The drug metabolizing ability is differentially altered during hepatic regeneration in rats – *in vitro* and *in vivo* assessments

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Abstract

Hepatic regeneration is essential for the success of living donor liver transplantation (LDLT) in which the residual liver in donor and the segment that is transplanted into the recipient will grow in size to accommodate the requirements of the donor and recipient. We hypothesized that the clearance of drugs will be drastically reduced during the immediate post operative period in donor and recipient due to reduced liver mass and the decreased activity of the drug metabolizing enzymes in the liver. The objectives of this dissertation are to evaluate 1) the mRNA expression and *in vitro* and *in vivo* activity (pharmacokinetics of tacrolimus) of CYP3A; 2) the effect of hepatotropic drugs such as cyclosporine and tacrolimus on the activity of CYP3A; 3) the expression, and *in vitro* and *in vivo* activity (pharmacokinetics of mycophenolic acid) of various uridine diphosphate glucuronosyltransferases; and 4) the *in vivo* metabolism of acetaminophen in rats, during hepatic regeneration after partial hepatectomy.

Our results indicate that 1) the activity of most of the drug metabolizing enzymes was decreased but recovered completely at different rates; 2) the mRNA expression mirrored the changes in *in vitro* activity of these enzymes; 3) the clearance of tacrolimus and

mycophenolic acid was decreased but recovered completely at different rates; 4) the magnitude of reduction in *in vivo* clearance of tacrolimus and mycophenolic acid was much less than what was predicted from the loss of liver mass and reduction in enzyme activity; 5) cyclosporine and tacrolimus did not have any significant effect on the recovery of activity of CYP3A, and 6) there was increased production of toxic metabolites of acetaminophen during regeneration.

The clinical implications of our study are as follows: 1) Drug dosing in LDLT patients should be carefully monitored. A less than proportionate decrease in dose relative to reduction in liver mass may be necessary for different drugs. 2) The drug elimination capacity of the liver recovers completely with time and normal hepatic function will be restored in subjects undergoing hepatic resection. 3) Recovery of hepatic function will proceed normally in presence of hepatotropic substrates such as cyclosporine A and tacrolimus.

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Preface

First, I would like to express my sincere gratitude to my advisor, Dr. Raman Venkataraman, for his guidance in this project; his patience and kindness in training me in the area of drug metabolism and pharmacokinetics; his tremendous time and energy in teaching me how to write and present scientific data; his assistance in job search for my future career; his respect for a student; his continuous financial support in completing the project and his unselfishness in treating a student. Without him, it is impossible for me to complete my Ph.D. at the University of Pittsburgh and find a direction for my future career in Pharmaceutical Sciences. I also want to thank my committee members, Dr. Stephen C. Strom who provided technical assistance, and lab space for mRNA and protein measurement in this project; Dr. Samuel M. Poloyac who provided the technical assistance in LC/MS and radioactivity measurements in this project; thanks to Dr. Samuel M. Poloyac and Dr. Stephen C. Strom for their assistance in my job application and thanks to Dr. Samuel M. Poloyac, Dr. Stephen C. Strom, Dr. Wen Xie and Dr. Michael A. Zemaitis for their time, effort and invaluable suggestions during the conduct of this project.

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Chapter 1 Introduction

Background:

Liver Transplantation

Liver transplantation is the treatment of choice for patients with end-stage liver disease or certain metabolic liver disorders where no other alternative therapies are available. Successful liver transplantation has led to a one year patient and graft survival rate exceeding 90%. Currently, the cadaveric livers are the primary source of organ for liver transplantation. However, the numbers of available cadaveric organs are not sufficient for the numbers of patients on the waiting list for liver transplantation. The increased number of patients requiring transplantation and the stagnant number of cadaveric organs available for transplantation has increased the waiting time and has led to a high mortality for patients on the waiting list (Shiffman *et al*, 2002).

A number of techniques have been developed to deal with the shortage of cadaveric organs (Wiesner *et al*., 2003): 1) Use of livers from marginal donors who are older than 55 years of age; who have fatty infiltration of the liver; who have diabetes mellitus; and who are hepatitis c virus (HCV) or hepatitis B virus (HBV) positive; 2) performing split liver transplantation in which a cadaveric liver is split into 2 pieces, with one piece being used for an adult recipient and the other being used for a pediatric recipient (Rogiers *et al*., 1996; Busuttil and Gross, 1999; Rela *et al*. 1998); 3) performing domino transplantation

in which the first patient receives a liver from a donor and donates her/his liver to another patient (Stangou *et al*., 1998); 4) performing living donor liver transplantation (LDLT) in which the right liver lobe (account for the removal of up to 60% to 65% of the liver), is removed from the donor and transplanted into the recipient (Seaman, 2001).

Living Donor Liver Transplantation

Adult living donor liver transplantation is based on the ability of the adult liver to regenerate to accommodate the requirements in both the donors and the recipients. Living donor liver transplantation has emerged as an effective therapy for some patients with end-stage liver diseases and is also a partial solution for the current shortage of cardaveric organs. The living donor liver transplantation was initiated originally with the concept of grafting a segment of a liver from a living donor into a child who needed liver transplantation. The success of living donor liver transplantation in the pediatric patients led to the application of this surgery to adult-to-adult living donor liver transplantation. Interestingly, the recipients in the LDLT program had higher survival rates than those receiving cadaveric grafts (Rosendale *et al*., 1997; Sindhi *et al*., 1999; Jurim *et al*., 1995) due to less cold ischemia, better quality of the graft because of the healthy status of the donor, appropriate size of the vessels, and stable status of the recipient in this relatively elective procedure (Seaman, 2001).

In the United States, the first series of adult living donor liver transplantation was conducted in 1998 (Wachs *et al*., 1998). Since then, there has been great interest in adult living donor liver transplantation not only within the transplant medical community, but also among patients and their families who desire to become liver donors (Shiffman *et al*., 2002).

Hepatic Regeneration

Hepatic regeneration is very critical for the success of the adult living donor liver transplantation. The unique ability of the liver to regenerate was first recognized in the Greek mythology of Prometheus. The regenerative responses are proportional to the amount of liver removed in animals and humans and even small resections (< 10%) can trigger a response that restores the liver to full size (Michalopoulos and DeFrances, 1997). The liver regeneration process involves proliferation of all the existing mature cellular populations within the liver, including hepatocytes, biliary epithelial cells, fenestrated endothelial cells, kupffer cells, and cells of Ito (Michalopoulos and DeFrances, 1997). After partial hepatectomy, hepatocyte proliferation first begins in the periportal region, which is the first to receive the portal blood flow.

The exact signals that trigger the onset of hepatic regeneration are still not well defined. However, it is believed that hepatocyte growth factor (HGF) and its receptor, c-Met are

key components for the hepatic regeneration since a dramatic increase in plasma concentration of HGF has been observed after hepatic resection both in humans and rats (Schmidt *et al*., 1995; Tomiya *et al*., 1992). The rapid rise of HGF in the plasma is believed to be the mitogenic stimulus leading hepatocytes into DNA synthesis (Michalopoulos and DeFrances, 1997). However, the causes for the increase in plasma HGF are not entirely clear. Several cytokines are also involved in hepatic regeneration. Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) are necessary for the remnant liver to regenerate after two thirds hepatectomy. A significantly increased IL-6 plasma concentration has been observed after partial hepatectomy in humans and rats (Matsunami *et al*., 1992; Fulop *et al*., 2001). IL-6 has been reported to be mitogenic and lack of IL-6 can suppress hepatocyte DNA synthesis (Kordula *et al*., 1990; Cressman *et al*., 1996). A lack of TNF-α (either due to treatment with antibodies or in TNF-α knock-out mice) also resulted in decreased DNA synthesis or inhibition of hepatic regeneration after partial hepatectomy (Diehl *et al*., 1994; Akerman *et al*., 1992; Yamada *et al*., 1997). In addition, epidermal growth factor, transforming growth factor, norepinephrine and insulin are also involved in triggering hepatic regeneration (Michalopoulos and DeFrances, 1997).

A large number of genes are either newly expressed or increased in their expression after partial hepatectomy. The first phase of gene expression after partial hepatectomy, namely

the immediate early phase, occurs very rapidly after PHx and lasts for approximately 4 hours (Fausto, 2000). As many as 70 genes participate in the immediate early response to partial hepatectomy (Haber *et al*., 1993). An important advance in understanding liver regeneration is the observation that transcription factors such as NF-κB, AP-1, C/EBP-β and STAT-3 are activated after partial hepatectomy (Fauto, 2000).

Our current understanding of liver regeneration forms the basis for the treatment of many liver diseases, living donor liver transplantation and the application of hepatic tissue engineering (Palmes and Spiegel, 2004). These advances have been achieved primarily by studies of liver regeneration in animals (Palmes and Spiegel, 2004).

Models for Studying Hepatic Regeneration

In general, two strategies have been adopted for the experimental initiation of liver regeneration in animals: surgical resection or injury by toxins (Palmes and Spiegel, 2004). Partial hepatectomy, portal branch ligation, portosystemic shunting and direct compensatory hyperplasia are surgical techniques that are used to initiate liver regeneration, while carbon tetrahydrochloride, D-galactosamin, thioacetamide, acetaminophen and ethanol are toxins that are used to initiate liver regeneration (Palmes and Spiegel, 2004). The 2/3 hepatectomy procedure (Figure 1) in the rat devised many

years ago by Higgins and Anderson (1931) has found broad acceptance (Palmes and Spiegel, 2004). The advantages of using a rat to study liver regeneration after 2/3 hepatectomy are: 1) a high level of accuracy (e.g., the liver deficit after 2/3 resection is nearly always 68%), and reproducibility due to the uniform and consistent anatomy of the rat; 2) a simple operation requring no more than basic surgical skills; 3) good tolerance by the rats without perioperative mortality (Palmes and Spiegel, 2004). This experimental approach was chosen for the current project.

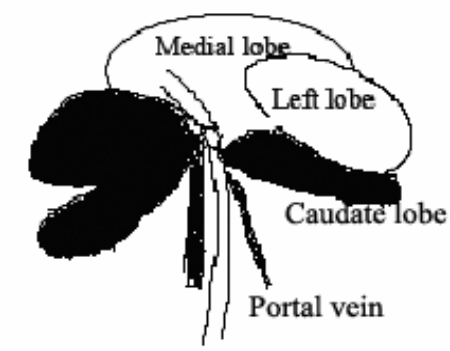


Figure 1. Surgical model of 2/3 partial hepatectomy. Medial and left lobes are surgically removed during PHx.

Drug Metabolism (aspects related to hepatic regeneration and living donor liver transplantation)

1. Drug metabolizing enzymes and transporters

Cytochrome P450 (CYP) is the most important phase I enzyme and refers to a

superfamily of heme-containing enzymes located in the membrane of the endoplasmic reticulum of the cell. CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A enzymes are responsible for the majority of phase I metabolism (Bertz and Granneman, 1997). Among these enzymes, CYP3A is the most abundant isoform in human livers. It constitutes about 30% of the CYPs in human liver followed by CYP2C, CYP1A2, CYP2E1 and CYP2D6, which represent 20%, 13%, 7% and 2%, respectively (Shimada *et al*., 1994; Eagling *et al*., 1998; Glue and Clement, 1999). Functionally, CYP3A4 metabolizes about 53% of the commonly prescribed drugs, followed by CYP2D6, CYP2C, CYP1A2 and CYP2E1 accounting for 25%, 18%, 3% and 1%, respectively (Bertz and Granneman, 1997).

The subfamily of CYP3A includes CYP3A4, CYP3A5, and CYP3A7. CYP3A4 is the major isoform in the liver as well as the small intestine of adult human. CYP3A5 is expressed in 20% of the population, while CYP3A7 is only expressed in the fetal liver (Venkatakrishnan *et al*., 2001). CYP3A is involved in the metabolism of tacrolimus, cyclosporine A, sirolimus and testosterone. In rats, CYP3A constitutes 17% of the total CYPs and includes CYP3A1, CYP3A2 and CYP3A23 (Waxman, 1999).

Uridine Diphosphate Glucuronosyltransfereases (UGTs) are an important superfamily of phase II membrane bound enzymes located in the endoplasmic reticulum. These enzymes catalyze glucuronidation of endogenous and exogenous compounds such as estradiol, acetaminophen, morphine, androsterone, testosterone and mycophenolic acid. UGTs exist in two subfamilies designated as UGT1A and UGT2B (Mackenzie *et al*., 1997). There are a total of 9 isoforms of UGT1 (UGT1A1, UGT1A2, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8 and UGT1A9) in rats. However, UGT1A4 and UGT1A9 are pseudogenes in rats. Even though there are some differences in UGT1A isoforms between humans and rats, most of UGT1A isoforms (UGT1A1, UGT1A3, UGT1A5, UGT1A6, UGT1A7 and UGT1A8) are common functional isoforms of UGT1A family in human and rats (Mackenzie *et al*., 1997). Rats have six isoforms of the UGT2B family (UGT2B1, UGT2B2, UGT2B3, UGT2B6, UGT2B8 and UGT2B12) (Mackenzie *et al*., 1997). Since isoforms of the UGT2B subfamily were named in the order that they were cloned, there is no information available on the corresponding isoforms of UGT2B in humans.

In addition to drug metabolizing enzymes, drug transporters also influence the pharmacokinetics of several drugs. Efflux of drugs from cells is mediated by several members of ATP-binding cassette (ABC) superfamily such as P-glycoprotein, multidrug resistance protein (Mrp) 1, Mrp2, Mrp3, Mrp4, Mrp5 and Mrp6. Among these tranporters,

P-glycoprotein, Mrp1, Mrp2 and Mrp3 contribute to a significant degree to the overall elimination of drugs. P-glycoprotein plays an important role in limiting the bioavailability of drugs from the gut, excretion of drugs into bile and protection of the brain by functioning as a blood-brain barrier (Kimura *et al*., 2004). Multidrug resistance protein 1 and Mrp3 are found in basolateral membranes and play a role in uptake of drugs in most tissues of human body. Multidrug resistance protein 2 is different from Mrp1 and Mrp3 in that it is located on the apical plasma membranes of polarized cells in liver, small intestine and brain (Haimeur *et al.*, 2004).

2. *Regulation of Drug Metabolism (cytokines released during hepatic regeneration may alter the activity of drug metabolizing enzyme)*

Different types of tissue damage (injury or infection) leads to induction of a series of secretory proteins, the acute-phase proteins (APP) (Morgan, 1997). The APP response to inflammation is mediated by cytokines such as IL-6, IL-1, and TNF α (Baumann and Gauldie, 1994; Koj, 1996; Moshage *et al*., 1990). An inflammatory response is initiated by the release of TNF α and IL-1 which will stimulate the further release of IL-1 and IL-6 (Baumann and Gauldie, 1994; Watkins *et al*., 1995). Even though IL-1 and TNF α are detectable in plasma, IL-6 levels are generally much higher (Lowry, 1993).

2.1 Cytochrome P450

Various reports have shown that IL-6, IL-1 and TNF α have selective suppressive effect on the expression and activity of different CYPs in *in vivo* and *in vitro* systems such as primary hepatocytes and microsomes (Morgan, 2001). The possible role of TNF-α in the down-regulation of CYP was studied using TNF-α receptor deficient mice. The results showed that TNF-α does not play a significant role in the down-regulation of CYP after administration of lipopolysaccharide (Warren *et al*., 1999). However, strong evidence indicated IL-6, the most important cytokine modulating acute-phase protein genes, to be a key player in the down-regulation of hepatic CYPs (Siewer *et al*., 2000).

In addition, nitric oxide generated from hepatocytes and kupffer cell in response to cytokines and inflammation is able to react with heme-containing proteins such as CYP enzymes and can decrease CYP-mediated activities (Khatsenko *et al*., 1993; Hakkola *et al*., 2003). Furthermore, nitric oxide can also inhibit the expression of some P450s (Khatsenko and Kikkawa, 1997; Hara and Adachi, 2002). Therefore, inflammatory cytokines and nitric oxide may contribute to the down-regulation of P450s during inflammation (Hakkola *et al*., 2003).

Since most of the suppressive effects of cytokines on CYPs take place at the transcription

level, there must be some regulatory factors involved in this process. Among many characterized transcription factors, liver-enriched transcription factors such as hepatocyte nuclear factor (HNF)-1α, HNF-4α and CCAAT/enhancer-binding proteins (C/EBPα) appear to be involved in the liver-specific expression of CYP genes (HNF-1α: CYP2E1, CYP7A1 and CYP1A2; HNF-4α: CYP2C9 and CYP2D6; C/EBPα: CYP2B) (Akiyama and Gonzalez, 2003). The nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), farnesol X receptor (FXR) are also involved in control of CYP genes (PXR: CYP3A4 and CYP2B6; CAR: CYP3A and CYP2B; FXR: CYP7A1) (Akiyama and Gonzalez, 2003).

Nuclear receptors such as CAR and PXR are responsible for the induction of some CYPs such as CYP2B and CYP3A at the transcriptional level. Phenobarbital and rifampicin are representative ligands for the activation of CAR and PXR. The functional feature of CAR and PXR is overlapped in the genes regulated by these receptors and the ligands to activate these receptors. However, recent findings pointed that the expression of CAR and PXR is down-regulated and these receptors are also responsible for the basal level of the expression of CYPs during acute phase response *in vitro* and *in vivo* (Pascussi *et al*., 2000; Beigneux *et al*., 2002).

2.2 Uridine Diphosphate Glucuronosyltransferases (UGTs)

Compared to the effects of cytokines and inflammation on the expression or activity of CYPs, there are limited reports of their effects on the expression or activity of UGTs. Levesque *et al*. (1998) reported that IL-1α inhibited the formation of dihydrotestosterone glucuronide in LNCaP cells. After turpentine injection in rats, the rate of p-nitrophenol glucuronidation (UGT1A6 in rats) by hepatic microsomes was not changed, while the formation of testosterone glucuronide (UGT2B1/3/6) was reduced to 65% of the control level (Strasser *et al*., 1998). The mRNA expression of both UGT1A1 and UGT2B3 was also decreased after injection of turpentine (Strasser *et al*., 1998). IL-6 suppressed the mRNA expression of UGT1A1 and UGT2B3 in primary rat hepatocytes at 50 u/ml (Strasser *et al*., 1998).

Studies on UGT gene expression and regulation have also been carried out in rodents. The promoter region of UGT2B1 contained HNF1 and CCAAT-Enhancer Binding Protein (C/EBP) binding sites (Mackenzie *et al*., 2003). Subsequent studies have shown that nuclear protein complexes containing HNF-1α or C/EBP α respectively bind to these sites and enhanced the transcriptional activity of the UGT2B1 promoter (Hansen *et al*., 1997; Hansen *et al*., 1998). Disruption of the C/EBP α gene results in complete loss of expression of UGT1A1 in mice (Lee *et al*., 1997). In addition to HNF-1α and C/EBP α, other transcription factors such as Octamer Transcription Factor 1 (Oct-1) and Pre B cell homeobox-2 (Pbx 2) regulate the expression of UGTs by interacting or interfering with HNF1 (Mackenzie *et al*., 2003). The expression of UGTs can also be induced through

activiation of nuclear receptors such as CAR and PXR by various inducers (ligands) (Mackenzie *et al*., 2003).

3. Effects of immunosuppressive drugs (drug therapy may alter the activity of drug metabolizing enzymes)

Cyclosporine A has been reported to have hepatotropic effect during hepatic regeneration (Mazzaferro *et al*., 1990). Cyclosporine A infusion stimulated cell renewal significantly and restored hepatocyte size in the infused lobes with a dose-response relationship from 0.6 mg/kg/day to 4 mg/kg/day in dogs (Mazzaferro *et al*., 1990). A study using male nude rats with T-cell deficiency showed that tacrolimus affected hepatic growth (DNA synthesis) by nonimmunoligical pathways (Francavilla *et al*., 1991). However, despite these reports indicating a tropic effect on the hepatic regeneration under immunosuppressive treatment, several studies have noticed that the liver weight restitution or liver growth was not affected by cyclosporine A during hepatic regeneration (Makowka *et al*., 1986; Kahn *et al*., 1990; Coughlin *et al*., 1987).

Chronic treatment with cyclosporine A at high doses (15 mg/kg subcutaneous dosing daily) suppressed CYP3A protein expression and *in vitro* activity (Brunner *et al*., 1998). Tacrolimus seems to interfere predominantly with the CYP isoforms 2A, 2B, 2C and 3A in rats and with the CYP subtypes 1A, 2B, 2C and 3A in man (Lupp *et al*., 2003). On the other hand, *in vitro* incubation with T cells showed that both cyclosporine A (100 nM)

and tacrolimus (10 nM) suppressed the expression of IL-2, IL-3, IL-4, c-myc, and TNF α (Tocci *et al*., 1989). This may modulate the effects of cytokines on drug metabolizing enzymes during hepatic regeneration. Conversely, since only serum levels of IL-6 and three soluble cytokine receptors (TNF α receptor I and II, IL-6 receptor) were significantly increased during hepatic regeneration (Fulop *et al*., 2001), the suppression of TNF α and other ILs by cyclosporine A and tacrolimus may have no obvious effect on the expression or activity of drug metabolizing enzymes. It is not clear whether the indirect effect or direct effect of cyclosporine and tacrolimus will predominate in a regenerating rat liver.

4. In vivo drug metabolism

Liver plays an important role in the elimination of endogenous and exogenous agents such as drugs, hormones and other chemicals. Hepatic metabolism of drugs can be influenced by hepatic blood fow (Q), intrinsic ability of the liver to metabolize drugs (Cl_{int}) and the unbound fraction of the drug in blood (f_u). In the eliminating organ, changes in blood flow will alter the clearance of drugs with a high extraction ratio (> 0.7, extraction ratio is calculated as total body clearance divided by blood or plasma flow), but will not alter the clearance of drugs with a low extraction ratio (< 0.2). Changes in f_u or Cl_{int} will change the clearance of low clearance drugs, but will not affect the clearance of high clearance drugs. Cl_{int} (ml/min) is calculated as maximal velocity (Vm (mol/min))

divided by Km (mol/ml). Due to the effect of cytokines and immunosuppressive drugs on drug metabolizing enzymes mentioned above, the activity of various drug metabolizing enzymes is likely to be changed during hepatic regeneration. Vm can be altered due to the altered activity of various drug metabolizing enzymes or the reduction in the mass of the eliminating organ. The unbound fraction of a drug in blood can be altered in certain disease states due to the change in the concentration of plasma proteins such as albumin and α-1-acid glycoprotein or the presence of competing endogenous chemicals such as a bilirubin and fatty acids. It has been reported that the concentration of albumin was decreased by 20%, 24 hours after hepatic regeneration and the concentration of alpha-1-acid glycoprotein was increased to 181%, 7 days after hepatic regeneration (Fouad *et al*., 1992). In summary, we can anticipate changes in Q, Cl_{int}, and f_u during hepatic regeneration and consequently changes in the pharmacokinetics of drugs that are metabolized in the liver.

5. Methods for studying drug metabolizing activity

Generally two strategies have been used to study drug metabolism: *in vitro* and *in vivo*. In the *in vivo* method, a probe substrate is administered to the subject or animal and multiple blood or any relevant biological fluids are sampled during specific time points. Total body clearance (or AUC) is then calculated based on concentration-time profile. This approach gives a good prediction of the activity of drug metabolizing enzyme under the

following conditions: 1) the substrate should be absorbed completely (IV administration); and 2) almost 100% of drug is metabolized by the liver only and not eliminated through the renal route (almost no parent drug recovered in the urine). *In vitro* methods typically involve whole liver perfusion, liver slices, isolated hepatocytes, or subcellular fractions. In the present study, we have used both *in vivo* method and *in vitro* method (subcellular fraction) to evaluate the effect of hepatic regeneration on drug metabolism.

Drug Therapy in LDLT Patients

Liver transplant patients are often treated with several drugs. Transplant patients receive one or more immunosuppressive drugs, antibiotics, antiviral drugs, antifungal drugs and others. Remarkable advances in immunology and the discovery of more selective immunosuppressive drugs have contributed significantly to the decreased incidence of acute rejection. Introduction of cyclosporine in the early 1980s, the introduction of tacrolimus in the mid-1990s and the introduction of mycophenolic acid in combination with cyclosporine and prednisone in the late 1990s have markedly increased the graft and patient survival (Wiesner *et al*., 2003). Currently, with tacrolimus-based immunosuppressive regimens, chronic rejection has decreased to 5% (Wiesner *et al*., 1999; Wiesner *et al*., 2003). Living donor liver transplant patients are normally treated with immunosuppressive drugs such as tacrolimus and mycophnolic acid. In addition,

acetaminophen is the most widely used analgesic in the USA and is sometimes used in the transplant patients for analgesia or fever reduction.

1. Tacrolimus

Tacrolimus (FK506, Prograf, Fujusawa, Osaka, Japan) is isolated from *Streptomyces tsukubasensis* and has a 23-member macrolide lactone structure (Figure 2).

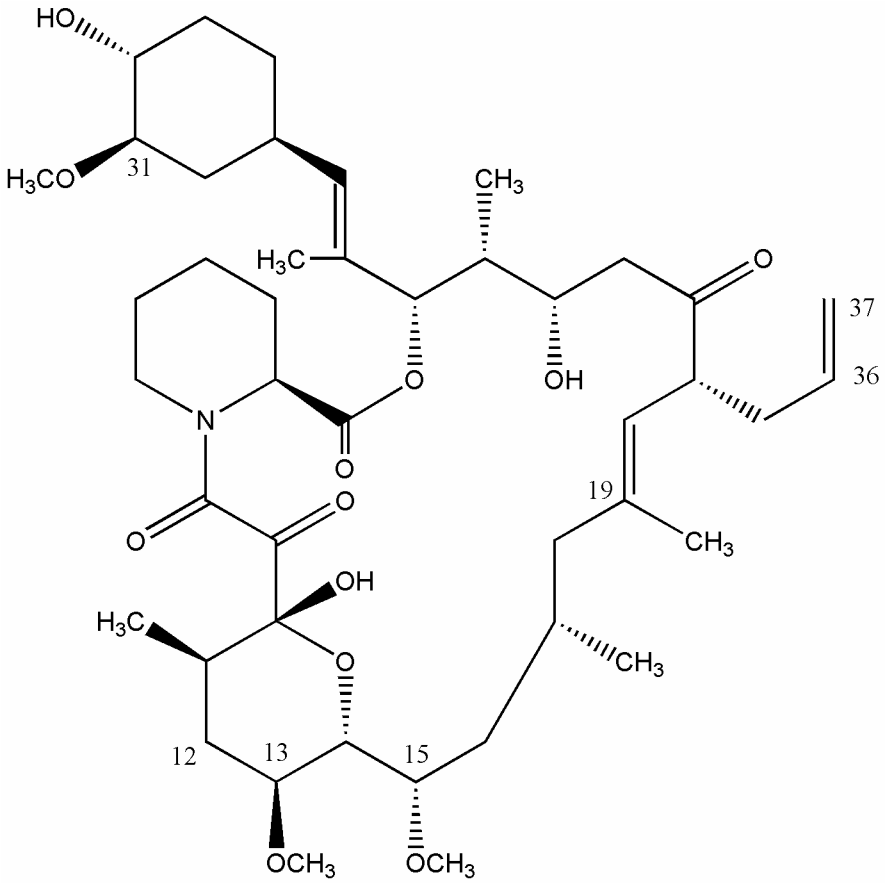


Figure 2. Chemical structure of tacrolimus.

Tacrolimus is a potent immunosuppressive drug. The structures of nine metabolites of

tacrolimus generated by liver microsomes of different species have been identified (Iwasaki *et al*., 1993; Lhoest *et al*., 1993; Iwasaki *et al*., 1995). The reactions involved in the metabolism of tacrolimus include, hydroxylation at C(12), C(19), and the C(36) and C(37) vinyl group, as well as demethylation at C(13), C(15) and C(31) (Lampen *et al*., 1995). Immunoinhibition with anti-CYP3A abolished the formation of almost all tacrolimus metabolites (Sattler *et al*., 1992). Subsequent study using liver microsome and cDNA expression system also documented that CYP3A is the major isoform involved in the metabolism of tacrolimus (Shiraga *et al*., 1994). Among the metabolites of tacrolimus, 13-demethylated metabolite (M1) is the major metabolite and accounts for about 75% of total metabolites in rats (Perotti *et al*., 1994).

Tacrolimus is metabolized extensively in rats, with limited excretion of the unchanged drug in the urine, bile or feces; biliary excretion is the major route of elimination of radioactively labeled drug and metabolites (Iwasaki *et al*., 1991). In humans, currently, most of the pharmacokinetic data available on tacrolimus are based on an enzyme-linked immunosorbent assay method. The rate of absorption of tacrolimus is variable with peak blood or plasma concentrations being reached in 0.5 to 6 hours. Approximately 25% of the oral dose of tacrolimus is bioavailable (Venkataramanan *et al*., 1995). Tacrolimus is extensively bound to red blood cells, with a mean blood to plasma ratio of about 15. Albumin and alpha 1-acid glycoprotein appear to primarily bind tacrolimus in plasma (Venkataramanan *et al*., 1995). Less than 0.5% of unchanged drug was detectable in feces

and urine (Moller *et al*., 1999). The mean disposition half-life is 12 hours and the total body clearance based on blood concentration is approximately 0.06 L/h/kg (Venkataramanan *et al*., 1995). The volume of distribution is within the range of 221 to 565 L (Staatz and Tett, 2002). The elimination of tacrolimus is decreased in the presence of liver impairment and in the presence of several drugs (Venkataramanan *et al*., 1995). Due to the interpatient and intrapatient variability in the pharmacokinetics and the narrow therapeutic index, monitoring of the trough concentration of tacrolimus is necessary to optimize dosing requirements.

The pharmacokinetic properties of tacrolimus have not been completely characterized in living related liver transplant patients and the clinical experience in adult living donor liver transplantation is limited. A recent population pharmacokinetic study (daily single blood sampling for each patient) demonstrated that the rate of metabolism of tacrolimus was quite different in recipients of partial liver grafts (Fukatsu *et al*., 2001). The use of reduced dose of tacrolimus in LDLT patients indicates that the clearance of tacrolimus is lower during the initial postoperative period in these patients (Fukatsu *et al*., 2001; Trotter *et al.,* 2002; Harihara *et al*., 2000; Troisi *et al*., 2002). Another study showed that adult living donor liver transplant patients receiving the right lobe required 50% less tacrolimus during the first 2 weeks postoperatively as compared with cadaveric liver recipients (Morgan *et al*., 2001; Troisi *et al*., 2002). This requirement is probably due to the reduced early postoperative hepatic function and the concomitant postoperative

metabolic overload (Fukatsu *et al*., 2001; Troisi *et al*., 2002). The pharmacokinetics of tacrolimus has not been completely characterized in LDLT patients.

2. Mycophenolic acid

Mycophenolic acid (Figure 3) (MPA, the active compound of the prodrug mycophenolate mofetil (MMF, CellCept, Roche, Nutley, NJ), an uncompetitive, selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), is used for immunosuppressive therapy after transplantation. MPA is primarily metabolized to 7-O-glucuronide (MPAG) in the body.

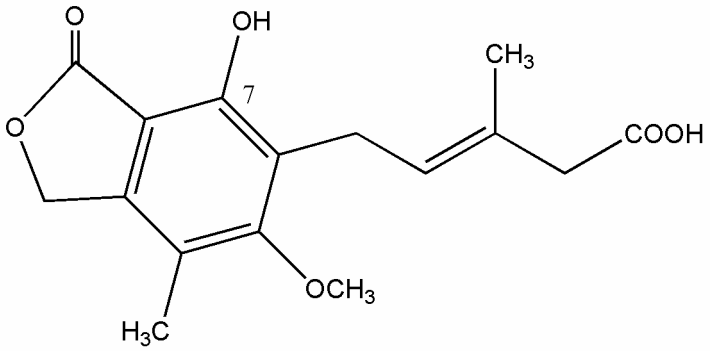


Figure 3. Chemical structure of mycophenolic acid.

Initially, UGT1A8, 1A9 and 1A10 were thought to be responsible for the formation of MPAG (Mackenzie, 2000). Since UGT1A8 and UGT1A10 were only found in the gastrointestinal tract (Mojarrabi and Mackenzie, 1998), MPA is thought to be the probe substrate for UGT1A9 in human livers. Subsequently, a study conducted using cDNA expressed UGTs showed that almost all human UGT1A and 2B are capable of

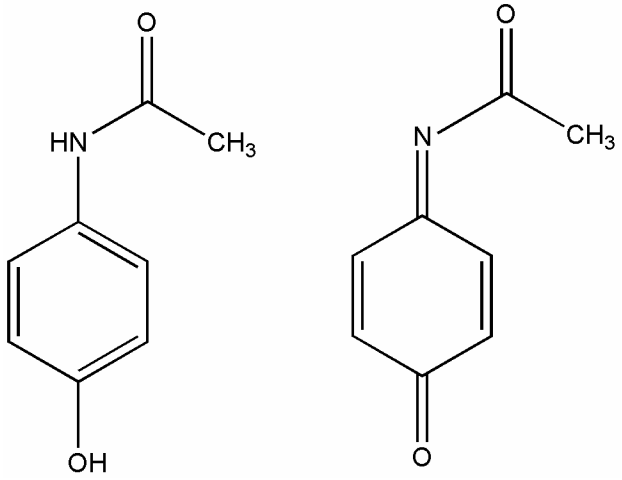
metabolizing MPA to MPAG (Shipkova *et al*., 2001). The most recent report clearly shows that UGT 1A9 is the main isoform involved, with at least 55% contribution to the hepatic MPAG production. UGT 1A1 and 1A6 probably account for a part of MPAG production in the liver, whereas UGT 1A7, 1A8, and 1A10 which are expressed in the small intestine, could contribute to the intestinal first-pass of MPA (Picard *et al*., 2005).

Over 95% of the administered dose is eventually excreted as MPAG in humans (Bullingham *et al*., 1996b; Morissette *et al*., 2001). A negligible amount of the drug is excreted as MPA (<1% of dose) in 48-hour urine (Bullingham *et al*., 1996a). When MPA was administered intravenously to Wistar or Sprague-Dawley rats, about 26% of the dose was excreted in the bile, suggesting that the biliary excretion of MPAG is extensive in these rat strains (Kobayashi *et al*., 2004) even though the glucuronide is preferentially excreted into the urine (about 70% of dose) in humans (Bullingham *et al*., 1996a). Mutidrug resistant protein 2 (Mrp2), an efflux transporter located on the apical side of hepatocytes, has been shown to be essential for the biliary excretion of MPAG based on studies in Mrp2 deficinent rats (Eisai hyperbilirubinemic rats) (Kobayashi *et al*., 2004). Mrp3, an uptake transporter located on the basolateral side of hepatocyte, is also capable of transporting several glucuronide conjugates (Hirohashi *et al*., 1999). It is likely that Mrp3 can efficiently transport MPAG across the sinusoidal membrane and back into the blood (Kobayashi *et al*., 2004). After biliary excretion, MPAG is either eliminated in the feces or is hydrolyzed to MPA by β-glucuronidases in the gut and is reabsorbed.

Mycophenolic acid repeatedly undergoes glucuronidation and Mrp2-mediated biliary excretion in the liver and deglucuronidation and subsequent intestinal reabsorption in the intestine until MPA molecules are finally excreted to the urine as MPAG (Kobayashi *et al*., 2004). At clinically relevant concentrations, MPA and MPAG are about 97% and 82% bound to albumin, respectively (Bullingham *et al*., 1998).

3. Acetaminophen

Acetaminophen (Figure 4) is primarily metabolized to glucuronide and sulfate conjugate by UGT and sulfonyltransferase (SULT). There is a cytochrome P450-catalyzed oxidative pathway of APAP metabolism, forming a reactive quinine form, N-acetyl-p-benzoquinone imine (NAPQI) (Figure 4)



acetaminophen **NAPQI**

Figure 4. Chemical structure of acetaminophen and NAPQI.

CYP3A and 2E1 have been reported to contribute to the formation of NAPQI in human

liver microsomes (Thummel *et al*., 1993). However, the Km (4.4 mM) for CYP2E1 was 25 times higher than Km (0.28 mM) for CYP3A4. The *in vitro* human liver microsomal inhibition data showed that CYP3A4 was, at most, a minor contributor to the formation of NAPQI (10%) at therapeutically relevant concentrations of APAP (0.1 mM) (Thummel *et al*., 1993). NAPQI can be further metabolized to its sequential metabolites: glutathione-, Cys- and NAC- (mercapturate-) conjugates.

After single dose, plasma acetaminophen concentrations in rats declined exponentially, with a half life that increased with increasing doses (Galinsky and Levy, 1981), indicating a non-linear pharmacokinetics in rats. The fraction of the dose that was converted to acetaminophen sulfate decreased with increasing doses (Jollow *et al*., 1974; Galinsky and Levy, 1981). Both acetaminophen glucuronide and acetaminophen sulfate formation in rats are capacity-limited, just as in humans (Levy and Yamada, 1971; Slattery and Levy, 1979; Galinsky and Levy, 1981).

A majority of the drugs used in LDLT patients are primarily eliminated by hepatic metabolism (Table 1). Therefore a thorough knowledge of hepatic metabolizing capacity in LDLT patients is of importance in optimizing drug therapy in this patient population.

Table 1. Common used drugs in transplantation.

Drug class	**Drug**	**Major elimination route**
Immunosuppressive drugs	Prednisone	Liver
	Cyclosporine A	Liver
	Tacrolimus	Liver
	Sirolimus	Liver
	Mycophenolic acid	Liver
Antibiotics	Trimethprim	Kidney
	Acyclovir	Kidney
	Ganciclovir	Kidney
Antifungal drugs	Itraconazole	Liver
	Ketoconazole	Liver
	Fluconazole	Liver
	Voriconazole	Liver
Antihypentensive drugs	Diltiazam	Liver
	Nifedipine	Liver

Central Hypothesis

Liver resection will produce a reduction in the *in vitro* hepatic functional activity via decreased expression of different drug metabolizing enzymes mediated by the acute

phase cytokines such as Il-6 and TNF-α. This in combination with a reduced liver mass will result in decreased clearance of drugs that are metabolized in the liver to a magnitude that is much greater than the loss of liver mass. Immunosuppressive drugs that are routinely used in liver transplant patient will increase activity of various metabolizing enzymes due to their effect on cytokines and their hepatotropic effect.

Hypothesis 1 (Chapter 2)

We hypothesize that the hepatic expression and *in vitro* activity of CYP3A will be impaired during the initial phase of hepatic regeneration due to the cytokines that are released, but will recover over time as the cytokine levels return to normal. We also hypothesize that treatment with cyclosporine A and tacrolimus will prevent the reduction in the expression or activity of CYP3A during hepatic regeneration due to their effect on proinflammatory cytokines and their hepatotropic effect. Partial hepatectomy will be performed in male Sprague-Dawley rats to induce hepatic regeneration since female Sprague-Dawley rats lack the expression of CYP3A or express it at very low levels. The mRNA expression, protein expression and *in vitro* activity of CYP3A will be measured using real-time PCR, western blot and liver microsomal incubations with probe substrate, testosterone, respectively. Effects of cyclosporine A and tacrolimus on the *in vitro* activity of CYP3A will also be evaluated at different time points during hepatic regeneration.

Hypothesis 2 (Chapter 3)

We hypothesize that the hepatic intrinsic clearance of tacrolimus, a substrate of CYP3A enzyme will be impaired and the clearance of tacrolimus will be significantly reduced during hepatic regeneration and the magnitude of reduction will be related to the reduction in the activity measured *in vitro* and the reduction in liver mass. Partial hepatectomy will be performed in male Sprague-Dawley rats to initiate hepatic regeneration. *In vitro* metabolism of tacrolimus will be assessed in liver microsomes based on the formation of 13-demethylated tacrolimus. Pharmacokinetics of tacrolimus after IV administration will be assessed at different time points during hepatic regeneration.

Hypothesis 3 (Chapter 4)

We hypothesize that the hepatic expression and *in vitro* activity of various forms of UGT will be decreased during the initial phase of hepatic regeneration due to decreased expression of transcription factors involved in their regulation as a result of increased levels of cytokines such as IL-6 and TNF-α, but will recover completely over time. Partial hepatectomy will be performed in male Sprague-Dawley rats to initiate hepatic regeneration. The mRNA expression (UGT1A1, 1A2, 1A3, 1A5, 1A6, 1A7, 1A8, 2B1, 2B2, 2B3, 2B6, 2B8, and 2B12) and *in vitro* activity (UGT1A1, 1A6/7, 2B1, 2B1/3/6, 2B2 and 2B12) of different UGTs will be measured using specific primers and specific substrates, respectively, at different time points during hepatic regeneration.

Expression of some of the transcription factors such as CAR, PXR, HNF-1, C/EBP α will also be evaluated.

Hypothesis 4 (Chapter 5)

We hypothesize that the hepatic intrinsic clearance of mycophenolic acid, a substrate for several UGTs, will be decreased and the clearance of mycophenolic acid will be significantly decreased during hepatic regeneration and the magnitude of reduction in clearance will be related to the reduction in UGT *in vitro* and the loss of liver mass. We also hypothesize that the clearance of MPAG will be significantly reduced due to the reduction in liver mass and correspondingly decreased total activity of transporters in the liver during hepatic regeneration. Partial hepatectomy will be performed in male Sprague-Dawley rats to initiate hepatic regeneration. *In vitro* metabolism of mycophenolic acid will be assessed in liver microsomes based on the formation of mycophenlic acid glucuronide. Pharmacokinetics of mycophenolic acid (UGT substrate) and its metabolite, mycophenolic acid glucuronide, will be evaluated at different time points during hepatic regeneration. The protein and mRNA expression of related drug transporters such as multidrug resistance associated protein (Mrp) 2 and Mrp 3 will be also measured at different time points during hepatic regeneration.

Hypothesis 5 (Chapter 6)

We hypothesize that the *in vivo* metabolism of acetaminophen will be altered during

hepatic regeneration with increased formation of NAPQI related toxic metabolite and APAP glucuronide and decreased formation of APAP sulfate due to the dose-dependent pharmacokinetics of APAP. Partial hepatectomy will be performed in male Sprague-Dawley rats to initiate hepatic regeneration. Twenty four-hour urine will be collected to measure various metabolites of acetaminophen with different doses at different time points during hepatic regeneration.

Hypothesis 6 (Chapter 2, 3, 4, 5, 6)

We hypothesize that the liver mass will recover to normal with time after partial hepatectomy. With a full recovery in liver mass and normalization of cytokine levels such as IL-6 and TNF-α, the activity of CYP3A, UGTs and clearance of tacrolimus and MPA and metabolism of APAP will recover to normal.

The studies proposed in this document will significantly increase our understanding of the pharmacokinetics of drugs during hepatic regeneration. Information gained from these studies will help us in designing future studies to optimize drug therapy in LDLT patients.

Chapter 2 Impaired Activity and Expression of CYP3A during Hepatic Regeneration in Rats

Abstract

Objective Hepatic regeneration is very critical to the success of living donor liver transplantation, which allows a reduced size liver to grow in size to accommodate the requirements of both the donor and the recipient. The objectives of this study were to evaluate 1) the activity and protein and mRNA expression of hepatic cytochrome P450 (CYP) 3A and 2) the effect of cyclosporine A and tacrolimus on the activity of hepatic CYP3A in rats at various time points after initiation of hepatic regeneration by partial hepatectomy (PHx).

Methods The activity of CYP3A was determined by the formation rate of 6β-hydroxytestosterone in liver microsomes incubated with the saturating concentration (200 μM) of testosterone. The protein expression of CYP3A was evaluated using Western blotting. The mRNA expression of CYP3A was detected by quantitative Real-time PCR. The effect of cyclosporine A and tacrolimus on CYP3A activity was studied by oral administration of cyclosporine A and tacrolimus immediately after PHx at a daily dose of 10 mg/kg, bid; and 2 mg/kg, bid, respectively for 24 hours, 6 days and 14 days.

Results The hepatic CYP3A activity, protein and mRNA expression were significantly decreased at 24 hours and day 6, but recovered back to normal by day 14. Cyclosporine A and tacrolimus didn't have any additional effect on the activity of CYP3A or on the recovery of CYP3A activity.

Conclusions During hepatic regeneration, the hepatic CYP3A was impaired transiently

but recovered completely with time. In spite of incomplete recovery of liver mass by day 18, the functional capacity of the liver returned to normal in the liver donors. Cyclosporine A and tacrolimus did not have any effect on hepatic CYP3A activity during hepatic regeneration. These results indicate that the clearance of CYP3A substrates will be transiently decreased in the LDLT patients, but will completely recover to normal with time. Reduction in doses of drugs that are metabolized by CYP3A are required during the first few weeks after transplantation. Further, hepatic regeneration, as determined by the activity of CYP3A, will proceed normally in presence of immunosuppressive drug therapy with cyclosporine A and tacrolimus.

Introduction

Liver transplantation is an accepted life-saving therapy for patients with end-stage liver diseases. The number of patients who need liver transplantation has increased 10-fold in the recent years, but the number of cadaveric organs that are available has been stagnant. In the year 2000, only about 25% of the 15,000 patients in the transplant waiting list received a donor organ. Nearly 1800 patients died while waiting for a liver (Wiesner *et al*., 2003). Living donor liver transplantation (LDLT) has emerged as an effective therapy for some selected patients and is a partial solution to the current severe shortage of cadaveric donor organs (Seaman *et al*., 2001). The number of LDLTs performed in the US has increased from less than 100 in the year 1998 to more than 500 in the year 2001 (Wiesner *et al*., 2003).

In LDLT, removal of the right hepatic lobe has become the preferred donor procedure (Wachs *et al*., 1998; Hayashi and Trotter, 2002). After surgery, the donor and recipient have a small liver that grows in size to accommodate the requirements of the donor and recipient, respectively due to hepatic regeneration. Liver regeneration after partial hepatectomy (PHx) involves proliferation of all the existing mature cellular populations, including hepatocytes, biliary epithelial cells, fenestrated endothelial cells, kupffer cells, and cells of Ito (Michalopoulos and DeFrances, 1997). Many genes such as c-fos, c-jun, c-myc, bclx, p53, p21, mdm2, cyclin D1, E, C and B are also activated during hepatic regeneration (Fausto, 2000). Hepatic regeneration is believed to be triggered or activated

by hepatocyte growth factor (HGF) (Lindroos *et al*., 1991), transforming growth factor-α (TGF-α) (Mead and Fausto, 1989), tumor necrosis factor-α (TNF- α) (Diehl *et al*., 1994) and interleukin-6 (IL-6) (Matsunami *et al*., 1992). Elevated concentrations of HGF and IL-6 are observed in plasma after PHx (Lindroos *et al*., 1991; Matsunami *et al*., 1992; Fulop *et al*., 2001; Iwai *et al*., 2001). Several cytokines that are known to be up-regulated during hepatic regeneration are known to alter the activity of some of the hepatic drug metabolizing enzymes (Chen *et al*., 1995; Monshouwer *et al*., 1996; Abdel-Razzak *et al*., 1993).

Cytochrome P450 3A plays a significant role in the metabolism of approximately 50% of the drugs in use including several immunosuppressive drugs used in liver transplant patients such as cyclosporine A, tacrolimus, and sirolimus (Combalbert *et al*., 1989; Bertault-Peres *et al*., 1987; Vincent *et al*., 1992; Sattler *et al*; 1992). The regulation of CYP3A activity during hepatic regeneration in LDLT patients is particularly important. Even though the rate of hepatic regeneration may be different between the donor and the recipient in LDLT program with the donor requiring more regeneration (60% liver regeneration for the donor and 40% liver regeneration for the recipient), both the donor and the recipient require hepatic regeneration to restore the liver size. Since cyclosporine A and tacrolimus have been reported to be hepatotropic during hepatic regeneration in cell renewal and DNA synthesis (Mazzaferro *et al*., 1990; Francavilla *et al*., 1991), treating donors in LDLT with cyclosporine A and tacrolimus may be beneficial in

accelerating the recovery of impaired drug metabolizing activity. On the other hand, recipients normally receive immunosuppressive drugs during the process of hepatic regeneration after transplantation, which may directly affect the drug metabolizing ability of these patients. Limited data based on systemic studies of the regulation of CYP3A activity, protein and mRNA expression levels during hepatic regeneration exist and nothing is known about the effect of immunosuppressive drugs such as cyclosporine A and tacrolimus on the hepatic CYP3A activity and expression during hepatic regeneration. A thorough understanding of the alterations in the activity and expression of CYP3A during hepatic regeneration and the effect of immunosuppressive drugs on the activity of CYP3A is important to optimize drug therapy in LDLT patients.

In this study, we have utilized partially hepatectomized rats to study the effect of hepatic regeneration and chronic treatment with cyclosporine A and tacrolimus on the *in vitro* activity, protein and mRNA expression of CYP3A enzyme. We hypothesized that the activity, protein content and mRNA expression of CYP3A will be decreased during the initial phase of heptic regeneration due to the increased concentration of IL-6 and that treatment with cyclosporine A and tacrolimus will prevent the reduction in the activity of CYP3A enzyme during hepatic regeneration due to their effect on proinflammatory cytokines and the hepatotropic effect on DNA synthesis.

Materials and Methods

Chemicals

Testosterone and 6β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI). Cyclosporine A injection (250 mg/5 ml) (Lot No. 380518) was purchased from Ben Venue Labs (Bedford, OH). Tacrolimus, 10 mg/ml tacrolimus injection (Lot No. 711337K), was a generous gift from Fujisawa Pharmaceutical Company (Osaka, Japan). CYP3A monoclonal antibody raised in mouse was obtained from BD Gentest (Woburn, MA). Horseradish peroxidase-conjugated sheep anti-mouse IgG was purchased from Amersham Biosciences, (Piscataway, NJ). Western Chemiluminescence reagent was bought from PerkinElmer Life Sciences, Inc. (Boston, MA). Reagents for reverse transcription were purchased from Promega (Madison, WI). Forward and reverse primers for CYP3A, beta-actin and beta-2-microglobulin (beta-2-m) were synthesized by Applied Biosystems (Forest city, CA). Rat IL-6 and TNF α kits were purchased from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents used were of HPLC grade.

Animals

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Partial hepatectomy was performed according to the method of Higgins and Anderson (1931). Male Sprague-Dawley rats weighing 225-250 g were anesthetized with methoxyflurane inhalation and the ventral surface was shaved along the mid line and swabbed with betadine. A midline incision of 3-4 cm was

made. Blood vessels supplying the medial and left lateral lobes of the liver were sutured and these lobes were excised. This resulted in the removal of 65-75% of the total liver, leaving the right lateral lobe and the small caudate lobe. For the sham operation (served as paired controls), the liver was manipulated similar to the partial hepatectomy procedure, but was not excised. After surgery, the rats had free access to food and water and were maintained on a 12-hour light and 12-hour night cycle. Rats were sacrificed by CO_2 inhalation at 24 hours, day 6 or day 14 after PHx. On the day of sacrifice, the livers from rats were perfused with ice-cold 0.15 M KCl, frozen in liquid nitrogen immediately and stored at -80ºC for extraction of total RNA and preparation of microsomes. In order to minimize the variability, all rats for each time points were ordered on the same day. Twelve rats were ordered every time (6 rats for PHx; 6 rats for sham) and the surgery was conducted between 9:00 am and 11:00 am. For the rats that were sacrificed at 24 hours, 3 ml blood were collected at the time of sacrifice. The whole blood was centrifuge immediately after collection at 3,000 rpm for 10 minutes and the serum was stored at -20ºC for cytokines measurement.

Liver Mass

The liver mass (L.M.) recovery was calculated in the following way: Wet weight of the remaining liver lobes after PHx / Estimated total L.M. (calculated by multiplying B.W. at the time of sacrifice X ratio of L.M. to B.W for normal rats). Here the ratio of L.M. to B.W. of 0.04 was used for normal rats (Davies and Morris, 1993).

Microsome Preparation

Liver microsomes were prepared by a differential centrifugation procedure. All instruments and buffers were kept on ice during this procedure. The whole liver lobe was used to prepare microsomes to avoid the possibility of uneven distribution of CYP3A. The CYP3A activity among different lobes from the same rat (N = 5 rats) was also evaluated. There was no difference in the CYP3A activity between the two different lobes (1.36 ± 0.20 vs 1.23 ± 0.17 nmol/mg protein/min, $P > 0.5$, paired t-test). Liver samples were placed in 3-4X their weight of homogenization buffer (50 mM Tris HCl buffer, 1.0 % KCl and 1 mM EDTA, pH 7.4) and homogenized using an electrical homogenizer. This homogenate was then centrifugated at 10,000 g for 20 min at 4ºC. Supernatants were then centrifuged at 105,000 g for 65 min at 4ºC. The pellets were reconstituted using a manual homogenizer in 2X their weight of a Tris HCl buffer (50 mM Tris HCl buffer, pH 7.4) containing 20% glycerol. Microsomes were aliquoted and stored at -80ºC until used in incubation studies (It has been shown that the activity of microsomal protein didn't change significantly with ten freeze/thaw cycles, Pearce *et al*., 1996). The protein content was determined by Lowry's method with bovine serum albumin as the standard (Lowry *et al*., 1951). Standards (200 µl) in triplicate and diluted samples (200 µl) in duplicate were mixed with alkine copper sulphate solution (2.5 ml). Then, Folin reagent (250 µl) was added to each tube and the samples were maintained for 45 min at room temperature. The absorbance was measured at 490 nm with a 96 well plate reader.

IL-6 and TNF-α measurement

For IL-6 and TNF-α measurement, fifty μl of pre-treatment buffer to each well followed by adding 50 μl of standards and samples. The plate was covered and incubated at room temperature for 2 hours (1 hour for TNF-α measurement). After incubation, the plate was washed three times. One hundred μl of biotinylated antibody reagent (50 μl for TNF-α measurement) was added to each well after washing. The plate was then covered and incubated at room temperature for 1 hour again. After washing the plate for three times, one hundred μl of prepared streptavidin-HRP solution was added to each well. After incubating for 30 min, the plate was washed for three times. Then 100 μl of premixed TMB substrate solution was added to each well. The plate was developed in the dark at room temperature for 30 min (10 min for TNF-α measurement). After development, the reaction was stopped by adding 100 μl of stop solution to each well. The absorbance was measured on a plate reader at 440 nm minus 540 nm.

Microsome Incubation with Testosterone

The formation of 6β-hydroxytestosterone from testosterone was used as a marker of CYP3A activity. The incubation was carried out in a glass culture tube containing a saturating concentration of testosterone (200 μM) for activity measurement and a series of concentrations of testosterone (0-250 μM) for Km determination, 0.5 mg/ml microsomal protein (linear to 0.5 mg/ml, Figure 5) and 10 mM $MgCl_2$, with the fluid

volume being adjusted to 0.5 ml by the addition of 100 mM phosphate buffer (pH 7.4). The tubes were pre-incubated for 10 minutes at 37°C in an oscillating water bath and then one mM β-NADPH was added to initiate the reaction. After incubation for 10 minutes (linear to 20 min, Figure 6), the reaction was stopped by adding an equal volume (0.5 ml) of ice-cold methanol. The reaction mixture was transferred to a fresh eppendorf tube and the tube was then centrifuged at 13,000 rpm for 5 minutes. The supernatant was analyzed for 6β-hydroxytestosterone using a HPLC method.

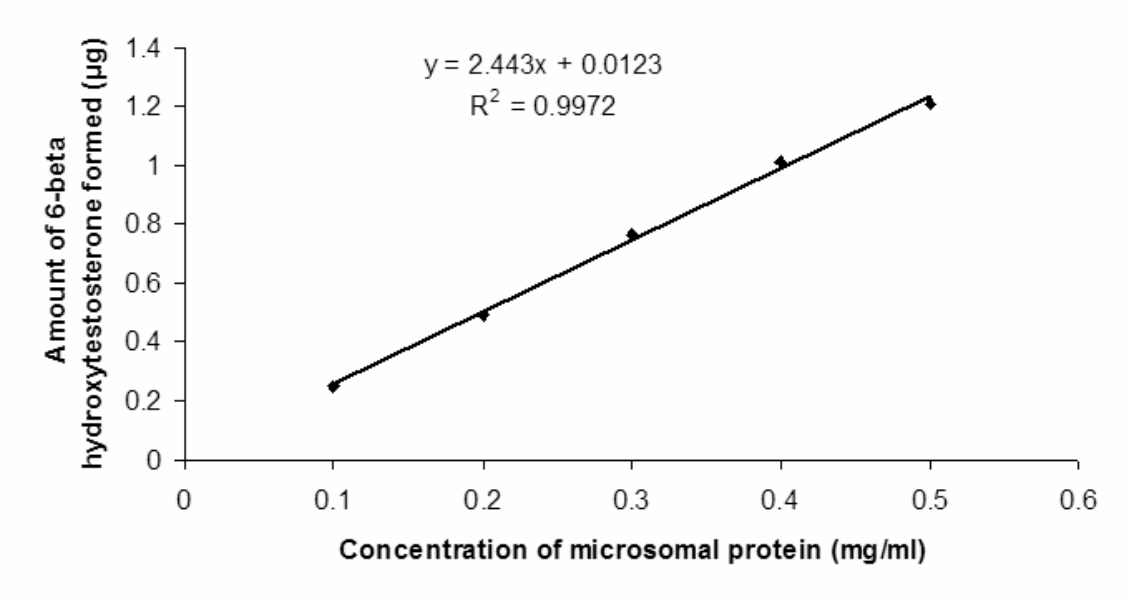


Figure 5. Relationship between microsomal protein concentration and the amount of 6β-hydroxytestosterone formed in rat liver microsomes. Concentration of testosterone: 200 µM; incubation time: 10 min.

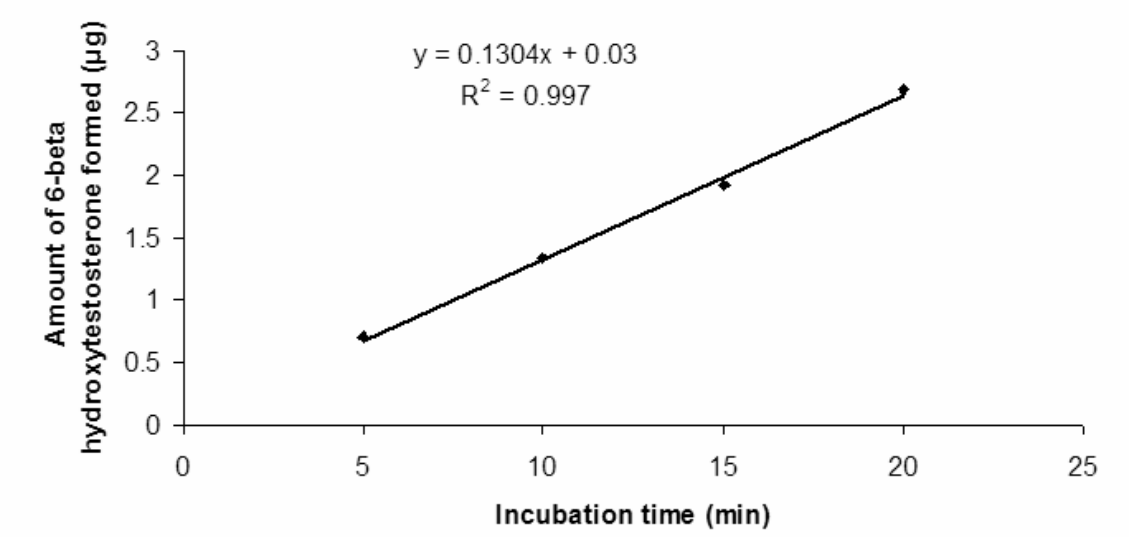


Figure 6. Relationship between time of incubation and the amount of 6β-hydroxytestosterone formed in rat liver microsomes. Concentration of testosterone: 200 µM; microsomal protein concentration: 0.5 mg/ml.

HPLC Analysis of 6β-hydroxytestosterone

6β-hydroxytestosterone was measured by the method developed in our laboratory (Kostrubsky *et al*., 1999). More specifically, one hundred µl of the incubation solution was injected onto a LiChrospher 100 RP-18 column (250 mm x 4.6mm, 5 µ) heated to 30ºC. A mobile phase of methanol/water (60/40) was used at a flow rate of 1.2 ml/min. The UV detector was set at 242 nm. The retention time for 6β-hydroxytestosterone was about 4.9 minutes and the total run time was 20 minutes. The standard curve was linear over the concentration of 0.25-5 µg/ml. The intra- and inter-day CV(%) at 0.25 µg/ml and 5 µg/ml was less than 3% (n =6).

Western blot

Microsomal proteins (20 µg/lane) were electrophoretically resolved using 10% Bio-Rad Ready gels and then transblotted for 3 hours at 4°C onto PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA) with Tris-glycine buffer containing 20% methanol and 0.1% SDS. The blots were then blocked for 1 hour in 5% blocking grade nonfat dry milk (Bio-Rad, Hercules, CA) in TBS-Tween buffer (15 mM Trizma base, 154 mM sodium chloride, 0.05% Tween 20, pH 7.4) at room temperature, incubated for 2 hours at room temperature with mouse anti-CYP3A monoclonal antibodies diluted in 0.5% nonfat dry milk (1:1000), and then washed three times for 15, 5 and 5 min, respectively, in TBS-Tween buffer. After the above washes, the blots were incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences Inc., Piscataway, NJ), diluted 1:10,000 in TBS-Tween buffer, and then subjected to three additional washes (15, 5, and 5 min respectively). CYP3A protein-antibody complexes were detected with ECL Western blotting reagents.

Extraction of RNA and Reverse Transcription

Total RNA was extracted from 50-100 mg livers with 1 ml Trizol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Extracted RNA was quantified spectrophotometrically at 260/280 nm and the integrity was checked using agarose gel. After removal of DNA using RNase-Free DNase, 2 µg of RNA was reversely transcribed using 0.5 µg of random hexamer (Promega, Madison, WI) heated to 70ºC for 5 minutes,

and then cooled to 4ºC. A reaction mixture containing 200 U Moloney murine leukemia virus reverse transcriptase, 1 mM dNTPs and 25 U RNasin (Promega, Madison, WI) was added to the previous mixture and incubated at 37ºC for 60 minutes. The resulting cDNA was diluted 10 folds and stored at -20ºC. The control samples were also prepared with the same procedure by replacing the reverse transcriptase with water (negative controls).

Real-Time PCR

Polymerase chain reaction (PCR) was performed on Applied Biosystems 7700 cycler using 5 µl of cDNA, 7.25 pmol of forward and reverse primers and 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) for a total volume of 25 µl. Forward and reverse primers, designed using Primer Express 2.0 (Applied Biosystems), are 5'-TCAAGGAGATGTTCCCCATCA-3' (Forward), 5'-TCTCCGCCTCTTGCTTCAA-3' (Reverse) for rat CYP3A; 5'-CTGGCCTCACTGTCCACCTT-3' (Forward), 5'-GGGCCGGACTCATCGTACT-3' (Reverse) for β-actin and 5'-CGTGCTTGCCATTCAGAAAA-3' (Forward), 5'-GAAGTTGGGCTTCCCATTCTC-3' for beta-2-m. The nucleotide-nucleotide Blast has confirmed that there was no match between the primers and the rat genome except for the target genes. Cycling conditions were 1 cycle at 95ºC for 10 min, followed by 50 cycles with 15 s at 95°C and 1 min at 60°C. The relative cDNA content was determined in duplicate using standard curves created from cDNA and normalized to beta-2-m for each sample. For each pair of primers, the control without reverse transcriptase was also used

for PCR reactions in duplicate to confirm that there was no genomic DNA contamination in the cDNA samples.

Data Analysis

All data are reported as mean ± SD. Student's t-test was used to evaluate the differences between the activities in the regenerating lobes and the lobes from sham group at a significant level of $P < 0.05$. Comparisons among groups were made via a one way analysis of variance ($P < 0.05$). For sample size calculation, the initial formation rate of 6β-hydroxytestosterone from the lobes of sham (24 hours) (1.72 ± 0.22 nmol/mg protein/min), was used. With a power of 80% and $\alpha = 0.05$, to detect a 25% difference, 4 rats were required. Experiments were completed with 4 to 6 rats in each group.

Results

Recovery of Liver Mass during Hepatic Regeneration.

Liver mass recovery after initiation of hepatic regeneration with or without cyclosporine A or tacrolimus administration is illustrated in Figure 7. Liver mass was 31.3 ± 2.0% at 24-hours, 50.5 ± 4.0% on day 6 and 72.3 ± 5.1% on day 14. The recovery of liver mass approached a plateau by day 18. Cyclosporine A or tacrolimus did not have any effect on the recovery of liver mass.

Serum concentration of IL-6 and TNF-α

The serum concentration of IL-6 was signicantly increased 24 hours after hepatic regeneration (PHx-24 hour vs. Sham: 124 ± 28 pg/ml vs. 47 ± 18 pg/ml, $P < .01$, student's t-test). However, the serum concentration of TNF-α was not altered 24 hours after initiation of hepatic regeneration (PHx-24 hour vs. Sham: 89 ± 20 pg/ml vs. 95 ± 18 pg/ml, $P > .05$, student's t-test).

In Vitro Activity of CYP3A as Measured by the Formation of 6ß-hydroxytestosterone from Testosterone

Figure 8 shows the activity of CYP3A enzyme during hepatic regeneration. CYP3A activity decreased significantly at the 24th hour compared to the activity in the sham group (t-test, $P < .01$, Figure 8) and didn't significantly differ between day 6 and 24 hours. The activity eventually returned to normal level by day 14 (t-test, $P > .05$, Figure 8). No significant difference in Km was observed at 24-hour or 14 days of hepatic regeneration compared to corresponding control values (24-hour: 34.7 ± 12.0 vs. 25.2 ± 7.7 μM; 14-day: 26.0 ± 8.7 vs. 28.0 ± 5.1 μM; t-test, $P > .05$).

Immunochemistry of CYP3A

The results of the western blot for CYP3A are shown in Figure 9. The regenerating lobes had a lower CYP3A protein at 24 hours and 6 days. The protein expression recovered almost completely by day 14.

Selection of Control Genes

The mRNA expressions of beta-actin and beta-2-m at different time points during hepatic regeneration are shown in Figure 10. The mRNA expression of beta-actin almost doubled at 24 hours (sham vs. PHx: 0.19 ± 0.03 vs. 0.32 ± 0.06, $P < .01$). The mRNA level of beta-actin in the PHx group was similar to the paired sham group by day six. The mRNA expression of beta-2-m, on the other hand was relative stable during hepatic regeneration and was not significantly different between paired sham groups and PHx groups. Therefore, beta-2-m was chosen as the normalization gene for the study of other target genes.

mRNA Expression of CYP3A

The mRNA expression of CYP3A after initiation of regeneration is shown in Figure 11. The mRNA expression of CYP3A was decreased to about 20% of control value in paired sham group by twenty four hours (t-test, $P < .01$). The mRNA expression of CYP3A stayed at the same level on day 6 (t-test, $P > .05$), and was lower than control level in paired sham group (t-test, $P < .05$). After regeneration for 14 days, the mRNA expression returned to control level (t-test, $P > .05$).

Effect of Tacrolimus and Cyclosporine

Twenty fours hours, six days and fourteen days of oral administration of cyclosporine A and tacrolimus didn't have any effect on the recovery of CYP3A activity (Figure 12).

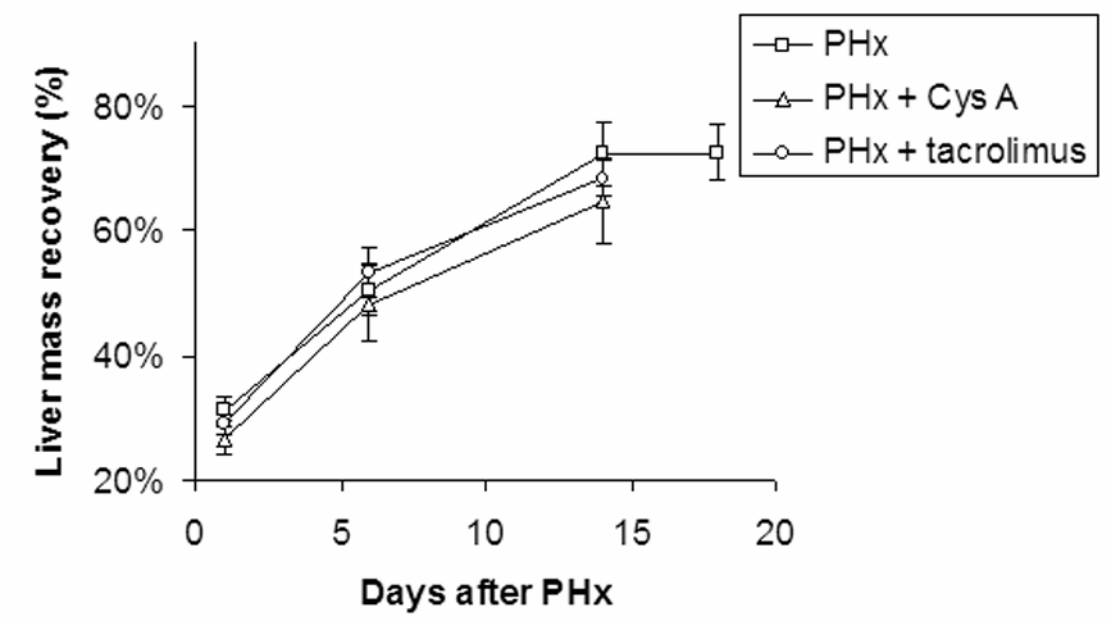


Figure 7. Recovery of liver mass during hepatic regeneration. The liver mass recovery was represented as percentage in the following way: Wet weight of the remaining liver lobes after PHx/Estimated total liver mass (calculated by multiplying body weight at the time of sacrificing X ratio of liver mass to body weight for normal rats). Here the ratio of liver mass to body weight for a normal rat was assumed to be 0.04. N = 5 to 6 rats.

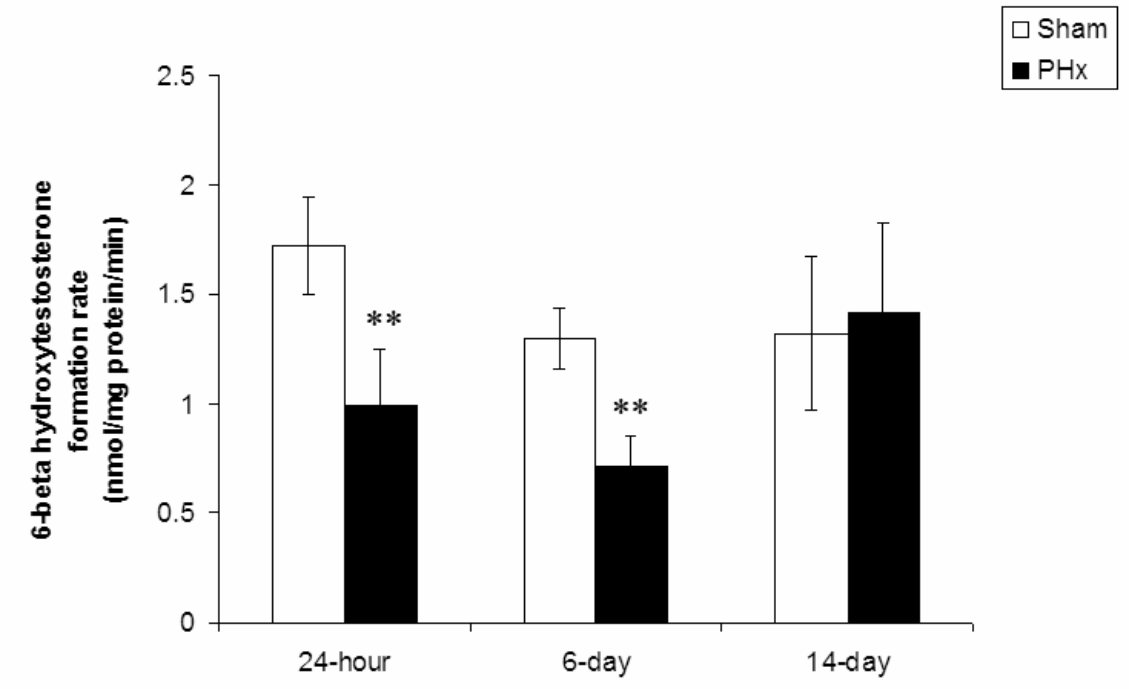


Figure 8. CYP3A activity at different time points during hepatic regeneration.

Sham: liver lobes from sham groups; PHx: regenerated liver lobes after PHx. The activity was measured using 6β-hydroxytestosterone formation rate at saturating testosterone concentration (200 uM). ** $P < .01$ vs. sham (student's t-test). N = 4 to 6 rats.

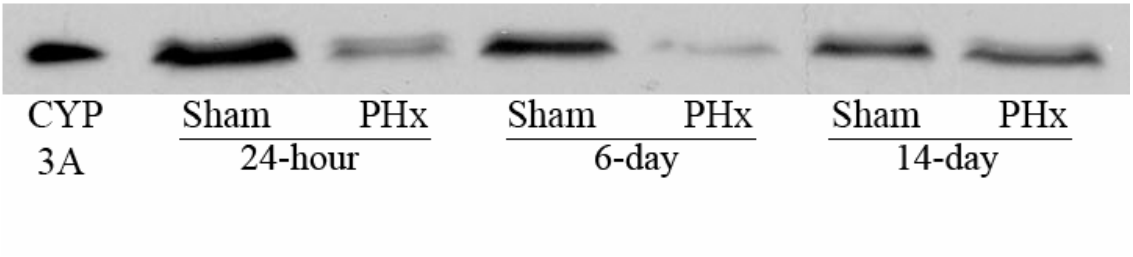


Figure 9. Immunochemical analysis of the CYP3A protein expression at different time points after initiation of regeneration. Equal amounts of microsomal protein were loaded in each lane. Sham: pooled lobes from sham groups; PHx: pooled regenerated lobes after PHx. Microsomal proteins from 6 rats were pooled together in each group.

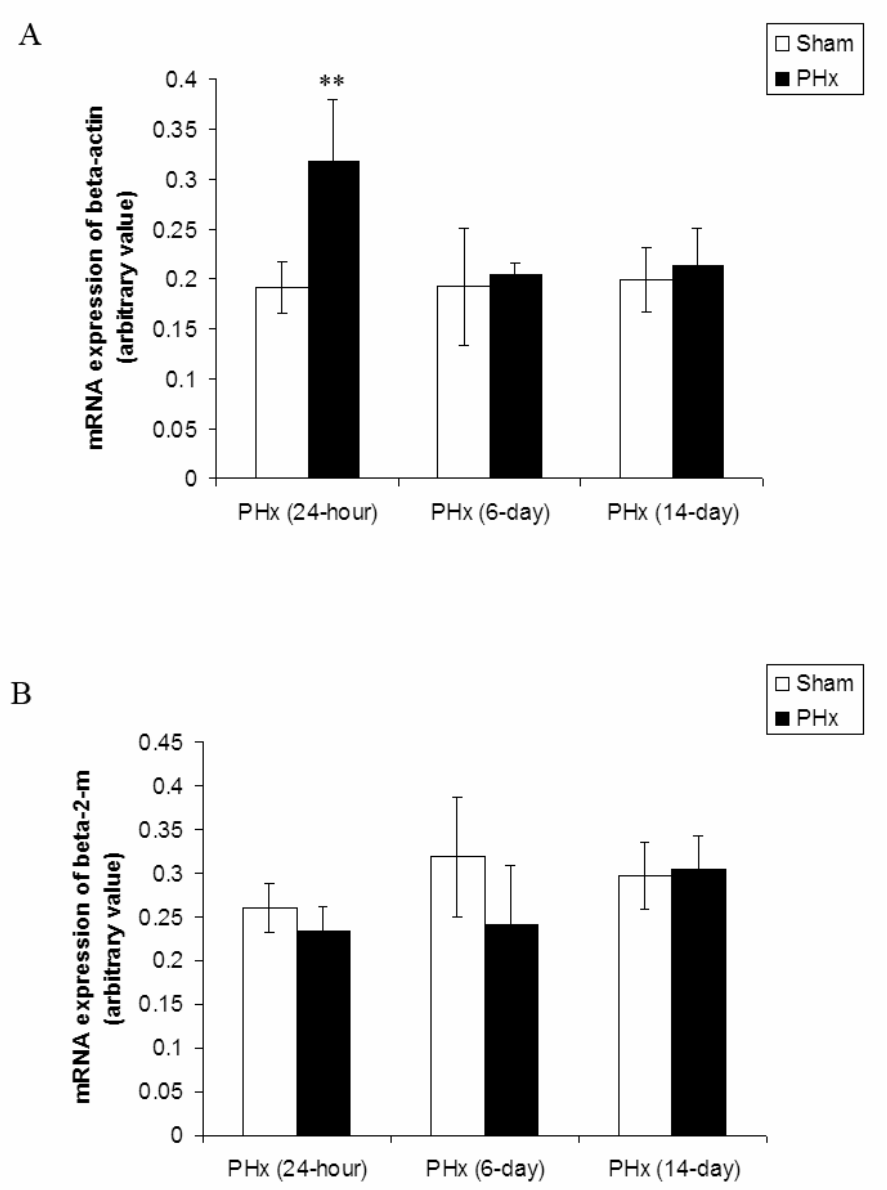


Figure 10. The mRNA expression of control genes at different time points after initiation of regeneration. Panel A: beta-actin; Panel B: beta-2-microglobulin. Sham: pooled lobes from sham groups; PHx: pooled regenerated lobes after PHx. The arbitrary mRNA values were determined by real time PCR as described in the Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. All data are expressed as mean ± SD. ** $P < .01$ vs. sham N = 4 rats.

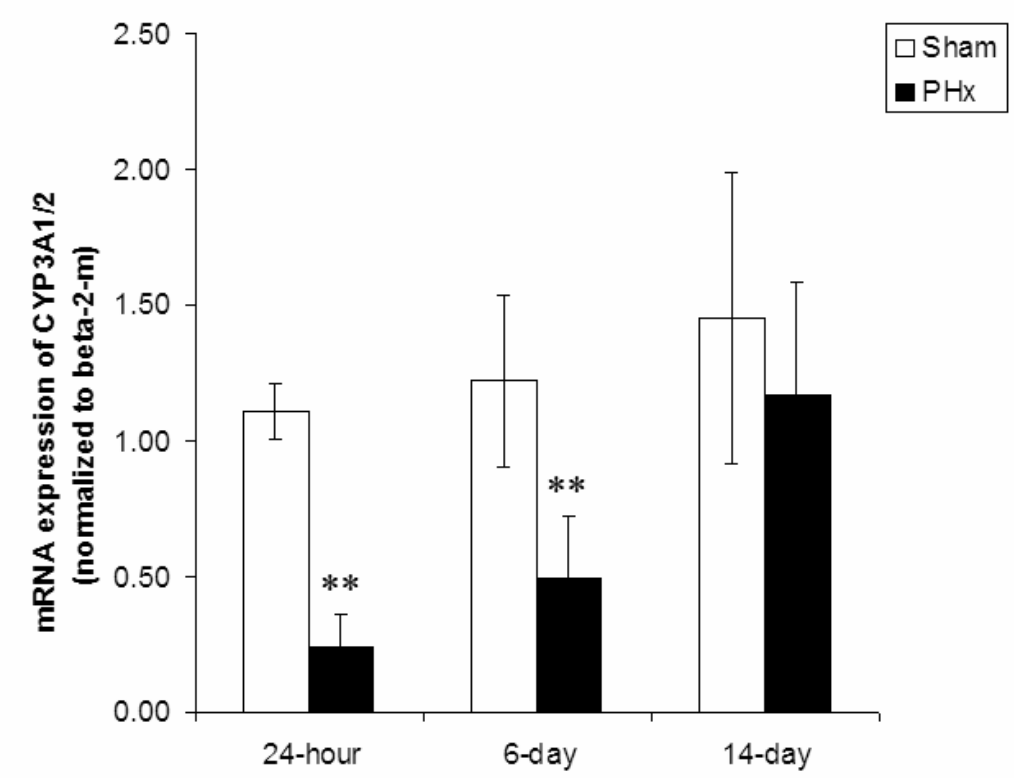


Figure 11. The mRNA expression of CYP3A at different time points after initiation of regeneration. Sham: pooled lobes from sham groups; PHx: pooled regenerated lobes after PHx. The relative mRNA level was determined by real time PCR as described in the Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. ** $P < .01$ vs. sham (student's t-test). N = 4 to 5 rats.

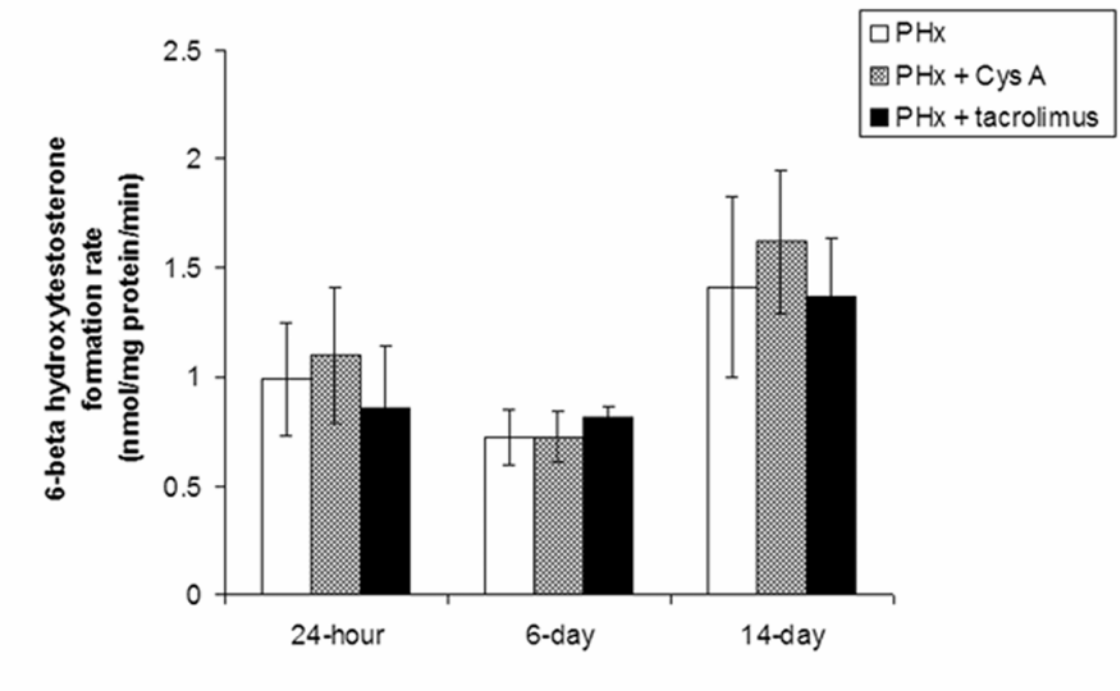


Figure 12. Effect of drug treatments on CYP3A activity during hepatic regeneration. PHx: regenerated liver lobes after PHx; Cys A: cyclosporine A (10 mg/kg/day, bid, po); tacrolimus: tacrolimus (2 mg/kg/day, bid, po). The activity was measured using 6β-hydroxytestosterone formation rate under saturating testosterone concentration (200 uM). $P > .05$, one-way ANOVA. N = 4 to 6 rats.

Discussion

In this study we have used partially hepatectomized rats to evaluate the effect of hepatic regeneration on the *in vitro* activity of CYP3A using the formation rate of 6β-hydroxytestosterone. We have also evaluated the effect of cyclosporine and tacrolimus on the CYP3A mediated drug metabolism in rats with regenerating livers as these drugs are known to be hepatotropic and are expected to increase hepatic regeneration (Mazzaferro *et al*., 1990; Francavilla *et al*., 1991).

Our results indicate that liver mass recovered gradually to about 72% of the original liver mass over a two week period. This value stayed the same on day 18. This agrees with the reported 78% liver mass recovery in rats by day 15 after PHx (Maza *et al*., 2001). This is also consistent with a recent observation of recovery of up to 78.6% of ideal liver volume in the donors in a living donor liver transplant program (Humur *et al*, 2004). Liver mass recovery in rats was not augmented by chronic treatment with cyclosporine A or tacrolimus, which is consistent with data published by Coughlin *et al* (Coughlin *et al*., 1987).

The activity of CYP3A as measured by 6 β-(OH) testosterone and the expression of CYP3A protein and mRNA were all reduced in the regenerating liver at 24 hours and on day 6, but recovered to normal by day 14.This indicates a definite time dependent change in CYP3A activity during hepatic regeneration. Our findings are consistent with some

previous studies indicating suppression of CYP3A during the initial part of liver regeneration (Starkel *et al*., 2000; Tamasi *et al*., 2001; Favre *et al*., 1998; Ishizuka *et al*., 1997). However, most of these studies reported a much earlier recovery of CYP3A activity using single point measurements. Our observation indicates that the activity and expression of CYP3A to be suppressed much longer and possibly until the liver regeneration is complete (14 days). The exact mechanism responsible for the lower CYP3A expression and activity during regeneration is currently not known and future studies are needed to address this. Interleukin-6 released during hepatic regeneration may contribute to the observed decrease in CYP3A expression and activity. It is also likely that newly divided cells may have a much lower enzyme expression and activity compared to existing liver cells.

Cyclosporine A and tacrolimus can augment DNA synthesis and enhance regeneration by non-immunological pathways (Francavilla *et al*., 1990). So we expected hepatotropic effect of cyclosporine A and tacrolimus to be reflected on the recovery of CYP3A activity. On the other hand, *in vitro* incubation with T cells showed that both cyclosporine A (100 nM) and tacrolimus (10 nM) suppressed the expression of IL-2, IL-3, IL-4, c-myc, and TNF α (Tocci *et al*., 1989). We also anticipated that the treatment with cyclosporine A and tacrolimus would prevent the reduction in the activity of CYP3A due to their effect on proinflammatory cytokines. However, in the present study, neither cyclosporine nor tacrolimus, at the doses used, altered the activity of CYP3A in rats with regenerating

livers. This indicates that any hepatotropic effect of cyclosporine and tacrolimus was not reflected in terms of the activity of CYP3A. Moreover, since only serum levels of IL-6 and three soluble cytokine receptors (TNF α receptor I and II, IL-6 receptor) were significantly increased during hepatic regeneration (Fulop *et al*., 2001), the suppression of cyclosporine A and tacrolimus on TNF α and other ILs may have no obvious effect on the expression or activity of drug metabolizing enzymes. Direct inhibition of CYP3A by cyclosporine A as published previously was observed only at doses much higher than what was used in the current study (Brunner *et al*., 1998).

To the best of our knowledge this is the first study to analyze the recovery profile of CYP3A enzyme over time after initiation of regeneration at the level of mRNA expression, protein expression and *in vitro* activity in an animal model. Our study points to several important conclusions. CYP3A expression and activity are significantly reduced after PHx but recovers completely with time. Second, in spite of an incomplete recovery in liver mass on day 14, the functional capacity of the liver (*in vitro* activity) returns to normal. Finally, chronic treatment with cyclosporine A or tacrolimus has no effect on CYP3A activity in the regenerating rat livers. The clinical implications of our study are 1) the clearance of CYP3A substrates will be transiently decreased in the LDLT patients, but will completely recover to normal with time; 2) reduction in doses of drugs that are metabolized by CYP3A is needed during the first few weeks after transplantation; 3) hepatic functional capacity will recover much earlier than the recovery of liver mass;

and 4) hepatic regeneration, as determined by the activity of CYP3A, will proceed normally in presence of immunosuppressive drug therapy with cyclosporine A and tacrolimus.

Chapter 3 *In Vitro* Hepatic Intrinsic Clearance and Pharmacokinetics of Tacrolimus Are Transiently Altered during Hepatic Regeneration in Rats

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Abstract

Objective The objective of this study was to evaluate the pharmacokinetics of tacrolimus in rats at various time points after initiation of hepatic regeneration by partial hepatectomy (PHx).

Methods The *in vitro* hepatic clearance of tacrolimus was measured with liver microsomes incubated with different concentrations of tacrolimus. The pharmacokinetics of tacrolimus was evaluated after intravenous administration of 0.6 mg/kg tacrolimus to partially hepatectomized rats. Two hundred microlitter of whole blood was collected at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours through a jugular vein catheter. The blood concentration of tacrolimus was analyzed by a microparticulate enzyme immunoassay (MEIA).

Results The hepatic intrinsic clearance of tacrolimus was decreased to 72% and 51% of that in the control rats at the 24th hour and the 6th day, respectively, but recovered to normal level by day 14. A two-compartment model (WinNonlin) fitted the data adequately. The total body clearance of tacrolimus was reduced transiently but recovered completely by day 18 (4.40 ± 0.50 ml/min/kg (24 hours); 8.64 ± 0.55 ml/min/kg (day 18) and 9.22 ± 0.71 ml/min/kg (control)). Other pharmacokinetic parameters such as the area under the blood concentration vs time curve (AUC), the terminal disposition rate constant

(β) and the terminal disposition half life (T1/2(β)) were also significantly different between control and PHx rats. The volume of distribution and other rate constants such as K_{12} and K_{21} were not altered at any time point after PHx.

Conclusions 1) During hepatic regeneration, the pharmacokinetics of tacrolimus was altered transiently. 2) The magnitude of reduction in *in vivo* clearance of tacrolimus was much less than what was predicted from the loss of liver mass and loss of enzyme activity as measured by *in vitro* studies. 3) The hepatic clearance of other CYP3A substrates will also be reduced transiently during the early hepatic regeneration process. 4) The magnitude of reduction in *in vivo* clearance of other CYP3A substrates will be much less than what was predicted than the loss of liver mass and loss of enzyme activity as measured by *in vitro* studies. 5) A reduction in the dose of CYP3A substrates that is less than the loss of liver mass is sufficient to achieve comparable blood levels of drugs in LDLT patients and cadaveric liver transplant patients. 6) The ability of the liver to clear drugs recovers completely with time and normal hepatic function will be restored in subjects undergoing hepatic resection.

Introduction

Cytochrome P450 3A enzymes account for more than 50% of the drugs metabolized through phase I pathways and is also involved in the metabolism of several immunosuppressive drugs such as cyclosporine A, tacrolimus and sirolimus. Even though the rate of hepatic regeneration may be different between the donor and the recipient in a LDLT program, with the donor requiring more regeneration (60% liver regeneration for the donor and 40% liver regeneration for the recipient), both the donor and the recipient require hepatic regeneration to restore the normal size of the liver. Recently, LDLT patients have been reported to achieve higher blood levels of tacrolimus and cyclosporine A for a given dose compared to cadaveric liver recipients (Trotter *et al*., 2002; Taber *et al*., 2002; Morgan *et al*., 2001). Previous studies (chapter 2) in rats have shown suppression of CYP3A during the initial phase of liver regeneration and complete recovery of the activity and expression of CYP3A within 2 weeks after initiation of hepatic regeneration. The impaired activity of CYP3A in the remaining liver along with a reduced liver mass will contribute to the reduced clearance and increased blood levels of drugs such as cyclosporine A and tacrolimus.

So it is important to understand whether the magnitude of change in the pharmacokinetics of immunosuppressive drugs metabolized by CYP3A during the hepatic regeneration process matches the predicted magnitude of change due to a decrease in liver mass and a decrease in the intrinsic activity of CYP3A. Nothing is currently known about the

pharmacokinetics of immunosuppressive drugs that are metabolized by CYP3A pathway, during hepatic regeneration. Such knowledge will help in optimizing not only the immunosuppressive drug therapy but also therapy with other drugs that are metabolized by CYP3A during hepatic regeneration. In this study, we hypothesized that the clearance and *in vitro* metabolism of tacrolimus will be decreased during hepatic regeneration due to the decrease in liver mass and a reduction in the activity of CYP3A in the remaining liver lobe.

Materials and Methods

Chemicals

Tacrolimus, (10 mg/ml tacrolimus injection - Lot No. 711337K) and 13-demethylated tacrolimus were generous gifts from Fujisawa Pharmaceutical Company (Osaka, Japan). Heparin injection (Lot No. 322024) was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA). IMx system and related reagents for measurement of tacrolimus blood concentration were obtained from Abbott Laboratories (Abbott Park, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

The study protocol was approved by the IACUC at the University of Pittsburgh. Partial hepatectomy was performed according to the method of Higgins and Anderson in male Sprague-Dawley rats weighing 225-250 g (Higgins and Anderson, 1931) as described in

Chapter 2 (For sham operation, please refer to ***Animals*** under **Methods and Materials** section in **Chapter 2**). Medial and left lateral lobes surgically removed during the partial hepatectomy procedure served as the control in this study.

Microsome Preparation

Liver microsomes were prepared by a differential centrifugation procedure as described under **Methods and Materials** section in **Chapter 2**.

Microsome Incubation with Tacrolimus

The formation of 13-demethlated tacrolimus (M1) from tacrolimus was also used as a marker of CYP3A activity. The incubation was carried out using methods described in ***Microsome Incubation with Testosterone*** under **Methods and Materials** section in **Chapter 2** with different concentration of tacrolimus (0-25 μM) and a fixed concentration of the microsomal protein (2.4 mg/ml) (linear to 2.4 mg/ml, Figure 13) for 20 minutes (linear to 20 min, Figure 14). The final solution was analyzed for concentration of M1 using HPLC.

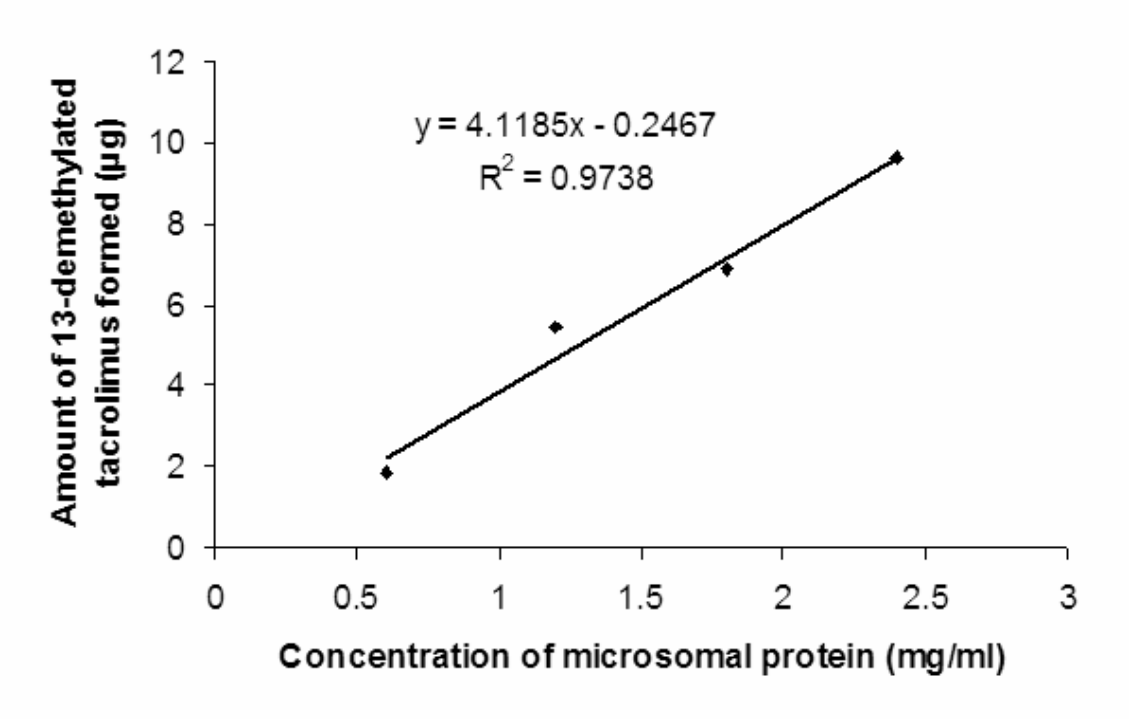

Figure 13. Relationship between microsomal protein concentration and the amount of 13-demethylated tacrolimus formed in rat liver microsomes. Concentration of tacrolimus: 25 μM; incubation time: 20 min.

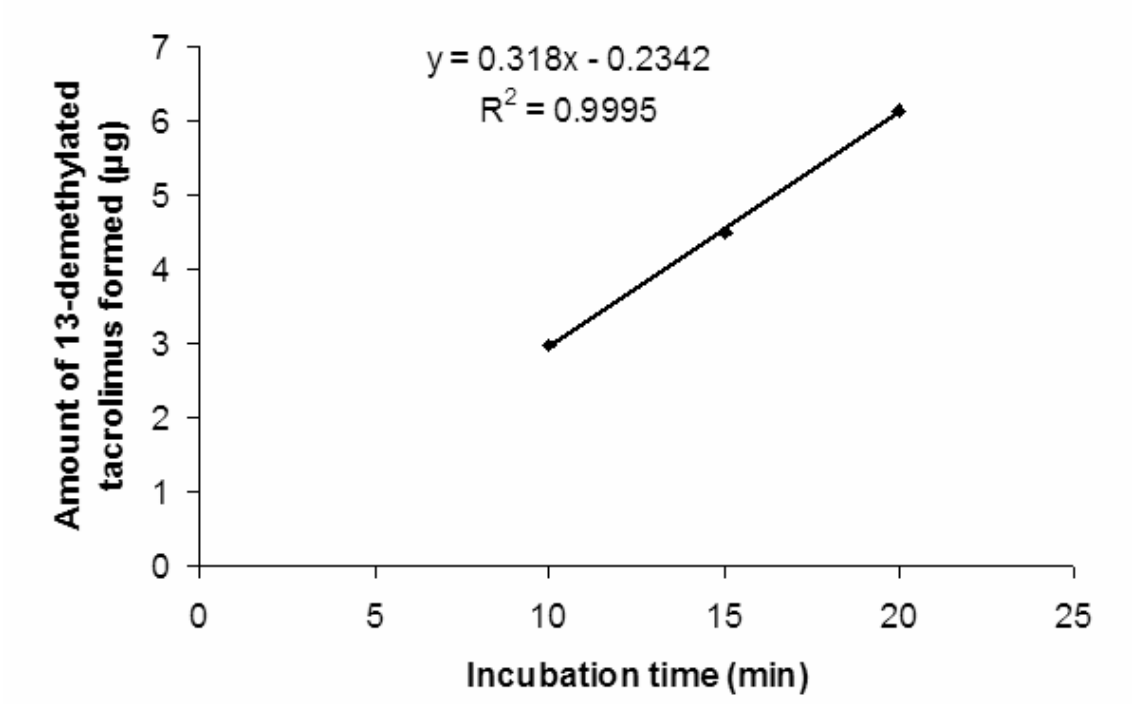

Figure 14. Relationship between time of incubation and the amount of 13-demethylated tacrolimus formed in rat liver microsomes. Concentration of tacrolimus: 25 μM; microsomal protein concentration: 1.8 mg/ml;

Pharmacokinetic Study of Tacrolimus

Pharmacokinetic studies were carried out in rats in the control group, 24 hours after PHx, 14 days after PHx and 18 days after PHx. Since there was no significant difference between control group and sham group in the *in vitro* intrinsic clearance of tacrolimus at any time, pharmacokinetic studies were not conducted in sham group (Table 2). A silastic tubing was inserted into the jugular vein 24 hours before the study. Rats were anesthetized with methoxyflurane inhalation (in control rats not undergoing PHx) or continued

methoxyflurane anesthesia in the rats undergoing surgery. Rats were restrained in the triangular shaped plastic bag with an opening at the tip for the rat to inhale the anesthetic. The extended toe pinch reflex was used to determine the depth of anesthesia. When anesthesia was appropriate, rats were turned on their back and an incision was made at the location of the jugular vein around the neck using sterile techniques with sanitized instruments. The jugular vein was revealed, the blood supply was stopped, and a small hole was made in the vein using fine tipped instruments. A PE-60 catheter tubing connected to a syringe filled with 100U/mL heparinated saline was inserted. A small plug was used to seal off the tubing. The tubing was secured with suture. A small incision was made at the top of the rat's back and the tubing was pulled through so the rat didn't damage the tubing.

On the study day, tacrolimus(0.6 mg/kg) was administered intravenously through the jugular vein catheter. A sterile syringe (1 cc) was used for the collection of each blood sample. First, fluid in the catheter and 0.1 ml blood was taken out by a 1cc syringe containing 0.2 ml 100 u/ml heparinized saline solution; then 0.2 ml blood sample was withdrawn for tacrolimus analysis; and finally the initial fluid plus blood together with 0.2 ml 100 u/ml heparin saline was injected back. Multiple blood samples were collected in heparinized tubes at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours after intravenous administration of tacrolimus.

HPLC Analysis of 13-demethylated Tacrolimus

As per the method described previously (Perotti *et al*., 1994b) with minor modifications, one hundred µl of the incubation solution was injected onto a LiChrospher RP-18 column (250 mm x 4.6 mm, 5 µ) heated to 60ºC. The UV detector was set at 214 nm. The mobile phase consisted of a mixture of acetonitrile/method/diluted *O*-phosphoric acid, pH 3.0, 49/3/48. The initial flow rate was 1 ml/min for the first 20 minutes, stepped up to 1.5 ml/min within 0.5 min and maintained for the subsequent 23 minutes. The retention time for M1 was 9.3 minutes and the total run time was 43 minutes. The standard curve was linear over a range of 0.25-5 µg. The intra- and inter-day CV(%) at 0.5 µg and 5 µg was less than 7% (n = 5).

Microparticulate Enzyme Immunoassay of Tacrolimus Blood Concentration

Tacrolimus concentration in the blood was measured by a microparticulate enzyme immunoassay (MEIA) using Abbott's IMx analyzer. Each blood sample, calibrator or control was individually mixed thoroughly. One hundred fifty µl of each sample (diluted to the linear range with blank blood when necessary), the calibrator or control was pipetted into a fresh 1.5 ml tube and 150 µl of IMx Tacrolimus II Whole Blood Precipitation Reagent was added. Each tube was vortexed and centrifuged at 13,000 rpm for 4 min. The tubes were uncapped and the supernatant was decanted into the sample well of an IMx reaction cell. The measurement was done automatically using IMx Tacrolimus II Reagent Pack containing mouse monoclonal anti-tacrolimus antibody

coated microparticles and tacrolimus alkaline phosphatase conjugate in a IMx system. The antibody used in the assay does not cross-react with the major metabolite, (representing more than 75% of total metabolites formed) M1. Cross reactivity has been observed only with a few minor metabolites. Based on this, the antibody used primarily measures only tacrolimus. The calibration curve, ranging from 2 ng/ml to 30 ng/ml, was generated using reagents supplied in the kit. The intra-day and inter-day CV (%) at 3 ng/ml, 12 ng/ml and 25 ng/ml was less than 14% (n =5). With each run of the samples, three controls with concentrations of 5 ng/ml, 11 ng/ml and 22 ng/ml were also run.

Data Analysis

Enzyme kinetic analysis was performed using Prism 3.0 (GraphPad Software Inc., San Diego, CA). The kinetic parameters (K_m and V_{max}) for the formation of 13-demethylated tacrolimus were calculated using nonlinear regression analysis. The intrinsic clearance (Cl_{int}) was calculated as V_{max}/K_m. Pharmacokinetics of tacrolimus was analyzed by fitting a biexponential equation to the data using WinNonlin 3.1 (Pharsight Co., Mountain View, CA). The selection of the kinetic model was made using AKAIKE information criterion (AIC) and the precision of the estimated parameters. All data are reported by mean ± SD. Comparisons among groups were made via a one way analysis of variance with Tukey post hoc analysis ($P < 0.05$). For sample size calculation, based on the initial measurement of AUC in control group, 1089 ± 83.05 hr*ng/ml, to observe a 20%

difference between groups, with a power of 80% and $\alpha = 0.05$, the sample size required was 3 rats. Experiments were completed with 4 to 6 rats in each group.

Results

Measurement of V_{max}, K_m, and CL_{int} for the Formation of 13-demethylated Metabolite of Tacrolimus in Hepatic Microsomes

Both Vmax and Km values were not different between control group and sham group at all time points studied (Table 2). The Vmax for the formation of M1 in hepatic microsomal fraction obtained at the 24th hour after PHx was significantly decreased compared to control value (Table 2). On day 6, the Vmax still remained at a lower level (50% of control level) but recovered completely by day 14 during hepatic regeneration (90% of control level). However, the Km values were similar among all the groups. The intrinsic clearance (CL_{int}) for the formation of M1 in the hepatic microsomal fraction was significantly decreased during hepatic regeneration at the 24th hour and on day 6.

Pharmacokinetics of Tacrolimus

The blood concentration vs. time curve of tacrolimus after intravenous administration of tacrolimus was well described by a biexponential process (Figure 15). The pharmacokinetic parameters of tacrolimus at different time points after initiation of the regeneration are summarized in Table 3. The area under the blood concentration vs time curve (AUC), the total body clearance (CL), the terminal disposition rate constant (β) and

the terminal disposition half life (T1/2(β)) were significantly different between control and PHx rats. The total body clearance of tacrolimus at the 24th hour was much lower than that in the control group. The clearance of tacrolimus increased significantly on day 14 from values observed at the 24th hour but was still lower than the clearance in the control group. There was no significant difference in total body clearance on day 18 and control group. The volume of distribution and other rate constants such as K_{12} and K_{21} were not altered at any time point.

Table 2. Mean (± SD) Vmax, Km and CL_{int} for the formation of 13-demethyled metabolite (M1) of tacrolimus in hepatic microsomes (N = 4-6 rats).

Time after PHx	Groups	Vm (nmol/mg protein/min)	Km (µM)	CL_{int} (ml/min/mg protein)
24-hour	Control	0.40 ± 0.06	4.87 ± 0.69	0.082 ± 0.007
	Sham	0.41 ± 0.03	5.28 ± 0.55	0.079 ± 0.011
	PHx	0.30 ± 0.03[b]	5.10 ± 0.85	0.059 ± 0.005[a]
6-day	Control	0.36 ± 0.07	5.29 ± 0.80	0.068 ± 0.008
	Sham	0.38 ± 0.05	5.16 ± 0.73	0.075 ± 0.018
	PHx	0.18 ± 0.02[a]	5.18 ± 0.74	0.035 ± 0.003[a]
14-day	Control	0.42 ± 0.03	5.04 ± 0.88	0.085 ± 0.018
	Sham	0.40 ± 0.03	5.00 ± 0.68	0.081 ± 0.013
	PHx	0.38 ± 0.04	4.90 ± 0.75	0.079 ± 0.011

[a] $P < .01$; [b] $P < .05$ (vs. control, Tukey post hoc analysis).

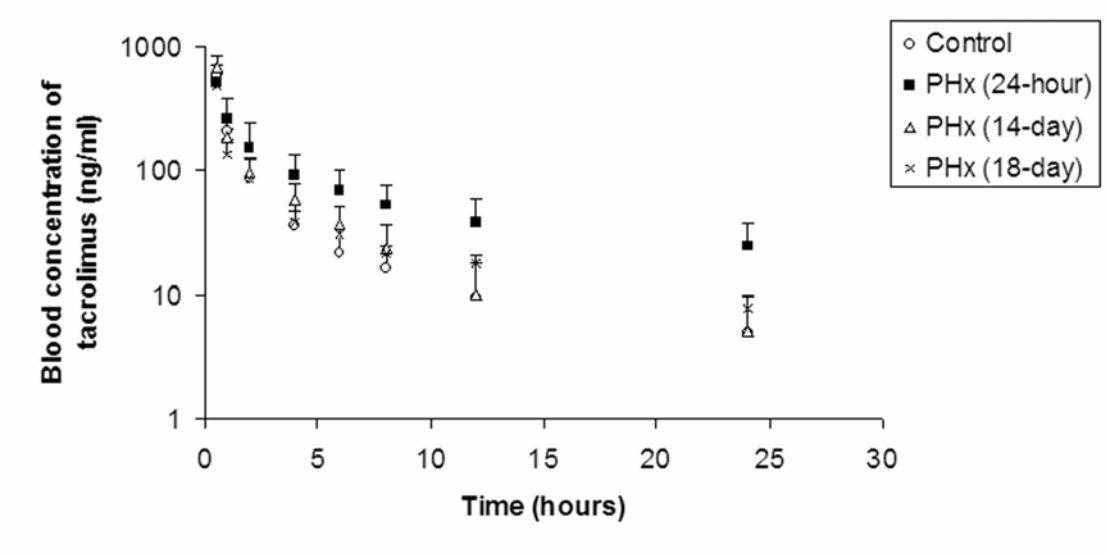

Figure 15. Blood concentrations of tacrolimus vs time profile at different time points after initiation of hepatic regeneration. Data was represented by mean + SD (N = 4 to 6 rats).

Table 3. Pharmacokinetic parameters of tacrolimus (0.6 mg/kg, i.v.) 24 hours, 14 days and 18 days after partial hepatectomy (N = 4 to 6 rats)

Parameters	**Control**	**PHx (24-hour)**	**PHx (14-day)**	**PHx (18-day)**
AUC (hr•ng/ml)**	1089.29 ± 83.05	2297.60 ± 260.51[a]	1575.97 ± 144.99[a]	1160.75 ± 74.62
CL (ml/min/kg)**	9.22 ± 0.72	4.40 ± 0.50[a]	6.39 ± 0.61[a]	8.64 ± 0.55
β ((hr^{-1}))*	0.31 ± 0.12	0.10 ± 0.05[b]	0.24 ± 0.04	0.24 ± 0.10
$t_{1/2,\beta}$ (hr)*	2.53 ± 0.97	8.67 ± 4.48[b]	2.98 ± 0.52	3.14 ± 1.10
K_{10} (hr^{-1})**	2.31 ± 0.74	0.83 ± 0.25[b]	2.88 ± 0.51	2.67 ± 0.28
K_{12} (hr^{-1})	1.12 ± 0.40	1.25 ± 0.17	1.03 ± 0.50	1.43 ± 0.54
K_{21} (hr^{-1})	0.40 ± 0.25	0.49 ± 0.20	0.35 ± 0.04	0.29 ± 0.07
Vss (ml/kg)	718.53 ± 258.20	895.74 ± 272.98	653.37 ± 192.74	658.31 ± 274.50

** $P < .01$, * $P < .05$ for ANOVA; [a] $P < .01$, [b] $P < .05$ (vs. control) was obtained from Tukey post hoc analysis.

Abbreviations: AUC, area under the blood concentration vs time curve; CL, total body clearance; $t_{1/2,\beta}$ = disposition half life; Vss = volume of distribution at steady state.

Discussion

In this study we have used partially hepatectomized rats to evaluate the effect of hepatic regeneration on the pharmacokinetics of tacrolimus. This study simulates what is likely to happen to the drug metabolizing capacity in the donor in a living donor liver transplant program. In addition to hepatic regeneration, additional factors such as cold ischemia, warm reperfusion injury and immunosuppressive drug therapy can also modify drug metabolizing capacity in the recipient. In this study, tacrolimus was used as a representative marker drug for CYP3A.

Tacrolimus is primarily eliminated by hepatic metabolism (hepatic clearance is close to total body clearance as only 0.5% unchanged tacrolimus was recovered in human after IV dosing of tacrolimus (Moller *et al*., 1999)) through the formation of M1 (representing 75% of the total metabolites, Perotti *et al*., 1994) and the CYP3A enzyme appears to be responsible for the formation of M1 (Shiraga T *et al*., 1994). Alterations in the hepatic function due to drug or disease state have been associated with altered ability of the liver to clear tacrolimus (Venkataramanan *et al*., 1995). In our studies, the magnitude in reduction in the liver mass at 24 hrs was 69%. The ability of CYP3A in the liver to metabolize testosterone *in vitro* (chapter 2) and to metabolize tacrolimus was also reduced in the regenerating liver at 24 hours and on day 6. Taking into consideration the reduction in liver mass and the decrease in the hepatic intrinsic clearance of tracrolimus normalized to protein amount (assuming that hepatic M1 formation clearance

approximates the hepatic intrinsic clearance of tacrolimus, because majority of tacrolimus is metabolized to M1 and that all other pathways of tacrolimus metabolism will behave similar to M1 pathways, as these metabolites are also formed by CYP3A), the whole liver intrinsic clearance for tacrolimus must decrease to 21% of normal liver at the 24th hour after initiation of hepatic regeneration (71% of control intrinsic clearance times 31% of normal liver mass 24 hours after PHx). Based on the total body clearance of tacrolimus in control rats (9.22 ml/min/kg) and the reported blood flow of 55.2 ml/min/kg (Davies and Morris, 1993), assuming lack of any change in the unbound fraction of tacrolimus as red blood cells are primarily responsible for binding tacrolimus in blood (Venkataramanan *et al*., 1995) and hematocrit did not change during hepatic regeneration (Okano *et al*., 2001; Kurata *et al*., 2000; Eguchi *et al*., 1998), the following relationship can be established for tacrolimus at 24 hrs:

$$CL = Q*f_u*Cl_{int}/(Q+f_u*Cl_{int}) \quad (1)$$

CL: hepatic clearance or total body clearance for tacrolimus

Q: hepatic blood flow

f_u: unbound fraction of tacrolimus

Cl_{int}: hepatic intrinsic clearance for tacrolimus

CL = 9.22 ml/min/kg (data from control group) (2)

Q = 55.2 ml/min/kg (data from Davies and Morris, 1993) (3)

f_u*Cl_{int} = 11.07 ml/min/kg (from equation 1, 2, and 3) (4)

At the 24th hour after PHx, we have:

$CL_{PHx,predicted} = Q*f_u*0.21Cl_{int}/(Q+ f_u*0.21Cl_{int})$ (5)

(Hepatic intrinsic clearance at the 24th hour after PHx was only 21% of that observed in the control group.) (remaining hepatic clearance (%) when normalized to per unit liver mass: 71% (Table 2); remaining liver mass (%): 31% (Figure 7))

$CL_{PHx,predicted}$: predicted total body clearance 24 hours after PHx = 2.22 ml/min/kg

At the 14th day after PHx, we have:

$CL_{PHx,\ predicted} = Q*\ f_u*0.67Cl_{int}/(Q+ f_u*0.67Cl_{int})$

(Hepatic intrinsic clearance at the 14th day after PHx was about 67.24% of that observed in the control group.) (remaining hepatic clearance (%) when normalized to per unit liver mass: 92% (Table 3); remaining liver mass (%): 72% (Figure 7))

$CL_{PHx,predicted}$: predicted total body clearance 14 days after PHx = 6.54 ml/min/kg

However, while the total body clearance of tacrolimus was significantly decreased (4.4 ml/min/kg) twenty four hours after PHx, the magnitude was much less than what was predicted based on *in vitro* data. Taking account of the low activity (undectable activity in microsomes) and small organ mass, the contribution of small intestine and kidney to the metabolism of tacrolimus is expected to be negligible in rats after intravenous

administration. When normalized to the predicted liver weight at the 24th hours, the clearance per unit liver weight calculated as shown below was increased significantly during hepatic regeneration (24-PHx vs. control: 0.33 ml/min/g vs. 0.23 ml/min/g).

Given the observed clearance in the control rats of 9.22 ml/min/kg, for a rat with 250 g body weight and an average liver weight of 10 g (N= 6 rats), the clearance will be 0.23 ml/min/g liver (CL = (9.22 ml/min/kg*0.25 kg)/10 g liver).

Given the observed clearance in the rats with a regenerating liver (24 hr) of 4.40 ml/min/kg, for a rat with 250 g body weight and an average liver weight of 3.3 g (N = 6 rats), the clearance will be 0.33 ml/min/g liver (CL_{PHx} = (4.40 ml/min/kg*0.25 kg)/3.3 g liver) at the 24th hour after PHx.

These observations point to a significant reserve capacity of the liver to clear drugs from the body during the regeneration process. Since tacrolimus is a low hepatic extraction ratio drug (0.167, total body clearance 9.22 ml/min/kg divided by reported blood flow 55.2 ml/min/kg (Davies and Morris, 1993)), increased blood flow per unit weight of the liver *per se* had little effect on the clearance of tacrolimus. It is interesting to note that while higher blood level of tacrolimus normalized to unit dose have been reported in LDLT recipients compared to those receiving cadaveric livers, this increase was also much smaller (26%) (Trotter *et al*., 2002) than what is expected based on the smaller liver

volume and the expected impairment in the metabolic activity of the liver. It is possible that increased blood flow per unit weight of the liver due to PHx increases percentage of hepatocytes involving in the drug metabolism (Under normal situation, a lower percentage of hepatocytes will be involved in the drug metabolism). The precise mechanism for this needs to be evaluated in future studies. Additionally, when comparing the values of K_{10}, K_{12} and K_{21}, the K_{21} has the smallest value which implies that the back distribution of tacrolimus from compartment 2 to compartment 1 is the rate limitation step of the elimination. This may also be the reason for the reserve capacity of the liver in the metabolism of tacrolimus.

To the best of our knowledge this is the first study to evaluate the pharmacokinetics of tacrolimus and to analyze the recovery profile of the pharmacokinetics of tacrolimus over time after initiation of hepatic regeneration in an animal model. Our study provides several implications for use of drugs metabolized by CYP3A in LDLT patients: 1) The hepatic clearance of CYP3A substrates will also be reduced transiently during the early hepatic regeneration process. 2) The magnitude of reduction in *in vivo* clearance of CYP3A substrates will be much less than what was predicted than the loss of liver mass and loss of enzyme activity as measured by *in vitro* studies. 3) A reduction in the dose of CYP3A substrates that is less than the loss of liver mass is sufficient to achieve comparable blood levels of drugs in LDLT patients and cadeveric liver transplant patients. 4) Caution must be exercised in using *in vitro* data to predict *in vivo* clearance of drugs by the

regenerating liver. 5) Living donor liver transplant patients would require a dose of CYP3A substrates that is not proportion to the loss of the liver mass. Finally, drug dosing in LDLT patients must be routinely monitored. 6) The ability of the liver to clear the drug the drug recovers completely with time and normal hepatic function will be restored in subjects undergoing hepatic resection.

Chapter 4 Activity and Expression of Various Isoforms of Uridine Diphosphate Glucuronosyltransferase (UGT) Are Differentially Regulated during Hepatic Regeneration in Rats

Abstract

Objective The objective of this study was to evaluate the activity and expression of various UGTs in rats at various time points after initiation of hepatic regeneration by partial hepatectomy (PHx).

Methods The mRNA expression of various UGTs was assessed using real-time PCR with specific primers. The *in vitro* activity of several UGTs was measured with liver microsomes incubated with different substrates such as estradiol (UGT1A1), acetaminophen (UGT1A6/7), morphine (UGT2B1), testosterone (UGT2B1/3/6), androsterone (UGT2B2), and (-)- borneol (UGT2B12).

Results While the activity of UGT1A1, UGT2B1, UGT2B2, UGT2B1/3/6 and UGT2B12 was decreased, the activity of UGT1A6/7 was preserved during hepatic regeneration. All UGTs with the altered activity or expression recovered differentially, with some returning to normal levels by day 6 (UGT1A3, UGT2B1, UGT2B2, and UGT2B12) and others recovering by day 14 (UGT1A1, UGT2B1/3/6 and UGT2B8) after initiation of hepatic regeneration.

Conclusion During hepatic regeneration, the hepatic UGT activity and the mRNA expression of several UGTs were decreased and different isoforms recovered differentially over time. The clearance of most of the substrates of UGT will be decreased significantly in LDLT patients due to the loss of liver mass and decreased activity of the

remaining liver mass. The activity of UGTs will normalize with time as the hepatic regeneration progresses. Individualized dosing regimen for different UGT substrates may be needed when using UGT substrates in LDLT patients.

Introduction

Living donor liver transplant patients are normally treated with drugs such as tacrolimus, sirolimus and cyclosporine, which are metabolized by phase 1 pathways and mycophenolic acid, acetaminophen, and morphine, which are metabolized through phase 2 pathways (glucuronidation). In addition, several endogenous compounds such as bilirubin, estradiol, androsterone, and testosterone are metabolized by glucuronidation. Cytokines that are released during the regeneration process are known to regulate uridine diphosphate glucuronosyltransferases (UGTs) in the liver (Monshouwer *et al*., 1996; Strasser *et al*., 1998). So we hypothesized that the activity and expression of all UGT isoforms will be altered during hepatic regeneration. While the expression and activity of phase 1 enzymes have been reported to be decreased during hepatic regeneration, there is controversial and incomplete information on the activity and expression of uridine diphosphate glucuronosyltransferases (UGTs) during hepatic regeneration. Some publications indicate a change, while others indicate a lack of change in UGT expression or activity (Catania *et al*., 1998; Iversen *et al*., 1985; Pellizzer *et al*., 1996; Zakko *et al*., 1996). Moreover, previous studies on the expression of UGTs have used northern blot. Compared to the conventional northern blot method for the measurement of RNA expression, real-time PCR is a more sensitive, quantitative, accurate and reliable assay for measurement of mRNA.

Systematic study of the activity and expression of UGTs is necessary to thoroughly understand the regulation of UGT isoforms during hepatic regeneration. This information is important in order to optimize drug therapy and understand the metabolism of various drugs and endogenous compounds in LDLT patients. We hypothesized that the expression and *in vitro* activity of various forms of UGT will be decreased during the initial pahse of hepatic regeneration due to decreased expression of transcription factors involved in their regulation as a result of increased cytokine levels. In this study, we have utilized partially hepatectomized rats and real-time PCR to systematically study the effect of hepatic regeneration on the activity and expression of different UGTs using specific markers and specific primers.

Materials and Methods

Chemicals

Estradiol, estradiol-3-glucuronide, acetaminophen, acetaminophen glucuronide, morphine sulfate, morphine-3-glucuronide, androsterone, testosterone glucuronide, (-)-borneol, and UDPGA were purchased from Sigma Chemical Co. (St. Louis, MO). Testosterone was obtained from Steraloids Inc. (Newport, RI). UDP-[U-^{14}C]glucuronic acid was bought from MP Biomedicals (Irvine, CA). Reagents for reverse transcription were purchased from Promega (Madison, WI). Forward and reverse primers for UGTs, constitutive androstane receptor (CAR), pregnane X receptor (PXR), hepatocyte nuclear factor 1 (HNF1) and beta-2-microglobulin (beta-2-m) were synthesized by Applied Biosystems

(Forest city, CA). Forward and reverse primers for C/EBP α were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All solvents were HPLC grade.

Animals

Partial hepatectomy was performed and livers were collected using the methods described in **Chapter 2**.

Preparation of Microsomes

Liver microsomes were prepared by differential centrifugation procedures described in **Chapter 2**.

Incubation of UGT substrates in Microsomes

The microsomes were incubated with various substrates to measure the *in vitro* activity (estradiol as a UGT1A1 marker; acetaminophen as a UGT1A6/7 marker; morphine as a UGT2B1 marker; testosterone as a UGT2B1/3/6 marker; androsterone as a UGT2B2 marker; (-)- borneol as a UGT2B12 marker (Senafi *et al*., 1994; King *et al*., 1996; Kessler *et al*., 2002; Mackenzie *et al*., 1996; Burchell, 1999). Incubation conditions were the same as those reported in the literature: estradiol (150 μM), acetaminophen (10 mM); morphine (10 mM); and testosterone (150 μM) (Alkharfy and Frye, 2002; Fisher *et al*., 2000; Narayanan *et al*., 2000).

For androsterone (200 μM) (reported Km: 7.2 μM, Rittmaster *et al*., 1989) and (-)-borneol (500 μM) (reported Km: 36 μM, Green *et al*., 1995), the incubation procedure was established as follows: A solution (250 μl) containing 1 mg/ml microsomal protein, 2 mM UDPGA (including 0.2 μCi UDP-[U-^{14}C]glucuronic acid/reaction for androsterone or 0.1 μCi UDP-[U-^{14}C]glucuronic acid/reaction for (-)- borneol), 10 mM $MgCl_2$, Brij 58 (0.1 mg/mg protein) and 200 μM androsterone or 500μM (-)- borneol was incubated for 60 min at 37°C in a shaking water bath. Then 25 μl of 6% perchloric acid was added to the incubation solution. After centrifugation at 13,000 rpm for 5 minutes, 100 μl of the supernatant was analyzed by high performance liquid chromatographic (HPLC) methods.

Assays

The concentration of the glucuronide metabolites in the supernatant was measured based on published HPLC methods, with minor modifications as shown in Table 4. The correlation coefficients (r^2) for the standard curves were ≥ 0.98, and the coefficient of variation was less than 3% for all the analytical methods used (n = 3). The peaks for both androsterone glucuronide and borneol glucuronide were identified by comparing samples incubated with or without radiolabeled UDPGA. No standard curves were established for the measurement of the radioactivity of the glucuronide of androsterone and borneol; however, the radioactivity was measured within the linear range of the detector.

Table 4. Methods for measuring the *in vitro* activity of UGTs

UGT isoform	S: Substrate M: Metabolite	HPLC Method	Reference
1A1	S: estradiol M: estradiol-3-glucuronide	Column: Alltima C18 (250 mm X 4.6 mm, 5 µ Mobile phase: acetonitrile/50 mM ammonium phosphate buffer (pH 3) (35/65, v/v), 1 ml/min Fluorescence detector: excitation (210 nm), emission (300 nm). Retention time: 4.5 min. Linear range: 30-750 pmol CV(%) (n = 3): less than 3%	Alkharfy and Frye, 2002 (with minor modification)
1A6/7	S: acetaminophen M: acetaminophen glucuronide	Column: LiChrospher RP-18 (250 mm X 4.6 mm, 5 µ Mobile phase: acetonitrile/10 mM phosphoric acid (pH 2.3) (3.75/96.25, v/v), 1.7 ml/min UV detector: 254 nm Retention time: 7.1 min Linear range: 0.4-8 nmol CV(%) (n = 3): less than 3%	Kessler *et al*., 2002 (with minor modification)
2B1	S: morphine M: morphine-3-glucuronide	Column: Alltima C18 (250 mm X 4.6 mm, 5 µ Mobile phase: acetonitrile/50 mM KH2PO4 (10/90, v/v), 1 ml/min Fluorescence detector: excitation (210 nm), emission (350 nm). Retention time: 3.7 min Linear range: 0.15-8 nmol CV(%) (n = 3): less than 3%	Fisher *et al*., 2000 (with minor modification)
2B2	S: androsterone	Column: LiChrospher RP-18 (250 mm X 4.6 mm, 5 µ	Rittmaster *et*

	M: androsterone glucuronide	Mobile phase: acetonitrile/0.1% trichloroacetic acid (40/60, v/v), 1 ml/min; scintillation cocktail: 3ml/min Radioactivity detector (Packard 500TR): 14C Retention time: 11.8 min	*al*., 1989 (with minor modification)
2B1/3/6	S: testosterone M: testosterone glucuronide	Column: Alltima C18 (250 mm X 4.6 mm, 5 µ Mobile phase: phase A, acetonitrile/50 mM ammonium phosphate (pH 4.5) (30/70, v/v); phase B, methanol. (0-7.5 min, 1 ml/min of phase A; 7.5-8 min, gradient from 1 ml/min phase A to 1.2 ml/min of phase B; 8-17 min, 1.2 ml/min of phase B; 17-17.2 min, gradient from 1.2 ml/min of phase B back to 1.2 ml/min of phase A; 17.2-27 min, 1.2 ml/min of phase A.) UV detector: 250 nm Retention time: 6.3 min Linear range: 0.15-4 nmol CV(%) (n = 3): less than 3%	Narayanan *et al*., 2000 (with minor modification)
2B12	S: (-)-borneol M: borneol glucuronide	Column: LiChrospher RP-18 (250 mm X 4.6 mm, 5 µ Mobile phase: acetonitrile/0.1% trichloroacetic acid (40/60, v/v), 1 ml/min; scintillation cocktail: 3ml/min Radioactivity detector (Packard 500TR): 14C Retention time: 6.6 min	Same as the method for androsterone glucuronide (UGT2B2)

Extraction of RNA and Reverse Transcription

Total RNA extraction and reverse transcription were conducted using the methods described in Chapter 2.

Real-Time PCR

PCR was performed as described in **Chapter 2**. Forward and reverse primers for UGT1A and 2B family, CAR, PXR, HNF-1, C/EBP α and beta-2-microglobulin (beta-2-m), were designed using the combination of Blast 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html), Amplify 1.2 (freeware from http://engels.genetics.wisc.edu/amplify/) and Primer Express 2.0 (Applied Biosystems), and are listed in Table 5.

Table 5. Primers for real-time PCR analysis of mRNA expressions

Gene	GenBank No.	Primers	Base Positions	Amplicon Size
Beta-2-m	NM_012512	Forward 5'-cgtgcttgccattcagaaaa-3' Reverse 5'-gaagttgggcttcccattctc-3'	58-77 113-133	76
UGT1A1	NM_012683	Forward 5'-gccatgcagcctggattt-3' Reverse 5'-ctcttgggcacgtaggacaac-3'	549-567 592-612	64
UGT1A2	D38066	Forward 5'-cgcaaattcttgtgcagctcta-3' Reverse 5'-accacatcgaaggaactggaa-3'	368-390 423-443	76
UGT1A3	D38067	Forward 5'-ggccatgtacctgcgtgttc-3' Reverse 5'-tgcttcaaattccagttcacaga-3'	473-493 521-543	71
UGT1A5	AF461734	Forward 5'-tcgacagttctcttaaggtcttgtatg-3' Reverse 5'-aaggagctggaattcagatgct-3'	395-422 451-472	78
UGT1A6	NM_057105	Forward 5'-ccgctatcgctcctttgg-3' Reverse 5'-ctgtactctcttagaggagccatcag-3'	356-374 403-428	73
UGT1A7	NM_130407	Forward 5'-cagaccccggtgactatgaca-3' Reverse 5'-caacgtgaagtctgtgcgtaaca-3'	750-771 800-822	73
UGT1A8	NM_175846	Forward 5'-gagggcatgaggtggtggta-3' Reverse 5'-cacggtaaaattcagcgactttc-3'	154-174 203-225	72
UGT2B1	M13506	Forward 5'-ctgaagcagagccctgagaga-3'	1626-1647	76

		Reverse 5’-gggaaggcactggcatga-3’	1684-1701	
UGT2B2	J02589	Forward 5’-ggcagggcagcagtcatc-3’	2182-2200	86
		Reverse 5’-cctacttcttgctcactctctgctt-3’	2243-2267	
UGT2B3/6	M31109 (2B3)	Forward 5’- atgccaagaaatgggatcca-3’	717-736	72
		Reverse 5’- tgcccattgtctcagctaagg-3’	768-788	
	M33746 (2B6)	Same primers as the pair for 2B3	728-747[a]	72
			779-799	
UGT2B8	U27518	Forward 5’-tgaacaaaatgttcgggcaat-3’	363-384	75
		Reverse 5’-aagttccttgtttgaaacaacttctct-3’	411-437	
UGT2B12	U06273	Forward 5’-tgctgcaaataagtttctgctttaa-3’	33-58	74
		Reverse 5’-tgactatattccatcggccatacc-3’	83-106	
CAR	NM_022941	Forward 5’-cggagtataaacagcgcatactca-3’	1190-1213	72
		Reverse 5’-aagcagcggcatcatagca-3’	1243-1261	
PXR	NM_052980	Forward 5’-cggctacctgcggtgttt-3’	725-742	63
		Reverse 5’-caacagtgaggcctgcagaa-3’	768-787	
HNF 1	X54423	Forward 5’-ctcctcggtactgcaagaaacc-3’	3061-3082	73
		Reverse 5’-ttgtcaccccagcttaagactct-3’	3111-3133	
C/EBP α	NM_012524	Forward 5’-tatagacatcagcgcctacatcga-3’	183-206	76
		Reverse 5’-ccggctgtgctggaagag-3’	241-258	

[a]one mismatch at position 738.

The specificity of each pair of primers was first evaluated using nucleotide-nucleotide Blast to confirm that there was no match between the primers described in Table 5 and the rat genome except for the individual UGTs (primers for UGTs) or for targeting genes (primers for CAR, PXR, HNF-1, C/EBP α, beta-2-m). The primer specificity was further checked (using Amplify 1.2) for any potential amplification of UGT isoforms other than the target isoform. Because of the high homology between UGT2B3 and UGT2B6, the primers were designed for both isoforms when using real-time PCR for specific measurements. For each pair of primers, the control without reverse transcriptase was also used for PCR reactions in duplicate to confirm that there was no genomic DNA contamination in the cDNA samples.

Data Analysis

All data are reported as mean ± SD. Comparisons between groups were made by student's t-test ($P < 0.05$). For sample size calculation, the initial formation rate of estradiol-3-glucuronide from liver lobes of sham (6 days) (258.61 ± 33.28 nmol/mg protein/min) was used. With a power of 80% and $\alpha = 0.05$, to detect a 25% difference, 4 rats were required. Experiments were completed with 4 to 6 rats in each group.

Results

Activity and Expression of UGT1A1 during Hepatic Regeneration

The activity of UGT1A1 was decreased 24 hours and 6 days after PHx and recovered by day fourteen (Figure 16, panel A). The mRNA expression of UGT1A1 was also decreased 24 hours after initiation of regeneration and returned to normal level by day 14 (Figure 16, panel B).

Activity and Expression of UGT1A6/7 during Hepatic Regeneration

The activity of UGT1A6/7 was preserved at all time points studied (Figure 17, panel A). The mRNA expressions of both UGT1A6 and UGT1A7 also stayed at the control level as measured in the paired sham group during hepatic regeneration (Figure 17, panel B).

Activity and Expression of UGT2B1 during Hepatic Regeneration

The activity of UGT2B1 was decreased 24 hours after PHx (Figure 18, panel A). The mRNA expression of UGT2B1 was also decreased 24 hours after regeneration and returned to normal level as measured in the paired sham group by day 6 (Figure 18, panel C).

Activity and Expression of UGT2B1/3/6 during Hepatic Regeneration

The activity of UGT1/3/6 measured using testosterone was decreased 24 hours and 6 days after PHx and recovered by day fourteen (Figure 18, panel B). The mRNA expression of UGT2B3/6 was also much lower at 24 hours and 6 days (Figure 18, panel D).

Activity and Expression of UGT2B2 during Hepatic Regeneration

Both the activity and mRNA expression of UGT2B2 were decreased after initiation of hepatic regeneration and recovered completely by day 6 (Figure 19).

Activity and Expression of UGT2B12 during Hepatic Regeneration

Both the activity and mRNA expression of UGT2B2 were decreased after initiation of hepatic regeneration and recovered completely by day 6 (Figure 20).

Expression of other UGTs (UGT1A2, UGT1A3, UGT1A5, UGT1A8 and UGT2B8) during Hepatic Regeneration

UGT1A2 and UGT1A3 mRNA was up-regulated compared to sham groups during hepatic regeneration (Figure 21, panel A and B). The mRNA expressions of UGT1A5, and UGT1A8, were not altered at any time during the regeneration process (Figure 21, panel C and D). UGT2B8 was down-regulated compared to sham groups (Figure 21, panel E).

The mRNA Expression of CAR, PXR, HNF1 and C/EBP α during Hepatic Regeneration

CAR, PXR and HNF1 genes were expressed stably 24 hours after PHx. The C/EBP α gene was down-regulated 24 hours after initiating hepatic regeneration (Figure 22) and recovered back to normal level by day 6.

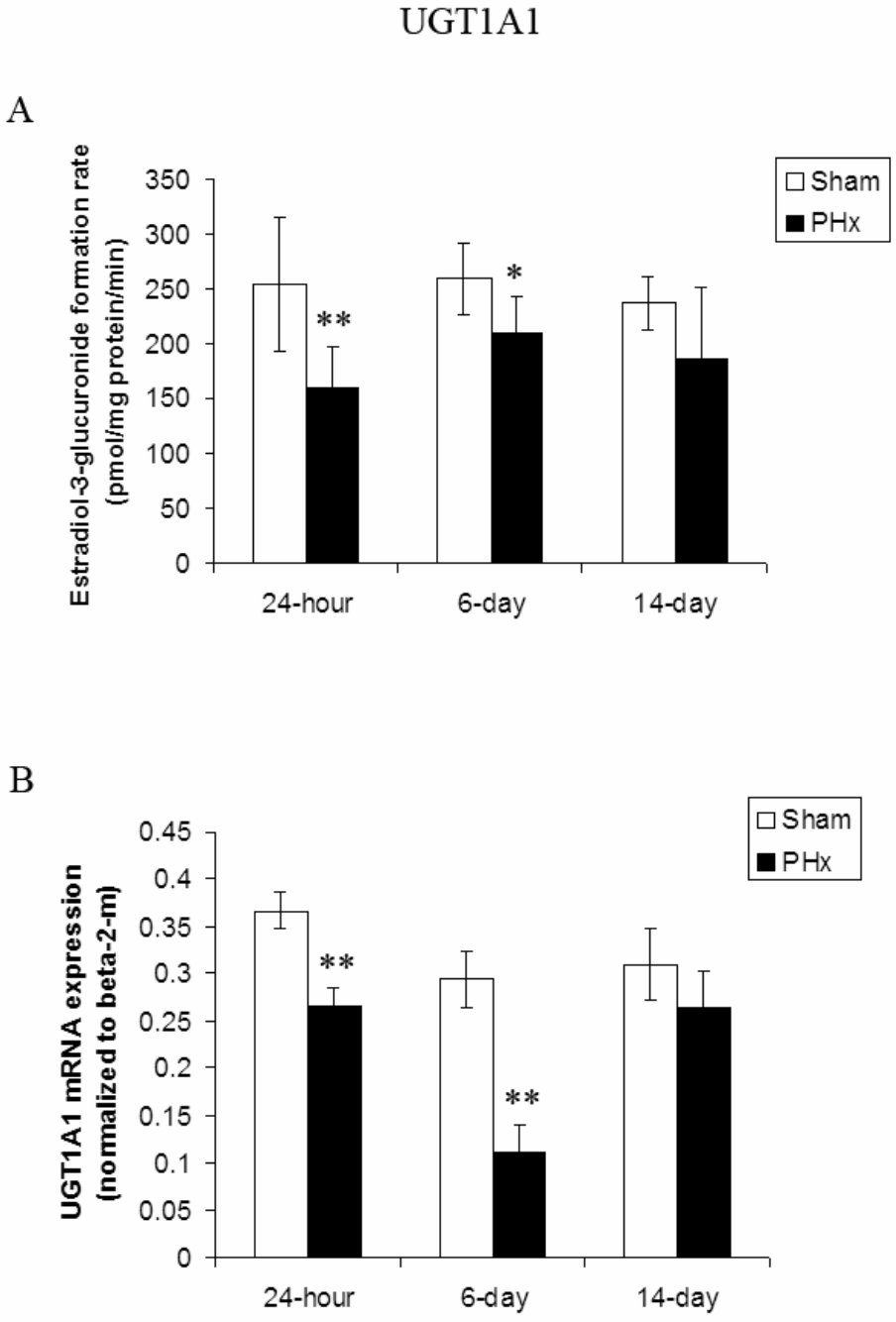

Figure 16. The activity and mRNA expression of UGT1A1 at different time points after PHx. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. The activity was measured using liver microsomes prepared as described in the Methods section. The relative mRNA level was determined by real time PCR as

described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. All data are expressed as mean ± SD. ** $P < .01$ vs. sham; * $P < .05$ vs. sham (student's t-test). N = 4 to 6.

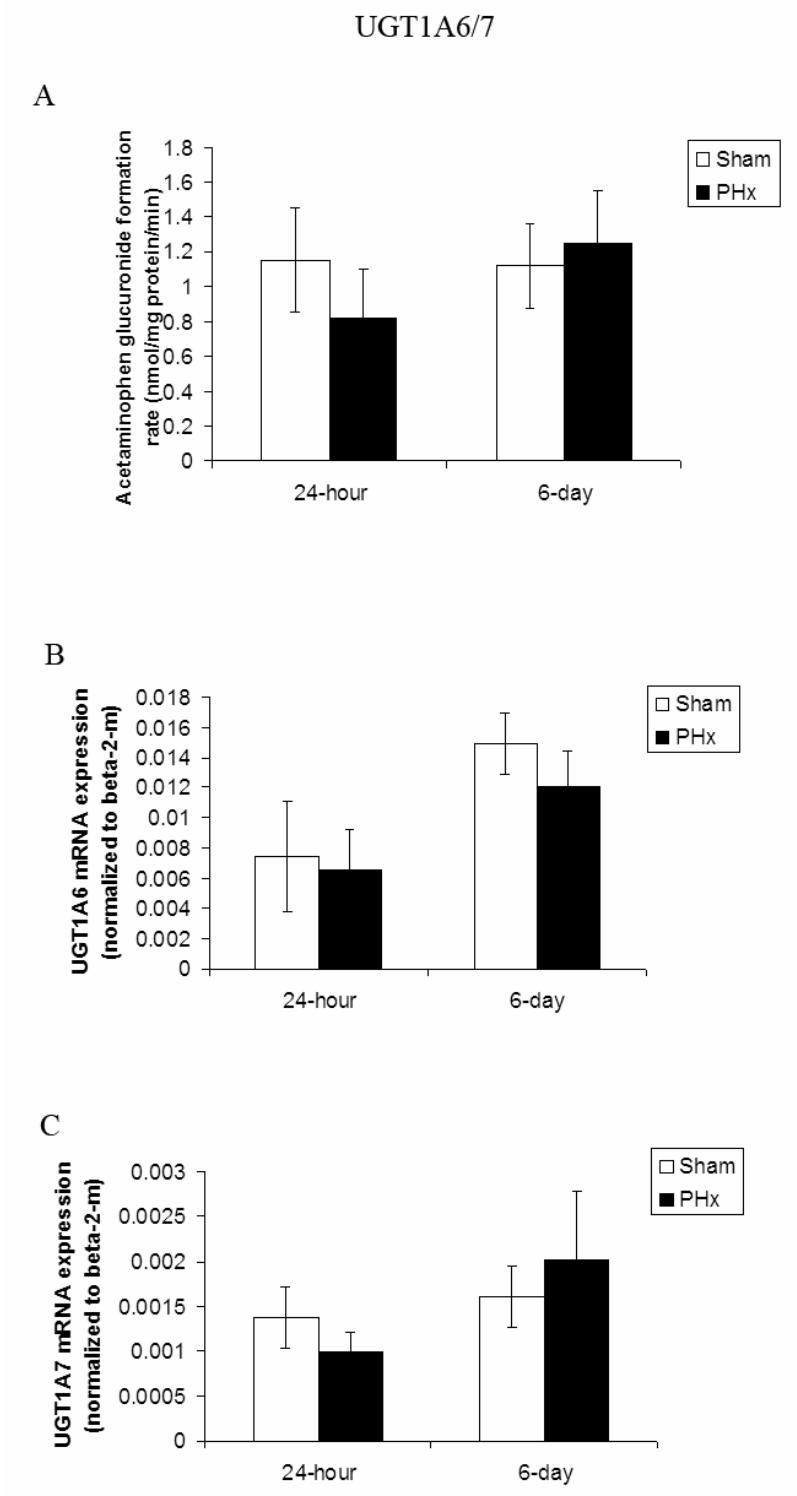

Figure 17. The activity and mRNA expression of UGT1A6/7 at different time points after PHx. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after

PHx. The activity was measured using liver microsomes prepared as described in the Methods section. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. All data are expressed as mean ± SD. $P > .05$ vs. sham (student's t-test). N = 4 to 6.

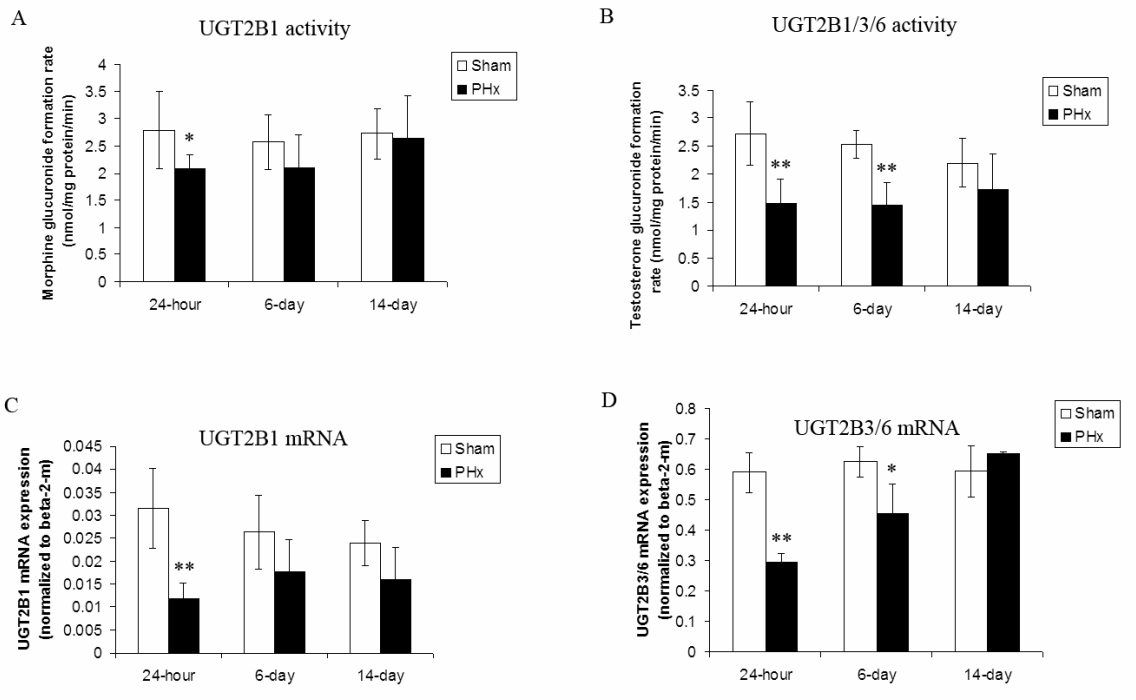

Figure 18. The activity and mRNA expression of UGT2B1/3/6 at different time points after PHx. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. The activity was measured using liver microsomes prepared as described in the Methods section. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. All data are expressed as mean ± SD. ** $P < .01$ vs. sham; * $P < .05$ vs. sham (student's t-test). N = 4 to 6.

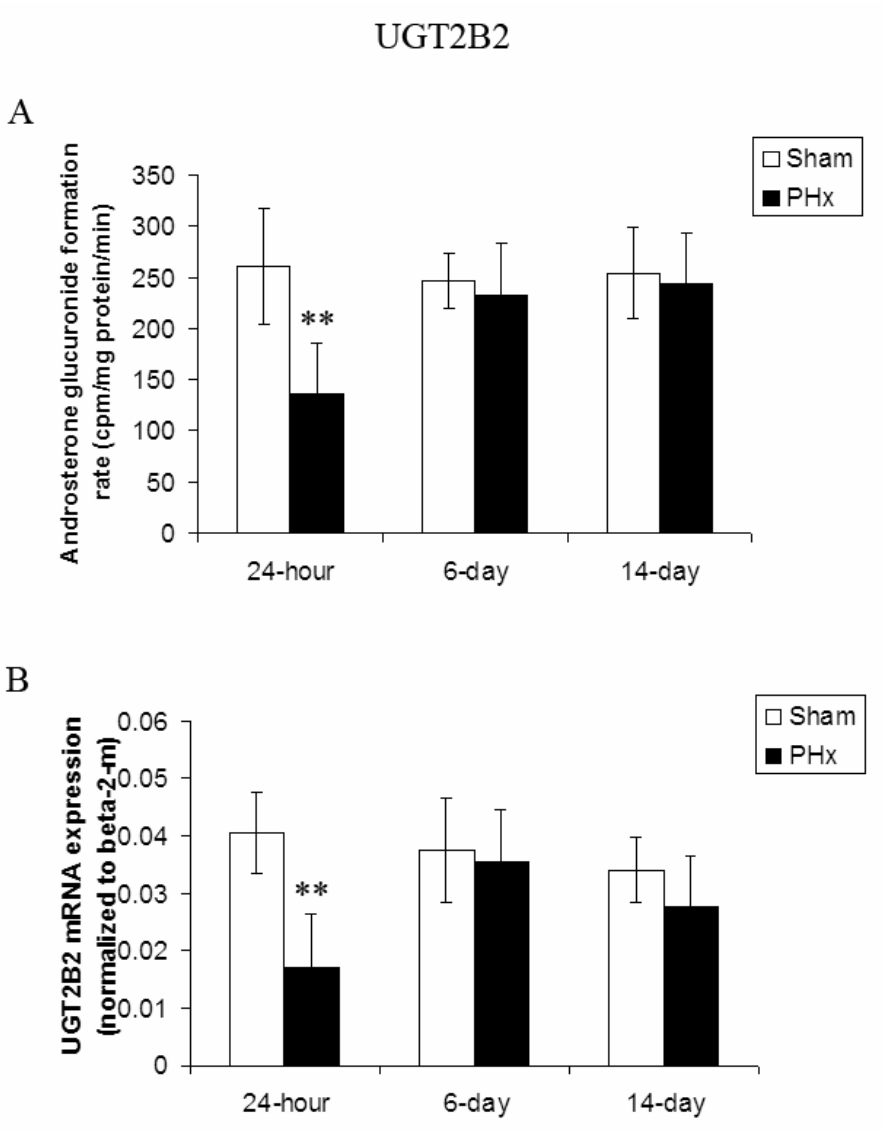

Figure 19. The activity and mRNA expression of UGT2B2 at different time points after PHx. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. The activity was measured using liver microsomes prepared as described in the Methods section. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective

beta-2-m values. All data are expressed as mean ± SD. ** $P < .01$ vs. sham (student's t-test). N = 4 to 6.

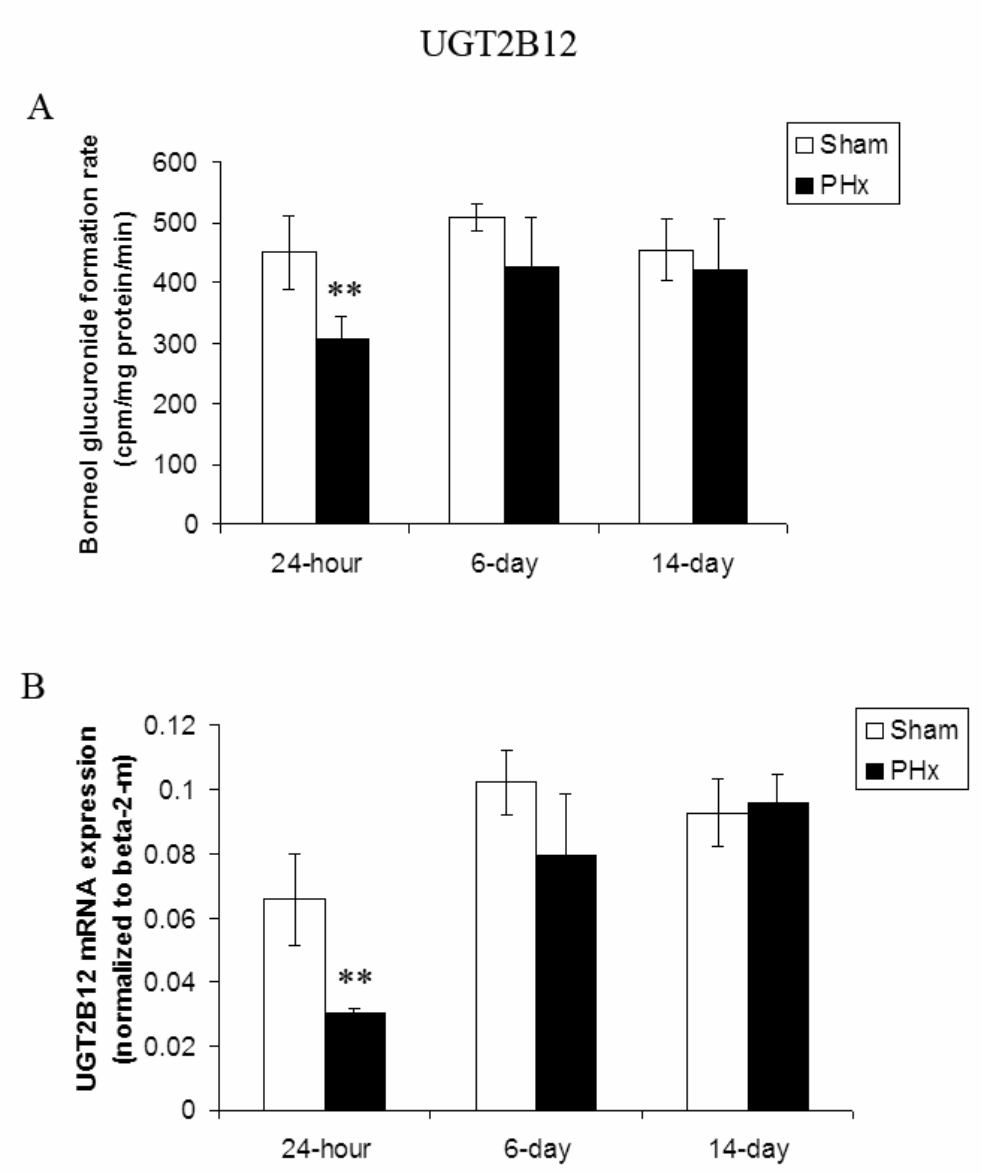

Figure 20. The activity and mRNA expression of UGT2B12 at different time points after PHx. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. The activity was measured using liver microsomes prepared as described in the Methods section. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective

beta-2-m values. All data are expressed as mean ± SD. ** $P < .01$ vs. sham; * $P < .05$ vs. sham (student's t-test). N = 4 to 6.

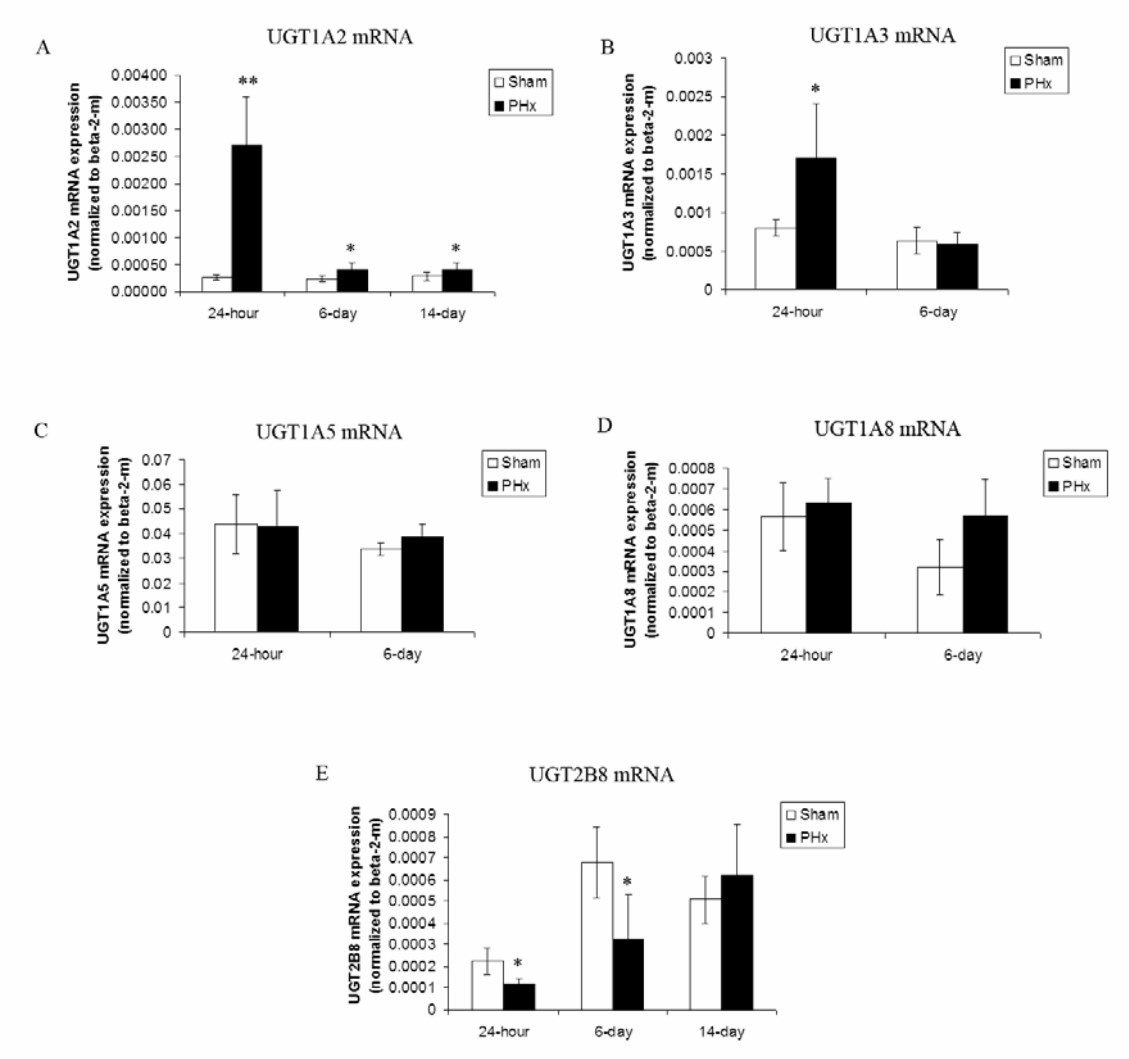

Figure 21. The mRNA expression of UGT1A2, 1A3, 1A5, 1A8, and 2B8 at different time points after PHx. The relative mRNA level was determined by real time PCR as described in the Methods section using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with respective to beta-2-m values. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham; * $P < .05$ vs. sham (student's t-test). N = 4 to 6.

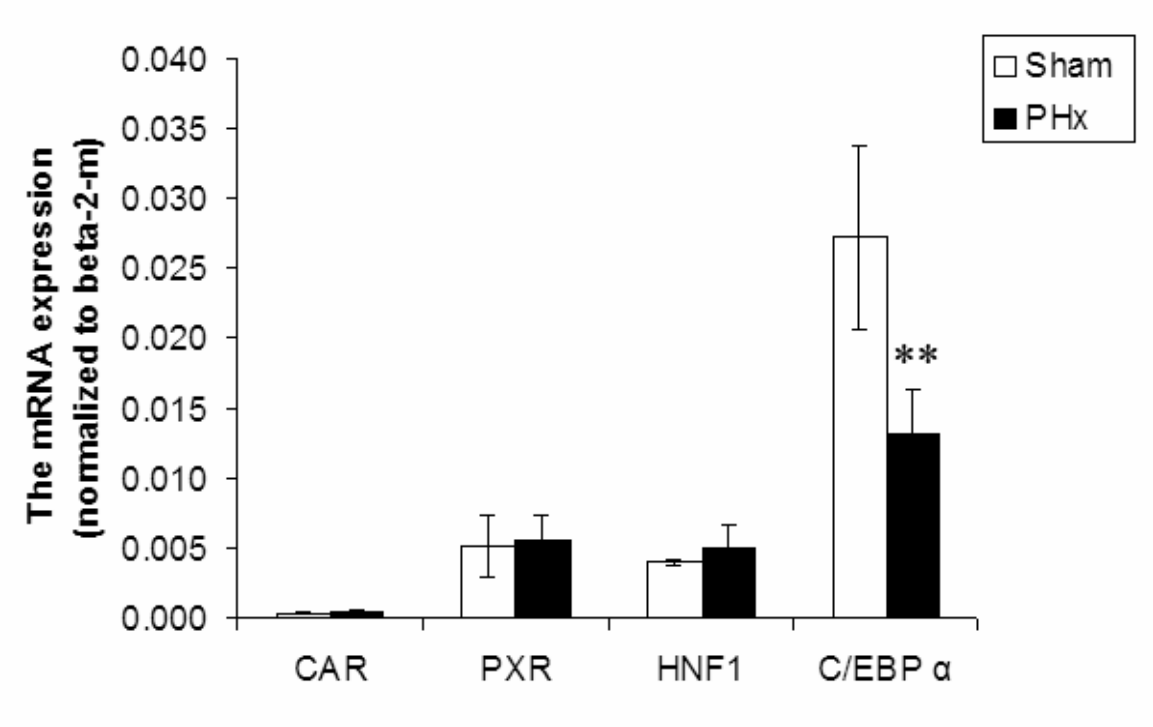

Figure 22. The mRNA expression of CAR, PXR, HNF1 and C/EBP α 24 hours after PHx. The relative mRNA level was determined by real time PCR as described in the Methods section using pooled cDNAs generated from total RNAs from 6 normal livers as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham (student's t-test). N= 5.

Discussion

Limited and conflicting data have been published on the effect of hepatic regeneration on the expression and the activity of UGTs. The conjugation of bilirubin (UGT1A1 activity) in the liver has been reported to be preserved in rats after PHx (Catania *et al*., 1998), while the UGT1A1 mRNA expression has been shown to be decreased (Pellizzer *et al*., 1996). In two studies, the conjugation of p-nitrophenol (UGT1A6 activity) was reported to be not altered after PHx in rats (Catania *et al*., 1998; Zakko *et al*., 1996). However, Iversen *et al*. reported decreased UGT1A6 (early stage) and induced UGT1A6 (later stage) activity for the glucuronidation of naphthol in rat livers at different time points after PHx (Iversen *et al*., 1985). The glucuronidation of morphine (UGT2B1 activity) has been reported to be decreased after PHx in rats (Iversen *et al*., 1985), while Pellizzer *et al*. reported the increased UGT2B1 mRNA expression in rat livers after PHx. The activity of rat UGT2B3 (testosterone) in rat livers has been reported to be altered after initiation of hepatic regeneration (Iversen *et al*., 1985) while Pellizzer *et al*. reported no change in the UGT2B3 mRNA expression in rat livers after PHx (Pellizzer *et al*., 1996). In these studies, the reported activity of different UGT isoforms during hepatic regeneration was inconsistent with the mRNA expression of these enzymes. No conclusive information about the regulation of UGTs can be obtained from published studies due to a lack of systematic studies. In this study, we used several currently available specific UGT markers to evaluate the activity of different UGT isoforms at different time points after initiation of hepatic regeneration. Real-time PCR provided more accurate, sensitive and

reliable measurements for the mRNA expression compared to northern blot. We used real time PCR to assess the effect of hepatic regeneration on the mRNA expression of these UGT isoforms. In addition, we measured the mRNA expression of additional UGTs using real-time PCR in this study because specific substrates or antibodies are not available for these isoforms. One major concern with mRNA measurements is the specificity of primers due to the high homology of different UGTs. We successfully designed the specific primers for all rat UGTs. The mRNA expression of all UGTs during hepatic regeneration was evaluated using specific primers.

The estradiol-3-glucuronide formation rate has been verified and used as the marker for the human UGT1A1 activity (Senafi *et al*., 1994). There was no direct documentation of the specificity of estradiol as a substrate for the UGT1A1 activity in rats; however, rat and human UGT1A1 share more than 70% identity in their deduced primary amino acid sequences. Accordingly, rat and human UGT1A1 exhibited similar enzymatic efficiencies toward estrogens (including estradiol), flavonoids, phenols, and several other class of chemicals (King *et al*., 1996). It has been concluded that rat and human UGT1A1 are functionally similar and can be considered orthologous enzymes (King *et al*., 1996). Consequently it is highly possible that estradiol is also a rat UGT1A1 probe substrate. Based on these data, we used the formation rate of estradiol-3-glucuronidation as the marker of the UGT1A1 activity in rats in this study.

In contrast to the conflicting reports published from different studies, our results showed consistent changes in both the activity and the mRNA expression for all isoforms that we have studied. In addition, we also found that the mRNA expression of UGT1A2 and UGT1A3 mRNA was up-regulated during hepatic regeneration. The significance of this finding is unknown at this time due to the lack of rat UGT1A2 and UGT1A3 probe substrates and unknown clinically relevant drugs metabolized by this isoform. Similarly, because of the lack of known clinically relevant drugs metabolized by UGT2B8, the significance of the down-regulation of UGT2B8 is unknown at this time.

Differential regulation of UGT isoforms has been reported in other systems. Our data of UGT1A6 and UGT2B1/3/6 were consistent with the observations in different systems. Acute-phase response induced by turpentine injection, leads to no reduction in the glucuronidation of *p*-nitrophenol (UGT1A6), while it impairs the glucuronidation of testosterone (UGT2B1/3/6) (Strasser *et al*., 1998). IL-6 can suppress the mRNA expression of UGT1A1 and UGT2B3 in rat hepatocytes (Strasser *et al*., 1998). Strasser *et al*. suggested that the promoter regions of some UGT isoforms may contain specific regulatory elements capable of responding to certain cytokines (Strasser *et al*., 1998). Several cytokines including TNF-α and IL-6 are involved in initiation of hepatic regeneration (Michalopoulos and DeFrances, 1997). Plasma concentration of IL-6 is significantly increased after initiation of hepatic regeneration (Matsunami *et al*., 1992; Fulop *et al*., 2001; Iwai *et al*., 2001). Cytokines such as TNF- α and IL-6 are reported to

inhibit the activity of UGT (Monshouwer *et al*., 1996; Strasser *et al*., 1998). So IL-6 is likely to be a contributor to the decreased activity and expression of UGTs after initiation of hepatic regeneration.

Two nuclear receptors CAR and PXR have been implicated in the acute phase response mediated decrease in CYP activity (Beigneux *et al*., 2002). Since both CAR and PXR are also involved in the regulation of UGTs (Mackenzie *et al*., 2003), we expected altered levels of CAR and PXR in rat livers during hepatic regeneration. However, there was no change in CAR or PXR in the regenerating livers.

There is also direct evidence showing that the C/EBP α knock-out is the cause for the loss of expression of UGT1A1 (bilirubin UGT) in mouse liver. In addition, HNF1 α binding site has been found in human UGT2B7 (Lee *et al*., 1997; Ishii *et al*., 2000). This would imply that C/EBP α and HNF1 α can also regulate the expression of UGTs (Mackenzie *et al*., 2003). We tested the expression level of C/EBP α and HNF1 in rat livers during hepatic regeneration. Only C/EBP α was down-regulated significantly after PHx. This suggests that C/EBP α is possibly an important factor responsible for the lower level of expression of UGTs during hepatic regeneration, even if not the only one. The differential activity and expression of different isoforms of UGT is probably due to the fact that UGTs are regulated by different factors. Additionally, it is likely that newly divided cells may have a much lower enzyme expression and activity compared to existing liver cells.

This study systematically evaluated the activity and the mRNA expression of various UGTs during hepatic regeneration in rats and provided more consistent and accurate measurements of the regulation of different UGT isoforms. To the best of our knowledge, this is also the first study to measure various UGT isoforms in rats using real-time PCR with specific primers. Congiu *et al*. documented the specific primers for measuring human UGT isoforms using real-time PCR (Congiu *et al*., 2002); however, there were several mismatches between the primers and the target isoforms and the size of amplicons was out of the optimum range of 50-150 bp (efficient amplification within the range) for most UGT isoforms. Our study points to several important conclusions. 1) During hepatic regeneration, the mRNA expression of UGTs mirrors the activity of the corresponding isoform during hepatic regeneration; 2) The lower activity of UGT1A1 can decrease the conjugation of bilirubin and increase the concentration of biliribin in bile or serum during the early post operative period in the LDLT patients; 3) The doses of drugs that are metabolized by UGT will be decreased during early phase of LDLT; 4) Differential adjustment in doses of drugs metabolized by UGT is necessary in LDLT patients.

Chapter 5 *In Vitro* Hepatic Intrinsic Clearance and Pharmacokinetics of Mycophenolic Acid Are Transiently Altered during Hepatic Regeneration in Rats

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Abstract

Objective The objectives of this study were to evaluate 1) the hepatic metabolism of an immunosuppressive drug, mycophenolic acid (MPA), and 2) the pharmacokinetics of mycophenolic acid at various time points after initiation of hepatic regeneration in rats by partial hepatectomy (PHx).

Methods The *in vitro* hepatic clearance of MPA was measured with liver microsomes incubated with different concentrations of MPA. The pharmacokinetics of mycophenolic acid was evaluated after intravenous administration of 20 mg/kg mycophenolic acid to partially hepatectomized rats. One hundred microlitter of blood was collected at 0, 1, 5, 10, 15, 20, 30, 60, 90, 120, 240 and 480 minutes through a jugular vein catheter. The blood concentrations of MPA and MPA glucuronide (MPAG) were measured by a HPLC method. The plasma concentration vs. time profile was analyzed by a non-compartment model using WinNonlin. The glucuronidation of MPA in small intestine and kidney was assessed using kidney and intestine microsomes. The expression of multidrug Resistance-Associated Protein (Mrp) 2 and Mrp 3 was evaluated using western blot and quantitative PCR, respectively.

Results The hepatic intrinsic clearance of MPA was decreased to 52% and 51% of that in

control rats at the 24th hour and the 6th day, respectively but recovered to normal level by day 14. The total body clearance of MPA was reduced at twenty four hour after PHx but recovered by day 6. The elimination clearance of MPA glucuronide was also impaired during hepatic regeneration but recovered to normal with time.

Conclusions During hepatic regeneration, the *in vitro* hepatic intrinsic clearance was significantly decreased and the clearance of MPA was also significantly but transiently decreased. By day 6 after initiation of hepatic regeneration, the intrinsic clearance of MPA recovered completely to normal. The ability of liver to clear MPA recovered much earlier than the complete recovery of liver mass. The magnitude of reduction in *in vivo* clearance of MPA was much less than what was predicted from the loss of liver mass and loss of enzyme activity in the residual liver. Overall glucuronide conjugation capacity of the regenerating liver recovered earlier than the CYP mediated metabolic capacity of the regenerating liver.

Introduction

The liver plays an important role in the elimination of drugs and xenobiotics. During hepatic regeneration, the reduction in total liver mass due to partial hepatectomy (PHx) and the decreased activity of some UGTs are expected to reduce the ability of the liver to metabolize drugs. UGT is involved in the metabolism of the immunosuppressive drug, mycophenolic acid (MPA). The activity and expression of various forms of UGT were differentially regulated during hepatic regeneration (chapter 4). Therefore, it is important to understand whether the intrinsic activity of UGT is altered and whether the magnitude of change in the pharmacokinetics of immunosuppressive drugs metabolized by UGT during the hepatic regeneration process agrees with the predicted changes due to a decrease of liver mass and a decrease in the intrinsic activity of UGT during hepatic regeneration. Nothing is known about the pharmacokinetics of immunosuppressive drugs metabolized by UGT pathway during hepatic regeneration. Such knowledge will help in optimizing not only the immunosuppressive drug therapy but also therapy with other drugs that are metabolized by UGT pathway. We hypothesized that the *in vitro* intrinsic clearance of MPA will be reduced and that the *in vivo* systemic clearance of MPA will be reduced more than the loss of liver mass. We also hypothesized that the clearance of MPAG will be significantly decreased due to a reduction in liver mass during hepatic regeneration. In this study, we have utilized partially hepatectomized rats to study the effect of hepatic regeneration on the hepatic intrinsic clearance and the pharmacokinetics of MPA.

Materials and Methods

Chemicals

Mycophenolic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Mycophenolic acid glucuronide was a generous gift from Roche Bioscience (Palo Alto, CA). Heparin injection (Lot No. 322024) was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA). Mouse anti-human M_2III-6 monoclonal antibody was obtained from ID Labs Inc. (London, ON, Canada). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Amersham Biosciences, (Piscataway, NJ). Western Chemiluminescence reagent was obtained from Perkin Elmer Life Sciences, Inc. (Boston, MA). All solvents used were of HPLC grade and were obtained from Fisher Scientific Inc. (Pittsburgh, PA).

Animals

The study protocol was approved by the IACUC at the University of Pittsburgh. Partial hepatectomy was performed according to the method of Higgins and Anderson in male Sprague-Dawley rats weighing 225-250 g (Higgins and Anderson, 1931) as described in Chapter 2. (For sham operation and definition of controls, please refer to ***Animals*** under **Methods and Materials** section in **Chapter 2** also). Livers and kidneys were harvested as mentioned earlier (***Animals*** under **Methods and Materials** section in **Chapter 2**) and small intestines were collected and processed immediately using the method described in

the next section (***Preparation of microsomes***).

Preparation of Microsomes

The livers and kidneys were prepared using the method mentioned in chapter 2 (***Microsome Preparation*** under **Methods and Materials**). Intestinal microsomes were prepared by the method described previously (Emoto *et al*., 2000): Immediately after excision of the small intestine, it was placed on a ice-cold stainless dish and cut longitudinally and washed with ice-cold salt solution containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA. The mucosal cells were gently scraped off with a micro-cover glass. The scraped sample was mixed with 3X volumes ice-cold 50 mM Tris-HCl buffer containing 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml trypsin inhibitor, 10 μM leupeptin, 0.04 unit/ml aprotinin, 1 μM bestatin. After homogenization, the solution was centrifuged using the same procedure as that for preparation of liver microsomes. The pellets were reconstituted in 50 mM Tris-HCl buffer containing 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml trypsin inhibitor, 10 μM leupeptin, 0.04 unit/ml aprotinin and 1 μM bestatin. Microsomes were stored at -80°C before use. Microsomal protein concentrations were determined by Lowry's method using bovine serum albumin as a standard (Lowry *et al*., 1951). Microsomes were stored at -80ºC until used in incubation studies.

Measurement of V_{max}, K_m, and CL_{int} for the Formation of Mycophenolic Acid Glucuronide in Hepatic Microsomes

The incubation was carried out in a glass culture tube using varying concentrations of mycophenolic acid (MPA) (0-7.5 mM), 0.4 mg/ml microsomal protein (linear to 1 mg/ml, Figure 23), 10 mM $MgCl_2$ and Brij58 (0.1 mg/mg microsomal protein) with the final volume adjusted to 0.2 ml by the addition of 0.05 M phosphate buffer (pH 7.4). The tubes were pre-incubated for 5 minutes at 37ºC and 4 mM UDPGA was added to initiate the reaction. After incubation for 30 minutes (linear to 60 min, Figure 24), the reaction was stopped by the addition of equal volume (0.2 ml) of ice-cold methanol. The tubes were centrifuged at 13,000 rpm at 4ºC for 5 minutes and the supernatants were frozen immediately at -80ºC. The formation of MPA glucuronide (MPAG) was measured using a HPLC method developed in our laboratory. (Column: LiChrospher column, C18, 5 µ, 250 mm x 4.6 mm; column temperature: 38ºC; mobile phase: 25% acetonitrile:75% water containing 0.05% phosphoric acid, 1.00 ml/min; UV detector at 254 nm; injection volume: 30 µl; retention time: 11.8 min; total run time: 57 min) The standard curve was linear over the range of 1-100 µg/ml. The intra-day and inter-day CV(%) at 2.5 µg/ml, 25 µg/ml and 100 µg/ml was less than 4% (n = 4).

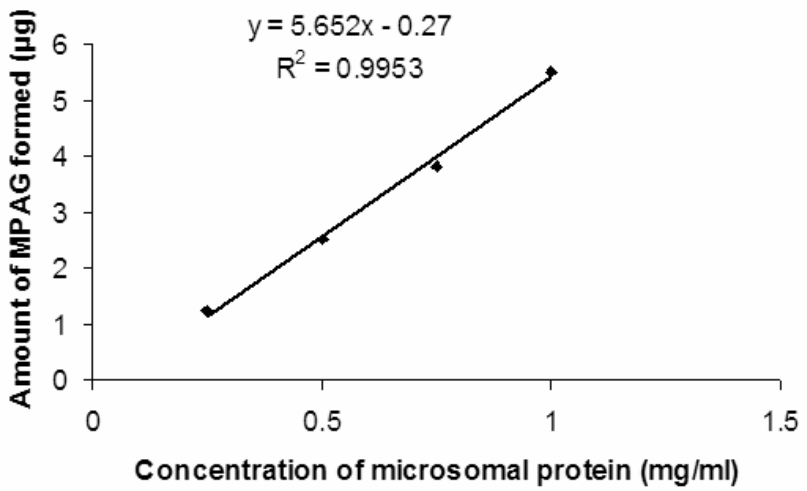

Figure 23. Relationship between microsomal protein concentration and the amount of MPAG formed in rat liver microsomes. MPA concentration: 2.5 mM; incubation time: 30 min.

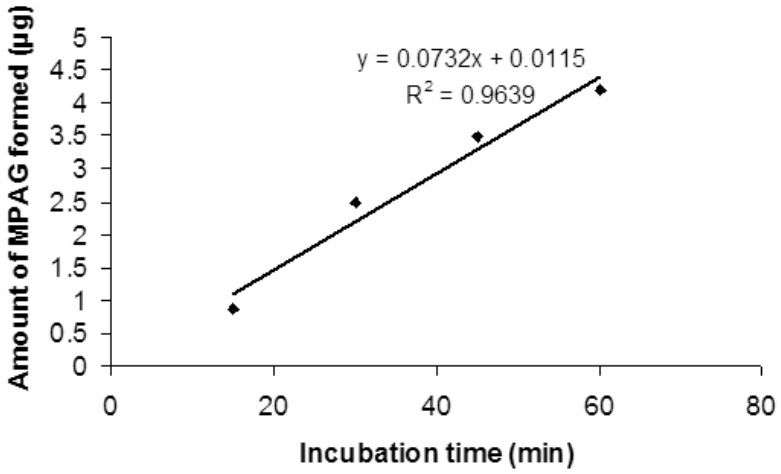

Figure 24. Relationship between time of incubation and the amount of MPAG formed in rat liver microsomes. MPA concentration: 2.5 mM; concentration of microsomal protein: 0.5 mg/ml.

Measurement of the Formation of Mycophenolic Acid Glucuronide in Small Intestine and Kidney Microsomes

The incubation was conducted using the method described above with saturating MPA concentration of 5 mM (Km is less than 1 mM for both small intestine and kidney microsomes). The formation of MPAG was measured using the HPLC method as described above.

Pharmacokinetics Study of MPA

Pharmacokinetic studies were carried out in rats in the control group, 24 hours after PHx, 6 days after PHx and 13 days after PHx. Since there was no significant difference between control group and the sham group in the *in vitro* intrinsic clearance of MPA at any time during hepatic regeneration, pharmacokinetic studies were not conducted in sham group. A silastic tubing was inserted into the jugular vein 24 hours before the study. Mycophenolic acid (20 mg/kg) was administered intravenously as a bolus through the jugular vein catheter. Multiple blood samples (150 µl) were collected in heparinized tubes at 0, 1, 5, 10, 15, 20, 30, 60, 90, 120, 240 and 480 minutes after intravenous administration of MPA. The concentration of MPA and MPAG in plasma was determined using a published method (Shipkova *et al*., 1998) with minor modifications as follows: to 50 µl plasma, 50 µl of acetonitrile containing 100 µg/ml diazepam (internal standard) was added to precipitate the proteins. After centrifugation at 13,000 rpm for 5 min, 50 µl of supernatant was injected onto a LiChrospher RP-18 column (250 mm x 4.6mm, 5 µ)

heated to 38ºC. The mobile phase consisted of solution A (250 ml of acetonitrile and 750 ml of 20 mmol/L phosphate buffer, pH 3.0) and solution B (700 ml of acetonitrile and 300 ml of 20 mmol/L phosphate buffer, pH 6.5) that formed the following gradient:0-4.5 min, 3% B; 4.5-5 min, 30% B; 5-12 min, 100% B; 12-17.5 min, 100% B; 17.5-18 min, 3% B; 18-25 min, 3% B. The flow rate was 1.2 ml/min. The UV detector was set at 215 nm. The retention time for MPAG, MPA, and diazepam was 5.7, 15.4 and 17.7 minutes, respectively, and the total run time was 25 minutes. The linearity of the method was from 2 µg/ml to 100 µg/ml for both MPA and MPAG in plasma. The intra-day and inter-day CV(%) at 2 µg/ml, 25 µg/ml and 100 µg/ml was less than 7% (n = 4).

Western Blot for Multidrug Resistance-Associated Protein (Mrp) 2 Protein Expression

Liver membrane preparations were made as previously described (Ogawa *et al*., 2000): Liver was homogenized in 5 vols of 0.1 M Tris · HCl buffer (pH 7.4) containing 1 µg/ml leupeptin and pepstatin A and 50 µg/ml phenylmethylsulfonyl fluoride with a homogenizer. After an initial centrifugation at 1,500 *g* for 10 min, the supernatant was centrifuged at 100,000 *g* for 30 min. The pellet was suspended in Tris · HCl buffer and again centrifuged at 100,000 *g* for 30 min. The crude membrane fraction was resuspended in 0.1 M Tris · HCl buffer (pH 7.4) containing the proteinase inhibitors. Protein concentration was measured using Lowry's method (Lowry *et al*., 1951). Western blot was performed using the published method (Slitter *et al*., 2003) with minor modifications: 45 µg of membrane protein (without boiling, note: this is very critical) were

electrophoretically resolved using Bio-Rad 7.5% Ready gels and then transblotted overnight at 4°C onto PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA) with Tris-glycine buffer containing 20% methanol and 0.1% SDS. The blots were then blocked for 1 hour in 5% blocking grade nonfat dry milk (Bio-Rad, Hercules, CA) in TBS-Tween buffer (15 mM Trizma base, 154 mM sodium chloride, 0.05% Tween 20, pH 7.4) at room temperature, incubated overnight at 4°C with mouse anti-human M_2III-6 monoclonal antibodies diluted in 0.5% nonfat dry milk (1:2000), and then washed three times for 15, 5 and 5 min, respectively, in TBS-Tween buffer. After the above washes, the blots were incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences Inc., Piscataway, NJ), diluted 1:10,000 in TBS-Tween buffer, and then subjected to three additional washes (15, 5, and 5 min respectively). Mrp2 protein-antibody complexes were detected using ECL Western blotting reagents.

Real-Time PCR for Mrp3 mRNA Expression

The total RNA extraction, reverse transcription and real-time PCR were performed using the methods mentioned in **chapter 2**. Forward and reverse primers, designed using Primer Express 2.0 (Applied Biosystems), are 5'-TCCCACTTCTCGGAGACAGTAAC-3' (Forward), 5'-CTTAGCATCACTGAGGACCTTGAA-3' (Reverse) for Mrp3 and 5'-CGTGCTTGCCATTCAGAAAA-3' (Forward),

5’-GAAGTTGGGCTTCCCATTCTC-3’ for beta-2-microglobulin (beta-2-m). Cycling conditions were 1 cycle in 50ºC for 2 min, 1 cycle at 95ºC for 10 min, 50 cycles at 95ºC with 1 min annealing at 60ºC. The relative cDNA content was determined in duplicate using standard curves created from cDNA and normalized to beta-2-m for each sample. For each pair of primers, the control without reverse transcriptase was also used for PCR reactions in duplicate to confirm that there was no genomic DNA contamination in the cDNA samples.

Data Analysis

Enzyme kinetics analysis was carried out by nonlinear regression analysis using Prism 3.0 (GraphPad Software Inc., San Diego, CA). The intrinsic formation clearance (CL_{int}) was calculated by dividing the V_{max} by the K_m. Pharmacokinetics of MPA was analyzed by a non-compartmental model using WinNonlin 3.1 (Pharsight Co., Mountain View, CA). Area under curve (AUC) was calculated using the trapezoidal method. All data are reported as mean ± SD. Comparisons among multiple groups were made by one way analysis of variance with Tukey post hoc analysis ($P < 0.05$). Based on the initial measurement of AUC in control group, 2174.00 ± 229.02 min*μg/ml, to observe a 25% difference between groups, with a power of 80% and $\alpha = 0.05$, the sample size required was 4 rats. Experiments were completed with 4 to 6 rats in each group.

Results

Measurement of V_{max}, K_m, and CL_{int} for the Formation of MPAG in Hepatic Microsomes

Both Vmax and Km values were not different between control group and sham group at all time points studied. The Vmax for the formation of MPAG in hepatic microsomal fraction obtained at 24 hours after PHx was significantly decreased compared to control value (Table 6). On day 6, the Vmax still remained at the lower level (51% of control level), but recovered completely by day 14 with hepatic regeneration (96% of control level). However, the Km values were similar among all the groups. The intrinsic clearance (CL_{int}) for the formation of MPAG in hepatic microsomal fraction was significantly decreased during hepatic regeneration at the 24th hour and on day 6.

Pharmacokinetics of MPA

The plasma concentration vs. time curves of MPA and MPAG after intravenous administration of MPA are shown in Figure 25. The pharmacokinetic parameters of MPA at different time points after initiation of hepatic regeneration are summarized in Table 7. The area under the plasma concentration vs time curve (AUC) for MPA, the total body clearance (CL) for MPA, the mean residence time (MRT) for MPA, the area under the plasma concentration vs time curve (AUC) for MPAG and the total body clearance (CL) for MPAG were significantly different between control and PHx rats. The total body clearance of MPA and MPAG at the 24th hour was significantly lower than that in the

control group. The clearance of MPA and MPAG recovered completely by day 6. The volume of distribution at steady state (Vss) was not altered.

Measurement of Expression of Mrp2 and Mrp3

The expression of Mrp2 was comparable among all the groups at the 24th hour after initiation of hepatic regeneration (Figure 26, panel A). The mRNA expression of Mrp3 also remained similar among groups at the 24th hour after PHx (Figure 26, panel B).

Measurement of the Formation of Mycophenolic Acid Glucuronide in Small Intestine and Kidney Microsomes

The formation of MPAG by small intestine microsomes at the 24th hour after initiation of hepatic regeneration was not different from that in sham group (N = 5 rats); (PHx 24-hour vs. sham: 1.15 ± 0.50 vs. 1.04 ± 0.48 nmol/mg protein/min, $P > .05$, t-test). The formation of MPAG by the kidney microsomes from PHx 24-hour group was similar to that in sham group (N = 5 rats). (PHx 24-hour vs. sham: 0.29 ± 0.07 vs. 0.23 ± 0.06 nmol/mg protein/min, $P > .05$, t-test)

Table 6. Mean (± SD) Vmax, Km and CL_{int} for the formation of mycophenolic acid glucuronide (MPAG) in hepatic microsomes (N = 4-6 rats).

Time after PHx	Groups	Vm (nmol/mg protein/min)	Km (mM)	CL_{int} (µl/min/mg protein)
24-hour	Control	1.75 ± 0.40	1.07 ± 0.22	1.62 ± 0.06
	Sham	1.88 ± 0.24	1.07 ± 0.18	1.79 ± 0.33
	PHx	0.89 ± 0.17[a]	1.06 ± 0.21	0.85 ± 0.20[a]
6-day	Control	1.84 ± 0.23	1.00 ± 0.19	1.87 ± 0.35
	Sham	1.90 ± 0.29	0.99 ± 0.18	1.98 ± 0.57
	PHx	0.94 ± 0.10[a]	1.00 ± 0.23	0.96 ± 0.17[a]
14-day	Control	1.84 ± 0.23	0.90 ± 0.88	2.08 ± 0.36
	Sham	1.77 ± 0.30	0.94 ± 0.16	1.96 ± 0.60
	PHx	1.76 ± 0.19	1.01 ± 0.24	1.80 ± 0.38

[a] $P < .01$ (vs. control, Tukey post hoc analysis).

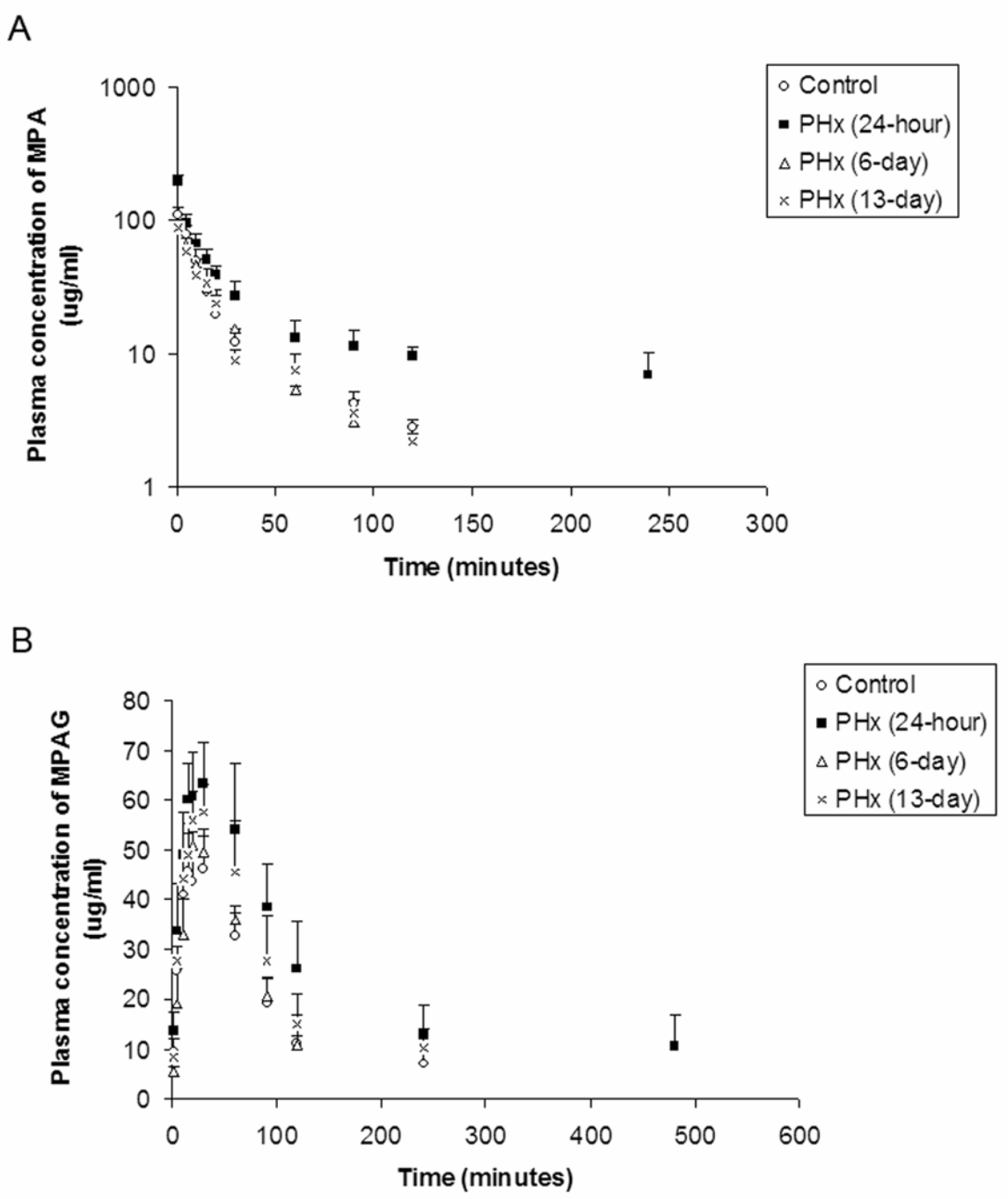

Figure 25. Plasma concentration of MPA (panel A) and MPAG (panel B) vs time profile at different time points after initiation of hepatic regeneration. Data was represented by mean + SD **(**N = 4 to 5 rats).

TABLE 7. Pharmacokinetic parameters of MPA (20 mg/kg, i.v.) 24 hours, 6 days and 13 days after partial hepatectomy (N = 4 to 5 rats)

Parameters	**Control**	**PHx (24-hour)**	**PHx (6-day)**	**PHx (13-day)**
AUC (min•μg/ml) **	2174.00 ± 229.02	3411.10 ± 250.01[a]	1821.50 ± 124.67	1987.03 ± 70.71
CL (ml/min/kg)**	9.29 ± 1.07	5.89 ± 0.42[a]	10.67 ± 0.43	10.07 ± 0.24
MRT (min)**	37.84 ± 2.07	53.04 ± 8.41[a]	33.87 ± 9.40	36.37 ± 3.93
Vss (ml/kg)	348.43 ± 44.18	293.01 ± 60.39	369.97 ± 88.76	429.03 ± 107.59
$(AUC_{MPAG})_{MPA}$ (min*μg/ml)**	4417.91 ± 850.02	7667.38 ± 1088.84[a]	4283.66 ± 569.63	5153.61 ± 455.53
CL_{MPAG} (ml/min/kg) **	4.66 ± 0.90	2.72 ± 0.41[a]	4.73 ± 0.57	3.90 ± 0.34

** $P < .01$ for ANOVA; [a] $P < .01$, [b] $P < .05$ (vs. control) was obtained from Turkey post hoc analysis.

** $P < .01$ for ANOVA; [a] $P < .01$, [b] $P < .05$ (vs. control) was obtained from Turkey post hoc analysis. CL_{MPAG} was calculated using $CL_{MPAG} = fm*AUC*CL/(AUC_{MPAG})_{MPA}$ (assuming fm = 1 because more than 95% MPA is metabolized to MPAG (sum of the amount excreted in urine and bile), Bullingham *et al.*, 1996b).

Abbreviations: AUC, area under the plasma concentration of MPA vs time curve; CL, total body clearance of MPA; MRT = mean residence time of MPA ; Vss = volume of distribution at steady state; $(AUC_{MPAG})_{MPA}$, area under the plasma concentration of MPAG vs time curve after intravenous administration of MPA; CL_{MPAG}, total body clearance of MPAG after intravenous administration of MPA.

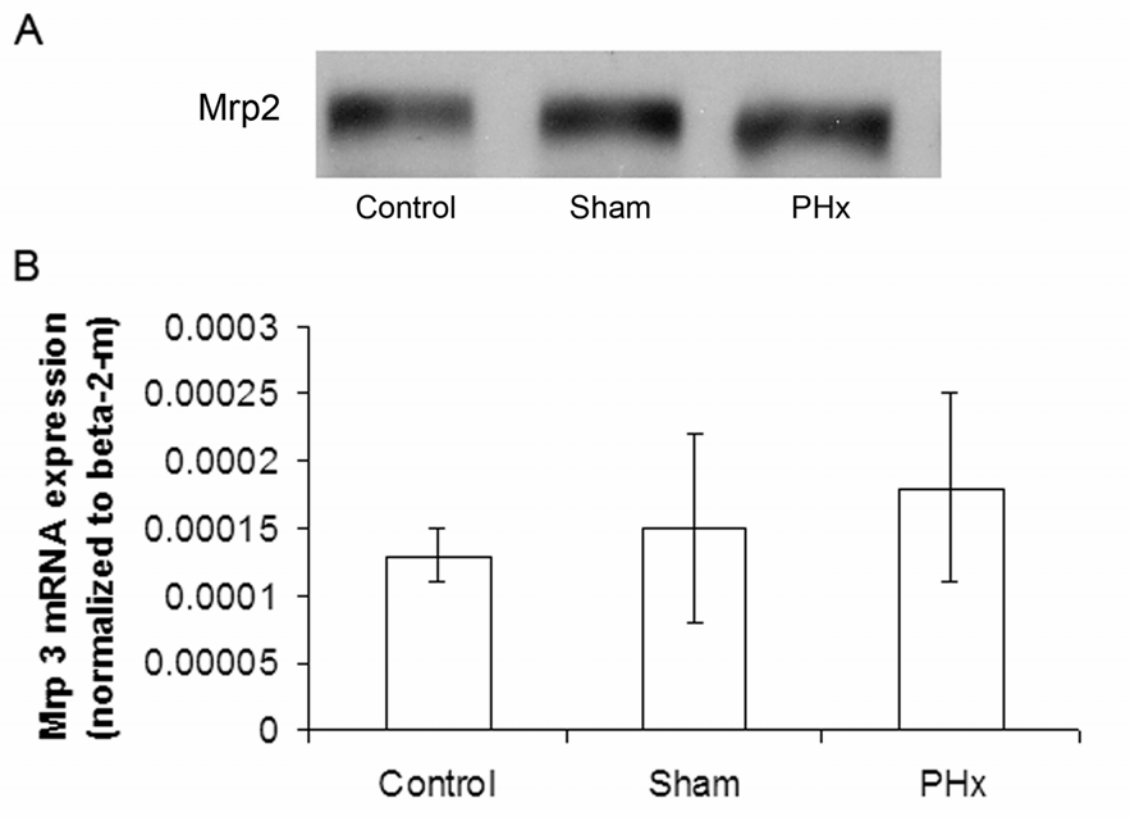

Figure 26. Immunochemical analysis of Mrp2 protein expression (panel A) and mRNA expression of Mrp3 (panel B) from control livers, livers from the sham group, and livers from the 24-hour PHx group. Equal amounts of protein were loaded in each lane. Proteins from 6 rats were pooled together in each group. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers with different dilutions as the standard. The arbitrary mRNA values were normalized with their respective beta-2-m values. N = 4-5.

Discussion

In this study we have used partially hepatectomized rats to evaluate the effect of hepatic regeneration on the metabolism and pharmacokinetics of MPA. This study simulates what is likely to happen to the drug metabolizing capacity in the donor in a living donor liver transplant program. A recent study showed that human UGT 1A9 is the main isoform involved in the metabolism of MPA, with at least 55% contribution to the hepatic MPAG production. Additionally, UGT 1A1 and 1A6 probably account for a part of hepatic production of MPAG. UGT 1A7, 1A8, and 1A10, which are located in the small bowel, could contribute to intestinal first-pass effect of MPA (Picard *et al*., 2005). In this study, MPA was used as a representative marker drug for UGT enzyme(s).

The magnitude of change in the total body clearance of MPA (37%) was much less than that in the liver mass (70%) at the 24th hour. So we evaluated the ability of the liver to metabolize MPA *in vitro*. The metabolism of MPA was also reduced in the regenerating liver at 24 hours and on day 6, but recovered to normal by day 14. Taking into consideration the decrease in the hepatic intrinsic clearance of MPA normalized to protein amount (assuming that hepatic MPA glucuronide formation clearance approximates the hepatic intrinsic clearance of MPA because 95% MPA is primarily metabolized to MPA glucuronide and only less than 1% of MPA was recovered in 48-hour urine after both PO and IV dosing of MPA in humans, Bullingham *et al*., 1996a; Bullingham *et al*., 1996b) and the reduction in liver mass, the whole liver intrinsic

clearance for MPA must decrease to 16% of normal liver at the 24th hour after initiation of hepatic regeneration. Based on the total body clearance of MPA in control rats (9.29 ml/min/kg) and the hepatic plasma flow 25.4 ml/min/kg in rats (estimated based on the reported hepatic blood flow of 55.2 ml/min/kg and hematocrit of approximately 46% in rats (Davies and Morris, 1993)), assuming lack of significant change in the unbound fraction of MPA as albumin, the total body clearance of MPA at the 24th hour should have been at most 2.12 ml/min/kg (If accounting for the three times increase of blood flow per unit liver weight (only 30% liver remaining), the total body clearance should have been at most 2.25 ml/min/kg) (using same equations and methods in the discussion part of chapter 3 to calculate this), and the total body clearance of MPA at the 6th day should have been at most 2.66 ml/min/kg. However, while the total body clearance of MPA was significantly decreased twenty four hours after PHx, the magnitude of reduction was much less than what was predicted based on *in vitro* data. Based on *in vitro* studies, even though our data showed that small instestines have the same metabolizing ability as livers (about four fold higer than that of kidneys) in rats when normalized to per unit organ mass, it was clear that extrahepatic pathways did not change and did not compensate for the reduction in hepatic metabolic capacity due to a much smaller organ mass. When normalized to the predicted liver weight at the 24th hours, the clearance per unit liver weight was increased significantly during hepatic regeneration (24-PHx vs. control: 0.49 ml/min/g vs. 0.23 ml/min/g). This further supported the hypothesis of the presence of

significant reserve capacity of the liver to clear drugs due to increased percentage of hepatocytes to metabolize drugs compared to normal situation.

Even though the pharmacokinetics of tacrolimus (**chapter 3**) and MPA were altered in a similar manner at the 24th hour during hepatic regeneration, the recovery profile for the pharmacokinetic parameters of tacrolimus and MPA was different at later stages of hepatic regeneration: 1) the total body clearance of MPA recovered much earlier than that of tacrolimus (6 days for MPA vs. 18 days for tacrolimus); and 2) total body clearance of MPA recovered earlier than *in vitro* metabolism of MPA (6 days for *in vivo* vs. 14 days for *in vitro*), but this is not the case for tacrolimus (18 days for *in vivo* vs. 14 days for *in vitro*). The reason for the differential recovery of the pharmacokinetic profile of tacrolimus and MPA may be due to the different reserve capacity of the regenerating livers for different metabolic pathways in which different metabolizing enzymes with different abundance are involved.

In addition, we also observed a lower total body clearance of MPAG at the 24th hour during hepatic regeneration. In order to determine the reason(s) for this decrease, we evaluated the expression of Mrp2 and Mrp3, two transporters that are involved in the biliary excretion of MPAG (Kobayashi *et al*., 2004). However, no change was detected in the expression of Mrp2 or Mrp3 at the 24th hour, which ruled out any possible roles of Mrp2 and Mrp3 in the reduced clearance of MPAG. This observation is also consistant

with the published data showing the lack of change in the protein expression of Mrp 2 (Chang *et al*., 2004; Vos *et al*., 1999). It is likely that the decrease in total body clearance of MPAG is partially due to the dramatic reduction in the number of hepatocytes leading to decreased bile formation in the regenerating liver. However, due to the small fraction of the MPAG being excreted in the bile (26% of the dose), the decreased bile formation is not the only mechanism or reason for the approximately two fold decrease in the total body clearance of MPAG during hepatic regeneration. There are other unknown mechanism(s) involved that are not apparent from this study such as the impaired function of both Mrp2 and Mrp3 (even though we observed the preserved expression of both transporters) because it takes time for newly generated cells to relocate to apical side of hepatocytes.

To the best of our knowledge this is the first study to evaluate the pharmacokinetics of MPA and to analyze the recovery profile of the pharmacokinetics of MPA over time after initiation of hepatic regeneration in an animal model. Our study provides several implications for use of drugs metabolized by UGTs in LDLT patients. First, since the UGT activity is decreased after PHx, lower doses of UGT substrates may be necessary during the early postoperative period for LDLT patients. Second, the magnitude of the reduction in doses should be less than the magnitude of reduction in liver mass. Third, caution must be exercised in using *in vitro* data to predict *in vivo* clearance of drugs by the regenerating liver due to significant reserve capacity of the liver to clear drugs.

Finally, the recovery is different between oxidative pathway and conjugation pathway.

Chapter 6 Transiently Altered Acetaminophen Metabolism during Hepatic Regeneration in Rats

Abstract

Objective The objectives of this study were to evaluate 1) the dose-dependent *in vivo* metabolism of acetaminophen (APAP) 24 hours after hepatic regeneration, and 2) the time-dependent *in vivo* metabolism of APAP during hepatic regeneration.

Methods The metabolism of APAP was evaluated by collecting urine (24 hrs) after intravenous administration of different doses of APAP to partially hepatectomized rats. The amount of APAP glucuronide, sulfate and mercapturate in the urine was measured by HPLC and LC/MS.

Results The fraction of the dose that is converted to APAP sulfate was decreased 24 hours after regeneration at the doses of 100 mg/kg and 300 mg/kg. The fraction of the dose that is metabolized to APAP glucuronide and mercapturate was significantly increased 24 hours after regeneration at the doses of 10 mg/kg and 100 mg/kg. At a dose of 10 mg/kg, the formation of APAP glucuronide and mercapturate significantly increased on day 6 and recovered on day 14, while the formation of APAP sulfate was not altered at any time during the hepatic regeneration.

Conclusions The metabolism of APAP was altered during hepatic regeneration. There may be a higher risk for the APAP-induced toxicity during the early postoperative period in LDLT patients. Acetaminophen must be avoided during hepatic regeneration. The altered metabolism of APAP recovered to normal level by day 14 after initiation of

regeneration. It is safe to use APAP once the regeneration process is recovered completely.

Introduction:

Acetaminophen (APAP) is the most widely used analgesic in the USA and overdoses of APAP are the leading causes of hospital admission for acute liver failure (Gill and Sterling, 2001). APAP is primarily metabolized by glucuronidation and sulfation in human, accounting for approximately 50% and 35% of a therapeutic dose, respectively (Forrest *et al*., 1982). Less than 10% of a therapeutic dose is metabolized to a reactive quinine form, N-acetyl-p-benzoquinone imine (NAPQI), mainly by CYP2E1 (Manyike *et al*., 2000), which can cause hepatotoxicity by forming adducts with critical proteins in the liver (Hinson *et al*., 1995; Holtzman, 1995). At low subtoxic doses, NAPQI is inactivated by GSTs through conjugation with reduced glutathione (GSH) (with subsequent conversions to cysteine and mercapturate conjugates of acetaminophen) (Moldeus, 1978; Van De Straat, 1986). Under an overdose situation, GSH is depleted so that NAPQI accumulates and binds to proteins (Davis *et al*., 1974; Jollow *et al*., 1974; Potter *et al*., 1974).

Humans have a limited capacity to conjugate APAP with sulfate. APAP sulfate formation may become capacity-limited (saturating) upon administration of a 2 g dose to adults (Levy and Yamada, 1971). A model that consists of two saturating biotransformation pathways (conjugation of APAP with glucuronic acid and with sulfate) as well as two apparent first-order processes (renal excretion of acetaminophen and oxidation of the drug to NAPQI) has been proposed to describe the pharmacokinetics of APAP (Slattery

and Levy, 1979). The limited capacity of the direct conjugation pathways causes a greater than proportional increase in the formation of NAPQI with increasing doses.

In rats, after a single dose, the plasma APAP concentrations declined apparently exponentially, with a T1/2 that increased with increasing dose (Siegers *et al*., 1978; Galinsky and Levy, 1981). Studies have also confirmed that in rats, just as in man (Davis *et al*., 1976), the fraction of the dose that is converted to APAP sulfate decreases with increasing doses. So the APAP kinetics is dose-dependent. In several species, the metabolites of APAP are excreted through bile and urine. In principle, alterations in hepatobiliary or urinary excretion of APAP metabolites, as determined in *in vivo* animal studies, are indicative of changes in either APAP biotransformation or transport processes for these metabolites.

During hepatic regeneration, the *in vivo* drug metabolizing activity of different enzymes or isoforms may be altered differentially as discussed in previous chapters, causing a shift in the relative contribution of different pathways to the overall disposition of a drug. Moreover, the reduction of liver mass will further decrease the drug metabolizing ability of the liver. Since the pharmacokinetics of acetaminophen is nonlinear and dose-dependent, we hypothesized that the formation of acetaminophen sulfate will decrease and the formation of glucuronide and NAPQI related metabolites (cysteine and mercapturate) will increase with an increase in dose or a reduction in the metabolizing

ability of the liver during hepatic regeneration. This change in the metabolic pathway, may predispose a LDLT patients to induced toxicity due to acetaminophen.

Materials and Methods

Chemicals

Acetaminophen and acetaminophen glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO). Acetaminophen sulfate, 3-cysteinyl acetaminophen, acetaminophen mercapturate, $^{2}H_3$-labeled 3-cysteinyl acetaminophen and $^{2}H_3$-labeled acetaminophen mercapturate were purchased from Toronto Research Chemicals (North York, ON, Canada).

Experiment Procedure

The study protocol was approved by the IACUC at the University of Pittsburgh. The sham operation and PHx were conducted as described in **Chapter 2**. Ten rats were ordered every time (5 rats for PHx; 5 rats for sham) and the surgery was conducted between 9:00 am and 11:00 am. APAP (APAP powder was dissolved in polyethylene glycol-0.9% saline (50:50, v/v) to make a final concentration of 5 mg/ml, 50 mg/ml and 150 mg/ml) was injected through the jugular vein in sham operated (N = 5) and PHx (N = 5) rats. Urine samples were collected for 24-hour using metabolic cages after IV administration of APAP. The urine samples were stored at -20^{o} C until analysis.

HPLC method

After centrifugation at 13,000 rpm for 5 minutes, 40 µl of the supernatant (1 to 5 dilution for 100 mg/kg dosing or 1 to 15 dilution for 300 mg/kg dosing) was injected into HPLC. Acetaminophen glucuronide and sulfate were quantified by HPLC, as described previously (Wilson *et al*., 1982) (Column: LiChrospher column, C18, 5 µ, 250 mm x 4.6 mm; mobile phase: methanol/glacial acetic acid/0.1 M KH_2PO_4 (7/0.75/92.25, v/v/v); UV, 248 nm; retention time: 7.2 min for glucuronide and 13.3 min for sulfate). Quantitation was performed by comparison of the peak area for unknown samples to standard samples (linear from 0.8 µg to 6 µg for glucuronide; from 2.4 µg to 24 µg for sulfate) for APAP glucuronide and sulfate. The intra- and inter-day CV (%) for APAP glucuronide at 0.8 µg, 2 µg and 6 µg were less than 9% (n =3). The intra- and inter-day CV (%) for APAP sulfate at 2.4 µg, 6 µg and 24 µg were less than 7% (n = 3).

LC/MS method

After centrifugation at 13,000 rpm for 5 minutes, 6 µl of supernatant (1 to 10 dilution for 100 mg/kg dosing or 1 to 100 dilution for 300 mg/kg dosing), 6 µl of internal standard ($^2H_3$-labeled 3-cysteinyl acetaminophen, 40 µg/ml or $^2H_3$-labeled acetaminophen mercapturate, 8 µg/ml) and 48 µl of water were mixed. 20 µl of the mixture was injected into LC/MS. The analysis of acetaminophen cysteine and mercapturate was performed on a Thermo Finnigan MSQ LC/MS, operated in the positive ion electrospray mode with selective ion monitoring. A YMC-AQ 2.1-µm, C18 (150 mm X 2 mm) column was used

for nominal chromatographic separation. The mobile phase consisted of methanol/1% acetic acid (20/80, v/v) delivered at a flow rate of 150 µl/min. The following mass spectrometric conditions were used: capillary and cone voltages of 3.0 kV and 50 V (for mercapturate) or 100 V (for cysteine), and a temperature of 300^{o} C. The following pairs of ions (unlabeled and deuterated internal standard) were detected by selective ion monitoring: mass-to-charge ratio (*m/z*) 271 and 274 (3-cysteinyl) and *m/z* 313 and 316 (mercapturate). The retention time for 3-cysteinly APAP and APAP mercapturate was 4.7 min and 11.7 min, respectively.

The concentrations of all the metabolites were measured by comparison of the peak area ratios after the unknown samples to the standard curves for each metabolite. For measurement of 3-cysteinyl APAP and APAP mercapturate in urine, the respective inter- and intra-day CV (%) was less than 13% and 8% (n = 4), respectively.

Data analysis

The amount of APAP equivalent to the amount of each metabolite in the 24-hour urine was calculated by considering the difference in molecular weight between the metabolite and the parent drug. The fraction excreted in 24-hour urine for each metabolite was calculated as equivalent APAP amount divided by the IV dose of APAP. All data are reported as mean ± SD. Comparisons between two groups were made by student's t-test ($P < 0.5$). For sample size calculation, the initial fraction of the formation of APAP

mercapturate from the sham group (24-hour time point) at the APAP dose of 10 mg/kg, 1.01 ± 0.1%, was used. With a power of 80% and $\alpha = 0.05$, to detect a 25% difference, 4 rats were required. Experiments were completed with 5 rats in each group.

Results

Formation of Acetaminophen Glucuronide (Dose-dependent effect)

The fraction of the dose that is excreted as APAP glucuronide in the 24 hr urine increased gradually with the increase in doses from 10 mg/kg to 300 mg/kg in the sham groups (Figure 27). During regeneration, there was a significant increase in the fraction of APAP glucuronide excreted in 24-hour urine at the doses of 10 mg/kg and 100 mg/kg compared to sham groups dosed with same amount of APAP. However, at a higher dose level, 300 mg/kg, the formation of APAP glucuronide was the same for the PHx (24-hour) group as that for the sham group.

Formation of Acetaminophen Sulfate (Dose-dependent effect)

The fraction of the dose that is excrted as APAP sulfate in the 24 hr urine decreased with an increase in dose from 10 mg/kg to 300 mg/kg in the sham groups (Figure 28). Twenty four hours after the initition of regeneration, the fraction of APAP sulfate in 24-hour urine was not altered at the dose of 10 mg/kg. However, 24 hours after initiation of regeneration, the formation of APAP sulfate was significantly decreased at the doses of 100 mg/kg and 300 mg/kg.

Formation of Acetaminophen Mercapturate (Dose-dependent effect)

The formation of APAP mercapturate also showed a dose-dependency (Figure 29). With an increase in the dose, the fraction of APAP converted to APAP mercapturate was increased in sham groups. At 24 hr after initiation of hepatic regeneration at the dose of 10 mg/kg, the formation of APAP mercapturate was much higher in PHx (24-hour) group than in the sham group. The formation of APAP mercapturate was also much higher in PHx (24-hour) group than that in the sham group at the dose of 100 mg/kg. However, 24 hr after initiation of hepatic regeneration, the formation of APAP mercapturate was not changed at the dose of 300 mg/kg.

Formation of Acetaminophen Glucuronide (Time-dependent effect)

At the dose of 10 mg/kg, greater amount of APAP was excreted as APAP glucuronide at the 24th hour and 6th after regeneration than that observed in sham groups (Figure 30). However, the percentage of dose excreted as APAP glucuronide returned to control level 14 days after regeneration.

Formation of Acetaminophen Sulfate (Time-dependent effect)

At a dose of 10 mg/kg at the all time points after initiation of regeneration, the percentage of dose excreted as APAP sulfate was not changed when compared to sham groups (Figure 31).

Formation of Acetaminophen Mercapturate (Time-dependent effect)

The formation of APAP mercapturate was significantly increased 24 hours and 6 days after regeneration (Figure 32), but recovered to control level (as measured in the sham group) 14 days after initiation of regeneration.

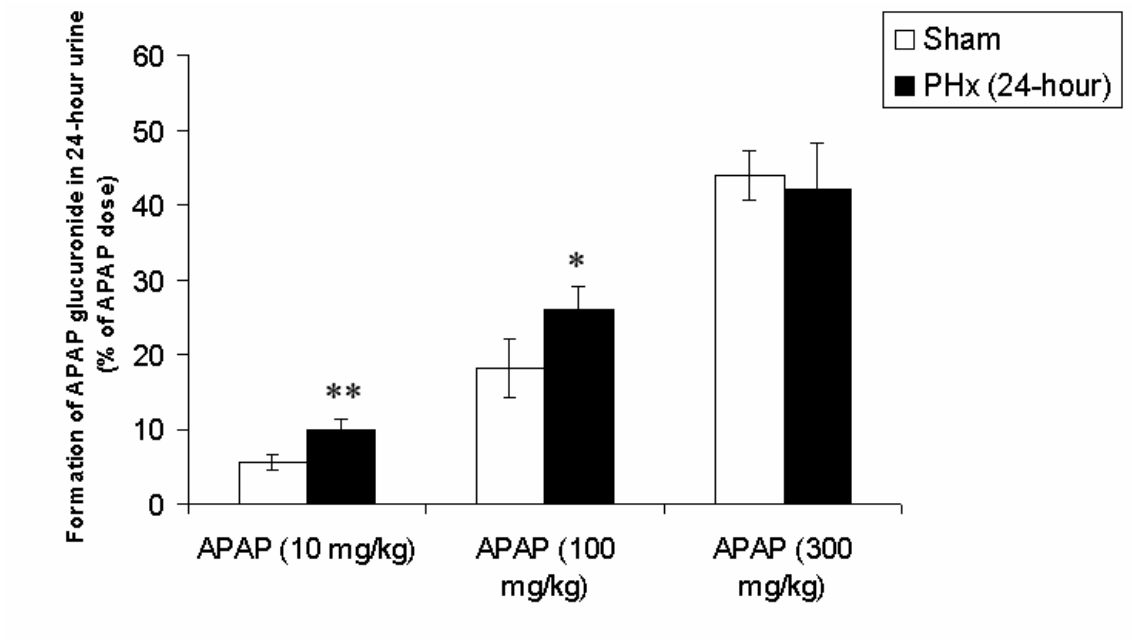

Figure 27. Formation of APAP glucuronide in 24-hour urine (dose-dependent effect).

Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham; * $P < .05$ vs. sham (student's t-test). N = 5 rats.

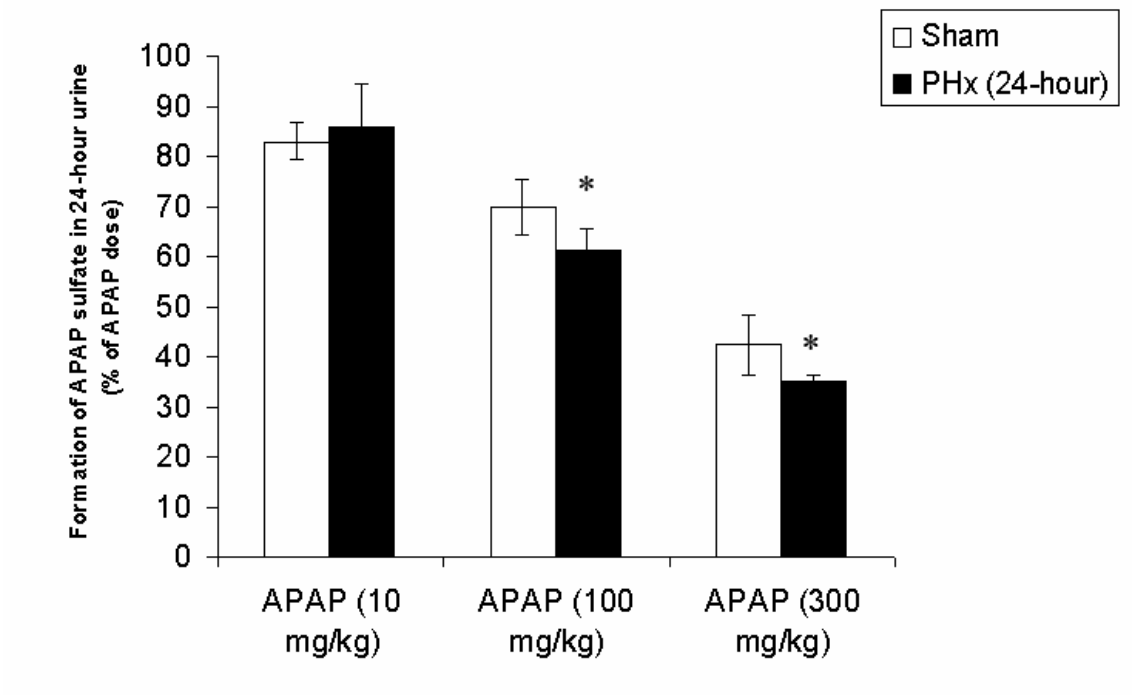

Figure 28. Formation of APAP sulfate in 24-hour urine (dose-dependent effect).

Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. * $P < .05$ vs. sham (student's t-test). N = 5 rats.

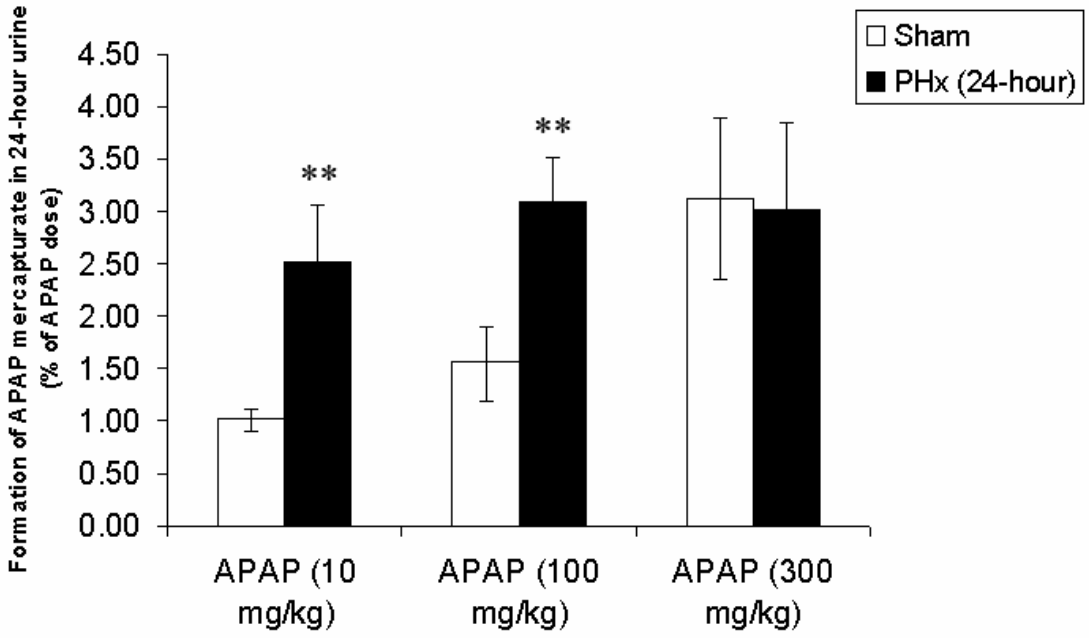

Figure 29. Formation of APAP mercapturate in 24-hour urine (dose-dependent effect). Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham (student's t-test). N = 5 rats.

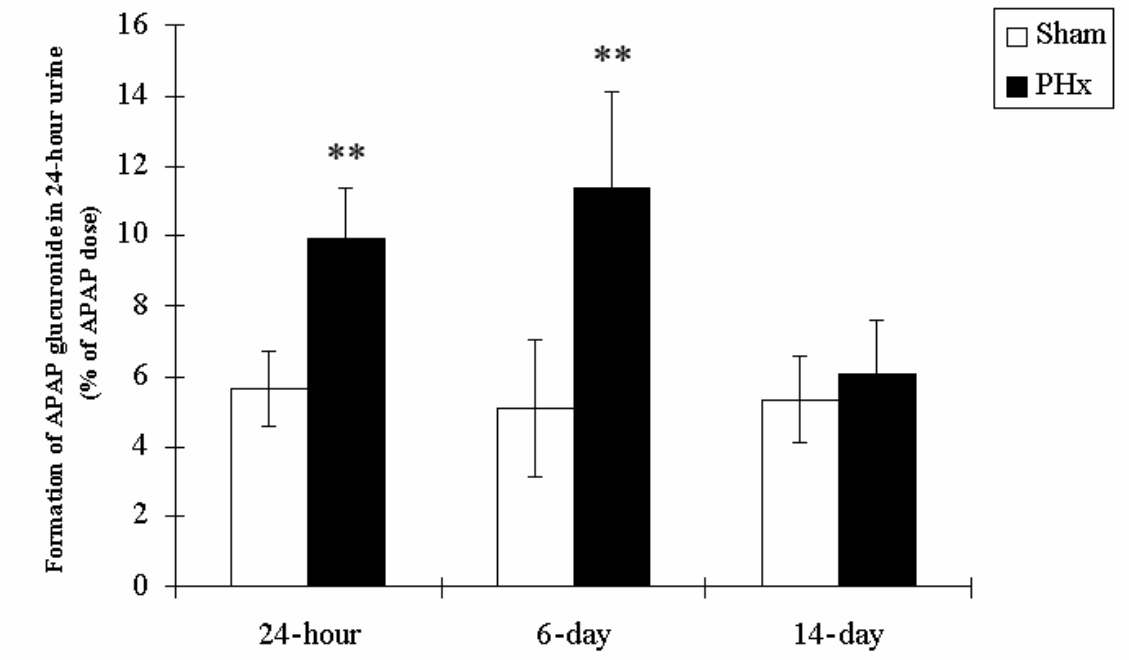

Figure 30. Formation of APAP glucuronide in 24-hour urine at the dose of 10 mg/kg (time-dependent effect). Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham (student's t-test). N = 5 rats.

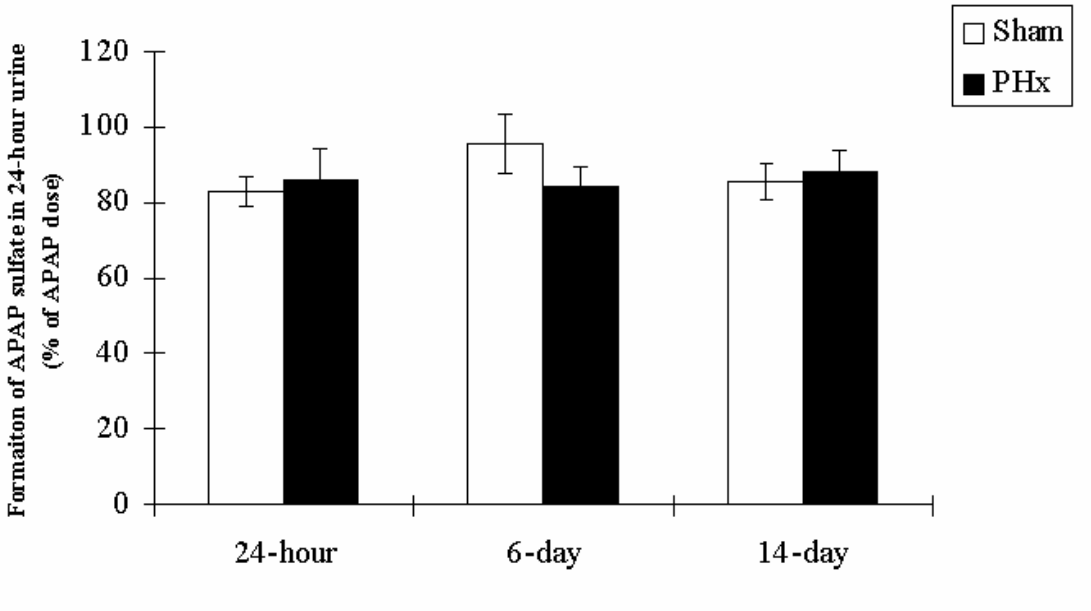

Figure 31. Formation of APAP sulfate in 24-hour urine at the dose of 10 mg/kg (time-dependent effect). Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. $P > .05$ vs. sham (student's t-test). N = 5 rats.

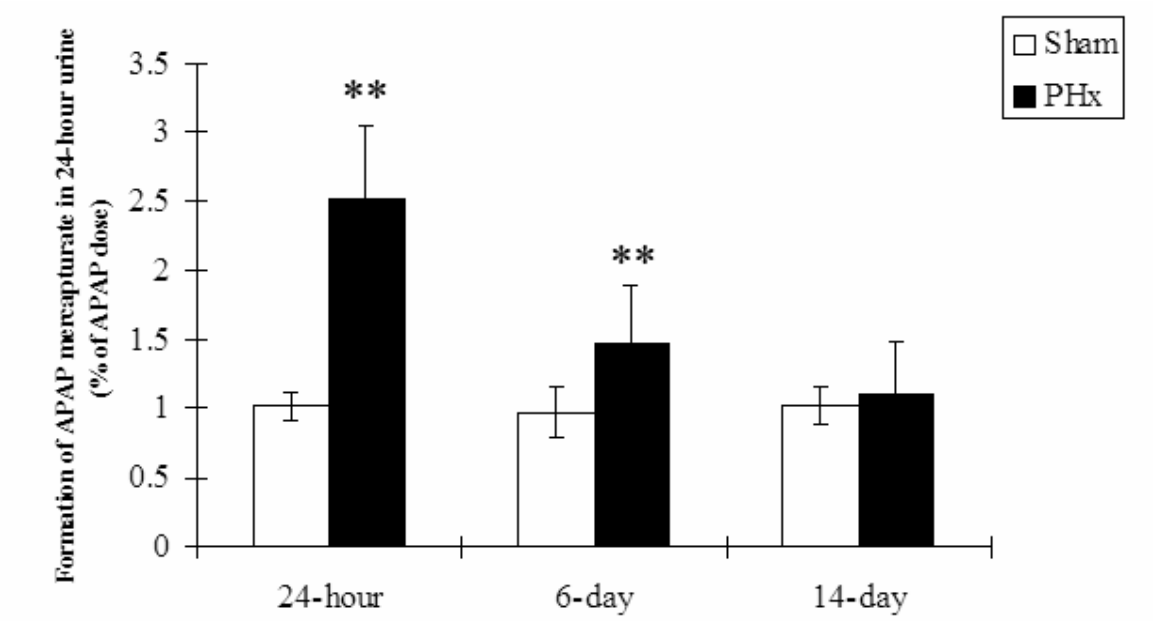

Figure 32. Formation of APAP mercapturate in 24-hour urine at the dose of 10 mg/kg (time-dependent effect). Sham: liver lobes from sham groups; PHx, the regenerated liver lobes after PHx. All data are expressed as mean ± SD. ** $P < .01$ vs. sham (student's t-test). N = 5 rats.

Discussion

Acetaminophen is commonly recommended for analgesia or fever reduction. APAP toxicity, due to overdose, has been well documented. We used partially hepatectomized rats to study the *in vivo* metabolism of APAP during hepatic regeneration. We used a clinically relevant dose, 10 mg/kg, to study the time profile of APAP metabolism during hepatic regeneration. To study the dose-dependent effect 24 hours after initiation of regeneration, we used a clinically relevant dose of 10 mg/kg and two higher doses: subtoxic dose 100 and toxic dose 300 mg/kg.

In this study, we observed dose-dependent formation of APAP glucuronide, APAP sulfate, and APAP mercapturate in the sham group, which is consistent with the reported non-linear pharmacokinetics of APAP in rats (Galinsky and Levy, 1981). A recent study showed significant time-dependent changes in APAP metabolism after liver transplantation (Park *et al*., 2003). At the early postoperative period (day 2 and day 10), the fraction of the dose that is excreted as 3-cysteinyl and mercapturate was significantly higher than day 180. The formation of APAP sulfate and glucuronide in the 24 hr urine was much lower on day 2 and 10. This may be due to impaired glucuronidation and sulfation and increase in the activity of CYP2E1 (Park *et al*., 2003; Burckat *et al*., 1998)

We observed a higher fraction of APAP glucuronide and mercapturate being formed at

doses of 10 mg/kg and 100 mg/kg 24 hours after initiation of regeneration, however, the mechanism(s) responsible for our observations are different from those for the altered APAP metabolism after liver transplantation. This is due to 1) the increased amount of APAP that is perfused through the liver when normalized to unit liver mass and the result of non-linear pharmacokinetics of APAP (increased drug amount per unit liver mass will cause more APAP glucuronide and mercapturate to be formed). 2) the dramatic decease in the number of hepatocytes caused by the reduction of the liver mass will lead to decreased bililary excretion of APAP glucuronide (A study conducted in rats showed that the biliary excretion of APAP glucuronide (Mrp 2 substrate) (6.0-10 % of the dose) was equal to the urinary excretion of APAP glucuronide (6.0-10 % of the dose) at the dose of 10 -100 mg/ml (Watari *et al*., 1983; Brouwer and Jones *et al*., 1990.). At the highest dose of 300 mg/kg, we didn't see the change in the formation for both metabolites (glucuronide and mercapturate) 24 hours after regeneration, probably because of the saturation of both pathways to a similar magnitude. Even though APAP mercapturate is also excreted through the biliary excretion and is a Mrp 2 substrate, in rats, the percentage of the dose excreted in bile (less than 0.2% of the dose at the dose of 150 mg/kg to 300 mg/kg) is much less than what is excreted in urine (1.2 – 2% of the dose at the dose of 150 mg/kg to 300 mg/kg) (Kwak *et al*., 1998; Chen *et al*., 2003). So the decreased biliary excretion due to the decreased number of hepatocytes did not alter the urinary excretion of mercapturate. 3) the activity of drug metabolizing enzymes such as the CYP2E1, UGT1A6/7 and sulfotransfereases (SULTs) were altered differentially and will lead to the

shift in the relative contribution of different pathway to the overall elimilation of APAP. The CYP2E1 activity as measured by the formation of 6-hydroxychlorzoxazone was decreased to about the 57% of the control level as measured in sham group (PHx-24 hr vs. sham: 0.40 ± 0.12 vs. 0.69 ± 0.23 nmol/mg protein/min, $P < .05$, student's t-test; N = 6 rats, our observations), while the glucuronidation of acetaminophen was preserved after initiation of hepatic regeneration as shown in Chapter 5. Currently, there is no information on the activity of SULTs during hepatic regeneration; however, the mRNA expression of the isoforms of phenol SULT family (SULT1A1, 1B1, 1C1, and 1E2) which may be responsible for the formation of APAP sulfate has been showed to be decreased after initiation of hepatic regeneration (Dunn *et al*., 1999). Since the glucuronidation of APAP was preserved (chapter 4) and the activity of CYP2E1 (our observations) and expression SULTs were decreased (Dunn *et al*., 1999), there was increased amount of APAP glucuronide excreted in urine after initiation of hepatic regeneration.

A lower fraction of the dose was excreted as APAP sulfate 24 hours after initiation of regeneration at doses of 100 mg/kg and 300 mg/kg compared to sham group. This is likely due to increased amount of drug being delivered per unit liver mass and the nonlinear pharmacokinetics of APAP (with the increase in dose, the fraction excreted as sulfate will decrease). This may be related to higher fraction (about 80%) of APAP excreted as APAP sulfate and inability to differentiate small changes.

This is the first study to document the altered metabolism of APAP during hepatic regeneration. Our data point to several important conclusions: 1) The metabolism of APAP will be altered during hepatic regeneration; 2) The formation of APAP glucuronide and mercapturate will be increased during hepatic regeneration at a clinically relevant dose; 3) There may be a higher risk for the APAP-induced toxicity in LDLT patients during the early post operative period; 4) The altered metabolism of APAP will recover to normal some time after regeneration and 5) Acetaminophen must be avoided during the hepatic regeneration in LDLT patients.

Chapter 7 Summary and Conclusions

The goal of this dissertation is to evaluate the drug metabolizing capacity in regenerating liver in rats.

Current observations

The liver has a unique ability to regenerate. During hepatic regeneration, the clearance of several drugs are expected to be significantly altered due to 1) reduction in liver mass, 2) reduction in metabolic capacity of the phase I and phase II drug metabolizing enzymes, 3) increased hepatic blood flow (ml/kg) and 4) changes in plasma protein binding of a drug. Based on the inhibitory effects of some of the cytokines released during hepatic regeneration on the *in vitro* activity of both CYP and UGT isoforms and the changes in the expression of many known and unknown regulatory factors and other genes due to the acute phase response triggered by partial hepatectomy, we hypothesize that the *in vitro* activity of both CYP and UGT isoforms will be decreased during hepatic regeneration. Taking into consideration the reduction in liver mass, we further hypothesize that the *in vivo* clearance of drugs will be reduced more than the loss of liver mass during hepatic regeneration. The direction and magnititude of changes in clearance will be drug specific and will be influenced by changes in blood flow and changes in free fraction of drugs.

In intial experiments, we evaluated the effects of hepatic regeneration on the *in vitro* activity of CYP3A, the most significant CYP isoform, which contributes to the metabolism of more than 50% of the marketed drugs. We also measured the protein and mRNA expression level of CYP3A over a similar time period. Since during the hepatic regeneration process, the patients are expected to take immunosuppressive drugs such as cyclosporine A and tacrolimus which are known to be inhibitors of CYP3A but inducers of DNA synthesis, we also evaluated the effect of cyclosporine A and tacrolimus on CYP3A activity during hepatic regeneration. The changes in CYP3A during hepatic regeneration were consistent at the levels of activity, protein content and mRNA expression. The activity of CYP3A enzyme was impaired during the initial phase of hepatic regeneration but recovered completely at a later time. At the doses used, cyclosporine A and tacrolimus didn't have any effect on CYP3A recovery in the regenerating liver.

Secondly, in order to evaluate the feasibility of predicting *in vivo* changes in clearance during hepatic regeneration, we evaluated the pharmacokinetics of a CYP3A substrate, tacrolimus. The hepatic intrinsic clearance calculated as Vm/Km for the metabolism of tacrolimus and total body clearance of tacrolimus were significantly decreased 24 hours after PHx. Even though the hepatic intrinsic clearance for the metabolism of tacrolimus recovered completely 14 days after PHx, the total body clearance of tacrolimus returned to normal level only by day 18. Moreover, the observed clearance of tacrolimus was

greater than the predicted total body clearance based on our *in vitro* data. This indicates caution in using *in vitro* data to predict the *in vivo* clearance of CYP3A substrates because of the reserved hepatic capacity to metabolize drugs during hepatic regeneration.

Thirdly, glucuronidation of several drugs has been reported to be differentially altered during hepatic regeneration. In this study, we evaluated either activity and/or mRNA expression level of various UGT isoforms after initiation of regeneration. The activity of different isoforms was altered differentially during hepatic regeneration with some having altered activity or expression while activity or expression of others was preserved. The mRNA expression of different UGT isoforms mirrored the activity of these isoforms. Moreover, different isoforms also recovered differentially during hepatic regeneration with some recovering 6 days after regeneration and others recovering 14 days after regeneration. The differential regulation of different UGT isoforms may be due to the differential expression of HNF-1 α and C/EBP α.

Fourthly, we evaluated the clearance and *in vitro* metabolism of MPA, an immunosuppressive drug, during hepatic regeneration. The hepatic intrinsic clearance for the metabolism of MPA and the total body clearance of MPA were much lower 24 hours after regeneration. However, the recovery of the pharmacokinetic parameters including total body clearance was much earlier than the hepatic intrinsic clearance *in vitro*. The observed *in vivo* clearance of MPA during hepatic regeneration was greater than what was

predicted based on *in vitro* metabolism of MPA. The extrahepatic metabolism of MPA didn't account for the increased clearance of MPA as the *in vitro* glucuronidation of MPA in small intestine and kidney was not altered during hepatic regeneration. Additionally, the total body clearance of MPA glucuronide (MPAG) was also significantly decreased during hepatic regeneration due to the dramatically decreased number of hepatocytes leading to decreased biliary excretion of MPAG. Two transporters, namely Mrp2 and Mrp3, which are involved in the biliary excretion of MPAG didn't contribute to the decreased clearance of MPAG.

When comparing the *in vitro* and *in vivo* data for the metabolism of both tacrolimus and mycophenolic acid, we also observed that 1) there was a difference between the *in vivo* recovery of oxidative pathway and conjugation pathway and 2) there was a disconnect between the *in vitro* recovery and the *in vivo* recovery. The clearance of CYP3A and UGT substrates studied eventually returned to normal in spite of incomplete recovery of liver mass.

Finally, we evaluated the *in vivo* metabolism of APAP during hepatic regeneration. The dose-dependent effect of APAP metabolism with a higher fraction of APAP glucuronide and mercapturate was observed after regeneration at low, subtoxic and toxic doses of APAP. The time-dependent effect of APAP showed more APAP glucuronide and mercapturate formation at the early stage of regeneration. The altered metabolism of

APAP returned to normal even before the complete recovery of liver mass.

Note: The summary of recovery of liver mass and *in vitro* activity of different drug metabolizing enzymes is shown on Figure 33.

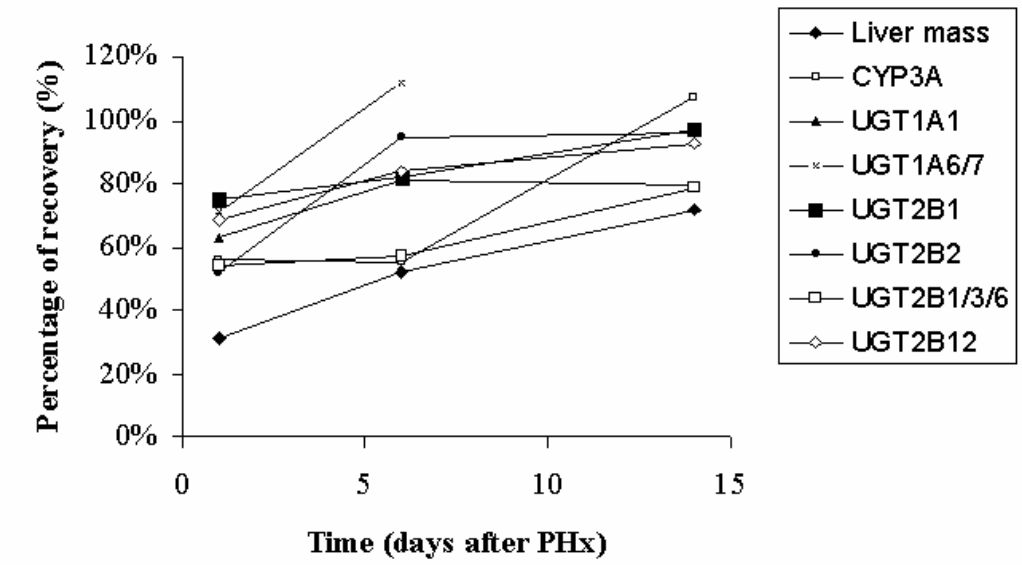

Figure 33. Summary of recovery of liver mass and *in vitro* activity of different drug metabolizing enzymes after hepatic regeneration. Percentage was calculated using mean value of PHx group divided by mean value of sham group.

Clinical implications:

1) Hepatic functional capacity will recover prior to the recovery of liver mass.
2) Reduction in doses of drugs that are metabolized by CYP3A or UGT is necessary during the first few weeks after transplantation in living donor liver transplant donors and recipients. Additional factors such as cold ischemia, warm reperfusion injury and

immunological response may further alter the dosing regimen of drugs in the recipients.

3) Reduction in clearance of the drug *in vivo* is not proportional to the reduction in liver mass. LDLT patients would require a dose of CYP3A or UGT substrates that is not in proportion to the loss of the liver mass.
4) Differential adjustment in doses of drugs metabolized by different UGTs as compared to CYPs is necessary, as UGT pathway appear to recover faster than CYPs.
5) The lower activity of UGT1A1 may lead to decreased conjugation of bilirubin and increase in the concentration of biliribin in bile or serum during the early part of hepatic regeneration process.
6) Liver regeneration with regards to drug metabolizing ability proceeds normally in the presence of immunosuppressive therapy with cyclosporine A or tacrolimus.

Future directions

The studies presented in this dissertation have evaluated the drug metabolizing ability during hepatic regeneration. This studies conducted in the partially hepatectomized rats have significantly enhanced our understanding of both *in vitro* and *in vivo* drug metabolizing ability with regarding to phase I and phase II pathways. Some of this knowledge may be applied to LDLT patients, however, additional studies related to the mechanism and clinical relevance of our findings are needed in the future for better understanding the drug metabolizing ability during hepatic regeneration:

A. The direct cause for the decreased activity and expression of some CYP and UGT isoforms needs to be identified and elucidated. Even though some cytokines are known to decrease the activity and expression of certain drug metabolizing enzymes, the direct role of cytokines as the gene regulation mediators during hepatic regeneration remains to be determined.

B. Even though the differential regulation of UGTs during hepatic regeneration to be probably due to the differential expression of HNF-1 α and C/EBP α, the data presented in this dissertation is premature and the functional activity data of both regulatory factors in the UGT isoforms need to be extensively studied. Currently, there is only one functional activity study of the role of C/EBP α in the expression of UGT1A1 using C/EBP α knock-out mice. More UGT isoforms need to be evaluated using knock-out animals.

C. The UGT isoforms we studied are rat isoforms. Even though humans and rats share most of UGT1A1 isoforms such as UGT1A1, 1A3, 1A5, 1A6, 1A7 and 1A8, not a single UGT2B isoform is shared between humans and rats. However, the substrates used for rat UGT2B isoforms in the studies are also known to be metabolized by glucuronidation in humans. So the change in specific human UGT isoforms may need to be evaluated using specific substrates in humans.

D. We interpretated the discrepancy between the *in vitro* metabolism and *in vivo* clearance using the reserved hepatic capacity to metabolize drugs during hepatic regeneration. However, there is no any direct evidence to support this notion and the mechanism of hepatic capacity preservation is still unkown.

E. The pharmacokinetic study using high clearance drug with extraction ratio near 1 is needed to see the effect of changes in blood flow during hepatic regeneration on drug clearance.

F. We studied only IV dosing of drugs in this project. However, the change in clearance of drugs given orally due to the decreased first-pass effect caused by PHx needs to be evaluated during hepatic regeneration due to the decreased intrinsic clearance of drugs during hepatic regeneration. We anticipate that there will be more difference between PHx and control group in the clearance of drugs administrated orally than intravenously administration.

G. The animal model used in this project reflects the donors and not the recipients. Other factors such as preexisting disease, cold ischemia, warm reperfusion injury and drug therapy such as antifungal drugs and antiviral drugs may also affect the drug metabolizing enzymes *in vitro* or *in vivo* in the recipients. The situations are expected

to be more complex in recipients and studies need to be conducted in different animal models reflecting the recipients.

H. In addition to the enzyme that were evaluated in this project, other phase I and II enzymes also contribute to the clearance of drugs used in transplantation. Future studies should evaluate regulation and activity of such enzymes.

I. In this study we primarily addressed hepatic drug metabolism. In addition to drug metabolism, drug transporters also play a significant role in the overall pharmacokinetics of drugs. Future studies should evaluate the regulation and activity of drug transporters during hepatic regeneration.

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