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Short and Long Term Effects of Cytoskeleton-Disrupting Drugs on Cytochrome P450 *Cyp1a-1* Induction in Murine Hepatoma 1c1c7 Cells: Suppression by the Microtubule Inhibitor Nocodazole

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SUMMARY

Cultured murine hepatoma 1c1c7 cells were treated with either the actin filament-disrupting drug cytochalasin D or the microtubule inhibitors colchicine and nocodazole (NOC) to assess the role of the cytoskeleton in the process of cytochrome P450 *Cyp1a-1* induction. Indirect fluorescence analyses demonstrated that microtubule or actin networks were disrupted within 1 hr of treatment and remained altered as long as cultures were maintained in the presence of the drugs. Treatment of cultures with cytochalasin D, colchicine, or NOC for 1 hr before the addition of dibenz[*a*,*c*]anthracene had no effect on *Cyp1a-1* induction, as monitored by measurements of CYP1A1 mRNA. Pretreatment with NOC for ≥18 hr produced populations of cells that had either a flat or rounded morphology. Both populations, when isolated 20–24 hr after NOC treatment, were arrested in the G_2/M phase of the cell cycle (83–98% in G_2/M versus ~7–10% in nontreated or solvent-treated cultures). *Cyp1a-1* induction was suppressed in both of these populations, as monitored by measurement of CYP1A1 mRNA content (reductions of >68%), 7-ethoxyresorufin *O*-deethylase activity (reductions of >80%), or microsomal CYP1A1 protein content (reductions of >80%). In contrast, overall [^{3}H]leucine incorporation into protein was not affected. Cytosol prepared from these NOC-treated cultures bound ~39% of the radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin bound by cytosol isolated from solvent-treated cultures. Nuclear extracts prepared from cultures treated with NOC for 20–24 hr before *in vivo* exposure to inducer and cytoplasmic extracts isolated from similarly NOC-treated cultures that were exposed to inducer *in vitro* demonstrated reductions of ≥54% and ≥55%, respectively, in their abilities to bind to DNA, when analyzed by gel retardation analyses using an oligonucleotide corresponding to dioxin-responsive element D of the *Cyp1a-1* gene. These studies suggest that ligand-dependent induction of *Cyp1a-1* transcription is unaffected by short term disruption of the microfilament or microtubule network. However, long term exposure to microtubule inhibitors causes cells to pause in the G_2/M stage of the cell cycle and modulates processes involved in the induction of *Cyp1a-1* in these cells.

The *Cyp1a-1* gene[1] encodes a protein that is involved in the metabolism of a variety of xenobiotics and can be induced by exposure to environmental chemicals such as PAHs and polychlorinated dibenzo-*p*-dioxins (1). Its induction is mediated by PAH/polychlorinated dibenzo-*p*-dioxin binding to the cytoplasmic AhR and subsequent translocation of the activated receptor to the nucleus, where it binds to DRE sequences in the 5′ regulatory region of the *Cyp1a-1* gene (2). The latent cytosolic AhR consists of the ALBS protein, an unspecified number of HSP90 molecules, and possibly other less well characterized factors (3, 4). HSP90 appears to be required for the binding of PAH/TCDD to the ALBS (5). Upon ligand binding, the AhR complex undergoes a transformation in which the ALBS dissociates from HSP90. This facilitates translocation and interaction with ARNT (6). The resulting heterodimer is capable of binding to DREs *in vitro* (7) and is thought to be the protein complex responsible for the *in vivo* induction of *Cyp1a-1* by PAHs and TCDD. Recent studies have demon-

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[1] In accordance with the proposed nomenclature for the cytochrome P450 genes (66), the murine and nonmurine genes encoding cytochrome P4501A1 are designated *Cyp1a-1* and *CYP1A1*, respectively.

ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; AhR, aromatic hydrocarbon receptor; ALBS, aromatic hydrocarbon ligand-binding subunit; ARNT, aryl hydrocarbon receptor nuclear translocator; COL, colchicine; CYT, cytochalasin; DB[*a*,*c*]A, dibenz[*a*,*c*]anthracene; DRE, dioxin-responsive element; EROD, 7-ethoxyresorufin *O*-deethylase; GR, glucocorticoid receptor; HSP, heat shock protein; NOC, nocodazole; PBS, phosphate-buffered saline; PKC, protein kinase C; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VDR, vitamin D receptor; α-MEM, minimum essential medium; DMSO, dimethylsulfoxide; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

strated that the ARNT protein is required for the binding of the liganded ALBS to the DRE (7) and that it is normally found in the nucleus (8).

The shuttling of proteins from the cytoplasm to the nucleus entails an interaction of nuclear localization signal-binding proteins with the nuclear localization signal sequence of the protein to be imported or its associated chaperone protein, accumulation of the protein at the nuclear pore, and subsequent energy-dependent transport through the nuclear pore channel (9). The cytoskeleton has been implicated in the initial steps of cytoplasm/nucleus shuttling of several steroid hormone receptors. Specifically, immunocytochemical studies have demonstrated that cytoplasmic GR and VDR are both associated with microtubules (10, 11). Disruption of the microtubule lattice by treatment with the microtubule-depolymerizing drug NOC prevents the calcitriol-induced nuclear accumulation of cytoplasmic VDRs (11). Similarly, the microtubule disrupter COL has been reported to disrupt the shuttling of the GR (12). HSP90, a component of the latent AhR complex, colocalizes with microtubules in a variety of cell lines (13) and binds to actin microfilaments *in vitro* (14, 15). The cytoplasmic 8 S GR complex, which contains HSP90 (15), interacts *in vitro* with actin filaments via the association of HSP90 with actin (14).

Several groups have reported that treatment of cultured cells, before the addition of inducer, with the PKC inhibitor H7 or concentrations of TPA sufficient to down-regulate PKC suppresses the induction of *Cyp1a-1* (16–18). Similarly, *in vivo* treatment of rodents with TPA has been shown to suppress the induction of *Cyp1a-1* in murine skin (19) and liver (20). Because components of the AhR are phosphoproteins (21, 22) and *in vitro* treatment of the ALBS-ARNT complex with phosphatases eliminates its ability to bind to DRE oligonucleotides (17, 18, 23), it has been hypothesized that components of the AhR complex must be phosphorylated to bind to the DRE (18, 23). Kinase inhibitor and reconstitution studies (16–19) circumstantially implicate PKC as being the kinase responsible for this phosphorylation. What is generally not appreciated is that both TPA and H7 dramatically affect the cytoskeleton (24, 25). The effects of TPA on actin microfilaments and intermediate filaments are presumably mediated through its activation of PKC. PKC phosphorylates several proteins of the peripheral cellular cytoskeleton, subunits of intermediate filaments, and cytomatrix cross-linkers (26–29). Although the mechanism of action is not known, H7 causes a collapse of the actin microfilament network, resulting in morphological changes similar to those seen in TPA-treated cells (24). Interestingly, induction of glucocorticoid-responsive genes and the translocation of the GR are suppressed in rat hepatocytes that have been exposed to H7 (30).

Given the aforementioned disruptive effects of H7 and TPA on the cytoskeleton and their suppressive effects on *Cyp1a-1* induction (16–20, 24, 25), the association of HSP90 (a component of the latent AhR) with actin filaments (14, 15), the role of the cytoskeleton in the translocation of the GR and VDR (10, 11), and the ability of H7 to inhibit GR translocation (30), it seems quite possible that the cytoskeleton may play a role in processes associated with the TCDD/PAH-dependent induction of *Cyp1a-1*. If so, one would predict that disruption of actin and microtubule networks might suppress *Cyp1a-1* induction. In the current study we investigated the effects of the actin-disrupting drug CYT D and the microtubule inhibitors COL and NOC on *Cyp1a-1* induction in murine hepatoma 1c1c7 cells. Such an approach has been used to investigate the role of the cytoskeleton in the mechanism of gene induction mediated by receptor proteins that bind to inducible enhancer elements (10–12).

Materials and Methods

Chemicals. [^{3}H]TCDD (29 Ci/mmol), TCDD, and 2,3,7,8-tetrachlorodibenzofuran were generous gifts from Dr. S. Safe (Texas A & M University). 7-Ethoxyresorufin and 7-hydroxyresorufin were purchased from the Pierce Chemical Co. and Aldrich Chemical Co., respectively. Trypsin, α-MEM, fetal bovine serum, and penicillin-streptomycin solution were purchased from GIBCO-BRL. 1,2,3,4-Dibenzanthracene, paraformaldehyde, DMSO, COL, NOC (methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate), CYT D, 4′,6-diamidino-2-phenylindole, and TRITC-phalloidin were obtained from Sigma Chemical Co. Monoclonal anti-chicken β-tubulin and polyclonal FITC-sheep anti-mouse IgG were obtained from Amersham. [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [^{3}H]leucine were purchased from DuPont-NEN.

Cell culture and treatment. Wild-type Hepa 1c1c7 cells (obtained from Dr. J. Whitlock, Stanford University) were cultured at 32° as described previously (16). Cells were plated at densities of 2×10^5/60-mm culture dish for EROD determinations and 8×10^5/100-mm culture dish for RNA isolations. Cells were seeded at $1–2 \times 10^6$ in 75-cm^2 tissue culture flasks for nuclear or cytoplasmic extract preparations. Cultures were exponentially growing and 2–3 days old at the time of initiation of drug treatments. DB[*a*,*c*]A, COL, NOC, and CYT D were dissolved in DMSO. The medium content of DMSO never exceeded 0.1%, a value that is below that reported to induce *Cyp1a-1* in cultured rat hepatocytes (31). NOC or COL treatment resulted in the generation of cells having either flat or rounded morphology. Both cell types remained attached to the culture dishes. However, cells having the rounded morphology could be detached by vigorously pipeting medium through a Pasteur pipet onto the culture surfaces. The remaining attached cells could be released by exposure of cultures to trypsin. Cell numbers were estimated by counting with a hemacytometer. Viability was assessed as the ability to exclude trypan blue dye.

Fluorescence microscopy. Cells were cultured on glass coverslips. For tubulin detection, fixations were carried out in 3.7% paraformaldehyde (in PBS) for 30 min at room temperature, followed by extraction with 0.2% Triton X-100 (in PBS) for 15 min. Fixed cells were incubated overnight at 4° with primary monoclonal antibodies to tubulin. After extensive rinsing with PBS the cells were further incubated with polyclonal FITC-sheep anti-mouse IgG (1/50) for 2 hr at 37° and mounted on slides with 50% glycerol/PBS (supplemented with 0.1% *n*-propylgallate to reduce fading). For actin detection fixations were carried out as described for tubulin. After fixation the cultures were first incubated for 45 min with 1% bovine serum albumin (in PBS) and then incubated for 1 hr at room temperature in a PBS solution containing 0.1% saponin and 0.1 μg/ml TRITC-phalloidin. After extensive rinsing with PBS/0.1% saponin, the coverslips were again treated for 10 min with 3.7% formaldehyde (in PBS). After fixation the coverslips were washed three times with PBS/0.1% saponin and mounted on glass slides. Fluorescence was visualized with a Nikon Optiphot microscope equipped with a fluorescence source and filters necessary for detection of FITC or tetramethylrhodamine isothiocyanate (TRITC-phalloidin). Photographs were taken using a 100× oil immersion objective and Plus-X-Pan or TMAX100 film.

For analysis of nuclei, fixed cells were stained with 4′,6-diamidino-2-phenylindole (1 μg/ml in PBS) for 5 min at room temperature, mounted in glycerol/PBS, and viewed with a Zeiss confocal microscope.

Isolation of microsomes. Cultures were washed with PBS and then scraped into a buffer (250 μl/100-mm culture plate) containing 0.1 M Tris-acetate, pH 7.4, 0.1 M KCl, 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 0.1 mg/ml leupeptin, and 0.1 mg/ml aprotinin. The cell suspension was homogenized with a Brinkmann Polytron (setting 3, 4

× 10 sec) and then centrifuged for 20 min at 11,000 × *g*. The resulting supernatant fluid was recentrifuged for 1 hr at 105,000 × *g* to pellet the microsomes. The microsomal pellet was carefully rinsed with and then suspended in 50 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 20% glycerol. The suspended microsomes were homogenized with an Omni-1000 homogenizer (at high speed for 2 × 20 sec) and then recentrifuged for 20 min at 11,000 × *g*. The supernatant fluids were used as a source of microsomes and were stored at −70°. The isolation procedure was performed at 4°.

Western blot analyses. Microsomes were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of Laemmli (32), and then electrophoretically transferred from the gel to nitrocellulose. After transfer, the blots were incubated with blocking solution (5% Carnation nonfat milk in PBS/0.05% Tween 20) for 1 hr at room temperature, with gentle rocking. Polyclonal rabbit anti-rat cytochrome P4501A1 (gift of Dr. F. P. Guengerich, Vanderbilt University School of Medicine) was added to the blocking solution, and the blots were incubated overnight at room temperature. The following morning the blots were rinsed with PBS/0.05% Tween 20 and then washed three times (5 min each) with the same solution. After the final wash the blots were incubated for 1 hr with an anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody in blocking solution. At the end of 1 hr the blots were again washed four times with PBS/0.05% Tween 20. Antigen-immunoglobulin conjugates were detected with an Amersham enhanced chemiluminescence detection kit and recorded on X-ray film.

EROD determination. EROD activity was assayed as described previously, except that 25 μM dicumarol was included in the assay cocktail, and the reactions were terminated by lysis of the cultures with sodium dodecyl sulfate (33). Specific activities are expressed as picomoles of product/hour/10^6 cells.

RNA preparation and CYP1A1 mRNA detection. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi (34). Northern blot analyses were performed as described previously (16). A 700-base pair fragment corresponding to the 3′ end of the *Cyp1a-1* gene (obtained from J. Whitlock, Stanford University) was used for detection of CYP1A1 mRNA. A radiolabeled *Bam*HI fragment of a murine 7 S cDNA clone (16) was used to normalize the amounts of RNA on nylon membranes (Nylon-1; GIBCO-BRL) after capillary transfer. Quantitation of autoradiographs was performed with a Molecular Dynamics densitometer and Image Quant software.

Nuclear extract preparation. An unpublished procedure of Denison,[2] with slight modification, was used for the preparation of nuclear extracts. Cultures of 1c1c7 cells were rinsed twice with cold 10 mM HEPES, pH 7.5, and allowed to swell on ice for 15 min. Cells were collected by scraping into 5 ml of MDH buffer (25 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM PMSF) and were gently homogenized with a Dounce homogenizer (pestle B). Nuclei were pelleted by centrifugation and washed twice with MDHK buffer (MDH buffer with 0.1 M KCl) before being transferred into microfuge tubes and pelleted by centrifugation. The resulting nuclear pellet was resuspended in 25–50 μl of HDK (25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 M KCl), left on ice for 20 min, and briefly vortexed every 5 min. Glycerol was added to 10% and the nuclear extract was cleared by two centrifugations (15 min at 11,000 × *g* and 1 hr at 110,000 × *g*). Samples of nuclear protein extracts were stored at −80°.

Cytoplasmic extract preparation and *in vitro* transformation of the AhR. Cytosol was prepared as described by Harper *et al.* (35), with the following modifications. Cells were collected by trypsinization (2–3 min at 37°), washed three times with cold PBS (containing 0.5 mg/ml soybean trypsin inhibitor), and homogenized with a Dounce homogenizer (pestle B, 20 strokes) in HED buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM PMSF). One volume of HEDG (HED buffer plus 20% glycerol) was added and cytoplasmic extracts were clarified by successive centrifugations for 15 min at 11,000 × *g* and 1 hr at 11,0000 × *g*. Cleared cytoplasmic extracts were aliquoted and stored at −80°. Protein concentrations in nuclear and cytoplasmic extracts were determined with Bradford reagent (Bio-Rad), using bovine serum albumin as a standard. For *in vitro* transformation of the AhR, 98 μl of extract (1.3–2 μg of protein/μl; amounts were similar for different groups in the same experiment but varied between experiments) were incubated with 1 μM DB[*a*,*c*]A (2 μl of a 50 μM stock solution, freshly diluted in HEDG) for 3 hr at room temperature.

Gel shift analyses. Gel retardation analyses were performed according to the procedure detailed by Harper *et al.* (35). Complementary oligonucleotides 5′-GATCCGGCTCTTCTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′ containing DRE site D (36) (core recognition sequences are underlined) were annealed and radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. In general, samples of nuclear or cytosolic extracts (5 μl) were mixed with 15 μl of HEDGK (HEDG buffer supplemented with 0.15 M KCl) and 4 μl of poly(dI·dC) (250 ng total) and incubated for 15 min at room temperature before the addition of 1 μl of labeled oligonucleotide (100,000 dpm, ~0.2 ng). After an additional 20-min incubation, samples (20 μl) were run on a 4% polyacrylamide gel with recirculating 1× TAE buffer (6.7 mM Tris·HCl, pH 8, 3.3 mM sodium acetate, 1 mM EDTA). Gels were vacuum dried and exposed at −80° to Fujichrome-X film. Quantitation of autoradiographs was performed with a Molecular Dynamics densitometer.

[^{3}H]TCDD binding assay. AhR-dependent binding of [^{3}H]TCDD in cytosolic extracts was quantitated using an hydroxylapatite adsorption assay, as described by Gasiewicz and Neal (37). The concentration of TCDD used in the assays was 2 nM. Radioactivity was determined with a Beckman scintillation counter. The difference in the amount of [^{3}H]TCDD bound in the absence and presence of a 200-fold excess of unlabeled 2,3,7,8-tetrachlorodibenzofuran was used to calculate the amount of specific binding to the AhR. Analyses of individual samples were generally performed with at least two different amounts of cytosolic protein, to ensure proportionality. Binding activity was expressed as femtomoles of [^{3}H]TCDD bound/milligram of cytosolic protein.

[^{3}H]Leucine incorporation into protein. An unpublished procedure from the laboratory of Dr. J. Pounds (Wayne State University) was used to quantitate the incorporation of exogenously added leucine into cellular protein. Culture dishes (35 mm) were seeded with 3 × 10^5 cells, in α-MEM supplemented with 5% fetal bovine serum, and cells were grown at 32° for ~48 hr before the initiation of any treatments. To label the cells with leucine, the cultures were washed three times with PBS and then pulsed for 1 hr at 32° in 1.5 ml of α-MEM containing 1.5 μCi of [^{3}H]leucine (168 Ci/mmol) but no serum. At the end of the 1-hr period the medium was aspirated and the cells were rinsed twice with cold PBS. The rinsed cultures were fixed by exposure to cold 5% trichloroacetic acid for a minimum of 1 hr. After fixation the cultures were washed twice (5 min each) with cold 5% trichloroacetic acid. The cultures were then extracted three times (5 min each) with methanol/ether (3:1, v/v) at room temperature. After air drying, the extracted cells were dissolved in 0.75 ml of 0.2 M NaOH. Duplicate samples of the dissolved cell solution were analyzed for radioactivity by liquid scintillation counting. A second set of dishes (two/treatment) were treated with trypsin to estimate cell numbers. [^{3}H]Leucine incorporated into protein is expressed as dpm/10^3 cells. Before performance of the studies reported herein, we determined that [^{3}H]leucine incorporation into protein was linear with respect to both cell number (0.1–3 × 10^6 cells) and pulse time (0.5–2 hr).

Flow cytometry. Nontreated, DMSO-treated, or NOC-treated cultures were trypsinized, washed three times with PBS, and fixed by dropwise addition of 1 volume of ice-cold 100% ethanol. Fixed cells were then washed in PBS and resuspended (1–2 × 10^6 cells/ml) in PBS containing 1 μg/ml RNase. After a 30-min incubation at 37° the cells were washed again with PBS, and DNA was stained by incubation of

[2] M. Denison, University of California, Davis, unpublished procedure.

the fixed cells in PBS containing propidium iodide (50 μg/ml). Cells were analyzed with a Becton Dickinson FACScan instrument, and the percentage of cells in G_1, S, and G_2/M stages of the cell cycle was determined with a DNA histogram-fitting program (MODFIT; Verity Software).

Statistical analyses. Data were analyzed by either the Student *t* test or analyses of variance using the Fisher protected least significance difference test. Differences were considered statistically significant for $p < 0.05$.

Results

Detection of actin filaments and microtubules in inhibitor-exposed cultures. The mechanisms of cytoskeleton disruption by CYT D, NOC, and COL are well characterized. CYT D binds to the barbed end of F-actin and inhibits both the addition and dissociation of G-actin, which results in disruption of the supramolecular organization of actin networks (38, 39). The microtubule-inhibiting drugs COL and NOC bind directly to tubulin and shift the assembly/disassembly equilibrium to favor disassembly (40, 41).

To verify the effectiveness of the cytoskeletal inhibitors, 1c1c7 cultures grown on coverslips were exposed to medium containing 10 μM CYT D, COL, or NOC. Within 1 hr of CYT D treatment the actin cytoskeleton had totally collapsed (Fig. 1, compare A and D). However, even after 24 hr of continuous CYT D exposure the cells remained attached to the culture dishes and were generally impermeable to trypan blue (Fig. 2, A and B). Exposure of cultures to COL or NOC for 1 hr was sufficient to cause disassembly of the microtubule lattice (Fig. 1, B, C, and E). In the continuous presence of either microtubule inhibitor the cells flattened and the cytoplasm became translucent (flat morphology). Cellular rounding (round morphology) of some cells began within 6 hr of treatment. Within 18–24 hr of treatment an appreciable proportion of the cells had the rounded morphology (Fig. 3, A and D). After 24 hr of continuous exposure to NOC, microtubules did not repolymerize in cells having either flat or rounded morphology (Fig. 1F). Similar results were seen in COL-treated cultures.[3]

The concentration of chemicals (10 μM) used in Fig. 1 to disrupt the cytoskeleton was in excess of the minimum concentrations necessary to obtain maximum morphological changes. Maximum morphological changes could be achieved with concentrations of CYT D, COL, and NOC that ranged from 1 to 10, from 0.5 to 20, and from 0.2 to 20 μM, respectively.[3] Furthermore, cell proliferation was suppressed by all three chemicals, and the concentrations mediating maximum morphological changes caused comparable suppressions of proliferation.[3]

EROD activities in cultures treated with cytoskeleton inhibitors. Measurements of EROD activity are commonly used to monitor the induction of *Cyp1a-1* (16, 19, 31). EROD activities were not elevated after 7 hr of exposure to DB[*a*,*c*]A but were markedly elevated after 14 hr of exposure to inducer

[3] J. J. Reiners, Jr. and A. Schöller, unpublished studies.

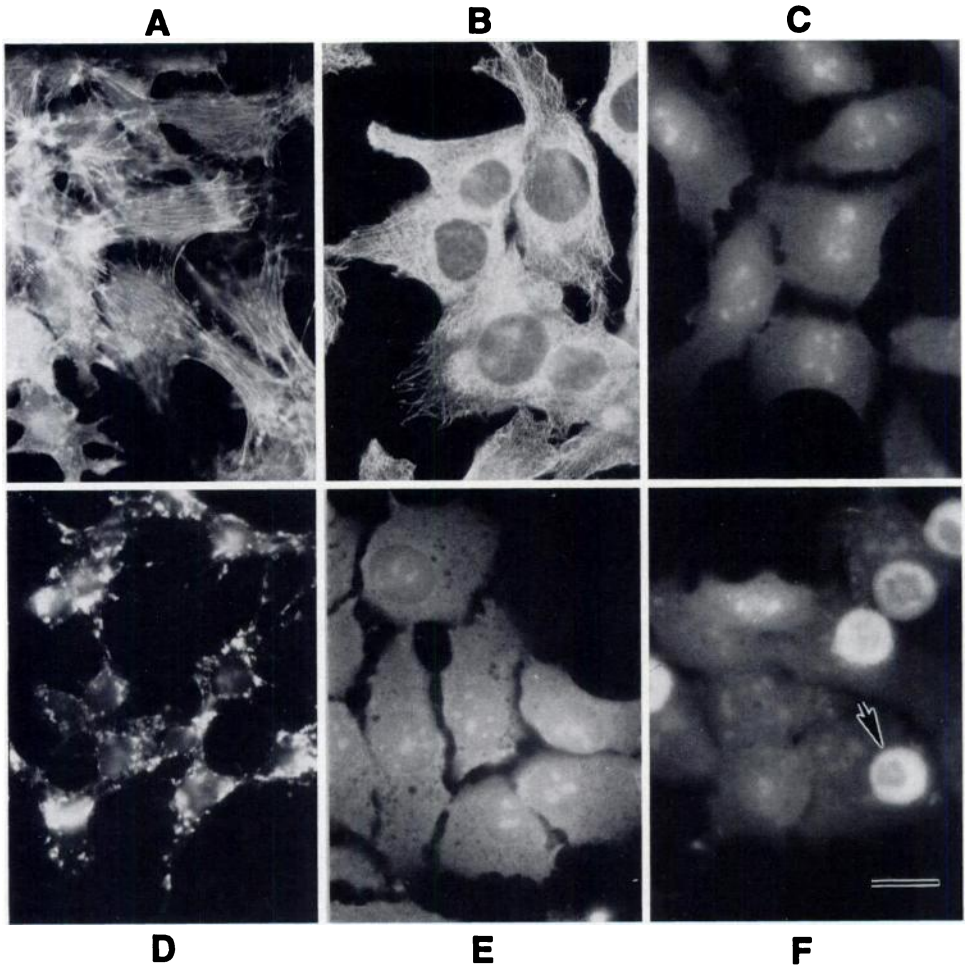


Fig. 1. Indirect fluorescence detection of actin and tubulin in cultures treated with cytoskeletal inhibitors. Cultures grown on coverslips were pretreated for 1 hr with DMSO (A and B) or 10 μM CYT D (D), COL (E), or NOC (C) or for 24 hr with NOC (F) before fixation and processing. Cultures in A and D were stained for actin. Cultures in B, C, E, and F were stained for tubulin. *Arrow* (F), NOC-treated cell having the rounded morphology. *Bar* (F) = 20 μm.

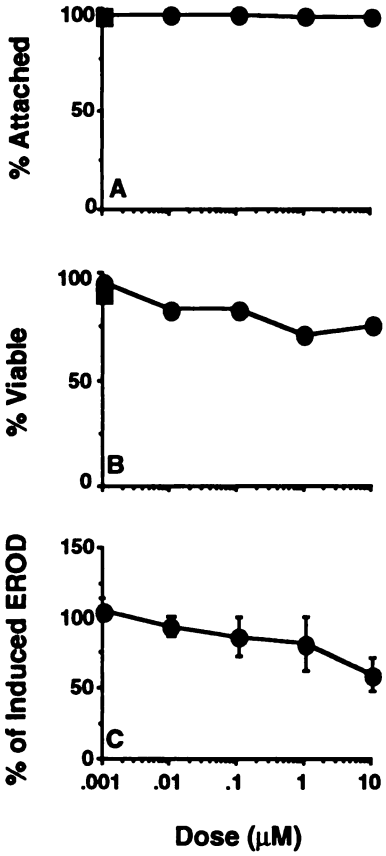


Fig. 2. Modulation of EROD induction by CYT D. Cultures were treated with CYT D for 3 hr before the addition of DB[*a*,*c*]A (3 μM). Cells were harvested for analyses of attachment, viability, and EROD activities ~20–23 hr after addition of inducer. Data represent the means ± standard errors for eight plates (two experiments). EROD activities of noninduced, DMSO-treated controls and DB[*a*,*c*]A-induced controls were 39 ± 2 and 981 ± 45 pmol/hr/10^6 cells, respectively. The values for DMSO-treated controls were subtracted from all induced values before calculation of percentage of induced EROD.

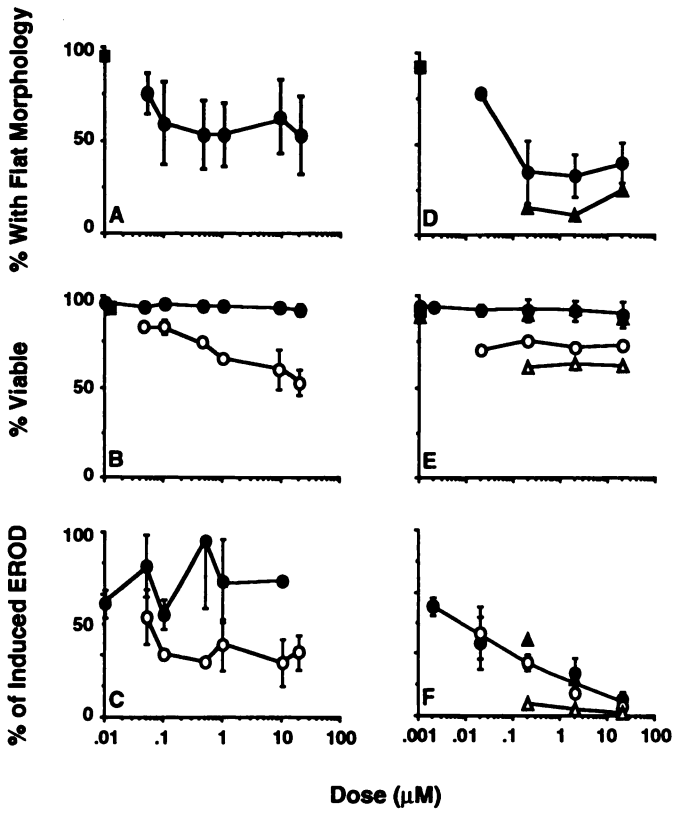
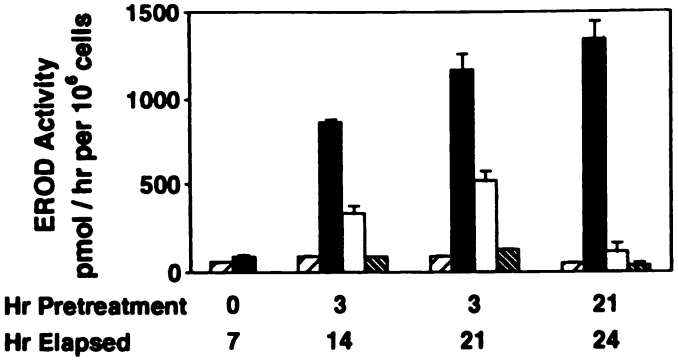


Fig. 3. Modulation of EROD induction by 10 μM COL (A, B, and C) or 10 μM NOC (D, E, and F). Cultures were treated with cytoskeletal inhibitors for 3 hr (*circles*) or 24 hr (*triangles*) before the addition of DB[*a*,*c*]A (3 μM). Cells were harvested for EROD analyses and assessments of morphology and viability ~20–24 hr after induction. Cells having a flat morphology (*closed symbols*) remained attached to the culture dishes upon vigorous passage of medium over the surfaces of the cultures, whereas cells having the rounded morphology (*open symbols*) detached from the culture plates. Data represent the means ± standard errors for eight plates (two experiments), except for the 0.001 μM NOC data, which entailed analyses of four plates. EROD activities of DMSO-treated controls and DB[*a*,*c*]A-induced controls were 48 ± 9 and 1213 ± 103 pmol/hr/10^6 cells, respectively. The values for DMSO-treated controls were subtracted from all induced values before calculation of percentage of induced EROD.

and continued to increase for at least an additional 7–10 hr (Fig. 4, *top*). The increases in EROD activities were paralleled by increases in the amount of microsomal protein recognized by an antibody prepared against CYP1A1 (Fig. 4, *bottom*). Reciprocally, we have been unable to detect a cross-reacting protein in microsomes isolated from 1c1c7 cultures either 6 or 7 hr after the addition of DB[*a*,*c*]A (three experiments).[3] Consequently, the induced EROD activities measured in DB[*a*,*c*]A-treated 1c1c7 cultures most likely reflect cellular CYP1A1 content.

Treatment of 1c1c7 cultures with CYT D for 3 hr before the addition of inducer resulted in a concentration-dependent suppression of EROD induction (Fig. 2C), However, this suppression paralleled the relative viabilities of the cultures (compare Fig. 2, B and C). Consequently, the suppressive effects of CYT D on EROD induction could not be separated from its cytotoxic effects.

Treatment of 1c1c7 cultures with COL 3 hr before the addition of inducer suppressed EROD induction in cells with the rounded morphology and had marginal inhibitory effects on cells with the flat morphology (Fig. 3C). However, the inhibitory effects of COL on EROD induction essentially paralleled the relative viabilities of the cultures (compare Fig. 3, B and C). In marked contrast, treatment of cultures with NOC either 3, 20, or 24 hr before the addition of inducer strongly suppressed EROD induction in cells having either flat or rounded morphology (Figs. 3F and 4). NOC-dependent suppression of EROD induction was paralleled by marked reductions (>80%) in the microsomal contents of CYP1A1 protein (Fig. 4, *bottom*, and a second independent experiment). Suppression of EROD induction in cells having the flat morphology occurred in the absence of observable cytotoxicity (Fig. 3, E and F). Although viability was somewhat reduced in cells having the rounded morphology, complete suppression of EROD induction could be achieved with conditions that were only modestly cytotoxic (compare Fig. 3, E and F). It should be emphasized that exposure of cultures to DB[*a*,*c*]A did not cause the cells to round up and did not alter the kinetics of formation or the distribution of flat and rounded cells in COL- and NOC-treated cultures.[3] Consequently, the distributions of flat and rounded cells depicted in Fig. 3, A and D, reflect the effects of COL and NOC, respectively. Furthermore, cultures treated with only



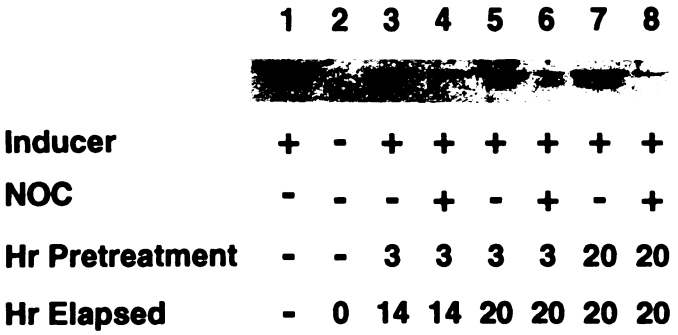


Fig. 4. Time-dependent induction of EROD and its suppression by NOC. Cultures were pretreated with solvent (DMSO) or 10 μM NOC for 3, 20, or 21 hr before the addition of DB[*a*,*c*]A (3 μM). Cultures were harvested for analyses of EROD activities (*top*) or CYP1A1 protein content (*bottom*) 7, 14, 20, 21, or 24 hr after the addition of inducer. *Top*, *columns* represent DMSO-treated cultures (■), DMSO- plus DB[*a*,*c*]A-treated cultures (■), flat cells from NOC- plus DB[*a*,*c*]A-treated cultures (□), and rounded cells from NOC- plus DB[*a*,*c*]A-treated cultures (▨). *Bottom*, the Western blot analyses were performed with 1 μg of 3-methylcholanthrene-induced rat liver microsomes (*column 1*) and 12 μg of 1c1c7 microsomes (*columns 2-8*). Microsomes prepared from NOC-treated cultures contained both flat and rounded cells.

NOC (10 μM) for either 24 or 44 hr had viabilities of ~92% and ~88%, respectively (three experiments).[3] Similarly, cultures treated with only DB[*a*,*c*]A for 20–24 hr (the length of exposure used for EROD induction) had viabilities of 90–92%. Consequently, the percentages of NOC-pretreated cells that were viable at the times of addition of inducer were very high (88–92%), and the reductions in viability noted in Fig. 3E reflect the combined effects of the inducer and NOC.

CYP1A1 mRNA levels in cultures treated with cytoskeletal inhibitors. Steady state CYP1A1 mRNA contents dramatically increase in 1c1c7 cells within 6 hr of addition of DB[*a*,*c*]A (16). The studies reported in Fig. 1 demonstrate that a 1-hr pretreatment with the cytoskeleton-depolymerizing drugs resulted in complete disruption of the actin and microtubule networks. Induction of the *Cyp1a-1* gene, as analyzed by measurements of steady state CYP1A1 mRNA contents, was not suppressed when inducer was added to cultures that had been pretreated with the cytoskeletal inhibitor drugs for 1 hr (Fig. 5). These results suggest that the translocation of the activated AhR from the cytoplasm to the nucleus and the *Cyp1a-1* induction process are independent of the integrities of the microtubule and actin microfilament lattices.

Cyp1a-1 induction, as measured by Northern blot analysis, was still unaffected if cultures were treated with NOC for 5 hr

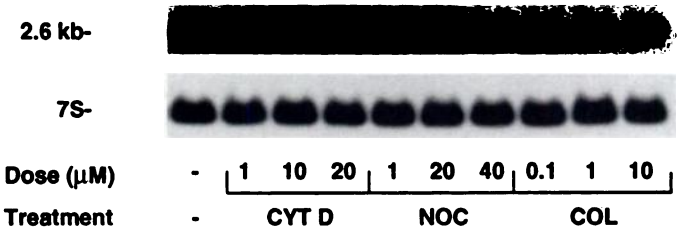


Fig. 5. Effects of short term treatment with cytoskeleton-depolymerizing drugs on induced CYP1A1 steady state mRNA contents. Cultures were treated for 1 hr with different concentrations of CYT D, COL, or NOC before the addition of DB[*a*,*c*]A. Total RNA was prepared from cultures 6 hr after the addition of inducer or solvent. RNA (20 μg) was separated on agarose-formaldehyde gels, transferred to nylon membranes, and probed with labeled *Cyp1a-1* or 7 S cDNA.

before the addition of DB[*a*,*c*]A (Fig. 6). However, accumulation of CYP1A1 mRNA was markedly suppressed in cultures treated with NOC for 21–23 hr before the addition of DB[*a*,*c*]A (Fig. 6). This suppression was seen in cells having either flat or rounded morphology. Pretreatment of DB[*a*,*c*]A-induced cultures with 0.2 μM NOC (two experiments) suppressed CYP1A1 mRNA accumulation in flat and rounded cells by 55 ± 0.5 and 68 ± 0.5%, respectively, whereas pretreatment with 10–20 μM NOC (four experiments) significantly suppressed CYP1A1 mRNA accumulation in flat and rounded cells by 68 ± 2 and 74 ± 4%, respectively. Consequently, under certain conditions pretreatment with NOC could suppress the DB[*a*,*c*]A-dependent accumulation of CYP1A1 mRNA.

It has been hypothesized that *CYP1A1* induction can be mediated by either the AhR complex or the 4 S PAH-binding protein (42–44). DB[*a*,*c*]A is a high affinity ligand for both receptors, whereas TCDD has a very high affinity for the AhR and is not a ligand for the 4 S binding protein (45). Pretreatment of cultures with 10 μM NOC for 23–24 hr before the addition of TCDD significantly suppressed the accumulation of CYP1A1 mRNA in flat and rounded cells, by 77 ± 6% and 77 ± 5%, respectively (Fig. 6 and a second independent experiment). Consequently, NOC affects induction being mediated through the AhR.

Nuclear AhR in NOC-treated cells. Induction of *Cyp1a-1* by DB[*a*,*c*]A requires that the PAH binds to the ALBS and that the ALBS forms a complex with the ARNT protein. Induction is mediated by subsequent interaction of the ALBS-ARNT complex with DRE sequences in the 5′ regulatory region of the *Cyp1a-1* gene. Gel retardation analyses of nuclear extracts isolated from DB[*a*,*c*]A-treated cultures indicated the presence of a predominant protein-DNA complex that could be eliminated by performing the initial incubation in the presence of an excess of unlabeled DRE (Fig. 7). Formation of this complex was dependent upon exposure of the cultures to DB[*a*,*c*]A.[3] The DNA-binding activities of nuclear extracts isolated from cultures that had been pretreated with 10 μM NOC for 20–23 hr before the addition of DB[*a*,*c*]A were suppressed by 77 ± 10% and 54 ± 12% (three experiments) in flat and rounded cells, respectively, relative to the intensity occurring with extracts prepared from DMSO-treated cultures (Fig. 7 and two additional experiments).

***In vitro* AhR transformation and TCDD-binding activity.** The reductions in ALBS/ARNT-DNA binding noted in Fig. 7 could reflect a loss of DRE binding capacity, an inhibition of the translocation of the AhR, or alterations in the cellular content of the ALBS or its ability to bind ligand.

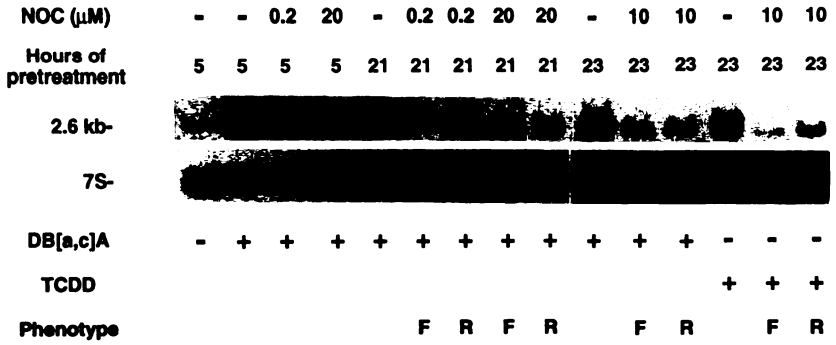


Fig. 6. Effects of long term treatment of cultures with NOC on *Cyp1a-1* induction. Cultures were treated for 5, 21, or 23 hr with 0.2, 10, or 20 μM NOC before the addition of DB[*a,c*]A (3 μM) or TCDD (5 nM). Total RNA was isolated, either 5 hr (TCDD) or 6 hr (DB[*a,c*]A) after the addition of inducer or solvent, from cells having normal, flat (*F*), or rounded (*R*) morphology, and 20 μg of RNA were analyzed by Northern blot analysis as described for Fig. 5. Data depict two independent experiments with the last 6 columns representing the second experiment.

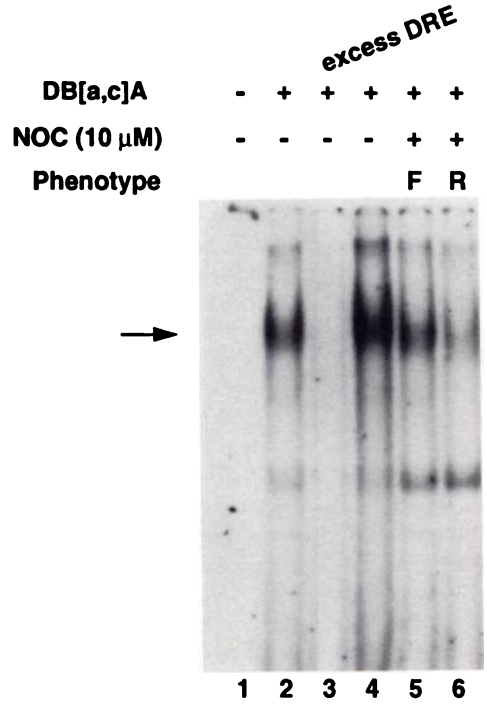


Fig. 7. AhR-DRE interactions in nuclear extracts. Cultures were treated with DMSO or NOC (10 μM) for 24 hr before the addition of DB[*a,c*]A. Cultures were harvested 1 hr after the addition of inducer and were used to prepare nuclear extracts, which were incubated with ^{32}P-labeled DRE-containing oligonucleotides and analyzed by gel retardation electrophoresis. The sources of extract and the amounts of protein analyzed were as follows: no extract (*lane 1*), 2 μg of nontreated extract (*lanes 2* and *3*; *lane 3* with a 100-fold excess of nonlabled DRE), 2.9 μg of DMSO-pretreated extract (*lane 4*), and 2.9 μg of NOC-pretreated extract from cells having the flat (*lane 5*) or rounded (*lane 6*) morphology. *Arrow*, position of the AhR-DRE complex.

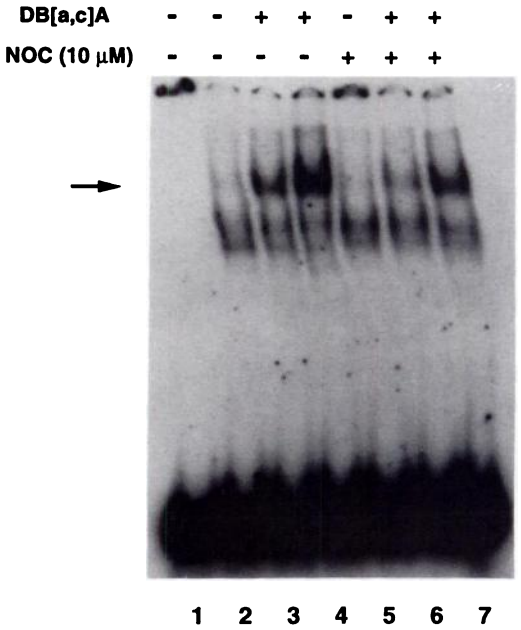


Fig. 8. *In vitro* AhR transformation and DRE binding. Cytoplasmic extracts were prepared from cultures that had been pretreated for 24 hr with either DMSO or 10 μM NOC. Extracts were incubated with DB[*a,c*]A (1 μM) for 3 hr at room temperature before being incubated with ^{32}P-labeled DRE oligonucleotide and analyzed by gel retardation electrophoresis. Lanes contained no extract (*lane 1*), 11 μg of noninduced control extract (*lane 2*), 11 μg (*lane 3*) or 22 μg (*lane 4*) of induced control extract, 11 μg of noninduced, NOC-treated extract (*lane 5*), and 11 μg (*lane 6*) or 22 μg (*lane 7*) of induced, NOC-treated extract. *Arrow*, position of the AhR-DRE complex.

Cytosol isolated from DMSO-treated cultures bound ~56 ± 16 fmol of [^{3}H]TCDD/mg of protein (five different extract preparations). This value is in reasonable agreement with the values published by Okey *et al.* (46) using 1c1c7 cells. TCDD binding was reduced an average of 61 ± 3% (three experiments, *p* values of 0.03, 0.06, and 0.09) in extracts prepared from cultures that had been pretreated with NOC for 24 hr. *In vitro* AhR transformation-DNA binding analyses yielded results that complemented the TCDD binding assay data (Fig. 8). Specifically, *in vitro* incubation of cytosols derived from DMSO-treated 1c1c7 cultures with DB[*a,c*]A and a radiolabeled DRE oligonucleotide resulted in a predominant protein-DNA complex that could be detected by gel retardation analysis. The amount of this complex was decreased ~55% (Fig. 8) in studies using cytosolic extracts isolated from cultures that had been pretreated with 10 μM NOC for 20–24 hr before harvest and extract preparation. A reduction of ~62% was measured in a second experiment.[3] Consequently, a correlation existed between the amount of [^{3}H]TCDD bound by cytosolic proteins and the relative amounts of transformed AhR complex capable of binding to a DRE oligonucleotide.

Cell cycle analysis of NOC-treated cells. The microtubule inhibitors colcemid and NOC are widely used to arrest cultured mammalian cells in the G_2/M phase of the cell cycle (47, 48). Treatment of asynchronous cultures of exponentially growing 1c1c7 cells with DMSO for 24 hr resulted in a cell cycle distribution pattern that was similar to that observed in nontreated cultures harvested before DMSO addition (Fig. 9, compare *A* and *B*) or 24 hr later.[3] In contrast, flow cytometric analyses clearly demonstrated a cell cycle synchronization in NOC-treated cells, with 83% and 98% of the cells having the flat and rounded morphologies being tetraploid, respectively

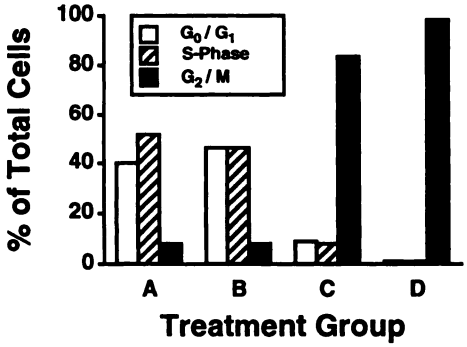


Fig. 9. Cell cycle analyses of NOC-treated cultures. Cultures were harvested at the beginning of the experiment (*A*) or exposed to DMSO (*B*) or 10 μM NOC (*C* and *D*) for 24 hr before harvesting and analyses of DNA content by flow cytometry. NOC-treated cultures were separated into populations having flat (*C*) or rounded (*D*) morphology. The data represent values derived from a DNA histogram-fitting program and are expressed as the percentage of cells within the histogram limits.

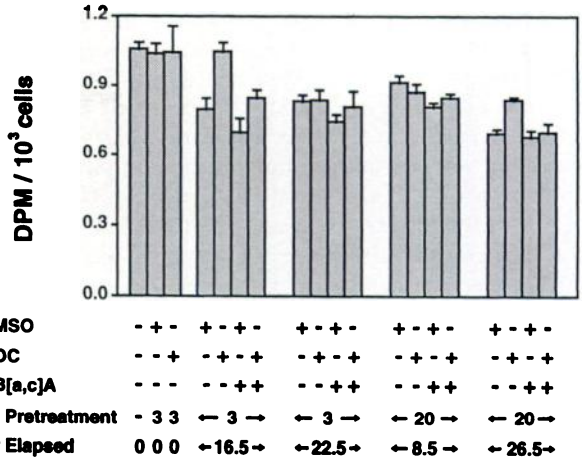


Fig. 10. Effects of NOC pretreatment on [^{3}H]leucine incorporation into protein. Some cultures were treated with nothing (*first column*) or pretreated with solvent or NOC (10 μM) for 3 hr (*second* and *third columns*) before pulsing with [^{3}H]leucine and harvesting. Other cultures were pretreated with NOC or solvent for 3 or 20 hr before addition of DB[*a,c*]A (3 μM) or solvent. These latter cultures were pulsed with [^{3}H] leucine 8.5, 16.5, 22.5, or 26.5 hr after the addition of inducer. Data are from two experiments and represent means ± standard errors of six to eight culture dishes/treatment group (three or four plates/experiment). NOC-treated cultures were not separated into cell types having the flat or rounded morphology.

(Fig. 9). DNA staining with 4′,6-diamidino-2-phenylindole revealed that NOC-treated cells had one nucleus, with diffuse or compact chromatin in cells having the flat and rounded morphologies, respectively.[3]

Kung *et al.* (47) recently reported that treatment of cultured rodent cell lines with colcemid or NOC does not cause a sustained arrest in the G_2/M phase of the cell cycle. Rather, these agents facilitate an initial pausing and accumulation of cells in G_2/M, which is followed by subsequent reentry into the cell cycle. Progression through subsequent cell cycles, in the absence of cell division, results in serial doubling of cellular DNA content and the generation of polyploid cells. In our studies, within 48 hr of NOC treatment a small percentage of the cells with flat morphology were octoploid.[3] Within 71 hr of NOC treatment ~19% and 30% of the cells with rounded and flat morphologies were octoploid, respectively.[3] Consequently, NOC treatment of 1c1c7 cultures caused an initial pausing and accumulation of cells in G_2/M and not a permanent cell cycle arrest.

Translation in NOC-treated cultures. The observation that short term pretreatment of cultures with NOC did not affect the accumulation of CYP1A1 mRNA (Fig. 5) but suppressed the induction of EROD activity and the expression of the CYP1A1 protein (Figs. 3 and 4) suggested that NOC might post-transcriptionally modulate the *Cyp1a-1* induction process. To determine whether NOC had a generalized effect on translation, we monitored the incorporation of [^{3}H]leucine into cellular protein. The studies reported in Fig. 10 demonstrate that incorporation of leucine into cellular protein was not significantly affected by pretreatment of cultures with NOC for 3 or 20 hr. Furthermore, relative to the incorporation occurring in DMSO-treated control cultures, neither the concentration of DB[*a,c*]A used for *Cyp1a-1* induction nor the combined use of NOC and DB[*a,c*]A altered leucine incorporation. Consequently, NOC did not cause a general suppression of translation in cultured 1c1c7 cells. It should be emphasized that the lack of a generalized effect of NOC on leucine incorporation into protein is not without precedent. Specifically, it has been reported that neither polyribosome profiles nor the rates, patterns, or absolute amounts of protein synthesis are markedly affected in cells treated with the microtubule inhibitors COL or colcemid (49–51).

Discussion

Conventional cellular fractionation in conjunction with TCDD binding assays indicates a cytoplasmic location of the latent AhR, which upon ligand binding rapidly translocates into the nuclear compartment (46). This ligand-dependent translocation of the AhR has recently been directly documented by indirect immunofluorescence microscopy using anti-ALBS and anti-ARNT antibodies (8). The current study was designed to investigate the role of the cytoskeleton in the *Cyp1a-1* induction process. Several lines of research provide a precedent for our study. First, microtubule inhibitors suppress the translocation of at least two different classes of hormone receptors (11, 12). Second, cytoplasmic HSP90 molecules have been reported to associate with actin microfilaments (14, 15), and HSP90 is a component of the latent AhR (4, 5). Third, exposure of cultured cells or animals to TPA or the kinase inhibitor H7 has been reported to suppress *Cyp1a-1* induction (16–20). Furthermore, in one study suppression of *Cyp1a-1* induction by TPA was correlated with reduced accumulation of the nuclear ALBS-ARNT complex (20). TPA and H7 have profound effects on components of the cytoskeleton (24–26), and H7 has been reported to suppress GR translocation (30). To analyze a possible involvement of the cytoskeleton in the processes of *Cyp1a-1* induction, we characterized the effects of CYT D, COL, and NOC on *Cyp1a-1* induction in cultured murine hepatoma 1c1c7 cells. All three drugs facilitated rapid and dramatic disruptions of either the actin or microtubule cytoskeletal network. However, at a time during which there was unequivocal disruption of the cytoskeleton we were unable to observe any significant suppression of *Cyp1a-1* induction, as monitored by measurements of CYP1A1 mRNA. This result suggests that nuclear import of the AhR and activation of *Cyp1a-1* transcription do not require intact microtubules or actin microfilaments.

In addition to the AhR, there are several other proteins whose cytoplasm/nucleus shuttling appears to be independent

of the cytoplasmic cytoskeleton. Although the progesterone receptor resides primarily in the nucleus, irrespective of its hormone occupancy, Perrot-Applanat *et al.* (52) have constructed a cell line having a mutated progesterone receptor that is functional, resides in the cytoplasm, and translocates to the nucleus upon hormone stimulation. Translocation of this progesterone receptor is not affected by exposure to NOC, phalloidin, CYT B, or CYT D or cotreatment with colcemid and CYT B (52). The nuclear affinity of the estrogen receptor is not diminished by treatment with the microtubule inhibitor vinblastine or the actin inhibitor CYT B in rat uterus (53). Similarly, microinjected HSP70 molecules and adenovirus EIA proteins are translocated to the nucleus in the presence of the cytoskeleton-disrupting drugs CYT E or colcemid (51). The basis for the differential involvement of the cytoskeleton in the cytoplasm/nucleus shuttling of various proteins, including hormone receptors, is poorly understood.

Although CYP1A1 mRNA accumulation was not suppressed by treatment of cultures with NOC for 1–5 hr before the addition of DB[*a*,*c*]A, induction was suppressed in cultures treated with NOC for at least 20 hr before exposure to inducer. The noted reductions in induced steady state CYP1A1 mRNA contents could reflect either a suppression of transcription or an acceleration of mRNA turnover. Our studies do not distinguish between the two possibilities, but there is precedent for the former. First, gel retardation assays demonstrated that nuclear extracts of NOC-treated cells had less ALBS-ARNT complex and/or the complex present had a reduced capacity for binding to DRE sequences. Transcriptional activation of the *Cyp1a-1* gene is dependent upon the binding of ALBS-ARNT complexes to DREs in the *Cyp1a-1* gene (2, 7). Second, [^{3}H]TCDD binding analyses suggested that either the cellular content of the ALBS was reduced in NOC-treated cells or its ability to bind TCDD was inhibited. Irrespective of which possibility is correct, induction of *Cyp1a-1* transcription has been shown to correlate with the amount of ALBS and its ability to bind TCDD (7, 54). In the future we intend to use immunological techniques to quantitate ALBS in NOC-treated cells. Such an approach should establish whether the reductions in TCDD binding represent an altered capacity for ligand binding or a reduction in actual ALBS content.

Agents such as COL and NOC cause mitotic arrest and are commonly used to synchronize cultured cells in G_2/M (47, 48). NOC treatment of 1c1c7 cultures resulted in the time-dependent development of cell populations having either flat or rounded morphology. Flow cytometric analyses of cultures treated with NOC for 24 hr demonstrated that both of these populations consisted primarily of tetraploid cells. Although cultured cells often round up during mitosis, we have insufficent data to conclude that the flat and rounded cells are in the G_2 and M phases of the cell cycle, respectively. Nevertheless, given the dramatic suppression of *Cyp1a-1* induction in the flat and rounded cells but an absence of suppression in cultures treated with NOC for 1–5 hr (a time insufficient for a majority of the cells to accumulate in G_2/M), it is tempting to speculate that *Cyp1a-1* inducibility might be cell cycle dependent and does not occur in cells in G_2/M.

Regulation of the effects of hormones as a function of the cell cycle has been clearly established in the case of the GR. Specifically, studies performed with synchronized cultured cells demonstrated that several glucocorticoid-responsive genes cannot be induced in the G_2 phase of the cell cycle (55–57). Hsu *et al.* (55) have shown that the suppression occurring in G_2 correlates with an inhibition of GR nuclear accumulation and site-specific alterations in GR phosphorylation. NOC-arrested cell lines have sustained and elevated levels of cyclin B and $p34^{cdc2}$ (47), which together with other kinases affect transcription by phosphorylating transcription factors (58). For example, the transcription factors Oct1 (59) and Ets-1, but not Ets-2 (60), are hyperphosphorylated in NOC-treated cells. The phosphate groups are removed in G_1. Translocation of the SWI5 protein in *Saccharomyces cerevisiae* from the cytoplasm to the nucleus is suppressed during S and G_2/M by phosphorylation catalyzed by CDC28-dependent kinase (61). Although the idea is extremely speculative and based upon analogy to the aforementioned examples, it is conceivable that a cell cycle-specific phosphorylation/dephosphorylation of ALBS may be associated with the mechanism of NOC suppression of *Cyp1a-1* induction. If such an hypothesis is correct, then one would anticipate that results comparable to those obtained with NOC should occur with other agents that cause G_2/M arrest. Although somewhat compatible with this prediction, our studies with COL are inconclusive because of our inability to clearly separate the cytotoxic effects of the agent from the biological processes being measured. Consequently, the question of whether the effects of NOC reflect cell cycle regulation of the *Cyp1a-1* induction process) or a NOC-specific but cell cycle-independent process, is still an unresolved issue.

Measurements of EROD activity are commonly used to monitor ligand-dependent induction of *Cyp1a-1* transcription (19, 31). Both EROD activities and CYP1A1 mRNA contents were suppressed in samples treated with NOC for 22–24 hr before the addition of inducer. In contrast, EROD activities, but not CYP1A1 mRNA accumulation, were suppressed in samples treated with NOC for 1–5 hr before exposure to inducer. Thus, in the latter situation EROD measurements must have reflected post-transcriptional events affecting the *Cyp1a-1* induction process, as opposed to transcriptional activation of the gene. These results emphasize the limitations of using EROD analyses to monitor the transcriptional activation of the *Cyp1a-1* gene.

The basis for the reductions in ligand-induced EROD activities that occur in short term NOC-treated cells is not known. However, the reductions do not reflect alterations in the early stages of RNA processing, because the relative contents of mature 2.6-kilobase CYP1A1 mRNA were similar in induced cultures treated either with or without NOC. Furthermore, they do not reflect a generalized suppression of translation. Several recent studies have implied a role for the microtubule component of the cytoskeleton in nuclear/cytoplasmic mRNA transport (reviewed in Refs. 62 and 63). More specifically, disruption of microtubules with COL has been reported to repress the transport of specific mRNAs from the nucleus to the cytoplasm in *Xenopus* oocytes (64). It is conceivable that the suppressive effects of NOC on EROD induction may in part be mediated in a similar fashion.

In summary, the current investigation demonstrates that disruption of the actin and microtubule cytoskeletal network does not suppress the ligand-dependent induction of *Cyp1a-1* transcription. Furthermore, the current study suggests, albeit extremely speculatively, that *Cyp1a-1* induction may be cell cycle dependent. Determination of the validity of such a hy-

pothesis will require comparisons of *Cyp1a-1* induction in cells in the various stages of the cell cycle. If the hypothesis is correct, it takes on considerable significance given the use of cell cycle-arresting drugs (e.g., taxol and estramustine) in cancer therapy and the ability of some environmental pollutants (e.g., methyl mercury) to disrupt microtubules and cause mitotic arrest (65). Furthermore, if the lack of *Cyp1a-1* inducibility in G_2/M-phase cells reflects alterations in AhR content or function, then one would predict that the induction of a variety of TCDD/PAH-inducible genes and processes may also be cell cycle dependent.

Acknowledgments

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