time PCR reaction. Amplification of the proximal promoter was achieved using primers that span a 250-base pair region that includes the transcription start site (forward 5'-AGA-AAG-GGC-CCA-GAA-GT-3' and reverse 5'-TCC-AAT-CCC-AGA-GACC-AG-3'). Results are displayed as raw Ct values and representative of three independent experiments.

**Statistical Analysis.** All experiments were performed in triplicate. Statistical analysis was performed where indicated using two-tailed t test assuming unequal variances. Differences were determined to be significant if P ≤ 0.05.
Effect of As\textsuperscript{3+} on Apoptosis and Cell Cycle. As\textsuperscript{3+} has been shown to interrupt normal cell function by interfering with cell cycle control and by initiating apoptosis. To examine the impact of As\textsuperscript{3+} on HepG2 cells, we treated cells with a range of As\textsuperscript{3+} concentrations and measured cell viability by MTT analysis (Fig. 1A) and apoptosis by detecting caspases activated PARP-1 cleavage (Fig. 1B). Comparisons were made over a range of As\textsuperscript{3+} concentrations that also included cotreatment with 10 nM TCDD. At concentrations of As\textsuperscript{3+} ranging from 0.5 to 25 \(\mu\) M, no changes in cell viability or the initiation of apoptosis were noted. TCDD alone or in combination with As\textsuperscript{3+} at these concentrations did not affect cell function. However, cell viability was reduced 30% in HepG2 cells treated with 50 \(\mu\) M As\textsuperscript{3+}, which correlated with a mild increase in caspases activated PARP-1 cleavage. When cell cycle status was evaluated, As\textsuperscript{3+}-treated HepG2 cells revealed an increase in G2/M cell cycle arrest at concentrations that exceeded 12.5 \(\mu\) M (Fig. 1C). Analysis of HepG2 cells treated with As\textsuperscript{3+} alone was consistent with the results shown in Fig. 1C, indicating that the increase in G2/M arrest was attributed solely to the actions of As\textsuperscript{3+}.

As\textsuperscript{3+} Treatment Blocks TCDD Induction of CYP1A1. The cotreatment of HepG2 cells with As\textsuperscript{3+} and TCDD elicited significant inhibition of TCDD-dependent induction of EROD activity over a concentration range from 5 to 50 \(\mu\) M As\textsuperscript{3+} (Fig. 2A). An excellent correlation was observed when EROD activity was measured both in whole cells as well as in HepG2 cell microsomal preparations (Fig. 2B), indicating that the expression of CYP1A1 was dramatically impaired. This was confirmed by Western blot analysis of induced CYP1A1. The pattern of EROD activity correlated with a concentration-dependent reduction in CYP1A1 protein (Fig. 3A).

Quantitation of TCDD inducible CYP1A1 mRNA by real-time RT-PCR demonstrated that the levels of mRNA after TCDD and As\textsuperscript{3+} treatment were concordant with reductions seen in CYP1A1 by Western blot analysis. At 0.5 \(\mu\) M As\textsuperscript{3+}, a concentration that has no detectable effect on cell cycle control, CYP1A1 mRNA induction decreased by 61% of the levels observed with TCDD treatment alone (Fig. 3B). At 50 \(\mu\) M As\textsuperscript{3+} cotreatment, a 90% decrease in TCDD induction of CYP1A1 mRNA was observed.

The levels of As\textsuperscript{3+} needed to block induction of CYP1A1 were 10-fold lower than those shown to stimulate G2/M arrest, indicating that those events associated with cell cycle control may have limited impact on induction of CYP1A1. However, we cannot exclude the possibility that analysis of cell cycle control in the presence of lower concentrations of As\textsuperscript{3+} may lie outside the detection limits of flow cytometry. To compensate for this possibility, an experiment was conducted using cultured mouse liver hepatocytes isolated from transgenic CYP1A1\textsuperscript{-/-} mice (Gal-
jatovic et al., 2004). When primary hepatocytes are placed in culture, they become quiescent so cell cycle control cannot be credited with alterations in gene expression patterns. Because CYP1A1N+/− mice express the full-length human CYP1A1 gene, induction of mouse Cyp1a1 and human CYP1A1 can be evaluated simultaneously. As shown in Fig. 3C, treatment of hepatocytes with TCDD resulted in a marked induction of Cyp1a1 and CYP1A1. As3+ cotreatment inhibited in a dose-dependent manner TCDD induction of both mouse Cyp1a1 and human CYP1A1. The inability of lower concentrations of As3+ to inhibit cell cycle control in HepG2 cells combined with the observations that As3+ can inhibit induction of CYP1A1 in CYP1A1N+/−-derived primary hepatocytes indicates that the cellular mechanisms initiated by As3+ on cell cycle control do not influence those events that lead to inhibition of CYP1A1 induction by Ah receptor ligands.

The Actions of As3+ on TCDD-Induced Transcriptional Control of CYP1A1. The reduction in TCDD enhancement of CYP1A1 by As3+ might indicate that cellular control of the Ah receptor is a potential target for the actions of As3+. When cytosolic preparations from hepa1c1c7 cells are incubated with TCDD, the Ah receptor forms a complex with Arnt, generating a transcriptional complex capable of binding to DNA. Ah receptor activation can be demonstrated by receptor binding to XRE sequences, as demonstrated by EMSA. In Fig. 4, the addition of TCDD to hepa1c1c7 cytosol leads to the identification of an Ah receptor (AhR)/XRE complex (lane 5). The binding of activated Ah receptor was shown to be specific by the reduction in labeled protein/DNA interactions when the reaction was incubated with excess of unlabeled XRE oligonucleotide (Fig. 4, lane 6). Incubation with an inhibitor of Ah receptor ligand binding (α-naphthoflavone) also demonstrates a specific reduction in binding (Fig. 4, lane 7). However, when increasing concentrations of As3+ were included in the binding reaction, no inhibition of Ah receptor binding to the XRE sequences was noted.

To examine the impact of As3+ on TCDD-induced nuclear accumulation of the Ah receptor, HepG2 cells were treated with TCDD or cotreated with TCDD and As3+ for 18 h, and nuclear accumulation of the Ah receptor was determined by EMSA. The treatment of HepG2 cells with 10 nM TCDD leads to the accumulation of nuclear Ah receptor, which can be specifically identified by disruption of binding to XRE sequences when antibodies to the Ah receptor and Arnt protein are included in the binding reaction (Fig. 5B). When HepG2 cells are cotreated with TCDD and varying concentrations of As3+ for 18 h, no disruption in the accumulation of nuclear Ah receptor complex is observed (Fig. 5A).

To examine directly the impact of As3+ on TCDD-initiated CYP1A1 transcription, TV101L cells that express the human CYP1A1 promoter upstream of the firefly luciferase reporter gene were used. Treatment with TCDD induced luciferase activity 50-fold over that of untreated cells (Fig. 6A). When TV101L cells were cotreated with 10 nM TCDD and varying concentrations of As3+ for 18 h, As3+ had negligible effects on TCDD-initiated induction of CYP1A1-luciferase activity.

Because activation of the Ah receptor and the initiation of transcription are not influenced by As3+ exposure, we elected to determine whether the rate of CYP1A1 transcription was altered by measuring the levels of CYP1A1 hnRNA RNA. Quantization of hnRNA is a measurement of the abundance of nuclear transcripts and reflects the rate

![Fig. 4. Activation of Ah receptor is unaffected by the presence of As3+. Cytosolic extracts (50 μg) from untreated hepa1c1c7 cells were incubated with 20 nM TCDD and indicated As3+ concentrations for 20 h at 4°C. Gel shift was performed as described in text. Control reactions are as follows: free probe (lane 1), water (lane 2), DMSO (lane 3), 5 μM As3+ (lane 4), 20 nM TCDD (lane 5), 20 nM TCDD and 200× unlabeled XRE (lane 6), and 20 nM TCDD + 1 μM β-naphthoflavone (lane 7). Cojunction of TCDD and As3+ is indicated (lanes 8–12). Activated AhR/Arnt heterodimer is indicated. Gel shift is representative of three independent experiments.](image-url)
of RNA synthesis at any steady-state level. In this experiment, cells were treated for 1 h with either TCDD or cotreated with TCDD and \( \text{As}^{3+} \), and the nuclear CYP1A1 RNA quantitated by real-time RT-PCR using primers that amplify the exon-intron boundary of exon 1 (Hestermann and Brown, 2003). Treatment of HepG2 cells with TCDD induced CYP1A1 hnRNA after 1-h treatment (Fig. 6B). When cells were cotreated with TCDD at either 0.1 or 0.5 \( \mu \text{M As}^{3+} \), reduction in the abundance of the hnRNA transcript was noted. The low concentrations of \( \text{As}^{3+} \) were comparable with those that inhibited the induction of mRNA and protein. Thus, \( \text{As}^{3+} \) seems to interfere with the transcriptional processes that promote induction of CYP1A1.

Confirmation of the inhibition of transcription on the CYP1A1 promoter was obtained by the chromatin immunoprecipitation assay. HepG2 cells were treated for 1 h with TCDD or TCDD with either 0.5 \( \mu \text{M As}^{3+} \) or 5 \( \mu \text{M As}^{3+} \). The pull down was performed with an antibody specific to RNA polymerase II (Pol II), and real-time PCR was performed with primers specific

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**Fig. 5.** Ah receptor nuclear translocation and DNA binding activity is unchanged by \( \text{As}^{3+} \) treatment. A, nuclear protein was prepared from HepG2 cells treated for 18 h with 10 nM TCDD and increasing concentrations of \( \text{As}^{3+} \) as described under Materials and Methods. Ten micrograms of nuclear protein was incubated with \( ^{32}\text{P}-\text{XRE} \) (5 \( \times \) 10^5 cpm). Controls are as indicated: free probe (lane 1), DMSO treatment (lane 2), 5 \( \mu \text{M As}^{3+} \) treatment (lane 3), 10 nM TCDD treatment (lane 4), and TCDD treatment incubated with 200\( \times \) unlabeled XRE as a specific competitor control (lane 5). Shifts of TCDD and \( \text{As}^{3+} \) cotreatments are indicated (lanes 6–10). Gel shown is representative of three independent shifts. B, supershift analysis was performed using 10 nM TCDD and 10 nM TCDD with 25 \( \mu \text{M As}^{3+} \) nuclear protein preparations. Activated AhR/Arnt binding complex was competed with 200\( \times \) unlabeled XRE (lanes 2 and 7). Specificity of binding was determined by incubation with 200 ng of anti-Ah receptor (lanes 4 and 9) and 100 ng of anti-Arnt (lanes 5 and 10) antibodies. Nonspecific antibody binding was determined using excess anti-UGT (lanes 6 and 11).
to the proximal promoter of CYP1A1. Because of the exponential nature of PCR, the C(t) value difference of 2 between DMSO- and TCDD-treated cells indicates an approximately 4-fold enrichment of promoter sequences detected in TCDD-treated cells (Fig. 6C). Cotreatment with 5 μM As3⁺ raised the C(t) value to almost basal levels, indicating a reduction in the amount of Pol II recruited to the promoter. These results suggest in conjunction with the hnRNA assay that As3⁺ inhibits the recruitment of the basic transcription machinery necessary for TCDD induction of CYP1A1.

**Discussion**

Our results indicate that As3⁺-initiated cell cycle arrest and the inhibition of CYP1A1 induction by TCDD are not associated with a common regulatory event. The disparity in these events can be observed by the very large differences in As3⁺ concentrations needed to induce G2/M arrest and the inhibition of TCDD-initiated CYP1A1 induction. Very clear reductions in the induction of CYP1A1 mRNA and protein are observed at 0.5 μM As3⁺, whereas the early events leading to G2/M arrest are not detectable until 12.5 μM As3⁺ exposure. One may speculate that As3⁺ is capable of initiating cell cycle arrest at very low concentrations, but the sensitivity of detecting G2/M arrest exceeds the limits of our techniques. Yet, when we analyzed the potential for cell arrest by monitoring changes in cell cycle regulatory proteins such as the cyclins B1 and D1 (Park et al., 2001; Zhao et al., 2002), p21, cdc25A, and cdk1 (Park et al., 2000), no observable changes in these proteins were detected with low concentrations of As3⁺ (data not shown). Furthermore, studies conducted in primary hepatocytes would question the role of cell cycle arrest in As3⁺-inhibition of TCDD-mediated CYP1A1 induction. Hepatocytes are quiescent cells in the intact liver but are capable of one to two progressions through the cell cycle during liver injury. In culture, primary hepatocytes progress through G1 independent of stimulation but arrest in mid-G2 after 42 h in culture (Loyer et al., 1996; Talarmin et al., 1999). The hepatocytes in our study were cultured for 48 h before treatment, allowing for cessation of cycling. Inhibition of TCDD-mediated CYP1A1 induction by As3⁺ was still observed and closely resembled the dose-response established in HepG2 cells. Thus, CYP1A1 induction was still dramatically inhibited in a quiescent cell model. These results demonstrate that the actions of As3⁺ on blocking CYP1A1 induction by TCDD are initiated through alterations in CYP1A1 transcription and are independent of the regulatory mechanisms initiated by As3⁺-induced cell arrest.

The reduction in TCDD-induced CYP1A1-specific EROD activity by As3⁺ has been clearly established. Similar results have been observed when cells were cotreated with PAHs and As3⁺ (Jacobs et al., 1998, 1999; Vakharia et al., 2001; Vernhet et al., 2003). It has been postulated that this inhibition is the result of interference by As3⁺ with the catalytic potential of CYP1A1, either through reduction in cellular heme pools or by direct binding of As3⁺ to CYP1A1. However, changes in cellular heme pools would not lead to the changes in CYP1A1 transcription and the resulting reduction in CYP1A1 as determined by Western blot analysis.

The unique ability of As3⁺ to bind thiol groups is well established in vitro, whereas relevant cellular models of binding are more difficult to determine. As3⁺ has been shown to inhibit steroid binding to the glucocorticoid receptor (Simons et al., 1990; Stancato et al., 1993) and to inactivate the catalytic loop in IκB kinase β subunit, thereby reducing nuclear factor-κB activity (Kapahi et al., 2000). Members of the cytochromes P450 superfamily contain a conserved cysteine residue that serves as the axial ligand for heme iron (Johnson, 2003). However, P450s contain very few cysteine residues and in the crystal struc-
turer of CYP2C5, no other cytochrome is found in proximity to the heme binding cytochrome. Because coupling to adjacent cytochrome residues is a prerequisite for inhibition of protein function by As³⁺ and given the evidence presented here on down-regulation of CYP1A1 by a transcriptional mechanism, the inhibition of EROD activity is not the result of As³⁺ directly associating with CYP1A1.

Recent work has demonstrated a similar pattern of PAH-induced CYP1A1 inhibition by chromium (Wei et al., 2004). This work suggests that heavy metals may be acting in a similar manner at the promoter to inhibit induction of CYP1A1. However, chromium and As³⁺ show distinct patterns of gene expression alteration. As³⁺ alters the expression of a unique set of genes, most notably up-regulation of heme oxygenase 1, metallothionein, and NAD(P)H:quinone oxidoreductase, whereas chromium inhibits the expression of metallothionein and NQO1 (Maier et al., 2000; Andrew et al., 2003; Majumder et al., 2003; Zheng et al., 2003). Chromium’s ability to cause DNA-protein cross-links has been suggested as a potential mechanism for HDAC1 sequestration on chromatin surrounding CYP1A1, a mechanism that has been proposed to inhibit CYP1A1 transcription. However, As³⁺-induced cross-links and DNA damage have been repeatedly found using primarily very high cytoxic concentrations (Yih and Lee, 2000; Mouron et al., 2001; Guillamet et al., 2004). Thus, it is doubtful that the low concentrations of As³⁺ used in our studies would impact on HDAC1 sequestration.

Our results suggest that the actions of As³⁺ leading to inhibition of CYP1A1 induction in HepG2 cells occur after TCDD-initiated activation of the Ah receptor and binding of the receptor to DNA. Because the nuclear concentrations of Ah receptor or the binding potential of the Ah receptor/Ant complex to DNA is not compromised, the reductions in CYP1A1 hnRNA indicate that the rate of transcription initiated by TCDD may be slowed. Because of the low concentrations of As³⁺ needed to inhibit TCDD induction of CYP1A1, we would predict that As³⁺ is modifying essential regulatory proteins involved in the maintenance of polymerase-initiated transcription. In support of this, chromatin immunoprecipitation assays revealed a reduction in Pol II recruitment to the CYP1A1 proximal promoter. Given the specificity of As³⁺ inhibition to CYP1A1, this suggests a block of a signaling event involved in the recruitment of Pol II to the gene.

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References


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