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Colchicine Down-Regulates Cytochrome P450 2B6, 2C8, 2C9, and 3A4 in Human Hepatocytes by Affecting Their Glucocorticoid Receptor-Mediated Regulation

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ABSTRACT

The xenobiotic-mediated induction of three major human liver cytochrome P450 genes, CYP2B6, CYP2C9, and CYP3A4, is known to be regulated by the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). CAR and PXR are regulated, at least in part, by the glucocorticoid receptor (GR) and the hypothesis of a signal transduction cascade GR-[CAR/PXR]-P450 has been proposed. This study was aimed at testing this hypothesis in primary human hepatocytes by using the tubulin network disrupting agent colchicine. Colchicine (COL) decreased both basal and rifampicin- and phenobarbital-inducible expression of CYP2B6, CYP2C8/9, and CYP3A4. A parallel down-regulation of mRNA expression of CAR, PXR, and tyrosine aminotransferase, a prototypic gene directly regulated by GR, was observed. COL affected neither the level of GR mRNA nor ligand binding to GR. To evaluate the effect of colchicine on GR-mediated gene transactivation, HeLa cells stably or transiently transfected with a GR-responsive element-dependent luciferase reporter gene were used. COL decreased the dexamethasone-induced luciferase expression in stably transfected cell line by 50%, whereas GR transactivation in transiently transfected cells was not affected by COL. In contrast, ligand-dependent GR translocation in the human embryonic kidney 293 cell line transiently transfected with GFP-GR was inhibited by COL. We conclude that alteration of the signal transduction mediated through the GR-[CAR/PXR]-P450 cascade by colchicine is responsible for the down-regulation of CYP2C9 and CYP3A4, implicating cytoskeleton as necessary for correct functioning of this cascade under physiological conditions.

The superfamily of cytochromes P450 comprises several gene families, of which families CYP1, -2, and -3 are the most important for drug metabolism in man (Maurel, 1996). Expression of these enzymes is altered by a variety of molecules, which may or may not be substrates of the enzymes, through the activation or deactivation of various nuclear receptors and/or transcription factors. Some of the receptors and their link to P450 regulation were identified more than a decade ago [e.g., the Ah receptor regulating CYP1A expression (Nebert et al., 1984)]. Others, notably those regulating *CYP2* and *CYP3A* genes, were found more recently, including the human pregnane X receptor (PXR; NR1I2) (Lehmann et al., 1998, Goodwin et al., 1999) and the constitutive androstane receptor (CAR; NR1I3) (Negishi and Honkakoski, 2000). Both PXR and CAR are members of the hormone nuclear receptor superfamily and form heterodimers with the retinoic acid receptor (RXR; NR2B1). PXR is activated by a number of xenobiotics and steroids (Goodwin et al., 2002) and controls *CYP3A* gene induction by targeting specific responsive elements present in the regulatory region of these genes (Sueyoshi and Negishi, 2001; Goodwin et al., 2002). In human *CYP3A4*, for example, these include a proximal PXR responsive element (also called ER6, for everted repeats separated by six nucleotides), located at −160, and a distal xenobiotic response element, located between −7800 and −7200 (Goodwin et al., 1999), consisting of two direct repeats separated by three nucleotides and an ER6 motif. CAR is sequestered in the cytoplasm and translocates into the nu-

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ABBREVIATIONS: P450, cytochrome P450; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; COL, colchicine; DR4, death receptor 4; GR, glucocorticoid receptor; DEX, dexamethasone; hsp90, 90-kDa heat-shock protein; LDH, lactate dehydrogenase; TAT, tyrosine aminotransferase; GAPDH, glyceraldehyde phospate dehydrogenase; PBS, phosphate-buffered saline; DTT, dithiothreitol; GRE, glucocorticoid responsive element; GFP, green fluorescent protein chimera; HMLN, HeLa cells stably transfected with glucocorticoid responsive reporter gene construct MMTV-Luc-SVNeo; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; RIF, rifampicin; NOC, nocodazole; HELN, HeLa cells stably transfected with estrogen responsive reporter gene construct MMTV-Luc-SVNeo; RU-486, mifepristone.

cleus upon xenobiotic-mediated activation, notably in response to phenobarbital (Sueyoshi and Negishi, 2001). The phenobarbital-response element module consists of two DR4 motifs (termed NR1 and NR2) (Honkakoski et al., 1998; Sueyoshi et al., 1999). Both DR4 motifs of *CYP2B* and one DR4 motif of human *CYP2C9* have been shown to be essential for phenobarbital induction (Gerbal-Chaloin et al., 2002).

While investigating the mechanisms by which glucocorticoids could interfere with P450 gene induction, we recently observed that the expression of PXR, CAR, and RXR is under the transcriptional control of the glucocorticoid receptor (GR) (Pascussi et al., 2000a,b). It explained the previously unresolved issue of the enhancement of xenobiotic-mediated induction of *CYP2B6*, *CYP2C8/9*, and *CYP3A4* by nanomolar concentrations of dexamethasone (DEX). Its dual role in CYP3A gene induction was clarified: DEX is an indirect CYP3A inducer by up-regulating PXR/CAR/RXR levels at submicromolar concentrations through activation of GR and a direct CYP3A inducer at supramicromolar concentrations through activation of PXR (Pascussi et al., 2001). The hallmark of these studies is that the expression of *CYP2* and *CYP3A* genes maybe controlled by a cascade of signal transmission: GR-[PXR/CAR/RXR]-P450s. If this hypothesis is correct, we can anticipate that any process or stimulus that should negatively affect either the expression or activity of GR will produce a down-regulation of P450s through down-regulation of PXR and CAR nuclear receptors. Indeed, this could open the way to elucidating mechanisms of various apparent relationships linking pathophysiological situations and the detoxication function. To verify this hypothesis, we decided to evaluate the effect of colchicine on the expression of P450 genes in primary cultures of human hepatocytes.

Colchicine binds very strongly to tubulin, which results in cytoskeleton disruption (Ben-Chetrit and Levy, 1998). Many intracellular signaling pathways are thought to need the microtubules and microfilament networks to become specific and efficient (Pratt et al., 1999, Willard and Crouch, 2000). Disruption of the cytoskeleton will undoubtedly result in signal impairment, but answers to mechanistic questions (e.g., Which point in the signal pathway is stricken? Can the point be bypassed, thus diminishing some toxic effects? Can the point be simply ignored?) are unclear. GR is one of the prime suspects to be influenced by cytoskeleton status, because its inactive form is located in the cytoplasm, where it is bound to the hsp90, a known chaperone protein. After steroid hormone binding, GR dissociates from hsp90 and translocates into the nucleus (Pratt et al., 1989), and it has been proposed that this process requires functional microtubules (Pratt, 1993). Therefore, disruption of the microtubule network by compounds such as colchicine should strongly affect GR translocation to the nucleus and eventually lead to the down-regulation of the battery of genes whose transcriptional activity is GR-dependent.

In this work, we investigated the effect of colchicine on the expression of CYP2 and CYP3A mRNA, and on the expression and/or transcriptional activity of PXR, CAR, and GR in primary human hepatocyte cultures. Our results suggest that colchicine down-regulates CYP3A4, CYP2B6, and CYP2C9 by altering the signal transduction cascade GR-[PXR/CAR/RXR]-P450s.

Materials and Methods

Materials

Cell culture media and supplements, dexamethasone, rifampicin, phenobarbital, RU-486, colchicine, methylthiazoletetrazolium, dithiothreitol, horseradish peroxidase-conjugated secondary antibodies, and fetal calf serum were purchased from Sigma (St. Louis, MO). Lactate dehydrogenase (LDH) assay kit was purchased from Promega (Madison, WI). An enhanced chemiluminescence kit including Hyperfilm photographic paper, nucleotides ([α-^{32}P]dCTP, dATP, dGTP, dTTP, [α-^{32}P]UTP, CTP, ATP, and GTP), [1,2,4-^{3}H]dexamethasone, DNA polymerase Klenow fragment, and RNA polymerases T3, T7, and SP6 were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). TRIzol reagent, phenol/chloroform reagents, RNase A, RNase T1, protease K, spermidine, endonucleases *Pst*I, *Nco*I, *Kpn*I, *Apa*I, *Eco*RI, *Pvu*II, *Bam*HI, *Dde*I, and corresponding buffers were purchased from Invitrogen (Cergy Pontoise, France). FuGENE 6 transfection reagent was obtained from Roche (Mannheim, Germany). All other chemicals and reagents were of the highest quality commercially available.

Primary Cultures of Human Hepatocytes

Liver samples were obtained from surgery of hepatic tumors or from multiorgan donors who met an accidental death. Tissue acquisition protocol was in accordance with the requirements issued by local ethical commissions in France and the Czech Republic. Hepatocytes were isolated according to a published protocol (Pichard et al., 1990). After isolation, the cells were plated on collagen-coated culture dishes using cell density of 14×10^4 cells/cm^2. Culture medium used was as described previously (Isom et al., 1985) enriched for plating with 5% fetal calf serum (v/v). The medium was exchanged for a serum-free medium 4 to 6 h later, and the culture was allowed to stabilize for an additional 24 h. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator.

Detection of P450, TAT, GAPDH, PXR, CAR, and GR mRNAs

Cell cultures were treated for 24 h with colchicine (0.01–1 μM, final), nocodazole (10 and 40 μM final), rifampicin (10 μM final), phenobarbital (500 μM final), and DMSO (as vehicle for control) as described in detail under *Results*. Total RNA was isolated using TRIzol reagent from 10^7 cultured hepatocytes according to the manufacturer's instructions. Concentration of RNA was quantified by spectrometry at 260 nm, and purity was assessed from the ratio of absorbances $A_{260\ nm}/A_{280\ nm}$. For quality control, 30 μg of total RNA were analyzed by Northern blot using [α-^{32}P]dCTP-labeled rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe (J.M. Blanchard, Institut de Genetique Moleculaire de Montpellier, France). Levels of CYP3A4 and tyrosine aminotransferase (TAT) mRNAs were determined by Northern blot using [α-^{32}P]dCTP-labeled cDNA probes (INSERM U128, Montpellier, France) and mouse TAT cDNA (gift from Dr. T. Grange, Institute J. Monod, Paris, France), respectively. To determine CYP2B6, CYP2C8, CYP2C9, PXR, CAR, and GR mRNAs, the total RNA was analyzed by ribonuclease protection assay as described previously (Greuet et al., 1997). Preparation of riboprobe plasmids for CYP2B6, CYP2C8, CYP2C9, PXR, CAR, and GR were described elsewhere (Pascussi et al., 2000b). Antisense RNA probes were prepared according to general protocol (Melton et al., 1984). Blotted membrane or dried gel were exposed using PhosphorImager storage screens, which were subsequently scanned and the scans stored as TIF files. Detected signals were quantified by ImageQuant software (Amersham Biosciences). Autoradiography was carried out as well for permanent record by exposing the membrane or dried gel to Kodak X-AR film.

Glucocorticoid Receptor Nuclear Translocation in Human Hepatocytes

Primary human hepatocytes cultures were maintained in culture medium in the absence of dexamethasone for 16 h before the treatment. Thereafter, cells were pretreated for 1 and 3 h with colchicine (1 μM final) and then treated for 25 min with dexamethasone (0.1 μM final). Nuclear protein extracts were prepared as described elsewhere (Carcamo et al., 2002) with minor modifications. Briefly, after the treatment, hepatocytes were washed two times with 2 ml of ice-cold PBS and scraped into 1 ml of PBS ($\sim 4 \times 10^6$ cells). Suspension was centrifuged (1,500*g* for 5 min at 4°C) and the pellet was resuspended by pipetting in 300 μl of ice-cold buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.1% (v/v) Nonidet P-40]. The mixture was incubated for 10 min on ice and then centrifuged (12,000*g* for 10 min at 4°C). After removal of supernatant, which contains cytosolic fraction, the pellet was vigorously resuspended by syringe/needle in 3 volumes of ice-cold buffer B [20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 25% (v/v) glycerol] and incubated 30 min on ice. After the centrifugation step (12,000*g* for 20 min at 4°C), the supernatant (nuclear extract) was collected and stored at −80°C. Protein content in extracts was determined by bicinchoninic acid method (Stoscheck, 1990). SDS-polyacrylamide gel electrophoresis gels (7.5%) were run on a Hoefer apparatus following the general procedure (Laemmli, 1970). Protein transfer onto nitrocellulose membrane was carried out as described previously (Towbin et al., 1979). The membrane was stained with amidoblack dye for control of transfer and then saturated with nonfat dried milk (8%) overnight. Blots were probed with primary antibodies against human glucocorticoid receptor [GR(E-20)X rabbit polyclonal; dilution 1/300] and actin [Actin (I-19) goat polyclonal; dilution 1/300], both of which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chemiluminescence detection using horseradish peroxidase-conjugated secondary antibodies and Amersham Biosciences enhanced chemiluminescence kit was performed.

Cytotoxicity Assays

HEK293 cells were seeded on 24-well dishes at a density of 1×10^5 cells/well using Dulbecco's modified Eagle's medium supplemented with glucose (4.5 g/l), pyruvate (final concentration, 1 mM), nonessential amino acids [100× diluted (Invitrogen)], penicillin (final concentration, 100 U/ml), streptomycin (final concentration, 100 μg/ml final), glutamine (2 mM final), and fetal calf serum [10% (v/v)]. After 16 h of stabilization, medium was exchanged for serum-free medium, and cells were treated for 4 and 14 h with colchicine (1 μM final) or DMSO as vehicle control. LDH leakage into the medium (Promega) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay were measured to assess membrane damage and viability, respectively.

Cell Lines and Transfections

Plasmids. The pSG5 Δ^{ATG}-hPXR and pBSEN-hCAR expression plasmids have been described elsewhere (Gerbal-Chaloin et al., 2002). Plasmid p(2B6–5NR1)$_5$)-tk-LUC was generated by cloning five copies of the NR1 motif of human CYP2B6 (Sueyoshi et al., 1999) upstream of a luciferase reporter gene driven by the thymidine kinase promoter in pGL3-TK vector (Pascussi et al., 2003). The pTAT-(GRE)2-TK-luc plasmid containing two copies of the consensus glucocorticoid responsive element (GRE) upstream of a minimal herpes simplex virus thymidine kinase promoter and a luciferase reporter gene was kindly provided by Dr. L. Poellinger (Karolinska Institute, Stockholm, Sweden). The wild hGR expression vector (pSG5-hGR) was kindly provided by Dr. J. C. Nicolas (INSERM, Montpellier, France). Polymerase chain amplification was employed using pSG5-hGR, *Pfu* DNA polymerase, and specific sets of primers to GR cDNA (1–777; GenBank accession number NM_000176) with newly created *Xho*I and *Eco*RI sites at the 5′ and 3′ ends, respectively, to generate GFP-hGR expression plasmid. Amplified fragment was digested with these two enzymes and inserted into pEGFP-C1 vector (BD Biosciences Clontech) to give an in-frame N-terminal fusion with the green fluorescent protein (GFP).

HMLN. The stably transfected cell line was obtained as described previously (Balaguer et al., 1999). Briefly, for obtaining HMLN cells, we transfected HeLa cells with the glucocorticoid responsive reporter gene MMTV-Luc-SVNeo. Control cultures of HeLa cells were transfected with the estrogen-responsive reporter gene MMTV-Luc-SV-Neo to assess GR-independent effects. Selection by G418 was done at 1 mg/ml. Luminescent and inducible clones were identified using photon-counting cameras [Argus 50 from Hamamatsu (Bridgewater, NJ) or NightOWL from Berthold (Bad Wildbad, Germany)] and the most responsive clones were isolated. The cultures were treated for 24 h with DMSO or DEX (0.1 μM) in the presence or absence of colchicine (final concentration, 1 μM). Luciferase activity was measured and standardized per milligram of protein.

HEK293. HEK293 cells transiently transfected by lipofection (FuGENE 6) with chimera GR-GFP or with GFP alone were cultured for 24 h using dishes with immersed glass coverslips. After 4 and 14 h of pretreatment with colchicine (1 μM) and/or DMSO, cells were treated for 15 min with DEX (final concentration, 0.1 μM). After the fixation of cells in formaldehyde, the GR translocation was evaluated using fluorescent microscopy.

For reporter assays, HEK293 cells were transiently transfected by lipofection (FuGENE 6) with 100 ng expression plasmid (pSG5 Δ^{ATG}-hPXR, pBSEN-hCAR, GFP or GFP-hGR) together with 300 ng luciferase reporter construct [p(2B6-(NR1)$_5$)-tk-LUC or pTAT-(GRE)2-tkLUC)] and with 50 ng of β-galactosidase for transfection quality control. After a 16-h incubation period, cells were treated for 24 h as described in the legend to Fig. 7. After the treatments, cells were lysed, and luciferase and β-galactosidase activities were measured.

Ligand Binding Assay. Ligand binding was performed according to a modification of a procedure described by Herr et al. (2000). Simian kidney cells COS-1 cells (American Type Culture Collection, Manassas, VA) were transfected with the hGR-expressing plasmid pSG5. After 16 h, cells were washed twice with PBS, scraped in 1 ml of binding buffer/10 cm Petri dish, centrifuged, and resuspended in total 1 ml of binding buffer (5 mM Tris-HCl, pH 7.4, 5% glycerol, 1 mM EDTA, 10 mM $Na_2MoO_6(2H_2O)$, and 2 mM β-mercaptoethanol). Cells were incubated 10 min on ice, well homogenized, centrifuged (35,000 rpm for 4°C at 1 h; Ti 70.1 rotor), and the supernatant, the cytosolic fraction, was collected. Then 143.5 μl of the cytosol was mixed with 5 μl of 300 nM [^{3}H]DEX alone or in combination with DMSO, 1 μM unlabeled DEX, 1 μM colchicine, or 3 μM RU-486, and incubated overnight at 4°C. With 100 μl of the incubation reaction, bound and free steroids were separated by gel filtration using Sephadex LH-20 (Pharmacia, Guyancourt, France) and radioactivity was measured with 600 μl of the total eluate of 1000 μl in a liquid scintillation counter (Tri-Carb 2100TR; PerkinElmer Life Sciences, Savigny-le-Temple, France). Presence of GR in the cytosolic fraction was verified using Western blot.

Statistics

Results were expressed as mean ± S.D. Student's paired *t* test was applied to all analyses. More detailed analysis using one-way analysis of variance with Scheffé and Tukey-Kramer tests was applied to the cell expression systems and ligand binding assay. All calculations were performed by use of NCSS statistical software (Kaysville, UT).

Results

Effect of Colchicine on Inducible Expression of CYP2B6, CYP2C8, CYP2C9, and CYP3A4 mRNA. As we reported previously (Dvořák et al., 2002), COL decreased CYP2C9 and 3A4 basal expression at the levels of mRNAs,

protein content, and activities in culture. We verified that under the treatment conditions that were used with no modifications for this work as well, no acute cytotoxicity was observed as assessed by light microscopy and by albumin production. Here, we followed COL effect on inducible expression of mentioned isoforms and furthermore on CYP2C8 isoform, expression of which is known to be coregulated together with CYP2C9 (Gerbal-Chaloin et al., 2002) and CYP2B6, which is regulated in similar fashion.

Twenty-four hours after plating, the cultures were pretreated for 3 h with 1 μM COL or dimethyl sulfoxide (DMSO) as the vehicle for control and then for 24 h with 10 μM rifampicin (RIF), 500 μM phenobarbital, and DMSO in the presence or absence of 1 μM COL. At the end of treatments, cells were harvested and total RNA was isolated. Ribonuclease protection assays for CYP2C8, and CYP2C9 mRNAs and Northern blot for CYP3A4 mRNA, as well as quantitative expression by histograms, are shown in Fig. 1. COL clearly suppressed both the basal and inducible expression of all tested P450 mRNAs. In a comparison of the effect of COL on *CYP2C* gene expression, the down-regulation of CYP2C8 mRNA was more pronounced than that of CYP2C9 mRNA.

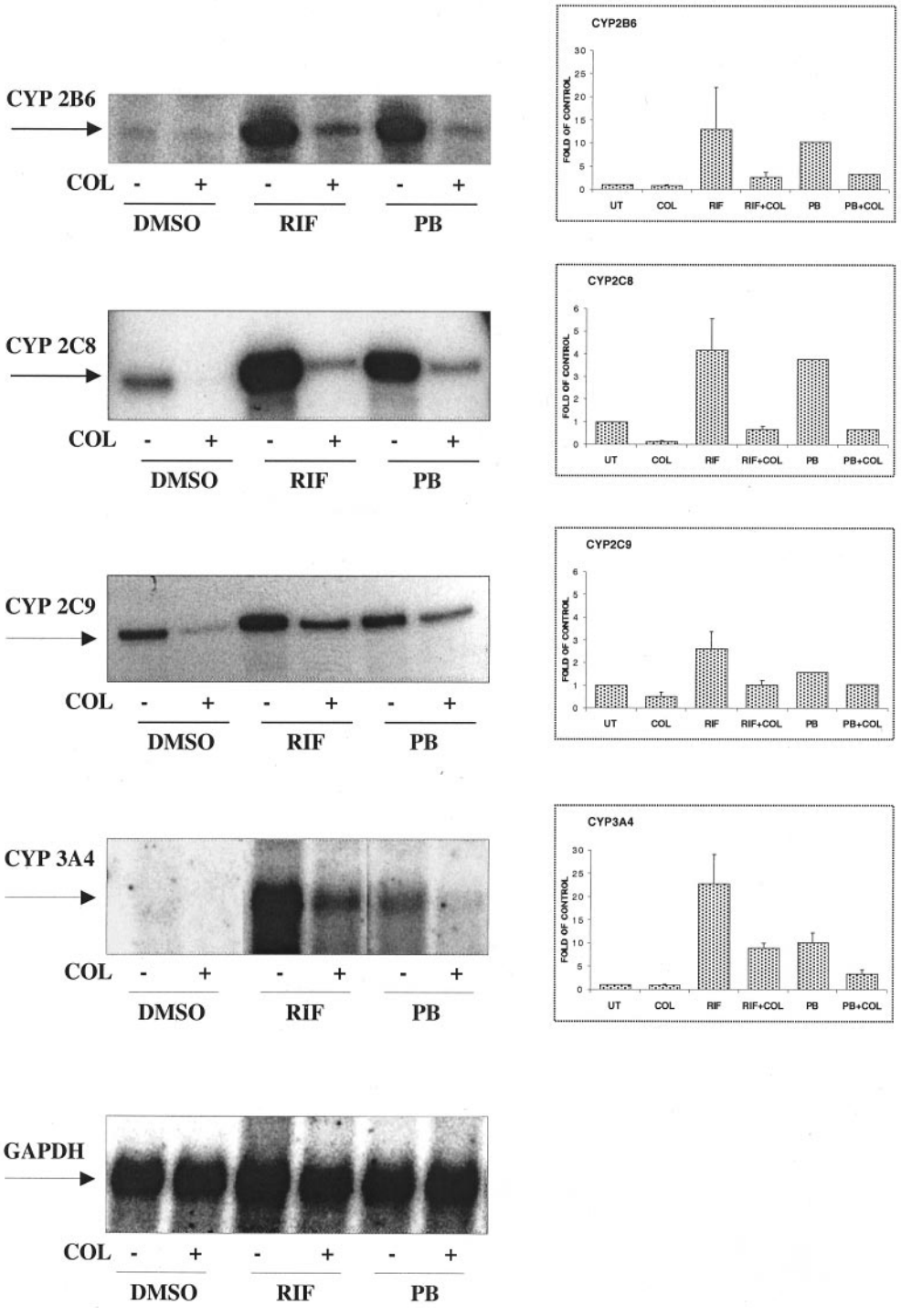

Fig. 1. Effect of colchicine on inducible expression of CYP2B6, CYP2C8, CYP2C9, and CYP3A4 mRNA by rifampicin and phenobarbital. Shown are representative ribonuclease protection assays (CYP2B6, CYP2C8, and CYP2C9 mRNAs) and Northern blots (CYP3A4 and GAPDH mRNAs). Culture was pretreated for 3 h with COL (1 μM) or DMSO. Thereafter, the culture was treated for 24 h with RIF (10 μM), phenobarbital (500 μM), and DMSO in the presence or absence of COL (1 μM) as described under *Results*. Similar results were obtained from three additional cultures. The histograms shown at right are quantifications of corresponding band intensities from all cultures tested. Bars, means $\pm$ S.D. ($n = 4$). Some errors are too small to be visualized.

Similarly, we tested COL effect on CYP2B6, which is under CAR regulation (Sueyoshi et al., 1999). Exactly the same phenomenon was observed; i.e., both the basal and inducible levels of CYP2B6 were suppressed by COL treatment (Fig. 1). Note that level of GAPDH mRNA did not vary significantly among the samples.

Effect of Colchicine on the Expression of PXR, CAR, and GR Receptors mRNAs. The induction of CYP2B6, CYP2C8–9, and CYP3A4 mRNAs by xenobiotics, such as rifampicin and phenobarbital, is known to be controlled by the PXR and/or CAR (Lehmann et al., 1998; Honkakoski and Negishi, 2000). We tested COL effect on CAR and PXR transcriptional activity in transient transfections. CAR, which is constitutively active, was unaffected by 1 μM COL treatment (Fig. 2A). PXR, which requires the presence of rifampicin to become transcriptionally active, was unaffected as well (Fig. 2B). Although these results do not rule out binding of COL to the two nuclear receptors, the data show a lack of significant effect on transcriptional activity of the two receptors.

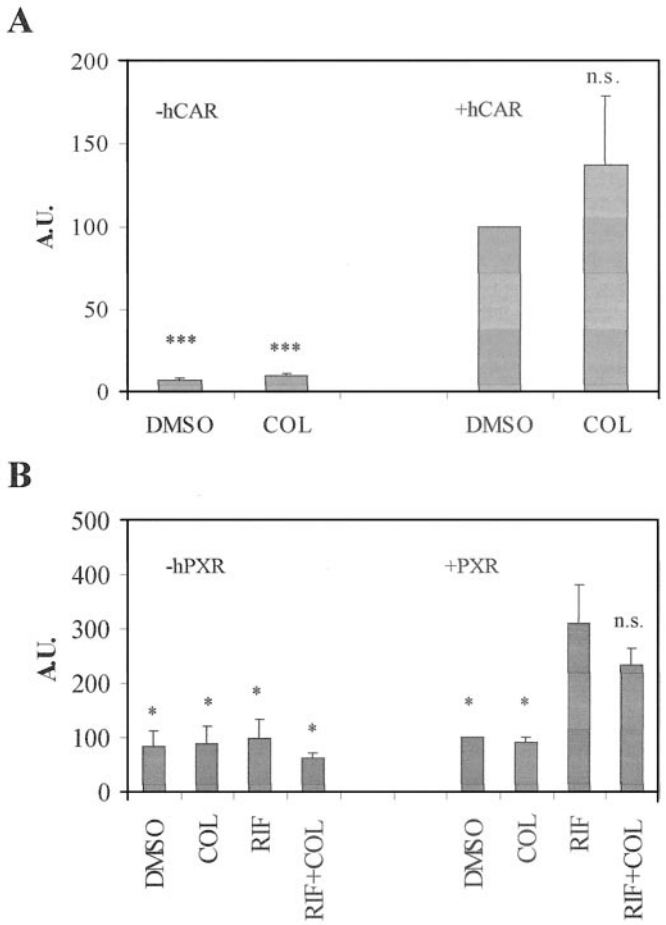

Fig. 2. Effect of colchicine on pregnane X receptor and constitutive androstane receptor activation in HEK293 cells. Bar graphs summarizing the CAR and PXR transcriptional activity in transiently transfected HEK293 cells. A, HEK293 cells transfected with pBSEN-hCAR (+hCAR) or empty vector (−hCAR) were incubated for 24 h with 1 μM COL or DMSO as vehicle control. Chemiluminescent activity of luciferase reporter gene in cell lysates was measured and normalized to β-galactosidase activity. ***, significantly different from constitutive hCAR activity ($p < 0.001$); n.s., not significant. B, HEK293 cells transfected with pSG5 Δ^{ATG}-hPXR (+hPXR) or empty vector (−hPXR) were incubated for 24 h with 1 μM COL, 10 μM RIF, COL+RIF, or DMSO as vehicle control. Chemiluminescent activity of luciferase reporter gene in cell lysates was measured and normalized to β-galactosidase activity. *, significantly different from RIF-induced hPXR activity ($p < 0.05$), n.s., not significant.

We have shown previously that the expression of both PXR and CAR is controlled at least in part by the GR (Pascussi et al., 2000a,b; 2003). Therefore, we suspected that colchicine could affect the expression and/or activity of these receptors. To test this possibility, cultures were treated for 24 h with colchicine (final concentration, 1 μM) and/or DMSO as vehicle, and the levels of PXR, CAR, and GR mRNAs were determined by ribonuclease protection assays. The data obtained reveal that COL strongly suppressed CAR mRNA and PXR mRNA only weakly but significantly (45 and 20% inhibition, respectively). In contrast, the level of GR mRNA remained unaffected (Fig. 3).

Time- and Dose-Dependent Effect of Colchicine on Tyrosine Aminotransferase, PXR, CAR, and GR mRNA Expression. Because GR mRNA expression was not affected by colchicine, we suspected that the transactivating function of GR is altered by the drug. In parallel with CAR and PXR mRNA, we tested the effect of COL on TAT mRNA expression, a gene controlled directly by GR. For this purpose, after a 24-h stabilization period, the cultures were incubated for 16 h in DEX-free medium. Cells were then treated for 24 h with different COL concentrations (0.01, 0.1, and 1 μM) or DMSO in the presence of 0.1 μM DEX. The results in Fig. 4 show that colchicine strongly inhibited the DEX-inducible expression of TAT (inhibition by 65%), CAR, and PXR mRNAs in a dose-dependent manner; the suppression was noticeable even at the nanomolar concentration. Effect of COL on CAR expression was again more pronounced than the one on PXR expression (compare with Fig. 3). Note that TAT mRNA was not detectable in cells cultured in the absence of DEX (Fig. 4, lane UT, − DEX). COL did not affect the expression of GR mRNA as observed in previous experiments (Fig. 3). Likewise, there was no effect of DEX withdrawal on GR mRNA expression (Fig. 4, lane UT, − DEX).

In a second series of experiments, we studied the time course of TAT, PXR, and CAR mRNAs down-regulation by COL. After the stabilization period, the cultures were treated with colchicine (COL 1 μM) or DMSO in the presence or absence of DEX in the medium. The treatments were stopped at 0, 6, 12, and 24 h and the level of TAT mRNA was determined (Fig. 5). Colchicine inhibited TAT mRNA expression within the entire evaluated period. Lower TAT mRNA down-regulation after 6 h, compared with 12 and 24 h, suggests the existence of a lag phase for TAT mRNA down-regulation in cells cotreated with COL and DEX (Fig. 5). This delay is probably caused by the time needed to generate the inhibitory process (1–3 h) by COL. Finally, TAT mRNA was not detectable even after 6 h when DEX was absent from the medium during the entire treatment of cells. In parallel, we investigated the time course of CAR and PXR mRNA expression in the presence of DEX within the period of 0 to 24 h. Similar to the TAT expression, the levels of CAR and PXR mRNAs decreased in COL-treated cells in the time-dependent manner with the time lag of inhibition between 4 and 8 h, suppression of CAR being stronger than that of PXR (Fig. 5). In sum these data show that colchicine does not affect the expression of GR at the mRNA level, but does affect its biological function, as assessed by TAT, PXR, and CAR mRNA down-regulation.

Because the main activity of COL is disruption of microtu-

bule network by binding to the β tubulin, we also tested another microtubule-disrupting agent, nocodazole (NOC), in an identical series of experiments. NOC caused an effect very similar to that of COL; however, a much higher concentration of NOC (40 μM) was necessary. Down-regulation of TAT mRNA by NOC is shown in Fig. 6. All P450 and receptor mRNAs, except GR, were affected by 40 μM NOC as well (data not shown).

Effect of Colchicine on GR-GFP Translocation in HEK293 Cell Line. To test the effect of COL on the translocation of activated GR to the nucleus, we used HEK293 cells transiently transfected with the chimeric GR-GFP and/or GFP only. The GR-GFP construct was fully functional as monitored by pTAT-(GRE)2-tkLUC reporter gene induction by dexamethasone (Fig. 7A). The data obtained by fluorescence microscopy reveal that GR translocation was inhibited in cells pretreated for 14 h with 1 μM COL but not in 4-h pretreatments, despite microtubule disruption at both times (Fig. 7B). For quantification, one hundred cells from randomly selected locations in each sample were examined for intracellular distribution of fluorescence. Of the cells treated with DMSO, COL for 4 h, and COL for 14 h, 11.1, 7.4, and 10.5%, respectively, showed fluorescent signal in the nucleus. In the presence of DEX, 100, 88, and 18% of the cells treated with DMSO, COL for 4 h, and COL for 14 h, respectively, displayed signal in the nucleus. In control experiments, distribution of GFP was affected neither by COL nor by DEX (data not shown). We can assume that GR translocation is inhibited by COL; the consequence of microtubule disruption and thus transport process alteration when the alkaloid is present for periods longer than 4 h.

Morphology of the cells treated for longer time periods with COL changes from flat to spherical. The majority of such cells apparently lose proper attachment to the support material, which is glass in cover-slips or plastic in culture dishes, and easily detach. This is reflected in somewhat conflicting viability tests. Whereas LDH leakage demonstrated 94% of undamaged cells (i.e., with intact cell membrane), even after 14 h of COL treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay demonstrated 78 and 38% viable cells after 4 and 14 h of treatment, respectively. The latter assay detects only viable cells firmly attached; those stained

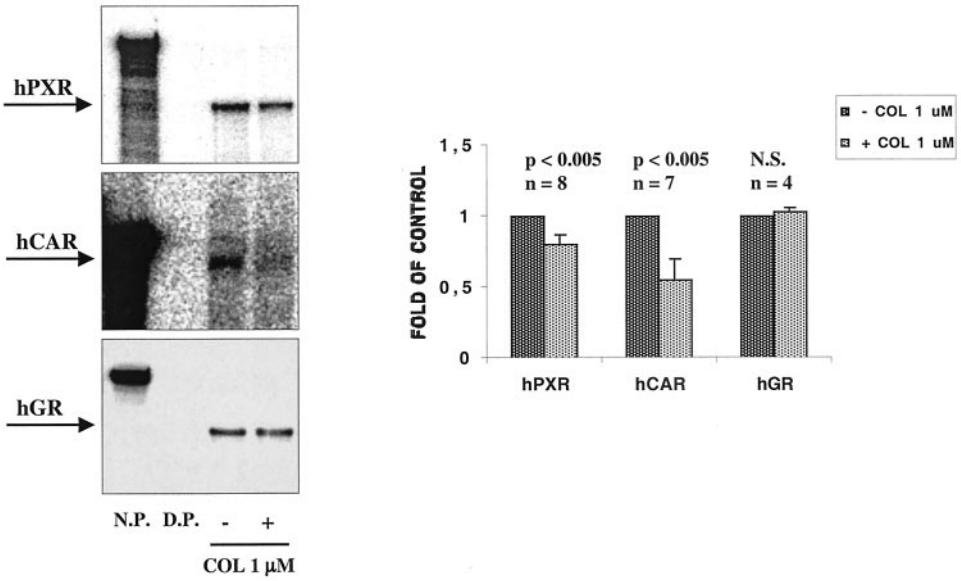

Fig. 3. Effect of colchicine on hPXR, hCAR, and hGR mRNA expression. Shown are representative ribonuclease protection assays of hPXR, hCAR, and hGR mRNAs. The culture was treated for 24 h with COL (1 μM) and/or DMSO as described under *Results*. Similar results were obtained from six additional cultures. The histogram shown at right is a quantification of corresponding band intensities from all cultures tested; the actual number of cultures is noted above the each graph bar. Bars represent means ± S.D. N.P., native probe; D.P. digested probe.

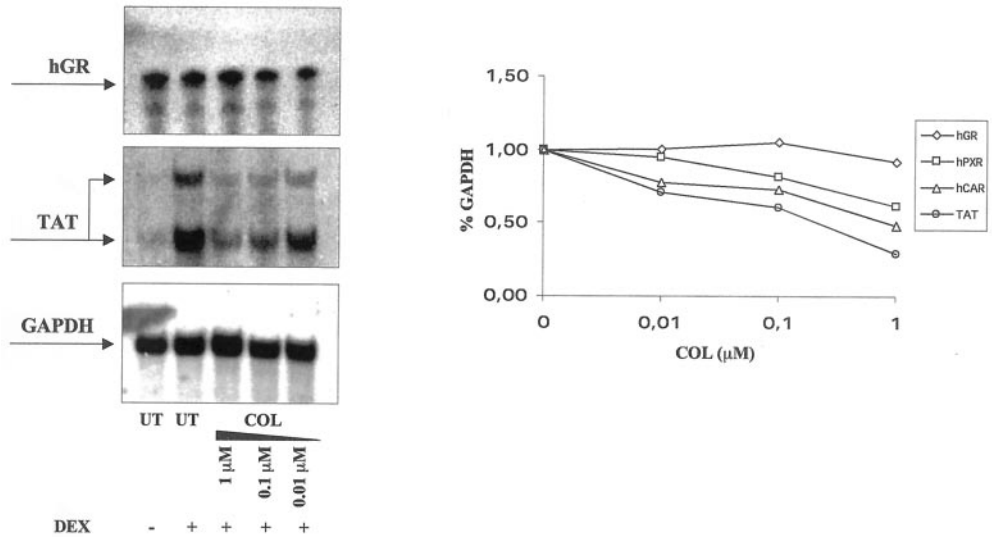

Fig. 4. Dose-response effect of colchicine on TAT, PXR, CAR, and GR mRNA expression. Shown are representative ribonuclease protection assay (GR) and Northern blots (TAT, GAPDH) of determined mRNAs. Data in the band intensity plot (GR, PXR, CAR, and TAT) were normalized to corresponding GAPDH mRNA value. Cultures were incubated 16 h in DEX-free medium and then pretreated with COL (0.01, 0.1, and 1 μM), or DMSO. Thereafter, the cultures were treated for 24 h with COL (0.01, 0.1, and 1 μM) or DMSO in the presence of DEX as described under *Results*. Similar results were obtained from two different cultures.

but removed with the medium before formazan stain solubilizing step are unaccounted for. Our estimate of stained cells lost in this fashion after 14 h of COL treatment is 35% of the total cell number, which is approximately equal to number of stained cells remaining attached. Consequently, cells that were attached to the coverslip after all the treatments and handling must have been viable because of the firm attachment and despite showing different morphology in the fluorescence microscope.

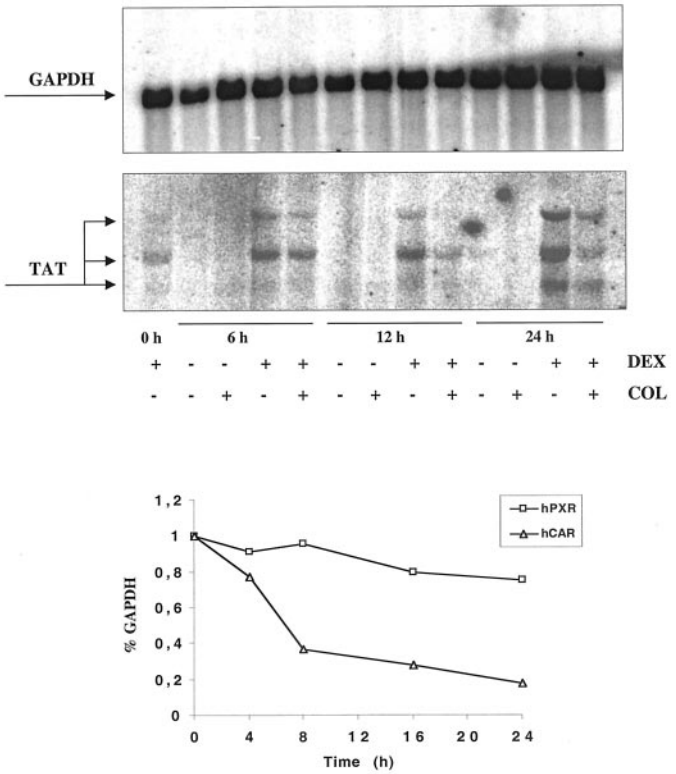

Fig. 5. Time course of colchicine effect on TAT, PXR, and CAR mRNA expression. Shown are representative Northern blots of TAT and GAPDH mRNAs. Data in the band intensity plot (PXR and CAR) were normalized to corresponding GAPDH mRNA value. TAT and GAPDH, after the stabilization period, the cultures were treated with COL (1 μM) or DMSO in the presence or absence of DEX in the medium. The treatments were terminated after 0, 6, 12, and 24 h of incubation as described under *Results*. PXR and CAR, after the stabilization period, the cultures were treated with COL (1 μM) or DMSO in the presence of DEX. The treatments were terminated after 0, 4, 8, 16, and 24 h of incubation as described under *Results*.

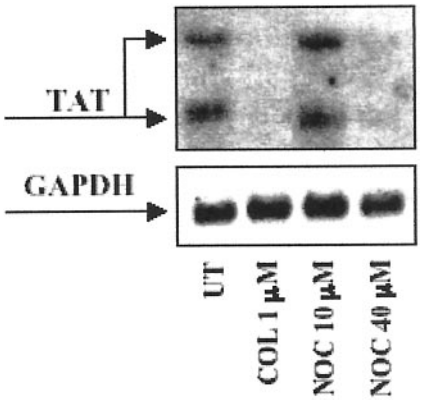

Fig. 6. Effect of nocodazole on tyrosine aminotransferase expression. Shown is a representative Northern blot of TAT and GAPDH mRNAs. Cultures were incubated for 16 h in DEX-free medium and then pretreated with COL (1 μM), NOC (10 and 40 μM), or DMSO. Thereafter, the cultures were treated for 24 h with COL (1 μM), NOC (10 and 40 μM), or DMSO in the presence of DEX as described under *Results*. Similar results were obtained from two additional cultures.

Effect of Colchicine on Glucocorticoid Receptor Activation. We next investigated the effect of COL on the ligand-dependent GR activation. For this purpose, we analyzed the effect of COL and other compounds on the binding of [^{3}H]DEX to in vitro translated GR. The results are shown in Fig. 8. The glucocorticoid antagonist RU486 as well as nonlabeled DEX strongly decreased the binding of [^{3}H]labeled DEX to GR, as expected. In contrast, COL had no effect on this binding. The data obtained reveal that COL is not a ligand for the glucocorticoid receptor and/or does not affect the binding of glucocorticoids to the receptor.

Effect of Colchicine on GR Activity in Model Cell Lines. In a final series of experiments, we determined whether colchicine affects the transactivation function of GR. For this purpose, we used HeLa cells transfected stably with a GRE-dependent luciferase gene (HMLN cells). The primary reason for using stably transfected cells was that the use of transiently transfected cells might lead to complications arising from GR overexpression, as noted by others (Galigniana et al., 1999). To control for GR independent effects, we used HeLa cells stably transfected with estrogen receptor responsive element because this cell line does not contain an intrinsic estrogen receptor (HELN cells). HMLN and HELN cells were treated for 24 h with DMSO or 0.1 μM DEX in the presence or absence of 1 μM COL. The results in Fig. 9, bars

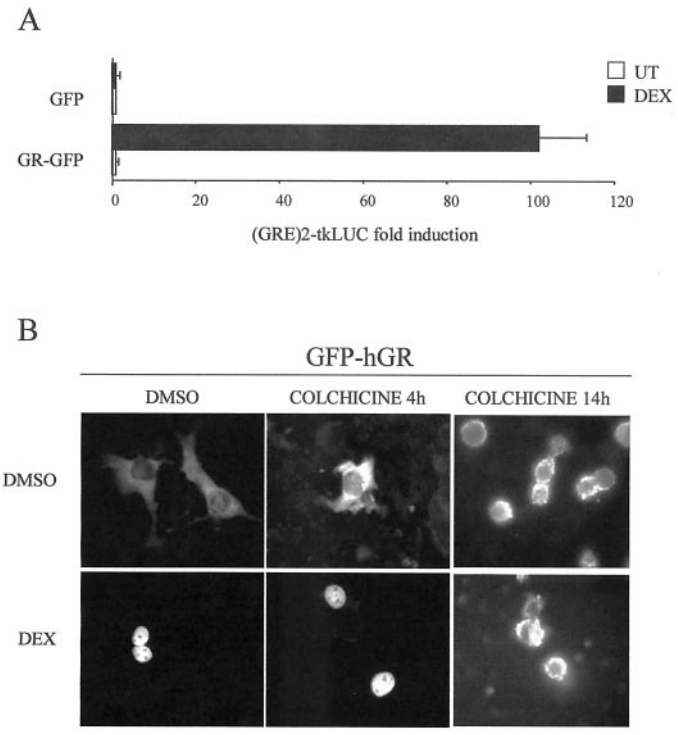

Fig. 7. Effect of colchicine on GR-GFP translocation in HEK293 cells. The cells were cultured for 24 h using dishes with immersed glass coverslips. After 4- and 14-h pretreatment with COL (1 μM) or DMSO, cells were treated 15 min with DEX (0.1 μM final). After the fixation of cells in formaldehyde, the GR translocation was evaluated using fluorescent microscopy. A, test on the functionality of GR-GFP construction by the response of (GRE)2-tkLUC induction. B, representative fluorescent micrographs of HEK293 cells transiently transfected with GR-GFP.

1 through 4, show that COL decreased the DEX-induced luciferase expression by more than 50% in HMLN cells. We suspected that this was a consequence of the effect of COL on the structure of microtubule filaments, a necessary network for nuclear translocation of GR. In control experiments, using a HELN cell line in which the luciferase gene is under estrogen receptor control, we verified that COL had no effect regardless of DEX presence (Fig. 9, bars 5–8). These results show that COL blocks the transcriptional activity of GR, presumably through tubulin network perturbation. The potent inhibition of transcriptional activity of GR by COL explains, at least in part, the down-regulation of P450s, CAR, PXR, and TAT observed in this study.

Effect of Colchicine on GR Translocation in Human Hepatocytes. In a final series of experiments, we tested the influence of COL on GR trafficking by following the time course of GR presence in the nucleus of primary human hepatocyte cultures. Treatment with 1 μM COL causes the disappearance of GR from the nuclear extract in the presence of DEX, whereas no change in GR level is observed in the absence of DEX (Fig. 10). The incubation times necessary for the GR vanishing act in the nucleus upon COL addition vary from culture to culture. Nevertheless, they are consistent with results on TAT mRNA down-regulation, where 6 h is the minimum for COL effect to take place, and GFP-GR translocation experiments, where more than 4 h of treatment is necessary.

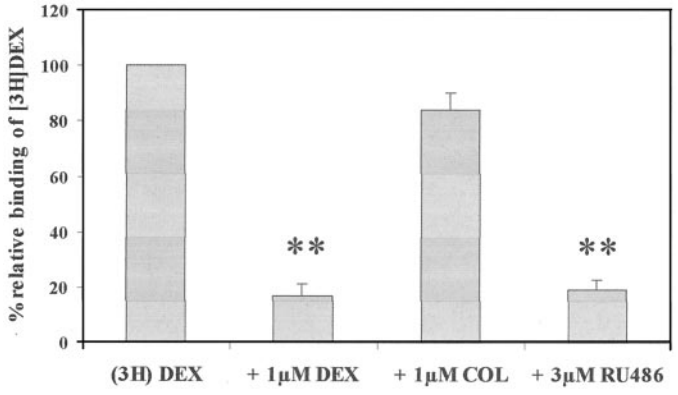

Fig. 8. Effect of colchicine on DEX binding to glucocorticoid receptor. Cytosolic fraction from COS-1 cells expressing glucocorticoid receptor was incubated overnight with 10 nM [^{3}H]DEX in the presence of DMSO, 1 μM unlabeled DEX, 1 μM colchicine, and 3 μM RU486 as described under *Materials and Methods* section. The data are expressed as percent of relative [^{3}H]DEX binding normalized per total protein in sample. Bars are mean ± S.D. from duplicate measurements for three independent cytosolic fraction preparations. **, significantly different from control ($p < 0.01$).

Discussion

In this work, we showed that colchicine decreases both the basal and inducible expression of CYP2B6, CYP2C9, and CYP3A4, one minor and two major forms of cytochrome P450 in the adult human liver, by altering the GR-[PXR/CAR/RXR]-P450 signal transduction cascade. This is supported by the following points: 1) the decrease in P450 expression is accompanied by a concomitant and massive decrease in TAT and CAR mRNAs and a significant but more modest decrease in PXR mRNA expression; 2) neither GR mRNA expression nor DEX-GR binding is affected by colchicine; 3) DEX-inducible transcriptional activity of GR in HeLa cells stably transfected with a GRE-driven reporter gene is inhibited by colchicine; 4) the basal expression of CYP2C9, a GR primary-responsive gene (Gerbal-Chaloin et al., 2002), is strongly repressed by colchicine at the mRNA levels.

The finding that colchicine affects neither the expression of GR mRNA, nor DEX binding to this receptor implies that the alkaloid cannot compete with other GR agonists or antagonists (e.g., RU486) and does not affect the stabilization of a GR-hsp90 complex such as geldanamycin (Whitesell et al., 1994). The absence of effect on GR expression and ligand binding suggested that transcriptional activity for this receptor could be the critical target of colchicine. Indeed, disruption of the microtubule network by compounds such as colchicine should strongly affect GR translocation to the nucleus and eventually lead to the down-regulation of the battery of genes whose transcriptional activity is GR-dependent.

We selected COL, rather than RU486 or geldanamycin, to

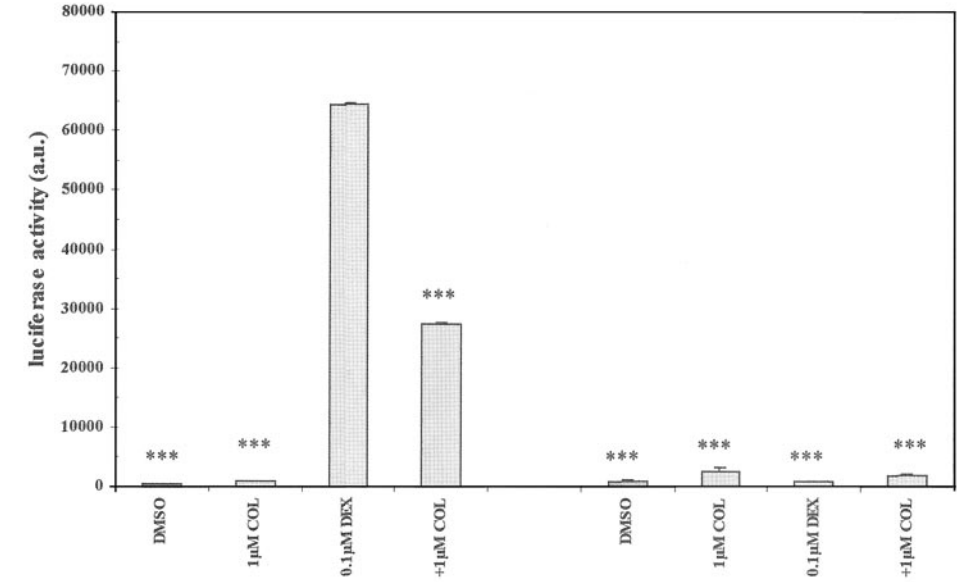

Fig. 9. Effect of colchicine on glucocorticoid receptor activation in HeLa cell lines. HMLN cell line (bars 1–4) stably transfected with GRE-dependent luciferase gene and HELN control cell line (bars 5–8) stably transfected with estrogen receptor responsive element (ERE)-luc gene, were treated for 24 h with DMSO or DEX (0.1 μM) in the presence or absence of COL (1 μM) as described under *Materials and Methods*. The data are expressed in arbitrary luciferase activity units per milligram of protein as the mean ± S.D. from triplicate measurements for three independent experiments. ***, significantly different from DEX-induced GR activity ($p < 0.001$).

test in parallel the involvement of cytoskeleton in the GR-[PXR/CAR/RXR]-P450 cascade. Although it was suggested that cytoskeleton does not affect GR translocation or activity (Szapary et al., 1994), a later report concludes that cytoskeleton is involved in GR translocation under physiological conditions (Galigniana et al., 1999). It must be noted that both research groups used overexpression systems. When attempting to test COL effect on GR activity in an overexpression system using the HepG2 cell line, we obtained the same result as Szapary et al. (1994) (i.e., lack of COL effect; data not shown). We did not attempt to quantify the amount of GR present in primary hepatocytes versus transiently or stably transfected cells, allowing us to conclude that no COL effect was caused by different GR levels. However, our ongoing work with GR translocation suggests that the effect of COL may depend on the actual GR level in cells and is reflected in the time-dependence of COL effect on GR translocation, which varies from culture to culture. The obvious reason, in our view, to explain no COL effect on GR in overexpression systems is that it does not reflect physiological conditions.

Important evidence in favor of this came from monitoring the expression of TAT mRNA. We found previously that COL down-regulates TAT gene expression in primary cultures of human hepatocytes (Dvořák et al., 2002). It is directly regulated by GR, which, after binding to a palindromic GRE located in the TAT upstream regulatory region (Jantzen et al., 1987), activates the gene transcription. Hence, any effect on GR activity should result in TAT mRNA expression alteration (activation/inhibition). TAT mRNA was strongly down-regulated by COL treatment, and the response was dose- and time-dependent (Figs. 4 and 5). The same phenomenon was observed for CAR and to a lesser extent for PXR mRNA expression. Transfection experiments using a GRE stably transfected cell line confirmed this view. Indeed, colchicine did not affect the basal reporter gene activity in these cells but did strongly interfere with the DEX-mediated induction of this activity (Fig. 9). The present results therefore confirm the existence of the GR-[PXR/CAR]-P450 cascade and illustrate the central contribution of GR and this cascade to the maintenance of a significant level of xenobiotic metabolism in the liver. Therefore, any compound, process, or stimuli that will affect the transcriptional activity and/or expression of GR will produce a concomitant change in P450 expression and eventually in the liver detoxication function.

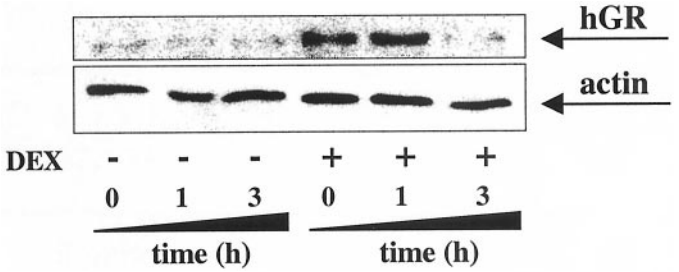

Fig. 10. Effect of colchicine on hGR translocation in primary human hepatocytes. Representative Western blot of hGR protein presence in nuclear extract. Primary human hepatocytes cultures were maintained in culture medium in the absence of dexamethasone 16 h before the treatment. Thereafter, cells were pretreated (0, 1, and 3 h) with 1 μM colchicine and then treated for 25 min with dexamethasone (final concentration, 0.1 μM) or DMSO as vehicle for control. Similar results were obtained from two different cultures. For loading control, the level of actin was determined.

The primary mechanistic explanation of COL effect, in agreement with our COL selection criterion, is impairment of hormone-dependent GR trafficking from cytoplasm into nucleus by cytoskeleton disruption. Many of the data presented here are in support of this with the demonstration of GR disappearance from the nucleus upon COL treatment being the strongest (Fig. 10). The less potent effect of NOC, another cytoskeleton-disrupting agent, lends further support and is consistent with the findings on mouse *Cyp2b10* gene induction, where 1 μM COL and 10 μM NOC were shown to be slightly inhibitory (Honkakoski and Negishi, 1998). One fact, however, leaves a reasonable doubt: expression of PXR is down-regulated only modestly, whereas CYP3A4, its regulatory target, is down-regulated strongly. However, CYP3A4 gene expression depends on several factors that are affected by colchicine (GR and CAR notably); thus, the overall effect could be more drastic then what would be expected based on the small PXR decrease.

An alternative explanation for COL effect is its well known ability to arrest cell cycle in the G_2/M phase (Kung et al., 1990). GR transactivation is severely impaired in G_2-synchronized cells, although not all nuclear functions of GR are disrupted (Hsu and DeFranco, 1995). This scenario is very likely to apply to cell lines used in transfection experiments. It may account for the lack of COL effect in a cell line lacking intrinsic GR (e.g., GR transfected HepG2), in which the machinery affecting GR activity during cell cycle may be incomplete or missing. Such machinery probably exists in HeLa cells containing GR, allowing for COL inhibition of GR activity. Primary human hepatocytes are nonproliferating and the effects of mitotic arrest in their case are questionable. However, suggested implication of cell cycle-dependent P450 expression is very tempting and intriguing for stem cell research.

A third, and remote, possibility to explain COL effect is the alkaloid preventing formation of di- or multiprotein transcription complexes by disrupting an intranuclear cytoskeleton network. Such a cytoskeleton, containing β-tubulin, which is the COL binding site, seems to exist (Woulfe and Munoz, 2000).

Despite some uncertainties about the mechanistic aspects of COL effect, we can conclude that the existence of GR-[PXR/CAR]-P450 cascade was established and that correct functioning of this cascade is cytoskeleton dependent.

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