Evaluation of the HC-04 Cell Line as an In Vitro Model for Mechanistic Assessment of Changes in Hepatic Cytochrome P450 3A during Adenovirus Infection

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ABSTRACT

HC-04 cells were evaluated as an in vitro model for mechanistic study of changes in the function of hepatic CYP3A during virus infection. Similar to in vivo observations, infection with a first generation recombinant adenovirus significantly inhibited CYP3A4 catalytic activity in an isoform-specific manner. Virus (MOI 100) significantly reduced expression of the retinoid X receptor (RXR) by ∼70% 96 hours after infection. Cytoplasmic concentrations of the pregnane X receptor (PXR) were reduced by 50%, whereas the amount of the constitutive androstane receptor (CAR) in the nuclear fraction doubled with respect to uninfected controls. Hepatocyte nuclear factor 4α (HNF-4α) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) were also reduced by ∼70% during infection. Virus suppressed CYP3A4 activity in the presence of the PXR agonist rifampicin and did not affect CYP3A4 activity in the presence of the CAR agonist CITCO [6-(4-chlorophenyl) imidazo [2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime], suggesting that virus-induced modification of PXR may be responsible for observed changes in hepatic CYP3A4. The HC-04 cell line is easy to maintain, and CYP3A4 in these cells was responsive to known inducers and suppressors. Dexamethasone (200 μM) and phenobarbital (500 μM) increased activity by 230 and 124%, whereas ketocazole (10 μM) and lipopolysaccharide (LPS) (10 μg/ml) reduced activity by 90 and 92%, respectively. This suggests that HC-04 cells can be a valuable tool for mechanistic study of drug metabolism during infection and for routine toxicological screening of novel compounds prior to use in the clinic.

Introduction

The liver plays a major role in maintaining physiologic homeostasis through the uptake, metabolism, conjugation, and excretion of a variety of natural biomolecules, toxins, and carcinogens (Sevior et al., 2012). The liver also provides an immunologic function through the reticuloendothelial system by phagocytosis and clearance of microorganisms from the portal blood (Crispe, 2009). Considering that this organ is the primary site for potential drug-drug interactions and that its dysfunction can significantly affect other organ systems, it would seem practical for screening technologies dedicated to the characterization of the safety profiles of new compounds to be centered around this organ. As a result, a wide spectrum of in vitro liver-derived systems currently exist (Fasinu et al., 2012). However, there remains to be one solid, “gold-standard” system that is amenable to large-scale, reproducible screening of novel molecular entities intended for therapeutic use.

For more than two decades, it has been known that microbial infection suppresses the activity and expression of hepatic cytochrome P450 3A (CYP3A) (Croyle, 2009; Gandhi et al., 2012; Zanger and Schwab, 2013). Because infections with novel viruses are emerging in the clinic at an unprecedented rate and the use of viruses for gene transfer and immunization purposes is on the rise (Furuse et al., 2011; Vannucci et al., 2013), understanding how the expression and function of CYP3A and other metabolic enzymes are altered during virus infection is an important public health concern. Currently, the manner by which CYP3A is altered during virus infection has not been fully elucidated. Data gathered from initial studies investigating this phenomenon have suggested that cytokines and nitric oxide associated with the immune response play a role in this effect during the acute stage of infection (Zanger and Schwab, 2013). We found that systemic administration of a single dose of a modified adenovirus with a reduced immunogenic profile still inhibited expression and function of hepatic CYP3A for a period of 14 days in male Sprague-Dawley rats (Callahan et al., 2008a). This led us to believe that adenovirus-mediated changes...
in CYP3A may not be caused solely by factors related to the immune response. In an effort to investigate this further in the absence of inflammatory mediators, an in vitro system that would produce reproducible and reliable results was needed.

Primary hepatocytes, when cultured under conditions that promote normal hepatocellular morphology and expression of liver-specific genes, demonstrate changes in cytochrome P450 (P450) expression and function in a manner similar to in vivo observations, making them one of the most relevant models for the study of drug metabolism and toxicity (Sahai et al., 2010). Although many different variables can significantly affect the phenotype and performance of primary hepatocytes in vitro (Takeba et al., 2011), most protocols favor establishing cultures at a high density between two layers of collagen or Matrigel for long-term use (Sellaro et al., 2010). We found this approach difficult to use effectively in a model of virus infection, because the matrix prevents virus-cell interactions. This, coupled with the potential for contamination of cultures with Kupffer cells, subtle differences in rat physiology, and the relatively low yield of cells attained from this complicated and expensive process made continual use of primary hepatocytes impractical for our purposes.

Taking this into consideration, we believe that a cell line suitable for modeling of P450 expression during virus infection and routine toxicological screening of therapeutic compounds should meet the following criteria. 1) It must constitutively express CYP3A4 (and other P450 isoforms) that are responsive to known inducers and inhibitors. 2) Expression of P450 must be at a level at which changes are easily detectable with standard techniques. 3) P450 expression must be consistent as cells are subcultured. 4) Culture media must not be complex and addition of specific reagents not required to maintain artificially P450 expression and function. 5) Cells must express virus receptors and other microbial pattern recognition receptors. 6) A continual, reliable source of cells must be available. The HC-04 cell line (ATCC MRA-975), an immortalized cell line developed from normal human liver tissue without genetic manipulation, retains normal hepatocellular morphology and expression of liver-specific genes (Sattabongkot et al., 2006). HC-04 cells express a large panel of hepatocellular transcription factors, transporters, and drug metabolizing enzymes at a level equivalent to that of primary human hepatocytes in standard culture medium (Lim et al., 2007). Transcriptional regulators of CYP3A4 are naturally found in the cytoplasmic and nuclear fractions of these cells. Thus, the aim of this study was to evaluate the HC-04 cell line as a suitable in vitro model for mechanistic study of CYP3A4 activity during adenovirus infection.

**Materials and Methods**

Acemannan was purchased from Fort Dodge Laboratories (Atlanta, GA). Ketamine was purchased from Pfizer (New York, NY). Phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), xylazine, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP), 1,10-phenanthroline, sorbitan mono-9 octadecenoate poly (oxy-1,1-ethanediyl) (Tween 20), cytosporine A, bacterial lipopolysaccharides (LPS, *Escherichia coli* serotype 0127:B8), phenobarbital, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO). Protocell acrylamide was purchased from National Diagnostics (Atlanta, GA). Ketaconazole and rifampicin were purchased from Fisher (Atlanta, GA). Ketoconazole and rifampicin were purchased from Fisher (St. Louis, MO). All primary antibodies for Western blots were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotide primers were custom synthesized by Sigma Life Science (Woodlands, TX). All other chemicals were of analytical reagent grade and purchased from EMD Chemicals (Gibbstown, NJ) unless specified otherwise.

**Cell Culture: Primary Hepatocytes.** All procedures were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and are in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals. Hepatocytes were isolated from adult male Sprague-Dawley rats (~300 g) by a modified two-step collagenase procedure (Smedsrod and Pertot, 1985). Briefly, deep plane anesthesia was established with a single intramuscular injection of a preparation consisting of 1:1:1 (v/v/v) ratio of ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml). A peristaltic pump (Harvard Apparatus, Inc., Holliston, MA) was used to perfuse the liver with 300 ml of liver perfusion media (Invitrogen, Carlsbad, CA) via the portal vein. The liver was then perfused with an equal volume of liver digest media (Invitrogen) followed by an additional 50 ml of liver perfusion media. The liver was excised, placed in Leibovitz’s L-15 Medium (Invitrogen) using a sterile scalpel and forceps, and pressed through a stainless steel screen (50 mesh, Sigma). Hepatocytes were purified on Percoll gradients (Amersham Biosciences, Piscataway, NJ). To remove Kupffer cells, a portion of the purified cell fraction was incubated with magnetic OX-6 beads and run through an XS column (Milenyi Biotec, Cambridge, MA). Hepatocytes were seeded onto rat tail collagen-coated plates (BD Biosciences, San Jose, CA) at a density of 1.5 × 10^5 cells/cm² in William’s E media (Invitrogen) supplemented with penicillin/streptomycin (1%, Mediatech, Manassas, VA), t-glutamine (2 mM, Hyclone, Logan, UT), dexamethasone (1 mM, BD Biosciences), and gentamicin (0.5 µg/ml, Cambrex Biosciences, Walkersville, MD) and maintained at 37°C and 5% CO₂ for 3 hours. Media were then replaced with HepatoZYME-SFM (Invitrogen) alone or HepatoZYME-SFM containing 12.5 µg/cm² rat tail collagen (BD Biosciences) for “sandwiched” cultures. The presence of Kupffer cells in purified cultures was determined by histochemical staining with a peroxidase (myeloperoxidase) kit (Sigma Diagnostics, St. Louis, MO) according to the manufacturer’s instructions.

Cells were infected with adenovirus 24 hours after plating.

**HC-04 Cells.** HC-04 cells (MRA-975) were purchased from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium/ Ham’s F12 50/50 mix (Gibco Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 2 mM t-glutamine (Gibco). Cells of passage 64–74 were used in the studies outlined in this manuscript.

**Virus Production.** First-generation adenovirus serotype 5 expressing E. coli beta-galactosidase under the control of a cytomegalovirus promoter was amplified in human embryonic kidney 293 cells (ATCC CRL-1573) and purified from secondary lysates according to established methods (Croye et al., 2000).

**Virus Purification.** Virus was purified from cell lysates by banding twice on cesium chloride gradients and desalted on an Econo-Pac 10DG disposable chromatography column (BioRad, Hercules, CA). Equilibrated with phosphate-buffered saline, pH 7.4. Virus concentration was determined by UV spectrophotometric analysis at 260 nm and by a standard limiting dilution assay (Callahan et al., 2008b). All experiments were performed with freshly purified virus.

**Virus Infection.** For each study, a minimum of three plates of HC-04 cells were trypsinized and counted using a standard hemocytometer (Hauser Scientific, Horsham, PA). Freshly purified virus was diluted to the appropriate concentrations in serum-free media. Three milliliters of each preparation was placed on cells seeded in 100-mm dishes. Cells were incubated at 37°C with 5% CO₂ for 2 hours, after which 7 ml of complete culture media was added to each plate for the remainder of the infection period. When infection was complete, cells were fixed with 0.5% glutaraldehyde (Sigma), and beta-galactosidase activity was determined by incubation with the substrate 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal) for 4 hours at 37°C in the dark. Staining medium was removed, and blue-colored, positive cells were observed with an Axiophot microscope and photographed using a Nikon Coolpix 4500 digital camera and Nikon View (Eastman Kodak Co., Rochester, N) software.

**Chemical Suppression/Induction of CYP3A4.** Substances known to suppress or induce CYP3A4 activity were added to culture media daily over a period of 3 days. Stock solutions of each compound were prepared in DMSO.
and diluted to a working concentration in standard culture media. The final DMSO concentration in each preparation added to cells for these studies was 0.1%. This did not interfere with the assay and did not significantly impact CYP3A4 activity. CYP3A4 activity was assessed using a P450-Glo CYP3A4 Luciferin-IP assay kit according to the manufacturer’s instructions (Promega, Madison, WI).

**Cytotoxicity Assay.** Cytotoxicity was assessed by measuring the amount of adenosine triphosphate (ATP), an indicator of metabolically active cells, in cultures using a Cell Titer-Glo Luminescent Cell Viability assay kit from Promega. Data generated from this assay were used to evaluate the cytotoxicity of the virus and to normalize data generated from the P450-Glo CYP3A4 Assay kit.

**Testosterone Hydroxylation Assay.** Production of the isoform-specific metabolite of testosterone, 6β-hydroxytestosterone, was used to assess changes in CYP3A activity during virus infection (Nallani et al., 2001). Reactions were performed directly in cultured cells plated on individual 100-mm tissue culture plates. Prior to initiating the assay, cells were washed once with sterile PBS and then incubated with complete culture medium containing 250 μM testosterone for 30 minutes at 37°C. After addition of an internal standard (2.5 μg 11α-hydroxyprogesterone), media was removed and the reaction quenched with 5 ml d-methane. The organic phase was evaporated under a constant stream of air and dissolved in 75 μl of solution consisting of methanol and water in a 1:1 ratio. The primary CYP3A testosterone metabolite, 6β-hydroxytestosterone, and 16α-hydroxytestosterone, the primary metabolite of CYP2D, were separated and quantified by high-performance liquid chromatography (HPLC) as previously described (van der Hoeven, 1984).

**Preparation of Whole Cell, Nuclear, and Cytoplasmic Extracts.** For whole cell lysates, cells were washed twice with PBS and then scraped from the culture surface with 5 ml of ice-cold PBS. Fractions were then cleared by centrifugation at 14,000 g for 5 minutes. The supernatant was then removed and replaced with 250 μl of ice-cold lysis buffer (RIPA buffer, Pierce Biotechnology, Rockford, IL) containing protease inhibitors (HALT Protease and Phosphatase Inhibitor Cocktail, Pierce). Cells were lysed by centrifugation through a 20-gauge needle (Becton Dickinson, Franklin Lakes, NJ) into a 1-ml syringe (Becton Dickinson) 25 times and placed on ice for 40 minutes. Lysates were then cleared by centrifugation at 14,000 g for 20 minutes at 4°C and stored at −80°C. Nuclear and cytoplasmic extracts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer’s instructions.

**Western Blot Analysis.** Protein (50 μg) from whole cell lysates, nuclear, or cytoplasmic extracts were fractionated by size on an 8% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane. Protein blots were incubated for either 1 hour at room temperature or overnight at 4°C in blocking buffer containing 5% non-fat dry milk and 0.05% (v/v) Tween 20 in Tris-buffered saline. After blocking, membranes were incubated with polyclonal rabbit anti-human RXRα antibody (D20, sc-553), polyclonal goat anti-mouse PXR antibody (H-160, sc-25381), or polyclonal rabbit anti-mouse CAR antibody (M-127, sc-13065) each at a 1:1,000 dilution overnight at 4°C. To evaluate changes in CYP3A4 protein, samples were run on a 12% polyacrylamide gel and membranes incubated with a monoclonal mouse anti-human CYP3A4 antibody (HL3, sc-53850) at a 1:2,000 dilution for 2 hours at room temperature. Each membrane was then incubated with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody (1:3,000 dilution, MP Cappell, Solon, OH), peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:3,000 dilution, Cell Signaling Technology, Danvers, MA), or peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10,000 dilution, Santa Cruz Biotechnology) in blocking buffer for 1–2 hours at room temperature. Immune complexes were detected with the SuperSignal West Pico chemiluminescent substrate (Pierce). Band density was determined by exposure of the nitrocellulose membrane to Kodak Biomax film. Blot densities were measured using a flatbed scanner (Microtek, Carson, CA) and analyzed using Kodak 1D image analysis software. The intensity of protein levels in nuclear and cytoplasmic extracts was quantified relative to signals obtained for lamin B (lamin B rabbit polyclonal antibody, 1:1,000 dilution) and β-actin (β-actin rabbit monoclonal antibody, 1:1,000 dilution, Cell Signaling Technology) on the same blot, respectively.

**RT-PCR.** RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Isolated RNA was reverse transcribed with random primers using the SuperScript III first-strand synthesis system (Invitrogen). PCR was carried out in a Master Cycler Pro thermal cycler (Eppendorf AG, Hamburg, Germany) using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA) under the following conditions: 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute for 32 cycles. Cycling was initiated at 94°C for 3 minutes and terminated at 72°C for 10 minutes. Primer sequences for human CAR, PXR, RXRα, and CYP3A4 were 5′-GCA AGG GTT TCT TCA GGA GAA C-3′ (forward) and 5′-CTT CAC AGC TTC CAG CAA AGG-3′ (reverse); 5′-CAG GCG GAA AA GAG TGA AGG-3′ (forward) and 5′-CTG GTG CTC GAT GGG CAA GTC-3′ (reverse); 5′-CTT TTC TCG GTC ATC AGC TC-3′ (forward) and 5′-CTC GCA GCT GTA CAC TTC AT-3′ (reverse); and 5′-TAG ATT TCT TCT TAA TGT GC-3′ (forward) and 5′-CTT CAT CCA ATG GAC GCA TAA AT-3′ (reverse), respectively. QuantumRNA 18S internal standards (Ambion Life Technologies, Carlsbad, CA) were coamplified in individual reaction tubes. Reaction products were visualized on a 1.5% agarose gel containing ethidium bromide. The intensity of each band was determined by densitometric analysis using Kodak image analysis software.

**Statistical Analysis.** Statistical analysis of data was performed using SigmaStat (Systat Software Inc., San Jose, CA). The statistical significance of differences between samples was calculated using a one-way analysis of variance followed by a Bonferroni/Dunn post hoc test. Differences were considered to be significant when the probability of chance explaining the results was reduced to less than 5% (P < 0.05).

**Results**

**Evaluation of CYP3A4 Activity in Response to Adenovirus Infection: Primary Hepatocytes.** When primary hepatocytes, isolated from male Sprague-Dawley rats and seeded on standard tissue culture plates, were infected with a first generation adenovirus containing a beta-galactosidase transgene (AdlacZ), CYP3A4 activity was reduced by 80% (Fig. 1A). In contrast, the rate of production of the CYP3A4 metabolite, 6β-hydroxytestosterone, in uninfected (control) and infected cells sandwiched between a collagen matrix was not statistically different (P = 0.2, Fig. 1A). Histochemical staining of cells for expression of the beta-galactosidase transgene revealed that the cells between the collagen matrix prevented the virus from infecting hepatocytes (Fig. 1B). When primary hepatocytes from male Sprague-Dawley rats were infected with different concentrations of the virus, CYP3A4 activity was suppressed to a level similar to that observed in vivo (Callahan et al., 2005b; Fig. 1C). Concentrations of 1,000, 2,500, and 5,000 virus particles per cell reduced activity by 34, 65, and 85%, respectively, whereas multiplicities of infection (MOIs) of 7500 and 10,000 almost completely blocked CYP3A4 activity (~100% reduction) 48 hours after infection. The 10,000 MOI concentration caused significant cellular toxicity, as indicated by measurement of lactate dehydrogenase (35 times saline control, data not shown) in culture medium. Although cells were purified over a Percoll gradient, Kupffer cells (KC) were not completely removed from cell isolates, as indicated by the presence of endogenous peroxidase activity (Widmann et al., 1972; Fig. 1D). This was a significant concern because the presence of these cells, which induce potent inflammatory responses against pathogens, could confound our results (Milosevic et al., 1999). Modification of the standard two-step collagenase perfusion technique (Smedsrød and Pertoft, 1985) to remove KCs from the hepatocyte fraction by magnetic bead separation was effective and did not alter the metabolic activity of the cells and their ability to respond to virus infection (Fig. 1D).

**Effect of Treatment with Known Inhibitors of CYP3A.** To determine if changes in CYP3A could be easily assessed in HC-04 cell line, cells were first cultured in the presence of compounds known to affect enzyme activity in vivo in vitro and in vivo settings. Ketoconazole (10 μM), a strong CYP3A4 inhibitor (Dagliyan et al., 2009), suppressed activity of the enzyme by 89.65 ± 1.2% (Fig. 2A, P ≤ 0.001).
Erythromycin (100 μM), a moderate inhibitor of CYP3A4, suppressed activity by 68.1 ± 1.3%, whereas weak inhibitors, isoniazid (100 μM) and cyclosporine A (4.2 μM) suppressed activity by 35.6 ± 10.4% and 40.3 ± 5.5%, respectively. A final test of the responsiveness of CYP3A4 in the culture system involved incubation of cells with bacterial lipopolysaccharides (LPS, *E. coli* serotype 0127:B8) at two different concentrations. Low concentration LPS (1 μg/ml) suppressed CYP3A4 activity by 51.2 ± 3.3%, whereas a concentration of 10 μg/ml suppressed enzyme activity by 92.2 ± 4.2% (Fig. 2A). When known CYP3A4 inducers dexamethasone (200 μM) and phenobarbital (500 μM) were added to cultures, CYP3A4 activity increased by 230 ± 12.5 and 124 ± 9.1%, respectively (*P* ≤ 0.001, Supplemental Fig. 1). Adenovirus at 100, 500, and 1000 MOI suppressed CYP3A4 activity in the presence of phenobarbital by 42.8, 71.8, and 76%, respectively.

Fig. 1. Primary hepatocytes isolated from male Sprague-Dawley rats respond to adenovirus infection in a manner similar to that observed in vivo when seeded on standard tissue culture dishes. (A) A first generation adenovirus containing a beta-galactosidase transgene (AdlacZ) did not significantly alter CYP3A activity of primary hepatocytes sandwiched between a collagen matrix. (B) Histochemical stain for the beta-galactosidase transgene indicates that the sandwich matrix prevented virus infection. (C) CYP3A activity of primary hepatocytes cultured on standard untreated tissue culture dishes responds to virus infection in a dose-dependent manner (MOI, multiplicity of infection). (D) Representative images of hepatocytes after histochemical staining for endogenous peroxidase activity before and after removal of Kupffer Cells (KC) through magnetic bead separation. Cells containing a black/blue precipitate exhibited endogenous peroxidase activity, a characteristic unique to KCs and absent in hepatocytes (Widmann et al., 1972). In each panel, cells were seeded at a density of 5 × 10⁵ cells/mm. (A and C) (n = 6 replicates/group) *P* ≤ 0.05, **P* ≤ 0.01, Bonferroni Dunn post hoc test. Magnification: 100× (B), 400× (D).

HC-04 cells respond to known CYP3A4 suppressors and respond to adenovirus infection in a CYP3A4 isoform-specific manner. (A) Substances known to suppress CYP3A4 activity were added to culture media daily over a period of 3 days. Ketocon, ketoconazole; Erythro, erythromycin; LPS, bacterial lipopolysaccharides; CyA, cyclosporine A. (B) Cells were infected with 500 MOI AdlacZ over time. In vitro catalytic activity was measured by incubation of infected cultures with testosterone for 30 minutes and quantitation of the CYP3A isoform-specific metabolite, 6β-hydroxytestosterone by HPLC. (C) Cells were treated as described for (B) except that the CYP2D-specific testosterone metabolite 16α-hydroxytestosterone was measured by HPLC. In each panel, results are reported as the mean ± standard error of the mean of data generated from three 100 mm culture plates per condition replicated in three separate experiments. Statistical significance was determined between individual treatment groups and saline-treated controls by one-way analysis of variance with a Bonferroni/Dunn post hoc test. *P* ≤ 0.05, **P* ≤ 0.01, ***P* ≤ 0.001.
Effect of Adenovirus Infection on Nuclear Receptors in HC-04 Cells. Pilot infection studies revealed that concentrations of virus greater than 1000 MOI were extremely cytotoxic (data not shown). In a secondary study, cells were infected with virus at a MOI of 500, and CYP3A activity was assessed over a period of 4 days. No significant difference was found between the CYP3A activity in infected and uninfected cells within the first 24 hours of infection (Fig. 2B). A slight (~17%) drop in activity was noted 48 hours after infection. CYP 3A4 activity in cultures infected for 72 and 96 hours significantly dropped by 47 and 42%, respectively, compared with saline-treated controls. Thus, an infection time of 96 hours was selected for additional mechanistic studies in this cell line. To determine if this effect was specific to CYP3A alone, the concentration of 16α-hydroxytestosterone, a testosterone metabolite primarily generated by CYP2D-mediated hydroxylation, was also monitored over the same time period (Fig. 2C). Concentrations of 16α-hydroxytestosterone in infected cultures were not significantly different from uninfected controls at all time points (P = 0.08).

To further refine protocols for infection of HC-04 cells and determine if results obtained from this model reflected those reported in vivo (Callahan et al., 2005b, 2008a; Wonganan et al., 2011), cells were infected with various concentrations of AdlacZ. Virus at a MOI of 100 modestly reduced CYP3A activity by 25%, whereas MOIs of 500 and 1000 reduced activity by 48.6 and 53.5%, respectively (Fig. 3A, P ≤ 0.001). These latter concentrations induced the most cytotoxicity (MOI 500, 8%; MOI 1000, 18%; data not shown). Virus infection also suppressed CYP3A4 mRNA levels in a similar manner (Fig. 3B), with MOIs of 100 and 500 exerting the most effect (61 and 72% reduction, respectively). Significant changes in CYP3A4 protein content in whole cell lysates was not detected by Western blot at any of the virus concentrations tested (Fig. 3C, P = 0.1). Histochemical staining revealed that transgene expression in primary hepatocytes infected with virus at MOI 500 (Fig. 4B) and MOI 1,000 (Fig. 4C), the highest concentrations of virus tolerated by HC-04 cells, was consistently lower than that found in HC-04 cells treated with the same amount of virus (Fig. 4, E and F).

Effect of Adenovirus Infection on Nuclear Receptors in HC-04 Cells. Hepatic CYP3A4 expression is predominantly regulated by several nuclear receptors, including the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the retinoid X receptor (RXR), and hepatocyte nuclear factor 4α (HNF-4α) (Croyle, 2009). Although we have clearly shown that adenovirus infection alters hepatic CYP3A in several in vivo systems, it is not clear how virus infection affects these important transcriptional regulators.

RXX. RXR, primarily located in the nucleus, is a heterodimeric partner required for DNA binding of a number of nuclear receptors and plays a key role in the regulation of many metabolic processes (Dawson and Xia, 2012). When paired with either PXR or CAR it binds to several regions along the CYP3A promoter to drive gene expression (Brtko and Dvorak, 2011). Nuclear RXR protein was moderately affected by AdlacZ, because concentrations of 10, 100, and 500 MOI suppressed protein levels by 12, 22, and 25%, respectively (Fig. 5A). MOIs of 100 and 500 suppressed RXR in the cytoplasm by 31.6 and 71.7%, respectively (Fig. 5B).

PXR. PXR, often referred to as the “master regulator” of CYP3A expression, is naturally found in the cytoplasm, held in check by the cytoplasmic CAR retention protein and heat shock protein 90 (Squires et al., 2004). Interaction with a PXR-specific ligand releases PXR and transports it directly to the nucleus where it forms a heterodimer complex with RXR (Lehmann et al., 1998). The RXR-PXR complex binds to the distal xenobiotic responsive enhancer molecule (XREM) and drives CYP3A expression in concert with the proximal PXR responsive element (Chai et al., 2013). To determine if changes in CYP3A during adenovirus infection were the result of aberrant PXR expression, HC-04 cells were infected with different concentrations of virus. Ninety-six hours after treatment, cells were harvested and Western blots of nuclear and cellular extracts were evaluated for PXR (Fig. 6). PXR protein levels in nuclear extracts were not significantly affected by infection (Fig. 6A). In contrast, the virus had a more profound effect on cytoplasmic PXR as protein levels were reduced by

![Fig. 3. Adenovirus infection inhibits CYP3A activity and mRNA levels in HC-04 cells. (A) Catalytic activity of CYP3A in HC-04 cells after virus infection. Activity was measured by quantitation of the CYP3A-specific testosterone metabolite 6β-hydroxytestosterone by HPLC. (B) CYP3A4 expression and representative gel of RT-PCR products. cDNA was amplified under the following conditions: 95°C for 45 seconds, 57°C for 45 seconds, and 75°C for 1 minute for 28 cycles. Data are reported as the ratio of band intensity of CYP3A4 with respect to 18S with the ratio for uninfected controls (MOI 0) normalized to 1. (C) Immunoblot analysis of CYP3A4 protein. A representative blot illustrating band intensity for each virus concentration is included under the plot summarizing the change in CYP3A4 protein concentration with respect to beta-actin. Data in each panel was collected 96 hours after virus infection. Data are reported as the mean ± standard error of the mean of three 100 mm culture plates per condition from three separate experiments. Statistical significance was determined between data generated from individual treatment groups and uninfected (MOI 0) controls by one-way analysis of variance with a Bonferroni/Dunn post-hoc test. **P < 0.01 and ***P < 0.001.](image-url)
50% after infection with 10 and 100 MOI (Fig. 6B). A MOI of 500 reduced PXR protein levels in the cytoplasm by 70.2%.

CAR. CAR, like PXR, is primarily located in the cytoplasm (Wang et al., 2012). Unlike PXR, CAR is constitutively active in the cytosol and can enter the nucleus in the absence of CAR-specific ligands (di Masi et al., 2009). Evaluation of Western blots for the presence of CAR in nuclear extracts of cells infected for 96 hours with AdlacZ indicated a general increase in the amount of protein present with samples from cells infected with 500 MOI of virus containing approximately twice that found in uninfected cells (*P*, 0.01, Fig. 7A). CAR also appeared to increase in the cytoplasmic fraction in response to increasing amounts of virus (Fig. 7B). This, however, was not found to be statistically significant (*P* = 0.07). A second protein band that crossreacted with the CAR antibody was found in cultures infected with 500 MOI (arrow, Fig. 7B).

**Coactivators of CYP Expression: HNF-4α and PGC-1α.** Although RXR, PXR, and CAR are critical determinants of CYP3A4 expression, other trans-acting factors and their corresponding cis-acting elements also affect enzyme function. Hepatocyte nuclear factor 4α (HNF-4α), when coactivated by peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), is critically involved in PXR- and CAR-mediated regulation of CYP3A4, because it facilitates binding of the RXR-PXR/CAR heterodimer to the XREM and ER6 regions of the CYP3A4 promoter (Tirona et al., 2003). Because initial Western blots of cellular extracts revealed that both HNF-4α and PGC-1α were expressed at high levels in HC-04 cells (Fig. 8, *t* = 0), changes in both of these cofactors were evaluated for 96 hours after infection with 500 MOI of AdlacZ. A general decline in both HNF-4α and PGC-1α was noted as early as 24 hours after infection. Within 72 hours, HNF-4α was reduced by 72% (*P*, 0.05, Fig. 8A). PGC-1α was suppressed to a similar degree at the 96-hour time point (*P* < 0.05, Fig. 8B). The virus had the most significant impact on HNF-4α expression at the 96-hour time point (80% reduction, *P*<0.05, Fig. 8A).

**Fig. 4.** Transgene expression patterns in cultured cells 48 hours after infection. Cells were infected with a first generation recombinant adenovirus expressing beta-galactosidase (AdlacZ) at MOIs of 0 (A and D), 500 (B and E), and 1000 (C and F). Primary rat hepatocytes are shown in (A–C). HC-04 cells are shown in (D–F). Transgene expression in HC-04 cells was highly concentrated throughout the cell, whereas in primary hepatocytes was concentrated in the nucleus and diffuse throughout the cytoplasm. Uninfected cultures in (A) and (D) received histochemical treatment as did infected cells to detect endogenous levels of beta-galactosidase. None was detected in either cell type. Magnification: 250× (A); 100× (B–F).

**Fig. 5.** Adenovirus infection significantly alters nuclear and cytoplasmic levels of the RXR nuclear receptor 96 hours after infection in HC-04 cells. Immunoblot analysis of nuclear (A) and cytoplasmic (B) extracts for RXR protein expression after infection with a first generation recombinant adenovirus. Representative blots illustrating band intensity for each treatment condition are shown under plots. Protein levels are reported in arbitrary units of relative density as compared with a known protein standard. Data are reported as the mean ± standard error of the mean of three 100 mm culture plates per condition collected from three separate experiments. Statistical significance was determined between individual treatment groups and saline-treated controls (MOI 0) by one-way analysis of variance with a Bonferroni/Dunn post hoc test. *P* ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001.
Role of CAR and PXR in Suppression of CYP3A4 during Adenovirus Infection. To determine if changes in the nuclear receptors responsible for regulation of CYP3A4 could be assessed at the transcriptional level in this cell line, RNA was isolated from HC-04 cells infected for 96 hours (Fig. 9). A concentration of 500 MOI significantly reduced RXR mRNA levels by approximately 30% (P < 0.05, Fig. 9A). RXR mRNA levels were not significantly altered by the virus at any of the concentrations tested (P = 0.085, Fig. 9B). A general trend of increasing amounts of CAR mRNA was detected in infected samples, although none were found to be statistically significant with respect to uninfected controls (Fig. 9C, P = 0.092).

In an effort to further determine how CAR and PXR are involved in the downregulation of CYP during virus infection, HC-04 cells were treated with 100 nm CITCO [6-(4-chlorophenyl) imidazo[2,1-b][1,3] thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime], a known CAR agonist (Maglich et al., 2003). In the presence of this compound, different concentrations of virus did not significantly affect CYP3A4 activity (Fig. 10, P = 0.078). Virus at MOIs of 100, 500, and 1000 reduced activity in the absence of the compound by 23, 38, and 48%, respectively (control, Fig. 10). In contrast, when cells were treated with 20 μM rifampicin, a known agonist of PXR (Maglich et al., 2003), CYP3A4 activity was reduced by 28, 52.5, and 57.9% after infection with 100, 500, and 1000 MOI virus, respectively.

Discussion

One of the most obvious and cost-effective models for screening novel compounds and evaluating their impact on CYP expression and function is an established, continuous cell line. However, cells that originate from tumors or which are established through oncogenic immortalization lack many liver-specific functions, especially CYP-related enzyme activities due to low basal expression or mislocation of transcriptional regulators like CAR and PXR (Guguen-Guilluzo and Guillouzo, 2010). HepG2 (ATCC HB-8065), the most widely used human hepatoma cell line in metabolic research, contains functional CYP, but expression levels of most isoforms (CYP2B6, 2C9, 3A4) are 2–3 orders of magnitude below that of primary hepatocytes (Rodriguez-Antona et al., 2002). Thus, changes in CYP are difficult to detect in these cells without reconstituting each component of the transcriptional network through transfection with a series of plasmid constructs and pairing them with specific reporter genes to monitor response to treatments over time (Honma et al., 2010). Although this system may be useful for our particular application, transfection with multiple plasmids is a stressful procedure that is often not compatible with virus infection. This and the fact that gene expression profiles in transfected cells can vary from experiment to experiment motivated us to continue the search for a suitable in vitro model to study how virus infection affects drug metabolism.

HepaRG cells, derived from a human hepatocellular carcinoma associated with chronic hepatitis C (Gripon et al., 2002), have morphology and metabolic activity of differentiated hepatocytes (Hart et al., 2010). When seeded at a low density, they acquire an undifferentiated morphology, actively divide, and once they reach confluency, differentiate to form colonies of hepatocyte-like cells surrounded by biliary epithelial-like cells. HepaRG cells express various CYP isoforms (CYP1A2, 2B6, 2C9, 2E1, and 3A4) and key enzymes related to drug metabolism.

Fig. 6. Adenovirus infection significantly alters PXR protein levels in the cytoplasm of HC-04 cells. Immunoblot analysis of nuclear (A) and cytoplasmic (B) extracts for PXR protein expression 96 hours after infection with a first generation recombinant adenovirus (AdlacZ). Representative blots illustrating band intensity for each treatment condition are shown under plots for each virus. Protein levels are reported in arbitrary units of relative density compared with a known protein standard specific for each cellular compartment. Data are reported as the mean ± standard error of the mean of three 100 mm culture plates per condition collected from three separate experiments. Statistical significance was determined between individual treatment groups and saline-treated controls (MOI 0) by one-way analysis of variance with a Bonferroni/Dunn post hoc test. **P < 0.01, and ***P ≤ 0.001.

Fig. 7. Recombinant adenovirus increases CAR in nuclear extracts of HC-04 cells. Immunoblot analysis of nuclear (A) and cytoplasmic (B) extracts for CAR protein 96 hours after infection with adenovirus. Representative blots illustrating band intensity for each virus concentration are shown. Arrow indicates aberrant band found in samples infected with 500 MOI of virus. Protein levels are reported in arbitrary units of relative density compared with a known protein standard suitable for each cellular compartment. Data are reported as the mean ± standard error of the mean of three 100 mm culture plates per condition collected from three separate experiments. Statistical significance was determined between individual treatment groups and saline-treated controls (MOI 0) by one-way analysis of variance with a Bonferroni/Dunn post hoc test. **P < 0.01.
transcriptional regulators at levels comparable to primary human hepatocytes (Andersson et al., 2012). They also express several phase II enzymes and drug transporters, respond to selective CYP inducers, and have been used in virology research (Marion et al., 2010). Although they seem to be the most viable alternative to primary cells for our purposes, specific supplements are required to maintain their differentiated morphology and to keep CYP at levels that are responsive to induction (Marion et al., 2010). These cells were also not readily available in the United States until recently and are sold under a limited use license.

HC-04 cells, grown in standard medium supplemented with fetal bovine serum without any additional additives, responded in a graded manner to compounds known to suppress or induce CYP3A4 in vivo (Fig. 2). They were also easily transduced in a dose-dependent manner by a recombinant adenovirus (Fig. 4). These cells responded to virus infection in the context of cytochrome P450 metabolism in a manner similar to what we have observed in the rat and primate (Callahan et al., 2005b; Wonganan et al., 2011) without the addition of exogenous inflammatory mediators. The HC-04 cell line is also more sensitive to virus infection than primary hepatocytes isolated from the rat. Virus concentrations as low as 100 MOI significantly suppressed CYP3A activity in HC-04 cells, whereas 1,000 MOI only modestly reduced CYP activity in the primary cells (Figs. 1C and 3A). This may be attributed to differences in the species of origin of the cells, because it is well known that human adenoviruses do not replicate and cause fulminant infection in rodents (Wold and Toth, 2012). Suppression of CYP3A by adenovirus infection was also found to be isoform specific, because CYP2D activity was not affected by the presence of the virus (Fig. 2). We also found that expression of key CYP3A regulators, RXRα, PXR, and CAR as well as their coactivators HNF-4α and PGC-1α, was altered to some degree during infection (Figs. 5–8). This is the first time to our knowledge that changes in these transcriptional elements have been noted in response to virus infection in any in vitro model of hepatic drug metabolism.

Adenovirus infection mildly suppressed RXRα mRNA levels (Fig. 9, A and D). Although this may contribute to the observed reduction in protein levels of this nuclear receptor in infected cells (Fig. 5), the stability, turnover, and degradation process of RXRα during
adrenovirus infection also must be considered. Soon after it was reported that RXRα undergoes ubiquitination and subsequent proteasome-mediated degradation (Boudjelal et al., 2000), several groups found that activation of several cell signaling pathways like the c-Jun N-terminal kinase, mitogen-activated protein kinase, and interferon regulatory factor 3 (IRF3) pathways suppress RXRα-mediated processes through this mechanism (Chow et al., 2006; Lefebvre et al., 2010; Hoshikawa et al., 2011). Considering that recombiant adrenoviruses also stimulate these pathways (McCaffrey et al., 2008) and that other inflammatory mediators induce cytoplasmic relocation of RXRα (Ghose et al., 2004), we believe that activation of these pathways during adrenovirus infection may play a role in translocation of this nuclear receptor to the cytoplasm, where it is then tagged for post-translational modification and subsequent degradation. Additional studies using the HC-04 cell line to further dissect the molecular processes associated with RXRα suppression during infection are currently underway.

Of all nuclear receptors investigated, PXR was the most profoundly affected by adrenovirus infection. Although it is known that nuclear factor κB, activated by adrenovirus infection (McCaffrey et al., 2008), suppresses CYP3A activity by interfering with the binding of the PXR-RXR heterodimer to the XREM and ER6 regions of the promoter (Gu et al., 2006), the substantial reduction of PXR in the cytoplasmic fraction suggests that this may play a minor role in the results obtained from our studies. Instead, posttranslational modifications of PXR (phosphorylation, ubiquitination, SUMOylation, or acetylation) through various cell signal transduction pathways are most likely responsible for this result. Although it is well known that activation of protein kinase C and the phosphoinositide 3-kinase-Akt pathway phosphorylate PXR and its coactivators required for CYP expression (Pondugula et al., 2009), very little is currently known about the degradation process for this key nuclear receptor (Staudinger et al., 2011), making additional studies using this cell line warranted.

A marked increase in CAR protein in nuclear fractions of infected cells was also noted (Fig. 7). Because CAR works in tandem with PXR to modulate the metabolism of xenobiotics, endogenous steroids, and dietary factors (di Masi et al., 2009) and it, unlike PXR, can translocate to the nucleus without direct binding of ligands (Wang et al., 2012), we believe this increase to be a compensatory, protective mechanism to maintain CYP activity despite the decline in PXR protein levels during adrenovirus infection. The observation that CAR expression is induced by metabolic stress and subsequent phosphorylation of Elk-1 via the stress-activated protein kinase inhibitor pathway provides a basis for additional mechanistic studies since this pathway has also been shown to be activated by adrenovirus in non-immune cell types (Fejer et al., 2008; Osabe et al., 2009). Given that mRNA levels of PXR were largely unaffected during adrenovirus infection in HC-04 cells, it seems that post-translational modification of PXR and its cofactors may play a role in the suppression of CYP3A4 activity during virus infection. Data generated from infected cells treated with CAR and PXR agonists also support this conclusion (Fig. 10).

In this context, it is also important to note that the observed increase in nuclear CAR is not mirrored by a concurrent increase in cytoplasmic fractions of infected HC-04 cells despite the fact that transcription of CAR appears to also increase during adrenovirus infection (Fig. 9). The presence of a second cross-reactive band on Western blots of cytoplasmic fractions suggests that CAR may undergo adrenovirus-mediated post-translational modification that prevents it from supporting CYP3A4 metabolism. While post-translational modification of CAR is currently not well understood, recent findings that adrenoviral proteins foster proteolysis and degradation of host cell proteins and organelles allow us to envision a model in which CAR that accumulates in the nucleus is rendered inactive in the presence of virus in the cytoplasm (Schreiner et al., 2012). Additional studies to characterize the status of CAR during adrenovirus infection in HC-04 cells are also currently underway in our laboratories.

One of the most extraordinary observations generated from these cells was that, although CYP3A4 activity and mRNA levels were suppressed by the virus, protein levels of the enzyme remained unchanged throughout the course of infection (Fig. 2). Initial screens for CYP3A2 protein by Western blot of lysates from adrenovirus-infected primary hepatocytes, using a different primary antibody than what was used in these studies, revealed CYP protein remained unchanged or increased slightly during virus infection (Callahan, 2005a). Although the reason for this disconnect between in vitro and in vivo observations is not overtly apparent, we realize that cultured cells represent an isolated system that has metabolic properties that may be very different from hepatocytes in vivo because of the presence of biochemical and physiologic feedback loops in a living organism. The presence of protein in the absence of activity suggests that the virus induces a unique set of conditions that create a stable, yet inactive, form of the protein similar to what has been observed in cases of virus-induced stress and the unfolded protein response (Hetz, 2012; Zhang and Wang, 2012). This response is very sensitive to the amount of virus in contact with the cell. For example, one concentration can elicit a cytoprotective response, whereas another can make the response apoptotic. This effect may not be prevalent in vivo because contact time between virus and hepatocyte is not as prolonged as that in a static culture system. It may also be counterbalanced by extracellular factors in vivo. This, paired with the recent observation that HC-04 cells contain notable levels of inducible enzymes that play a key role in posttranslational modification of proteins like small ubiquitin-like modifier (SUMO) suggest that additional posttranslational modifications to the CYP3A4 protein may also occur during virus infection (Tao et al., 2014). This may also explain why, despite increases in CAR mRNA and protein, CYP3A4 activity is not enhanced by treatment with a CAR agonist during virus infection. Additional studies to determine if this phenomenon is something that
is unique to the HC-04 cell line or is something common to in vitro systems because of differences in metabolic patterns between cultured cells and hepatocytes in a living organism are highly warranted and are currently underway.

This work summarizes a series of studies designed to assess the suitability of HC-04 cells for mechanistic evaluation of how hepatic CYP3A expression is altered during virus infection. Cells were maintained in standard culture media with minimal supplementation. Under these conditions, the cells inherently expressed measurable levels of CYP and associated nuclear receptors. CYP3A4 in these cells could respond to a panel of compounds known to have inductive and inhibitory properties and was suppressed during virus infection in a concentration-dependent manner, which correlates with results previously obtained from several in vivo models. These and other results outlined in this manuscript were consistently reproducible over multiple passages and could be validated in other cell lines with established reporter assays. Although the HC-04 cell line is clearly a suitable model for our very specific application, we believe it may also be useful for generation of relevant data required for preclinical evaluation of novel drug candidates. More importantly, they can easily be maintained and incorporated in more complex physiologic models of hepatic metabolism for high-throughput screening platforms and in the study of disease pathogenesis (Esch et al., 2011; van Midwoud et al., 2011; Messner et al., 2013).

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Wonganan, Jonsson-Schmunk, Choi, and Croyle.

References

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