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2017

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**A Mechanistic Study of How Hepatic Cytochrome P450 3A4 is
Regulated During Infectious and Non-Infectious Conditions**

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Regulated During Infectious and Non-Infectious Conditions**

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2017

Dedication

To my husband, David, for your love and support in all my efforts. Without you, this experience would not have been as much fun.

Acknowledgements

First, I would like to extend my sincerest gratitude to my advisor, Dr. Maria Croyle. Thank you for your support, guidance and encouragement throughout my time as a graduate student. I have learned so much from you and I cannot wait to apply the knowledge I have gained in my future endeavors. Also, a thank you to our lab manager, Stephen Schafer, for always being eager to teach me about how everything in the lab works.

I would also like to thank my lab members, both past and present. Thank you for all the discussions and laughter. I would especially like to thank Jin Huk Choi for being an exceptional mentor and for teaching me so much in such a short amount of time. And thank you to Irnela Bajrovic, though our projects are very different, I appreciate your fresh perspective and our friendship in the lab.

I also extend my sincerest thanks to my committee members for their insightful discussions and suggestions for how to improve my project. I truly believe it has made a great impact on my work.

In addition, a thank you to my parents for supporting me in all of my crazy decisions. Your love and inspiration has made me into the person I am today.

Finally, to my husband David, thank you for cheering me on during my successful moments, being there with chocolate and encouragement during the less good days, and for always being my number one supporter.

A Mechanistic Study of How Hepatic Cytochrome P450 3A4 is Regulated During Infectious and Non-Infectious Conditions

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The University of Texas at Austin, 2017

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It has been established that adenovirus infection alters the expression and function of hepatic cytochrome P450 3A (CYP3A) for 14 days in several animal models. To determine the mechanism behind adenovirus-mediated alterations in CYP3A4 and if this effect was relevant to humans, we characterized a human hepatocyte cell line, HC-04, as an *in vitro* model to study virus-mediated changes in human CYP3A4. Similar to *in vivo* observations, infection with a first-generation recombinant adenovirus (AdlacZ) significantly suppressed CYP3A4 catalytic activity in an isoform-specific manner in this cell line. HC-04 cells were subsequently used to determine how vaccines to be used in pandemic outbreaks impact CYP3A4 expression and function. Infection with a recombinant adenovirus-based Ebola vaccine or influenza A virus subtypes H1N1 and H3N2 significantly suppressed human hepatic CYP3A4 catalytic activity by 34%, 91%, and 90%, respectively. This effect is important to understand as these vaccines are given to people of all ages. Mechanistic study of adenovirus infection in HC-04 cells indicated that engagement of integrin receptors is key in the initiation of processes responsible for changes in CYP3A4 during infection. Mice infected with AdlacZ experienced a 70% reduction in CYP3A activity 24 hours after infection. While infection with a mutant virus with integrin-binding arginine-glycine-aspartic acid (RGD) sequences deleted from the

viral capsid (AdΔRGD) did not alter CYP3A activity at the same timepoint. CYP3A mRNA and protein levels in AdlacZ-treated animals were also suppressed, whereas those of mice given AdΔRGD were similar to uninfected control mice. Silencing of the integrin β -subunit reverted adenovirus-mediated CYP3A4 suppression *in vitro*. Silencing of the α -subunit did not. This led us to believe that interactions with the β -tail of the integrin receptor is important for virus-mediated suppression of CYP3A4. Talin-1, a cytoplasmic protein that specifically binds to the β -tail of integrins therefore became the focus of remaining studies. Knockdown of talin-1 increased CYP3A4 catalytic activity by 190%. Genes associated with post-translational modifications (PTMs) were downregulated in talin-silenced samples and analysis during viral infection revealed a significant increase in PTMs of CYP3A4. These initial experiments indicate a role of talin-1 and PTMs in the regulation of CYP3A4, however further experiments are needed to confirm these findings. Understanding changes in CYP3A4 during wild-type infection and immunization is clinically important, especially for low metabolizers. In this population, suppression of CYP3A4 could lead to accumulation of drugs that normally would be metabolized and cleared via this pathway, leading to serious therapeutic failure or adverse effects.

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CHAPTER ONE

Introduction: Hepatic Drug Metabolism and Infection¹

1.1 HEPATIC DRUG METABOLISM

The liver plays a major role in biotransformation of endogenous and exogenous compounds through phase I catalysis, principally by the cytochrome P450 (CYP) enzyme family, as well as by phase II biotransformation enzymes. Biotransformation via the phase I pathway, involves oxidation, reduction or hydrolysis reactions [1], while the phase II pathway is characterized by conjugation of exogenous molecules, including glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation, with endogenous cofactors [2]. Many phase I products also become substrates for phase II enzymes [1]. CYP3A4 metabolizes a wide range of drugs due to a binding pocket that is highly diverse in substrate specificity, which could lead to undesirable drug-drug interactions and toxic side effects [3]. When taking drugs concurrently, one drug could inhibit or induce CYP3A4 which would affect the metabolism of another drug if it is metabolized by the affected enzyme. Understanding any aberrations of hepatic drug

¹ Large portions of this chapter are part of a manuscript accepted to the Journal of Pharmaceutical Investigation.

Jonsson-Schmunk K, Schafer SC, Croyle MA. Impact of nanomedicine on hepatic cytochrome P450 3A4 activity: things to consider during pre-clinical and clinical studies.

Contribution KJS: wrote the manuscript.

metabolism is especially of importance as an increasing number of prescription drugs become available on the market, to promote increased lifespans and quality of life. On average close to 50% of the U.S. population is using at least one prescription drug, 21% is using three or more prescription drugs, and 10% is using five or more prescription drugs [4]. With increased usage of prescription pharmaceuticals any aberration in drug metabolism could induce drug-drug interactions, cause medication levels to fall in the sub-therapeutic range or toxic reactions that can be life threatening due to drug accumulation. This has a terrible social cost through greater expenses related to increased hospital visits and lost time at work. Adverse drug reactions in the USA are currently estimated to cost 30.1 billion USD annually, with an estimated 12 day increase in hospital stay [5]. For cancer patients taking several concomitant medications, 58% had potential drug interactions [6], indicating a high prevalence of drug interactions in special patients.

1.2 THE CYTOCHROME P450 ENZYME FAMILY

In 1958, a carbon monoxide-binding pigment of liver microsomes was discovered [7]. It was later determined that this new pigment was a type of cytochrome [8] that can bind carbon monoxide (CO). The bound CO compound of the reduced pigment had a peak spectral absorbance of 450 nm. Hence, it was given the name “cytochrome P450” [9]. The CYP enzyme superfamily contains structurally diverse and functionally different enzymes that are integral endoplasmic reticulum-anchored hemoproteins involved in the metabolism

of endogenous and exogenous compounds. There are more than 15,000 known CYP genes found in virtually all organisms [10]. In humans, two thirds of the CYPs have important roles in biosynthesis or catabolism of endogenous substances, including steroid hormones, sterols and fatty acids, while the other third is mostly expressed in the liver where they are involved in biotransformation of xenobiotics [11]. The CYP enzyme family is responsible for the majority of metabolism in the liver through activation, inactivation, and circulatory clearance of several endogenous and exogenous compounds [12]. The human genome contains 18 CYP families, which are divided into 41 protein-coding subfamilies that encodes 57 genes (Table 1.1) [13]. The genes of this diverse family of enzymes are subdivided and classified on the basis of amino acid identity, phylogenic criteria, and gene organization. Division of CYP families is based on proteins with more than 40% amino acid sequence identity, and these are classified with a number after the general root “CYP”. Subfamilies are named with a letter, and are classified as amino acid sequences with greater than 55% identity, which is followed by a number for the gene [14]. In humans, CYP3A enzymes constitute over 35% of all CYP isoforms (Figure 1.1A). The human CYP3A subfamily, which is the only member of the CYP3 family, has a size of 231 kb and is located on chromosome 7q22.1 [15]. The *CYP3A* locus is comprised of four CYP genes: 3A4, 3A5, 3A7 and 3A43 with a relative expression of 85.4%, 5.4%, 3.4%, and 5.8%, respectively, in an average human adult liver (Figure 1.1B: [16]). In 1986, CYP3A4 was the first human *CYP3A* gene to be identified [17, 18]. Subsequently, CYP3A5 was identified [19-21] but only approximately 20% of human livers express this isoform, most commonly due to a splice site mutation [22]. After CYP3A7 was identified [23, 24] and

cloned [25], it turned out that this isotype accounts for 30-50% of total CYP in the fetal liver [26] and is expressed at very low levels in the human adult liver [16]. The most recently discovered CYP3A isoform is CYP3A43 [27-29], which has a 75% and 71% amino acid sequence similarity with CYP3A4 and CYP3A5, respectively. CYP3A43 is expressed at low levels in the human liver [16] and its function is currently unknown [22], however evidence indicates that it might be involved in steroid metabolism [27]. Out of all CYP isoforms, CYP3A4 is the most active drug-metabolizing enzyme, responsible for metabolizing over 30% of currently marketed drugs (Figure 1.1C: [15]). Most of these medications are chemically unrelated compounds, spanning over 38 different therapeutic classes of medicinal agents. Since CYP3A4 constitutes about 30% of all CYP isoforms, subtle changes in this single enzyme can lead to alteration in the therapeutic response of drugs [30].

Table 1.1 Overview of human cytochrome P450s including primary expression and function/substrates metabolized by specific CYPs.

CYP	Primary Expression	Function/Substrates
CYP1A1	Extrahepatic tissues including epithelia of the lung, skin, and gastrointestinal tract, and placenta, fetus, and embryo	Metabolizes endogenous substrates and drugs, activates certain toxins and environmental pollutants, polycyclic aromatic hydrocarbons (PAHs) [31].
CYP1A2	Liver	Metabolic activation of several carcinogens, among them aromatic and heterocyclic amines, nitroaromatic compounds, mycotoxins and estrogens [32].
CYP1B1	Extrahepatic sites including lung and kidney	Metabolizes endogenous compounds including 17 β -estradiol, retinals, arachidonic acid, and melatonin [33].
CYP2A6	Liver, lung, several extrahepatic sites including nasal mucosal	Drug metabolism and synthesis of cholesterol, steroids and other lipids. Known to hydroxylate coumarin, and also metabolizes nicotine, aflatoxin B1, nitrosamines, bilirubin, and some pharmaceuticals [34].
CYP2A7	Liver	Unknown function and substrates [35].
CYP2A13	Respiratory tract	Metabolic activation of hexamethylphosphoramide, N,N-dimethylaniline, 2'-methoxyacetophenone, N-nitrosomethylphenylamine, and the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [36].

Table 1.1 Continued.

CYP2B6	Liver, kidney, small intestine	Metabolism of drugs including artemisinin, bupropion, cyclophosphamide, efavirenz, ketamine, and methadone [37].
CYP2C8	Liver	Oxidative metabolism of many clinically available drugs from a diverse number of drug classes (e.g., thiazolidinediones, meglitinides, NSAIDs, antimalarials and chemotherapeutic taxanes) [38].
CYP2C9	Liver	Involved in oxidation of both xenobiotic and endogenous compounds. 15-20% of all drugs undergoing phase I metabolism including (NSAIDs, statins, sulfonylurea, anticoagulant) [39].
CYP2C18	Liver	Low protein expression, no significant effect on drug metabolism [15].
CYP2C19	Liver	Metabolizes drugs including proton pump inhibitors, anticoagulant, antidepressants. Exogenous substrates progesterone and melatonin [40].
CYP2D6	Liver	Typical substrates are largely lipophilic, include antidepressants, antipsychotic, antiarrhythmic, beta-blockers, opioids, 25% of current market [41].

Table 1.1 Continued.

CYP2E1	Liver, lung and other tissue	Metabolism of aliphatic and aromatic hydrocarbons, solvents and industrial monomers (i.e. alkanes, alkenes, aromatic and halogenated hydrocarbons) [42].
CYP2F1	Lung	Bioactivation of many potent environmental toxicants such as 3-methylindole, naphthalene, styrene and benzene [43].
CYP2J2	Lung	Epoxygenase that catalyzes epoxide formation at the site of a carbon–carbon double bond in the substrate. Metabolizes therapeutic agents ebastine, astemizole, terfenadine, diclofenac, and bufurarol [44].
CYP2R1	Liver	Vitamin D 25-hydroxylase [45].
CYP2S1	Lung	Dioxin-inducible, substrates include retinoic acid [46], cyclooxygenase- and lipoxygenase-derived eicosanoids [47].
CYP2U1	Identified in human	Catalyzes ω - and (ω -1)-hydroxylation of fatty acids, hydroxylation of debrisoquine and terfenadine derivatives [48].
CYP2W1	Identified in human	Converts all-transretinoic acid (atRA) to 4-hydroxy atRA and all-transretinol to 4-OH all-transretinol, and it also oxidizes retinal [49].

Table 1.1 Continued.

CYP3A4	Liver, small intestine	Substrates include referentially lipophilic and bulky compounds from almost all therapeutic categories, including tacrolimus, cyclosporine A, erythromycin, ifosfamide, tamoxifen, benzodiazepines, several statins, antidepressants, and opioids [50].
CYP3A5	Liver, lung	Overlapping substrate specificity with CYP3A4. Ex tacrolimus, hydroxylation of midazolam, and lidocaine demethylation [51].
CYP3A7	Fetal liver	Major fetal form, rarely expressed in adults. Involved in estriol biosynthesis, metabolizes 6- α -hydroxylation of dehydroepiandrosterone 3-sulphate [52].
CYP3A43	Liver	Function is currently unknown [22].
CYP4A11	Liver	Metabolism of lauric acid [53].
CYP4A22	Liver, kidney	Involved in ω -hydroxylation of saturated fatty acids including lauric acid, myristic acid (tetradecanoic acid), palmitic acid (hexadecanoic acid) and the unsaturated fatty acids oleic acid [(Z)-octadec-9-enoic acid] and arachidonic acid (all- <i>cis</i> -5,8,11,14-eicosatetraenoic acid) [54].

Table 1.1 Continued.

CYP2B1	Lung	Metabolizes many xenobiotics, including the anti-cancer drugs cyclophosphamide (CPA) and ifosfamide (IFA) and environmental contaminants such as polychlorinated biphenyls [55].
CYP4F2	Liver	ω -hydroxylase that catalyzes the first step in vitamin E metabolism, functions as a vitamin K(1) oxidase [56].
CYP4F3	Leukocytes	Involved in ω -hydroxylation of C18-epoxides, oxidation of fatty acid epoxides, leukotriene B ₄ (LTB ₄) and prostaglandins [57].
CYP4F8	Seminal vesicles	Metabolizes prostaglandin (PG)H ₁ and H ₂ to 19-hydroxy-PGH ₁ and 19-hydroxy-PGH ₂ [58].
CYP4F11	Liver	Involved in synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) from arachidonic acid, erythromycin, benzphetamine, ethylmorphine, chlorpromazine, and imipramine [59].
CYP4F12	Liver	Oxidizes arachidonic acid to 18-hydroxyarachidonic acid, and the omega-side chain PGH ₂ analogs 11,9-epoxymethano-PGH ₂ and 9,11-diazo-15-deoxy-PGH ₂ [60].

Table 1.1 Continued.

CYP4F22	Skin	Substrates are ultra-long-chain fatty acids. Involved in fatty acid ω -hydroxylase required for acylceramide synthesis [61].
CYP4V2	Lung, liver, kidney, pancreas, retina	Selective ω -hydroxylase of saturated, medium-chain fatty acids with relatively high catalytic efficiency toward myristic acid [62].
CYP4X1	Brain and vasculature	Metabolize arachidonic acid to form epoxyeicosatrienoic acids [63].
CYP4Z1	Mammary gland	Catalyzes ω -hydroxylation of fatty acids and is mainly implicated in metabolism of arachidonic acid, fatty acids, prostaglandins, and other eicosanoids [64].
CYP5A1	Platelets	Metabolizes the cyclooxygenase product PGH_2 into thromboxane A_2 [65].
CYP7A1	Liver	Involved in bile acid biosynthetic pathway, functions as a cholesterol 7α -hydroxylase [66].
CYP7B1	Liver, reproductive tract, brain	Hydroxylates carbons 6 and 7 of the B ring of oxysterols and steroids [67].
CYP8A1	Aorta, others	Prostacyclin I_2 synthase, acts as an isomerase and catalyze the formation of PGH_2 into PGI_2 [68].
CYP8B1	Liver	Sterol 12α -hydroxylase, catalyzes the synthesis of cholic acid and controls the ratio of cholic acid over chenodeoxycholic acid in the bile [69].

Table 1.1 Continued.

CYP11A1	Adrenal, testis, ovary, and placenta	First step and rate limiting enzyme in the steroidogenic pathway. It cleaves the side chain of cholesterol, converting it to pregnenolone [70].
CYP11B1	Adrenals	Steroid 11 β -hydroxylase, catalyzes the last step in cortisol production, the hydroxylation of deoxycortisol in the 11 β -position [71].
CYP11B2	Adrenals	Aldosterone synthase, catalyzes the terminal steps of aldosterone synthesis in the zona glomerulosa cells of the adrenal [72].
CYP17A1	Steroidogenic tissue	Two distinct types of substrate. Oxidation: via its hydroxylase activity, it catalyzes the 17 α -hydroxylation of pregnenolone to 17 α -OH pregnenolone and through its C17,20lyase activity, it can further convert 17 α -OH pregnenolone to the androgen dehydroepiandrosterone [73].
CYP19A1	Steroidogenic tissue, adipose, brain	Aromatase, irreversibly catalyzes androgens into estrogen in the steroidogenesis pathway [74].
CYP20A1	Identified in human liver	Unknown function and substrates [13].

Table 1.1 Continued.

CYP21A2	Steroidogenic tissue	21-hydroxylase, essential role in the biosynthesis of aldosterone and cortisol. Converts progesterone and 17-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively [75].
CYP24A1	Kidney	24-hydroxylase, responsible for the five-step 24-oxidation pathway from 1,25-dihydroxyvitamin D ₃ to calcitroic acid [76].
CYP26A1	Intestine, liver, endothelia	Retinoic acid-4-hydroxylase, oxidizes retinoic acid to more polar metabolites, promoting its removal from tissues [77].
CYP26B1	Brain	Oxidizes retinoic acid (RA) to including 4-hydroxy-RA, 18-hydroxy-RA, and 4-oxo-RA [78].
CYP26C1	Identified in human	Catabolism of retinoic acid [79].
CYP27A1	Liver	Sterol 27-hydroxylase, mitochondrial enzyme with broad substrate specificity for C27 sterols including 7-ketocholesterol (7kCh), catalyzes bile acid hydroxylations, activates vitamin D [80].
CYP27B1	Kidney	25-hydroxyvitamin D3 1 alpha-hydroxylase, converts extracellular substrate 25-hydroxyvitamin D (25OHD) to 1,25-dihydroxyvitamin D (1,25(OH)2D) [81].

Table 1.1 Continued.

CYP27C1	Liver, kidney	Catalyzes 3,4-desaturation of retinoids [82].
CYP39A1	Liver	24-HC 7 α -hydroxylase, involved in generation of oxysterols from cholesterol [83].
CYP46A1	Brain, retina	Cholesterol-24S-hydroxylase, catalyzes the hydroxylation of cholesterol at position C24 [84].
CYP51A1	Liver, testes	Lanosterol 14 α -demethylase, involved in early step in cholesterol biosynthesis, namely the 14 α -demethylation of lanosterol [85].

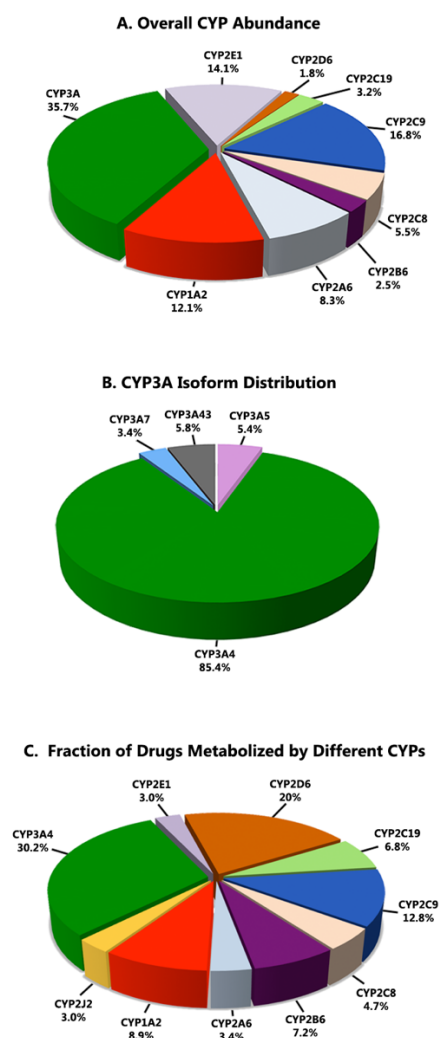


Figure 1.1 Abundance and fraction of drugs metabolized by different cytochrome P450 isoforms in an average human liver (A) The CYP3A subfamily constitutes 35.7% of the total overall CYP expression in the human liver. (B) In an average human liver, out of the CYP3A family (CYP3A4, CYP3A5, CYP3A7, and CYP3A3) 85.4% of the expression is due to CYP3A4. (C) The CYP3A family metabolizes approximately 30% of clinically used drugs across most therapeutic categories. Data extracted and compiled from [15, 16, 86].

1.2.1 Catalytic activity of the cytochrome P450 enzyme family

The CYP enzymes are heme-containing proteins. A thiolate anion of a cysteine residue serves as the axial ligand for the heme, which functions as an oxygen activator [87]. The monooxygenase activity of most CYPs requires molecular oxygen and nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) to introduce an oxygen molecule into a compound [88]. The heme molecule is located at the bottom of the large hydrophobic substrate-binding pocket of the CYP enzyme [89]. The biochemical reaction of CYP3A4-catalyzed oxygenation begins with the binding of a substrate molecule to the oxidized form of CYP (Figure 1.2: step 1). This binding induces a conformational change in the active site of CYP that fosters transition of the heme iron from its low-spin Fe(III) form to high spin Fe [90]. The high spin form favors CYP to be reduced by an electron supplied from NAD(P)H via the cytochrome P450 reductase [91] (Figure 1.2: step 2). This is followed by covalent binding of molecular oxygen to the distal axial coordination position of the ferrous iron of the heme [92] (Figure 1.2: step 3). A second electron from NAD(P)H is transferred, which reduces the dioxygen molecule to a negatively charged peroxo group, initiating O-O cleavages, which for most cytochrome P450s is the rate-determining step of their hallmark metabolic processes (Figure 1.2: step 4). Local transfer of protons from neighboring amino acid side chains results in the release of one molecule of water [93] (Figure 1.2: step 5). The metabolic substrate is finally oxidized and released from the active site (Figure 1.2: step 6) and a water molecule is taken up to occupy the distal position of the heme until the process starts over again [94]. The potent oxidizing properties of the

activated oxygen permit oxidation of a large number of substrates, which usually results in hydroxylated products or epoxides [87].

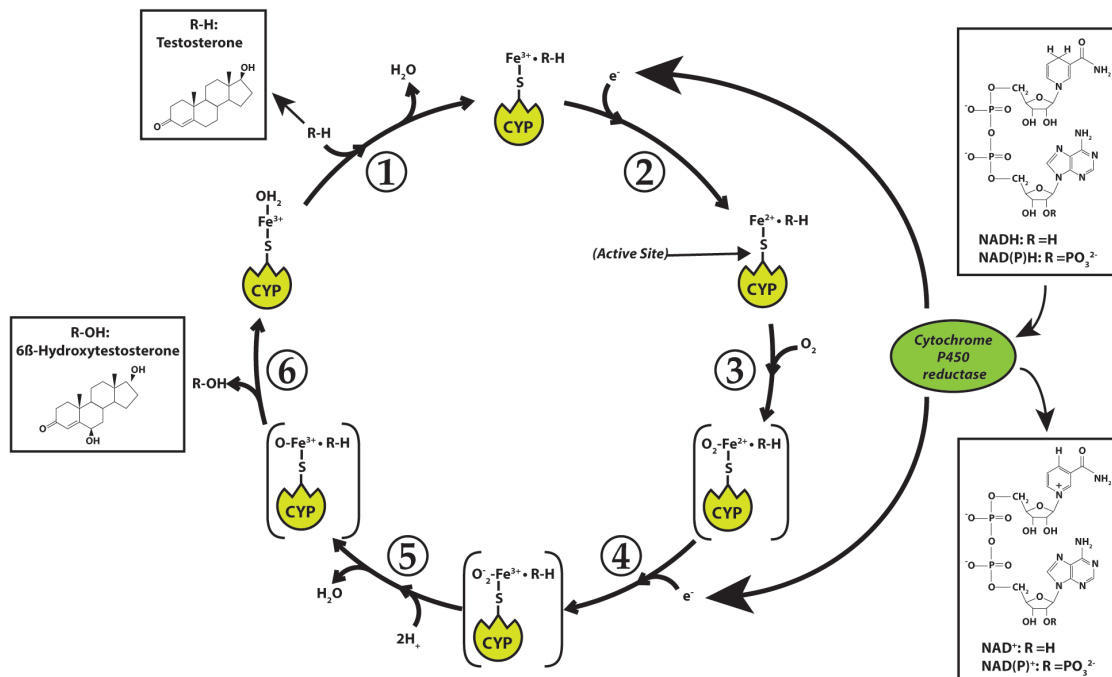


Figure 1.2 Catalytic mechanism of cytochrome P450 3A4 enzymes. The substrate, shown as testosterone, binds to the active site of CYP3A4, which induces a conformational change that displaces a water molecule from the axial ligand of CYP3A4 heme and modifies the heme iron to assume a high spin conformation (step 1). Transfer of an electron via cytochrome P450 reductase, an enzyme expressed in the endoplasmic reticulum of the liver in proximity of CYP3A4, from NAD(P)H reduces the ferric iron to its ferrous state (step 2). Molecular oxygen binds to the heme iron (step 3) followed by a second electron transfer from NAD(P)H (step 4) forming a peroxo group, which is rapidly protonated twice (step 5). Hydroxylation of testosterone and the release of the metabolite 6β-hydroxytestosterone is followed by the binding of a water molecule to the distal axial coordination position of the heme iron (step 6) before the process starts again.

1.3 REGULATION OF HEPATIC CYTOCHROME P450 3A4

1.3.1 Transcriptional regulation of CYP3A4

On a transcriptional level CYP3A4 is regulated by several signaling pathways that can be naturally expressed and influenced by certain xenobiotics (Figure 1.3). Constitutively CYP3A4 expression is mediated by both positive and negative regulators including transcription factors like the CCAAT enhancer binding protein (C/EBP) α [95] and C/EBP β [96-98], hepatocyte nuclear factor (HNF) -1 α and -3 γ [97], HNF-4 α [99-101], and upstream stimulatory factor 1 (USF1) [102]. HNF-4 α and HNF-1 α , together with transcription factors such as USF1 and activator protein 1 (AP1), bind to the constitutive liver enhancer module 4 (CLEM4) to constitutively induce CYP3A4 (Figure 1.3A) [99, 103, 104]. C/EBP α synergistically transactivates CYP3A4 together with HNF-3 γ (Figure 1.3B) and C/EBP β modulates CYP3A4 expression together with liver activating protein (LAP) or liver inactivating protein (LIP) (Figure 1.3C) [97, 98]. Transcriptional regulation is induced by a wide variety of structurally diverse xenobiotics. The mechanism is mainly driven by three cis-acting modules within the well-studied 12 kb region of the 35.8 kb CYP3A 5'-promoter sequence. These include the proximal PXR responsive element (prPXRE, -172 bp to -149 bp), the distal xenobiotic responsive enhancer module (dXREM, -7.8 kb to -7.2 kb) and CLEM4 (-11.4 kb to -10.5 kb) [98, 103, 105, 106]. Nuclear receptors, including the pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor and HNF-4 α , are primarily responsible for regulating hepatic CYP3A through interactions at specific points within the CYP3A4 5' promoter

sequence [12]. PXR is a transcriptional activator of at least 40 genes, including biologically important drug transporters and metabolic enzymes. PXR, which has broad substrate specificity, has been indicated as the master regulator of CYP3A, and the root cause of 60% of all clinically relevant CYP3A4 mediated drug-drug interactions [107]. In the cytoplasm, PXR is naturally bound to the cytoplasmic CAR retention protein (CCRP) and heat shock protein 90 (Hsp90). The CCRP-Hsp90 complex is released upon interaction with a PXR-specific ligand (Figure 1.3D) [108]. Once free, PXR can enter the nucleus where it forms a heterodimer complex with the retinoid X receptor (RXR) [109], which binds to the dXREM and the prPXRE to drive expression of CYP3A4 (Figure 1.3E and 1.3F, respectively) [104]. HNF-4 α facilitates binding of the PXR-RXR heterodimer to the dXREM and prPXRE of the CYP3A4 promoter after co-activation by peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) and the steroid receptor coactivator-1 (SRC-1) and has also been implemented in CAR binding (Figure 1.3E and F, respectively) [99, 110]. CAR is also naturally located in the cytosol and is held in check by CCRP and Hsp90 [111, 112]. Unlike PXR, CAR is constitutively active and can enter the nucleus upon removal of a phosphate group by protein phosphatase-2 (PP2A) [111] in the absence of a CAR specific ligand (Figure 1.3G) [113]. Like PXR, CAR also heterodimerizes with RXR in the nucleus, and initiates CYP3A expression by binding to the proximal everted repeat 6 (pER 6) of the prPXRE and to distal nuclear response element 3 (d-NR3) of the dXREM (Figure 1.3 H and I, respectively) [114].

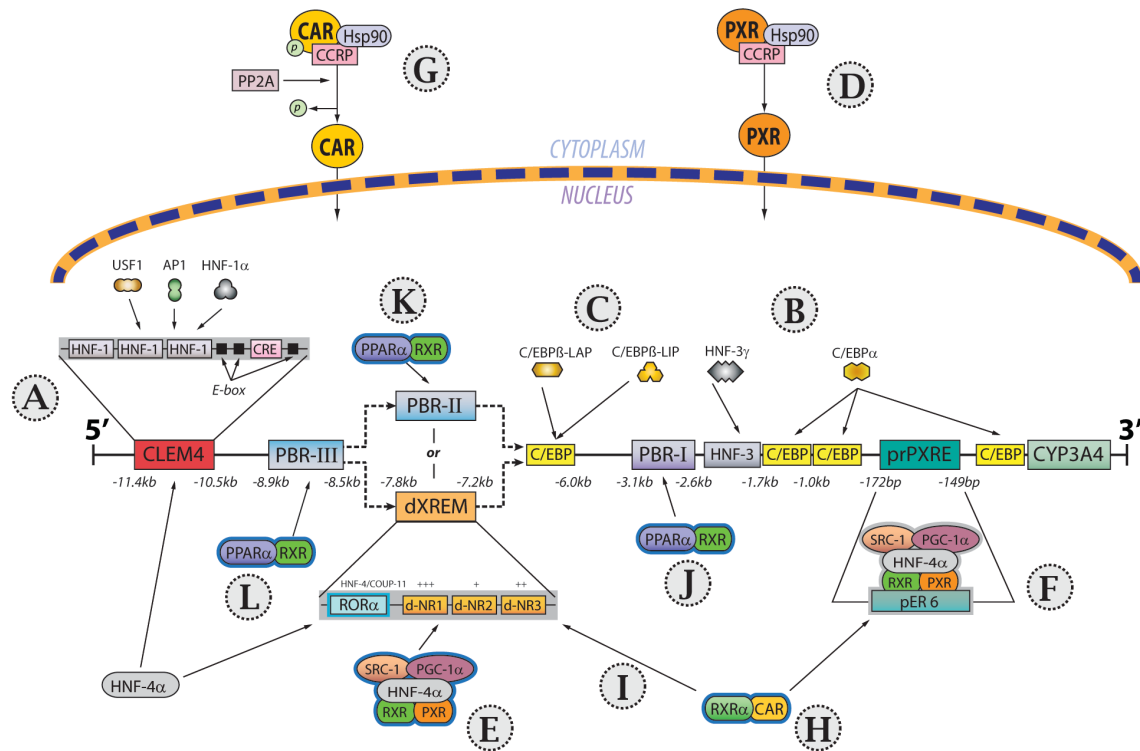


Figure 1.3 Cytochrome P450 3A4 is constitutively expressed and can be influenced by multiple transcription factors and regulatory elements. Binding sites on the CYP3A4 promoter region for transcriptional regulation of CYP3A4 by nuclear receptors regulate CYP3A4 by certain xenobiotics and natural compounds. See text for detailed explanation of these processes, figure adapted from [12].

The innate structure of the ligand binding domain of RXR allows it to assume multiple conformations, which dimerize with different nuclear receptors [115-117]. In addition, its flexible dimerization allows RXR to bind to direct repeat motifs on CYP3A4 while bound to several different dimerization partners [118]. RXR has three distinct isoforms, α , β and γ , but the α -isoform is most highly expressed in the liver [119] where it is responsible for actively regulating bile acid, cholesterol, fatty acid, steroid and xenobiotic metabolism [120]. RXR is essential for DNA binding of a number of nuclear receptors to various transcriptional elements and RXR can also form homodimers to drive CYP3A gene expression [121]. Cytoplasmic glucocorticoid receptors also regulate CYP3A4 by forming homodimers with their nuclear counterparts and subsequently binding with glucocorticoid-rich elements in the CYP3A promoter region [122]. Physiological concentrations of certain glucocorticoids like dexamethasone will increase CAR and PXR levels via glucocorticoid receptor-mediated pathways [123] while supraphysiological levels directly activate PXR and possibly CAR [107, 124]. Constitutive and inducible regulation of CYP3A4 was also shown to also be regulated by lipid homeostasis regulator peroxisome proliferator-activated receptor alpha (PPAR α) [125]. Three functional PPAR α binding regions, PBR-I, -II, and -III, are found in the CYP3A4 upstream sequence, to which PPAR α binds to in a heterodimer complex with RXR α (Figure 1.3J, K and L, respectively). Genome-wide time resolved data collected from human hepatocytes treated with various statins not only confirmed that PPAR α plays a role in CYP3A4 expression [126] but that single nucleotide polymorphisms (SNPs) in this region dictate distinct CYP3A4 phenotypes [127]. A significant induction of CYP3A4 was seen in these studies with a PPAR α agonist while an

antagonist or knockdown of PPAR α lead to suppression of CYP3A4, suggesting that nutritional status can affect drug biotransformation since PPAR α regulated the metabolism of lipids, carbohydrates and amino acids [126, 127].

1.3.2 Regulation of CYP3A4 by signal transduction pathways

Several signal transduction pathways have been shown to impact expression of the primary nuclear receptors that drive CYP3A4 expression. Signal transduction pathways associated with nuclear factor kappa-light-chain-enhancer of activated B Cells (NF κ B) [128] and the mitogen-activated protein kinase (MAPK) family, which comprise of extracellular-signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 mitogen-activated protein kinase (p38 MAPK) [124, 129, 130], have been especially indicated in CYP3A4 regulation. During inflammation, activation of ERK is involved in changes of CYP3A expression possibly via direct changes in the expression of RXR and potentially C/EBP β by phosphorylation of HNF-4 α [131]. Modulation of CYP3A expression by the JNK pathway is achieved through suppression of CAR via glucocorticoid receptor activity depression [124], alteration of nuclear transport of RXR α via IL-1 β activation [132], phosphorylation of HNF-4 α [133], and activation and altered nuclear transport of PXR [134]. In a recent phase I study of a selective p38 MAPK inhibitor in patients with advanced cancer, a potential induction of CYP3A4 activity was seen following treatment. The drug does not induce PXR *in vitro* hence it is believed that the effect seen on CYP3A4 activity is due to the pharmacological inhibition of p38 MAPK

[135]. NF κ B can alter CYP3A activity by inhibiting PXR activity [128], by preventing RXR α from heterodimerizing with PXR [136], and by repressing glucocorticoid receptor transcription thereby inhibiting CAR [137]. Although the ubiquitin-proteasome system is a major pathway in CYP3A4 protein degradation, inhibition of the NF κ B pathway decreased CYP3A4 protein stability via inhibition of the proteasome while CYP3A4 mRNA increased. This mechanism of CYP3A4 protein regulation may be related to NF κ B-mediated suppression of intracellular oxidative stress [138]. It was recently discovered that the WNT/ β -catenin signaling pathway is an important endogenous regulator of hepatic drug metabolism. Activation of the WNT/ β -catenin pathway determines hepatic zonation of CYP expression, the spatial separation of different metabolic pathways along the liver sinusoids [139-144], and is involved in regulating CYP transcription in response to xenobiotic agonists for several nuclear receptors, including PXR and CAR [145-147]. Activation of this pathway also prevented PPAR α -mediated induction of CYP3A4 [148].

1.3.3 Post-transcriptional regulation of CYP3A4 by miRNAs

It has been estimated that up to 60% of genes in the human genome are controlled by microRNAs (miRNAs) [149-151]. miRNA is a family of short, non-coding RNA whose final product is a 22-nucleotide functional RNA molecule, that function as a guide molecule in RNA silencing [152]. They play an important role in regulation of genes by binding to complementary regions of mRNA transcripts to repress the translation of encoded genes and occasionally in triggering degradation of the bound mRNA [153]. PXR

is regulated by microRNA-148a (miR-148a). PXR protein levels were decreased by overexpression of miR-148a and were increased by inhibition of miR-148a in human hepatocellular carcinoma cell lines. This correlated to changes of the inducible and/or constitutive levels of CYP3A4 in the human liver [154]. RXR α gene expression is downregulated by the binding of miR-27a and miR-27b to the RXR α 3' untranslated region (UTR), which is also translated to reduced protein levels of RXR α [155]. Suppression of CYP3A4 protein expression has been directly associated with the binding of miR-27b to the CYP3A4 3' UTR [156]. Since a single gene is usually regulated by multiple miRNAs [157], a recent study used large-scale screening methodology, including *in silico*, *in vitro* and *in vivo* approaches, to demonstrate that human miR-577, miR-1, miR532-3p and miR-627 all repress translation of CYP3A4 mRNA to active enzyme in human liver samples. Expression of these miRNAs correlate with various cancers and could be involved in chemosensitivity after chemotherapy, which could be explained by their influence on CYP enzymes needed to clear chemotherapeutic drugs [158]. The miRNA field is an ongoing area of exciting research and the full picture of how CYP3A4 is regulated via miRNAs is not yet fully elucidated.

1.3.4 Post-translation modifications of CYP3A4

It was originally believed that, once expressed, CYPs did not experience post-translational modifications. However, recent discoveries have shown that certain CYPs can be modified after translation of the protein under certain conditions. CYP3A4 has been

shown to be modified by phosphorylation and ubiquitination [10]. Generally, phosphorylation of CYPs leads to interference with substrate binding, substrate specificity, and their catalytic activity. For CYP3A4 specifically, phosphorylation is also believed to enhance lysine ubiquitination of the protein, which results in aggregation of ubiquitin tagged CYP3A4 in microsomal membranes and enhanced protein degradation [159]. Ubiquitin-dependent 26S proteasomal systems, including E2 ubiquitin-conjugating-E3 ubiquitin ligase complexes [160] and protein kinase (PK)A and PKC [161, 162], are involved in CYP3A4 degradation. Additionally, phosphorylation by PKA and PKC alters nuclear localization patterns of transcription factors involved in CYP3A4 activation resulting in suppression of CYP3A4 expression and function [129, 163, 164]. Hepatic CYP3A undergoes ubiquitin-dependent 26S proteasomal degradation [159, 165] and ubiquitination by components of E3 systems including gp78, an UBC7-dependent polytopic RING-finger E3, and the UbcH5a-dependent cytosolic E3 CHIP has been observed *in vitro* [159, 166].

1.3.5 Genetic and non-genetic factors affecting CYP3A4

The clinical impact of CYP polymorphism is important as loss-of-function variants lead to reduced clearance and increased plasma concentrations while gain-of-function variants lead to increased clearance and lower drug concentrations. This is significant as increased or decreased drug effect could induce drug-related toxicity or sub-therapeutic levels. mRNA levels of CYP3A4 are generally expressed in higher levels in women

compared to men and most clinical studies show that women metabolize drugs faster than men [167]. Hepatic protein levels of CYP3A4 is 2-fold higher in human females, which results in substrate-dependent pharmacokinetic differences between 20-50% [168-171]. Age is an important factor for the function and expression of drug-metabolizing enzymes. Neonates have substantially lower capacity due to immaturity of CYPs, which fully develop during the first year of life [172, 173]. CYP3A7 is the primary expressed CYP3A enzyme in the human fetus [174], which becomes silenced in the perinatal period within the first postnatal week. In some individuals, the enzymatic activity of CYP3A7 continues throughout adulthood. CYP3A7 catalyzes 6- α -hydroxylation of dehydroepiandrosterone 3-sulphate, an intermediate metabolite in the estriol biosynthesis [52]. In the elderly, the ability to clear drugs is decreased, which could be due to reduced liver blood flow [167]. Changes in drug metabolism due to age have been observed in rats [175] and long-lived mutant mice that are homozygous for a missense mutation in the growth hormone releasing hormone receptor (*Ghrhr*) gene [176]. Population variability of CYP3A4 is greater than 100-fold and several polymorphisms in the *CYP3A4* gene have been identified [15]. The most common polymorphism is the proximal promoter variant CYP3A4*1B (–392A>G, rs2740574), which occurs in 2-9% in Caucasian populations and at higher frequencies, 54-72%, in African populations [50]. This variant has been associated with higher tumor grade and stage in prostate cancer [177]. Another recent discovered polymorphism is CYP3A4*22 (intron 6 variant C > T, rs35599367) which accounts for 7% of mRNA expression variability of CYP3A4 [178, 179]. Patients with

CYP3A4*22 variant treated with atorvastatin, simvastatin, or lovastatin required 1.7- to 5-fold reduced statin doses compared to non-carriers [178].

1.4 THE EFFECT OF INFECTION AND INFLAMMATION ON CYP3A4

1.4.1 Bacterial vectors

Reduction in circulatory clearance of drugs due to inflammation was first seen in 1953 in rats that were treated with trypan blue, which resulted in prolonged pentobarbital sleeping time [180]. Bacterial inflammation in mice induced by components from Gram-negative (lipopolysaccharide, LPS) or Gram-positive (lipoteichoic acid, LTA) bacteria, reduced CYP3A protein expression in mice by 95% and 60%, respectively, and CYP3A activity by 70% [181]. In another study, murine hepatic CYP3A mRNA was downregulated after infection with *C. rodentium* [182], a Gram negative bacteria, which is most likely due to increased tumor necrosis factor (TNF)- α levels [183].

1.4.2 Cytokines

Cytokines produced in response to inflammation during the acute phase of the immune response, including interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, interferon (IFN)- γ , TNF- α and transforming growth factor (TGF)- β , have been shown to significantly suppress hepatic CYP3A function and expression *in vitro* and *in vivo* [184-187]. Individual CYPs respond differently to specific cytokines, hence changes in CYP3A4 expression will

vary depending on type of inflammatory disease state [188]. Changes in CYP3A have been observed due to induction of CAR by IL-1 β [137], IL-6 induced C/EBP [96], and activation of the NF κ B signal pathway has been associated with PXR changes [136]. In addition, pro-inflammatory cytokines induce nitric oxide synthase (NOS) and subsequent NO production *in vivo* and *in vitro* via the NF κ B signal pathway [189], which can cause nitrosylation of CYP [190] or NO-dependent polyubiquitination of CYP [11]. Cytokine-mediated downregulation of CYP3A4 is also clinically important as tumors in cancer patients are a source of systemically circulating cytokines [191, 192], which is especially important to monitor as cancer patients take on average 5.5 prescription drugs per day [193]. Suppression of CYP3A4 in any of these patient populations through cytokine-mediated pathways could lead to drug levels outside of the therapeutic range and increased risk of drug-drug interactions.

1.4.3 Viral vectors

Viral vectors are promising tools for vaccines and gene therapy with advantages over other systems including high efficiency of gene transduction, specificity of delivery to target cells and induction of a robust immune response and increased cellular immunity. Viral vectors have been evaluated in clinical trials as vaccines or therapeutic vaccines for many disease states including HIV-1 [194, 195], hepatitis B [196], influenza A [197], malaria [198], tuberculosis [199], Ebola virus disease [200] and cancer [201]. Common viral vectors include, adenovirus, adeno-associated virus (AAV), vaccinia virus, retrovirus

vectors, lentivirus, and cytomegalovirus [202]. In 1963, virus-induced hepatitis was reported to change hexobarbital and strychnine metabolism in mice, which was the first time that changes in drug metabolism were described during virus infection [203]. Correlations between changes in drug metabolism during inflammation with changes in hepatic CYP enzymes were first reported in 1976, when interferon (IFN) inducers suppressed drug metabolism in rodents and changes in CYP occurred after the production of IFNs during viral infection [204, 205]. It is currently known that microbial infection and inflammation suppress the expression and function of hepatic CYP3A, although the specific mechanism of this phenomenon is not fully understood. Mediators of the immune response, such as cytokines and nitric oxide, have been implicated with this effect during the acute stage of infection [188]. However, it has been found through *in vitro* [206, 207] and *in vivo* [206, 208-211] studies that CYP3A expression and function is significantly suppressed for an extended period of time by a single dose of a recombinant adenovirus. A single dose of a modified adenovirus, with a reduced immunogenic profile, inhibited expression and function of hepatic CYP3A for 14 days in Sprague-Dawley rats [208] suggesting mediators other than those associated with the immune response are involved in adenovirus-mediated CYP3A suppression.

Other viruses including hepatitis B virus (HBV) and hepatitis C virus (HCV), Newcastle disease virus, encephalomyocarditis virus, retrovirus, and influenza virus, have also been associated with aberrations in CYP expression and/or function. In HCB- and/or HCV-infected livers, CYP3A gene expression was significantly upregulated [212].

However, for patients that had developed hepatocellular carcinoma CYP3A4 activity and protein levels were significantly decreased [213]. Virus infection causing increased interferon levels have been associated with decreased CYP levels in both Newcastle disease virus (NVD) [214] and encephalomyocarditis virus [215], indicating an interaction between virus infection and CYP-dependent drug transformation. Increased resistance to acetaminophen-induced hepatotoxicity was seen in mice with chronic retrovirus infection [216], which was due to enhanced elimination of acetaminophen by activation of metabolic detoxification pathways [217]. Decreased theophylline clearance has been observed in healthy volunteers following influenza vaccination [218].

1.5 INTEGRIN RECEPTORS

A wide panel of pathogens including bacteria, viruses and parasites, express adhesive proteins on their outer surface that directly or indirectly associate with integrins [219]. This association establishes a close contact with host tissue and triggers cellular responses including bacterial [220] and viral internalization. Several viruses have RGD sequences displayed on the exposed loops of the viral capsid proteins that are recognized by a variety of integrin receptors [221]. The integrin family consists of 24 membrane-spanning receptors. Each consists of a heterodimeric non-covalently associated combination of one of the 18 alpha (α) and one of the 8 beta (β) protein subunits. They mediate adhesion to the extracellular matrix (ECM) and play a role in stimulation of signaling pathways associated with an immunoglobulin superfamily of molecules.

Integrins are divided into five subfamilies based upon the major protein recognized, type of cell expressing the receptor or the presence of an I-domain in the alpha subunit: arginine-glycine-aspartic acid (RGD)-binding integrins, $\alpha 4$ family of integrins, leukocyte adhesion integrins, laminin-binding integrins, and I-domain collagen-binding integrins. All integrin subunits have a single membrane-spanning helix and usually a short unconstructed cytoplasmic tail [222, 223]. Integrins are regulated by extracellular stimuli, initiating intracellular signals, which convert the extracellular domains into a high-affinity ligand-binding state, known as an inside-out response. Elicitation of this response results in cell adhesion and migration, and ECM assembly (Figure 1.4). Integrins lack intrinsic enzymatic signaling, instead they are activated by binding to extracellular ligands, which results in clustering and changes in the ligand-binding affinity of integrins on the cell surface [224]. Activation causes changes in the tertiary and quaternary structure of integrins, which propagate across the membrane to activate cytoplasmic kinase- and cytoskeletal-signaling cascades. This is known as the outside-in response, which initiates changes in cell polarity, survival, proliferation, cytoskeletal structure and gene expression [222, 223] (Figure 1.4).

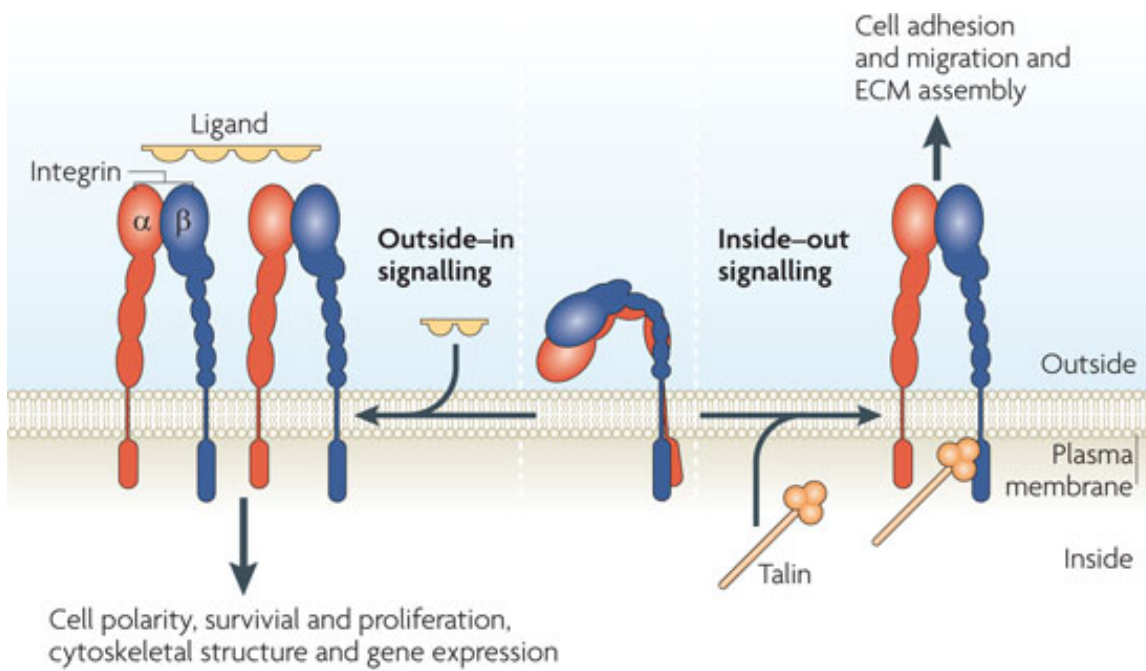


Figure 1.4 Outside-in and inside-out integrin signaling. During inside-out signaling, the intracellular activator talin binds to the β -integrin tail leading to conformational changes that result in increased affinity for extracellular ligands. Integrins also behave like traditional extracellular signaling receptors in transmitting information into cells by outside-in signaling resulting in integrin clustering (Figure adapted from reference [225]).

1.6 OBJECTIVES

It has been well established that the expression and function of hepatic CYP3A is altered by microbial infection and inflammation, which is mediated by cytokines and chemokines. We have shown that systemic administration of a first-generation recombinant adenovirus vector expressing beta-galactosidase altered the function and expression of hepatic CYP3A over a period of 14 days. This is long after cytokines and inflammation are cleared, suggesting an alternative mechanism for the long-lasting effect adenovirus has on CYP3A function and expression. Gene expression patterns in the liver of animals given a single dose of different live and inactivated recombinant adenoviruses were analyzed and five pathways were found to consistently be upregulated by the presence of virus, all involved in CYP3A regulation and associated with integrin pathways (Figure 1.5A). In another pilot study, CYP3A activity was suppressed in primary hepatocytes isolated from rats (male Sprague-Dawley) treated with an arginine-glycine-aspartic acid (RGD) peptide, known to engage integrins in the absence of virus infection (Figure 1.5B) [208]. The role of integrin receptors in CYP3A4 expression and function during adenovirus infection could either be triggered by the virus binding to the integrin receptors, signaling from the outside in, or after the virus has entered the cell via integrin inside-out signaling. In addition, viral vectors such as HIV, adenovirus, human papillomavirus and rubulavirus, rely on cellular ubiquitin pathways for efficient replication and survival while herpes virus encodes its own ubiquitin ligase [226, 227], which could be another way for these viruses to alter CYP3A function as ubiquitination of CYP3A is known to lead to enhanced protein

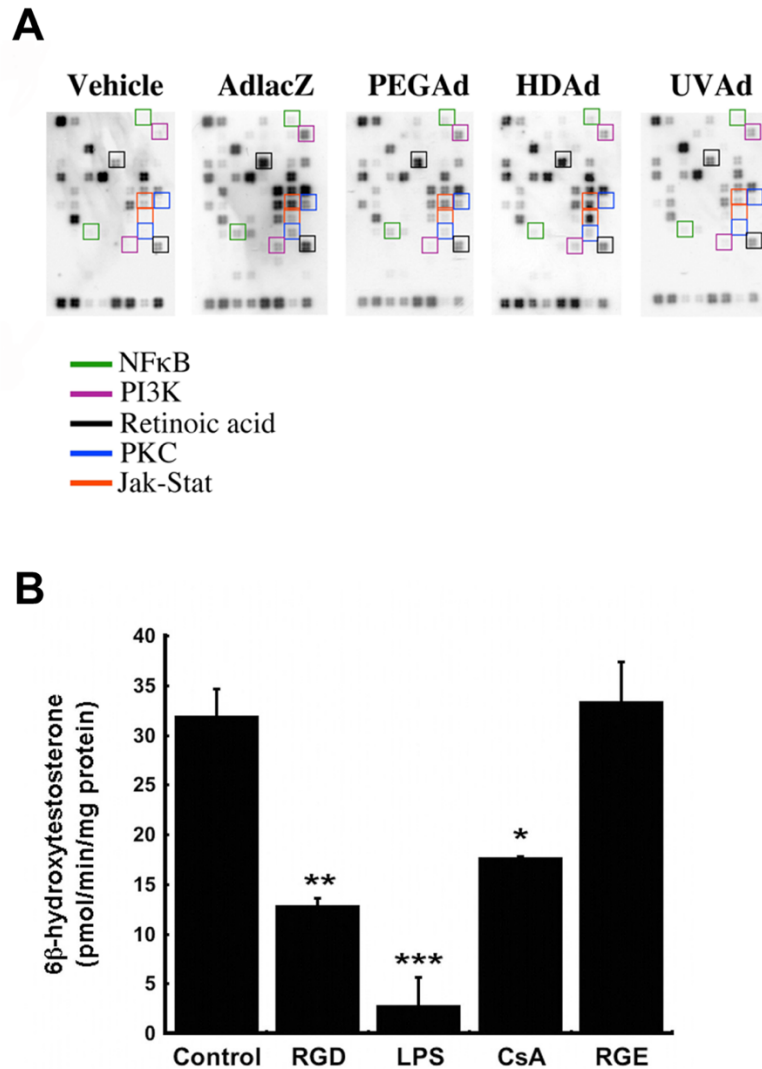


Figure 1.5 Genes affected by adenovirus infection related to CYP3A and integrin pathways and effect of integrin engagement on CYP3A activity. (A) Live and inactivated recombinant adenoviruses activated five signal transduction pathways in the liver. (B) Engagement of integrin receptors with a non-infectious RGD peptide suppressed CYP3A activity in primary rat hepatocytes. The hypothesis that integrin receptors are involved in CYP3A regulation, upon which this thesis was built, was inspired by these two figures (Adapted from [207, 208]).

degradation [228] or protein stabilization [229]. Given this, the following hypothesis was generated: Engagement of integrin receptors through an outside-in and/or inside-out mechanism is involved in the regulation of hepatic CYP3A4 during infectious and non-infectious conditions. The activation of integrin receptors by adenovirus infection triggers several different cellular pathways involved in CYP3A4 regulation, including the ubiquitin pathway.

More specifically, the aims of the project were:

- i. **Characterization of the HC-04 cell line as an *in vitro* model for mechanistic assessment of changes in hepatic CYP3A4 during virus infection.** HC-04 cells, a human hepatocyte cell line, was assessed for suitability for mechanistic studies of human CYP3A4 during virus infection. Pharmacological relevance of this cell line was evaluated by treating the cells with known inducers and inhibitors of CYP3A followed by measurement of changes in CYP3A activity. HC-04 cells were also infected with virus and CYP3A activity, mRNA levels and protein expression was determined. Additionally, the effect of virus infection on nuclear receptors regulating CYP3A4 expression was assessed. Results obtained from this cell line was compared with previous *in vivo* studies to validate its utility for our future studies (Chapter 2).

- ii. **Evaluation of influenza A virus infection and immunization using an adenovirus-based vaccine on CYP3A4 activity.** While the majority of studies of virus-mediated changes in CYP3A focus on pathogens that cause disease, few have focused on the impact immunization has on hepatic CYP3A. To determine if these responses mimic natural infection or impact CYP3A in a different manner, *in vivo* and *in vitro* infection with two types of influenza A virus strains (H1N1 and H3N2) or immunization using an adenovirus-based vaccine expressing a full-length Ebola glycoprotein sequence (AdEBO) was used to assess changes in CYP3A expression and function. Hepatic CYP3A4 activity was suppressed by 55% in mice infected with H1N1 influenza virus and by 44% in mice immunized with an adenovirus-based vaccine expressing the Ebola-glycoprotein (AdEBO). Changes in gene and protein expression of nuclear receptors regulating CYP3A4 was determined in the HC-04 cell line. Understanding the impact of adenovirus-based vaccines and wild-type infections with influenza A virus on CYP3A4 is of critical importance as, on average, 48.5% of the global population uses at least one prescribed medication. The effect of virus infection on host transcription and potential influence of the transgene on CYP3A4 function and expression was also determined (Chapter 3).
- iii. **Evaluation of the role of integrin receptors in the regulation of hepatic cytochrome P450 3A during infectious and non-infectious conditions.** The influence of integrin receptors on the expression and function of CYP3A was determined by infecting mice with two recombinant adenoviruses that differ only

in their ability to bind integrin receptors. Additionally, individual integrin subunits responsible for changes in CYP3A was evaluated to determine the specific pathways associated with integrin regulation of CYP3A (Chapter 4).

- iv. **Assessment of regulation of CYP3A by talin-1 and post-translational modifications during infectious and non-infectious conditions.** Talin-1, an intracellular signaling molecule that binds to the β -tail of the integrin receptor, was silenced using siRNA techniques to study the effect this protein might have in the regulation of CYP3A4. Silencing of talin-1 followed by infection with adenovirus revealed that genes involved in the ubiquitin pathway were downregulated, hence changes in ubiquitin and other post-translational modifications of CYP3A was studied (Chapter 5).

CHAPTER TWO

Evaluation of the HC-04 Cell Line as an *In Vitro* Model for Mechanistic Assessment of Changes in Hepatic Cytochrome P450 3A During Adenovirus infection²

2.1 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 [1]. The liver also provides an immunologic function through the reticuloendothelial system by phagocytosis and clearance of microorganisms from the portal blood [230]. 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 [231]. 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.

² Large portions of this chapter have been previously published.

Wonganan P, Jonsson-Schmunk K, Callahan SM, Choi JH, Croyle MA. 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. *Drug Metab Dispos.* 2014; 42: 1191-1201.

Contribution KJS: Planned and performed experiments, analyzed data, and helped write the manuscript.

For more than two decades, it has been known that microbial infection suppresses the activity and expression of hepatic cytochrome P450 3A (CYP3A) [12, 232, 233]. 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 [234, 235], 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 [233]. 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 [208]. 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 (CYP) 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 [236]. Although many different variables can significantly affect the phenotype and performance of primary hepatocytes *in vitro* [237], most protocols favor establishing cultures at a high density between two layers of collagen or Matrigel for long-term use [238]. 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 [239]. 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 [240]. 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.

2.2 MATERIAL AND METHODS

2.2.1 Materials

Acepromazine 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), 11 α -hydroxyprogesterone, sorbitan mono-9 octadecenoate poly (oxy-1,1-ethanedyl) (Tween 20), cyclosporine A, bacterial lipopolysaccharides (LPS, *Escherichia coli* serotype 0127:B8), phenobarbital, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO). Protogel acrylamide was purchased from National Diagnostics (Atlanta, GA). Ketoconazole and rifampicin were purchased from Fisher Scientific (Pittsburgh, PA). Erythromycin, isoniazid, and CITCO [6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)-oxime] were purchased from MP Biomedicals (Solon, OH), Acros Organics (Geel, Belgium), and Tocris Bioscience (Bristol, UK), respectively. Dimethylsulfoxide (DMSO) was purchased from

Malinckrodt Baker, Inc. (Phillipsburg, NJ). Testosterone metabolites were purchased from Steraloids Inc. (Wilton, NH). 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was purchased from Gold Biotechnology (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.

2.2.2 Cell culture

2.2.2.1 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 [241]. Briefly, deep plane anesthesia was established with a single intramuscular injection of a preparation consisting of a 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 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 (Miltenyi 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), l-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.

2.2.2.2 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, catalog number 26140, lot number 676548) and 2 mM l-glutamine (Gibco).

Cells of passage 64–74 were used in the studies outlined in this manuscript.

2.2.3 Virus production and purification

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 [242]. 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 [243]. All experiments were performed with freshly purified virus.

2.2.4 Virus infection

For each study, a minimum of three plates of HC-04 cells were trypsinized and counted using a standard hemocytometer (Hausser Scientific, Horsham, PA). Freshly purified virus was diluted to the appropriate concentrations in serum-free media. Three milliliters of each preparation were 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-galactoside (X-gal) for 4 hours at 37°C in the dark. Staining medium was removed, and blue-colored, positive cells were observed with a MicrosOptics IV900 microscope and photographed using a Nikon Coolpix 4500 digital camera and Nikon View (Eastman Kodak Co., Rochester, N) software.

2.2.5 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-IPA assay kit according to the manufacturer's instructions (Promega, Madison, WI).

2.2.6 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.

2.2.7 Testosterone hydroxylation assay

Production of the isoform-specific metabolite of testosterone, 6 β -hydroxytestosterone, was used to assess changes in CYP3A activity during virus infection [244]. 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 μ M testosterone for 30 minutes at 37°C. After addition of an internal standard (2.5 μ g 11 α -hydroxyprogesterone), media was removed and the reaction quenched with 5 ml dichloromethane. The organic phase was evaporated under a constant stream of air and dissolved in 75 μ l of solution consisting of methanol and water in a 1:1 ratio. The primary CYP3A testosterone metabolite, 6 β -hydroxytestosterone, and 16 α -hydroxytestosterone, the primary metabolite of CYP2D, were separated and quantified by high-performance liquid chromatography (HPLC) as previously described [245].

2.2.8 Preparation of whole cell, nuclear, and cytoplasmic extract

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 combined and centrifuged at

2,000 *g* at 4°C 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 extrusion through a 20-gauge needle (Becton Dickinson, Franklin Lakes, NJ) attached to 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.

2.2.9 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 Cappel, 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.

2.2.10 RT-PCR

RNA was isolated using TRIzol™ reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNA was reverse transcribed with random nanomers 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 minutes 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, RXR α , 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' -CAA GCG GAA GAA AAG TGA ACG -3' (forward) and 5' -CTG GTC CTC GAT GGG CAA GTC-3' (reverse); 5' -CCT TTC TCG GTC ATC AGC TC-3' (forward) and 5' -CTC GCA GCT GTA CAC TCC AT-3' (reverse); and 5' -TAG ATT TCT CCT 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.

2.2.11 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% ($P < 0.05$).

2.3 RESULTS

2.3.1 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), CYP3A activity was reduced by 80% (Figure 2.1A). In contrast, the rate of production of the CYP3A4 metabolite, 6 β -hydroxytestosterone, in uninfected (control) and infected cells sandwiched between a collagen matrix was not statistically different ($P = 0.2$, Figure 2.1A). Histochemical staining of cells for expression of the beta-galactosidase transgene revealed that sandwiching the cells between a collagen matrix prevented the virus from infecting hepatocytes (Figure 2.1B). When primary hepatocytes from male Sprague-Dawley rats were infected with different concentrations of virus, CYP3A4 activity was suppressed to a level similar to that observed *in vivo* ([211]; Figure 2.1C). 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 CYP3A 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 ([246]; Figure 2.1D). This was

a significant concern because the presence of these cells, which induce potent inflammatory responses against pathogens, could confound our results [247]. Modification of the standard two-step collagenase perfusion technique [241] 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 (Figure 2.1D).

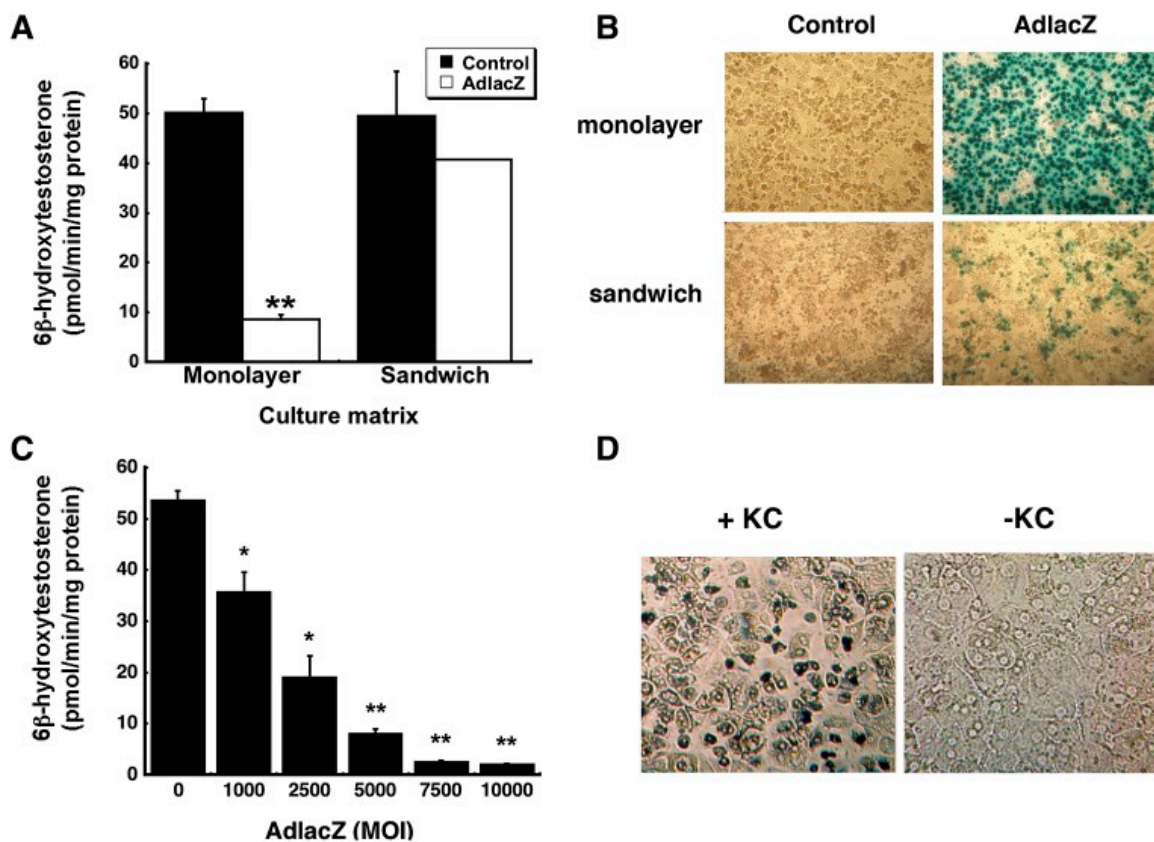


Figure 2.1 Primary hepatocytes isolated from male Sprague-Dawley rats respond to adenovirus infection in a manner similar to that observed *in vivo* when seeded on standard tissue culture dishes. (A) AdlacZ did not significantly alter CYP3A activity of primary hepatocytes sandwiched between a collagen 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. In each panel, cells were seeded at a density of 5×10^5 cells/mm.

2.3.2 Effect of treatment with known inhibitors of CYP3A4

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 *in vitro* and *in vivo* settings. Ketoconazole (10 μ M), a strong CYP3A4 inhibitor [248], suppressed activity of the enzyme by $89.65 \pm 1.2\%$ (Figure 2.2A, $P \leq 0.001$). Erythromycin (100 μ M), a moderate inhibitor of CYP3A4, suppressed activity by $68.1 \pm 1.3\%$, whereas weak inhibitors, isoniazid (100 μ M) and cyclosporine A (4.2 μ M) suppressed activity by $35.6 \pm 10.4\%$ and $40.3 \pm 5.5\%$, 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 μ g/ml) suppressed CYP3A4 activity by $51.2 \pm 3.3\%$, whereas a concentration of 10 μ g/ml suppressed enzyme activity by $92.2 \pm 4.2\%$ (Figure 2.2A). When known CYP3A4 inducers dexamethasone (200 μ M) and phenobarbital (500 μ M) were added to cultures, CYP3A4 activity increased by 230 ± 12.5 and $124 \pm 9.1\%$, respectively ($P \leq 0.001$, Figure 2.3). Adenovirus at 100, 500, and 1000 MOI suppressed CYP3A4 activity in the presence of phenobarbital by 42.8, 71.8, and 76%, respectively. CYP3A4 activity in infected cells treated with dexamethasone followed the same trend (Figure 2.3).

2.3.3 Effect of adenovirus infection on CYP3A activity 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

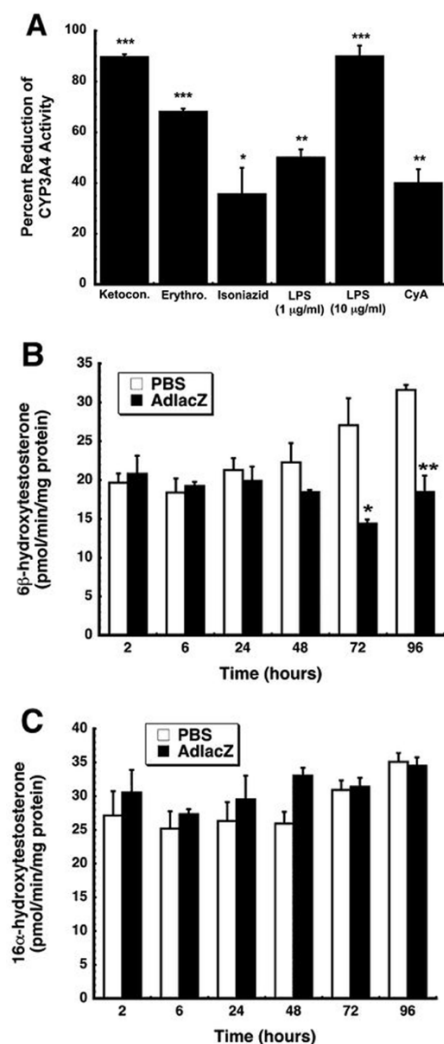


Figure 2.2 HC-04 cells respond to known CYP3A4 suppressors and respond to adenovirus infection in a CYP3A4 isoform-specific manner. (A) Substances known to suppress CYP3A4 activity. Ketocon, ketoconazole; Erythro, erythromycin; LPS, bacterial lipopolysaccharides; CyA, cyclosporine A. (B) Infection with AdlacZ (MOI 500) over time. *In vitro* catalytic activity was measured by incubation of infected cultures with testosterone and quantitation of 6 β -hydroxytestosterone by HPLC. (C) Cells were treated with a CYP2D-specific metabolite and 16 α -hydroxytestosterone was measured by HPLC.

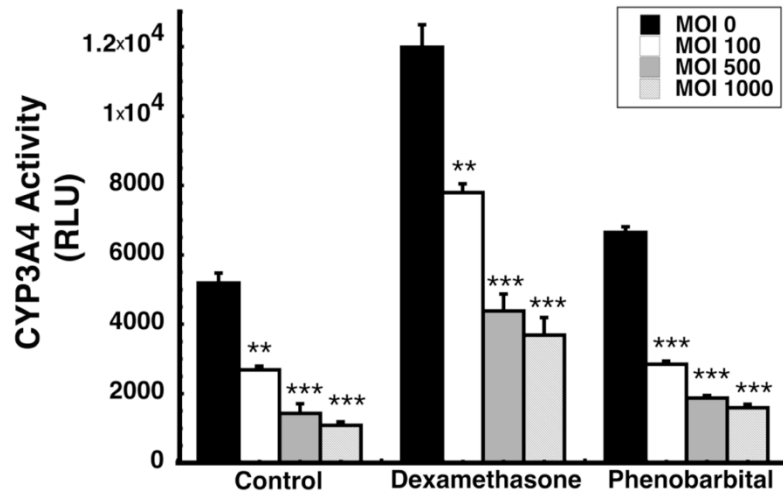


Figure 2.3 Adenovirus-mediated suppression of CYP3A4 activity is not reversed by treatment with compounds known to induce enzymatic activity. Substances known to induce CYP3A4 activity were added to culture media daily over a period of 3 days. Cells were infected with different concentrations of adenovirus for 24 hours. CYP3A4 activity was assessed using a P450-Glo™™ CYP3A4 Luciferin-IPA assay kit according to the manufacturer's instructions. Control cells are those infected with virus in the absence of a chemical CYP3A4 inducer. Results are reported as the mean $\pm$ 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. ** $p \leq 0.01$, *** $p \leq 0.001$.

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 (Figure 2.2B). A slight (~17%) drop in activity was noted 48 hours after infection. CYP3A4 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 16 α -hydroxytestosterone, a testosterone metabolite primarily generated by CYP2D-mediated hydroxylation, was also monitored over the same time period (Figure 2.2C). Concentrations of 16 α -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* [208, 211, 249], 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 (Figure 2.4A, $P \leq 0.001$). 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 (Figure 2.4B), 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 (Figure 2.4C, $P = 0.1$). Histochemical staining revealed

that transgene expression in primary hepatocytes infected with virus at MOI 500 (Figure 2.5B) and MOI 1,000 (Figure 2.5C), 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 (Figure 2.5E and 2.5F).

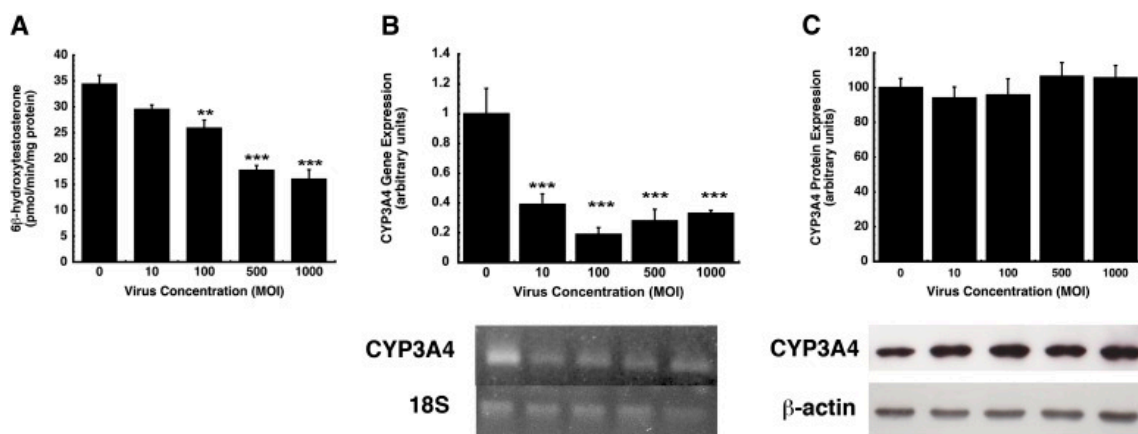


Figure 2.4 Adenovirus infection inhibits CYP3A activity and mRNA levels in HC-04 cells. (A) Catalytic activity of CYP3A in HC-04 cells after virus infection. Activity was measured by quantitation of the CYP3A-specific testosterone metabolite 6β-hydroxytestosterone by HPLC. (B) CYP3A4 expression and representative 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. 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.

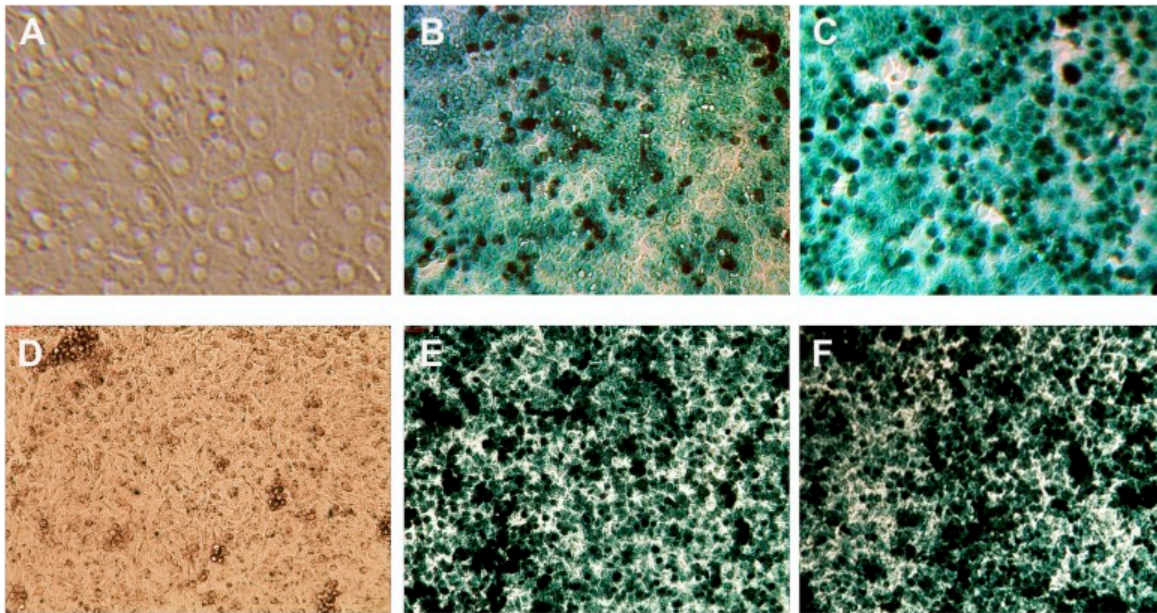


Figure 2.5 Transgene expression patterns in cultured cells 48 hours after infection.

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 that 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).

2.3.4 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 4 α (HNF-4 α) [12]. 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.

2.3.4.1 RXR

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 [250]. When paired with either PXR or CAR it binds to several regions along the CYP3A promoter to drive gene expression [251]. 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 (Figure 2.6A). MOIs of 100 and 500 suppressed RXR in the cytoplasm by 31.6 and 71.7%, respectively (Figure 2.6B)

2.3.4.2 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 [108]. Interaction with a PXR-specific ligand releases PXR and transports it directly to the nucleus where it forms a heterodimer complex with RXR [252]. The RXR-

PXR complex binds to the distal xenobiotic responsive enhancer molecule (dXREM) and drives CYP3A expression in concert with the proximal PXR responsive element [253]. 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 (Figure 2.7). PXR protein levels in nuclear extracts were not significantly affected by infection (Figure 2.7A). In contrast, the virus had a more profound effect on cytoplasmic PXR as protein levels were reduced by 50% after infection with 10 and 100 MOI (Figure 2.7B). A MOI of 500 reduced PXR protein levels in the cytoplasm by 70.2%.

2.3.4.3 CAR

CAR, like PXR, is primarily located in the cytoplasm [254]. Unlike PXR, CAR is constitutively active in the cytosol and can enter the nucleus in the absence of CAR-specific ligands [255]. 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 ($P < 0.01$, Figure 2.8A). CAR also appeared to increase in the cytoplasmic fraction in response to increasing amounts of virus (Figure 2.8B). 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 virus at 500 MOI (arrow, Figure 2.8B).

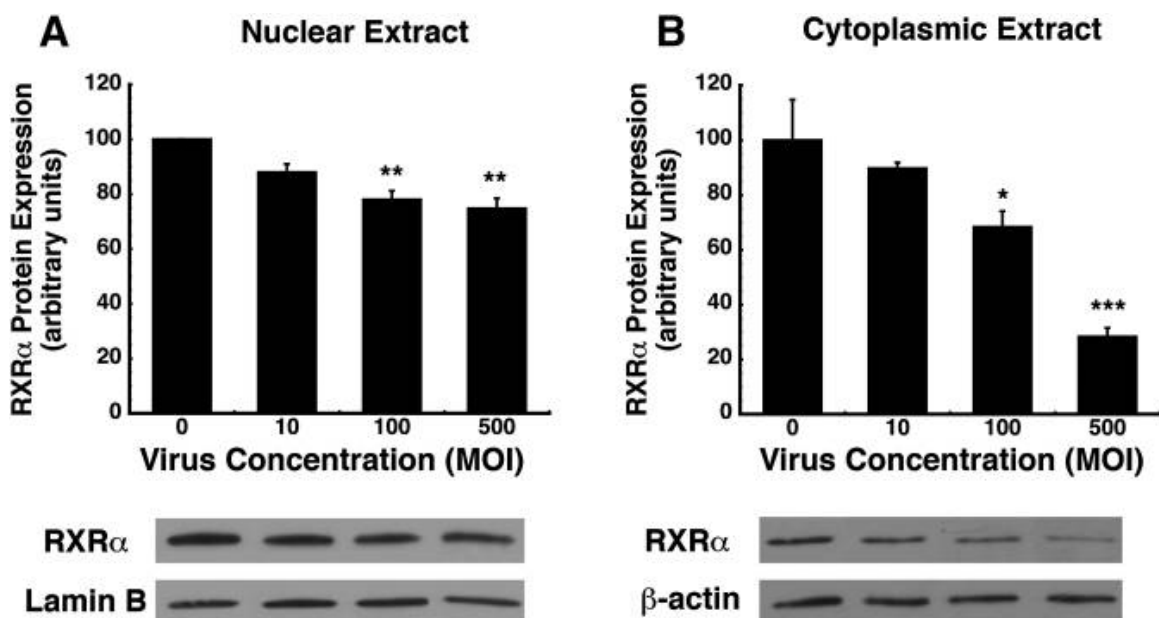


Figure 2.6 Adenovirus infection significantly alters nuclear and cytoplasmic levels of the RXR nuclear receptor 96 hours after infection in HC-04 cells. Immunoblot analysis of nuclear (A) and cytoplasmic (B) extracts for RXR protein expression after infection with a first- generation recombinant adenovirus. 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 $\pm$ 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. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

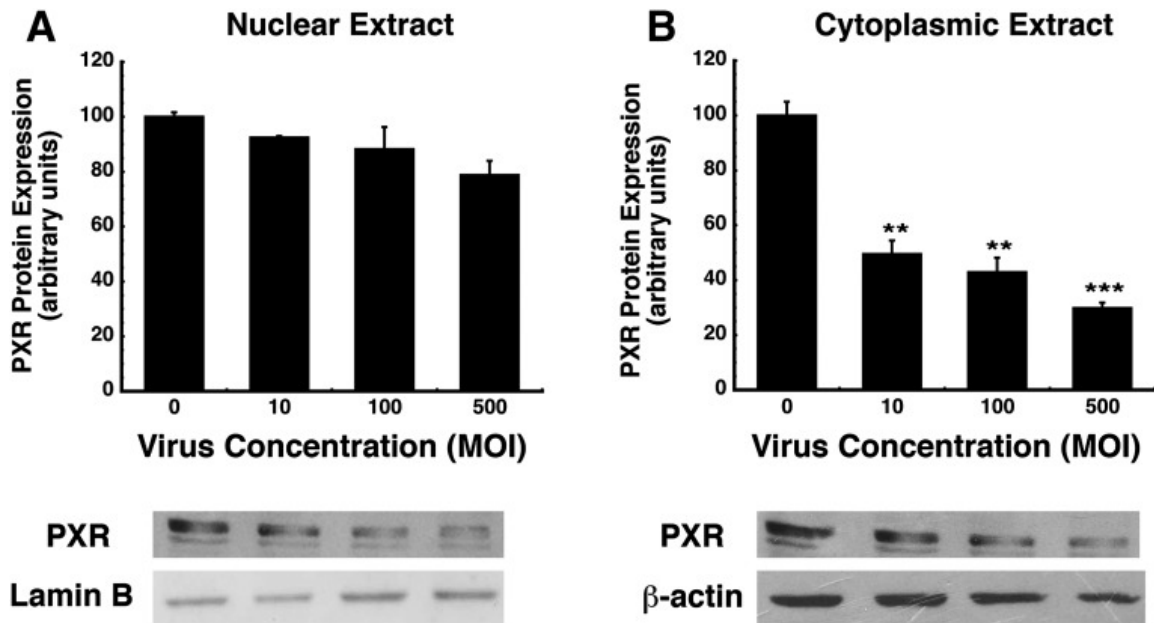


Figure 2.7 Adenovirus infection significantly alters PXR protein levels in the cytoplasm of HC-04 cells. Immunoblot analysis of nuclear (A) and cytoplasmic (B) extracts for PXR protein expression 96 hours after infection with a first-generation recombinant adenovirus (AdlacZ). Representative blots illustrating 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 $\pm$ 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. ** $P \leq 0.01$, and *** $P \leq 0.001$.

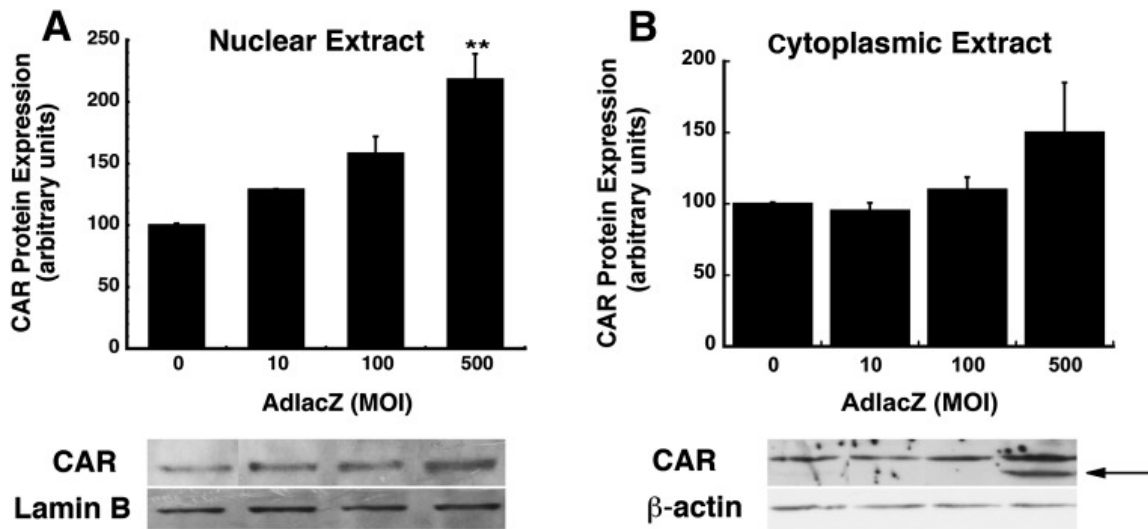


Figure 2.8 Recombinant adenovirus increases CAR in nuclear extracts of HC-04 cells.

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 $\pm$ 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. $**P \leq 0.01$.

2.3.4.4 Coactivators of CYP Expression: HNF-4 α and PGC-1

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 4 α (HNF-4 α), when coactivated by peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), 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 [99]. Because initial western blots of cellular extracts revealed that both HNF-4 α and PGC-1 α were expressed at high levels in HC-04 cells (Figure 2.9, $t = 0$), changes in both of these cofactors were evaluated for 96 hours after infection with 500 MOI of AdlacZ. A general decline in both HNF-4 α and PGC-1 α was noted as early as 24 hours after infection. Within 72 hours, HNF-4 α was reduced by 72% ($P < 0.05$, Figure 2.9A). PGC-1 α was suppressed to a similar degree at the 96-hour time point ($P < 0.05$, Figure 2.9B). The virus had the most significant impact on HNF-4 α expression at the 96-hour time point (80% reduction, $P < 0.05$, Figure 2.9A).

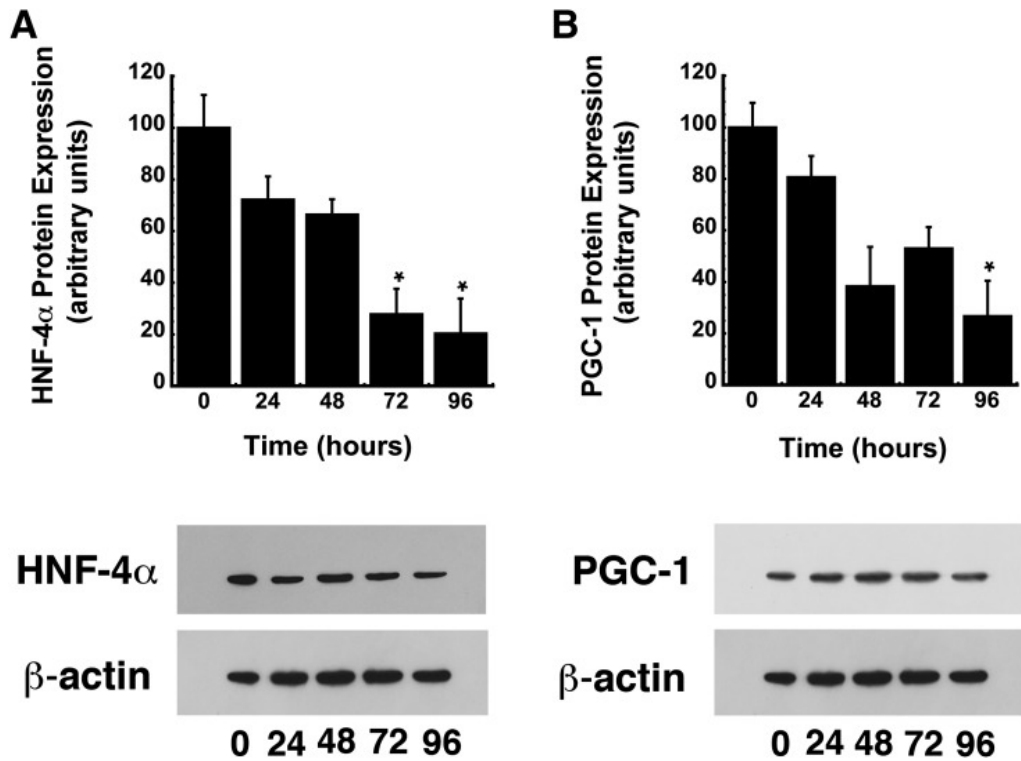


Figure 2.9 Adenovirus infection reduces HNF-4 α and PGC-1 α late in the infection process. Immunoblot analysis of (A) HNF-4 α and (B) PGC-1 after infection with adenovirus for 24-96 hours. Representative blots illustrating band intensity for each time point are shown under each plot. Data are reported as the mean $\pm$ 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 uninfected controls ($t = 0$) by one-way analysis of variance with a Bonferroni/Dunn post hoc test. * $P \leq 0.05$.

2.3.5 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 (Figure 2.10). A concentration of 500 MOI significantly reduced RXR mRNA levels by approximately 30% ($P < 0.05$, Figure 2.10A). PXR mRNA levels were not significantly altered by the virus at any of the concentrations tested ($P = 0.085$, Figure 2.10B). 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 (Figure 2.10C, $P = 0.092$).

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 [256]. In the presence of this compound, different concentrations of virus did not significantly affect CYP3A4 activity (Figure 2.11, $P = 0.078$). Virus at MOIs of 100, 500, and 1000 reduced activity in the absence of the compound by 23, 38, and 48%, respectively (control, Figure 2.11). In contrast, when cells were treated with 20 μ M rifampicin, a known agonist of PXR [256], CYP3A4 activity was reduced by 28, 52.5, and 57.9% after infection with 100, 500, and 1000 MOI virus, respectively.

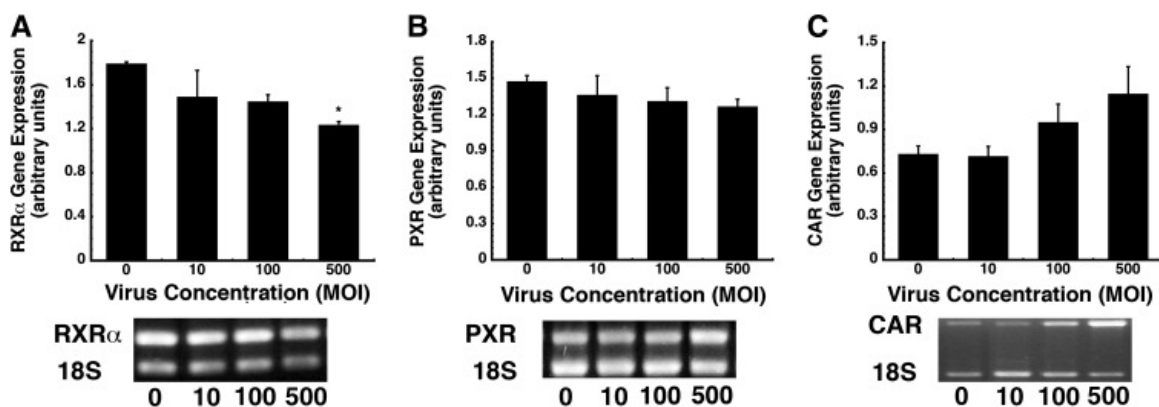


Figure 2.10 Changes in gene expression patterns induced by virus infection can be detected in HC-04 cells. mRNA levels for each nuclear receptor and representative agarose gels from extracts collected 96 hours after infection with adenovirus at MOIs of 0, 10, 100, and 500. Results are reported as the mean $\pm$ 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 uninfected controls (MOI 0) by one-way analysis of variance with a Bonferroni/Dunn post hoc test. * $P \leq 0.05$.

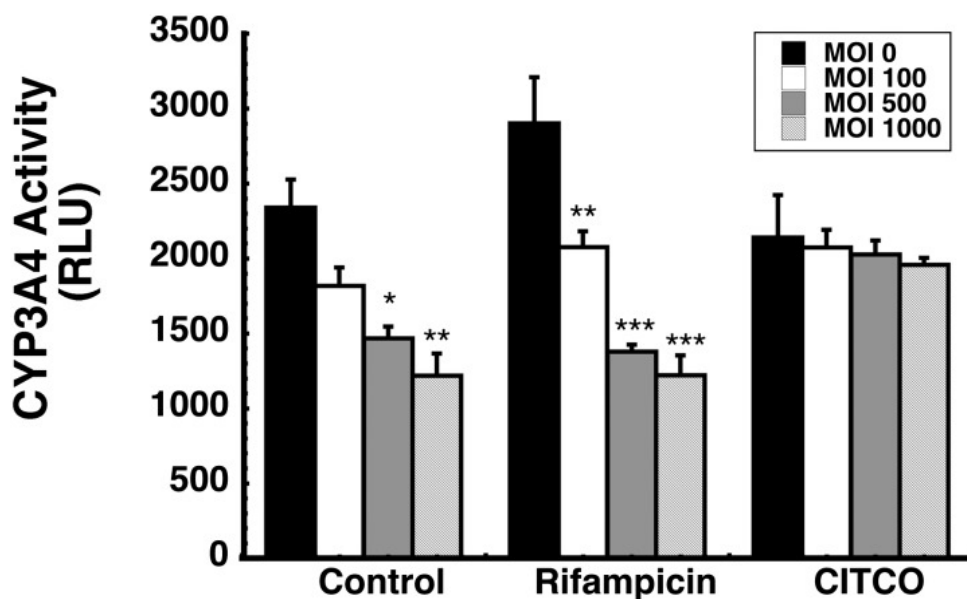


Figure 2.11 Adenovirus overrides the effects of a PXR agonist and suppresses CYP3A activity in HC-04 cells. Stock solutions of rifampicin and CITCO were prepared in DMSO. Compounds were diluted to working concentrations (100 nM CITCO, 20 μ M rifampicin) in complete culture media. The final DMSO concentration in these solutions was 0.1%. Cells were treated with each compound for 48 hours prior to infection with adenovirus. Twenty-four hours after infection, CYP3A4 activity was assessed using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit according to the manufacturer's instructions. Results are reported as the mean $\pm$ standard error of the mean of three culture plates per condition. Statistical significance was determined between individual treatment groups and uninfected controls by one-way analysis of variance with a Bonferroni/Dunn post hoc test. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

2.4 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 [257]. 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 [258]. 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 [259]. 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 [260], have morphology and metabolic activity of differentiated hepatocytes [261]. 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 transcriptional regulators at levels comparable to primary human hepatocytes [262]. They also express several phase II enzymes and drug transporters, respond to selective CYP inducers, and have been used in virology research [263]. 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 [263]. 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* (Figure 2.2). They were also easily transduced in a dose-dependent manner by a recombinant adenovirus (Figure 2.4). 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 [211, 249] 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 (Figure 2.1C and Figure 2.4A). 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 [264]. 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 (Figure 2.2). We also found that expression of key CYP3A regulators, RXR α , PXR, and CAR as well as their coactivators HNF-4 α and PGC-1 α , was altered to some degree during infection (Figures 2.6-9). 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.

From *in vivo* experiments we have determined that using 5.7×10^{12} virus particles/kg of AdlacZ, a dose corresponding to that used in human clinical trials [265], had the most severe impact on CYP3A in the rat. At this dose, approximately 100% of the hepatocytes were transduced by the virus [211]. The total number of hepatocytes in a rat liver is approximately 7×10^8 cells [266], hence using 5.7×10^{12} virus particles/kg of AdlacZ corresponds to an MOI of approximately 2,500. This is a high dose of adenovirus, higher than that given to a patient that died in a gene therapy trial using an adenovirus vector at a dose of 6×10^{11} virus particles/kg, which was administered into the right hepatic artery [267]. Therefore, when conducting the *in vitro* studies an MOI of 500-1,000 was used, since in these studies lower MOIs resulted in a significant suppression of CYP3A. An MOI of 500 corresponds to approximately 1.1×10^{12} virus particles/kg. Recombinant adenoviruses used in gene therapy or as vaccines for disease states such as cancer and human immunodeficiency virus (HIV), have been tested in clinical trials at various

concentrations. In the STEP clinical trial, where an adenovirus-based vaccine against HIV was evaluated, up to 1×10^{11} virus particles were used in repeated intramuscular injections. Several clinical trials using recombinant adenoviruses for the treatment of cancer have used various amounts of adenovirus ranging from 2.5×10^{11} to 7.5×10^{13} virus particles per dose [268]. Hence, the MOIs that have been tested in this study correlate to amounts that have been given to patients. It is therefore clinically important to understand how CYP3A4 is suppressed during a virus infection. Especially as cancer patients take on average 5.5 different prescription drugs a day [193], virus-induced suppression of CYP3A4 could lead to unpredictable drug-drug interactions that can delay, decrease or enhance the absorption of drugs taken concurrently. This can either increase or decrease the pharmaceutical effect of drugs and potentially cause severe adverse effects or toxic drug levels.

Adenovirus infection mildly suppressed RXR α mRNA levels (Figure 2.10A and 2.10D). Although this may contribute to the observed reduction in protein levels of this nuclear receptor in infected cells (Figure 2.6), the stability, turnover, and degradation process of RXR α during adenovirus infection also must be considered. Soon after it was reported that RXR α undergoes ubiquitination and subsequent proteasome-mediated degradation [269], 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 RXR α -mediated processes through this mechanism [270-272]. Considering that recombinant adenoviruses also stimulate these pathways [273] and that other inflammatory mediators induce cytoplasmic relocalization

of RXR α [132], we believe that activation of these pathways during adenovirus infection may play a role in translocation of this nuclear receptor to the cytoplasm, where it is then tagged for posttranslational modification and subsequent degradation. Additional studies using the HC-04 cell line to further dissect the molecular processes associated with RXR α suppression during infection are currently underway.

Of all nuclear receptors investigated, PXR was the most profoundly affected by adenovirus infection (Figure 2.7). Although it is known that nuclear factor κ B, activated by adenovirus infection [273], suppresses CYP3A activity by interfering with the binding of the PXR-RXR heterodimer to the XREM and ER6 regions of the promoter [136], 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 [274], very little is currently known about the degradation process for this key nuclear receptor [275], making additional studies using this cell line warranted.

A marked increase in CAR protein in nuclear fractions of infected cells was also noted (Figure 2.8). Because CAR works in tandem with PXR to modulate the metabolism of xenobiotics, endogenous steroids, and dietary factors [255] and it, unlike PXR, can

translocate to the nucleus without direct binding of ligands [254], we believe this increase to be a compensatory, protective mechanism to maintain CYP activity despite the decline in PXR protein levels during adenovirus 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 adenovirus in non-immune cell types [276, 277]. Given that mRNA levels of PXR were largely unaffected during adenovirus 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 (Figure 2.11).

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 adenovirus infection (Figure 2.10). The presence of a second cross-reactive band on western blots of cytoplasmic fractions suggests that CAR may undergo adenovirus-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 adenoviral 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 [278]. Additional studies to

characterize the status of CAR during adenovirus 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 (Figure 2.2). Initial screens for CYP3A2 protein by western blot of lysates from adenovirus-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 [279]. 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 [280, 281]. 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 [282]. 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 suppressive and inductive 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 [283-285].

CHAPTER THREE

Impact of an Ebola Vaccine and Influenza on Hepatic Cytochrome P450: Effects to be Considered During Management of Immunization Campaigns and Wild Type Infections

3.1 INTRODUCTION

The complex and constantly evolving dynamic of microbial pathogens has allowed once controlled diseases to re-enter the forefront of medical practice while new infections are being reported at an unprecedented rate [286]. Approximately one half of these involve viruses [287]. Although much attention is given to development of technology for pathogen detection and public health policies to respond to widespread disease, the subtle effects of infection on processes that maintain physiological homeostasis, like hepatic metabolism and transport of drugs and endogenous substances, must also be considered. This is of critical importance as, on average, 48.5% of the global population uses at least one prescribed medication [288]. This number increases further with respect to use of over the counter [289] and homeopathic medications [290].

In the United States, approximately two-thirds of the currently approved standard, non-traveler's vaccines are routinely administered within the first years of life to prevent 18 dangerous and deadly communicable diseases [291]. Globally, an estimated 86% of

infants receive three doses of diphtheria-tetanus-pertussis (DTP3) vaccine, 85% receive one dose of the measles vaccine by their second birthday, 46% are immunized with the rubella vaccine and 23% received the rotavirus vaccine [292]. Several of these vaccines, in addition to others, are recommended for secondary administration later in life to reinforce childhood immunization and to prevent infections from pathogens that pose serious threats to an aging immune system [291]. Human influenza infections, both pandemic and seasonal, are a constant opponent to public health due to the seasonal emergence of novel genetic strains and occasional cross-species infections [293, 294]. Serious influenza-related complications, including pneumonia, bronchitis and sinus infections, are often seen during seasonal infections in children under the age of 5 and elderly adults [295]. About 90% of influenza-related mortality occurs in people aged 65 years or older [296]. During the 2009 influenza pandemic, these statistics changed with those under the age of 60 experiencing a higher morbidity and mortality rate from infection [297]. This, coupled with practices to accelerate vaccine development and deploy them to diverse patient populations who may also be taking a variety of medications for unrelated disease states, highlight some unique differences between traditionally scheduled vaccines, those utilized under pandemic and epidemic conditions, and the populations in which they are administered.

The 2014-2016 Ebola outbreak in West Africa, with more than 15,000 laboratory confirmed infections and 11,310 deaths, represents one of the world's most recent and most serious infectious disease emergencies [298]. Three Ebola vaccine platforms currently in clinical testing utilize recombinant adenoviruses expressing the Ebola virus glycoprotein

(E-GP) [299-302]. During our effort to develop a recombinant adenovirus-based vaccine against this deadly pathogen, we evaluated the toxicological and pharmacological profiles of the vaccine [303-307]. Based upon our prior work demonstrating that recombinant adenoviruses significantly alter hepatic drug metabolism in several different animal and human models of infection [207-211, 249], studies evaluating the impact of our vaccine on hepatic drug metabolism were warranted. The cytochrome P450 3A4 enzyme (CYP3A4) was the focus of our studies as it is the most active drug-metabolizing enzyme, it is responsible for metabolizing over 60% of medications currently on the market and it plays an important role in the metabolism of many pharmaceutical and traditional medicines utilized in areas where Ebola outbreaks occur [308, 309]. Additional studies focused on the effect on CYP3A4 by two influenza A subtypes currently in circulation [310], one of which originated in the 2009 influenza pandemic and is currently recommended for inclusion in the 2017-2018 influenza seasonal vaccines [311]. To our knowledge this is the first report describing how vaccines utilized under emergency response plans and their associated virus infections impact hepatic drug metabolism. Given the prevalence of prescription medications in the United States and around the world, subtle changes in hepatic CYP3A4 induced by vaccination or naturally acquired virus infections could underline certain drug-drug interactions, treatment failures due to subtherapeutic levels of medications with narrow therapeutic indexes, and unwarranted toxic adverse effects. Understanding how drug metabolism is altered during immunization and virus infection will improve public health through incorporation of plans for adjustment of therapeutic regimens of those participating in immunization campaigns and those experiencing wild type infections

during infectious disease emergencies now and in the future.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Phosphate-buffered saline (PBS) was purchased from Sigma-Aldrich (St. Louis, MO). Protogel[®] acrylamide was purchased from National Diagnostics (Atlanta, GA). Purified 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.

3.2.2 Recombinant adenovirus production

An E1/E3 deleted recombinant adenovirus serotype 5 vector expressing a codon optimized full-length Ebola virus glycoprotein sequence under the control of the chicken β -actin promoter (Ad-CAGoptZGP, also referred to as AdEBO throughout the remainder of this manuscript; [304]) was amplified in human embryonic kidney (HEK) 293 cells. Cell lysates were purified by banding twice on cesium chloride gradients and desalted on Econo-Pac 10 DG disposable chromatography columns (BioRad, Hercules, CA) equilibrated with sterile 100 mM PBS (pH 7.4) [307]. Positive fractions were collected and the number of virus particles determined by UV spectrophotometric analysis using the

method of Maizel et al. [312] with the following formula: virus particle/ml = (absorbance at 260 nm) $\times$ (dilution factor) $\times 1.1 \times 10^{12}$.

3.2.3 Influenza A viruses

Mouse-adapted influenza A, A/Puerto Rico/8/1934 virus was prepared at the Trudeau Institute by two serial infections of C57BL/6 mice in which naïve mice were nasally challenged with an infective dose of the clinical isolate. Lungs of each animal were then harvested 3 days later, homogenized, pooled and stored at -80°C. Lung homogenate (6.25×10^6 EID₅₀/ml) was then utilized to inoculate the allantoic cavity of 10-day-old embryonated hen's eggs according to standard protocols [313]. Harvested virus stock was determined to be free of mycoplasma and endotoxin and the titer was determined by hemagglutination assay [314]. HC-04 cells were infected with virus stocks generated with pooled allantoic fluid from specific pathogen free (SPF) embryonated chicken eggs infected with either A/Puerto Rico/8/1934 (H1N1; NR-348; BEI Resources, Manassas, VA) or A/Victoria/361/2011 (H3N2; NR-44022; BEI Resources). Infectious titers were determined by a Chicken Embryo Infectious Dose 50 (CEID₅₀) assay in 9-11 day-old SPF embryonated chicken eggs as described by the manufacturer.

3.2.4 Animal studies

All procedures were approved by the Institutional Animal Care and Use Committee

of the University of Texas at Austin and are in accordance with the guidelines established by the National Institutes of Health for humane treatment of animals.

3.2.4.1 AdEBO immunization

Male C57BL/6 mice (6 weeks at time of immunization; Jackson Laboratories, Bar Harbor, ME) were maintained in a 12-hour light/dark cycle environment with free access to standard mouse chow (Harlan, Indianapolis, IN) and deionized water. Following a 7-day acclimation period, mice were immunized intranasally with a dose of 5×10^8 infectious virus particles (IVP) of AdEBO. Twenty-four hours later, animals were euthanized and the liver immediately excised and hepatic microsomal proteins were isolated according to established protocols [211].

3.2.4.2 Influenza A virus infection

Male BALB/c mice (8 weeks at time of infection; Jackson Laboratories) were maintained as described above. Mice were infected with 100,000 CEID₅₀ of influenza A virus, A/Puerto Rico/8-9VMC3/1934 (H1N1; NR-29028) in a total volume of 20 μ l, divided equally between each nostril. Twenty-four hours later, animals were euthanized and the liver immediately excised, snap frozen in liquid nitrogen and stored at -80°C. Hepatic microsomal proteins were isolated as described above.

3.2.5 Cell culture

The HC-04 cell line (MRA-975, MR4, ATCC[®], Manassas, VA), spontaneously

immortalized from normal human liver tissue, is an established *in vitro* model for studying changes in drug metabolism due to virus infection [206, 207]. HC-04 cells retain normal hepatocellular morphology [239] and expresses hepatocellular transcription factors, drug transporters and drug metabolizing enzymes at levels comparable to human primary hepatocytes [240]. HC-04 cells were maintained in Dulbecco's modified Eagle's medium/F12 (1:1 ratio, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, catalog number F0926, Lot number 17A412) and 2 mM L-glutamine (Corning Life Sciences, Tewksbury, MA) during routine maintenance and prior to initialization of experiments. Cells were enzymatically released from tissue culture plates with 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA, Corning Life Sciences) every 2-3 days when a cell density of 90% was achieved in standard 100 mm plates. Cells of passage 4-10 were used in the studies outlined in this manuscript.

3.2.6 CYP3A activity

CYP3A activity was measured for samples collected *in vivo* and *in vitro* using modified protocols for the P450-Glo™ CYP3A Luciferin-IPA assay kit according to the manufacturer's instructions (Promega, Madison, WI).

3.2.6.1 Mouse microsomal protein

Enzymatic activity was assessed in mouse liver by incubating 1 µg of microsomal protein, suspended in 0.01M Tris buffer containing 20% glycerol (pH 7.4), with 8 µM of CYP3A substrate in 100 mM KPO₄ buffer (pH 7.4) for 10 minutes prior to initiating the reaction by adding a NADPH regeneration system (Promega). After 10 minutes of incubation at 37°C, 50 µl of Luciferin detection reagent was added, samples were incubated at room temperature for 20 minutes, and luminescence was determined by a GloMax[®]-Multi Detection System (Promega).

3.2.6.2 HC-04 cells

HC-04 cells were seeded at a density of 20,00 cells/well in a 96-well plate 24 hours prior to infection with either AdEBO at a multiplicity of infection (MOI) of 10-1000 for 72 hours or H1N1 or H3N2 influenza A viruses at a MOI of 50 or 100 for 48 hours. Cells were then washed with PBS and fresh medium containing 3 µM of Luciferin-IPA CYP3A4 substrate added for 60 minutes at 37°C. Analysis was performed as described above. Cell viability assays were performed with the CellTiter-Glo[®] Luminescent assay kit according to the manufacturer's instructions (Promega) and data utilized to normalize CYP3A4 activity.

3.2.6.3 Treatment of HC-04 cells with Ebola virus glycoprotein

HC-04 cells were seeded in 96-wells at a density of 20,000 cells per well and 24-hours later treated with 0.4 mg/ml of purified Ebola virus GP₃₃₋₆₃₇ΔTM-HA [315]. Since

the E-GP carries an HA-tag, cells were also treated with 04 mg/ml of an HA peptide (YPYDVPDYA, GenScript, Piscataway, NJ) to eliminate any effect of HA-tag on CYP3A4 activity. CYP3A4 activity was measured 24, 48, and 72-hours post treatment as described above.

3.2.7 Western blot

Infected and uninfected control HC-04 cells were washed twice with ice-cold PBS before adding 500 µl of ice-cold lysis buffer (radioimmunoprecipitation assay buffer; Thermo Fisher Scientific, Waltham, MA) containing 1% protease inhibitors (HALT Protease and Phosphatase Inhibitor Cocktail; Pierce, Rockford, IL). Cells were then placed on ice for 5 minutes. Cell lysis was then performed by aspirating the cells through a 20-gauge needle (Becton Dickinson, Franklin Lakes, NJ) attached to a 1-ml syringe (Becton Dickinson) 25 times. After remaining on ice for an additional 40 minutes, lysates were cleared by centrifugation at 14,000 *g* for 15 minutes at 4°C and isolated protein stored at -80°C until analysis. Fifty µg of protein was separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked overnight at 4°C in a blocking buffer containing 3% bovine serum albumin (BSA) in 0.05% (v/v) Tween-20 in Tris-buffered saline (TBS-T). Membranes were then incubated for two hours with mouse monoclonal anti-CYP3A4 antibody (1:2,000 dilution, HL3, sc-53850) followed by one hour of incubation with a goat anti-mouse IgG-HRP antibody (1:10,000 dilution, sc-2005,

Santa Cruz Biotechnology). Alternatively, membranes were incubated for two hours with a mouse monoclonal anti-PXR antibody (G-11, sc-48403), polyclonal rabbit anti-RXR α antibody (D20, sc-553), or polyclonal rabbit anti-CAR1/2 antibody (M-127, sc-13065) at a dilution of 1:800 followed by one hour of incubation with the following corresponding secondary antibodies: m-IgG κ BP-HRP (sc-516102) for PXR primary antibody and goat anti-rabbit IgG-HRP antibody (sc-2004; Santa Cruz Biotechnology) for RXR α and CAR primary antibodies, all at a dilution of 1:10,000. Furthermore, to detect total protein levels and phosphorylated levels of eIF4e, membranes were incubated with rabbit anti-eIF4e (#9742) or anti-phospho-eIF4E (Cell Signaling Technology, Danvers, MA, #9741) for 2 hours at a dilution of 1:1,000 followed by one hour of incubation with a goat anti-rabbit IgG-HRP antibody (1:10,000 dilution, sc-2004, Santa Cruz Biotechnology). Immune complexes were detected by chemiluminescence (SuperSignal[™] West Pico Chemiluminescence Substrate, Fisher Scientific) using a G:BOX Chemi XX6 chemiluminescence imaging system (Syngene, Frederick, MD). The intensity of protein bands was quantified, relative to the signals obtained for β -actin (mouse monoclonal anti- β -actin antibody (AC-15, sc-69879, 1:1,000 dilution) with goat anti-mouse IgG-HRP antibody (1:10,000 dilution, sc-2005, Santa Cruz Biotechnology)) run on the same gel, using Kodak 1D image analysis software (Eastman Kodak Co., Rochester, NY).

3.2.8 RNA isolation, RT-PCR and qRT-PCR

Total RNA was isolated using TRIzol[®] reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Isolated RNA (2-5 µg) was reversed transcribed with random hexamers using the SuperScript[®] III first-strand synthesis system (Thermo Fisher Scientific) and a Master Cycler Pro thermal cycler (Eppendorf AG, Hamburg, Germany). Quantification of H1N1 and H3N2 influenza virus genomic, CYP3A4, PXR, RXR α , CAR, and 18S (QuantumRNA[™] 18S internal standards; Thermo Fisher Scientific) mRNA was performed using the SYBR[®] GreenER[™] qPCR SuperMix (Thermo Fisher Scientific) and the ViiA7[™] Real-Time PCR system with the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds (see Table 3.1 for primer sequences). Reaction products from qRT-PCR were confirmed through visualization on a 2% agarose gel containing ethidium bromide. Quantification of adenovirus genomes was determined using primers for the hexon capsid protein (Table 3.1) and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) with a ViiA7[™] Real-Time PCR system under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 41 cycles of 95°C for 15 seconds and 62°C for 60 seconds. The real-time data to determine mRNA levels were analyzed by the comparative C_T method as described previously [316].

Table 3.1 Primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR).

Target gene	Primer	Sequence 5' to 3'	Reference
A/Puerto Rico/8-9VMC3/1934 (H1N1) hemagglutinin	Forward	GTCCGGCATCATCACCTCAA	[317]
	Reverse	ACCGGCAATGGCTCCAAATA	
A/Victoria/361/2011 (H3N2) neuraminidase	Forward	GCTGGTCCAACCCTAAGTCC	[317]
	Reverse	ACCTGAGGTGCCACAAAACA	
CYP3A4	Forward	GATTGACTCTCAGAATTCAAAAGAACTGA	[318]
	Reverse	GGTGAGTGGCCAGTTCATACATAATG	
PXR	Forward	TGCGAGATCACCCGGAAGAC	[154]
	Reverse	ATGGGAGAAGGTAGTGTCAAAGG	
RXR α	Forward	CCT TTC TCG GTC ATC AGC TC	[240]
	Reverse	CTC GCA GCT GTA CAC TCC AT	
CAR	Forward	GCAAGGGTTTCTTCAGGAGAAC	[319]
	Reverse	CTTCACAGCTTCCAGCAAAGG	
Adenovirus serotype 5 hexon	Forward	ACTATATGGACAACGTCAACCCATT	[307]
	Reverse	ACCTTCTGAGGCACCTGGATGT	
	Probe	6FAM-ACCACCGCAATGCTGGCCTGC-TAMRA	

3.2.9 Statistical analysis

Statistical analysis of data was performed using SigmaStat (Systat Software Inc., San Jose, CA). Differences with respect to treatment were calculated by a one-way analysis of variance followed by a Bonferroni/Dunn *post hoc* test. Differences were determined to be significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$).

3.3 RESULTS

3.3.1 Impact of immunization with an adenovirus-based vaccine or mouse-adapted influenza A virus infection on hepatic CYP3A activity in mice

Intranasal immunization of mice using an adenovirus-based vaccine carrying a transgene encoding the Ebola virus glycoprotein (AdEBO) resulted in a significant decrease in hepatic CYP3A activity, 44% compared to uninfected control mice, 24-hours post immunization (Figure 3.1A). Additionally, during a pre-clinical evaluation of a novel adjuvant for use with influenza vaccines, BALB/c mice were challenged intranasally with a dose of 100,000 CEID₅₀ of mouse-adapted influenza A virus (PR8/H1N1). To assess the impact that influenza virus infection alone may have on CYP3A catalytic activity, livers from challenged but not vaccinated animals were collected 24 hours after exposure. Hepatic CYP3A activity in these animals was suppressed by 55% when compared to healthy, uninfected, age-matched controls (Figure 3.1B). These striking results served as

the basis for further investigation regarding how immunization with an adenovirus-based vaccine or influenza A virus infection may impact human CYP3A4 in an established human hepatocyte model.

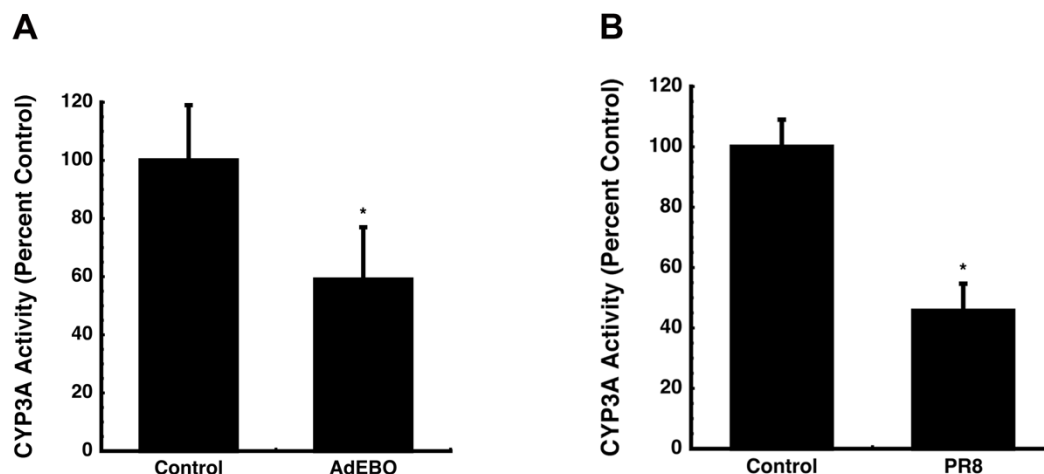


Figure 3.1 Immunization with an adenovirus-based vaccine or infection with a mouse-adapted influenza A virus (PR8/H1N1) significantly suppresses hepatic CYP3A activity. Six-week old C57BL/6 mice were intranasally immunized with an E1/E3 deleted recombinant adenovirus serotype 5 vector expressing a codon optimized full-length Ebola virus glycoprotein sequence under the control of the chicken β -actin promoter (AdEBO; A) and eight-week old BALB/c mice were infected intranasally with 100,000 CEID₅₀ of influenza A virus, A/Puerto Rico/8-9VMC3/1934 (B). Twenty-four hours later, animals were euthanized along with uninfected, healthy age matched mice and livers immediately excised. Following isolation of microsomal proteins, CYP3A catalytic activity was determined using a P450-Glo™ CYP3A Luciferin-IPA assay kit (Promega). Data are reported as the mean $\pm$ standard error of values obtained from three mice for each treatment group. * $p < 0.05$ with respect to uninfected, saline treated mice.

3.3.2 Effect of an adenovirus based-vaccine and influenza A virus infection on human CYP3A4 activity *in vitro*

We have previously shown that CYP3A4 activity is suppressed by adenovirus infection using a first-generation adenovirus containing a beta-galactosidase transgene (AdlacZ) both *in vivo* [208, 211, 249] and *in vitro* [206, 207]. To test if this effect is observed with an adenovirus carrying a different transgene for immunization purposes, HC-04 cells were infected with several different concentrations of an E1/E3 deleted recombinant adenovirus vector expressing a codon-optimized, full-length Ebola virus glycoprotein sequence (Ad-CAGoptZGP or AdEBO). After 72 hours of infection, AdEBO infection was confirmed by qRT-PCR to be 11.2 adenovirus genomes/cell for an MOI of 100. CYP3A4 activity was unaffected by the virus at an MOI of 10 when compared to uninfected controls. However, MOIs of 100, 500 and 1,000 suppressed CYP3A4 activity by 26, 34, and 36 %, respectively (Figure 3.2A). Since the degree by which MOIs of 100 and 1000 suppressed CYP3A4 were not significantly different, remaining experiments were conducted at an MOI of 100 to avoid generating results that could be attributed to cell death associated with use of higher amounts of virus.

The 2016-2017 influenza season has to date 21,970 confirmed cases of out of which over 90% is caused by influenza A viruses (2.5% H1N1, 96.1% H3 subtype) [320]. To understand if the two most prevalent subtypes of influenza A viruses can impact CYP3A4 activity, HC-04 cells were infected with either H1N1 (A/Puerto Rico/8/1934) or H3N2 (A/Victoria/361/2011) influenza A virus at an MOI of 50 and 100 for 48 hours. Infection

of HC-04 cells by H1N1 and H3N2 influenza A virus was confirmed by qRT-PCR to be 1,185 CEID₅₀/cell and 755 CEID₅₀/cell, respectively, for an MOI of 50. Infection with the H1N1 virus almost completely suppressed CYP3A4 activity (91% reduction for an MOI of 50 and 95% for an MOI of 100 with respect to uninfected cells; Figure 3.2B). Similar changes in CYP3A4 activity were observed for the H3N2 subtype (90% reduction, MOI 50, 87% reduction, MOI 100; Figure 3.2C). As for the adenovirus studies, the lowest MOI (MOI of 50) was used for remaining experiments in order to minimize results attributable to virus-mediated cell death.

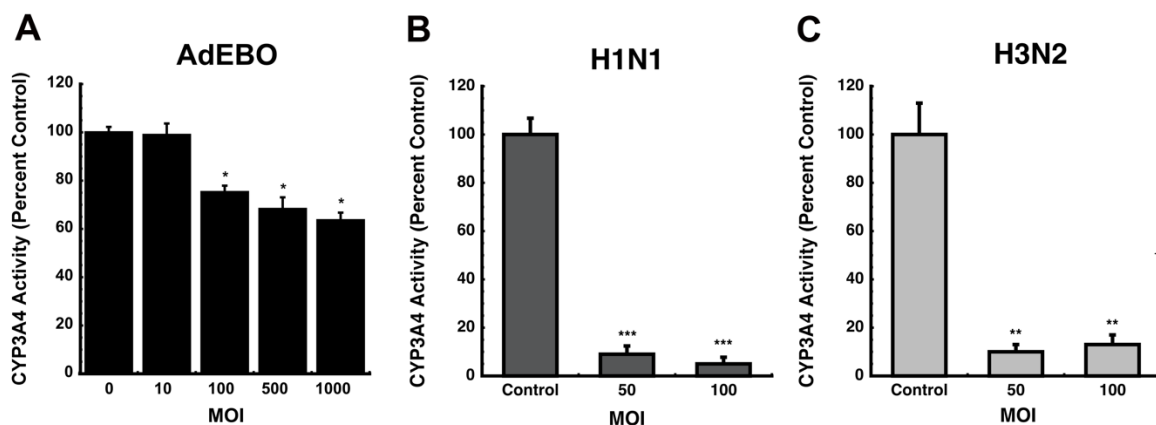


Figure 3.2 An adenovirus-based vaccine and influenza A viruses significantly suppress CYP3A4 activity in HC-04 cells. Catalytic activity of CYP3A4 72 hours after (A) AdEBO infection and 48 hours after (B) H1N1 and (C) H3N2 virus infection. CYP3A4 activity was determined using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit. Results are reported as the ratio of activity readings of infected cells over activity readings of uninfected cells of the same passage number. In each panel, data are reported as the mean $\pm$ standard error of values obtained from three culture plates per condition. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.01$ with respect to uninfected control cells.

3.3.3 Virus-specific changes in CYP3A4 protein and gene expression during infection

To determine if the suppression of CYP3A4 activity is due to virus-induced changes in CYP3A4 mRNA and/or protein levels, HC-04 cells were infected for 72 hours with the AdEBO virus or for 48 hours with the H1N1 or H3N2 virus. Infection with AdEBO did not significantly alter CYP3A4 protein levels (Figure 3.3A, $p = 0.08$) while infection with H1N1 or H3N2 significantly decreased CYP3A4 protein levels by 81% and 98%, respectively (Figure 3.3B). AdEBO infection resulted in a 4.9-fold decrease in CYP3A4 mRNA levels when compared to uninfected controls (Figure 3.3C). Infection with the H1N1 subtype reduced CYP3A4 mRNA levels by ~2 fold while the H3N2 subtype increased CYP3A4 mRNA levels by ~4 fold (Figure 3.3C).

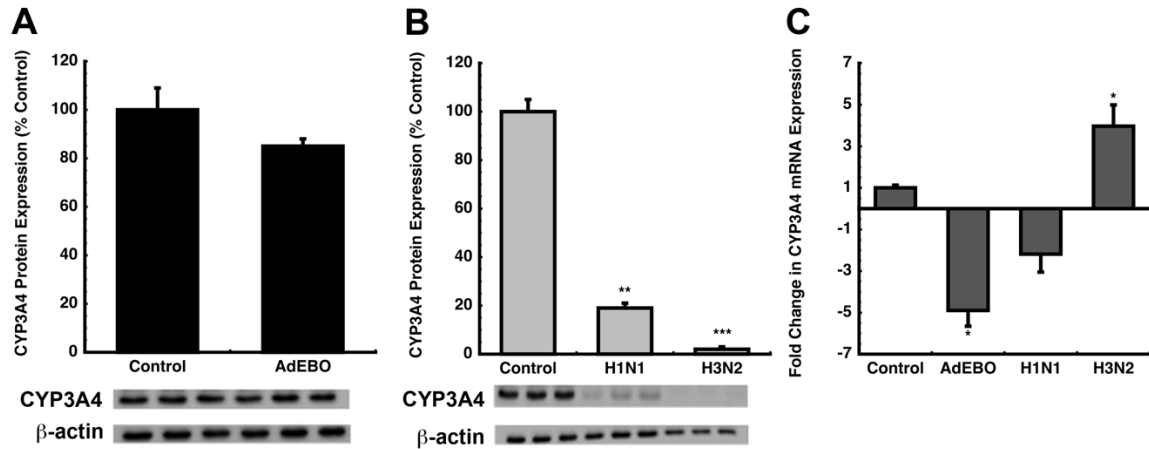


Figure 3.3 Influenza viruses significantly suppress CYP3A4 protein levels. CYP3A4 protein levels in (A) AdEBO infected HC-04 cells 72 hours after infection and (B) H1N1 or H3N2-infected cells 48 hours after infection. Representative blots illustrating band intensity for each treatment condition is shown under plots in the following loading order: (A) lanes 1-3 uninfected control, 4-6 AdEBO or (B) lanes 1-3 control, 4-6 H1N1 and 7-9 H3N2. (C) CYP3A4 mRNA levels 72 hours after infection with AdEBO or 48 hours after infection with H1N1 or H3N2 viruses. In all panels (A-D), data are reported as the mean $\pm$ standard error of values obtained from three culture plates per condition. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.01$ with respect to uninfected control cells, Bonferroni Dunn post hoc test.

3.3.4 Influence of adenovirus infection on nuclear receptors CAR, PXR and RXR α *in vitro*

Hepatic CYP3A4 is constitutively expressed on a transcriptional level and is predominantly regulated by several nuclear receptors, including constitutive androstane receptor (CAR), pregnant X receptor (PXR), and retinoid X receptor alpha (RXR α) [12]. In an effort to determine if AdEBO infection impacts these key regulators of CYP3A4, protein and gene expression levels were determined for each nuclear receptor 72-hours post infection. RXR α protein levels were decreased by 34% with respect to uninfected cells (Figure 3.4A). A similar decrease in protein levels, 45%, was also seen for PXR (Figure 3.4B). CAR protein levels were reduced by 16% by this virus (Figure 3.4C). AdEBO infection suppressed RXR α mRNA levels 4-fold. In sharp contrast, a 4.5-fold increase was seen in PXR gene expression while CAR expression was unaffected by the virus (Figure 3.4D).

3.3.5 Effect of influenza A H1N1 and H3N2 virus infection on nuclear receptors regulating CYP3A4 *in vitro*

Changes in mRNA and protein levels of nuclear receptors regulating CYP3A4 was determined 48-hours post infection with either H1N1 or H3N2 virus. Both viruses significantly reduced RXR α protein levels by over 60% compared to uninfected cells (Figure 3.5A). PXR protein levels were reduced by 51% during H1N1 virus infection and H3N2 infection had an even higher decrease in PXR protein levels with an 88% reduction

(Figure 3.5B). In contrast, CAR protein levels doubled in cells infected with H1N1 virus and H3N2 did not alter CAR protein levels (Figure 3.5C). Gene expression of RXR α was reduced 4-fold by the H1N1 virus and 3.1-fold by the H3N2 virus (Figure 3.5D). PXR mRNA levels were reduced 12.8-fold by H1N1 infection and by 3.4-fold by H3N2 infection (Figure 3.5D). Infection with the H1N1 virus reduced CAR mRNA levels by a factor of 4 while infection with the H3N2 virus did not have any effect (Figure 3.5D).

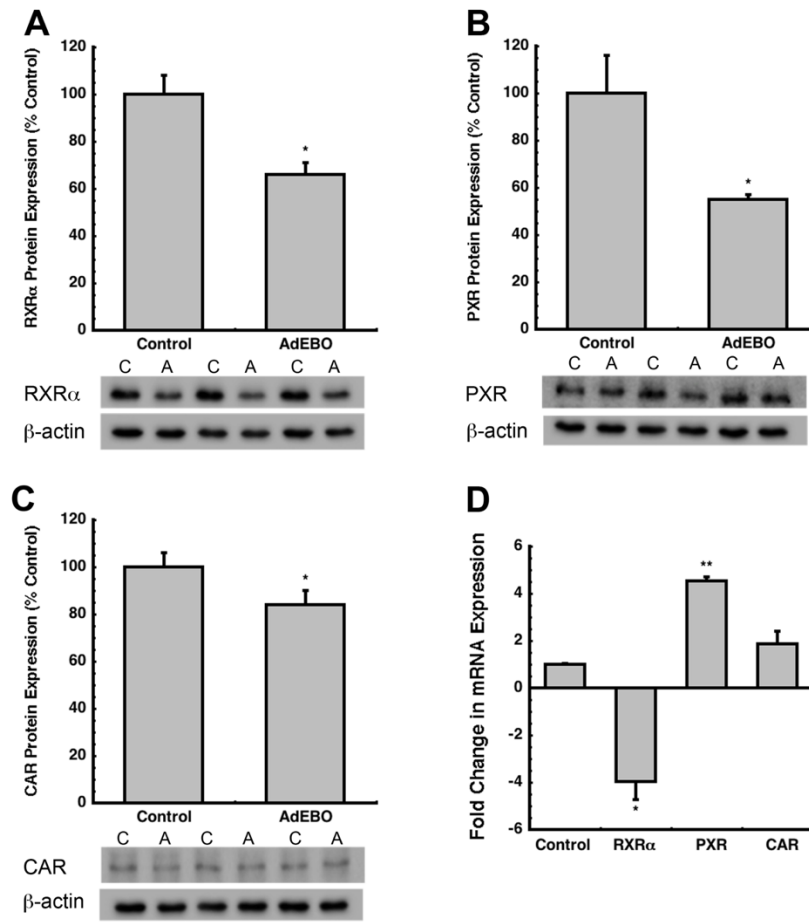


Figure 3.4 AdEBO infection has opposing effect on protein and mRNA levels of two key regulators of CYP3A4 expression. Seventy-two hours after treatment with 100 MOI of AdEBO, cell lysates were collected and analyzed for (A) RXR α , (B) PXR and (C) CAR protein content by western blot. Representative blots illustrating band intensity for each treatment condition is shown under plots in panels (A-C) with the following loading order: lanes 1, 3 and 5 uninfected control and 2, 4 and 6 AdEBO infected cells. (D) mRNA levels of nuclear receptors in infected cells with respect to uninfected cells (control) as determined by qRT-PCR.

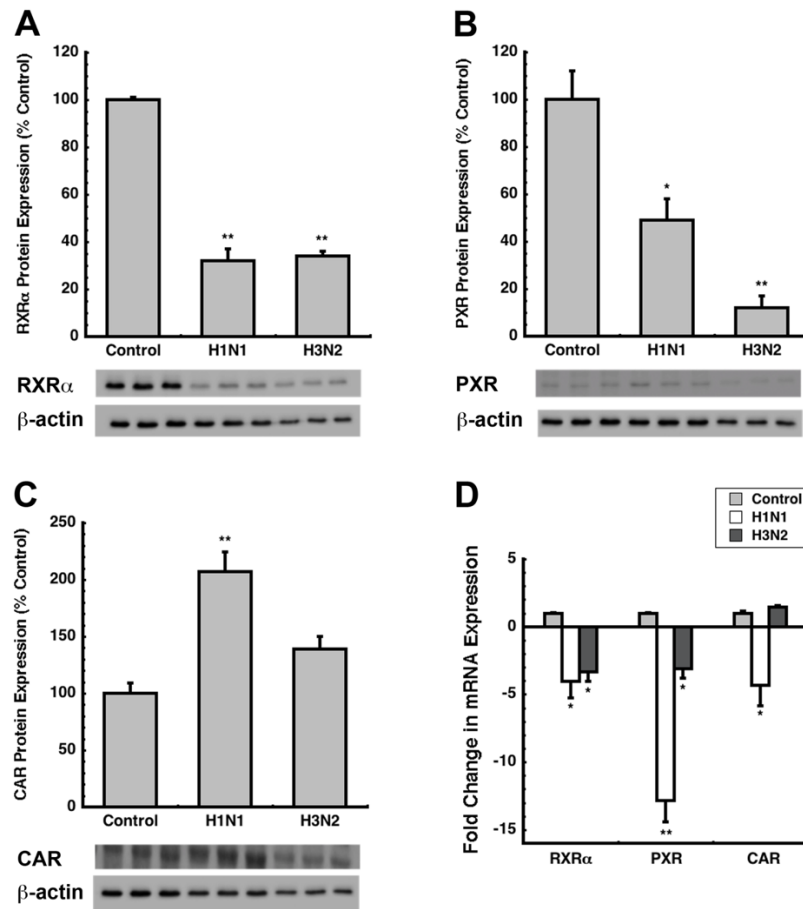


Figure 3.5 Infection with influenza A viruses significantly alters mRNA and protein expression patterns of nuclear receptors involved in the regulation of human hepatic CYP3A4. HC-04 cell lysates were collected 48 hours after infection with 50 MOI of either H1N1 or H3N2 virus and assessed for protein levels of nuclear receptors: (A) RXR α , (B) PXR and (C) CAR by western blot. Representative blots illustrating band intensity for each treatment condition is shown under each plot in panels A-C with the following loading order: lanes 1-3 control, 4-6 H1N1, 7-9 H3N2. (D) mRNA expression patterns for each nuclear receptor as determined from cellular extracts harvested 48 hours after influenza infection by qRT-PCR.

3.3.6 Impact on CYP3A4 activity by the Ebola virus glycoprotein *in vitro*

As the AdEBO vaccine carries a transgene that expresses E-GP, changes in CYP3A4 activity due to E-GP was assessed. In a preliminary study, HC-04 cells were treated with 0.4 mg/ml of E-GP and after 24 hours of treatment CYP3A4 activity was unchanged compared to untreated cells (Figure 3.6). A significant increase in CYP3A4 activity was detected 48 hours after treatment with the E-GP (increase of 5158% when compared to untreated cells) and increased CYP3A4 activity was also detected at 72-hours post treatment with E-GP but at a much lower level (204% increase compared to untreated control: Figure 3.6).

3.3.7 Effect of influenza A viruses on host translational synthesis *in vitro*

Since mRNA and protein levels did not always correlate after infection with either H1N1 or H3N2 virus, changes in host translation during influenza virus infection was determined. The eukaryotic translation initiation factor 4E (eIF4E), a rate-limiting determinant of protein synthesis, recognizes the 7-methylguanosine cap structure of the 5' end of mRNA and initiates recruitment of ribosomes [321]. Increased phosphorylation of eIF4e at Ser209 is associated with increased host translation and decreased levels with inhibition of host translation [322]. Protein levels of eIF4E and P-eIF4E were determined in the HC-04 cell line after 48 hours of infection with either H1N1 or H3N2 virus. Compared to uninfected cells only 30% of eiF4E was phosphorylated in cells infected with

H1N1 and 44% in cells infected with H3N2 (Figure 3.7). A general decrease in eIF4E was also seen in the influenza A virus infected cells compared to uninfected cells (Figure 3.7).

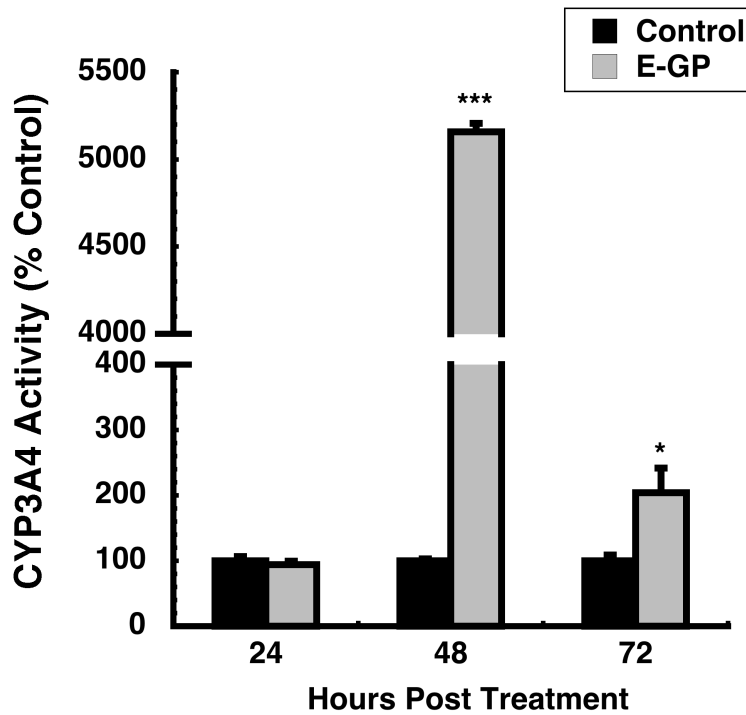


Figure 3.6 Hepatic CYP3A4 activity is significantly induced in human hepatocytes 48 and 72 hours after treatment with Ebola virus glycoprotein. HC-04 cells were treated with 0.4 mg/ml of purified Ebola virus GP₃₃₋₆₃₇ΔTM-HA and CYP3A4 activity was measured after 24, 48, and 72 hours using P450-Glo™ CYP3A Luciferin-IPA assay kit (Promega). Since the E-GP carries an HA-tag, HC-04 cells were also treated with 0.4 mg/ml of HA peptide to eliminate any potential effect on CYP3A4 activity. CYP3A4 activity was normalized against cell viability (CellTiter-Glo® Luminescent assay kit, Promega). Results are reported as the ratio of activity readings of infected cells over activity readings of uninfected cells of the same passage number. Data are reported as the mean ± standard error of values obtained from three wells per condition. * $p < 0.05$ and *** $p < 0.01$ with respect to untreated control cells.

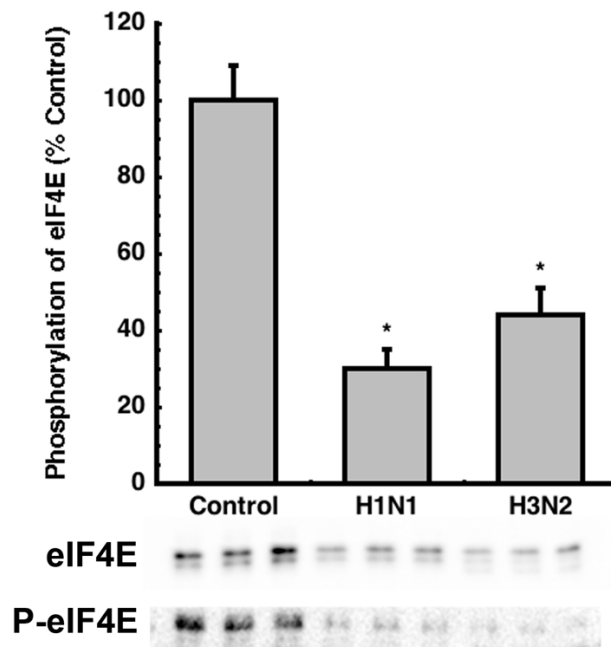


Figure 3.7 Influenza A virus infection inhibits host translational machinery via eIF4E in human hepatocytes. HC-04 cells were infected with either H1N1 or H3N2 influenza virus 48 hours before protein lysates were collected. Two different antibodies were used, one that detects eIF4E and one that specifically detects phosphorylated eIF4E at Ser209. Representative blots illustrating band intensity for each treatment condition is shown under the plot in the following loading order: lanes 1-3 uninfected control, lanes 4-6 H1N1-infected cells and lanes 7-9 H3N2-infected cells. Band intensities were measured using Kodak 1D image software and the ratio of phosphorylated eIF4E over total eIF4E was calculated. The control cells were set to 100% and each of the treatment groups were compared to this value. Data are reported as the mean $\pm$ standard error of values obtained from three culture plates per condition. * $p < 0.05$ with respect to uninfected control cells using Bonferroni Dunn post hoc test.

3.4 DISCUSSION

Despite advancement in immunization protocols, sanitation, and antimicrobial agents, viral infections are still increasing. This is highlighted in the emergence of the 2009 H1N1 influenza pandemic, caused by an H1N1 virus with a unique combination of influenza virus genes never previously identified in either animals or humans [323], as well as the reemergence of the Ebola virus in the 2014-2016 outbreak in West Africa [324] with several cases detected in other regions, including the United States [325]. Both of these outbreaks emphasized the need for effective vaccines. Exposure to infectious agents is expected to increase due to use of viruses as medicinal agents for gene therapy and vaccine applications. Since virus infections are unavoidable it is crucial to understand how virus infections impact drug metabolism, so that adjustments can be made in drug regimens for patients with viral infections taking pharmaceuticals, to avoid potential drug-drug interactions, subtherapeutic or toxic drug levels.

Adenovirus vectors administrated systemically will be cleared from the bloodstream and accumulate in the liver within minutes. Virus enter the liver via the portal vein, pass through the liver sinusoids and contact hepatocytes [326]. *In vitro*, adenovirus vectors infect cells in a two-step process, first by a high-affinity binding between the adenovirus fiber knob and the coxsackievirus-adenovirus receptor (Ad-CAR) [327] followed by binding of the viral penton arginine-glycine-aspartic acid (RGD) motifs to cellular integrins, which triggers viral internalization [328]. We have previously shown

that adenovirus infection suppresses CYP3A *in vivo* [208, 209, 211] and *in vitro* [207] using other transgenes, inactivated virus or wild-type virus. Here we show that a recombinant adenovirus-based vaccine carrying the gene for E-GP significantly suppresses CYP3A activity in mice by 44% compared to saline-treated mice (Figure 3.1A). Infecting a human hepatocyte cell line, HC-04 cells, with the same virus resulted in a dose dependent suppression of CYP3A4 activity (Figure 3.2A). Hence it is important to establish the effect of the virus itself on drug metabolism but also the transgene it is carrying.

To determine if the transgene, here E-GP, has any effect on CYP3A4 activity, HC-04 cells were treated with 0.4 mg/ml of purified E-GP for 24-72 hours in a preliminary study (Figure 3.6). The amount of E-GP used did not cause cell toxicity (data not shown). The concentration of 0.4 mg/ml is representative of the amount produced by AdEBO in cells, as large amounts of E-GP is produced with a peak production at 48 hours post-infection [304]. At 24-hours CYP3A4 activity was unaffected by the E-GP but at 48 hours a 5158% increase in CYP3A4 activity was observed and at 72 hours CYP3A4 activity was elevated by 204% compared to untreated cells (Figure 3.6). This is preliminary data using HC-04 cells, hence additional experiments *in vivo* are needed to confirm that the observed changes are not something unique to the cell line used. We have previously seen that transgene expression varies over time and it is also dependent on the transgene itself [209], suggesting that a large production of the transgene at 48 hours results in a significant induction of CYP3A4 activity. The suppression of CYP3A4 observed *in vivo* and *in vitro* by AdEBO is not as drastic as previously observed for a first-generation adenovirus containing a beta-galactosidase transgene (AdlacZ) [207-211, 249], suggesting that the transgene

plays an important role in CYP3A4 regulation. E-GP binds to $\alpha_5\beta_1$ -integrin receptors, which are required to promote virus penetration within an endocytic organelle by regulating endosomal cathepsins [329]. Additionally, E-GP has been found to downregulate β_1 integrins [330], which could explain the increase in CYP3A4 activity as integrins have been shown to be involved in hepatic CYP3A4 regulation [206]. Hence, overexpression of E-GP and its interaction with integrins may counteract adenovirus-induced suppression seen with other transgenes. Furthermore, we have previously shown that using a recombinant adenovirus expressing a transgene for erythropoietin [209], a glycoprotein hormone that promotes the formation of red blood cells, does not alter CYP3A activity. This suggests that the transgene plays an important role in aberrations seen in CYP3A4 expression and function and that glycoproteins seem to have an opposing effect compared to the adenovirus. This is important as several vaccine candidates currently in clinical trial for Ebola virus disease are carrying similar transgenes for the E-GP [331]. During Ebola virus disease, the tissue factor (TF) plays a key role in disseminated intravascular coagulation [332]. An excess of heme present in the blood has been associated with TF-induced hemolytic anemia [333], which could be due to the E-GP. Since CYP3A4 is a heme-based protein, the induction in CYP3A4 activity could be due to increased heme levels. Interestingly, erythropoietin has also been found to regulate heme-containing metabolic proteins [334]. This implies that heme could be an important factor in CYP3A4 regulation. Further experiments are warranted to elucidate the role of heme and the coagulation cascade in connection to different transgenes known to affect coagulation cascades to determine involvement of heme in CYP3A4 regulation.

Influenza virus infection in humans has been associated with mild hepatitis as implicated by elevation in serum aminotransaminase levels [335, 336], which is an indicator for liver injury [337]. In a murine study, using naïve mice infected with an H3N2 virus resulted in elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in 100% of the animals [338]. Here we show for the first time that infection of mice with a mouse-adapted PR8 influenza A virus (H1N1) significantly suppresses hepatic CYP3A activity 24-hours post infection (Figure 3.1B). We also show that infection of hepatocytes *in vitro* with either H1N1 or H3N2 virus significantly suppresses the catalytic activity of CYP3A4 *in vitro* by 91% and 90%, respectively (Figure 3.2B and 3.2C). We confirmed the presence of influenza virus in hepatocytes by qRT-PCR, 1185 CEID₅₀/cell for H1N1 and 755 CEID₅₀/cell for H3N2, which showed that influenza viruses infect hepatocytes. Influenza viruses infect ciliated epithelium cells of the conductive airways and infection in humans and monkeys show that influenza viruses infect several different types of airway epithelial cells [339]. Cellular attachment and entry is achieved by binding of the viral hemagglutinin (HA) protein to the sialic acid group of cellular surface glycoproteins. Multivalent attachment of influenza viruses to sialic acid triggers endocytosis of the virus that is contained in the endosome [340]. Human influenza viruses bind to α 2,6-linked sialic acid [341] and after initial infection forms continuous foci of infected cells, including nonciliated and ciliated cells, suggesting that at high local concentrations of the virus either cell type can be infected [342]. α 2,6-linked sialic acid is expressed on the surface of the liver [343] which provides an initial binding receptor for

influenza viruses. Also, it has been shown that binding of influenza virus to α 2,6-linked sialic acid requires host fibronectin [341], which is also expressed on hepatocytes [344]. The abundance of α 2,6-linked sialic acid and fibronectin on hepatocytes provides the necessary components for the influenza virus to infect human hepatocytes as seen in these experiments.

The influenza vaccine has been reported to alter the pharmacokinetic properties of CYP enzymes for several drugs [345]. Although a study in humans showed no significant change in CYP3A4 activity seven days after influenza vaccination using erythromycin breath tests (ERMBT) [346], ERMBT has two shortcomings; there is a lack of high correlation with other CYP3A4 probes and erythromycin is a substrate for P-glycoprotein [347, 348]. Hence even though no significant suppression was observed in this study, other probes like midazolam, might be able to detect changes in CYP3A4 activity. In another study, an age correlated suppression in CYP3A4 activity was observed after influenza vaccination, with older age correlating to less CYP3A4 catalytic activity [349]. Our study showed a significant suppression of CYP3A4 catalytic activity 24-hours post infection *in vivo* (Figure 3.1B) and 48-hours post infection *in vitro* (Figure 3.2B and 3.2C). This indicates that there is an early effect on CYP3A4 activity caused by influenza infection that might last for an extended time but for the influenza vaccine this effect is eliminated by day seven in humans. With the elderly population having a greater risk of CYP3A4 suppression, and being more likely to be taking multiple medications, many of which could have narrow therapeutic indices, it is critical to understand how either an influenza

infection or the influenza vaccine affects this important enzyme. It is also important to understand to what extent CYP3A4 activity is affected by virus infection, hence time-based experiments are currently underway.

Protein levels of CYP3A4 were almost eliminated in cells infected with H1N1 or H3N3 virus (81% and 98%; Figure 3.3B and 3.3C) while no significant difference was seen for AdEBO (Figure 3.3A). The phenomenon of suppression of CYP3A4 catalytic activity accompanied by no change in protein levels has been observed previously *in vitro* for a first-generation adenovirus containing a beta-galactosidase transgene (AdlacZ) [207]. Activation of various cell signal transduction pathways by adenovirus could lead to posttranslational modifications of CYP3A4, including phosphorylation, ubiquitination, SUMOylation and/or acetylation, inactivating the protein while protein levels remain unchanged. Gene expression levels of CYP3A4 was decreased almost 5-fold in cells infected in CYP3A4 (Figure 3.3C), indicating that adenovirus infection impacts CYP3A4 on a transcriptional level. In cells infected with H1N1 virus, a small reduction was observed, while H3N2 virus infection resulted in an almost 4-fold increase (Figure 3.3C). The increase in CYP3A4 mRNA expression could be due to a stress response by the infected cells to the drastic reduction of CYP3A4 protein cells infected with H3N2 virus.

To understand how CYP3A4 activity is suppressed by viral infection, we evaluated each of the nuclear receptors that are involved in CYP3A4 regulation. CYP3A4 is constitutively expressed on a transcriptional level but is also regulated by nuclear receptors

including pregnant X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptor alpha (RXR α). PXR is a 50-kDa protein that is primarily expressed in the liver and intestine that is often referred to as the “master regulator” of CYP3A4 [12]. PXR can bind to a wide range of ligands due to ability of the ligand-binding cavity to change shape, expand in volume or adopt unique conformational structures. Found in the cytoplasm, PXR is transported into the nucleus after interaction with a PXR-specific ligand [252]. In the nucleus PXR forms a heterodimer complex with RXR α , which binds to the distal xenobiotic responsive enhancer molecule (dXREM), and together with the proximal PXR responsible element drive CYP3A4 expression [253]. RXR α is a 50-kDa protein that is located in the nucleus where it can function as either a homodimer or as a heterodimeric partner required for DNA binding of several nuclear receptors [250]. Paired together with PXR or constitutive androstane receptor (CAR) RXR α binds to several regions of the CYP3A4 promotor driving gene expression [251]. CAR is primarily located in the cytoplasm [350] where it is constitutively active. It can enter the nucleus either in the absence of CAR-specific ligands or after binding of a specific ligand [255] where it forms a heterodimer pair with RXR α to regulate CYP3A4 gene expression [251].

PXR protein levels were suppressed by all three viruses while gene expression was decreased by both influenza viruses but increased by adenovirus infection (Figure 3.4 and 3.5). In a previous study, infecting HC-04 cells with AdlacZ, PXR gene expression was unaffected by virus infection [207], suggesting that the transgene, here glycoprotein, might

be involved in the impact on PXR. RXR α gene and protein levels were decreased by all three viruses, suggesting that this important heterodimer partner for CAR and PXR, might be a crucial player in the suppression of CYP3A4 (Figures 3.4 and 3.5). CAR protein levels were decreased in cells infected by AdEBO, unaffected by H3N3 infection and increased by H1N1 infection. Gene expression of CAR was unaffected by AdEBO and H3N2 but increased by H1N1 virus infection (Figure 3.4 and 3.5).

Discrepancies between observed mRNA levels and protein expression together with the almost complete elimination of CYP3A4 protein levels, with only 19% and 2% remaining 48 hours after H1N1 and H3N2 infection respectively, is most likely be due to the ability of negative-strand RNA viruses to shut off host cellular protein synthesis [351]. Cellular mRNAs possess a 7-methyl guanine structure (cap) at their 5' ends, which is a critical requirement for recruitment of the ribosome. This cap structure is recognized by the cytosolic cap binding protein eIF4E, a rate-limiting enzyme in protein synthesis [352]. Phosphorylation of eIF4E is associated with increased host translation and decreased levels with inhibition of host translation [321]. At 48-hours post infection, phosphorylation of eIF4E was decreased by 70% and 56% by H1N1 and H3N2 viruses, respectively, compared to uninfected control cells (Figure 3.7). Translation of viral genes proceed independently of a fully activated eIF4E [353], indicating that although host mRNA is available for translation, influenza virus infection caused a reduction in phosphorylation of eIF4E, that resulted in host cellular mRNA not becoming translated. Not only was there a difference in the phosphorylation state of eIF4E in the influenza infected cells, but at 48 hours post

infection, the overall protein levels of eIF4E were significantly reduced compared to uninfected control cells. These findings suggest that the shut off of host translation was already implemented at 48 hours post infection.

These studies show that adenovirus and influenza infection have a significant impact on CYP3A4 catalytic activity. This knowledge is important as the seasonal influenza virus infects millions of people annually, influenza vaccines are administered yearly, and several adenovirus-based vaccines will soon be available on the market. Understanding how the metabolism of drugs is changed during a virus infection, either by a natural infection or by vaccines, could help improve how drug regimens are planned to avoid toxic or subtherapeutic drug levels for patients.

CHAPTER FOUR

Integrin Receptors Play a Key Role in the Regulation of Hepatic CYP3A³

4.1 INTRODUCTION

The CYP3A enzyme family is unique in its capability of metabolizing a variety of compounds that span 38 different therapeutic indications in addition to natural biomolecules (steroids, cholesterol), toxins and carcinogens [1]. The performance of this rather diverse group of enzymes is also highly sensitive to microbial infection [12, 232]. While the primary paradigm of the field attributed this effect to the production of interferons, cytokines and chemokines [233], we found that the expression and function of hepatic CYP3A2 in the rat is suppressed for 14 days after a single dose of a recombinant adenovirus, long after these inflammatory mediators dissipate [211]. Additional studies revealed that reducing the immunogenicity of the virus by chemical and physical means did not mitigate this effect [208]. These findings, coupled with a clinical report detailing dramatic changes in hepatic CYP3A in response to cytokines produced during active infection while minimal changes were found in patients given exogenous cytokines in the

³ Large portions of this chapter have been previously published.

Jonsson-Schmunk K, Wonganan P, Choi JH, Callahan SM, Croyle MA. Integrin receptors play a key role in the regulation of hepatic CYP3A. *Drug Metab Dispos.* 2016; 44: 758-770.

Contribution KJS: Planned and performed experiments, analyzed data, and wrote the manuscript.

absence of infection [354], suggest that the presence of the microbial pathogen itself plays a role in the process that alters CYP during infection.

In an effort to identify the underlying mechanism by which virus infection alters CYP3A expression and function, gene expression patterns in the liver of animals given a single dose of different live and inactivated recombinant adenoviruses were analyzed over time using signal transduction microarrays (Figure 4.1, Table 4.1). Global evaluation of data identified 5 pathways that were consistently upregulated for 14 days after infection. The nuclear factor kappa B (NFκB) pathway was the most profoundly affected by all viruses when compared to saline treated controls. Other pathways upregulated by the presence of virus (in descending order of intensity) were: the phosphoinositide 3-kinase (PI3K), Janus kinase/signal transducers and activators of transcription (Jak-Stat), protein kinase C (PKC) and retinoic acid pathways. Each of these pathways has been documented to alter the expression and function of hepatic CYP3A in some capacity [121, 138, 163, 274, 355]. Further evaluation of this data with that from a pilot study in which CYP3A activity was suppressed in primary hepatocytes treated with an arginine-glycine-aspartic acid (RGD) peptide known to engage integrins in the absence of virus infection [208], solidified our hypothesis that engagement of integrin receptors by the virus at the start of infection may be sufficient to initiate processes that suppress hepatic CYP3A.

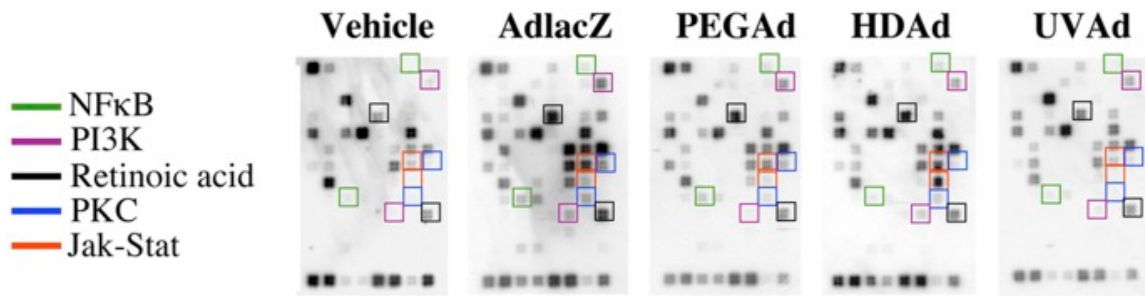


Figure 4.1 Live and inactivated recombinant adenoviruses activate certain signal transduction pathways in the liver. Representative gene expression arrays and densitometric profiles for each treatment group at 4 days post treatment. Highlighted boxes represent genes associated with five different pathways that were significantly elevated in 3 of the 4 treatment groups with respect to saline treated controls. Housekeeping genes are located in the bottom horizontal row of each array and were utilized for normalization and analysis. The primary virus used in this study was a first-generation adenovirus, expressing the *E. coli* beta-galactosidase transgene (AdlacZ). The early region 1 (E1, involved in virus replication) and early region 3 (E3, involved in evasion of the host immune response) in the genome were removed in this vector to accommodate the beta-galactosidase transgene cassette. A PEGylated version of this virus (PEGAd), which has a significantly lower immunological profile [356, 357] and an inactive control, AdlacZ inactivated by exposure to riboflavin and UV light (UVAd) [243], were included to study the effect of the immune response against virus capsid proteins and virus receptor interactions on signal transduction patterns during infection. A helper-dependent adenoviral vector (HDAd), devoid of all viral genes containing the beta-galactosidase transgene, was also included to fully study the effect of viral gene expression on signal transduction in the liver.

Table 4.1 Differential expression patterns of genes associated with various signal transduction pathways in the liver four days after administration of recombinant adenovirus.^a

Numbers represent the ratio of the intensity of gene expression in a sample obtained from an animal given virus with respect to an animal given saline (vehicle control). Gene expression was normalized based on minimal background subtraction and interquartile normalization using ScanAlyze software and the GEArray Expression Analysis Suite. Bolded colored text corresponds with colors of signal transduction pathways in arrays depicted in Figure 4.1.

Gene	Pathway	AdlacZ	PEG	HDAd	UVAd
A2m	Jak-Stat	0.754	3.232	0.710	1.705
Bax	p53	0.697	1.634	2.745	2.361
Bcl2a1	NFκB	1.815	6.477	15.443	2.761
Ccl2	LDL	0.865	1.971	0.944	1.074
Cend1	Wnt, PI3K	3.012	6.543	0.225	5.267
Cdkn1a	p53 and Androgen	0.685	1.573	0.044	2.682
Cebpb	Insulin	0.943	2.290	0.154	3.584
Fos	Mitogenic, stress, CREB, PKC, PLC	1.061	1.662	0.436	0.718
Ctsd	Retinoic acid, Estrogen	1.533	4.141	0.096	2.956
Egfr	Estrogen, Androgen	0.940	0.939	0.144	2.617
Egfr1	Mitogenic, CREB	3.146	6.875	0.289	0.937
Ei24	p53	0.647	2.132	1.023	1.634
Fasn	Insulin	0.641	1.169	0.170	1.606
Fn1	PI3 Kinase/AKT	0.628	1.480	0.029	1.954
Gadd45a	p53	1.119	0.865	0.238	1.045
Gys2	Insulin	0.746	1.614	0.059	1.563
Hnf3b	Hedgehog	1.223	1.532	0.400	1.363
Hspb1	Stress	1.482	2.556	0.227	2.398
Hspca	Stress	1.575	3.839	0.144	3.279
Icam1	NFκB and Phospholipase C	1.134	5.012	0.614	1.137
Igfbp3	p53	0.382	0.877	1.708	2.834
Il4r	Jak-Stat	1.085	1.674	0.211	1.814

Table 4.1 Continued.

Irf1	Jak-Stat	2.368	4.078	3.186	2.265
Jun	Mitogenic, Wnt, PI3K/AKT, Calcium and PKC	3.362	5.572	0.360	2.663
Klk3	Androgen	0.417	1.481	0.633	1.177
Tnfrsf6	p53, NFAT	1.646	0.756	0.794	0.781
Lta	NFκB	1.297	0.396	0.497	0.837
Mdm2	p53	4.117	4.066	3.854	2.040
Mig	Jak-Stat	3.984	3.395	7.851	1.862
Myc	Wnt, Stress, PKC	0.922	1.774	0.923	1.773
Nfkb1	NFκB	1.782	3.501	3.788	1.429
Nfkbia	NFκB	2.209	1.775	0.592	1.479
Nos2	Jak-Stat, NFκB, PLC	2.699	0.510	2.267	1.517
Odc1	PKC	3.527	2.822	4.258	1.311
Pten	PI3 Kinase/AKT	3.605	1.400	8.479	1.975
Rbp1	Retinoic acid	1.443	2.236	1.935	2.271
Vcam	NFκB, PKC, LDL	1.804	0.879	1.461	0.258

^aDescriptions of vectors can be found in the Figure 4.1 legend.

The integrin family consists of 24 membrane-spanning receptors. Each receptor consists of a heterodimeric, non-covalently associated combination of one of the 18 alpha and one of the 8 beta protein subunits [222]. In humans, integrins are expressed in almost every cell type and primarily mediate adhesion to the extracellular matrix (ECM) but also play a role in embryonic development and cell differentiation [358]. All integrin subunits have a single membrane-spanning helix and usually a short unconstructed cytoplasmic tail [225]. Integrins are also employed by a variety of enveloped and non-enveloped viruses [221], fungi [359] and bacteria [219] to gain entry and establish infection in host cells. Adenoviruses bind to several extracellular receptors, including integrins. Well-characterized integrins responsible for cellular entry of human adenovirus are integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Both integrin subtypes promote virus internalization through interaction with a conserved RGD motif in the adenovirus penton base [360]. Integrin receptors are attractive targets for pathogens in that they play a key role in endocytosis and intracellular trafficking throughout the cytoplasm [361]. Integrins are also involved in numerous disease states including cancer, thrombosis, multiple sclerosis, psoriasis, asthma, ulcerative colitis and acute coronary syndromes [362]. Hence, integrin receptors have evolved during the last few years as exciting therapeutic targets with approximately 300 anti-integrin drugs in various stages of clinical testing [224].

Given the importance of integrins in infection and other non-infectious disease states, the primary goal of the studies summarized in this manuscript was to further solidify the hypothesis that integrin receptors influence the expression and function of hepatic

cytochrome P450 3A *in vivo*. This was achieved by infecting mice with two recombinant adenoviruses that differ only in their ability to bind integrin receptors [363]. A secondary goal of these studies was to identify the manner by which integrin receptors alter changes in hepatic CYP3A. *In vitro* assessment of CYP3A activity and expression after knockdown of various α - and β - subunits of integrin receptors in a novel human-derived hepatocyte cell line [207] allowed us to prioritize the role each plays in the regulation of CYP3A and several transcription factors known to moderate this enzyme. To our knowledge, this is the first report describing a role for these ubiquitous receptors in the regulation of drug metabolism *in vivo* and in human hepatocyte cells.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Acetopromazine was purchased from Fort Dodge Laboratories (Atlanta, GA). Ketamine was purchased from Pfizer (New York, NY). Phosphate-buffered saline (PBS), ethylenediaminetetraacetic acid (EDTA), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, β -nicotinamide adenine dinucleotide phosphate sodium salt hydrate (NADP), 11 α -hydroxyprogesterone and testosterone were purchased from Sigma-Aldrich (St. Louis, MO). Protogel[®] acrylamide was purchased from National Diagnostics (Atlanta, GA). Testosterone metabolite standards were purchased from Steraloids Inc. (Wilton, NH). 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.

4.2.2 Recombinant adenovirus production

All viruses were amplified in human embryonic kidney (HEK) 293 cells except for the helper-dependent adenovirus (HDA_d) which was amplified in 293Cre cells with the use of the helper virus, AdLC8cLuc, and purified from secondary lysates as previously described [242, 364]. Viruses were purified by banding twice on cesium chloride gradients and desalted on Econo-Pac 10 DG disposable chromatography columns (BioRad, Hercules, CA) equilibrated with sterile 100 mM phosphate buffered saline (pH 7.4). Positive fractions were collected and the number of virus particles determined using the method of Maizel et al. [312] with the following formula: virus particles/ml = (absorbance at 260 nm) × (dilution factor) × 1.1×10^{12} .

4.2.3 Modification of recombinant adenoviruses

Virus capsid proteins were covalently modified with monomethoxypoly(ethylene) glycol, activated by tresyl chloride (Sigma Aldrich, St. Louis, MO) according to an established protocol [242]. Approximately 15,445 PEG molecules were associated with each virus particle in the studies outlined here as determined by a PEG-biotin assay [364]. Inactive control virus was produced by mixing freshly purified virus with riboflavin (50

μM, final concentration) and subsequent exposure of the mixture to UV light for one hour according to a protocol established in our laboratory [243]. Virus inactivation was confirmed by limiting dilution and infection of HeLa cells.

4.2.4 Administration of adenoviral vectors

All procedures were approved by the Institutional Animal Care and Use Committee of 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. For microarray analysis, catheters were surgically implanted in the right jugular vein of male Sprague-Dawley rats (9-10 weeks, Harlan Sprague Dawley, Inc. Indianapolis, IN). Twenty-four hours later, rats were given a single intravenous dose of 5.7×10^{12} virus particles per kilogram (vp/kg) of virus in a 0.5 ml volume or vehicle alone (phosphate buffered saline, PBS, 0.5 ml). Four days later, animals were sacrificed and the liver immediately excised and stored in RNAlater™ (Qiagen, Valencia, CA) at 4°C for microarray analysis.

For studies in the mouse, male C57BL/6 mice (6-8 weeks, Jackson Labs, Bar Harbor, ME) were maintained in a twelve-hour light/dark cycle environment with free access to standard mouse chow (Harlan, Indianapolis, IN) and deionized water. Following a seven-day acclimation period, a single dose of 1.5×10^{11} vp of virus in a volume of 100 μl or vehicle (PBS, 100 μl) was given to mice by tail vein injection. Animals (n = 5 per group) were sacrificed 24 and 48 hours after treatment. At this time, serum was collected

and a portion of the liver placed in Tissue-Tek[®] optimal cutting temperature (O.C.T[™]) compound (Sakura Finetek, Torrance, CA) for histological analysis. Remaining liver tissue was excised, rinsed in saline, snap frozen in liquid nitrogen and stored at -80°C prior to preparation of microsomes and isolation of nucleic acids for RT-PCR.

4.2.5 Histological analysis of transgene expression

Liver tissues were harvested and immersed in disposable peel-away molds containing Tissue-Tek[®] O.C.T[™] compound and stored at -80°C prior to analysis. Sections were fixed with 0.5% glutaraldehyde (Sigma), and beta-galactosidase activity determined by incubation with the substrate 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-gal, Gold Biotechnology, St Louis, MO) for 4 hours at 37°C in the dark. Staining medium was removed, and blue-colored, positive cells were visually tallied. For GFP analysis, sections of liver tissues were stained with a rabbit anti-GFP antibody (Molecular Probes, Eugene, OR) and with a horseradish-peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Abcam, Cambridge, UK). Spots were developed with 3-amino-9-ethylcarbazole and H₂O₂ substrate buffer (Sigma) and counterstained with hematoxylin (Sigma). Tissues were examined with a Leica DM LB microscope (Leica Microsystems Inc., Buffalo Grove, IL) and photographed using a Leica DFC 320 camera.

4.2.6 Testosterone hydroxylation assay -microsomes

Hepatic microsomes were prepared by ultracentrifugation according to established methods [365]. Two hundred micrograms of microsomal protein were incubated with testosterone, a NADPH regenerating system (5 mM NADP, 100 mM glucose-6-phosphate, 100 mM magnesium chloride) and 5 units of glucose-6-phosphate dehydrogenase for 15 minutes at 37°C with gentle agitation. The reaction was quenched with dichloromethane and 11 α -hydroxyprogesterone added as an internal standard. The organic phase was evaporated under a constant stream of filtered air and samples dissolved in methanol. Testosterone and its metabolites were separated and quantified by high-performance liquid chromatography as described [245]. Peak areas of the hydroxylated metabolites were measured and compared to peak areas of the internal standard within the same experimental run.

4.2.7 Western blot: microsomal proteins

Hepatic microsomes (50 μ g) were separated on 8% (CYP3A) or 12% sodium dodecylsulfate polyacrylamide gels by electrophoresis and transferred to nitrocellulose membranes (BioRad). Protein blots were blocked overnight at 4°C in blocking buffer containing 5% non-fat dry milk in 0.05% (v/v) Tween-20 in Tris buffered saline (TBS-T). After blocking membranes were incubated with polyclonal rabbit anti-RXR α antibody (D20, sc-553), anti-PXR antibody (H-160, sc-25381), or CAR1/2 antibody (M-127, sc-13065), each at a 1:800 dilution, they were then incubated with goat anti-rabbit IgG-HRP

secondary antibody (sc-2004, dilution 1:8,000, Santa Cruz Biotechnology, Dallas, TX). To evaluate changes in CYP3A protein, a polyclonal rabbit anti-CYP3A1 antibody (1:3,000 dilution, BD Gentest, Woburn, MA) was used together with a polyclonal anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (1:3,000, Cell Signaling Technology, Danvers, MA). Immune complexes were detected by chemiluminescence (SuperSignal™ West Pico Chemiluminescence Substrate, Fisher Scientific, Pittsburgh, PA). The intensity of protein bands was quantified relative to the signal obtained for an internal standard, β -actin (mouse anti- β -actin monoclonal antibody, AC-15, sc-69879, 1:1000 dilution, Santa Cruz Biotechnology), run on the same gel using Kodak 1D image analysis software (Eastman Kodak Co, Rochester, NY).

4.2.8 RNA isolation and RT-PCR: microsomes

RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Isolated RNA (1 μ g) was reverse transcribed with random hexamers using the SuperScript® III first-strand synthesis system (Invitrogen) in a Master Cycler Pro thermal cycler (Eppendorf AG, Hamburg, Germany). Changes in CYP3A mRNA expression in mouse liver microsomes were assessed using an AmpliTaq Gold PCR Master Mix kit (Applied Biosystems, Foster City, CA) with the following cycling conditions: 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute for a total of 32 cycles. Cycling was initiated at 94°C for 3 minutes and terminated at 72°C for 10 minutes. Primer sequences for mouse CYP3A11 were 5'-CTC AAT GGT GTG TAT ATC

CCC C-3' (forward) and 5'-CCG ATG TTC TTA GAC ACT GCC-3' (reverse) [366]. QuantumRNA™ 18S internal standards (Applied Biosystems/Ambion, Austin, TX) using proprietary competimer technology were co-amplified in individual reaction tubes. Reaction products were visualized on a 2% agarose gel containing ethidium bromide and the intensity of each band determined by densitometric analysis using Kodak 1D image analysis software (Eastman Kodak Co). PXR, RXR α , and CAR mRNA expression was assessed using SYBR® GreenER™ qPCR SuperMix (Invitrogen) analyzed on a ViiA7™ Real-Time PCR system (Applied Biosystems) with the following cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Primer sequences for mouse PXR, RXR α , and CAR were 5'- GTT CAA GGG CGT CAT CAA CT-3' (forward) and 5'-TTC TGG AAG CCA CCA TTA GG-3' (reverse); 5'-ACA AGG ACT GCC TGA TCG AC-3' (forward) and 5'-CAT GTT TGC CTC CAC GTA TG-3' (reverse); 5'-CTC AAG GAA AGC AGG GTC AG-3' (forward) and 5'-AGT TCC TCG GCC CAT ATT CT-3' (reverse), respectively [367]. This real-time PCR data was analyzed by the comparative C_T method as described previously [316].

4.2.9 Serum cytokines and transaminases

Cytokines, IL-6 and IL-12, were quantitated with commercially available ELISA kits according to the manufacturer's protocols (Invitrogen, BioSource International, Camarillo, CA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase

(AST) levels were determined using Vitros AST/SGOT and ALT/SGPT DT slides on a Vitros DTSC autoanalyzer (Ortho-Clinical Diagnostics, Rochester, NY).

4.2.10 Microarray evaluation of hepatic gene expression *in vivo*

The relative expression of genes associated with various signal transduction pathways after virus infection were assessed using oligo GEArray Rat Signal Transduction Pathway Finder microarrays (ORN-014; SuperArray, Frederick, MD). Array grade RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Biotin-labeled cDNA probes were synthesized using 3 µg of total isolated RNA and biotin-16-uridine-5'-triphosphate (Roche, Indianapolis, IN) for each array. The membrane was incubated with GEAhyb Hybridization Solution (SuperArray) for 3 hours at 60°C prior to adding 10 µg denatured cDNA probe for overnight hybridization at 60°C. The membrane was then washed twice with 2× saline sodium citrate (SSC) buffer containing 1% sodium dodecyl sulfate (SDS) followed by two additional washes with 0.1× SSC containing 0.5% SDS for 15 minutes each at 60°C. Arrays were blocked with GEArray blocking Solution Q (SuperArray) for 40 minutes at room temperature. Two milliliters of binding buffer (Buffer F, SuperArray), containing a 1:8000 dilution of alkaline phosphatase-conjugated streptavidin (SuperArray), was added for 10 minutes at room temperature. The array image was developed using the CDP-Star chemiluminescent substrate (SuperArray) and recorded on X-ray film after a 30-minute exposure. The image was scanned using a flatbed scanner (Microtek, Carson, CA).

Arrays were analyzed with ScanAlyze software (Michael Eisen, Lawrence Berkeley National Laboratory) and the GEM Express Analysis Suite. Expression levels for each gene on a given array were calculated using minimal background subtraction and interquartile normalization to an internal standard (lactate dehydrogenase A) on each membrane. Data in Table 4.1 reflect the average ratios of the intensities for a given gene from a given treatment group to that of control animals given phosphate buffered saline.

4.2.11 Cell culture

HC-04 cells (human hepatocytes: MRA-975, MR4, ATCC[®], Manassas, VA) were maintained in Dulbecco's modified Eagle's medium/Ham's F12 (50/50 ratio, Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, catalog number 26140, lot number 676548) and 2 mM L-glutamine (Hyclone, Logan, UT). HC-04 cells of passage 4-12 were used for this study.

4.2.12 RNA interference - HC-04 cells

HC-04 cells were seeded in either 6 well tissue culture dishes at a density of 1×10^5 cells per well or in 96 well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 6×10^3 cell per well in complete HC-04 culture medium 48 hours prior to transfection. Cells in each well were then transfected with 100 pmol (6

wells) or 5 pmol (96-wells) of siRNA targeting integrin α_v (sc-29373), β_3 (sc-29375) and/or β_5 (sc-35680) (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For double silencing studies, 50 pmol (6-wells) or 2.5 pmol (96-wells) of siRNA was used for each target. Efficiency of the knockdown was confirmed by qRT-PCR and compared with expression levels in cells treated with a non-specific siRNA control (sc-37007, Santa Cruz Biotechnology). Forty-eight hours after silencing, cells were infected with recombinant AdlacZ at a multiplicity of infection (MOI) of 500 for 72 hours prior to assessing CYP3A4 activity using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit (Promega, Madison, WI). The activity of CYP3A4 treated with siRNA control was $\pm$ 4.65% of the activity of untreated HC-04 cells, which is similar to the range found in a normal control population [368].

4.2.13 RNA isolation and qRT-PCR

RNA was isolated from HC-04 cells using TRIzol® and reverse transcribed using the SuperScript® III first-strand synthesis system as described above. SYBR® Green™ qPCR SuperMix (Invitrogen) was added to each sample before it was run on a ViiA7™ Real-Time PCR system. All samples were analyzed as described above for tissue samples. Primer sequences for human CYP3A4, PXR, RXR α , CAR, integrin α_v , integrin β_3 and integrin β_5 were 5'-GAT TGA CTC TCA GAA TTC AAA AGA AAC TGA-3' (forward) and 5'-GGT GAG TGG CCA GTT CAT ACA TAA TG-3' (reverse) [318]; 5'-AGA AGG AGA TGA TCA TGT CCG A-3' (forward) and 5'-GTT TGT AGT TCC AGA CAC TGC

C-3' (reverse) [369]; 5'-CCT TTC TCG GTC ATC AGC TC-3' (forward) and 5'-CTC GCA GCT GTA CAC TCC AT-3' (reverse) [240]; 5'-GCA AGG GTT TCT TCA GGA GAA C-3' (forward) and 5'-CTT CAC AGC TTC CAG CAA AGG-3' (reverse) [319]; 5'-AAT CTT CCA ATT GAG GAT ATC AC-3' (forward) and 5'-AAA ACA GCC AGT AGC AAC AAT-3' (reverse); 5'-CCG TGA CGA GAT TGA GTC A-3' (forward) and 5'-AGG ATG GAC TTT CCA CTA GAA-3' (reverse); 5'-GGA GCC AGA GTG TGG AAA CA-3' (forward) and 5'-GAA ACT TTG CAA ACT CCC TC-3' (reverse) [370], respectively.

4.2.14 Protein isolation and western blot - HC-04 cells

Cells were washed twice with ice-cold PBS prior to addition of 500 µl of ice-cold lysis buffer (RIPA buffer, Thermo Scientific) containing 1% protease inhibitors (HALT Protease and Phosphatase Inhibitor Cocktail, Pierce). After incubation on ice for 5 minutes cells were lysed by extrusion 25 times through a 20-gauge needle (Becton Dickinson, Franklin Lakes, NJ) attached to a 1-ml syringe (Becton Dickinson) and placed on ice for an additional 40 minutes. Lysates were then cleared by centrifugation at 14,000 g for 15 minutes at 4°C and stored at -80°C. Proteins (50 µg) were separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane (BioRad). Protein blots were blocked overnight at 4°C in 3% bovine serum albumin (BSA) in 0.05% TBS-T. Membranes were then incubated for 2 hours with mouse anti-CYP3A4 antibody (1:2000 dilution, sc-53850) followed by 1 hour of incubation with a goat anti-mouse IgG-HRP antibody (1:10,000 dilution, sc-2005, Santa Cruz

Biotechnology). Membranes were then analyzed as described above.

4.2.15 Statistical analysis

Statistical analysis of data was performed using SigmaStat (Systat Software Inc., San Jose, CA). Differences with respect to treatment were calculated using a one-way analysis of variance followed by a Bonferroni/Dunn *post hoc* test. Differences were determined to be significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$).

4.3 RESULTS

4.3.1 Effect of integrin-independent virus infection on hepatic CYP3A expression and function *in vivo*

To validate our hypothesis that virus-integrin interactions are required for infection-induced changes in CYP3A *in vivo*, mice were given a single dose of either a first-generation recombinant adenovirus containing the beta-galactosidase transgene (AdlacZ) or a similar virus in which the RGD motif within the penton capsid protein was deleted via PCR-directed mutagenesis [363]. Twenty-four hours after administration, mice given the AdlacZ vector experienced a notable reduction in CYP3A catalytic activity, approximately 70% compared to saline-treated mice (PBS, Figure 4.2A, $p < 0.01$). Forty-eight hours after treatment, CYP3A activity continued to be suppressed in the AdlacZ group (56.4% of

control, Figure 4.2B, $p < 0.01$). Conversely, the Ad Δ RGD vector did not alter CYP3A activity at either timepoint. A similar trend was noted with respect to CYP3A protein expression with reductions of 38% and 23% of control noted 24 and 48 hours after administration of AdlacZ respectively (Figure 4.2C and 4.2D, $p < 0.05$) while protein levels were unaltered by the Ad Δ RGD virus. CYP3A11 mRNA levels were also reduced by the AdlacZ vector (32.8% of control) 24 hours after treatment (Figure 4.2E, $p < 0.05$) and returned to baseline levels 48 hours after infection (Figure 4.2F, $p = 0.67$). CYP3A11 mRNA levels were unaffected by Ad Δ RGD throughout the study period.

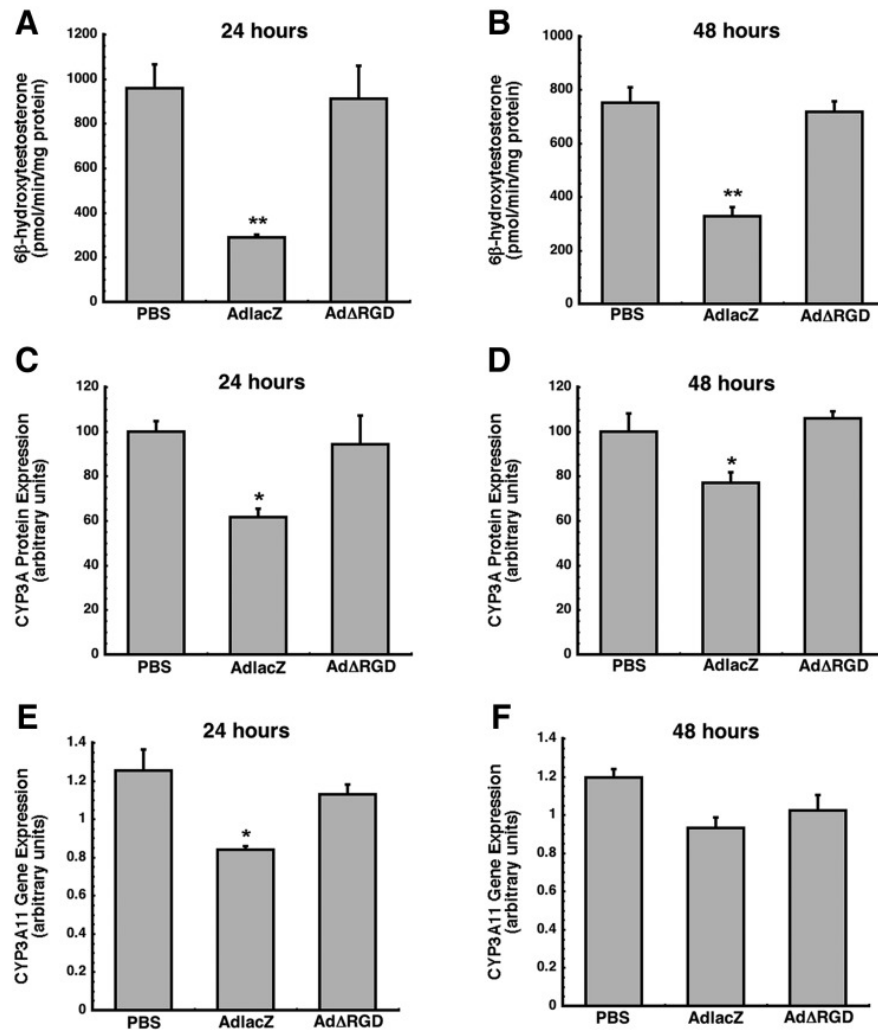


Figure 4.2 Systemic administration of a RGD-deleted recombinant adenovirus does not alter hepatic CYP3A expression and function in mice. Catalytic activity, protein and mRNA levels of hepatic CYP3A11 were evaluated 24 hours (Panels A, C and E respectively) and 48 hours (Panels B, D and F respectively) after systemic administration of 1×10^{11} particles of either a first-generation adenovirus expressing beta-galactosidase (AdlacZ) or a vector of the same generation expressing green fluorescent protein with all arginine-glycine-aspartic acid (RGD) residues removed from capsid proteins (AdΔRGD).

4.3.2 Histochemical evaluation of transgene expression

In order to determine that differences in CYP3A expression between the AdlacZ and the Ad Δ RGD virus were not simply due to disparities in the ability of each virus to infect the liver, tissue sections from mice given the AdlacZ vector were stained with the histochemical substrate for the beta-galactosidase transgene, X-gal, while livers from mice given Ad Δ RGD were stained with an antibody against the green fluorescent protein transgene (GFP, Figure 4.3). Staining of tissues obtained from animals given a saline bolus (vehicle, negative control) revealed minimal endogenous levels of either transgene in the liver (Figures 4.3A, 4.3E, and 4.3I). Twenty-four hours after administration of AdlacZ, approximately 80% of the cells were positive for beta-galactosidase (Figure 4.3B), while 15% of the cells in sections taken from mice given Ad Δ RGD were positive for GFP (Figure 4.3G). GFP expression increased in these animals at the 48 hour timepoint with approximately 80% of hepatocytes expressing the transgene (Figure 4.3K). Sections from animals given an adenovirus expressing GFP without the RGD mutation demonstrated similar transgene expression patterns at 24 and 48 hours (Figures 4.3H and 4.3L). Endogenous staining for beta-galactosidase was not present in any of the sections obtained from animals given viruses expressing GFP (Figures 4.3C and 4.3D) as was positive staining for GFP absent in sections obtained from animals given AdlacZ (Figures 4.3F and 4.3J).

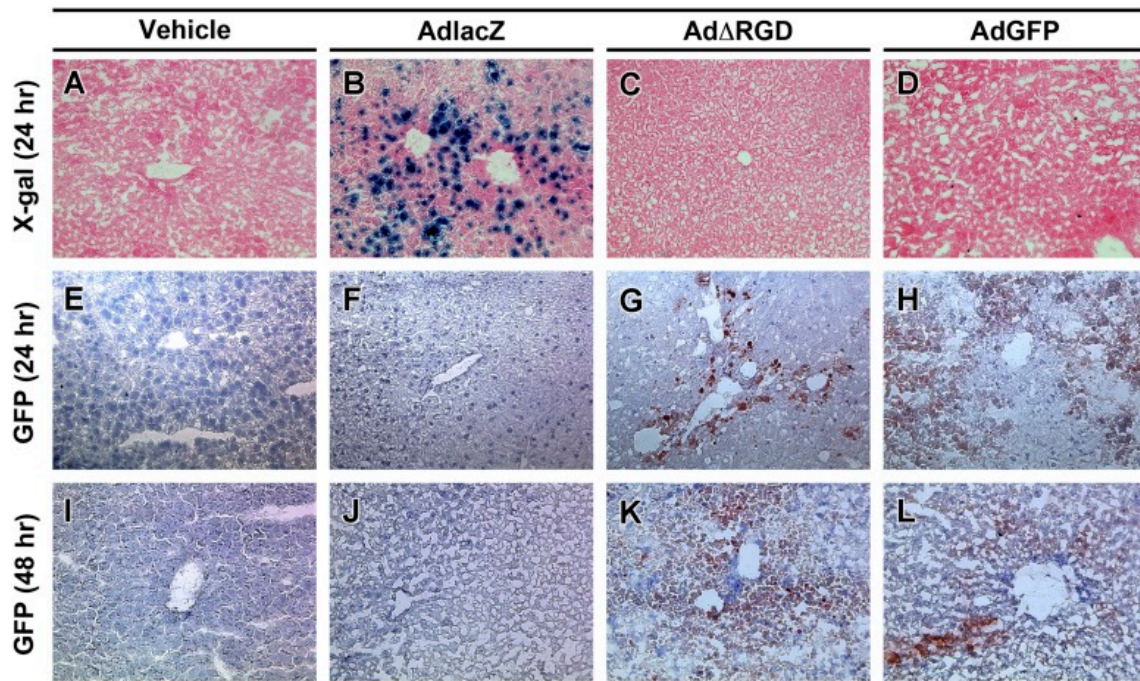


Figure 4.3 Hepatic transduction efficiency of a RGD-Deleted recombinant adenovirus in the mouse. Liver section collected 24 hours after administration of phosphate-buffered saline (A, E, I), AdlacZ (B, F, J), Ad Δ RGD (C, G, K) and AdGFP (D, H, L). Surface staining was done with X-gal (A-D), using an anti-GFP antibody at 24 hours (E-H) or an anti-GFP antibody at 48 hours (I-L). Magnification for all panels: 200 $\times$.

4.3.3 Effect of integrin-independent entry on the toxicology profile of recombinant adenovirus

Interleukin-6 (IL-6) and IL-12, commonly produced in response to systemic administration of adenovirus [371, 372], were measured 24 and 48 hours after virus administration. In a manner that has been noted previously in our laboratory and those of others, serum IL-6 was significantly elevated 6 hours after administration of the AdlacZ vector ($1,105 \pm 226.1$ pg/ml, Figure 4.4A). IL-6 levels were significantly lower in samples taken from animals given Ad Δ RGD at the same timepoint (340.4 ± 50.1 pg/ml). IL-6 declined to 38.2 ± 11.7 pg/ml by 24 hours after infection with AdlacZ. It was not detectable in samples taken from animals given Ad Δ RGD at this timepoint and in any sample collected 48 hours after treatment. The IL-12 profile for AdlacZ was similar to that for IL-6 in that levels spiked to $1,175.7 \pm 103.1$ pg/ml at 6 hours and declined to 109.2 ± 28.6 and 25.6 ± 17.9 pg/ml at the 24 and 48 hour timepoints respectively (Figure 4.4B). IL-12 in samples obtained 6 hours after administration of Ad Δ RGD was 3 times lower than those from mice given AdlacZ (392.1 ± 26.1 pg/ml), however, the concentration of this cytokine was significantly higher than that detected in the AdlacZ group at the 24 hour (247.6 ± 30.3 pg/ml) and 48 hour (270.0 ± 18.6 pg/ml) timepoints.

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels are often used as indicators for determining liver function during inflammation, injury, or disease [337]. Twenty-four hours following virus administration, AST levels were 4

(AdlacZ) and 3 (Ad Δ RGD) times that of saline-treated animals (Figure 4.4C). At this time, serum ALT was also 9 and 5 times above baseline in samples from mice given the AdlacZ and Ad Δ RGD vectors respectively (Figure 4.4D). At the 48 hour timepoint, serum AST levels in mice given the Ad Δ RGD vector were not significantly different from those given saline (127.7 ± 5.2 vs. 85.7 ± 20.9 $p = 0.18$), while those from mice given AdlacZ were still notably elevated (193.3 ± 27.1 U/L, Figure 4.4E). ALT levels were still significantly higher than baseline in samples taken from each treatment group at the 48-hour timepoint (Figure 4.4F, $p < 0.05$).

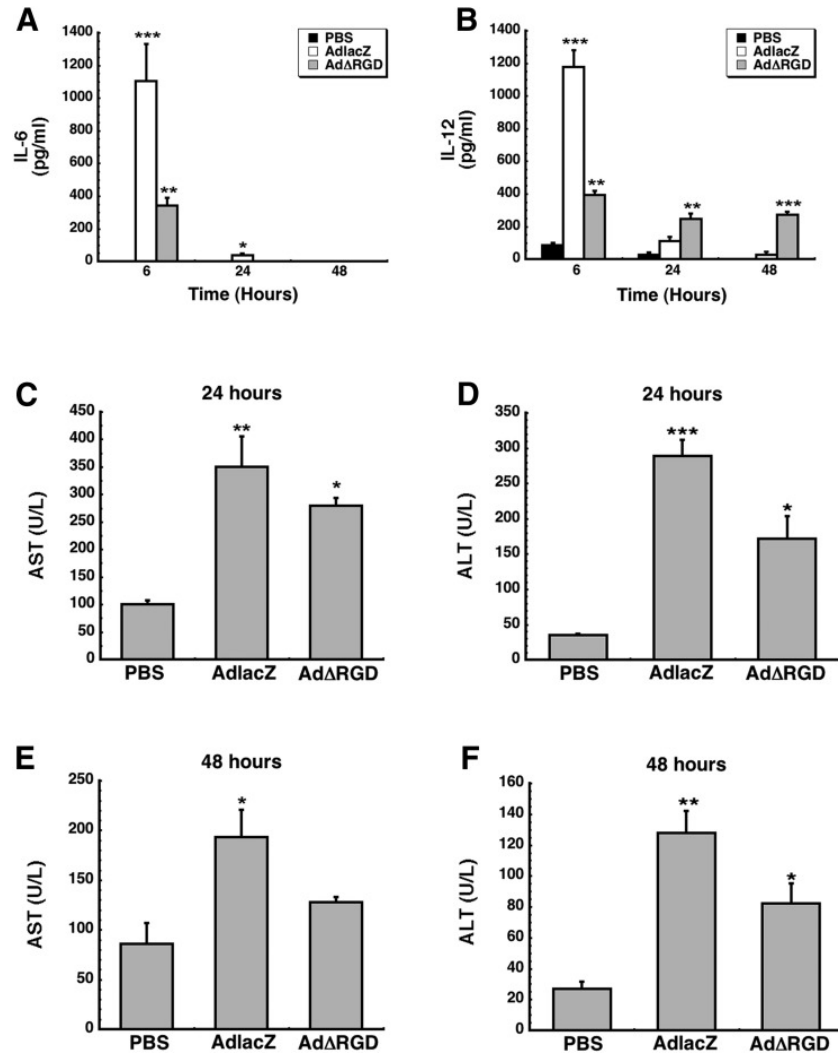


Figure 4.4 Removal of RGD epitopes from the adenovirus capsid alters the cytokine secretion profile and elicits a mild increase in serum transaminases in the mouse. Serum levels of (A) IL-6 and (B) IL-2 measured 6, 24 and 48 hours after a single dose of saline (PBS) or 1×10^{11} particles of either AdlacZ or AdΔRGD. (C) Serum aspartate aminotransaminase (AST) profile and (D) serum alanine aminotransaminase (ALT) profile 24 hours after a single dose of virus. (E) Serum AST levels 48 hours after administration of virus. (F) Serum ALT 48 hours after administration of virus.

4.3.4 Effect of integrin-independent entry on nuclear receptors regulating CYP3A expression

On a transcriptional level, hepatic CYP3A is constitutively expressed in the liver but several nuclear receptors, including the pregnane X receptor (PXR), constitutive androstane receptor (CAR), and retinoid x receptor (RXR) are primarily responsible for regulating CYP3A gene expression [12]. However, it remains to be known which is predominantly influenced by virus infection and ultimately responsible for our observed changes in CYP [207, 208]. In an effort to determine how integrin engagement changes each of these key regulators of CYP3A *in vivo*, gene and protein expression was determined 24 and 48 hours after infection with each virus (Figure 4.5). PXR gene expression was unaffected by AdlacZ 24 and 48 hours after infection. In contrast, a 2-fold increase was seen at 24 hours and a 2-fold decrease observed at 48 hours after infection with AdΔRGD with respect to uninfected saline controls (Figure 4.5A). PXR protein levels increased 24 hours after administration of both viruses and declined to baseline (AdΔRGD) and below baseline (AdlacZ) levels at 48 hours (Figure 4.5B, $p < 0.05$ and $p < 0.01$). RXR α gene expression was unaffected for animals treated with AdΔRGD for 24 and 48 hours. However, samples collected 48 hours after AdlacZ infection showed a 5-fold reduction in RXR α mRNA (Figure 4.5C). RXR α protein expression was reduced 27% (24 hours) and 63% (48 hours) for AdlacZ-infected animals with respect to saline controls (Figure 4.5D, $p < 0.05$ and $p < 0.01$). A 2-fold reduction was seen in CAR gene expression in animals treated with AdlacZ for 24 and 48 hours while treatment with AdΔRGD did not have any

effect on this nuclear receptor (Figure 4.5E). A general trend of suppressed protein levels was observed for CAR in animals infected with either virus (Figure 4.5F, $p < 0.01$).

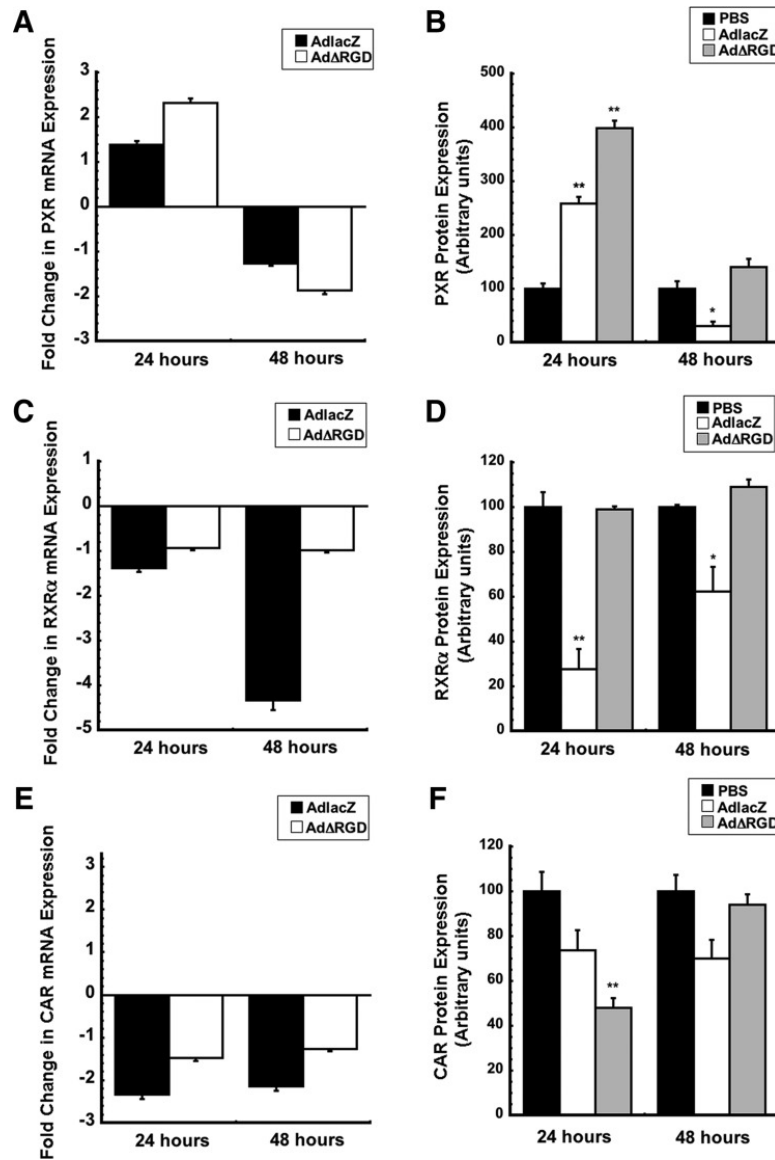


Figure 4.5 Virus-mediated changes in gene and protein expression of nuclear receptors regulating CYP3A4. Gene expression of nuclear receptors PXR, RXR α , and CAR (Panels A, C, and E, respectively) and protein levels (Panels B, D, and F, respectively) in mice after 24 or 48 hours of infection with either AdlacZ or Ad Δ RGD. Mice were given saline (PBS) or 1×10^{11} particles of either AdlacZ or Ad Δ RGD.

4.3.5 Treatment with an integrin-specific peptide inhibits CYP3A4 activity in the HC-04 cell line

To confirm that a human hepatic cell line (HC-04) can be used for mechanistic studies involving virus-integrin and CYP3A interactions, an initial pilot experiment was performed in which HC-04 cells were treated with a linear peptide containing a RGD sequence, a known integrin receptor ligand, or control RGE (arginine-glycine-glutamic acid) peptide that does not engage integrins at a concentration of 1.5 mg/ml. Treatment with the RGD peptide significantly suppressed CYP3A4 activity in HC-04 cells by 40% with respect to untreated controls while the RGE peptide did not affect CYP3A catalytic activity (Figure 4.6A) in the absence of virus infection. The amount of peptide used was sufficient to cover the majority of integrin receptors on the cells as, in a separate experiment, it reduced the transduction efficiency of AdlacZ by approximately 70% (Figures 4.6B and 4.6C). Results from this study confirm results from previous studies performed in primary rat hepatocytes [208], making this cell line useful for further mechanistic studies to determine how integrins regulate CYP3A4 in humans.

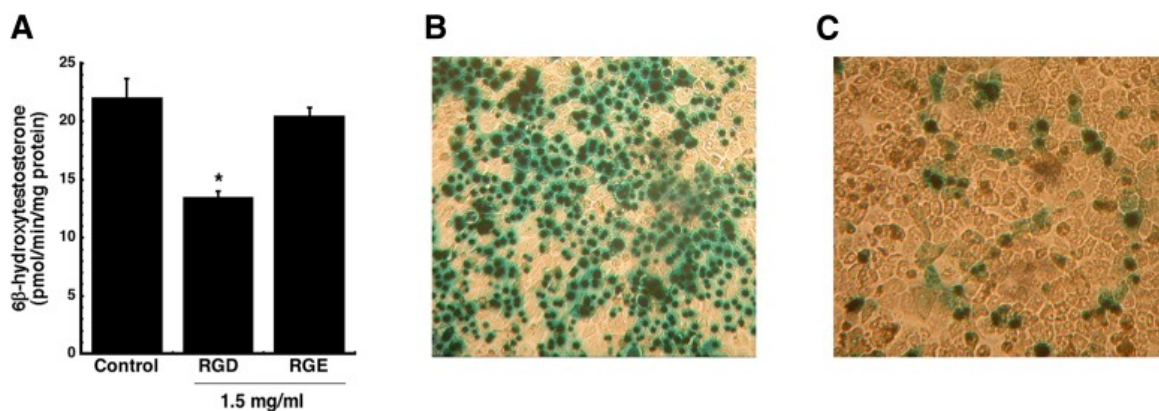


Figure 4.6 Engagement of integrin receptors with a non-infectious peptide suppresses CYP3A4 activity in HC-04 cells. (A) Catalytic activity of CYP3A after treatment with peptides containing either RGD or RGE sequences. Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per condition. $*p < 0.05$ with respect to saline-treated cells (control). (B) Histochemical staining for beta-galactosidase expression in hepatocytes infected with AdlacZ at a MOI of 100. Similar staining patterns were detected for cells treated with a control peptide containing an arginine-glycine-glutamic acid (RGE) sequence at a concentration of 1.5 mg/ml for 2 hours prior to infection. (C) Histochemical staining for beta-galactosidase expression in hepatocytes treated with a RGD peptide (1.5 mg/ml). Peptide was added to culture media for 2 hours at 4°C prior to infection with AdlacZ at a MOI of 100. Original magnification of each panel: 200 $\times$.

4.3.6 Impact of silencing of integrin expression on the catalytic activity of hepatic CYP3A4 *in vitro*

To determine which components of the integrin receptor regulate CYP3A4, expression of α_v , β_3 and/or β_5 subunits were knocked down via siRNA targeting in the HC-04 cell line. Forty-eight hours after transfection with optimized sequences, gene expression was confirmed to be reduced by 3.5-fold for integrin α_v and β_3 , and 4-fold for integrin β_5 (Figure 4.7A). When silencing both subunits at the same time, the $\alpha_v\beta_3$ combination allowed for the most maximal suppression (3.6 fold below cells transfected with an irrelevant siRNA sequence, Figure 4.7A). At this time, cells were infected with virus (AdlacZ, MOI 500) and, 72 hours later, CYP3A4 activity was assessed. Adenovirus-mediated suppression of CYP3A4 activity was still seen in cells treated with negative control siRNA and α_v siRNA (reduced by 70% and 75% with respect to uninfected controls respectively, Figure 4.7B). Very minimal reductions in CYP3A4 activity were detected when the β_3 or β_5 subunit was silenced (7% and 23% reduction, respectively). Less of an effect (6-10% reduction) was seen when both α_v and β subunits were silenced together (Figure 4.7B).

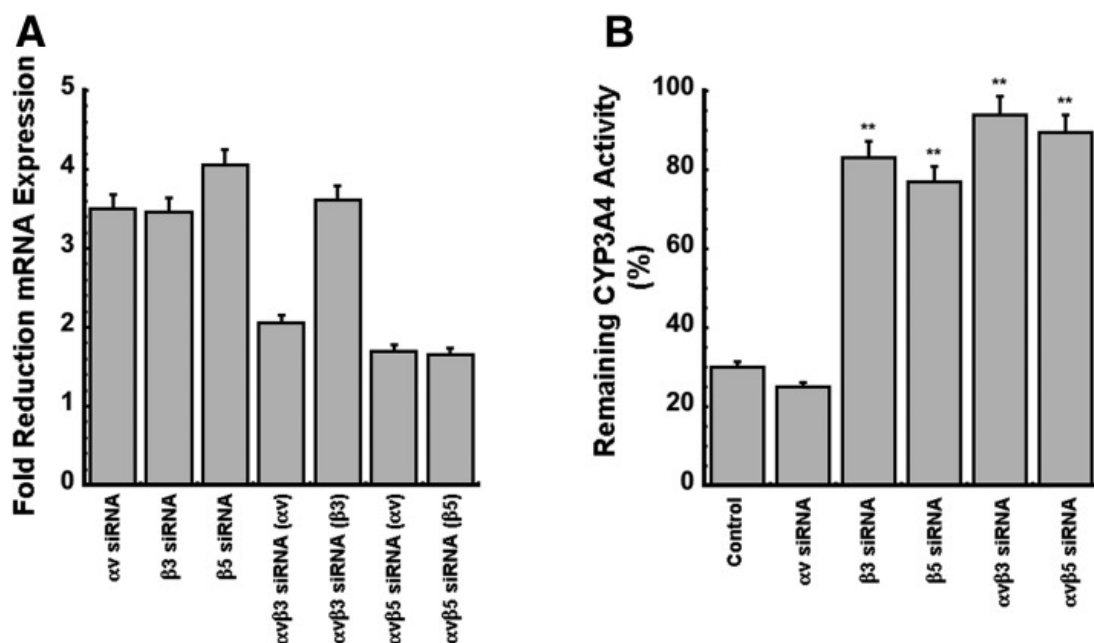


Figure 4.7 Silencing of β_3 and/or β_5 integrin subunits reverses adenovirus-mediated changes in CYP3A4. (A) Degree of silencing achieved for each integrin subunit in HC-04 cells. Cells were transfected for 48 hours with either non-targeting control siRNA (Control) or siRNA targeting integrin α_v , β_3 or β_5 . Silencing was confirmed by qRT-PCR using primers specific for the different integrins. (B) Remaining CYP3A4 catalytic activity in HC-04 cells infected with adenovirus (MOI 500) for 72 hours after integrin silencing. CYP3A4 activity was assessed using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit. Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per condition. ** $p < 0.01$ with respect to siRNA control-treated cells.

4.3.7 Silencing of integrin subunits alters CAR and PXR gene expression *in vitro*

Baseline gene expression levels of the nuclear receptors PXR, CAR and RXR α was determined in HC-04 cells 48 hours after knockdown of each integrin subunit (Figure 4.8). Silencing of subunits α_v and β_3 decreased expression of PXR by 6- and 5-fold, respectively, while silencing of the β_5 subunit had no effect (Figure 4.8A). Silencing of either β -subunit significantly decreased the gene expression level of CAR while silencing of α_v did not alter CAR mRNA levels (Figure 4.8B). Silencing with either α_v , β_3 or β_5 siRNA had no significant effect on RXR α , although the general trend was a small increase in the gene expression level of RXR α when silencing the α_v and β_5 subunits (Figure 4.8C).

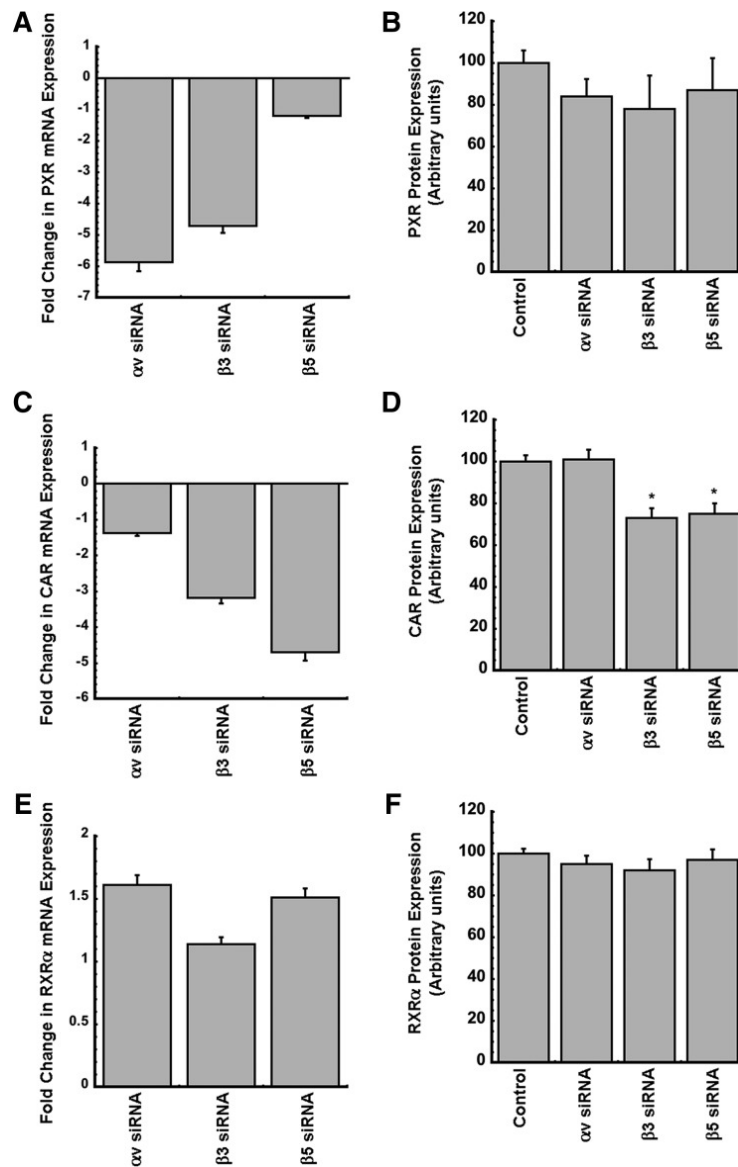


Figure 4.8 Silencing of α_v , β_3 or β_5 integrin subunits decreases gene expression level of PXR and CAR but not RXR α . Gene expression levels of key regulators of CYP3A including (A) PXR, (B) CAR, and (C) RXR α in HC-04 cells treated with siRNA targeting either the α_v , β_3 or β_5 subunit of the integrin receptor. Silencing of the different integrin subunits was confirmed by qRT-PCR (Figure 4.7A). Fold reduction was determined using the comparative C_T method as described previously [316].

4.4 DISCUSSION

Like many other pathogens, human adenovirus serotype 5 utilizes additional receptors like the coxsackie- and adenovirus receptor (AdCAR) and heparan sulfate proteoglycans (HSPGs) to enter cellular targets [373]. While integrins facilitate internalization of virus particles, AdCAR and HSPGs are responsible for attachment of the virus to the hepatocyte [360]. Until recently, HSPGs were the primary receptor in the liver to which adenovirus binds *in vivo* [374, 375]. However, studies in knockout mice suggest that the LDL receptor-related protein (LRP), may be responsible for adenovirus-mediated transduction of human liver and various animal models of disease *in vivo* [375, 376]. AdCAR is poorly expressed in the liver *in vivo* but has been shown to mediate infection in cultured cells [375]. It is through the utilization of these alternative receptors that Ad Δ RGD transduced the liver of mice in the outlined studies. The role of these receptors in the regulation of CYP3A seems minimal at best since our data clearly shows that elimination of the adenovirus-integrin interaction reverses any virus-induced changes in CYP previously noted with wild type virus.

Internalization of adenovirus via clathrin-mediated endocytosis is triggered by interactions between the RGD motif on the penton base of the virus and the α_v integrin subunit [377]. During internalization, the penton base will detach from the virus with other proteins, leaving uncoated particles to collect in the endosome [378]. Following endosomal escape, the viral genome complex travels to the nucleus via actin filaments dispersed

throughout the cell [379]. Integrins initiate cellular signals by two different pathways often referred to as “outside-in” and “inside-out” responses [222]. In this context, it is important to realize that integrins lack intrinsic enzymatic signaling, instead, they are activated by binding of extracellular ligands (like viruses and other microbes), which results in clustering and changes in the ligand-binding affinity of integrins on the cell surface [224]. Activation in this manner also changes the tertiary and quaternary structure of integrins, which propagate across the cell membrane to activate cytoplasmic kinase- and cytoskeletal-signaling cascades. This, commonly known as the outside-in response, initiates changes in cell polarity, survival, proliferation, cytoskeletal structure and gene expression [222]. Additionally, integrins are also regulated by internal stimuli, initiating intracellular signals, which convert the extracellular domains into a high-affinity ligand-binding state. This is known as the inside-out response [380]. Elicitation of this response results in cell adhesion, migration and extracellular matrix assembly [224]. Given that adenovirus infection is a complex, multistage process, infection with this virus induces a sustained effect on CYP3A expression and function by first inducing pathways stimulated by the outside-in response during virus internalization followed by induction of additional pathways stimulated by the inside-out response during nuclear transit and subsequent expression of virus genes throughout the cell. We also realize that, while simple engagement of integrins with the RGD peptide elicited a comparable reduction in CYP3A activity to that seen with adenovirus, this effect may be induced by a different mechanism as monomeric RGD-peptides are internalized via a fluid-phase endocytic pathway [381]. Additional *in vitro* and *in vivo* studies with non-infectious RGD-based integrin ligands and other integrin and non-

integrin dependent pathogens are currently underway in our laboratory to further define the role of the outside-in and the inside-out response on hepatic CYP3A expression and to confirm that our observations are not unique to adenovirus serotype 5.

The most interesting finding of these studies was that silencing of the β_3 - and β_5 -integrin subunits reversed the effect of adenovirus-mediated suppression of CYP3A4 in human hepatocytes (Figure 4.7). This is important since the β subunit is largely responsible for the inside-out response. The key activator of the inside-out response is the intracellular protein talin-1. Talin-1 binds specifically to the tail of the β -subunit of the integrin receptor [382] resulting in separation of the α - and β -tail ultimately leading to increased binding affinity of the integrin receptor [383]. Several viral proteins interact with talin-1 *in vivo* in various acts of self-preservation [384, 385]. Disruption of the β -subunit and talin-1 interaction by pUL135 protein suppresses integrin function and protects human cytomegalovirus from natural killer cells and T cells [384]. In contrast, knockdown of talin-1 enhances susceptibility to retroviral infection in human cells [385]. To date, adenovirus-talin-1 interactions have not been described. Hence, *in vitro* studies in which talin-1 is silenced and *in vivo* studies utilizing talin knockout animal models [386] should be performed to determine what role talin plays in the regulation of CYP3A4.

On a transcriptional level, hepatic CYP3A is constitutively expressed but can also be induced by a variety of structurally different xenobiotics. Nuclear receptors, including the pregnane X receptor (PXR), constitutive androstane receptor (CAR), and retinoid X

receptor (RXR), are primarily responsible for regulating hepatic CYP3A expression [12]. Analysis of changes in each of these receptors during infection *in vivo* revealed that RXR α , essential for binding of nuclear receptors to the dXREM and prPXRE regions of the CYP3A4 5' promoter sequence [121], was involved in the observed downregulation of CYP3A via integrin-virus interactions (Figure 4.5). This observation was not congruent with those made in cultured human hepatocytes where RXR α expression was not affected when β integrin expression was silenced (Figure 4.8). An interesting biphasic change (2 fold increase followed by a 2 fold decrease) in PXR expression was induced *in vivo* by both viruses, indicating that expression of this receptor was affected in an integrin-independent manner. Silencing experiments did not reflect these observations as PXR was notably suppressed when β integrin expression was silenced. It is not clear at this time if these disparities are due to species-specific differences or differences in inside-out/outside-in responses between *in vivo* and *in vitro* systems. Studies using both the AdlacZ- and Ad Δ RGD viruses in RXR α and PXR knockout mice, primary hepatocytes isolated from these animals and human hepatocytes in which each nuclear receptor is silenced will assist us further in making these distinctions. While notable reductions in CAR were observed in animals given the AdlacZ vector only, they were less profound than that of RXR, suggesting that CAR is less responsive to virus-induced changes in CYP initiated through integrin engagement. Unlike that of RXR α , this observation was also made during *in vitro* silencing experiments as CAR was significantly reduced when β integrin expression was silenced (Figure 4.8).

Integrin receptors have been implicated in the regulation of several functions in tumor cells such as migration, invasion, proliferation and survival. The diversity in these processes suggests that pathology of cancer, as well as other non-infectious diseases in which integrins play a role such as cardiovascular disease, diabetes and wound healing, may not be attributed to a single integrin receptor, but instead are the end result of crosstalk between several integrin receptors. Thus, drugs targeting single as well as multiple integrin receptors have been developed for treatment of cancer and other diseases [223, 358]. In this context, data generated in our studies suggest that hepatic drug metabolism patterns can be significantly altered in response to these medications which will most likely be given with other chemotherapeutic agents that are metabolized by CYP3A4; either resulting in an enhanced therapeutic effect or severe toxicities due to accumulation of unmetabolized agents over time. Various expression patterns of integrins have been shown to correlate with increased tumor progression and decreased patient survival in breast, cervical and colon cancers [358]. More recent findings have shown that cancer cells can change their integrin repertoire in response to drug treatment [223]. Given the link between integrin expression and CYP3A4 activity illustrated here, we believe that integrin receptors may play a notable role in the development of drug resistance. Experiments to further explore the correlation between integrin expression, CYP3A4 activity and tumor resistance are underway in our laboratory. Understanding this interplay could potentially be used to regulate integrin expression during the early stages of cancer detection to mitigate severity of disease or to modulate drug metabolism to improve therapeutic outcomes in patients through personalized drug therapies directed at integrin expression.

Toll-like receptors (TLRs) 2, 3 and 4 have been shown to be involved in CYP3A suppression [387-389] and therefore also need to be taken into consideration. TLRs are a family of type I transmembrane proteins with an extracellular domain containing leucine-rich repeats (LRRs) and a conserved cytoplasmic tail [390]. Most TLRs are located on the plasma membrane except TLR3, TLR7, TLR8, and TLR9 that are localized in the endosomal compartment. In humans, ten TLRs have been identified while twelve have been characterized in mice [391]. Adenoviruses are known to interact with TLRs 2, 4, 7, 8 and 9 [392]. To avoid excessive or prolonged activation of the innate immune response, which can lead to autoimmunity or a cytokine storm that can result in tissue damage and even lethal septic shock, TLR signaling is subjected to negative regulation and feedback inhibition [393]. The exact mechanism through which this occurs is an active area of research but several studies have shown that immune-receptor tyrosine-based activation motif (ITAM)-associated receptors play an important role in the regulation of TLR signaling [394]. Several cell surface receptors use ITAM-containing adaptors to relay extracellular signals to enable appropriate cellular changes, including β_2 integrins. Activation of the β_2 integrin CD11b by TLRs via inside-out signaling has been shown to limit TLR signaling [395]. This is achieved by inhibiting NF- κ B pathway activation and promoting p38 MAPK activation resulting in degradation of TLR adaptors MyD88 and TRIF, in such a way that fine-tunes TLR-induced inflammatory responses in cells of the innate immune system [396]. It is not known if TLRs and integrins are involved in a similar negative feedback loop with respect to drug metabolism. However, serotype 5 human adenovirus vectors elicit innate immune responses through MyD88 and TLR 9 [397] hence

activation of integrins via interaction with the adenovirus could activate this negative feedback loop to limit the innate immune response against the vector. Since only the involvement of TLRs 2, 3 and 4 have been studied in respect to CYP3A expression and function, further studies evaluating the connection between integrin receptors and TLRs 7, 8 and 9 in the involvement of hepatic CYP3A4 regulation is therefore needed to fully understand this potential interplay.

In this study we have shown, for the first time, a direct correlation between engagement of integrin receptors and the regulation of hepatic CYP3A activity. Although engagement of other intracellular receptors including Toll-like receptors (TLRs) have previously been implicated in the regulation of CYP3A during infection [398], here we introduce evidence to support the notion that extracellular receptors influence hepatic drug metabolism under infectious and non-infectious conditions. We also realize that these results may translate far beyond that of CYP3A activity as several of the nuclear receptors that regulate this enzyme (and were affected by integrin expression) also play notable roles in the regulation of other metabolic enzymes and drug transporters. As a result, assessment of the function of several key efflux and uptake transporters in the liver in both *in vivo* and *in vitro* systems in response to integrin expression are currently underway.

CHAPTER FIVE

Assessment of Regulation of Cytochrome P450 3A by Talin-1 and Post-Translational Modifications During Infectious and Non-Infectious Conditions

5.1 INTRODUCTION

The cytochrome P450 3A4 (CYP3A4) enzyme is the most important drug-metabolizing enzyme in humans due to its broad substrate specificity, which includes drugs from most therapeutic categories in addition to several endogenous substrates [50]. Any aberration in this single enzyme could have severe consequences including drug-drug interactions, sub-therapeutic drug levels or drug toxicity, especially for drugs with a low therapeutic index. We have previously shown that integrin receptors are involved in regulating hepatic CYP3A4 during infectious and non-infectious conditions [206]. Integrins are transmembrane cell-surface receptors essential for normal cell adhesion, migration, and assembly of the extracellular matrix [399]. There are 24 known human integrins, each consisting of one alpha and one beta subunit [400]. Many, but not all, integrin receptors recognize arginine-glycine-aspartic acid (RGD) sequences, which are often displayed on a variety of extracellular matrix proteins. Viruses also express RGD sequences on exposed loops of their capsid proteins to enable interaction with integrin receptors, which can result in attachment, cell entry, signaling and/or endosomal escape. Several bacteria, including *Staphylococcus aureus*, *Streptococcus pyrogens* and

Helicobacter pylori, express either integrin-binding adhesive proteins or they can capture physiological integrin ligands present in their host and in that manner, engage integrins. Diverse viral pathogens, including adenovirus, herpesvirus, hantavirus, picornavirus and reoviridae, are known to interact with integrin receptors [221]. Engagement of integrin receptors by adenoviruses, specifically integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [360], have been shown to significantly suppress CYP3A function and expression in C57BL/6 mice 24 and 48 hours after infection [206]. However, CYP3A function and expression was unaffected by administration of a similar adenovirus in which the RGD motif within the penton capsid protein was deleted [363]. Suppression of CYP3A4 has also been seen after treatment of hepatocytes with a non-infectious RGD peptide while a negative control RGE peptide, that does not bind to integrin receptors, did not alter CYP3A4 activity [206]. However, the specific mechanism of this phenomenon is unknown. The primary goal of these studies was to explore the underlying mechanism of CYP3A4 regulation by engagement of integrin receptors.

Similar to other cell surface receptors, integrins trigger intracellular signals in response to ligand binding via an outside-in response. However, they can also be activated by certain cues within the cell, creating an inside-out response [401]. Physiologically, inside-out signaling is important when cells are constantly in close proximity to their ligands but cell-ligand interactions only occur after integrins are activated in response to specific external signals. For example, integrin $\alpha_{IIb}\beta_3$ receptors on platelets are constantly exposed to their ligand, fibrinogen, therefore they need to be kept inactivate until

appropriate cues are given to prevent pathological platelet aggregation and thrombus formation [399]. Integrin activation through the inside-out mechanism is mediated by specific binding of the cytoskeletal protein talin-1 to the cytoplasmic tail of the β -subunit of the integrin receptor, which results in conformational rearrangement of the integrin extracellular domains, ultimately increasing their affinity for a variety of ligands [402]. In our previous studies, we found that silencing of integrin subunit β_3 and/or β_5 followed by adenovirus infection reverted adenovirus-mediated suppression of CYP3A4 while silencing of integrin subunit α_v did not [206]. This lead us to believe that interactions with the β -tail of the integrin receptor are highly important for virus-mediated suppression of CYP3A4. Talin-1, a cytoplasmic protein that specifically binds to the β -tail of integrin receptors therefore became our focus for this study. The talin-1 protein consists of a 50 kDa head domain that contains the high-affinity binding domain for integrin β subunits and a 220 kDa rod domain with multiple binding sites for actin and a second binding site for the β -subunit of the integrin receptor (Figure 5.1) [386]. One of the primary goals of this project is to determine the role that talin-1 plays in the regulation of hepatic CYP3A4 expression and function.

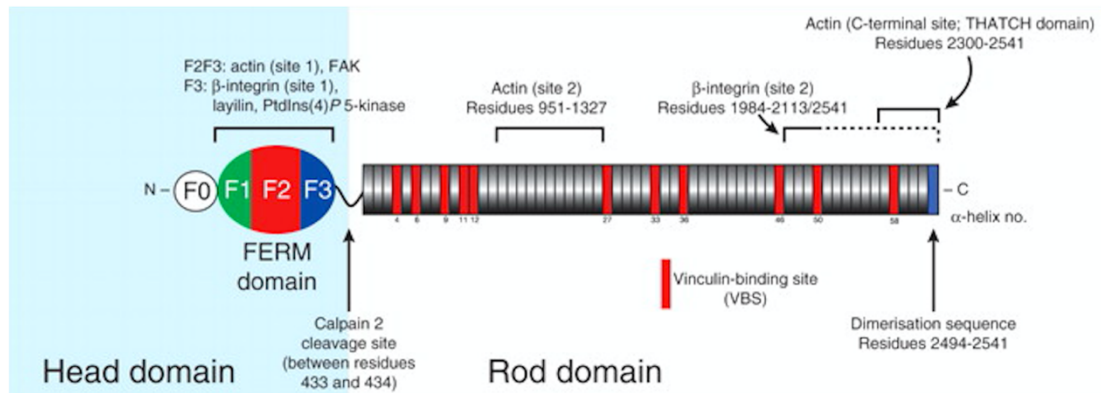


Figure 5.1 Schematic overview of talin-1 domains and binding sites of proteins. Talin consists of a globular N-terminal head region and a flexible rod domain. These two regions can be dissociated by the protease calpain 2. The head contains a FERM domain, divided into three subdomains: F1, F2 and F3. This region contains the binding sites for the cytoplasmic domains of β -integrins as well as for filamentous actin. The head also binds to two signaling molecules that regulate the dynamics of focal adhesions (FA). The talin rod contains an additional integrin-binding site, at least two actin-binding sites and several binding sites for vinculin, which itself has multiple partners. Picture adapted from [401].

Additional mechanistic studies conducted in our laboratory have revealed that even though adenovirus infection of human hepatocytes suppresses CYP3A4 activity, protein levels of the enzyme remain unchanged with respect to baseline [207]. A possible explanation for this phenomenon is that virus infection induces certain post-translational modifications (PTMs) of the CYP3A4 protein, such as phosphorylation, ubiquitination, and small ubiquitin-like modifiers (SUMOylation), rendering it inactive or with altered activity. Phosphorylation is an enzymatic process most often performed by kinases, which results in addition of an extraneous phosphate group, usually via an ester bond to specific serine and threonine amino acid residues of proteins. Phosphorylation also plays an essential role in regulating a variety of signaling pathways and cellular processes [403]. Ubiquitination involves the addition of the 76-amino acid protein ubiquitin. It starts with the attachment of a single ubiquitin molecule to a lysine residue on a substrate. The ubiquitin protein contains seven lysine residues, all of which can be further ubiquitinated, which gives rise to isopeptide-linked ubiquitin chains. This can then also be modified further through other post-translational mechanisms such as phosphorylation and SUMOylation [404]. Ubiquitination controls almost every biological process in the cell and depending on the type of ubiquitin modifier assigned to a given target, the ubiquitin molecules can assemble into many distinct conformations, each leading to a different outcome in the cell [405]. SUMOylation is a reversible post-translational protein modification that is involved in diverse cellular functions including cellular localization, altered protein activity, and in some cases the stability of the modified protein [406]. Any of these PTMs could result in changes in CYP3A4 activity without affecting the protein

levels of the enzyme. Thus, a secondary goal of this study was to determine if CYP3A4 changes during adenovirus infection could be the result of post-translational modification of the protein stimulated by the virus itself or through integrin/talin-1 mediated signaling processes.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Phosphate-buffered saline (PBS) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). Protogel[®] acrylamide was purchased from National Diagnostics (Atlanta, GA). Acetopromazine was purchased from Fort Dodge Laboratories (Atlanta, GA) and ketamine was purchased from Pfizer (New York, NY). Ketoconazole and rifampicin were purchased from Fisher Scientific (Pittsburgh, PA). Erythromycin was purchased from MP Biomedicals (Solon, OH), dexamethasone from BD Biosciences (San Jose, CA) and isoniazid from Acros Organics (Geel, Belgium). 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) was purchased from Gold Biotechnology (St. Louis, MO). 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 otherwise specified.

5.2.2 Recombinant adenovirus production

A first-generation recombinant adenovirus containing the beta-galactosidase transgene (AdlacZ) and a similar virus in which the RGD motif within the penton capsid protein was deleted via PCR-directed mutagenesis (Ad Δ RGD) [363] were amplified in human embryonic kidney (HEK) 293 cells and purified from secondary lysates as previously described [242, 364]. Virus purification was done by banding twice on cesium chloride gradients followed by desalting on Econo-Pac 10 DG disposable chromatography columns (BioRad, Hercules, CA) equilibrated with sterile 100 mM PBS (pH 7.4). Positive fractions were collected and the number of virus particles determined using the method of Maizel et al. [312] with the following formula: virus particles/ml = (absorbance at 260 nm) $\times$ (dilution factor) $\times 1.1 \times 10^{12}$.

5.2.3 Cell culture and RNA interference

HC-04 cells (human hepatocytes: MRA-975, MR4, ATCC[®], Manassas, VA) were grown in Dulbecco's modified Eagle's medium/Ham's F12 (50/50 ratio, Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, catalog number F0926, Lot number 17A412) and 2 mM L-glutamine (Hyclone, Logan, UT). All experiments were performed on HC-04 cells at passage six. HC-04 cells were seeded in either 6 well tissue culture dishes at a density of 1×10^5 cells per well or in 96 well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 6×10^3 cells per well, 48 hours prior to transfection. Cells in each well were then

transfected with 100 pmol (6 wells) or 5 pmol (96-wells) of small interfering RNA (siRNA) targeting talin-1 (sc-36610), integrin α_v (sc-29373), β_3 (sc-29375) and/or β_5 (sc-35680) (Santa Cruz Biotechnology, Dallas, TX) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Efficiency of the knockdown was confirmed by qRT-PCR and compared with expression levels in cells treated with a non-specific siRNA control (sc-37007, Santa Cruz Biotechnology). Twenty-four to 48 hours after transfection, cells were infected with a first-generation recombinant adenovirus containing the beta-galactosidase transgene (AdlacZ) at a multiplicity of infection (MOI) of 500 for 24-72 hours prior to assessing CYP3A4 activity using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit (Promega, Madison, WI). Normalization of cell numbers was achieved by using CellTiter-Glo® assay kit according to the manufacturer's instructions. Activity of CYP3A4 treated with negative control siRNA was $\pm 4.65\%$ of the activity of untreated HC-04 cells, which is similar to the range found in a normal control population [368].

5.2.4 Protein isolation and western blot

Cells were washed twice with ice-cold PBS prior to addition of 500 μ l of ice-cold lysis buffer (RIPA buffer, Thermo Scientific) containing 1% protease inhibitors (HALT Protease and Phosphatase Inhibitor Cocktail, Pierce). After incubation on ice for 5 minutes cells were lysed by extrusion 25 times through a 20-gauge needle (Becton Dickinson, Franklin Lakes, NJ) attached to a 1-ml syringe (Becton Dickinson) and placed on ice for an additional 40 minutes. Lysates were then cleared by centrifugation at 14,000 g for 15

minutes at 4°C and stored at –80°C. Fifty µg of protein were separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane (BioRad). Protein blots were blocked overnight at 4°C in blocking buffer containing 3% bovine serum albumin (BSA) in 0.05% TBS-T. Membranes were incubated for two hours with primary antibody followed by one hour in corresponding secondary antibody all from Santa Cruz Biotechnology (Table 5.1). Immune complexes were detected by chemiluminescence (SuperSignal™ West Pico Chemiluminescence Substrate, Fisher Scientific, Pittsburgh, PA). The intensity of protein bands was quantified relative to the signal obtained for an internal standard, β-actin on the same membrane using Kodak 1D image analysis software (Eastman Kodak Co, Rochester, NY).

Table 5.1 Primary and secondary antibodies used for western blot analysis

Primary Antibody and Dilution	Secondary Antibody and Dilution
Monoclonal mouse anti-talin-1 (sc-365875) Dilution 1:800	Goat anti-mouse IgG-HRP (sc-2005), dilution 1:8,000
Monoclonal mouse anti-CYP3A4 (sc-53850) Dilution 1:2000	Goat anti-mouse IgG-HRP (sc-2005), dilution 1:10,000
Monoclonal mouse anti-RXR α (sc-46659) Dilution 1:800	Goat anti-mouse IgG-HRP (sc-2005), dilution 1:10,000
Mouse monoclonal anti-PXR (sc-488403) Dilution 1:1000	Goat anti-mouse IgG-HRP (sc-2005), dilution 1:10,000
Polyclonal rabbit anti CAR1/2 antibody (sc-13065) Dilution 1:800	Goat anti-rabbit IgG-HRP (sc-2004), dilution 1:10,000
Mouse monoclonal anti- β -actin (sc-69879) Dilution 1:2000	Goat anti-mouse IgG-HRP (sc-2005), dilution 1:10,000

5.2.5 RNA Isolation and qRT-PCR

RNA was isolated from HC-04 cells using TRIzol[®] and reverse transcribed using the SuperScript[®] III first-strand synthesis system according to the manufacture's instructions. SYBR[®] GreenER[™] qPCR SuperMix (Invitrogen) was added to each sample before it was run on a ViiA7[™] Real-Time PCR system with the following cycling settings: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primer sequences for human Talin-1, CYP3A4, PXR, RXR α , CAR were 5'-TCT CCC AAA ATG CCA AGA AC-3' (forward) and 5'-TGG CTA TTG GGG TCA GAG AC-3' (reverse) [407], 5'-GAT TGA CTC TCA GAA TTC AAA AGA AAC TGA-3' (forward) and 5'-GGT GAG TGG CCA GTT CAT ACA TAA TG-3' (reverse) [318]; 5'-AGA AGG AGA TGA TCA TGT CCG A-3' (forward) and 5'-GTT TGT AGT TCC AGA CAC TGC C-3' (reverse) [369]; 5'-CCT TTC TCG GTC ATC AGC TC-3' (forward) and 5'-CTC GCA GCT GTA CAC TCC AT-3' (reverse) [240]; 5'-GCA AGG GTT TCT TCA GGA GAA C-3' (forward) and 5'-CTT CAC AGC TTC CAG CAA AGG-3' (reverse) [319]; 5'-AAT CTT CCA ATT GAG GAT ATC AC-3' (forward) and 5'-AAA ACA GCC AGT AGC AAC AAT-3' (reverse), respectively. All qRT-PCR data were analyzed by the established comparative threshold cycle (C_T) method [316].

5.2.6 Microarray evaluation of hepatic gene expression *in vitro*

HC-04 cells were seeded in 6-wells and transfected with control siRNA, talin-1

siRNA or integrin β 3 siRNA as described above. After 24 hours of transfection, cells were either infected with AdlacZ at a MOI of 500 for 24-hours or left uninfected. RNA was isolated using TRIzol[®] and cDNA reverse transcribed as described above. One μ g of cDNA was added to 19 μ l of 1X SYBR[®] GreenER[™] qPCR SuperMix (Invitrogen), which was added to each well of a 96-well TaqMan[™] Array Human Signal Transduction Pathways Microarray (ThermoFisher Scientific, Waltham, MA). Arrays were run on a ViiA7[™] Real-Time PCR system with the same cycling settings as described above. Data was analyzed according to the comparative threshold cycle method.

5.2.7 Chromatin immunoprecipitation (ChIP) assay

HC-04 cells were grown, transfected with talin-1 siRNA or control siRNA, and infected as described above. Chromatin immunoprecipitation (ChIP) was performed using ChIP-IT Express Enzymatic kit (Active Motif, Carlsbad, CA) according to the manufacturer's description. In brief, cells were incubated in formaldehyde to cross-link protein to DNA. Cells were then broken open and DNA was sheared by enzymatic shearing. Three μ g of primary antibody against PXR, CAR, or RXR α (Table 5.1) was added together with magnetic beads. Antibody-bound protein/DNA complexes were pelleted using a magnet and washed. Chromatin was eluted and separated from magnetic beads followed by reverse cross-linkage and digestion of protein with proteinase K. Isolated DNA was added to SYBR[®] GreenER[™] qPCR SuperMix (Invitrogen) and run at the

same settings as above using primers in Table 5.2 for different binding sites on the CYP3A4 promoter.

Table 5.2 Primers used to detect binding to prPXRE and dXREM sites on the CYP3A4 promoter by PXR, CAR and RXR α

Binding site	Forward Primer Reverse primer	Reference
prPXRE PXR	5'-ACA AGG GCA AGA GAG AGG CGA-3' 5'-AAGAGGCTTCTCCACTTGGA-3'	[408]
dXREM PXR	5'-GAG AGA TGG TTA CTT CCT TTC-3' 5'-TTA CCA TGT GCA CAT ATT ACC-3'	[408]
prPXRE CAR/RXR α	5'-GGC GAT TTA ATA GAT TTT ATG C-3' 5'-TGC TCT GCC TGC AGT TGG AA-3'	[409]
dXREM CAR/ RXR α	5'-CCC AAT TAA AGG TCA TAA A-3' 5'-CAG AAG TTC AGC TTG TGA ATT C-3'	[409]

5.2.8 Chemical suppression/induction of CYP3A4

Substances known to suppress or induce CYP3A4 activity were added to culture media after 24 hours of treatment with siRNA targeting talin-1, integrin α_v , integrin β_3 or integrin β_5 . 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%. CYP3A4 activity was assessed using a P450-Glo™ CYP3A4 Luciferin-IPA assay kit according to the manufacturer's instructions (Promega, Madison, WI).

5.2.9 Administration of adenoviral vectors

All procedures were approved by the Institutional Animal Care and Use Committee of 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. Male C57BL/6 mice (6-8 weeks, Jackson Labs, Bar Harbor, ME) were maintained in a 12-hour light/dark cycle environment with free access to standard mouse chow (Harlan, Indianapolis, IN) and deionized water. Following a seven-day acclimation period, a single dose of 1.5×10^{11} vp of AdlacZ or Ad Δ RGD in a volume of 100 μ l or 100 μ l of PBS was given to mice by tail vein injection. Animals (n = 5 per group) were sacrificed 24 and 48 hours after treatment. At this time, liver tissue was excised, rinsed in saline, snap frozen in liquid nitrogen and stored at -80°C prior to preparation of microsomes according to established protocols [365] and isolation of RNA for RT-PCR.

5.2.10 Immunoprecipitation of proteins

Five-hundred μg of total cellular protein from HC-04 cells or mouse microsomes was incubated with 10 μl of either a monoclonal mouse anti-CYP3A4 antibody (sc-53850) or anti-talin-1 antibody (sc-365875) for one hour at 4°C before adding 20 μl of protein A/G PLUS-Agarose Immunoprecipitation Reagent (sc-2003, Santa Cruz Biotechnology). Samples were incubated on a rotating device overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2,500 rpm for five minutes at 4°C and supernatant was discarded. Pellets were washed four times with one ml of RIPA buffer, each time repeating the centrifugation step. After the final wash, 40 μl of 1X electrophoresis buffer was added and samples were boiled for three minutes. This was followed by centrifugation as described above and 20 μl of the supernatant was separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to nitrocellulose membranes. Western blot protocol was followed as described above with the use of the antibodies described in Table 5.3.

Table 5.3 Antibodies and dilutions used in immunoprecipitation experiments

Primary Antibody and Dilution	Secondary Antibody and Dilution
Polyclonal rabbit anti-phospho-(Ser/Thr) Phe antibody (#9631, Cell Signaling Technology, Danvers, MA) Dilution 1:1000	Goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology), dilution 1:10,000
Monoclonal mouse anti-ubiquitin (sc-8017, Santa Cruz Biotechnology) Dilution 1:1000	Goat anti-mouse IgG-HRP (sc-2005, Santa Cruz Biotechnology), dilution 1:10,000
Polyclonal rabbit anti-SUMO-2/3 (#4971, Cell Signaling Technology) Dilution 1:1000	Goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology), dilution 1:10,000

5.2.11 Nuclear and cytoplasmic fractionation

Nuclear and cytoplasmic compartments were fractionated according to an established protocol [410]. In brief, HC-04 cells were infected with AdlacZ or Ad Δ RGD of an MOI of 500 for 96 hours. Cells were washed with ice-cold PBS, scraped off using a cell scraper, and centrifuged at 1,000xg for five minutes at 4°C. The pellet was resuspended in 500 μ l of cytoplasmic buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 0.1% triton-X100 and 1X HALT Protease and Phosphatase Inhibitor Cocktail, pH 7.8) and incubated for 30 min on ice followed by centrifugation at 3,500xg for five min at 4°C. The supernatant, which contained the cytoplasmic fraction, was saved at -80°C. The pellet containing the nuclear fraction was washed with 500 μ l of cytoplasmic buffer and the centrifugation step above was repeated. Five-hundred μ l of nuclear buffer (3.2 mM EDTA, 0.1% SDS) was added to the pellet followed by incubation on ice for 30 min. The lysate was then passed through a 21G needle ten times and cleared by centrifugation of 10,000 xg for 10 min at 4°C. Finally, the lysate was treated with 50 μ g/ml of DNase I (Invitrogen) for 20 min at 37°C and the nuclear fraction was stored at -80°C. Fifty μ g of protein was separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transferred to a nitrocellulose membrane as described above. Antibodies against PXR, CAR and RXR α were used as described in Table 5.1. Intensity of protein levels in nuclear extracts were quantified relative to signals obtained for lamin B (lamin B rabbit polyclonal antibody, 1:1,000 dilution, sc-20682) using a goat anti-rabbit IgG-HRP secondary antibody (1:10,000 dilution, sc-2004, Santa Cruz Biotechnology).

Levels in the cytoplasmic fraction were determined relative to β -actin (sc-69879, dilution 1:2000) using a goat anti-mouse IgG-HRP antibody (sc-2005, Santa Cruz Biotechnology).

5.2.12 Detection of infection of AdlacZ and Ad Δ RGD in HC-04 cells

Detection of AdlacZ was done by measuring β -galactosidase activity by incubating cells with the substrate X-gal (5-bromo-4-chloro-3-indolyl- β -galactoside) for 4 hours at 37°C in the dark. Cells infected with Ad Δ RGD were detected using the Adeno-X™ Rapid Titer kit (Clontech Laboratories, Inc., Mountain View, CA). Cells were examined with a Leica DM LB microscope (Leica Microsystems Inc., Buffalo Grove, IL) and were photographed using a Leica DFC 320 camera.

5.2.13 Statistical analysis

Statistical analysis of data was performed using SigmaStat (Systat Software Inc., San Jose, CA). Differences with respect to treatment were calculated using a one-way analysis of variance followed by a Bonferroni/Dunn *post hoc* test. Differences were determined to be significant when the probability of chance explaining the results was reduced to less than 5% ($p < 0.05$).

5.3 RESULTS

5.3.1 Effect of silencing talin-1 on hepatic CYP3A4 function and expression in the HC-04 cell line

To test our hypothesis that integrin receptors are involved in regulating hepatic CYP3A4 via interactions with intracellular proteins such as talin-1, HC-04 cells were transfected with siRNA targeting talin-1 to study the effect of reduced expression of talin-1 on CYP3A4 expression and function. Forty-eight hours after transfection, talin-1 gene expression was reduced by 2.3-fold compared to cells transfected with siRNA consisting of a scrambled sequence that does not lead to specific degradation of any cellular message (control, Figure 5.2A). At this same timepoint, protein levels of talin-1 were reduced by 60% compared to control cells (Figure 5.2B). This confirmed successful reduction in talin-1 mRNA and protein levels for at least 48 hours after silencing.

CYP3A4 mRNA and protein levels were also measured 48-hours post transfection. Silencing of talin-1 did not significantly impact CYP3A4 gene expression, producing only a 1.69-fold reduction in CYP3A4 expression compared to control siRNA (Figure 5.3A). Although CYP3A4 protein levels were increased by 33% when compared to control siRNA, this was found to not be a significant observation (Figure 5.3B, $P = 0.052$). CYP3A4 catalytic activity was measured at various timepoints for 72 hours after transfection (Figure 5.3C). Twenty-four hours after treatment with siRNA targeting either talin-1 or random sequence that does not lead to specific degradation, no significant change was seen in CYP3A4 activity. After 48 and 72 hours of transfection, CYP3A4 catalytic

activity was increased by 129% and 190% respectively, compared to cells treated with negative control siRNA (Figure 5.3C).

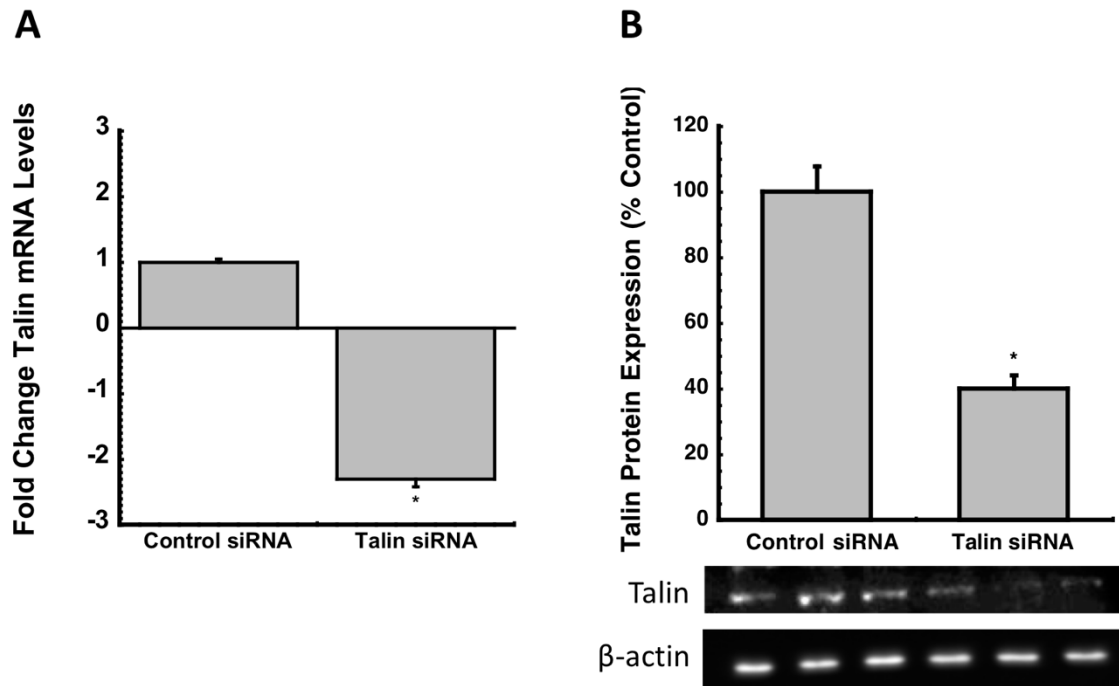


Figure 5.2 Transfection of HC-04 cells with siRNA targeting talin-1 significantly reduces gene and protein levels of talin-1. HC-04 cells were grown in 6-well plates until they reached 50% confluency. They were then transfected with siRNA using lipofectamine 2000. (A) Talin-1 gene expression and (B) protein levels of talin-1 were determined 48 hours post transfection with siRNA targeting talin-1 mRNA or siRNA consisting of scrambled sequence that does not lead to specific degradation of any cellular message (negative control). Data are reported as the means $\pm$ standard error of values obtained from 3 tissue culture plates per treatment group. * $p < 0.05$ with respect to negative control siRNA.

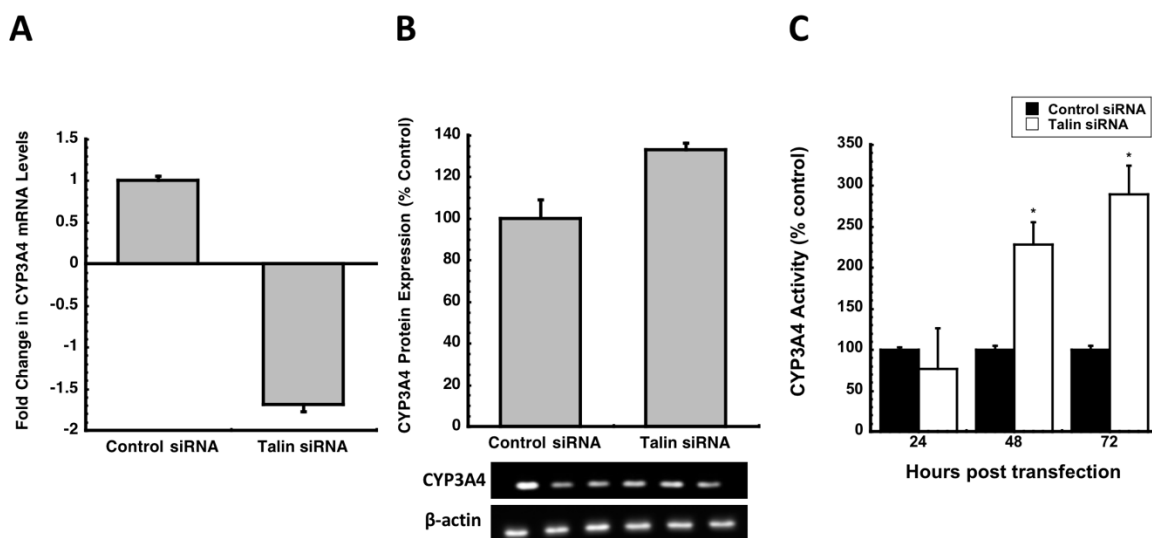


Figure 5.3 Talin-1 siRNA does not impact CYP3A4 gene and protein levels but does increase CYP3A4 activity in HC-04 cells. Forty-eight hours after transfection of HC-04 cells with siRNA targeting talin-1 mRNA or using a negative control siRNA, (A) fold change in CYP3A4 gene expression and (B) protein expression compared to control were determined using qRT-PCR and western blotting, respectively. (C) In addition, 24-72 hours post transfection CYP3A4 activity was determined using P450-Glo™ assay kit (Promega). Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per condition. * $p < 0.05$ with respect to siRNA control-treated cells.

5.3.2 Effect of silencing talin-1 on nuclear receptors regulating CYP3A4 *in vitro*

To understand how talin-1 is involved in CYP3A4 regulation, effect of silencing of talin-1 on nuclear receptors regulating CYP3A4 was determined. HC-04 cells were treated with talin-1 siRNA for 48 hours and mRNA and protein levels for nuclear receptors regulating CYP3A4, including pregnane X receptor (PXR) constant androstane receptor (CAR), and retinoid X receptor alpha (RXR α), were determined (Figure 5.4). There was no significant change in gene expression of PXR and RXR α (1.2 and 1.1-fold increase) or for CAR (1.2-fold decrease) compared to cells treated with negative control siRNA (Figure 5.4A). There was also no significant change in PXR and RXR α protein levels with 11% reduction for PXR (Figure 5.4B) and 7% increase for RXR α (Figure 5.4D). However, silencing of talin-1 resulted in a significant reduction (41%) in CAR protein levels compared to cells treated with negative control siRNA (Figure 5.4C). Since only CAR protein levels were significantly affected by the silencing of talin-1, another possibility for changes in CYP3A4 is that binding of the nuclear receptors to the CYP3A4 promoter could be impaired by removal of talin-1. To explore this mechanism, HC-04 cells were treated with talin-1 siRNA and a chromatin immunoprecipitation (ChIP) assay was performed using antibodies against CAR, PXR and RXR α and primers specific for two different binding sites on the CYP3A4 promoter; the distal xenobiotic responsive enhancer module (dXREM, marked with * in Figure 5.5A) and the proximal PXR responsive element (prPXRE, marked with ** in Figure 5.5A). Silencing of talin-1 did not alter the binding of RXR α to either the dXREM or prPXRE site of the CYP3A4 promoter (Figure 5.5B and 5.5C). Promoter occupancy by CAR and PXR increased at the dXREM site by 3.7-fold and

3.6-fold, respectively, in cells treated with talin-1 siRNA compared to those treated with negative control siRNA (Figure 5.5B). Both CAR and PXR promoter occupancy also increased at the prPXRE binding site, 4.4 and 4.8-fold, in cells with reduced expression of talin-1 compared to those with normal levels (Figure 5.5C).

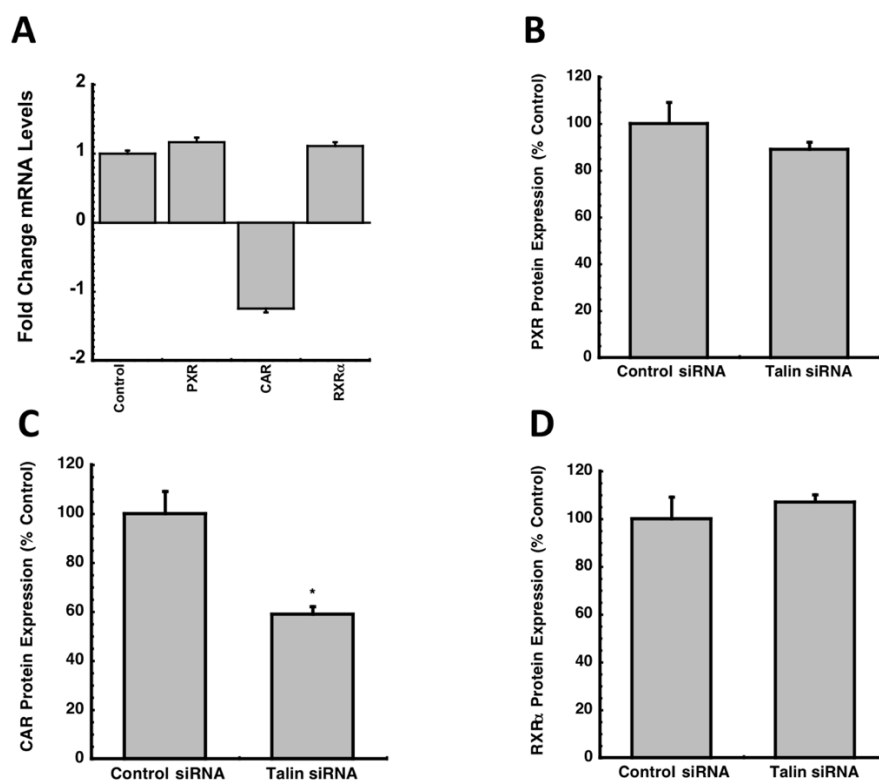


Figure 5.4 Silencing of talin-1 significantly reduces CAR protein expression in HC-04 cells. Gene expression levels of key regulators of CYP3A4 including (A) PXR, CAR, and RXR α and their respective protein levels (B-D) in HC-04 cells treated with talin-1 siRNA. HC-04 cells were transfected with talin-1-targeting siRNA or control siRNA for 48-hours. RNA was isolated, reverse-transcribed and the corresponding cDNA was analyzed using qRT-PCR and primers targeting PXR, CAR and RXR α . Protein lysates were isolated, separated, transferred to nitrocellulose membranes and analyzed using western blot with antibodies targeting PXR, CAR and RXR α . Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per condition. * $p < 0.05$ with respect to siRNA control-treated cells.

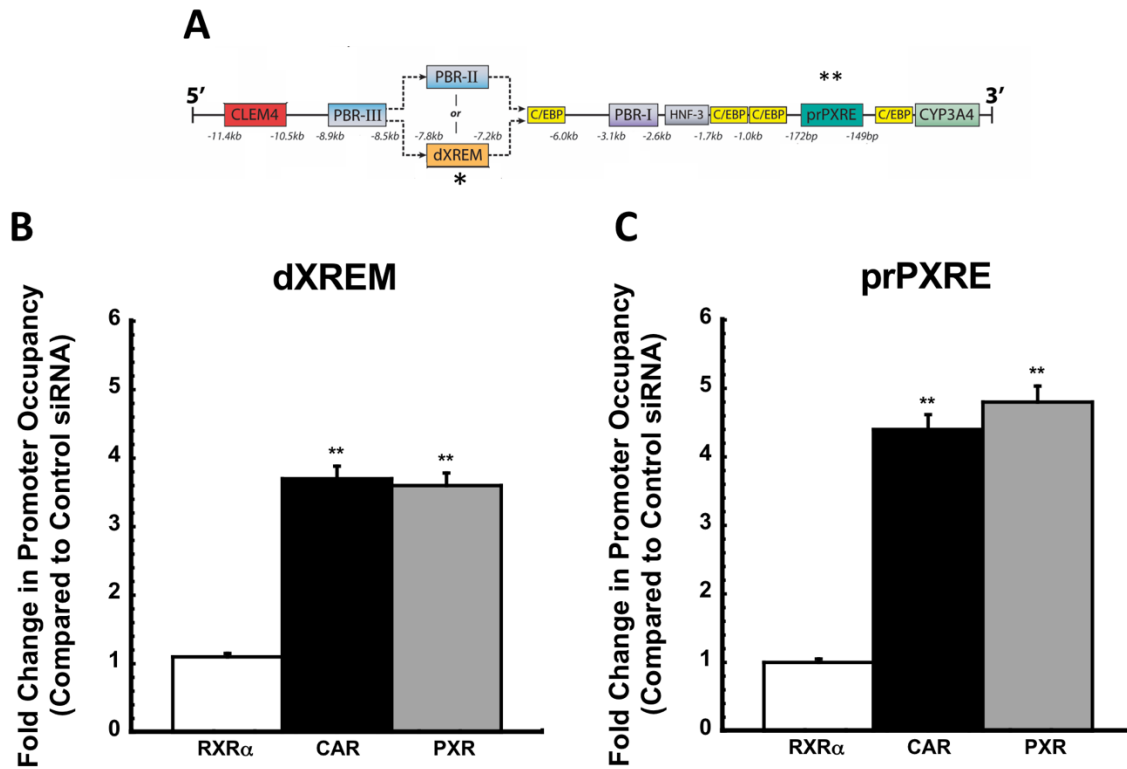


Figure 5.5 Significant increase in CAR and PXR binding to the dXREM and prPXRE sites of the CYP3A4 promoter after silencing talin-1. (A) Location of dXREM and prPXRE binding sites on the CYP3A4 promoter, where PXR, CAR and RXRα binds, indicated with a * and **, respectively. Degree of binding by RXRα, CAR and PXR to the (B) dXREM promoter site or (C) prPXRE site. HC-04 cells were treated with talin-1 siRNA for 48 hours followed by immunoprecipitation using antibodies against RXRα, CAR and PXR. Chromatin associated with the different nuclear receptors were analyzed using qRT-PCR with primers recognizing either the dXREM or prPXRE modules on the CYP3A4 promoter. Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per antibody. ** $p < 0.01$ with respect to siRNA control-treated cells.

5.3.3 Changes in human signal transduction pathways in cells with reduced talin-1 or integrin β_3 levels during adenovirus infection

In an effort to determine how integrin-mediated signaling pathways impact human CYP3A4 expression and function, HC-04 cells were treated with either negative control siRNA, integrin β_3 siRNA or talin-1 siRNA and infected 24 hours later with a first-generation recombinant adenovirus containing the beta-galactosidase transgene (AdlacZ) or left uninfected. Twenty-four hours after infection, RNA was isolated and global cell changes in gene expression was assessed with a human signal transduction array that monitored changes in 96 different genes. Analysis of array data revealed that, when comparing infected cells to uninfected cells with the same siRNA treatment, several genes were up- and downregulated by virus treatment (Table 5.4). Overall, treatment with siRNA targeting integrin β_3 or talin-1 had a more profound effect on gene expression compared to control siRNA. For several genes opposing effects were observed after treatment with β_3 siRNA compared to talin-1 siRNA. Since silencing of talin-1 is specific for inside-out signaling and integrin β_3 is involved in both outside-in and inside-out signaling, these results indicate that different responses are elicited depending on if the outside-in or inside-out pathway is activated by virus infection.

To study the impact of talin-1 and inside-out signaling by integrin receptors during virus infection, HC-04 cells were transfected with either control siRNA or talin-1 siRNA. Twenty-four hours later, cells were infected with AdlacZ for 24 hours before RNA was isolated. Analysis of array data (Table 5.5) revealed a significant decrease in two genes

involved in regulating ubiquitin in cells with reduced talin-1 levels compared to control cells. These two genes were UBC (ubiquitin C, >8-fold reduction) and BMP4 (bone morphogenic protein 4, 3-fold decrease). Additionally, KLK2 (kallikrein-related peptidase 2) was reduced by more than 8-fold in cells transfected with talin-1 siRNA compared to control cells. In this study, we have seen an effect on CYP3A4 activity that did not correlate to changes in the level of CYP3A4 gene or protein expression, which indicate that changes in CYP3A4 might occur at a post-translational level, which is supported by the array results.

Table 5.4 Gene expression of signal transduction pathways altered by integrin β 3 or talin-1 siRNA after virus infection (compared to uninfected cells with same siRNA treatment). The scale that was used to categorize the extent of increase or decrease in gene expression was as follow: a single plus or minus corresponds to an increase or decrease, respectively, of greater than or equal to 2 and less than 4 compared to control. Two plus or minus symbols identify a change of greater than or equal to 4 and less than 6, three symbols identify a change of greater than or equal to 6 and less than 8, and four plus or minus symbols identify a change in activity of 8-fold or greater. Change in gene expression less than 2-fold received a symbol of 0.

Target Name	Comment	Control siRNA	Integrin β 3 siRNA	Talin-1 siRNA
HPRT1	hypoxanthine phosphoribosyltransferase 1	0	++	0
GUSB	glucuronidase	0	++	0
ACTB	actin	0	+	0
B2M	beta-2-microglobulin	0	++	0
RPLP0	ribosomal protein	0	++	0
HMBS	hydroxymethylbilane synthase	0	++++	++++
TBP	TATA box binding protein	–	0	–
PGK1	phosphoglycerate kinase 1	+	++++	0
UBC	ubiquitin C	0	++	– – – –
PPIA	peptidylprolyl isomerase A (cyclophilin A)	0	+++	0
TFRC	transferrin receptor (p90)	0	+	++++

Table 5.4 Continued.

ATF2	activating transcription factor 2	0	+	–
BAX	BCL2-associated X protein	0	++	0
BCL2	B-cell CLL/lymphoma 2	0	+++	++++
BCL2A1	BCL2-related protein A1	0	+++	+
BCL2L1	BCL2-like 1	0	+	++++
BIRC2	baculoviral IAP repeat-containing 2	0	+	++++
BIRC3	baculoviral IAP repeat-containing 3	0	++++	0
BMP2	bone morphogenetic protein 2	–	+	– – – –
BMP4	bone morphogenetic protein 4	0	+	+
BRCA1	breast cancer 1	–	+	–
CCL20	chemokine (C-C motif) ligand 20	0	+	0
CCND1	cyclin D1	–	0	– – – –
CDK2	cyclin-dependent kinase 2	0	+	– – – –
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)	0	++	–
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27)	0	+	– – – –
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma	0	+	+
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15)	– –	– – – –	++++
CEBPB	CCAAT/enhancer binding protein (C/EBP)	– –	0	– – – –
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	– –	0	– – – –
CYP19A1	cytochrome P450	0	++	+
EGR1	early growth response 1	– – – –	– – – –	– – – –
FAS	Fas (TNF receptor superfamily)	–	0	– – – –
FASN	fatty acid synthase	0	++	0

Table 5.4 Continued.

FN1	fibronectin 1	0	+	--
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0	+	----
FOXA2	forkhead box A2	0	++++	0
GADD45A	growth arrest and DNA-damage-inducible	–	0	–
GREB1	GREB1 protein	0	++	0
GYS1	glycogen synthase 1 (muscle)	0	+++	0
HK2	hexokinase 2	0	+	0
HOXA1	homeobox A1	0	++++	0
HSF1	heat shock transcription factor 1	–	+	----
HSPB1	heat shock 27kDa protein 1	–	0	0
ICAM1	intercellular adhesion molecule 1	–	0	++
IGFBP3	insulin-like growth factor binding protein 3	0	++++	–
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells	–	0	0
IL4R	interleukin 4 receptor	–	0	–
IL8	interleukin 8	---	0	---
IRF1	interferon regulatory factor 1	0	+	–
JUN	jun oncogene	0	+	–
KLK2	kallikrein-related peptidase 2	0	0	----
LTA	lymphotoxin alpha (TNF superfamily)	–	0	0
MDM2	Mdm2 p53 binding protein homolog (mouse)	--	0	–
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	---	0	---
NAIP	NLR family	+	++++	++++
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0	0	–

Table 5.4 Continued.

NOS2	nitric oxide synthase 2	0	+++	+
NRIP1	nuclear receptor interacting protein 1	–	0	–
ODC1	ornithine decarboxylase 1	0	0	– – – –
PECAM1	platelet/endothelial cell adhesion molecule	0	++	0
PPARG	peroxisome proliferator-activated receptor gamma	0	++	0
PRKCA	protein kinase C	0	+	0
PRKCE	protein kinase C	–	0	– –
PTCH1	patched homolog 1 (Drosophila)	0	++++	++++
RBP1	retinol binding protein 1	0	+	+
SELPLG	selectin P ligand	0	+	0
TANK	TRAF family member-associated NFKB activator	–	0	0
TCF7	transcription factor 7 (T-cell specific)	–	0	– – – –
TERT	telomerase reverse transcriptase	0	+	+
TNF	tumor necrosis factor (TNF superfamily)	– – –	0	0
TP53	tumor protein p53	0	+	0
TP53I3	tumor protein p53 inducible protein 3	–	+	0
VCAM1	vascular cell adhesion molecule 1	0	– – – –	– – – –
VEGFA	vascular endothelial growth factor A	0	++	0
WNT1	wingless-type MMTV integration site family	–	+	0

Table 5.5 Gene expression of signal transduction pathways altered by virus infection in talin-1-silenced cells (compared to control-siRNA infected cells). Bolded genes are those that were significantly different in cells transfected with talin-1 siRNA compared to those transfected with control siRNA, both infected with AdlacZ. A single plus corresponds to an increase compared to the control of greater than or equal to 2 and less than 4, two pluses identify an increase of greater than or equal to 4 and less than 6, three pluses identify an increase of greater than or equal to 6 and less than 8 while four pluses identify an increase of 8x or greater. Likewise, a single minus corresponds to a decrease of greater than or equal to 2 and less than 4, two minuses identify a decrease of greater than or equal to 4 and less than 6, three pluses identify a decrease of greater than or equal to 6 and less than 8 while four minuses identify a decrease of 8-fold or greater compared to the control. Change in gene expression less than 2-fold received a 0 symbol.

Target Name	Comment	Talin-1 siRNA
HPRT1	hypoxanthine phosphoribosyltransferase 1	0
GUSB	glucuronidase	0
ACTB	actin	0
B2M	beta-2-microglobulin	0
RPLP0	ribosomal protein	0
HMBS	hydroxymethylbilane synthase	0
TBP	TATA box binding protein	0

Table 5.5 Continued.

PGK1	phosphoglycerate kinase 1	0
UBC	ubiquitin C	-----
PPIA	peptidylprolyl isomerase A (cyclophilin A)	0
TFRC	transferrin receptor (p90)	0
ATF2	activating transcription factor 2	0
BAX	BCL2-associated X protein	0
BCL2	B-cell CLL/lymphoma 2	0
BCL2A1	BCL2-related protein A1	0
BCL2L1	BCL2-like 1	0
BIRC2	baculoviral IAP repeat-containing 2	0
BIRC3	baculoviral IAP repeat-containing 3	—
BMP4	bone morphogenetic protein 4	0
BRCA1	breast cancer 1	0
CCL20	chemokine (C-C motif) ligand 20	0
CCND1	cyclin D1	0
CDK2	cyclin-dependent kinase 2	0
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)	0
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27)	0
CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma)	0
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15)	0
CEBPB	CCAAT/enhancer binding protein (C/EBP)	0
CYP19A1	cytochrome P450 19A1	0
EGR1	early growth response 1	0

Table 5.5 Continued.

FAS	Fas (TNF receptor superfamily)	0
FASLG	Fas ligand (TNF superfamily)	0
FASN	fatty acid synthase	0
FN1	fibronectin 1	0
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0
FOXA2	forkhead box A2	0
GADD45A	growth arrest and DNA-damage-inducible	0
GREB1	GREB1 protein	0
GYS1	glycogen synthase 1 (muscle)	0
HK2	hexokinase 2	0
HOXA1	homeobox A1	0
HSF1	heat shock transcription factor 1	0
HSPB1	heat shock 27kDa protein 1	0
ICAM1	intercellular adhesion molecule 1	0
IGFBP3	insulin-like growth factor binding protein 3	0
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells	0
IL4R	interleukin 4 receptor	0
IL8	interleukin 8	0
IRF1	interferon regulatory factor 1	0
JUN	jun oncogene	0
KLK2	kallikrein-related peptidase 2	----
LTA	lymphotoxin alpha (TNF superfamily)	0
MDM2	Mdm2 p53 binding protein homolog (mouse)	0

Table 5.5 Continued.

MMP7	matrix metalloproteinase 7 (matrilysin)	0
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	0
NAIP	NLR family	0
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0
NOS2	nitric oxide synthase 2	0
NRIP1	nuclear receptor interacting protein 1	0
ODC1	ornithine decarboxylase 1	0
PECAM1	platelet/endothelial cell adhesion molecule	0
PPARG	peroxisome proliferator-activated receptor gamma	0
PRKCA	protein kinase C	0
PRKCE	protein kinase C	0
PTCH1	patched homolog 1 (Drosophila)	0
RBP1	retinol binding protein 1	0
SELPLG	selectin P ligand	0
TANK	TRAF family member-associated NFKB activator	0
TCF7	transcription factor 7 (T-cell specific)	0
TERT	telomerase reverse transcriptase	0
TNF	tumor necrosis factor (TNF superfamily)	0
TP53	tumor protein p53	0
TP53I3	tumor protein p53 inducible protein 3	0
VEGFA	vascular endothelial growth factor A	0
WNT1	wingless-type MMTV integration site family	0

5.3.4 Effect of adenovirus infection on phosphorylation, ubiquitination and SUMOylation on whole cell lysate of HC-04 cells

The gene arrays indicated that genes involved in post-translational modifications (PTMs) of proteins are downregulated in cells with reduced talin-1 expression that are infected with adenovirus. As such, we decided to look at three of the most common PTMs, phosphorylation, ubiquitination and small ubiquitin-like modifiers (SUMO)ylation, to determine how each mechanism of post-translational modification impacts CYP3A4 during virus infection. HC-04 cells were infected for 96 hours with an MOI of 0, 100, 500 or 1000. A general trend of increased activity of post-translational modification of proteins was observed for each PTM studied (Figure 5.6). With increased amount of virus, the general pattern was that more cellular proteins were modified, indicating that post-translational modification is dose dependent. For phosphorylation, one major band between 100 and 140 kDa stood out as being phosphorylated and affected by virus infection (Figure 5.6A). For both ubiquitination and SUMOylation, bands that were in the size range of 50 kDa, which is the size of CYP3A4, PXR, CAR and RXR α , were seen to be affected by these two PTMs (Figure 5.6B and 5.6C). Hence remaining studies focused on changes in ubiquitin and SUMO patterns to assess whether these changes were applicable to CYP3A4 specifically.

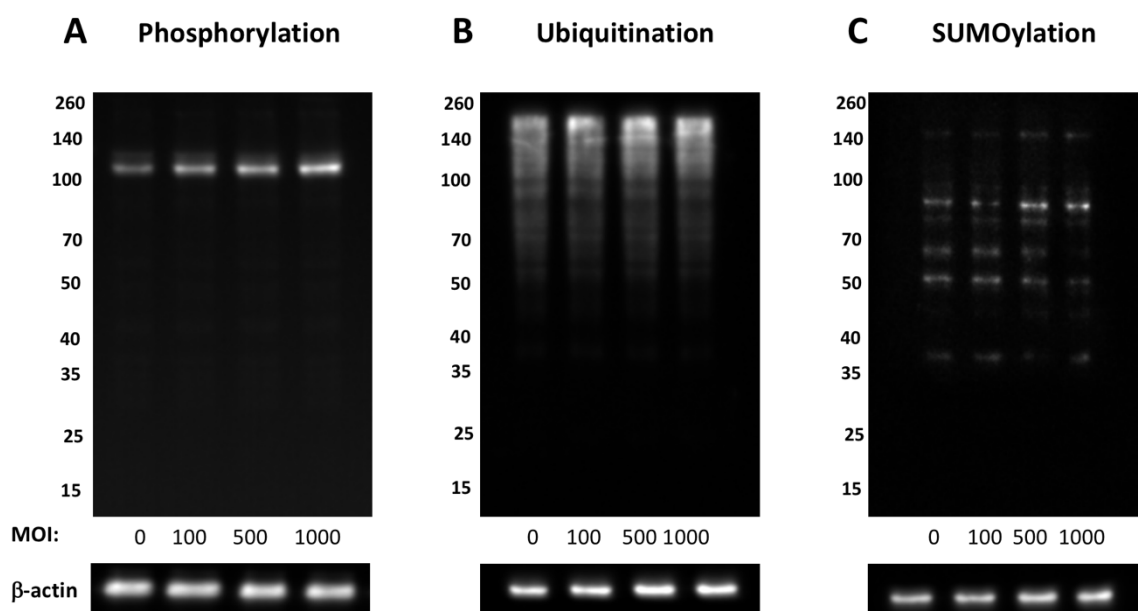


Figure 5.6 Adenovirus infection increases phosphorylation, ubiquitination and SUMOylation patterns of whole cell lysates from the HC-04 cell line. (A) Phosphorylation, (B) ubiquitination and (C) SUMOylation patterns of hepatic proteins after 96-hours of infection. Following 96-hours of infection with four different MOIs of adenovirus (MOI of 0, 100, 500 and 1000), whole cell lysates were separated on gels and protein transferred to nitrocellulose membranes. Western blot was performed with antibodies targeting phosphorylation sites, ubiquitin and SUMOylation to determine if virus infection impacts general patterns of PTMs of hepatic proteins. Loading control was performed using an antibody against β -actin. Representative blots are shown for PTMs and β -actin.

5.3.5 Effect of adenovirus infection on ubiquitination and SUMOylation of CYP3A4 *in vitro*

Changes in PTMs of CYP3A4 were assessed 96-hours post infection with AdlacZ at various MOIs by immunoprecipitation of CYP3A4 and western blot analysis using antibodies against ubiquitin or SUMO-2/3. CYP3A4 SUMOylation was significantly increased by 554%, 918% and 2337% for an MOI of 100, 500 and 10000, respectively, compared to uninfected cells (Figure 5.7A). At an MOI of 100 no change was seen in ubiquitin levels of CYP3A4 but at MOIs of 500 and 1000 ubiquitination of CYP3A4 increased by 71% and 216%, respectively, compared to uninfected control cells (Figure 5.7B). A general trend of reduced CYP3A4 protein levels was seen with increased amounts of virus for either PTM (Figure 5.7).

5.3.6 Impact of silencing of talin-1 on SUMOylation and ubiquitination of CYP3A4 in HC-04 cells

To confirm whether the observed changes in ubiquitin and SUMO patterns of the CYP3A4 protein are induced through a talin-1 mediated or inside-out integrin mediated mechanism, HC-04 cells were infected with AdlacZ 24 hours after treatment with siRNA targeting talin-1. Virus infection alone caused a significant increase in ubiquitination of CYP3A4 using negative control scrambled siRNA (147% compared to uninfected cells, Figure 5.8A). The amount of ubiquitinated CYP3A4 was increased further to 160% when talin-1 was silenced (Figure 5.8A). SUMOylation of CYP3A4 was also increased in virus

infected cells treated with negative control siRNA ($132\% \pm 16\%$) and this was also increased further with siRNA targeting talin-1 ($167\% \pm 18\%$; Figure 5.8B), however these were found not to be statistically significant ($P = 0.215$).

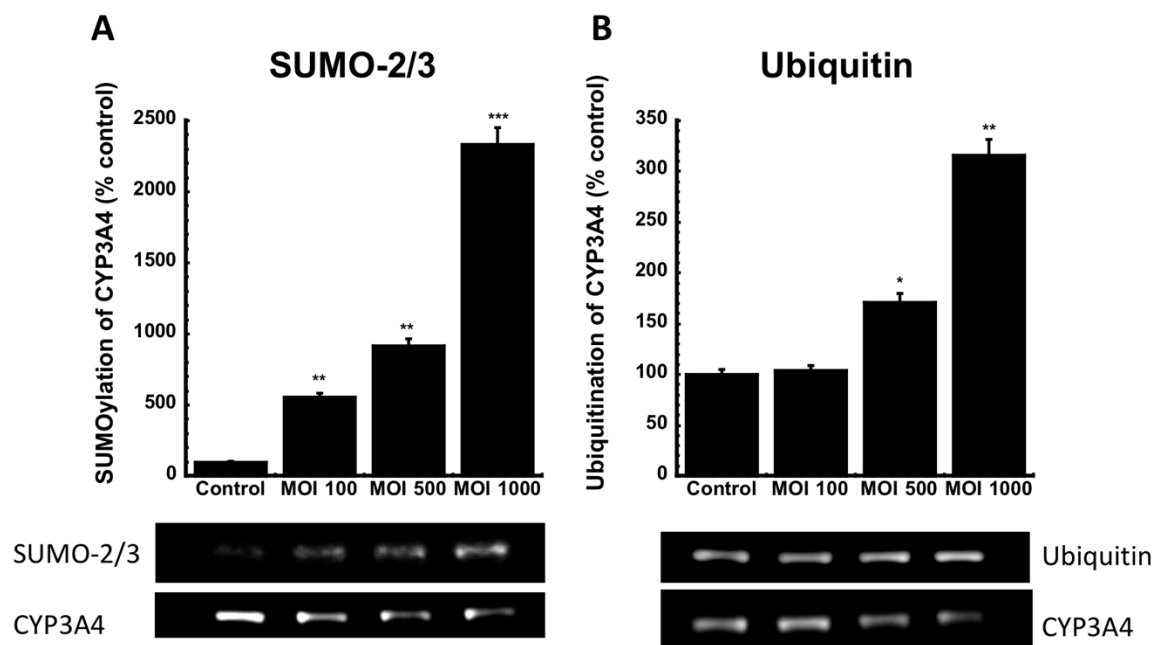


Figure 5.7 SUMOylation and ubiquitination of CYP3A4 is significantly increased by adenovirus infection in vitro. (A) SUMOylation or (B) ubiquitination of the CYP3A4 protein after 96-hours of adenovirus at a MOI of 0, 100, 500 or 1000. HC-04 cells were infected with AdlacZ at MOIs 0, 100, 500 or 1000 for 96-hours when protein lysates were collected, immunoprecipitated using an anti-CYP3A4 antibody, immunoprecipitated proteins were separated on gels and then transferred onto nitrocellulose membranes. Antibodies against SUMO-2/3 or ubiquitin were used to detect patterns of these two PTMs. Membranes were stripped and reprobed with an antibody against CYP3A4 to normalize observed levels of PTMs to amount of CYP3A4. Results are reported as the means $\pm$ standard error of values obtained from 3 culture plates per antibody. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ with respect to uninfected cells.

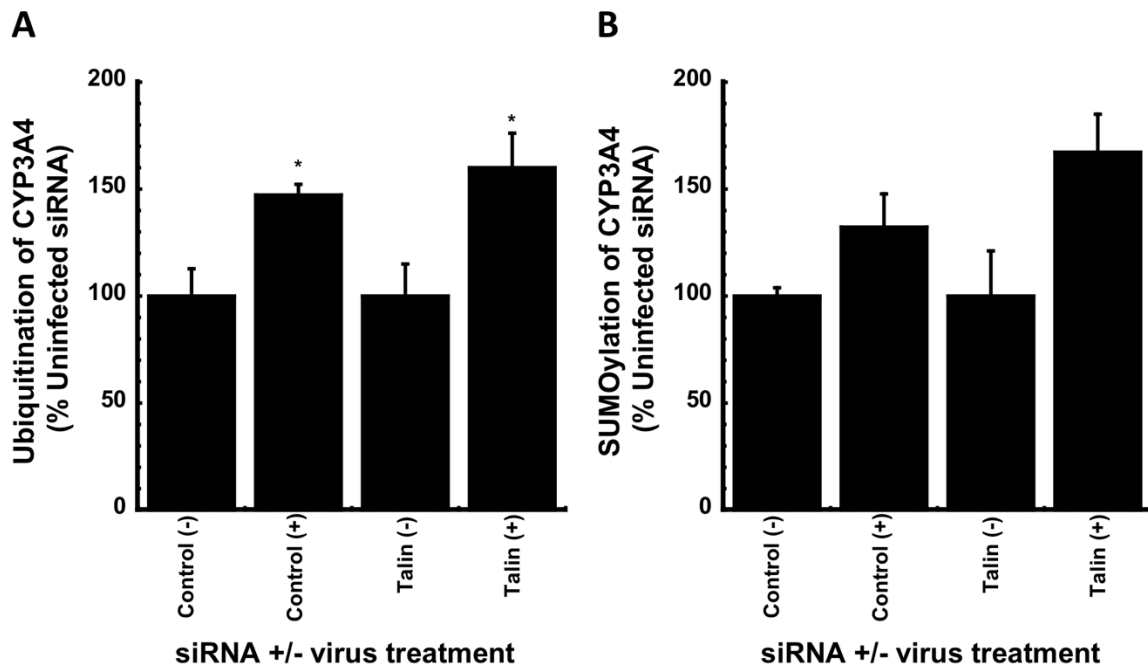


Figure 5.8 Silencing of talin-1 and adenovirus infection caused increased ubiquitination of CYP3A4 in HC-04 cells. (A) Ubiquitination and (B) SUMOylation of CYP3A4 in HC-04 cells transfected with control siRNA or siRNA targeting talin-1 for 24 hours followed by AdlacZ infection for 24 hours. Control cells were transfected for 48 hours but not infected with virus. Protein lysates were isolated following the treatment with siRNA and adenovirus, immunoprecipitation was performed using an anti-CYP3A4 antibody, immunoprecipitates were separated on a 12% gel, protein transferred to nitrocellulose membranes and western blot performed using antibodies against ubiquitin, SUMO-2/3 and to normalize against levels of CYP3A4, an anti-CYP3A4 antibody was used.

5.3.7 Impact of AdlacZ or Ad Δ RGD infection on ubiquitination of CYP3A4 *in vivo*

To test how engagement of integrin receptors via the outside-in mechanism during adenovirus infection impacts ubiquitination patterns of the CYP3A4 protein, C57BL/6 mice were infected for 24 or 48 hours with AdlacZ or a similar virus in which the RGD motif within the penton capsid protein was deleted via PCR-directed mutagenesis (Ad Δ RGD) [363]. At both 24 and 48-hours post infection with Ad Δ RGD ubiquitination of hepatic CYP3A was decreased, 37% and 46% respectively, compared to saline controls (Figure 5.9A and 5.9B). Infection with AdlacZ on the other hand did not impact the ubiquitination status of CYP3A4 at either 24 or 48 hours post infection compared to saline treated mice (Figure 5.9A and 5.9B).

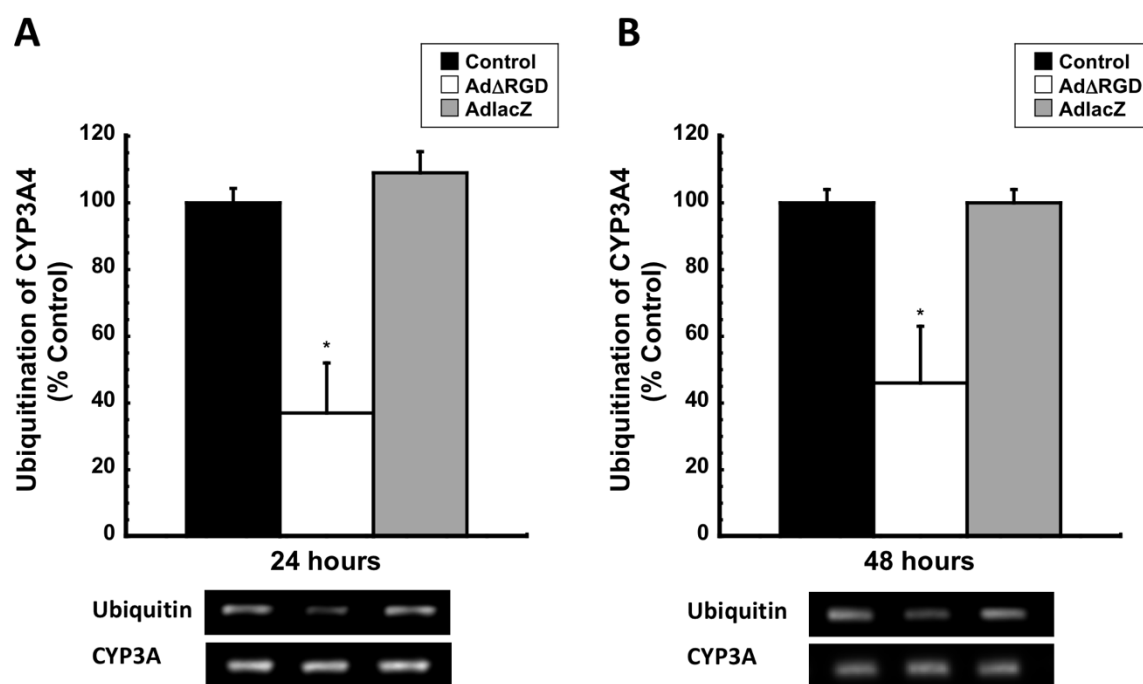


Figure 5.9 Infection of mice with an adenovirus without the ability to bind to integrin receptors causes decreased ubiquitination of CYP3A. Mice were infected with either AdlacZ, AdΔRGD or vehicle control (PBS) for (A) 24 hours or (B) 48 hours and level of ubiquitination of CYP3A was determined. Following 24 and 48 hours of infection, the liver was excised and microsomes were prepared and immunoprecipitated using an anti-CYP3A antibody. Protein lysate was separated on a 12% gel and transferred to nitrocellulose membranes and probed with antibodies against ubiquitin followed by an antibody against CYP3A to normalize ubiquitin levels to the amount of CYP3A present. Representative blots are shown below each figure, with the following loading order: PBS control, AdΔRGD infected mice and AdlacZ infected mice.

5.3.8 Effect on PXR, CAR and RXR α nuclear and cytoplasmic localization during AdlacZ or Ad Δ RGD infection *in vitro*

Since such different patterns in ubiquitination of CYP3A4 was seen for Ad Δ RGD and AdlacZ we investigated whether engagement of integrins would affect how CAR, PXR and RXR α is translocated from the cytoplasm to the nucleus. A drastic increase in RXR α protein levels were seen in the nuclear fraction of cells infected with Ad Δ RGD, 384% of control ($P = 0.0006$), while a trend of decreasing levels was observed in AdlacZ-infected cells (Figure 5.10A). An additional band above the RXR α band was observed only in cells infected with Ad Δ RGD, suggesting that the RXR α protein located in the nucleus of cells infected with Ad Δ RGD may be modified in some manner (Figure 5.10A). Significantly lower levels ($10\% \pm 14\%$, $P = 0.016$) of RXR α were observed in the cytoplasmic fraction in cells infected with Ad Δ RGD (Figure 5.10B). A trend of decreasing levels of RXR α in the cytoplasmic fraction was seen in cells infected with AdlacZ ($55\% \pm 17\%$, $P = 0.061$) compared to uninfected control cells (Figure 5.10B). Nuclear levels of the CAR protein were increased by 260% in cells infected with Ad Δ RGD and a trend of increased levels were also seen in AdlacZ-infected cells ($144\% \pm 22\%$, $P = 0.058$; Figure 5.10C). Cytoplasmic CAR protein levels were increased by 68% in cells infected with AdlacZ while no statistically significant change was seen after infection with Ad Δ RGD, although a trend of decreased levels of CAR was observed ($61\% \pm 16\%$, $P = 0.057$; Figure 5.10 D). Both Ad Δ RGD and AdlacZ infection reduced the level of PXR in the nuclear fraction, by

43% and 56% reduction respectively (Figure 5.10E). Cytoplasmic PXR protein levels were unaffected by Ad Δ RGD infection while AdlacZ infection caused a 35% decrease compared to uninfected cells (Figure 5.10F).

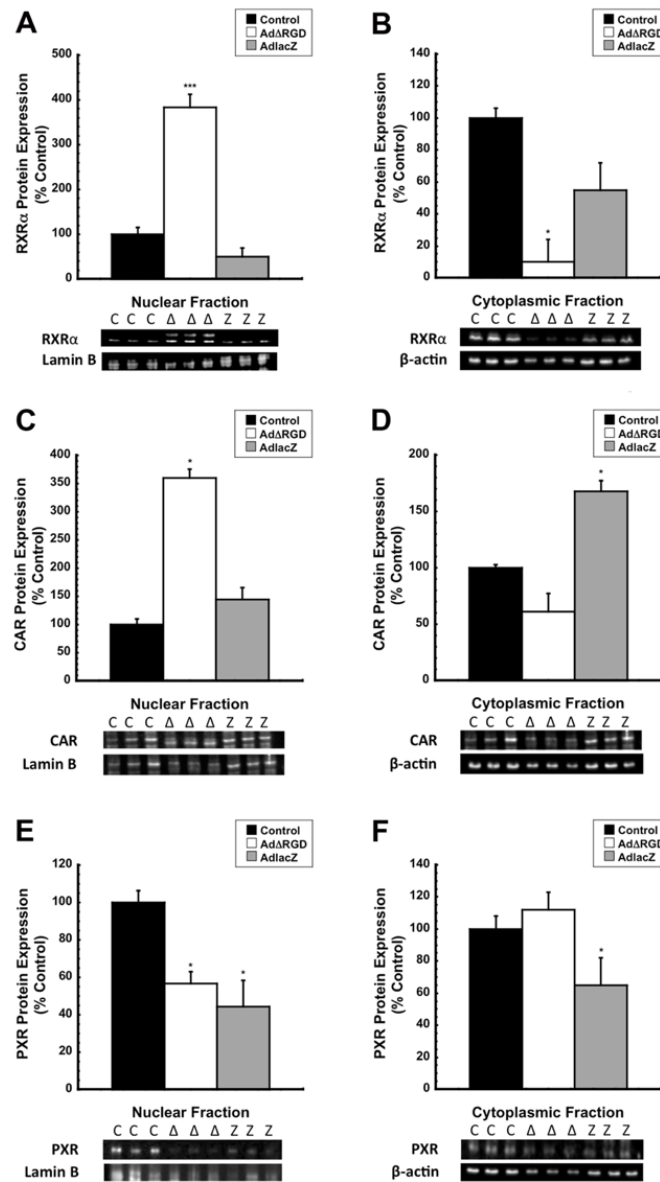


Figure 5.10 Localization of RXR α , CAR and PXR in the nucleus and cytoplasm is affected by AdlacZ and Ad Δ RGD in different manners *in vitro*. HC-04 cells were infected with AdlacZ or Ad Δ RGD, with an MOI of 500. Nuclear fractions were analyzed for levels of (A) RXR α , (C) CAR and (E) PXR protein levels. Similarly, protein levels of (B) RXR α , (D) CAR and (F) PXR were determined in the cytoplasmic extract.

5.3.9 Effect on SUMOylation of RXR α by AdlacZ and Ad Δ RGD infection of HC-04 cells

Since a double band was observed for RXR α in the nuclear fraction of cells infected with Ad Δ RGD we investigated this further to determine if this was due to SUMOylation since this is a known PTM of RXR α [411]. Ninety-six hours after infection with AdlacZ or Ad Δ RGD (MOI of 500), RXR α was immunoprecipitated from the nuclear fraction of virus infected and control uninfected cells. Immunoprecipitated protein was separated on an SDS-gel and then transferred to nitrocellulose membranes. An antibody against SUMO-2/3 was used to detect levels of SUMOylation and an RXR α antibody was used to confirm levels of RXR α . Significantly higher levels of RXR α were found in the nuclear fraction, which we had previously seen (Figure 5.10B), for cells infected with Ad Δ RGD compared to uninfected cells (Figure 5.11A). SUMO-2/3 levels were detected in a higher ratio in cells infected with Ad Δ RGD, 128% increase compared to uninfected cells, and unaffected by AdlacZ infection (Figure 5.11A). To confirm that the two different viruses are capable of establishing comparable levels of infection in HC-04 cells, an X-gal stain was performed to determine the proportion of the population that was infected with the AdlacZ virus (Figure 5.11B) and an adenovirus titer kit was used to do the same for the Ad Δ RGD virus (Figure 5.11C). At an MOI of 500 both viruses infected all cells, confirming that the difference seen in SUMOylation patterns of RXR α is not due to differences in infectivity between the two viruses.

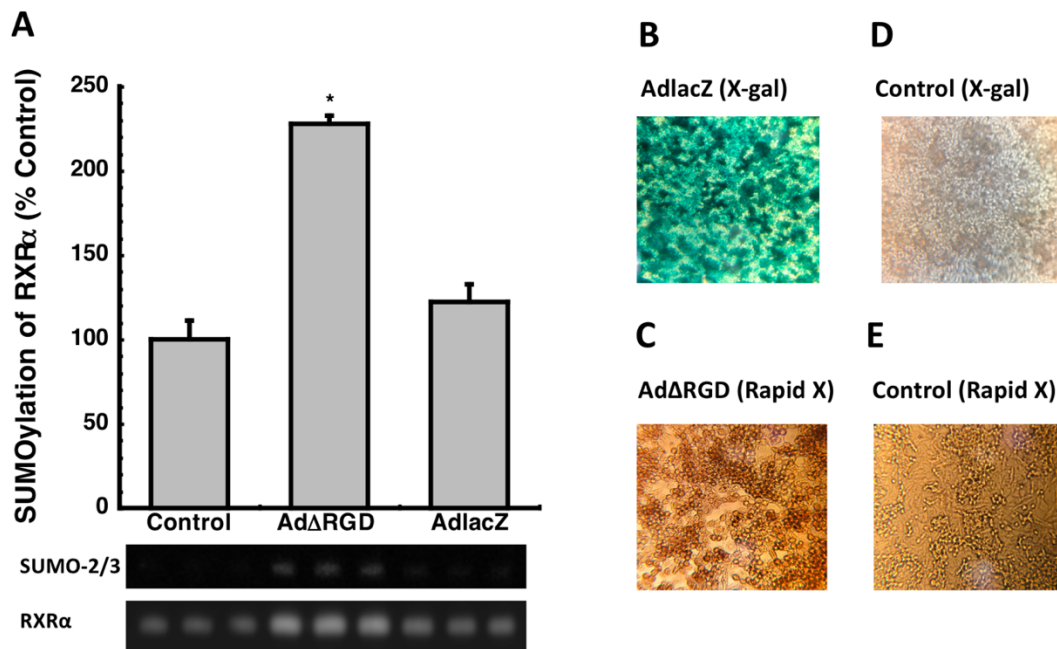


Figure 5.11 SUMOylation of RXR α in the nuclear fraction increased with Ad Δ RGD infection but not AdlacZ despite similar levels of infectivity in HC-04 cells. (A) Infection with either AdlacZ or Ad Δ RGD for 96-hours followed by extraction of the nuclear protein fraction and immunoprecipitation of these proteins using an antibody against RXR α . Immunoprecipitate was separated and transferred onto a nitrocellulose membrane, which was probed with an anti-SUMO-2/3 antibody. Equivalency of infectivity of the AdlacZ and Ad Δ RGD virus were confirmed by staining respective cultures with (B) an X-gal stain and (C) and Adeno-XTM Rapid Titer kit. Uninfected cells were also stained with (D) X-gal stain and (E) Adeno-XTM Rapid Titer kit to assess background signal that can be attributed to each staining method. Original magnification: 20 $\times$.

5.4 DISCUSSION

Integrin receptors are essential adhesion receptors that regulate diverse cellular functions involving cell migration, growth, differentiation and apoptosis [222]. Numerous pathogens have evolved to express adhesive proteins to directly or indirectly associate with integrins [412]. One such pathogen is the adenovirus, which expresses RGD peptides on its penton base proteins. These residues interact with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors promoting cell entry, signaling and endosomal escape [360]. From previous research, we have found that engagement of integrin receptors by adenovirus significantly suppresses CYP3A4 and that this effect is eliminated when the β -subunit of the integrin receptor is silenced. However, this was not seen for the α -subunit [207]. Hence, our intention with this study is to focus on the main protein that regulates integrin activation via the β -subunit: the intracellular protein talin-1.

Talin-1 exists in two different forms: a globular and an extended conformation. Activation of phosphatidylinositol phosphate kinase type I γ (PIPKI γ) by an extracellular agonist increases PIP2 production, which can bind to talin-1, resulting in release of talin-1 from its autoinhibited state. Activated talin-1 interacts with the cytoplasmic domain of the β -subunit of the integrin receptor via its F3 subunit, disrupting a salt bridge between the α - and β -integrin subunits, leading to separation of their cytoplasmic domains and activation of integrins from the inside out. For outside-in signaling, integrins are activated by binding to extracellular ligands, which results in clustering and changes in the ligand-binding affinity of integrins on the cell surface. Activation causes changes in the tertiary and

quaternary structure of integrins, which propagate across the membrane to activate cytoplasmic kinase- and cytoskeletal-signaling cascades. Adenovirus infection causes integrin clustering in a similar manner, which is required for virus internalization [413]. Signaling via integrins occurs mainly via recruitment and activation of the Src-family of kinases (SFKs) through the α subunit via the outside-in pathway but also occurs via the β subunit by recruitment of talin-1 and focal adhesion kinase (FAK) via the inside-out pathway. Via Src phosphorylation of p130^{cas} and paxillin, the Crk complex is recruited to FAK and Rac is activated resulting in activation of Pac, JNK, and ERK1/2. Recruitment and activation of paxillin results in activation of PI3K, which then activates NF κ B and Casp3. Pathways that overlap between CYP3A4 regulation and integrin signaling include PKC, PKA, JNK, PI3K and NF κ B (Table 4.1, Figure 5.12).

To understand the role of talin-1, siRNA was used to reduce protein levels of talin-1 in a human hepatocyte cell line, HC-04. Silencing of talin-1 resulted in a 2.3-fold reduction in talin-1 gene expression and a 60% reduction in protein levels of talin-1 (Figure 5.2). This is a partial knockdown of talin-1, as a full knockout of talin-1 results in loss of cell adhesion and embryonic death at embryonic day E8.5 in mice [386]. The knockdown of talin-1 had no impact on CYP3A4 gene or protein levels 48 hours post transfection (Figure 5.3A and 5.3B). However, with this partial knockdown, CYP3A4 activity was significantly increased during this time (Figure 5.3C). This was particularly surprising

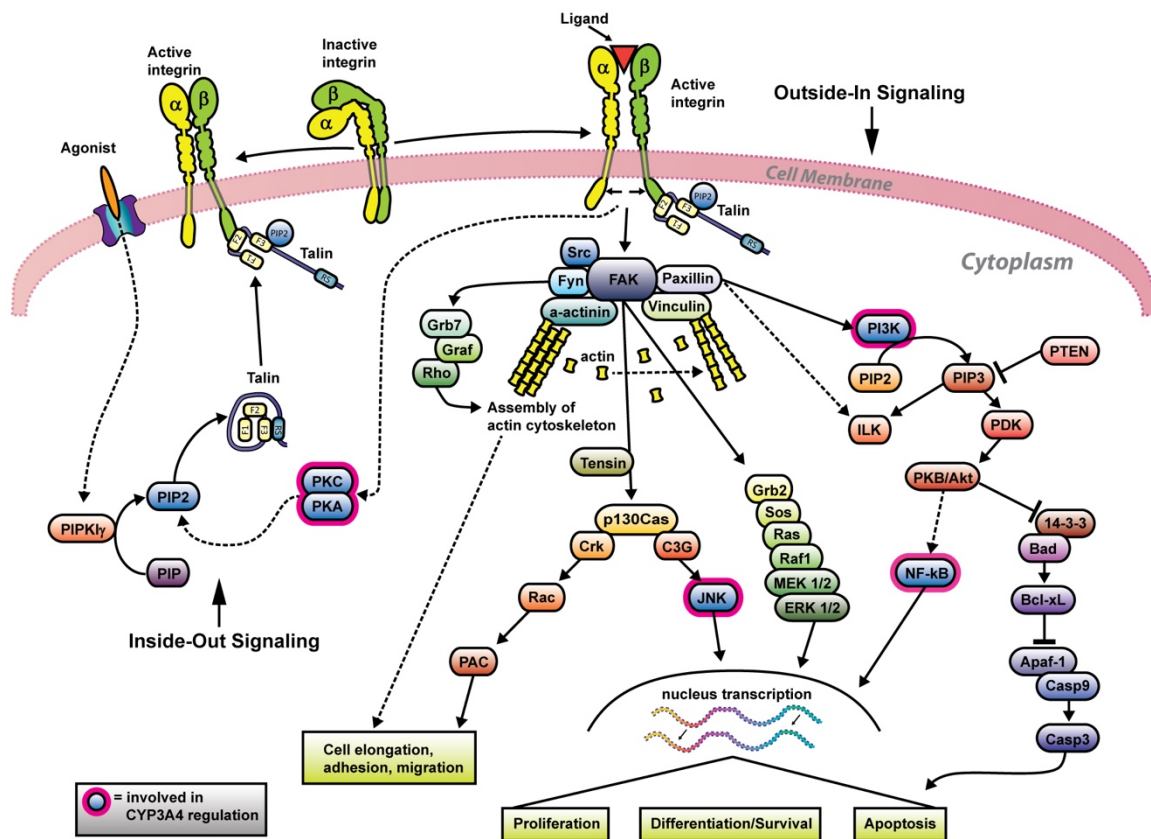


Figure 5.12 Bidirectional regulation of integrin receptors: inside-out and outside-in signaling pathways. Integrins can be activated by binding of ligands to the integrin receptor resulting in a change in the conformation of the receptor, which activates it. Integrins are also regulated by extracellular stimuli, initiating intracellular signals, which convert the extracellular domains into a high-affinity ligand-binding state, known as an inside-out response. Figure adapted from references [414, 415].

since we previously observed the opposite effect, suppression of CYP3A4 activity, during adenovirus infection with minimal change in protein levels in the same *in vitro* system [207]. In addition, mRNA and protein levels of PXR and RXR α were unaffected by talin silencing (Figure 5.4). While CAR was the only nuclear receptor significantly affected by knockdown of talin-1, it is important to realize that CAR and PXR both heterodimerize with RXR α [12]. Thus, observed decrease in CAR protein levels would most likely not be responsible for the profound changes in CYP3A4 activity observed in response to silencing talin-1.

Therefore, we focused on the specific interaction between the nuclear receptors regulating CYP3A4 and the CYP3A4 promoter. RXR α , CAR and PXR interact with the CYP3A4 promoter at two different binding sites, the distal xenobiotic responsive enhancer module (dXREM) and the proximal PXR responsive element (prPXRE, labeled with * and **, respectively; Figure 5.5A) [12]. Interaction with both promoter binding sites by RXR α did not change in cells with reduced talin-1 levels. However, binding by both CAR and PXR at both sites of the CYP3A4 promoter was significantly increased by talin-1 knockdown (Figure 5.5B and 5.5C). This implies that increased levels of CYP3A4 activity observed in talin-1-silenced cells may be due to the enhanced ability of PXR and CAR to bind to the CYP3A4 promoter. Since no increase in CAR or PXR protein levels were seen, the higher binding capacity to the two binding sites on the CYP3A4 promoter could be associated with alteration in interactions with proteins that form a complex with CAR and PXR at the promoter site or by post-translational modifications of the nuclear receptors.

The process of binding CAR-RXR α and PXR-RXR α to dXREM and prPXRE is facilitated by hepatocyte nuclear factor (HNF)-4 α after co-activation by peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) and the steroid receptor coactivator-1 (SRC-1) [99, 110]. We are currently developing a mass spectrometry approach to detect changes in this protein complex to determine if knockdown of talin-1 affects the association of this complex and hence the binding to the CYP3A4 promoter. This approach will also be used to determine if any other proteins are involved in the binding ability of PXR, CAR and RXR α to the two different CYP3A4 promoter binding sites.

Gene expression of 96 different genes common in human signal transduction pathways was determined in HC-04 cells after 24 hours of transfection using either control, integrin β_3 or talin-1 siRNA followed by either infecting the cells for 24 hours with AdlacZ (MOI of 500) or leaving them uninfected for the same amount of time (Table 5.4). Gene expression was compared between infected and uninfected cells treated with the same siRNA. Several genes were up- and downregulated in all three siRNA treatment groups after virus infection, with more changes seen in cells transfected with integrin β_3 or talin-1 siRNA. Opposing changes in levels of several genes were seen in samples treated with talin-1 siRNA compared to those treated with integrin β_3 siRNA, which could be due to the different influence of the integrin inside-out and outside-in pathways. Comparing data from this array with an array evaluating gene expression in rats four days after infection with

AdlacZ (Table 4.1) confirmed some of the changes seen in HC-04 cells but not all. The gene encoding the CCAAT/enhancer binding protein (CEBPB), a transcription factor known to regulate CYP3A4 expression [95], was downregulated in both arrays. Another gene that was downregulated in both arrays was v-myc myelocytomatosis viral oncogene homolog (MYC), which is involved in protein kinase C (PKC) and wingless-type MMTV integration site family (WNT) signaling, both of these pathways are also involved in regulating CYP3A4 expression [148, 163]. Levels of other genes such as CCL20, EGR1, FASN, JUN, NFkB1 and VCAM1, did not have the same trend in the arrays performed on samples from HC-04 cells compared to rat samples. This could be due to several factors including species differences (human vs. rat), using a cell line compared to *in vivo* data, and the difference in timepoints that gene expression was analyzed (two days for the HC-04 arrays and four days for the rat arrays).

In addition, an increase was seen in gene expression of CYP19A1, an enzyme that irreversibly catalyzes androgens into estrogen in the steroidogenesis pathway [74], in cells infected with AdlacZ and transfected with either talin-1 siRNA or integrin β_3 siRNA (Table 5.4). We have studied the effect of adenovirus infection on other CYPs and have seen an isoform-specific inhibition of CYP3A4 in the HC-04 cell line [207], but never specifically looked at CYP19A1. These results suggest that virus infection is affecting other CYPs in addition to CYP3A4, which in this case could cause an imbalance in estrogen levels. Hence, understanding how individual CYPs are affected by virus infection is warranted.

Gene expression in cells with reduced talin-1 expression was compared to cells expressing normal levels of talin-1, both after AdlacZ infection, to identify genes that are altered if the influence of the inside-out signaling pathway is minimized. Only three genes were significantly impacted by talin-1 silencing and AdlacZ infection compared to cells transfected with control siRNA and infected with the same virus. These genes were ubiquitin C, baculoviral IAP repeat containing 3 (BIRC3), and kallikrein-related peptidase 2 (KLK2) (Table 5.5). Ubiquitin C is a polyubiquitin gene that has been described as a stress-responsive gene. It supports constitutive monoubiquitin genes in maintaining cellular ubiquitin homeostasis [416]. BIRC3 acts as an E3 ubiquitin-protein ligase regulating NF κ B signaling involved in both caspases and apoptosis [417]. KLK2 is a subgroup of serine proteases involved in proteolytic mechanisms involved in maintaining appropriate protein levels by recognizing and degrading proteins that are misfolded or mislocalized [418]. All of these pathways are associated with protein degradation, two of which are directly associated with the ubiquitin pathway. Since CYP3A4 activity in cells with reduced talin-1 levels is not suppressed during adenovirus infection and the only difference between infected control cells and cells with reduced talin-1 levels are these three genes, our focus turned to assessing PTMs of CYP3A4 during virus infection specifically to elucidate the role of talin-1 activation of integrin receptors. If virus infection is causing altered ubiquitin activity and/or that of other post-translational modifiers then the overall pattern of these PTMs should be affected. Therefore, ubiquitin and two other common PTMs, phosphorylation and SUMOylation, that were not included in the microarray analysis, were assessed for changes by adenovirus infection. The general trend

for all three PTMs was an increase in the amount of modification with increased virus MOIs at 96-hours post infection (Figure 5.6). This confirmed our theory that adenovirus infection is impacting PTM patterns of cellular proteins. While phosphorylation patterns for proteins around the size of CYP3A4 could not be identified through screening of whole cell lysates, notable changes could be seen on screens for the presence of ubiquitin and SUMOylation, hence these two PTM motifs were the continued focus for the remainder of this study

Ubiquitin is an 8.5 kDa highly conserved protein that contains seven lysine residues [419]. The ubiquitin pathway is activated by the ubiquitin-activating enzyme E1 when a high-energy thioester bond is formed between the carboxy terminus of ubiquitin and an internal cysteine residue of E1. This process requires input of energy, in this case from ATP. The activated ubiquitin protein is transferred and conjugated to the active site of the ubiquitin-conjugating enzyme, E2. The ubiquitin ligase, E3, finally catalyzes the formation of an isopeptide bond between the target protein and the ubiquitin moieties [227]. De-ubiquitination reverses the process of ubiquitination, which entails cleavage of the ubiquitin moiety from the target protein, which is catalyzed by de-ubiquitination proteins or ubiquitin-specific proteases [420] (Figure 5.13). Several viruses encode gene products that mimic the function of cellular proteins, which interferes with cellular processes and host cell homeostasis [421]. Human wild-type adenoviruses encode early factors E1B-55K and E4orf6 that assemble into an E3 ubiquitin ligase complex. This complex sequesters specific cellular substrates, including p53 and integrin α 3, into the ubiquitin-dependent

proteasomal degradation pathway [278]. Human wild-type adenoviruses also encode the viral protease adenain (Avp), a cysteine proteinase, which exhibits de-ubiquitination activity against Lys48-linked poly-ubiquitin [422]. However, the recombinant adenovirus used in these studies does not express these proteins. It would therefore be warranted to study how wild-type adenovirus infection, where both of these proteins would be expressed, to understand the impact of these two proteins on the state of ubiquitination and/or SUMOylation of CYP3A4 to further our understanding of how PTMs are involved in CYP3A4 regulation.

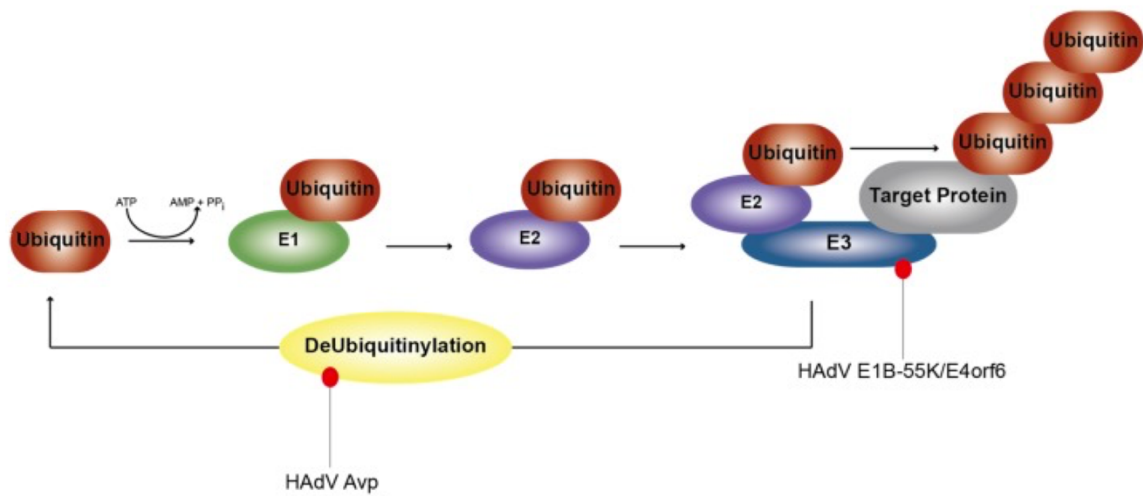


Figure 5.13 Ubiquitination pathway with potential interactions by human adenoviruses. Ubiquitination is a three-step process involving activation by E1, conjugation by E2 and finally ligation by E3, which result in mono- or polyubiquitination of a target protein. Deubiquitination is the reverse process of removing ubiquitin moieties from target proteins. Human adenovirus has evolved to modulate the E3 and deubiquitination step via viral proteins E1B-55K/E4orf6 and Avp, respectively. Figure adapted from [227].

SUMOylation is the covalent linkage of small ubiquitin-like modifier (SUMO) proteins to lysine residues of target proteins. There are four different SUMO paralogs, all around 12 kDa, known as SUMO-1, SUMO-2, SUMO-3 and SUMO-4 [423]. In this study, we chose to study SUMO-2 and 3 as they are about 95% identical to each other they cannot be distinguished using western blot techniques. The overall cellular concentration of SUMO-2 and 3 is greater than that of the other SUMO paralogs, hence it is likely that the majority of the SUMOylation that takes place involves SUMO-2 and 3 [424]. Similar to ubiquitination, SUMOylation involves a three-step enzymatic process. It begins with activation by a SUMO E1 enzyme, conjugation of E2 enzyme Ubc9, and finally SUMOs are ligated to target proteins via isopeptide bonds formed between the carboxyl groups of SUMO and the amino group of the lysine residue on the substrate protein via the E3 enzymes [421]. SUMOylation is reversible by deconjunction of SUMO moieties by a de-SUMOylation process, which create an on and off state that is essential for biological regulation [424] (Figure 5.14). The Avp protease of human adenoviruses is a cysteine protease that is also included in the same class as cellular SUMO proteases. Human adenovirus early protein E1B-55K has been reported to possess E3 SUMO ligase function [227]. However, in the recombinant adenovirus system used in these studies these two proteins are not expressed. Hence, studies with wild-type adenovirus where these proteins are expressed would further increase our understanding of how CYP3A4 is regulated by PTMs during virus infection.

To study changes in SUMOylation and ubiquitination of CYP3A4, HC-04 cells were infected with AdlacZ for 96 hours followed by immunoprecipitation of CYP3A4

(Figure 5.7). With increasing MOI of virus, the amount of CYP3A4 that was modified by these two PTMs increased. This implies that CYP3A4 is modified at a post-translational level, which is most likely partially responsible for the decrease in CYP3A4 activity observed during adenovirus infection that we have reported previously [208, 209, 211]. To test if this was linked to talin-1 interacting with integrin receptors via inside-out signaling, talin-1 was knocked down by transfection in HC-04 cells for 24 hours followed by infection with AdlacZ (MOI of 500) for 24 hours. Ubiquitination of CYP3A4 was still significantly increased compared to uninfected cells treated with talin siRNA (Figure 5.8A). However, no significant increase was seen in SUMOylation of CYP3A4 for either siRNA treatment (Figure 5.8B).

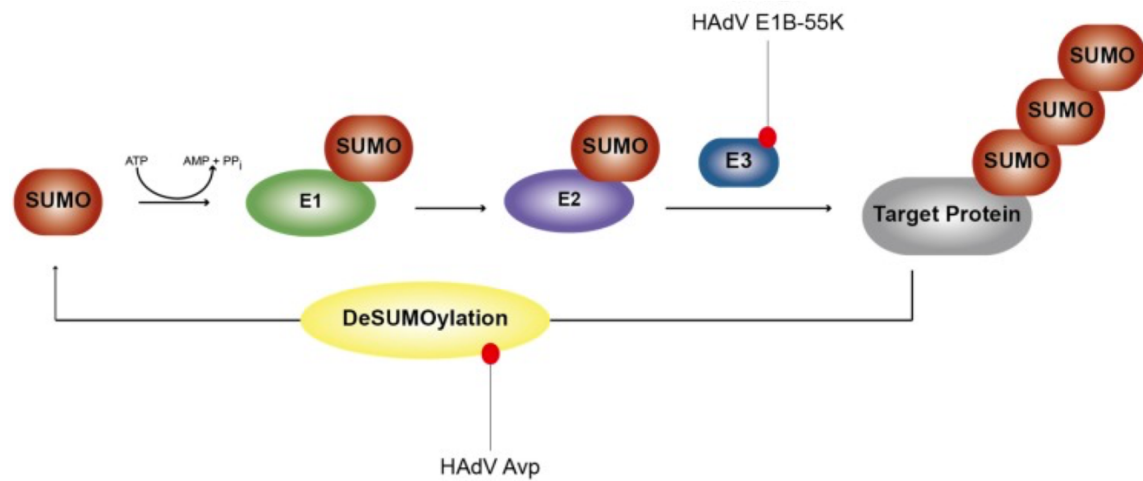


Figure 5.14 SUMOylation pathway with potential interactions by human adenoviruses. The SUMOylation pathway is similar to ubiquitination, where ATP is used to activate E1, followed by conjugation by E2 and ligation via E3. Human adenoviruses encode proteins Avp and E1B-55K that both exhibit functions of cellular proteins involved in SUMOylation. Figure adapted from [227].

These results indicate that talin-1 alone is not responsible for changes in PTMs. Therefore, involvement of integrin receptors via outside-in signaling was investigated in relation to ubiquitination of CYP3A4. Infection of mice with two types of adenoviruses, one that can bind to integrin receptors (AdlacZ), and a mutated form that cannot bind to integrin receptors due to deletion of the RGD sequence in the penton base (Ad Δ RGD), was used to determine changes in ubiquitination patterns of CYP3A4. At both 24 and 48 hours post infection, ubiquitination of CYP3A4 was reduced in mice infected with Ad Δ RGD and unaffected by AdlacZ infection (Figure 5.9). These results imply that at these early timepoints during Ad Δ RGD infection when the adenovirus is forced to enter cells through receptors other than integrins, such as the coxsackie- and adenovirus receptor (AdCAR) and heparan sulfate proteoglycans [373], other signaling pathways are affected resulting in a decrease in ubiquitination of CYP3A4.

The experiments described here also highlight the importance of timing when collecting samples. For example, the talin-1 experiment summarized in Figure 5.8 was conducted at 24 hours post infection where no significant changes were observed in ubiquitination of human CYP3A4. However, the most significant changes in PTM of CYP3A4 was only seen after 96 hours of infection (Figure 5.7). This indicates that significant change in PTMs will not be immediately detected early during the viral infection. However, in our siRNA transfection system, talin-1 knockdown peaks at 48 hours post transfection and returns to normal levels around 72 hours post transfection.

Therefore, another system would have to be developed to study changes at later timepoints during virus infection in cells with reduced talin-1 expression. Additionally, the *in vivo* experiment, summarized in Figure 5.9, measured ubiquitination of CYP3A at 24 and 48 hours post infection. Observations for AdlacZ infection *in vivo* did not correlate with amount of CYP3A4 that was ubiquitinated seen *in vitro* in the HC-04 cell line after 96 hours of infection with the same virus. This also supports the idea that PTM patterns may not be fully implemented until later timepoints during the infection. We have previously detected changes in CYP3A for up to 14-days post infection [208], this phenomenon could be explained by changes in PTMs of CYP3A and additional studies to test this hypothesis are underway in our lab.

We also determined whether changes in localization of nuclear receptors regulating CYP3A4 are involved in changes seen in CYP3A4 function and expression during AdlacZ and Ad Δ RGD infection in HC-04 cells (Figure 5.10). AdlacZ infection decreased protein levels of PXR in the cytoplasm but increased the level of CAR, which could be a compensation by the cell since the two nuclear receptors often regulate similar processes (Figure 5.10E and 5.10C). Infection with Ad Δ RGD decreased the level of RXR α in the cytoplasm with a corresponding increase in the nucleus (Figure 5.10A and 5.10B). Interestingly, a second protein band above the RXR α band was detected in the nuclear fraction of cells infected with Ad Δ RGD (Figure 5.10B). An increase in protein size as seen here is often associated with PTM of the protein. RXR α is a target for SUMOylation [411]

hence we determined whether the additional band in the nuclear fraction was due to this PTM. A significant increase in SUMOylation of RXR α was detected in the nuclear fraction after infection with Ad Δ RGD but not AdlacZ or uninfected cells (Figure 5.11A). RXR α is a target for the SUMO-specific protease SUSP1. SUMOylation of RXR α in this manner negatively modulates the transcriptional activity of RXR α [425], which could be a way for the cell to reduce the level of transcription of CYP3A4 by RXR α to compensate for the increase of RXR α in the nucleus. The increase in RXR α levels in the nuclear fraction of cells infected with Ad Δ RGD could compensate for any other impact of the adenovirus, balancing CYP3A4 function and expression in cells infected with this type of virus.

To understand the role of the outside-in and the inside-out signaling pathways, HC-04 cells were transfected with siRNA targeting integrin subunits α_v , β_3 , or β_5 or talin-1 followed by 24-hours of incubation with known inducers and suppressors of CYP3A4. Treatment with ketoconazole (10 μ M), a strong suppressor of CYP3A4, reduced CYP3A4 activity by 55% in cells treated with control siRNA and significantly reduced suppression of CYP3A4 activity in cells treated with α_v , β_5 or talin-1 siRNA (36%, 45% and 39%, respectively), while no significant difference was seen in cells treated with β_3 integrin siRNA ($41\% \pm 8\%$, Figure 5.15A). Erythromycin (100 μ M), a moderate inhibitor of CYP3A4, reduced CYP3A4 activity by 44% in control siRNA-treated cells while CYP3A4 activity in integrin α_v , β_3 , β_5 or talin-1 siRNA treated cells resulted in only a 12%, 6%, 22%, or 16% reduction, respectively, when compared to cells treated with the same siRNA

but without drug treatment (Figure 5.15B). Treatment with a mild suppressor of CYP3A4, isoniazide (100 μ M), reduced CYP3A4 activity by 46% in control cells. In cells treated with siRNA targeting integrin subunit α_v , β_3 , or β_5 , reduced suppression of CYP3A4 was seen after treatment with isoniazide (26%, 36% and 27%, respectively Figure 5.15C). Treatment with talin-1 siRNA did not significantly alter the suppression caused by treatment with isoniazide (41% reduction compared to cells treated with same siRNA but no drug, Figure 5.15C). Dexamethasone (200 μ M) is a strong inducer of CYP3A4 that increased CYP3A4 activity by 51% in cells treated with control siRNA (Figure 5.15D). Silencing of integrin α_v , β_3 , and β_5 or talin-1 followed by treatment with dexamethasone increased CYP3A4 activity by 131%, 121%, 234% and 113%, respectively (Figure 5.15D). Treatment with rifampicin (20 μ M), a known agonist of PXR, reduced CYP3A4 activity by 18% in cells treated with control siRNA (Figure 3E). In cells with reduced expression of integrin α_v , β_3 , and β_5 or talin-1, a more pronounced increase in CYP3A4 activity was seen after treatment with rifampicin (increase of 59%, 35%, 43%, and 20% compared to cells treated with same siRNA but no drug, Figure 5.153E).

The general trend for three different inducers was a further increase in CYP3A4 activity in cells with reduced levels of integrin subunits α_v , β_3 or β_5 or talin-1 (Figure 5.15A-5.15C). A similar trend was seen using suppressors of CYP3A4, where knockdown of integrin subunits or talin resulted in significantly lower reduction in CYP3A4 activity (Figure 5.15D-5.15E). Clustering of α_v integrins is induced by adenovirus and result in internalization of the virus via integrin receptors [426]. This step is important for integrin

outside-in signaling. Since similar levels of CYP3A4 activity were observed in α_v -silenced cells after treatment with inducers or suppressors of CYP3A4, this suggests that the outside-in pathway may also play a role in integrin-adenovirus mediated changes of CYP3A4 and further studies are warranted.

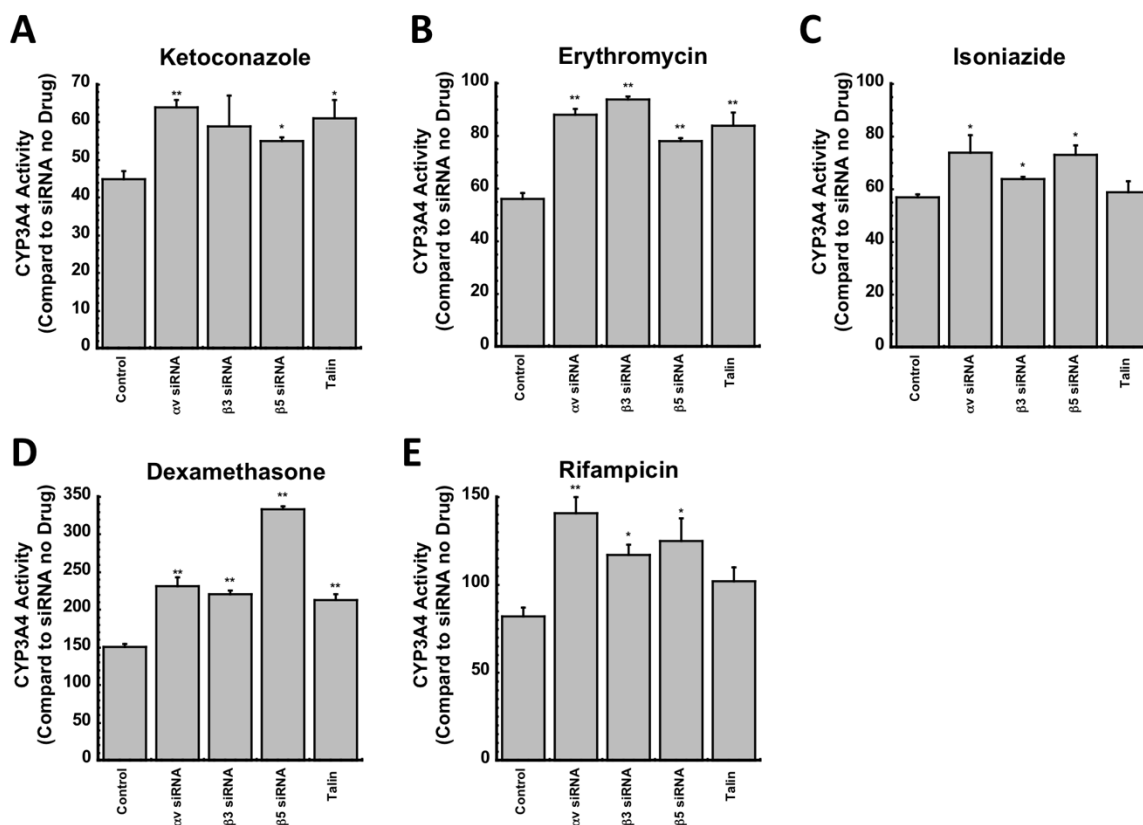


Figure 5.15 Silencing of talin-1 or integrin α_v , β_3 or β_5 induces CYP3A4 activity in cells treated with known inhibitors or inducers of CYP3A4. CYP3A4 activity was determined in cells treated with siRNA targeting of talin-1, integrin α_v , β_3 or β_5 and incubated with (A) ketoconazole, (B) erythromycin, (C) isoniazide, (D) dexamethasone or (E) rifampicin. After 24 hours of treatment with siRNA, cells were incubated with these known inducers or suppressors of CYP3A4 for an additional 24 hours before CYP3A4 activity was determined (P450-Glo™ assay: Promega). Differences in cell numbers were accounted for by using the CellTiter-Glo® assay kit (Promega). Data are reported as the means $\pm$ standard error of values obtained from 3 wells per treatment group. * $p < 0.05$ and ** $p < 0.01$ with respect to cells not treated with drug but transfected with the same siRNA.

Hepatic CYP3A4 is regulated via a complex network of signal transduction pathways, nuclear receptors and post-translational modifications. Several signal transduction pathways are affected by viral infection, which results in changes in CYP3A4 on different levels. Here we show for the first time that talin-1, an intracellular protein that interacts with the β -tail of integrin receptors, is involved in regulating CYP3A4 *in vitro*. Reduction of talin-1 levels resulted in an increase in CYP3A4 activity, suggesting that talin-1 could function as an inhibitor of CYP3A4 during physiological conditions. Knockdown of talin-1 followed by adenovirus infection suppressed gene expression for three genes involved in protein degradation, two of which are directly associated with the ubiquitin pathway, compared to cells treated with control siRNA and infected with the same virus. Ubiquitination and SUMOylation of CYP3A4 was significantly increased in HC-04 cells after 96-hours of infection with AdlacZ, which could explain why protein and gene expression can remain unaffected while CYP3A4 activity is suppressed by virus infection. However, at earlier timepoints changes in PTMs were not observed, which could be why this phenomenon has not been observed previously due to the timing of these experiments as they were evaluating CYP3A changes by cytokines and chemokines. Understanding how these different mechanisms change CYP3A4 is important to be able to adjust therapeutic regimens during a viral infection, especially for those patients that are taking multiple drugs concurrently, to avoid altered drug levels. Integrins are exiting targets for several different therapeutics, including treatment of cancer [358] and thrombosis [224], and consideration of the impact on CYP3A4 should be taken when designing drug

treatment plans for patients that are taking multiple medications, such as cancer patients, to avoid potentially toxic or subtherapeutic drug levels.

The talin-1 and post-translational modification experiments presented here are preliminary data mostly performed *in vitro* in the HC-04 cell line. It is necessary to confirm these results *in vivo* to determine if the observed regulation of CYP3A also takes place in an animal model. In addition, the post-translational modifications seen on CYP3A4 by ubiquitin and SUMO were only confirmed by Western Blot techniques. To fully understand what is happening to CYP3A4, these changes should be confirmed by using techniques such as mass spectrometry where the specific amino acids that have been modified can be identified. Using mass spectrometry, the exact extent of PTMs, such as poly-ubiquitination or poly-SUMOylation, can also be confirmed. This is important as the effect of ubiquitination or SUMOylation of proteins is diverse, including cellular localization and changes in protein activity, hence understanding where the protein is modified and by what specific PTM is warranted. Comparing how CYP3A4 is modified under infectious and non-infectious conditions could further our understanding of how CYP3A4 is regulated. In this study, an infection of 96 hours was used to study changes in PTMs of CYP3A4, at which point a significant increase in ubiquitination and SUMOylation was observed. This implies that the changes seen are not due to the initial infection and that this effect could be long-term. If the changes in PTMs of CYP3A4 is a long-term effect, this could lead to permanent aberrations in drug metabolism. Fast metabolizers, that might need a higher prescription dose to compensate for the high metabolism of drugs, could become slow

metabolizers and toxic drug levels could build up over time. Similarly, this would also be of concern for those that are slow metabolizers as this phenomenon would further slow down drug metabolism and drug levels could become toxic leading to unpredictable side effects. Additional studies are needed to determine how long these modifications are present to understand what is happening to CYP3A4 on a long-term basis.

CHAPTER SIX

Conclusions

This project was based on the observation that a single dose of recombinant adenovirus significantly suppressed the activity of hepatic CYP3A in rats for 14 days [211]. For decades, changes in CYP3A expression and function during infection has been attributed to the production of interferons, cytokines and chemokines [15], however by 14 days these inflammatory mediators have dissipated [211]. Additional studies revealed that reducing the immunogenicity of the virus by either chemical or physical means did not mitigate this effect [208]. These findings suggest that the presence of the microbial pathogen itself plays a role in the process that alters CYP3A during infection. However, it is unknown if this effect is translatable to humans and if it applies to adenovirus-based vaccines and other wild-type virus infections.

The studies outlined here sought to answer the following questions: 1) Is there an *in vitro* model that can be used to study virus-mediated changes of human CYP3A4?, 2) What is the impact of adenovirus-based vaccines and their transgene on CYP3A4 expression and function?, 3) Does wild-type infection with influenza viruses impact CYP3A4 function and expression?, 4) Is simple engagement of integrin receptors involved in regulating CYP3A4?, and 5) Are these changes mediated by intracellular proteins such as talin-1 and/or changes in post-translational modifications of CYP3A4?.

Previous work focused on changes in CYP3A in different animal models but to understand whether this translates to humans, a human hepatocyte cell line, HC-04, was evaluated as a suitable model to study the impact of virus infection on human CYP3A4. HC-04 cells were maintained in standard culture media with minimal supplementation. Under these conditions, the cells inherently expressed measurable levels of CYP3A4 and associated nuclear receptors (CAR, PXR and RXR α). CYP3A4 in these cells responded to a panel of compounds known to have suppressive and inductive properties and was suppressed during virus infection in a concentration-dependent manner, which correlates with results previously obtained from several *in vivo* models. Experiments in HC-04 cells were consistently reproducible over multiple passages and could be validated in other cell lines with established reporter assays. The HC-04 cell line was therefore determined to be a suitable model for studying virus-mediated changes in human CYP3A4.

Impact on hepatic CYP3A by immunization was determined using an adenovirus-based vector expressing Ebola glycoprotein (AdEBO) in mice and HC-04 cells. CYP3A4 catalytic activity was significantly suppressed in mice and HC-04 cells after infection with AdEBO for 24 and 48 hours, respectively. Evaluating the impact of the transgene, Ebola glycoprotein, further confirmed previous findings that the transgene itself can impact CYP3A4 activity. Wild-type infection with influenza H1N1 virus in mice significantly suppressed CYP3A activity for at least 24 hours. Infection of the HC-04 cell line with either H1N1 or H3N2 virus subtypes significantly suppressed CYP3A4 activity and protein levels for at least 72 hours. The reduction in protein levels was almost complete, which can be attributed to the ability of negative-strand RNA viruses to hijack cellular translational

machinery thereby not allowing the translation of CYP3A4 protein. These studies show that adenovirus and influenza infection have a significant impact on CYP3A4 catalytic activity. This knowledge is important as the seasonal influenza virus infects millions of people annually, influenza vaccines are administered yearly, and several adenovirus-based vaccines for Ebola will soon be available on the market. Using the information from these novel studies, drug regimens can be planned to avoid toxic or subtherapeutic drug levels for patients with viral infections to compensate for suppression in CYP3A4. Low clearance drugs (Extraction ratio (E) >0.3) are cleared slowly from the body due to the stability of the molecule or the low capacity or activity of the metabolizing enzyme. For these drugs, the clearance is proportional to the ability of the liver to metabolize the drug, hence subtle changes in CYP3A4 or other CYPs could have a significant clinical impact on the clearance. For high clearance drugs (Extraction ratio (E) <0.7) where the drug is extensively metabolized, suppression of CYP3A4 would also have a significant impact. For these drugs, the sudden decline in metabolism would result in extended clearance time. Hence the drug would be in the body for a longer time and the dosing regimen would have to be adjusted to avoid toxic drug levels.

Similar to previous observations, mice infected with a recombinant adenovirus (AdlacZ) experienced a significant reduction in hepatic CYP3A catalytic activity. However, infection with a mutant virus with integrin-binding arginine-glycine-aspartic acid (RGD) sequences deleted from the penton base protein of the virus capsid (Ad Δ RGD) did not alter CYP3A activity. CYP3A mRNA and protein levels in AdlacZ-treated animals were also suppressed, whereas those of mice given Ad Δ RGD were not significantly

different from uninfected control mice. Simple engagement of integrins with an RGD peptide elicited a comparable reduction in CYP3A activity to that seen with adenovirus, while an RGE peptide had no impact. Suppression of integrin subunits had a profound effect on nuclear receptors PXR, whereas RXR α was largely unaffected. To our knowledge, this is the first time that extracellular receptors, like integrins, have been indicated in the regulation of CYP3A. This finding has several implications owing to the important role of integrins in normal physiologic process and in many disease states.

To understand the specific mechanism by which integrin receptors are involved in CYP3A4 regulation, the different subunits of the integrin receptor were silenced. Silencing of the β -subunit reverted adenovirus-mediated CYP3A4 suppression *in vitro* while silencing of the α -subunit did not. Due to importance of the β -subunit and its role in inside-out signaling by integrin receptors, we evaluated the role of talin-1, a cytoplasmic protein that specifically binds to the β tail, in CYP3A4 regulation. We found that silencing of talin-1 increased CYP3A4 catalytic activity but did not affect CYP3A4 mRNA or protein levels. Using an array to detect changes in common human signal transduction pathways after silencing of talin-1 and infection with AdlacZ revealed significant suppression of three genes encoding for proteins involved in protein modifications. This led us to believe that post-translational modifications of the CYP3A4 protein is involved in the regulation of CYP3A4. Adenovirus infection did indeed significantly increase the level of ubiquitination and SUMOylation of CYP3A4. These are however initial experiments performed *in vitro* in the HC-04 cell line and further experiments *in vivo* are warranted to confirm these observations.

Throughout our studies, changes in nuclear receptors PXR and CAR have been observed during infection with a recombinant adenovirus but also during wild-type influenza A virus infection. This suggests that the impact of virus infection on CYP3A4 alone is not the only effect that needs to be taken under consideration during viral infection. Alterations in nuclear receptors PXR and CAR have been implicated in several human disease states including cancer, diabetes, metabolic disease and liver disease [427]. As such, the potential of the virus to change these nuclear receptors should also be studied to understand the long-term effect of chronic virus infection in terms of changes in the regulating processes that they are involved in as well as drug metabolism.

Our novel observations of how CYP3A4 is regulated by extracellular receptors, such as integrins, and intracellular proteins, like talin-1, implies a complex network of signaling pathways that are induced or suppressed by engagement of integrin receptors. To further elucidate the detailed mechanism of this phenomenon, we are investigating the effect of adenovirus infection or simple engagement by an RGD peptide on signaling pathways involved in integrin signaling and CYP3A4 regulation, such as protein kinase A (PKC), PKA, phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal kinases (JNK) and nuclear factor kappa beta (NF κ B), to determine if any of these common signaling pathways are responsible for observed changes in hepatic CYP3A4. Furthermore, to determine the role of PTMs such as ubiquitination, a drug that inhibits the E3 enzyme of the ubiquitin pathways will be used to determine whether adenovirus-mediated suppression of CYP3A4 occurs without the ubiquitin pathway. The impact of other viruses known to either engage with integrin receptors or not, will be studied to further understand the role of integrins in

CYP3A4 regulation

In summary, the experiments described here show that CYP3A4 is regulated at several levels during virus infection (Figure 6.1). We have shown that engagement of integrin receptors by the adenovirus suppresses CYP3A expression and function. Protein levels of PXR and RXR α , two nuclear receptors involved in CYP3A regulation, were also reduced, which could explain the suppression seen in CYP3A during adenovirus infection. Initial experiments looking at post-translational modifications (PTMs) of CYP3A4 during adenovirus infection revealed that ubiquitin and SUMO patterns of CYP3A4 were increased during adenovirus infection. Hence, PTMs of CYP3A4 are another possible mechanism of adenovirus-mediated suppression. Additional experiments are needed to confirm the involvement of PTMs in CYP3A4 regulation during adenovirus infection and the timing at which these factors play a role in CYP3A4 function.

Finally, we have also identified new potential pathways for regulation of CYP3A4 during non-infectious conditions (Figure 6.2). Engagement of integrin receptors using a non-infectious RGD peptide significantly suppressed CYP3A4. In preliminary studies, we found that reduced levels of talin-1, without virus infection, resulted in an increase in CYP3A4 activity. This suggests that talin-1 could function as a negative regulator of CYP3A4. By reducing talin-1 levels, the interaction of CAR and PXR with the CYP3A4 promoter sequence increased significantly. This could be another mechanism of CYP3A4 regulation under non-infectious conditions.

Understanding how CYP3A4 is regulated is especially important for those people that are already low metabolizers, due to genetics or age, as suppression of CYP3A4 during

a virus infection could further lower their drug metabolism. For drugs with a narrow therapeutic index, where CYP3A4 is responsible for the clearance of these drugs, further suppression of CYP3A4 could result in toxic drug levels. Hence, by understanding how CYP3A4 is changed during virus infection, the drug dose given to these patients could be adjusted to avoid serious therapeutic failures or adverse drug reaction.

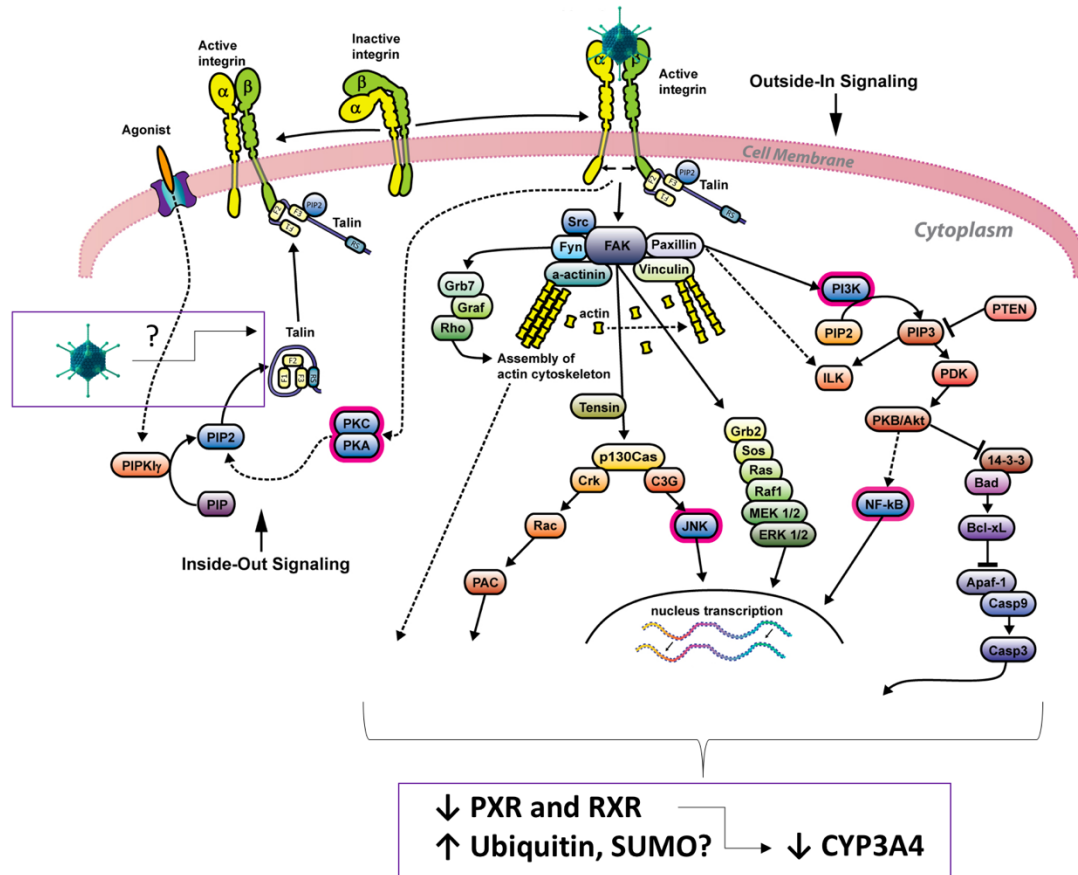


Figure 6.1. Summary of proposed mechanism of CYP3A4 suppression during adenovirus infection. Several different mechanisms have been found to regulate CYP3A4 during adenovirus infection including, engagement of integrin receptors which resulted in the downregulation of PXR and RXR, talin-1 mediated activation of integrins via integrin inside-out signaling and/or by addition of ubiquitin and/or SUMO moieties to the CYP3A4 protein.

APPENDIX

A.1 Limit of detection for antibodies used in western blot analysis

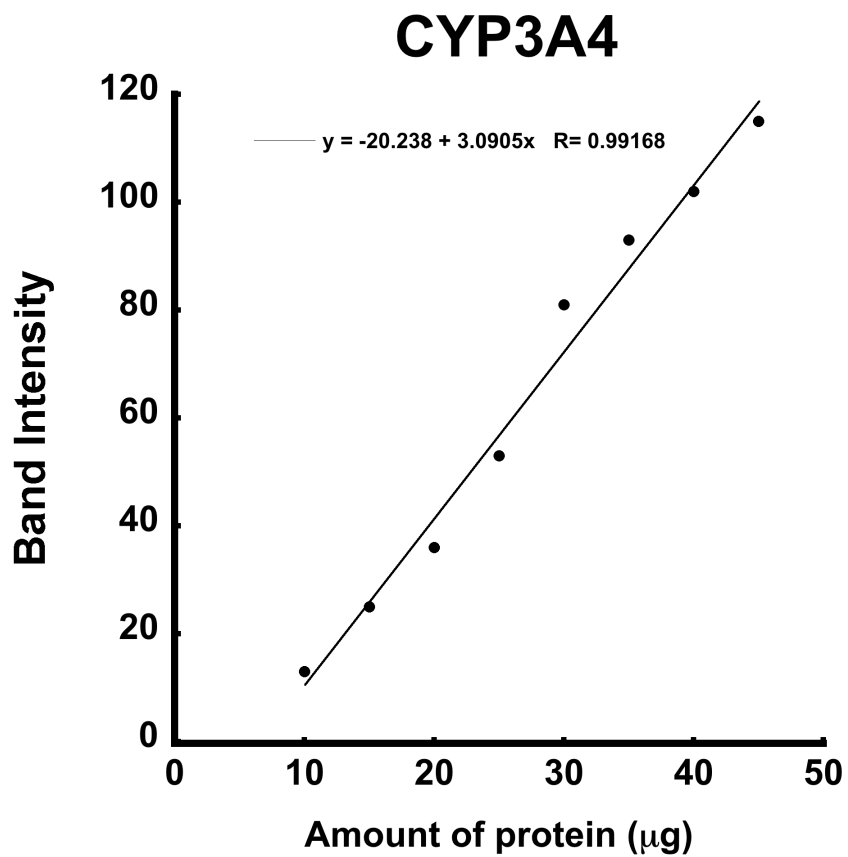


Figure A.1 Limit of detection of CYP3A4 by western blot analysis. Relationship between band intensity and amount of hepatic CYP3A4 as determined by western blot revealed linear relationship between 10-45 µg, with 10 µg being the lowest amount detectable with antibody concentrations as described previously.

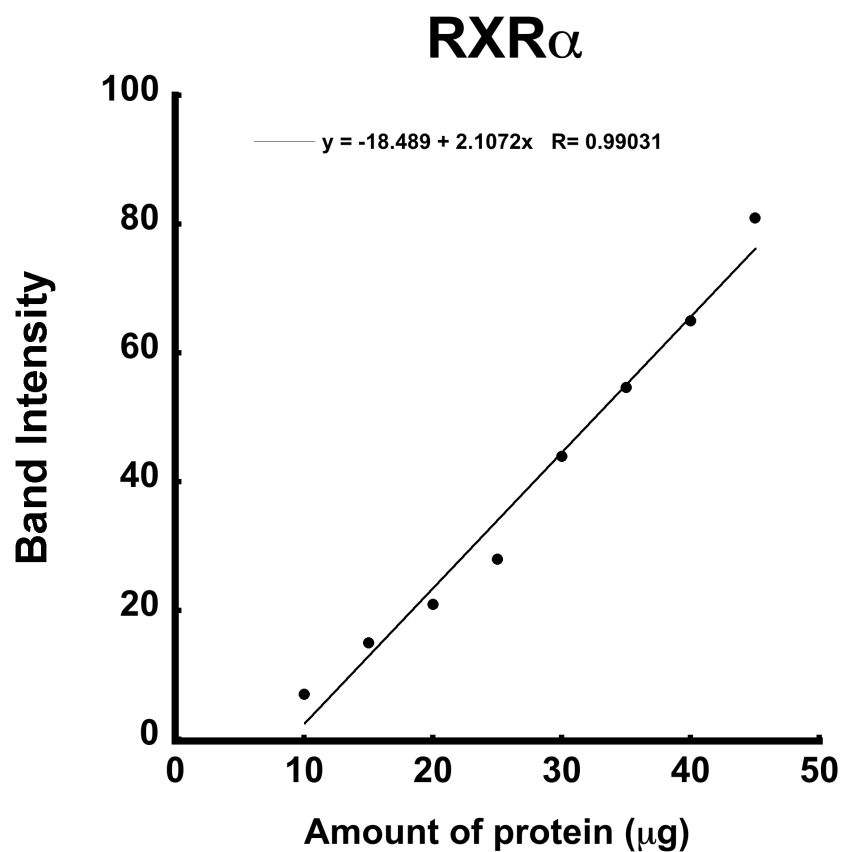


Figure A.2 Limit of detection of RXR α by western blot analysis. Linear relationship between band intensity and amount of hepatic RXR α as determined by Western Blot. Linear range was between 10 μ g and 45 μ g, with 10 μ g being the lowest amount detectable with antibody concentrations as described previously.

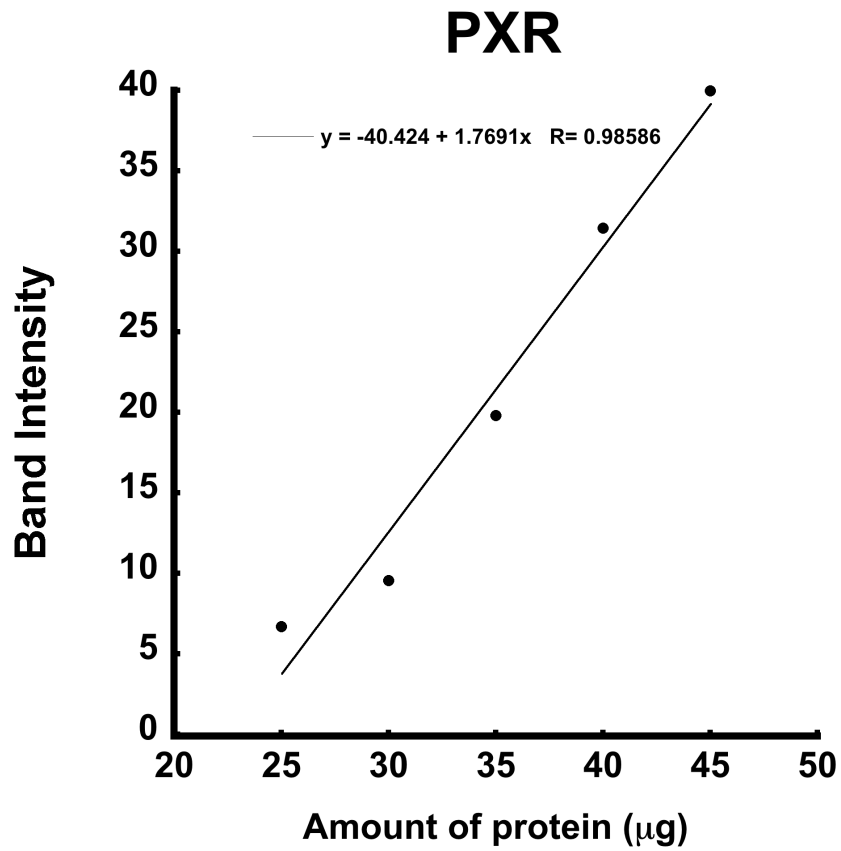


Figure A.3 Limit of detection of hepatic PXR by western blot techniques. Relationship between band intensity and amount of hepatic PXR as determined by Western Blot with antibody dilutions as described previously. PXR was detected at 25-45 μg and the limit of detection for PXR was 25 μg of total protein.

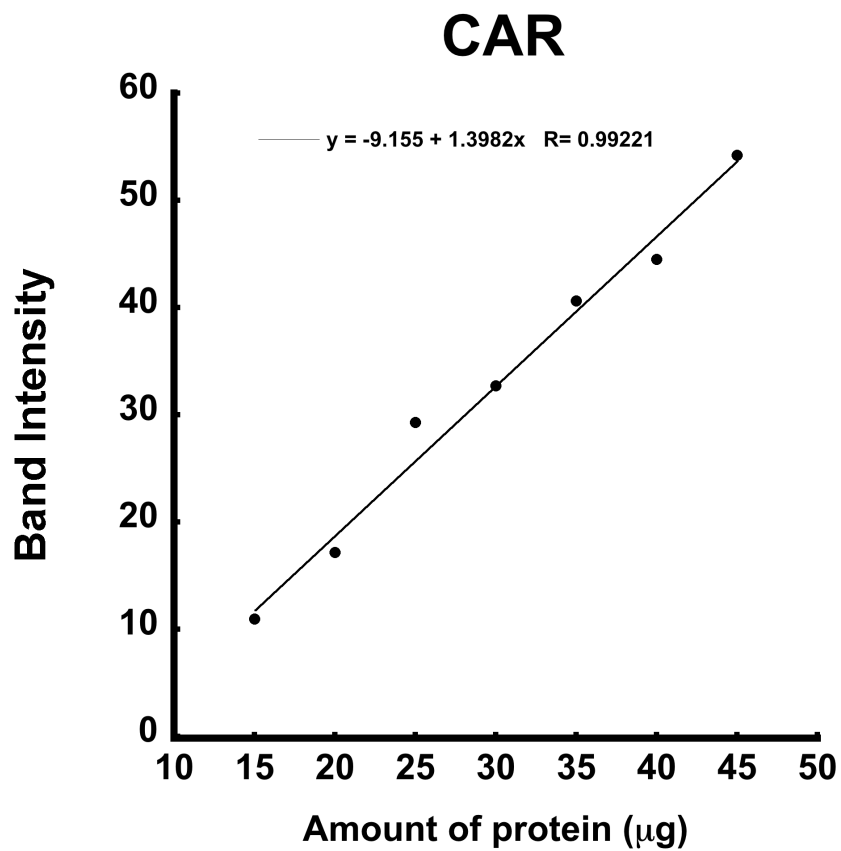


Figure A.4 Limit of detection of CAR by western blot analysis. Relationship between band intensity and amount of hepatic CAR as determined by Western Blot revealed linear relationship between 15-45 μg, with 15 μg being the lowest amount detectable with antibody concentrations as described previously.

A.2 Intra-assay, intraday and interday variability for western blot analysis of β -actin

Table A.1 Variability in western blot using HC-04 protein lysate

Intra-assay variability was determined by measuring band intensity of β -actin of three identical samples of protein from the same protein lysate. Intraday variability was determined by assessing band intensity for β -actin of three randomly selected samples in triplicate within the same day. Interday variability was determined by assessing band intensity of three randomly selected samples in triplicate for three days. Percent coefficient by variance was determined by dividing the standard deviation (SD) by the mean relative intensity. The high variance intra- and interday variability highlights the importance of using internal control, such as β -actin, on the same membranes as the protein of interest.

Variability	Mean $\pm$ SD	%CV
Intra-assay	188.39 $\pm$ 0.26	0.14%
Intraday	212.42 $\pm$ 31.32	14.75%
Interday	195.22 $\pm$ 26.07	13.36%

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