Cytochrome P450 2A5 and Bilirubin
Mechanisms of Gene Regulation and Cytoprotection

by
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ABSTRACT

CYTOCHROME P450 2A5 AND BILIRUBIN: MECHANISMS OF GENE REGULATION AND CYTOPROTECTION

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Murine cytochrome P450 2A5 (CYP2A5) is an interesting enzyme for its unique regulation and its involvement in liver injury caused by various well-known pathological conditions or hepatotoxins. It has been reported that CYP2A5 is upregulated following exposure to chemical hepatotoxins and during pathophysiological conditions in which the levels of most Cytochrome P450s are either unchanged or down-regulated. Recently bilirubin has been identified as the first endogenous substrate for CYP2A5 and it has been suggested that CYP2A5 plays a major role in bilirubin clearance as an alternative mechanism to BR conjugation by UGT1A1. This study investigated the mechanisms of gene regulation and cytoprotective role of CYP2A5 in response to bilirubin treatment in liver. Our results demonstrate that bilirubin induces CYP2A5 expression at the mRNA and protein levels by increasing CYP2A5 transcription via a mechanism that involves Nrf2 activation. Furthermore, our results suggest that induced CYP2A5 plays a cytoprotective role against bilirubin toxicity by directly lowering the cellular levels of bilirubin and by inhibiting caspase-3 activation.

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DECLARATION OF WORK PERFORMED

I declare that with exception of the item indicated below, all the work reported in this thesis was performed by me. HPLC analysis of Hepa 1-6 media samples for the 7-ethoxycoumarin deethylation assay was performed by Yanping Lou in the Department of Animal Science.

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LIST OF ABBREVIATIONS

3-MC	3-methylcholanthrene
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APAF1	Apoptotic protease activating factor
ARNT	Aryl hydrocarbon receptor nuclear translocator
β -NF	β -naphthoflavone
BIP	Binding immunoglobulin protein
BR	Bilirubin
BVR	Biliverdin reductase
CAR	Constitutive androstane receptor
CAR-RE	CAR-responsive element
CdCl ₂	Cadmium chloride
CHX	Cyclohexamide
CYP	Cytochrome P450
CO ₂	Carbon dioxide
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle's medium
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRP	Glucose-regulated protein
GST	Glutathione-S-transferase
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF-4 α	Hepatic nuclear factor-4-alpha
hnRNPA1	Nuclear ribonucleoprotein A1
HO	Heme oxygenase
HPLC	High-performance Liquid Chromatography
HRP	Horseradish peroxidase
iCa ²⁺	Intracellular calcium
ITS	Insulin-Transferrin-Selenium
Keap1	Kelch-like ECH associating protein 1
Mafs	Small musculo-aponeurotic fibrosarcoma proteins
M-MLV RT	Murine-Moloney Leukemia Virus reverse transcriptase
NF-I	Nuclear factor I
NNK	Tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone
Nrf2	Nuclear factor-E2 p45-related factor 2
PB	Phenobarbital
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGC-1 α	Peroxisome proliferator-activated receptor γ coactivator

POR	Porcine P450-oxidoreductase/P450 NADPH reductase
PXR	Pregnane X-receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Real-time reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
StRE	Stress response element
TCDD	2,3,7,8-tetracholorodibenzo- <i>p</i> -dioxin
UGT	Diphosphoglucuronate glucuronosyltransferase
UPR	Unfolded protein response
USF	Upstream stimulatory factor
XRE	Xenobiotic response element
XREM	Xenobiotic-responsive enhancer module

INTRODUCTION

Cytochrome P450s (CYPs) are a superfamily of oxidative heme-thiolate enzymes found in nearly all species. CYPs are essential enzymes involved in the metabolism of a wide range of endobiotics and xenobiotics. The substrates for CYPs are metabolized primarily through CYPs-catalyzed phase I reaction of drug metabolism, which involves dehydrogenation/hydrogenation, oxidation/reduction, hydrolysis, and monooxygenation (Porter and Coon, 1991; Meyer, 1996). These phase I reactions catalyzed by CYPs in general, convert the parent compounds into more hydrophilic metabolites to facilitate their excretion from the body (Testa and Kramer, 2007). However, biologically neutral parent substrates can also be bioactivated to form more toxic, mutagenic, or even carcinogenic products (Porter and Coon, 1991; Guengerich, 2001).

Since the first discovery, CYPs have been classified by their sequence homology, gene organization, and chromosomal location, and to date, more than 11,200 sequences have been identified in over 970 CYP families in animals, plants, and microorganisms (Nelson et al., 1996). These enzymes have been also shown to be involved in the metabolism of various drugs and endogenous compounds, and their expression is affected by pathological conditions, gender differences, and genetic polymorphisms.

The present study focuses on a specific CYP isozyme of the 2A class namely CYP2A5. CYP2A5 is a murine orthologue of the human nicotine-metabolizing enzyme, CYP2A6, which was first identified as coumarin 7-hydroxylase (Pelkonen et al., 2000). CYP2A5 is primarily located in the endoplasmic reticulum and mitochondrial membranes of the liver and olfactory mucosa of mice (Juvonen et al., 1988; Su et al., 1996; Piras et

al., 2003; Genter et al., 2006; Sangar et al., 2010). CYP2A5 is an interesting enzyme for its unique regulation and its involvement in liver injury caused by various well-known pathological conditions or hepatotoxins. It has been reported that CYP2A5 is upregulated following exposure to chemical hepatotoxins and during pathophysiological conditions in which the levels of most CYPs are either unchanged or down-regulated (Juvonen et al., 1985; Camus et al., 1996; Kojo et al., 1998; Kirby et al., 2011). The identified chemical inducers of CYP2A5 have varying structures and origin, and include various hepatotoxins, heavy metals, hormones, and porphyrinogenic compounds (Juvonen et al., 1985; Mangoura et al., 1989; Hahnemann et al., 1992; Viitala et al., 2001; Abu-Bakar et al., 2004). The increased CYP2A5 levels are also associated in various pathophysiological conditions such as hepatitis, hepatoma, malaria, fatty liver, and fasting (Kirby et al., 1994b; Chomarat et al., 1997; Sipowicz et al., 1997; Bauer et al., 2004; De-Oliveira et al., 2006, 2010; Fisher et al., 2009). Previous studies have shown that the regulation of CYP2A5 in these conditions involves many cellular pathways, but a commonality exists that CYP2A5 may play a role in protection against oxidative stress induced by these conditions. Furthermore, bilirubin and heme have been identified as the first endogenous substrates for CYP2A5 and CYP2A6 (Abu-Bakar et al., 2011, 2012; Lämsä et al., 2012).

Bilirubin (BR) is the end product of heme catabolism, and heme is found in hemoglobin or other hemoproteins such as cytochromes, catalase, and a small pool of free heme (Roy-Chowdhury et al., 2007). BR has dual physiological characteristics in that it has antioxidative properties at low concentrations ranging from 0.01 – 10 μ M (Stocker et al., 1987; Neuzil and Stocker, 1994; Dailly et al., 1998; Hatfield and Barclay, 2004), and cytotoxic properties in many cell types and intracellular organelles at higher

concentrations ($> 20 \mu\text{M}$) (Roy-Chowdhury et al., 2007). BR is generally metabolized by a single uridine diphosphoglucuronate glucuronosyltransferase isoform, UGT1A1 via the glucuronidation reaction of phase II drug metabolism (Bosma et al., 1994). Therefore, in conditions in which the function of UGT1A1 is compromised or BR levels are drastically increased due to jaundice, liver disease, and genetic disorders, the prolonged exposure and accumulation of BR can lead to serious cell damage or even death in various cell types (Dennery et al., 1995; Bhutani et al., 1999; McDonagh, 2010).

Cellular injury by BR starts at the cell membrane level, which is followed by the interaction with the intracellular membranes that surround various organelles, particularly mitochondria and the endoplasmic reticulum (ER). Previous studies have shown that BR exposure can induce alterations in the membrane content of cholesterol and lipids in erythrocytes (Brito et al., 2002). BR has also been shown to disrupt the membrane receptors, transporters and enzymes that play key roles in various functions such as endocytosis, glutamate uptake, and maintaining membrane potentials (Mayor et al., 1986; Silva et al., 2001; Brito et al., 2006, 2007). Upon the entrance of BR, its cytotoxic effects have been reported in intracellular organelles, particularly in mitochondria and the ER.

Many reports have shown BR disrupts the functions of mitochondria at the membrane level and also induce apoptosis via both intrinsic and extrinsic apoptotic pathway (Rodrigues et al., 2002; Seubert et al., 2002; Ostrow et al., 2004; Vaz et al., 2011; Brites and Brito, 2012). BR has been also reported to induce expression of regulatory genes and proteins for unfolded protein response (UPR), which is a key regulatory defense mechanism against ER stress (Calligaris et al., 2009; Oakes and Bend, 2010). Increased intracellular calcium (iCa^{2+}), reactive oxygen species (ROS), and

activities of pro-apoptotic protease caspase-3 has been also observed with BR treatment, suggesting that BR causes ER stress (Brito et al., 2006; Gao et al., 2011). Furthermore, many reports show that the cytotoxic effects of BR extend down to nucleus. Previous studies have demonstrated that BR induces DNA damage and disrupt cell growth via DNA oxidation and activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Ollinger et al., 2007; Nguyen et al., 2008; Deganuto et al., 2010).

Although the cytotoxic effects of BR involve numerous cell signaling pathways and molecules, the common mechanism that leads to apoptosis and/or necrosis of an subjected cell appears to be, in part, by production of ROS and reactive nitrogen species (RNS) (Brites and Brito, 2012). In addition, most studies have looked at nervous tissue for cytotoxic effects of BR for its implication in neonatal hyperbilirubinemia, but recent studies suggest liver is also significantly affected by BR (Seubert et al., 2002; Oakes and Bend, 2005, 2010).

Furthermore, Abu-Bakar et al. (2011) suggested an interesting theory that CYP2A5 plays a major role in BR clearance as an alternative mechanism to BR conjugation by UGT1A1 (Abu-Bakar et al., 2011). Abu-Bakar et al. (2011) demonstrated that BR treatment in mouse primary hepatocytes increased CYP2A5 protein and its activity levels, and that CYP2A5 protein is stabilized by BR (Abu-Bakar et al., 2011). Abu-Bakar et al. (2011) also established a recombinant yeast system overexpressing CYP2A5 and demonstrated CYP2A5 metabolizes BR into biliverdin as the main metabolite, suggesting that CYP2A5 is an inducible BR oxidase that it prevents the wasting of BR via glucuronidation and accumulation of BR in pro-oxidant levels (Abu-Bakar et al., 2011). Later, Abu-Bakar et al. (2012) further confirmed this notion in CYP2A6 by using the

same yeast expression system (Abu-Bakar et al., 2012). However, the role of CYP2A5 in BR metabolism needs to be confirmed *in vivo* model, and its regulation by BR also needs to be further investigated, as recent reports shows evidences that CYP2A5 may also be transcriptionally regulated by BR.

Cyp2a5 is regulated in a complex and unique manner. To study the gene regulation of *Cyp2a5*, a reporter construct containing the *Cyp2a5* 5' upstream promoter region from -3033 to +10 bp has been constructed (Ulvila et al., 2004). Since then, a number of transcription factors for *Cyp2a5* and their response elements in the *Cyp2a5* promoter region have been identified. The transcription factors identified to date, such as aryl hydrocarbon receptor (AhR), aryl hydrocarbon receptor nuclear translocator (ARNT), upstream stimulatory factors (USF-1 and USF-2), hepatic nuclear facor-4-alpha (HNF-4α), nuclear factor I (NF-I), constitutive androstane receptor (CAR), and nuclear factor-E2 p45-related factor 2 (Nrf2) have been shown to play an important role in cellular stress and the immune response, oxidative stress and detoxification of xenobiotics, cell cycle and proliferation, and lipid and glucose metabolism (Cai et al., 2002; Maglich et al., 2002; Abu-Bakar et al., 2004; Ulvila et al., 2004; Abu-bakar et al., 2007; Arpiainen et al., 2007, 2008; Kirby et al., 2011).

Nrf2 is the transcription factor that regulates the expression of numerous cytoprotective genes in response to oxidative stress and xenobiotic challenges. The target genes are specifically involved in glutathione synthesis, ROS clearance, xenobiotic metabolism, and drug transport (Itoh et al., 1999; Kobayashi et al., 2004). Nrf2 has been characterized to tightly regulate constitutive and inducible expression of *Cyp2a5* in response to oxidative stress caused by heavy metals or nitric oxide (Abu-Bakar et al.,

2004; Dhakshinamoorthy and Porter, 2004; Purdom-dickinson et al., 2007; Lämsä et al., 2010). Furthermore, recent work by Lämsä et al. showed that heme, the parent compound of BR, regulates *Cyp2a5* expression via Nrf2 activation (Lämsä et al., 2012). These findings suggest Nrf2 may be involved in BR induction of *Cyp2a5*.

Therefore, to confirm the role of CYP2A5 in BR metabolism and to further investigate its regulatory mechanism in response to BR, we hypothesized that CYP2A5 overexpression can protect hepatocytes against excessive BR and CYP2A5 is transcriptionally regulated by BR via activation of Nrf2. The first objective of this study was to examine the effects of excessive BR in CYP2A5 mRNA and protein expression, and investigate the regulatory mechanisms. The second objective of this study was to investigate if overexpression of CYP2A5 could protect cells against BR toxicity. Lastly, the third objective of this study was to confirm that cytoprotective role of CYP2A5 in BR toxicity is due to its direct clearance of BR. Our results indicate that BR upregulates CYP2A5 mRNA and protein expression and that this induction requires Nrf2 activation. Furthermore, overexpression of CYP2A5 directly degrades BR and partially blocks BR-induced apoptosis.

LITERATURE REVIEW

Cytochrome P450 enzyme

Cytochrome P450s (CYPs) enzymes are a superfamily of oxidative heme-thiolate enzymes found in nearly all species. These enzymes are responsible for metabolism of a wide range of drugs and xenobiotics, and are primarily involved in phase I reaction of drug metabolism, which includes dehydrogenation/hydrogenation, oxidation/reduction, hydrolysis, and monooxygenation (Meyer, 1996). The phase I reactions catalyzed by CYPs converts drugs or potentially harmful xenobiotics into more hydrophilic compounds, facilitating their excretion from the body (Testa and Kramer, 2007). However, biologically neutral parent substrates can be bioactivated to form more cytotoxic, mutagenic, or carcinogenic products (Porter and Coon, 1991; Guengerich, 2001). In mammals, CYPs have been found in the liver, brain, kidney, and many other tissues, and are primarily located in the endoplasmic reticulum, mitochondria, and plasma membrane compartments (Sangar et al., 2010).

Origin and Nomenclature

Evidence relating to the existence of CYPs was first reported in 1955 by Axelord and Brodie et al. where it was demonstrated that endoplasmic reticulum of the liver was able to oxidize xenobiotics (Axelord, 1955; Brodie et al., 1955). In 1958, Garfinkel and Klingenberg discovered a CO binding pigment in rat liver microsomes which had an absorption spectrum with a peak at 450 nm (Garfinkel, 1958; Klingenberg, 2003). This was soon demonstrated to be a hemoprotein of the b-type class by Omura and Sato in

1964, and proposed the name Cytochrome P450 after the strong feature of absorption spectrum of hemoprotein at 450 nm (Omura and Sato, 1964a, 1964b).

CYPs are classified by their sequence homology, gene organization, and chromosomal location. Generally, genes within the same family have 40% sequence identity and are denoted by a number (e.g. CYP2). The members of the same subfamily, denoted by a letter (e.g. CYP2A), have greater than 55% sequence identity, and are also clustered in the genome. Finally, different isoenzymes and various allele within the subfamily are denoted by numbers (e.g. CYP2A6*4) (Nelson et al., 1996). Up to date, more than 11,200 sequences have been identified in over 970 CYP families in animals, plants, and microorganisms (Cytochrome P450 homepage; drnelson.uthsc.edu/CytochromeP450.html, September 2012). CYP families 1-4 are typically classified as xenobiotic- and fatty acid-metabolizing CYPs, but the majority of them also have known endogenous substrates that can regulate their expression and activity (Guengerich 2008; Omiecinski, Rummel, and Hosagrahara 1999).

Cytochrome P450 2A5

Cytochrome P450 2A5 (CYP2A5) is a murine orthologue of the human nicotine-metabolizing enzyme CYP2A6 and is located primarily in the endoplasmic reticulum (ER) of the liver and olfactory mucosa of mice (Juvonen et al., 1985; Su et al., 1996; Piras et al., 2003; Sangar et al., 2010). CYP2A5 can be also found in mitochondrial membranes and the recent study suggests that mitochondrial CYP2A5 may play a role in the oxidative stress response (Honkakoski et al., 1988; Genter et al., 2006). CYP2A5, to date, has been extensively studied for its unique regulation. CYP2A5 is upregulated following

exposure to chemical hepatoxins and during pathophysiological conditions in which the levels of most CYPs are either unchanged or decreased (Juvonen et al., 1985; Camus et al., 1996; Kojo et al., 1998; Kirby et al., 2011). CYP2A5 was first identified as the catalyst for the 7-hydroxylation of coumarin and the deethylation of 7-ethoxycoumarin, and was later associated with the metabolism and activation of many other xenobiotics (Juvonen, Shkumatov, & Lang, 1988). Gender differences exist as well for CYP2A5 activity levels. CYP2A5 activities are significantly higher in female mice of DBA/2, 129/J, and CBA/Ca strains, but not in A/K, AKR, BALB/c, C3H/He, and C57BL/6 strains (Iersel et al., 1994).

Substrates and Inducers for CYP2A5

After CYP2A5 was first identified as the catalyst for coumarin and 7-ethoxycoumarin metabolism, a wide range of substrates have been identified. The substrates include nitrosamines, aflatoxin B1, the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), nicotine, 3-methylindole, methimazole, and acetaminophen (Juvonen et al., 1988; Camus et al., 1993; Kirby et al., 1994a, 1996; Pelkonen et al., 1994; Genter et al., 1998; Felicia et al., 2000; Siu et al., 2006; Xie et al., 2011; Zhou et al., 2012). A wide range of atypical CYP inducers of CYP2A5 has also been identified. Chemical inducers of CYP2A5 have varying structures, and include hepatotoxins, heavy metals, hormones, and porphyrinogenic compounds as shown in Table 1. Furthermore, recently Abu-Bakar et al. (2011, 2012) identified bilirubin (BR) as the first endogenous substrate for CYP2A5 and CYP2A6 using recombinant yeast system overexpressing CYP2A5 (Abu-Bakar et al., 2005, 2011, 2012).

Abu-Bakar et al. (2011) suggested CYP2A5 is potentially an inducible “bilirubin oxidase” where bilirubin post-translationally stabilizes CYP2A5 protein (Abu-Bakar et al., 2011).

Pathophysiological states associated with CYP2A5

Increased CYP2A5 levels are also associated with various pathophysiological states as shown in Table 1.2. The mechanism of the unique regulation of *Cyp2a5* during these conditions is unclear and remains to be investigated. The one demonstrated mechanism that is common in these conditions is, in part, oxidative stress (Kirby et al., 1994b; Chomarat et al., 1997; Nebert et al., 2000; Cheung et al., 2003; Gilmore et al., 2003; Gilmore and Kirby, 2004; Su and Ding, 2004; Weng et al., 2005; Chen and Siddiqui, 2007; Fisher et al., 2009; De-Oliveira et al., 2010). Furthermore, these conditions appear to be associated with increased serum bilirubin levels (Bonacini, 2004; Malaguarnera et al., 2005; Richardson et al., 2006b; Hansen, 2010).

Table 1.1 Chemical inducers of CYP2A5

Type	Name	Use	Reference
Hepatotoxin	Carbon tetrachloride	Fire extinguisher and cleaning agent	(Pellinen et al., 1993)
	Chloroform	Anesthetic/Teflon	(Camus et al., 1996)
	Cocaine	Topical anesthetic	(Pellinen et al., 1994a, 1994b)
	Ethanol	Fuel, beverage, and antiseptic	(Lu et al., 2010)
	Phenobarbital	Barbiturate and anticonvulsant	(Hahnemann et al., 1992; Salonpää et al., 1994)
	Pyrazole	Precursor for various medicines	(Juvonen et al., 1985; Nichols and Kirby, 2008)
Heavy metals	Cobalt	Metal in alloys	(Kocer et al., 1991; Hahnemann et al., 1992)
	Cerium	Additives to fuels	(Arvela et al., 1991)
	Cadmium	Batteries	(Abu-Bakar et al., 2004)
	Indium	Fusible alloy, solders, and electronics	(Mangoura et al., 1989)
	Lead	Building construction, fusible alloys, batteries	(Lämsä et al., 2010)
	Methylmercury	No use (environmental toxicant)	(Lämsä et al., 2010)
Porphyrinogenic compounds	Acifluorfen	Herbicide	(Salonpää et al., 1997)
	Aminotriazole	Herbicide and application in microbiology	(Salonpää et al., 1995)
	Griseofulvin	Antifungal drug	(Salonpää et al., 1995)
	Thioacetamide	No use (carcinogen)	(Salonpää et al., 1995)
	Heme	Prosthetic group for porphyrin	(Lämsä et al., 2012)
	Bilirubin	End product of heme catabolism	(Abu-Bakar et al., 2011)
cAMP modifier	Glucagon	Hormone from pancreas for increasing blood glucose	(Viitala et al., 2001)
	Forskolin	Activator of adenylyl cyclase	(Viitala et al., 2001)
	Isoproterenol	β -adrenergic agonist	(Viitala et al., 2001)
	Sp-cAMP	Specific activator of cAMP-dependent protein kinase	(Viitala et al., 2001)
Xenobiotics	1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)	Constitutive androstane receptor (CAR) ligand	(Cai et al., 2002)
	Buthionine sulfoximine	Drug used in chemotherapy to reduce levels of glutathione	(Gilmore et al., 2003)
	pregnenolone 16 α -carbonitrile (PCN)	Pregnane X receptor (PXR) agonist or antiglucocorticoid	(Cai et al., 2002)
	Rifampicin	Bactericidal antibiotics	(Donato et al., 2000)
	Trans-4,5-dihydroxy-1,2-dithiane (DTTox)	Intramolecular disulfide form of dithiothreitol; reducing agent	(Gilmore and Kirby, 2004)

Table 1.2. Pathophysiological states associated with increased CYP2A5 level

Pathophysiological states	Reference
Bacterial hepatitis by <i>Helicobacter hepaticus</i>	(Chomarat et al., 1997; Sipowicz et al., 1997)
Bacterial infection by <i>Citrobacter rodentium</i>	(Richardson et al., 2006a)
Fasting	(Bauer et al., 2004)
Hepatitis B antigen – Fulminant hepatitis	(Chemin et al., 1996)
Hepatitis B virus	(Kirby et al., 1994a)
Hepatoma	(Jounaïdi et al., 1994)
Malaria	(De-Oliveira et al., 2006, 2010)
Nonalcoholic Fatty Liver	(Fisher et al., 2009)
Trematode infection by <i>Ouke Fasciola hepatica</i>	(Montero et al., 1999)

Bilirubin

Bilirubin (BR) is the end product of heme catabolism, and heme is found in hemoglobin or other hemoproteins such as cytochromes, catalase, and a small pool of unbound free heme (Roy-Chowdhury et al., 2007). BR consists of a tetrapyrrole (Figure D1). In heme, however, the four pyrrole rings are connected into a larger ring, called a porphyrin ring (Bonnett, Davies, & Hursthouse, 1976; Grandchamp, Bissell, Licko, & Schmid, 1981; Figure D1). BR is formed through a series of oxidation-reduction reactions. The microsomal heme oxygenase (HO) enzymes catalyze the oxidation of heme, leading to opening of the tetrapyrrole ring and release of the iron molecule (Tenhunen et al., 1969). The resulting product, biliverdin is then subsequently reduced to BR by cytosolic biliverdin reductase (BVR) (Tenhunen et al., 1970).

Figure D1. Chemical structures of some nitrogen heterocycle substrates, inducers or inhibitors of CYP2A enzymes (Kirby et al., 2011).

Bilirubin metabolism

Erythrocytes are destroyed in the spleen when they get old or damaged; this releases hemoglobin, which is broken down into heme. The heme is then converted into unconjugated BR in the reticuloendothelial cells of the spleen (Tenhunen et al., 1969, 1970). The unconjugated BR has a low water solubility, which therefore requires binding to albumin in order to travel to the liver via bile duct (Roy-Chowdhury et al., 2007). Unconjugated BR is dissociated from albumin at the sinusoidal surface of the hepatocyte and is taken up by the hepatocytes by facilitated diffusion (Roy-Chowdhury et al., 2007).

A member of the organic anion transporter protein family, termed OATP2/8/C, has been suggested to mediate BR uptake, but it still remains questionable and requires confirmation (Briz et al., 2003; Wang et al., 2003b; Steeg et al., 2010; de Graaf et al., 2011). After entering the hepatocytes, BR binds to glutathione-S-transferases (GSTs), a major group of detoxification enzymes (Levi et al., 1969; Pickett, 1989; Strange et al., 2001). BR, however, is not a substrate for glutathione conjugation. The binding to GSTs reduces the efflux of BR from hepatocytes increasing its net uptake (Kamisaka et al., 1975; Wolkoff et al., 1978, 2012). BR is then converted into mono- and di-glucuronide predominantly by a single uridine diphosphoglucuronate glucuronosyltransferase (UGTs) isoform, UGT1A1 (Bosma et al., 1994). This converts water-insoluble unconjugated BR to be transformed into a more polar form that can be excreted in the bile and feces (Roy-Chowdhury et al., 2007).

Dual characteristics of bilirubin (BR)

BR is an interesting molecule for its cytoprotective potential at physiological levels and its cytotoxic characteristics at supraphysiological levels (Stocker et al., 1987; Roy-Chowdhury et al., 2007; Brites and Brito, 2012). Previous studies showed that BR has an antioxidant property that is equivalent to that of α -tocopherol in potency to inhibit lipid peroxidation. Antioxidative properties of BR have been reported at low concentrations ranging from 0.01 – 10 μ M (Stocker et al., 1987; Neuzil and Stocker, 1994; Dailly et al., 1998; Hatfield and Barclay, 2004). The antioxidative property of BR has been shown to be beneficial in protection against a various disease states including vascular, cardiovascular, and neural diseases (Doré et al., 1999; Ollinger et al., 2005;

Gazzin et al., 2012). Furthermore BR has been also implicated in hepatic protection against bile acid-induced apoptosis in rat hepatocytes, and hyperoxia in neonate Gunn rats (Dennery et al., 1995; Granato et al., 2003).

In contrast, BR at higher concentrations ($> 20 \mu\text{M}$) has been shown to be toxic to many cell type and intracellular organelles (Roy-Chowdhury et al., 2007). High BR concentration is observed in many pathobiological conditions, including biliary obstruction, neonatal jaundice, liver diseases, or some genetic disorders such as Gilbert's syndrome. In these conditions, plasma BR levels can be significantly increased ($> 300 \mu\text{M}$) above its homeostatic level ($\sim 20 \mu\text{M}$) (Dennery et al., 1995; Bhutani et al., 1999). In such circumstances, the prolonged exposure and accumulation of BR can lead to serious cell damage or even death (McDonagh, 2010). However, the molecular mechanisms underlying or contributing to BR toxicity in various cell types or tissues are not completely understood. Previous studies have reported that BR toxicity is mediated by disruption of several cellular functions rather than a single signaling pathway, suggesting BR toxicity involves complex mechanisms (Roy-Chowdhury et al., 2007; Brites and Brito, 2012).

Bilirubin toxicity

Cellular injury by BR starts at the cell membrane level, which is followed by interaction with the intracellular membranes that surround various organelles. Previous studies demonstrate that BR exposure to erythrocytes induce alterations in the membrane content of cholesterol and all the classes of phospholipids, which may facilitate hemolysis and erythrophagocytosis (Brito et al., 2002). In neurons, BR exposure impairs

endocytosis, glutamate uptake, and alterations in membrane potential (Mayor et al., 1986; Silva et al., 2001; Brito et al., 2006). Furthermore, disruption in efficiency of membrane receptors, transporters, and enzymes in neurons have also been reported as toxic effects of BR (Bruto et al., 2006, 2007). The mechanism of these toxic effects has been reported to be through production of reactive oxygen species (ROS) resulting in protein oxidation, and lipid peroxidation (Bruto et al., 2004, 2008a, 2008b).

Cytotoxic effects of BR have been also observed in intracellular organelles. Many reports suggest mitochondria are particularly vulnerable to toxic effects of BR. BR causes mitochondrial dysfunction with collapse of inner mitochondrial membrane potential, a diminished activity of cytochrome c oxidase, and the release of cytochrome c to the cytosol, which is an initiation of apoptosis via mitochondrial intrinsic pathway (Rodrigues et al., 2002; Ostrow et al., 2004; Brites and Brito, 2012). BR may also induce apoptosis via mitochondrial extrinsic pathway by activation of caspase-8, suggesting that BR interaction with a death receptors (e.g. Fas) may be involved (Seubert et al., 2002; Vaz et al., 2011). However, BR-induced apoptosis via extrinsic pathway remains to be confirmed as the induction may be cell type-specific (Keshavan et al., 2004). BR-induced mitochondrial apoptotic pathway is also the result of production of lipid peroxides, hydrogen peroxide, and hydroxyl radical, as well as a decrease in glutathione content and reduction of the mitochondrial membrane (Kumar et al., 2008; Brites and Brito, 2012). The increased production of ROS by BR has been observed in various cell types including HeLa, mouse embryonic fibroblasts, Hepa 1c1c7, astrocytes, and neurons (Seubert et al., 2002; Cesaratto et al., 2007; Brito et al., 2008a, 2008b).

Another mechanism of cell damage by bilirubin involves ER stress. The ER regulates the synthesis, folding and trafficking of newly formed secretory and membrane proteins, cellular responses to stress, and intracellular calcium (iCa^{2+}) (Ron and Walter, 2007). ER stress refers to any disruption that compromises protein folding and other functions of the ER. During ER stress, the unfolded protein response (UPR) is activated in the cell, which involves reducing the load of unfolded proteins through global attenuation of protein synthesis and translocation and by upregulating chaperones (Kaufman, 2002). BR has been reported to induce the expression of key regulatory genes and proteins required for the UPR (Calligaris et al., 2009; Oakes and Bend, 2010). Elevated iCa^{2+} along with ROS production has been observed in synaptosomes treated with BR (Brito et al., 2006). Increased iCa^{2+} disrupts intracellular homeostasis and may further produce more ROS and induce ER stress (Brites and Brito, 2012). A recent study also demonstrated that neurotoxic effects of BR in a hyperbilirubinemic mouse model that was associated with increased iCa^{2+} and activities of proapoptotic caspase-3 (Gao et al., 2011).

The cytotoxic effects of BR in various cell types as noted above also extend to the nucleus. BR-induced oxidative stress causes DNA damage and cell growth reduction in a human neuroblastoma cell line thereby impairing S-phase cell cycle progression and triggering apoptosis (Deganuto et al., 2010). It has also been reported that BR treatment induces cell cycle arrest by activation of extracellular signal-regulated kinase 1 and 2 (ERK 1/2) in the human colon cancer cell line (HRT-18), and the genes linked to the cell cycle are significantly repressed while genes linked to the inhibition of cellular kinases are induced in *Ugt*-null mice (Ollinger et al., 2007; Nguyen et al., 2008).

The cytotoxic effects of BR, as noted above, involve various cell signaling pathways and molecules. The consequences of high levels of BR can lead to apoptosis and/or necrosis of affected cell, and the production of ROS and reactive nitrogen species (RNS) appears to be the common mechanism involved, in part, for cell death (Brites and Brito, 2012). In addition, most studies have looked at nervous tissue for cytotoxic effects of BR, but recent studies suggest that the liver is also significantly affected by BR (Seubert et al., 2002; Oakes and Bend, 2005, 2010). Furthermore, Abu-bakar et al. (2011) demonstrated an alternative pathway for bilirubin clearance by CYP2A5, suggesting the need for further investigation of BR metabolism in liver (Abu-Bakar et al., 2011).

CYP2A5 in bilirubin metabolism

Evidence for the possible involvement of CYP2A5 in BR metabolism was first reported by Berry et al. (1972) that a fraction of BR is excreted as hydroxylated products in jaundiced Gunn rats, by the action of cytochrome P450 microsomes (Berryl et al., 1972; Kapitulnik and Ostrow, 1978). Later, Matteis et al. (1989) established an *in vitro* BR disappearance activity assay using the microsomal fraction of liver homogenates after treatment with powerful inducer of CYPs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in vivo* (Matteis et al., 1989). This approach was further utilized to identify specific isoforms of CYPs involved in BR degradation. Studies using TCDD and *Cyp1a2*-null mutant mice, suggested both *Cyp1a1* and *Cyp1a2* play a role in BR degradation (Matteis et al., 1991; Zaccaro et al., 2001).

However, *Cyp2a5* has been the most extensively studied CYPs to date and the direct involvement of CYP2A5 in BR degradation has been demonstrated by Abu-Bakar et al.

(Abu-Bakar et al., 2005). In their study, treatment of DBA/2J mice with Cadmium Chloride (CdCl_2) *in vivo* induced *Cyp2a5* and HO-1, an enzyme that catalyzes the rate-limiting step in heme catabolism, and this in turn, increased microsomal BR degradation activity (Abu-Bakar et al., 2005). They also showed that *Cyp1a2* was down-regulated by the CdCl_2 and microsomal COH activity was competitively inhibited by BR. This report provided indirect evidence that CYP2A5 may play a major role in BR clearance during the liver injury where BR levels are elevated following the induction of HO-1 (Abu-Bakar et al., 2005).

Recently, Abu-Baker et al. (2011) established a recombinant yeast system overexpressing CYP2A5, and identified oxidative metabolites of BR using a high-performance liquid chromatography/electrospray ionization mass spectrometry (Abu-Bakar et al., 2011). The report shows that recombinant yeast microsomes expressing CYP2A5 oxidize BR to biliverdin as the main metabolite, and to three other smaller products (Abu-Bakar et al., 2011). Aside from enzymatic degradation of BR, previous reports have showed BR degradation can also occur via interaction with reactive free radicals (Reed et al., 1985; Joshi et al., 1995). The BR oxidative metabolite profiles however, are different in chemical oxidation of BR. In chemical oxidation the smaller products are the main metabolites, suggesting the CYP2A5-mediated BR oxidation is selective, towards biliverdin production (Matteis et al., 2006; Abu-Bakar et al., 2011).

Abu-Bakar et al. (2011) further demonstrated that BR treatment in mouse primary hepatocytes increased the CYP2A5 protein and activity levels with no effects on mRNA expression. In addition, they also suggested CYP2A5 induction by BR was mediated by protein stabilization, using a protein synthesis inhibitor, cycloheximide (CHX)(Abu-

Bakar et al., 2011). Abu-Bakar et al. (2011) suggests CYP2A5 is an inducible BR oxidase, and its major role is to prevent wasting of BR via glucuronidation and accumulation of BR in pro-oxidant levels, by maintaining the cycle of BR conversion into biliverdin, and subsequent recovery of BR by biliverdin reductase (Abu-Bakar et al., 2011). Later, the role of CYP2A6 human orthologue of CYP2A5 in BR metabolism was confirmed in the recombinant yeast microsomes expressing CYP2A6, human orthologue of CYP2A5 (Abu-Bakar et al., 2012). However, the mechanism of CYP2A5 induction by BR remains to be further investigated.

Transcriptional regulation of *Cyp2a5*

CYP2A5 is regulated in a complex and unique manner. Ulvila et al. (2004) first cloned the *Cyp2a5* 5' upstream promoter region from -3033 to +10 bp into a luciferase (Luc) reporter construct and studied the promoter activity in mouse primary hepatocytes by deletion analysis (Ulvila et al., 2004). Since then, several transcription factors and cofactors have been identified for the constitutive and inducible expression of the *Cyp2a5* gene (Kirby et al., 2011). The functional response elements have been also identified in the *Cyp2a5* promoter region. To date, transcription factors that bind to these response elements include the aryl hydrocarbon receptor (Ahr), aryl hydrocarbon receptor nuclear translocator (ARNT), upstream stimulatory factors (USF-1 and USF-2), hepatic nuclear factor-4-alpha (HNF-4 α), nuclear factor I (NF-I), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor γ coactivator (PGC-1 α), and nuclear factor-E2 p45-related factor 2 (Nrf2) (Cai et al., 2002; Maglich et

al., 2002; Abu-Bakar et al., 2004; Ulvila et al., 2004; Abu-bakar et al., 2007; Arpiainen et al., 2007, 2008).

AhR is a ligand-activated transcription factor involved in the protection against oxidative stress and detoxification of xenobiotics. Previous studies show that Ahr directly regulates the Nrf2 antioxidant response pathway and also upregulates CYP1A1 and CYP1A2, which are xenobiotic detoxifying enzymes involved in oxidative stress response, cell cycle control, and apoptosis (Nebert et al., 2000; Miao et al., 2005). The AhR translocates from the cytoplasm into the nucleus upon its activation and forms a heterodimer complex with the AhR nuclear translocator (ARNT) (Hoffman et al., 1991). Arpiainen et al. (2005) identified a putative AhR response element (XRE) in the *Cyp2a5* promoter at the position -2514 to 2492, then also demonstrated that the AHR/ARNT complex binds to XRE when stimulated with AhR ligand, TCDD (Arpiainen et al., 2005). Later, Arpiainen et al. (2007) further demonstrated that ARNT can homodimerize and bind to the E-box which is located at position -2403 to -2398 in the *Cyp2a5* promoter (Arpiainen et al., 2007).

USF-1 and USF-2a are basic-Helix-Loop-Leucine Zipper transcription factors involved in response to stress, and the immune response, cell cycle and proliferation, and lipid and glucose metabolism (Corre and Galibert, 2005). Arpiainen et al. (2007) showed that USF-1 and USF-2a also bind to the E-box, but requires HNF-4 α to activate *Cyp2a5* promoter, while ARNT transactivation was independent of HNF-4 α (Arpiainen et al., 2007).

HNF-4 α is one of the liver-enriched transcription factors that plays an important role in liver-specific gene regulation (Schrem et al., 2002). HNF-4 α constitutively regulates

genes in a numerous important metabolic pathways involved in glucose, fatty acid, cholesterol metabolism, drug metabolism, and blood coagulation (Sladek, 1993; Chen et al., 1994; Stoffel and Duncan, 1997; Schrem et al., 2002; Onzalez, 2008). HNF-4 α was originally considered to be an orphan receptor, but recent studies showed that linoleic acid selectively binds to active site of HNF-4 α (Wisely et al., 2002; Yuan et al., 2009). However, the ligand occupancy appears to have no significant effect on HNF-4 α transcriptional activity and HNF-4 α is transcriptionally active even in the absence of its ligand (Yuan et al., 2009). Ulvila et al. (2004) demonstrated that HNF-4 α regulates constitutive expression of *Cyp2a5* and also identified its binding site in the *Cyp2a5* promoter at position -63 to -47 (Ulvila et al., 2004). However, the promoter region -63 to +10 produced very little transcriptional activity, while mutation of the promoter at positions -271 to -3033 strongly decreased transcriptional activity by HNF-4 α (Ulvila et al., 2004). In addition, PGC-1 α has been identified as a cofactor for HNF-4 α -mediated transcriptional activation of *Cyp2a5* in response to fasting, glucagon, and cyclic AMP (cAMP) (Arpiainen et al., 2008).

Transcription factor NF-I is involved in the initiation of transcription and DNA replication. NF-I has a conserved N-terminal region that mediates DNA binding and dimerization (Mermod et al., 1989). Ulvila et al. (2004) also identified a binding site for NF-I in the *Cyp2a5* promoter at -132 to -107, and demonstrated that NF-I is responsible for constitutive expression of *Cyp2a5* together with HNF-4 α (Ulvila et al., 2004).

CAR and PXR are nuclear receptors that regulate genes involved in important physiological pathways including xenobiotic, glucose, and fatty acid metabolism

(Maglich et al., 2002; Moreau et al., 2007; Konno et al., 2008). CAR and PXR have been also shown to modulate the immune response and the genes involved in lipid and cholesterol synthesis (Moreau et al., 2007; Roth et al., 2008). No definite physical ligand have been identified for both CAR and PXR, but previous reports show they exhibit the ability to bind multiple ligands (Tzamelis et al., 2000; Sueyoshi and Negishi, 2001; Willson and Kliewer, 2002; Maglich et al., 2003). Upon activation, CAR and PXR translocate to the nucleus and heterodimerizes with the retinoid X receptor (RXR), forming CAR/RXR or PXR/RXR complexes (Timsit and Negishi, 2007). A previous report shows that the CAR/PXR complex binds to the Phenobarbital-Responsive Enhancer Module (PBREM) and the distal CAR-responsive element (CAR-RE) (Honkakoski et al., 1998; Wang et al., 2003a). The CAR/RXR complex binding sites has been identified in the CYP2B6 promoter (Honkakoski et al., 1998; Wang et al., 2003a). The Xenobiotic-responsive enhancer module (XREM) has also been identified as the binding site for the PXR/RXR complex, in the CYP3A5 promoter (Goodwin et al., 1999). These binding sites have not been identified in CYP2A5 promoter, however the importance of CAR and PXR in the inducible and basal expression of *Cyp2a5* has been demonstrated, suggesting that the binding sites exist in CYP2A5 promoter (Cai et al., 2002; Maglich et al., 2002; Rosenfeld et al., 2003; Foretz et al., 2005).

Nrf2 is a basic-leucine zipper protein that regulates the expression of numerous cytoprotective genes in response to oxidative stress and xenobiotic challenges. Specifically, the target genes are involved in the glutathione synthesis, ROS clearance, xenobiotic metabolism, and drug transport (Okawa et al., 2006; Yates et al., 2009). Under physiological conditions, Nrf2 is bound to its repressor Kelch-like ECH associating

protein 1 (Keap1) in the cytosol, and is consistently degraded via ubiquitin-proteasome pathway in a Keap1-dependent manner (Itoh et al., 1999; Sekhar et al., 2002; McMahon et al., 2003; Cullinan et al., 2004; Kobayashi et al., 2004). Upon oxidative or electrophilic challenge, the Keap1 protein undergoes a conformational change that leads to disruption of the Keap1-Nrf2 binding (Kobayashi and Yamamoto, 2006). Nrf2 is released from the Keap1-Nrf2 complex and then translocates to the nucleus, then heterodimerizes with small musculo-aponeurotic fibrosarcoma proteins (Mafs) (Itoh et al., 1997). The resulting heterodimer has been shown to bind Maf recognition elements, the antioxidant response element, or the stress response element of various genes (Rushmore et al., 1991; Kataoka et al., 1994; Choi and Alam, 1996). The binding site for the Nrf2-Maf heterodimer in the *Cyp2a5* promoter was first identified by Abu-Bakar et al. (2007) at positions -2467 to -2269 (Abu-bakar et al., 2007).

As noted above, the transcriptional regulation of *Cyp2a5* does not involve a single pathway, rather involves numerous signaling pathways and transcriptional factors depending on the physiological states of the subject. However, the majority of the factors involved in *Cyp2a5* transcriptional activation appear to play significant role in protection against oxidative stress.

Post-transcriptional regulation of *Cyp2a5*

Although it appears that CYP2A5 regulation is most commonly transcriptional, previous studies show that *Cyp2a5* can be also regulated post-transcriptionally. The post-transcriptional regulation of *Cyp2a5* identified to date, include mRNA stabilization and protein stabilization to increase expression and activity. The evidence of *Cyp2a5* mRNA

stabilization was first reported by Aida and Negishi (Aida and Negishi, 1991; Hahnemann et al., 1992). Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), a multifunctional protein involved in transcriptional regulation and cell growth, has been identified as the RNA-binding protein responsible for the *Cyp2a5* mRNA stabilization (Tilloy-ellul et al., 1999; Raffalli-Mathieu et al., 2002). These studies have shown that hnRNP A1 functions as an mRNA stabilizing *trans*-acting factor on the 3' UTR, and also acts as a transcriptional activator by interacting with the proximal promoter (Tilloy-ellul et al., 1999; Raffalli-Mathieu et al., 2002; Glisovic et al., 2003). CYP2A5 protein stabilization has been also reported. The evidence for CYP2A5 protein stabilization was first reported by Juvonen et al. (1988) that coumarin, the substrate for CYP2A5 with high affinity, effectively protected CYP2A5 protein from degradation and increased its catalytic activity (Juvonen et al., 1988). Furthermore, very recently, Abu-Bakar et al. (2011) in their study of the role of CYP2A5 in BR metabolism, showed that BR stabilizes CYP2A5 via its substrate function (Abu-Bakar et al., 2011).

RATIONALE

Bilirubin (BR) is the end product of heme catabolism with cytoprotective potential at physiological levels and cytotoxic potential at supraphysiological levels (Roy-Chowdhury et al., 2007). The majority of BR is metabolized by UGT1A1 via glucuronidation but previous studies have suggested that BR is also metabolized by CYP2A5 as an alternative pathway (Bosma et al., 1994; Abu-Bakar et al., 2011). Abu-Bakar et al (2011) recently showed that CYP2A5 is an inducible oxidase that metabolizes BR into biliverdin as its major product, which can potentially prevent accumulation of BR in the cell (Abu-Bakar et al., 2011). However, the regulatory mechanism of CYP2A5 in BR metabolism still remains unclear and the role of CYP2A5 *in vivo* further remains to be determined.

Recently, Lämsä et al. (2012) showed that heme, the parent compound of BR, regulates *Cyp2a5* expression via Nrf2 activation (Lämsä et al., 2012). Nrf2 is one of the transcription factors identified for *Cyp2a5*, and is involved in regulation of expression of numerous cytoprotective genes in response to oxidative stress and xenobiotic challenges (Itoh et al., 1999; Kobayashi et al., 2004). The cytotoxic effects of BR involve various cell signaling pathways, but it has been suggested that the common mechanism of BR-induced apoptosis or necrosis is in part through production of ROS and RNS (Brites and Brito, 2012). Hence, these findings lead to hypothesis that the regulation of CYP2A5 by BR is mediated by Nrf2 activation and that CYP2A5 prevents the toxicity of BR by direct clearance of BR.

Objective 1. Determine the effect of BR on CYP2A5 gene expression and the associated regulatory mechanisms.

Approach: Mouse hepatocytes in primary culture were treated with BR and CYP2A5 expression analyzed by real-time RT PCR and western blot. Luciferase reporter assays were used to identify the response elements and transcription factors recruited by BR.

Objective 2. Investigate the cytoprotective role of CYP2A5 in BR toxicity.

Approach: The effect of transient CYP2A5 overexpression on BR-induced apoptosis was assessed in Hepa 1-6 cells transfected with a V5-His-CYP2A5 expression plasmid or the empty vector pcDNA3.1.

Objective 3. Determine if the cytoprotective effect of CYP2A5 on BR-induced cytotoxicity is due to increased bilirubin metabolism.

Approach: BR degradation was assessed *in vitro* using microsomes from Hepa 1-6 cells transiently overexpressing V5-His-CYP2A5 expression plasmid or the empty vector pcDNA3.1.

MATERIALS AND METHODS

PART 1. Animal model and cell culture

Mice

Male C57BL/6 mice were purchased from Charles River Canada (St. Constant, QC). All the mice were given food and water ad libitum, and were housed at 23.8°C with a 12 h photoperiod. All mouse perfusions were conducted in accordance with the guidelines of Canadian Council on Animal Care and the Animal Care Committee of the University of Guelph.

Mouse liver perfusion

Mice were anesthetized by an intraperitoneal injection of 0.1 mL pentobarbital (54.7 mg/mL, Somnotol, MTC Pharmaceuticals). Once sufficiently anesthetized (3-8 minutes), mice were secured in dorsal recumbency. The abdominal and thoracic areas of the mice were wiped with 70% ethanol. A medial incision was made from the pubis to the sternum using a blunt ended mayo scissors. The intestines were reflected dorsally to the left, and the hepatic portal vein was exposed. The rib cage was then removed and the inferior vena cava was located. A single loose ligature (3-0 silk) was placed around the inferior vena cava, as close to the right atrium as possible.

A 23-gauge catheter was made from polyethylene tubing, beveled at one end and attached to Tygon tubing (Fisher Scientific, Ottawa, ON) that ran through a peristaltic pump from the solutions in a 40°C water bath. The blanching solution (Appendix I), consisting of 1X Hanks' balanced salt solution (HBSS, without Ca^{2+} , Mg^{2+} , HCO_3^- and

phenol red; Invitrogen, Burlington, ON), 1 mM EGTA (Sigma-Aldrich, Oakville, ON), and 10 mM HEPES (Sigma-Aldrich, Oakville, ON), was pre-run at a flow rate of 2.0 mL/min to exclude air bubbles from the tubing. The inferior vena cava was catheterized via the right atrium, by grasping the left ventricle with small rat tooth forceps and making a small incision into the atrium with iris scissors. The suture was then placed around the inferior vena cava to secure the catheter. The hepatic portal vein was cut once the catheter was in place to allow the solutions to exit after perfusing through liver. The blanching solution was pumped retrograde to blood flow through the liver for 2.5 minutes.

Once the liver was successfully perfused with the blanching solution, as indicated by clearance of blood in liver, the perfusate was changed to 50 mL of collagenase medium (Appendix I), consisting of 100 U/mL mouse collagenase type I (Sigma-Aldrich, Oakville, ON), 10 mM HEPES (Sigma-Aldrich, Oakville, ON), and 1.325 μ L/mL 7.5% bovine serum albumin (Sigma-Aldrich, Oakville, ON) in Williams' Medium E, pH 7.4 (Sigma-Aldrich, Oakville, ON). The collagenase perfusion medium was run at a flow rate of 4.0 mL/min for approximately 8-10 minutes, which allowed hepatocytes to be fully dissociated.

Preparation of primary cultures of mouse hepatocytes

The liver was removed from the mouse, the gallbladder was excised, and the liver was placed into a sterile 100 mm Petri dish (Corning, NY). The liver was rinsed with cold attachment medium (Appendix I), which contained 10 mM HEPES pH 7.4 (Sigma-Aldrich, Oakville, ON), ITS (Roche Diagnostics, Laval, QC; 5 μ g/mL insulin, 5 μ g/mL transferrin, and 5 ng/mL sodium selenite), 7.5 μ g/mL geneticin (Invitrogen, Burlington,

ON), 10% fetal bovine serum (FBS; Sigma-Aldrich, Oakville, ON), 50 nM dexamethasone (Sigma-Aldrich, Oakville, ON) in Williams' Medium E, pH 7.4 (Sigma-Aldrich, Oakville, ON). The liver capsule was then gently scored with scissors, and the cells were pipetted through sterile gauze into a 50 mL conical tube (Corning, NY). The cell suspension was centrifuged at 50 g for 2 min. The pellet of hepatocytes was re-suspended in 25 mL of fresh attachment media.

Isolated mouse primary hepatocytes were plated in 6-well plates (Corning, NY) at a density of 1.0×10^6 cells/well or 24-well plates (Corning, NY) at a density of 0.25×10^5 cells/well in attachment media. After 5 hours of incubation, the attachment media was removed and replaced with fresh serum-free media (Appendix I), which contained 10 mM HEPES pH 7.4 (Sigma-Aldrich, Oakville, ON), ITS (Roche Diagnostics, Laval, QC; 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite), 10 µg/mL gentamicin (Invitrogen, Burlington, ON) in Williams' Medium E, pH 7.4 (Sigma-Aldrich, Oakville, ON) and the cells were incubated in a humidified incubator with 5% CO₂ at 37°C for 19 hours.

Hepa 1-6 cell line

The Hepa 1-6 cell line was obtained from ATCC (Manassas, VA). Hepa 1-6 is a mouse hepatoma cell line derived from the BW7756 tumour that arose in a C57/L mouse. The cells were cultured and maintained with Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich, Oakville, ON) supplemented with 1% (v/v) Penicillin/Streptomycin (Invitrogen, Burlington, ON) and 10% (v/v) FBS (Sigma-Aldrich, Oakville, ON) in 75 cm² canted neck flasks with vented caps (Corning, NY), in

a humidified incubator (5% CO₂, 37°C). The cells were counted using a hemocytometer and plated at variable cell densities according to the needs of the experiments.

Bilirubin solubilization

Bilirubin powder was purchased from Sigma-Aldrich (Oakville, ON). Bilirubin was first dissolved using fixed amount in 0.1 N NaOH (10% of final volume) and followed by addition of an appropriate volume of PBS, pH 7.4. All experiments involving bilirubin treatment were protected from exposure to light.

PART 2. Regulation of CYP2A5 by BR

Treatment

Cells in 6-well plates were treated with bilirubin (0, 1, 10, 25, 50 µM) or its vehicle (1% v/v 0.1 N NaOH PBS, pH 7.4) in serum-free media for 24 hours.

RNA isolation and reverse transcription

After 24-hour of treatment, the media was removed from the culture plate and the cells were washed with PBS. One mL of TRIzol reagent (Invitrogen, Burlington, ON) was then added directly to the hepatocytes and the TRIzol-cell homogenate was scrape-transferred to 1.5 mL RNase-free Eppendorf tubes. Samples were incubated in room temperature for 5 minutes and 200 µL of chloroform (Fisher Scientific, Ottawa, ON) was added. The samples were vortexed gently for 15 seconds and then incubated for 2 minutes at room temperature (RT). The samples were centrifuged at 12,000 g for 15 minutes at 4°C and the resulting supernatant (approximately 500 µL) was transferred to

new 1.5 mL Eppendorf tubes. To each tube, an equal volume (500 μ L) of cold isopropanol (Fisher Scientific, Ottawa, ON) was added to precipitate RNA and mixed by inverting several times. The samples were then incubated at RT for 10 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. The RNA pellets were washed with 1 mL 75% ethanol. The samples were centrifuged at 12,000 g for another 10 minutes and the remaining ethanol was removed. The RNA pellets were then air dried at RT until the pellets turned from opaque to clear, and the pellets were finally dissolved in 12 μ L of nuclease-free water, and incubated for 10 minutes at 55°C. The RNA concentration and purity of each sample was determined with a NanoDrop ND-1000 spectrophotometer (Thermoscientific, Ottawa, ON).

Once the mRNA was isolated, 1 μ g of RNA was treated with 1 unit of DNase (RQ1 RNase-Free DNase; Promega, Madison, WI) at 37°C for 30 min, and was reverse transcribed using 200 units of Murine-Moloney Leukemia Virus reverse transcriptase (M-MLV RT; Promega, Madison, WI), 20 units of RNase inhibitor (RNasin; Promega, Madison, WI), and 0.1 μ g of (Invitrogen, Burlington, ON) primers for 1 hour at 37°C.

Real-time RT-PCR

CYP2A5 mRNA analysis was conducted by relative real-time reverse transcription polymerase chain reaction (RT-PCR) using a Roche Molecular Biochemicals Lightcycler (Indianapolis, IN) and the FastStart SYBR Green Master Mix (Roche Diagnostics, Mississauga, ON). PCR was performed in a 10 μ L volume containing a 2 μ L sample of cDNA, 1 μ L of SYBR Green Master Mix, 2 mM MgCl₂, and 5 μ M of each primer. The PCR parameters were as follows: denaturation (95°C for 10

min), 45 cycles of PCR (95°C, 15 sec; 70, 5 sec; 72°C, 15 sec). The threshold cycle at which the fluorescent signal reached an arbitrarily set threshold near the middle of the log-linear phase of amplification for each reaction was calculated and relative quantities of mRNA for the *Cyp2a5* gene were analyzed. The mRNA levels were normalized against mRNA levels of the housekeeping gene GAPDH. The gene specific primers for *Cyp2a5* and GAPDH were as follows:

Cyp2a5 (1,746 bp)

Forward 5'-GGACAAAGAGTTCCTGTCCTGCTTC-3' (606– 631)

Reverse 5'-GTGTTCCACTTTCTTGGTTATGAAGTCC-3' (759– 786)

GAPDH (1,002 bp)

Forward 5'-ACAGTCCATGCCATCACTGCC-3' (581–601)

Reverse 5'-GCCTGCTTCACCACCTTCTTG-3' (826–846)

Western Blotting

After 24 hours of treatment, the medium was removed from the culture plate and the cells were washed with PBS. One hundred µL of cell lysis buffer (Appendix I) was directly added to hepatocytes and incubated for 5 minutes at RT. The cell homogenate was then scraped, transferred to 1.5 mL Eppendorf tubes and centrifuged at 10,000 g for 10 minutes at 4°C. The resulting supernatant, total protein concentration was measured by the Bradford method using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Mississauga, ON).

Hepatic CYP2A5 protein expression was analyzed by western blot analysis as described previously (Gilmore and Kirby, 2004). Briefly, sample proteins (25 µg protein per lane) were separated by SDS-PAGE on a 10% gel and transferred to Amersham Hybond ECL membrane (GE Healthcare, Oakville, ON). Membranes were blocked in blocking buffer (5% v/v powdered skim milk in Tris-buffered saline with 0.5% Tween-20, TBS) for 1 hour at RT. Membranes were then incubated with chicken anti-mouse Cyp2a5 (1:10,000; 2 h; Dr.Juvonen, Kuopio, Finland) or mouse anti-β-actin (1:5000; 30 min; Sigma-Aldrich, Oakville, ON) polyclonal antibodies in blocking buffer at RT, followed by HRP-conjugated goat anti-chicken IgG (1:10,000; 1 h; Sigma-Aldrich, Oakville, ON) or HRP-conjugated goat anti-mouse IgG (1:2000; 30 min; Sigma-Aldrich, Oakville, ON) in blocking buffer at RT. Protein bands were detected with ECL plus (Amersham Biosciences, Baie d'Urfe, QC) and the molecular mass was confirmed with precision plus molecular weight markers (Bio-Rad Laboratories, Mississauga, ON). The bands were scanned with a Typhoon 9410 scanner (GE Health Sciences, Piscataway, NJ) and the band intensity was quantified with ImageQuant TL (GE Health Sciences, Piscataway, NJ). The band intensity was normalized to β-actin and shown as fold-change.

Luciferase reporter constructs

A number of various expression vectors and reporter constructs were used in transient transfection assay. A reporter construct consisting of the luciferase gene under the control of a 3033 bp fragment of the *Cyp2a5* promoter region (*Cyp2a5* 5'-3033/+10) was provided by Dr. Jukka Hakkola (University of Oulu, Finland). Various deletion constructs of the *Cyp2a5* promoter (-2603/+10, -2263/+10, -746/+10, -432/+10) were

provided by Andrea Leclair (Leclair, 2004). The *Cyp2a5* 5'-3033/+10 promoter construct with site-directed mutation of the Nrf2 response element (*Cyp2a5* 5'-3033/+10-ΔNrf2) was provided by Kathleen Nichols (Nichols, 2009).

The promoter constructs were cotransfected either with the empty expression plasmid pcDNA3.1 or with the mouse Nrf2 expression plasmid (pcDNA3.1-mNrf2). The Nrf2 expression plasmid was provided by Dr. Ah-Ng (Tony) Kong (University of New Jersey, USA).

Transfection of primary mouse hepatocytes

Cyp2a5 5'-3033/+10 promoter construct, 5'-truncated fragments, and Nrf2 response element mutant were used in transient transfection assays. The promoter constructs were co-transfected either with pcDNA3.1 or with the mouse pcDNA3.1-mNrf2. The primary mouse hepatocytes cultured in 24-well plate were transiently transfected using Lipofectamine 2000 (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Cells in each well were transfected with 0.5 µg of *Cyp2a5* 5' reporter construct, 0.3 µg of pcDNA3.1 or pcDNA3.1-mNrf2, and 0.2 µg of *Renilla* (pRL3-TK). The transfected cells were cultured for 24 hours and treated with 50 µM BR or its vehicle for another 24 hours.

Dual Luciferase Activity Assay

The luciferase activities were assessed on a FLUOstar OPTIMA using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, the media from the cultured cells were

removed and the cells were washed with PBS. The cells were passively lysed with Passive Lysis Buffer for 15 min on shaker. The cell lysate were collected to Eppendorf microcentrifuge tubes and 20 μ L of each sample was transferred to 96-well plate (flat bottom, white polystyrene). The firefly luciferase activity was first measured after 100 μ L of Luciferase Assay Substrate injection and then Renilla luciferase activity was subsequently measured after the injection of 100 μ L Stop & Glo Reagent. The resulting values are normalized against luciferase activity in cells transfected with the empty vector, pRL3-TK.

PART 3. Cytoprotective role of CYP2A5 in BR toxicity

Transfection of Hepa 1-6 with CYP2A5 overexpression vectors

CYP2A5 overexpression in Hepa 1-6 was achieved via transfection with pcDNA3.1/V5-His-TOPO into which *Cyp2a5* cDNA had been cloned (pCYP2A5-V5), a kind gift from Dr. Masahiko Negishi (NIEHS, USA). The cells were also co-transfected with an expression vector, pcDNA3.1/V5-His-TOPO, containing cDNA for porcine cytochrome P450 oxidoreductase (pPOR-V5) provided by Dr. Jim Squires (University of Guelph, ON) to supply electrons for the overexpressed CYP2A5 protein. Hepa 1-6 cells were cultured in 6-well plates (Corning, NY) at a density of 0.4×10^6 cells/well in DMEM supplemented with 1% (v/v) Penicillin/Streptomycin (Invitrogen, Burlington, ON) and 10% (v/v) FBS (Sigma-Aldrich, Oakville, ON). The cells were incubated in a 5% CO₂, humidified incubator at 37°C for 24 hours to achieve 70-80% confluency. At confluency, the cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Cells in each well were

transfected with 2.0 µg of pcDNA3.1 or CYP2A5-V5 plasmid. The cells were also cotransfected with 0.4 µg pcDNA3.1 or POR. The transfected cells were cultured for 24 hours and further treated with BR (0, 1, 10, 25, 50 µM) or the vehicle (1% v/v 0.1 NaOH PBS, pH 7.4 in serum-free media for 24 hours.

CYP2A5 activity assay: 7-ethoxycoumarin deethylation assay

To confirm the activity of overexpressing CYP2A5 protein, the metabolism of coumarin was measured in CYP2A5 and POR overexpressing Hepa 1-6 cells. At 24 hours after transfection, 7-ethoxycoumarin (Sigma-Aldrich, Oakville, ON) was added in fresh media to the 6-well plate. After incubation for 2 hours, the media was collected and the cells were harvested. For media samples, trichloroacetic acid (20% v/v) was added to precipitate out protein and was centrifuged at 15,000 g for 15 minutes at 4°C. The resulting supernatant was injected to a HPLC on a Luna 5 µ 250 mm x 4.6 mm reverse phase C-18 column (Phenomenex, Torrance, CA) using a mobile phase of 5% acetonitrile, 95% water, 0.2% acetic acid at a flow rate of 1 mL/min. The equipment consisted of a Spectra Physics HPLC system (Spectra-Physics, San Jose, CA) equipped with a Spectra 100 UV–Vis Detector set at 320 nm. 7-hydroxycoumarin was used as internal control. The retention time for coumarin, 7-hydroxycoumarin, and 7-ethoxycoumarin were 11 min, 7.7 min, and 16 min respectively. The concentration of metabolites were first normalized to the internal control, and then normalized to total protein concentration from the cells harvested previously.

Western Blotting

To determine BR-induced apoptosis in Hepa 1-6 cells, caspase-3 cleavage was demonstrated using western blot analysis. Briefly, sample proteins (40 µg protein per lane) were separated by SDS-PAGE on a 10% gel and transferred to Amersham Hybond ECL membrane (GE Healthcare, Oakville, ON). Membranes were then incubated with rabbit anti-mouse caspase-3 (1:1000; overnight; Cell signaling) or mouse anti-β-actin (1:5000; 30 min; Sigma-Aldrich, Oakville, ON) polyclonal antibodies in blocking buffer at RT, followed by HRP-conjugated goat anti-rabbit IgG (1:2000; 1 h; Sigma-Aldrich, Oakville, ON) or HRP-conjugated goat anti-mouse IgG (1:2000; 30 min; Sigma-Aldrich, Oakville, ON) in blocking buffer at RT. Also to confirm the protein expression of transfected pcDNA3.1/V5-His-TOPO expression plasmids, the membranes were also incubated with mouse anti-V5 (1:5000; Invitrogen, Burlington, ON) for 1 hour at RT, followed by HRP-conjugated goat anti-mouse IgG (1:2000; Sigma-Aldrich, Oakville, ON) for 1 hour at RT.

Protein bands were detected with ECL plus (Amersham Biosciences, Baie d'Urfé, QC) and the molecular mass was confirmed with precision plus molecular weight markers (Bio-Rad Laboratories, Mississauga, ON). The bands were scanned with a Typhoon 9410 scanner (GE Health Sciences, Piscataway, NJ) and the band intensity was quantified with ImageQuant TL (GE Health Sciences, Piscataway, NJ). The band intensity was normalized to β-actin and shown as fold-change.

PART 4. Bilirubin disappearance activity

Transfection of Hepa 1-6 with CYP2A5 overexpression vectors

Hepa 1-6 cells were cultured in 100 mm dish (11 dish per each plasmid; Corning, NY) at a density of 3.0×10^6 cells/well in DMEM supplemented with 1% (v/v) Penicillin/Streptomycin (Invitrogen, Burlington, ON) and 10% (v/v) FBS (Sigma-Aldrich, Burlington, ON). The cells were incubated in a 5% CO₂, humidified incubator at 37°C for 24 hours to achieve 70-80% confluency. Once confluency was reached, cells were transiently transfected with 13.0 µg pcDNA3.1 or pCYP2A5-V5 plasmids using Lipofectamine 2000 (Invitrogen, Burlington, ON) according to the manufacturer's protocol. Cells were also cotransfected with 1.2 µg pcDNA3.1 or pPOR-V5. The transfected cells were cultured for 24 hours.

Microsomal protein extraction

After 24 hours transfection of cells, the media was removed from the culture plate and the cells were washed twice with ice-cold PBS. Cells were scraped and pulled into one sterile 15 mL conical tube (Fisher Scientific, Ottawa, ON). The cell suspension was centrifuged at 200 g for 2 minutes and supernatant was aspirated. The weight of pellet was then measured and the pellet was resuspended into 3x (w/v) homogenization buffer (Appendix I; 20% sucrose, 100 mM Tris-HCl, pH 7.4, 4°C). The suspended cells were transferred into 5 mL glass homogenizer coated with teflon, and homogenized with 50 strokes. The homogenate was centrifuged at 10,000 g for 30 minutes at 4°C and the supernatant was transferred to a 3.0 mL polycarbonate tube, and recentrifuged at 105,000 g for 1 hour at 4°C. The supernatant was aspirated and the pellet was resuspended in 1x

(v/v) microsomal storage buffer (Appendix I; 20% glycerol, 100 mM Tris-HCl, pH 7.4, 4°C). Protein concentration was measured by the Bradford method using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Mississauga, ON). Freshly made microsomal protein was used within 2 hours for the BR disappearance assay.

Bilirubin disappearance assay

The bilirubin disappearance assay was determined as previously described (Abu-Bakar et al., 2005) with a slight modification. Briefly, incubation buffer (0.1 M Tris-HCl pH 8.2, 26 mM KCl, 2mM EDTA) was mixed with CYP2A5-overexpressing microsomes (1 mg/mL final protein concentration) in a total volume of 200 μ L was placed in 96-well plate (Corning, NY) in triplicate. After 5 min pre-incubation, NADPH (2.0 mM) was added to both sample and control wells, and blank absorbance was taken at 450 nm. Immediately, BR in DMSO (10 μ M final concentration) was added to the sample plate and absorbance at 450 nm was recorded for 3 cycles at 10 min/cycle. The rate of BR disappearance is expressed as pmol BR disappearing/hr/mg protein, using a $\epsilon = 35.127 \text{ cm}^{-1}\text{mM}^{-1}$, which was obtained experimentally under the conditions of the assay. Additional wells containing the above mixture without NADPH were also run to control for the spontaneous disappearance of BR.

PART 5. Statistical analysis

All data are shown as mean $\pm$ SEM. One-way repeated measures analysis of variance (ANOVA), followed by Dunnett's *post-hoc* test was used to determine significance between experimental groups with one independent variable (IV). Two-way

repeated measures ANOVA, followed by Bonferroni's *post-hoc* test was used to determine significance between experimental groups with two or more IVs (Prism 5.0, GraphPad Software Inc., La Jolla, CA, USA). A probability level of $P < 0.05$ was considered significant.

RESULTS

BR induces CYP2A5 mRNA and protein expression in mouse primary hepatocytes

To investigate whether BR regulates CYP2A5 at both the transcriptional and translational level, isolated primary hepatocytes from C57BL/6 mice were treated with vehicle (1% v/v 0.1 NaOH PBS, pH 7.4) or BR (0, 1, 10, 25, 50 μ M) in serum-free media for 24 hours. CYP2A5 mRNA levels were determined by RT-PCR and were normalized with GAPDH mRNA expression. CYP2A5 mRNA levels did not change at low-dose BR (1, 10 μ M) treatment, but was significantly increased (2- and 3-fold induction; $P < 0.0001$) at higher-dose BR (25, 50 μ M) treatment (Figure 1). CYP2A5 protein expression was determined by Western blot analysis using chicken anti-mouse CYP2A5 or anti- β -actin antibody. Western blotting for CYP2A5 in mouse primary hepatocytes showed similar results with protein levels corresponding to the changes in mRNA transcript levels. CYP2A5 protein expression did not change at low-dose BR (1, 10 μ M) treatment, but was significantly increased (1.7- and 2-fold induction; $P < 0.05$ and $P < 0.001$) at higher-doses of BR (25, 50 μ M) (Figure 2).

Figure 1. Effect of BR on CYP2A5 mRNA expression. Mouse primary hepatocytes were treated with 1, 10, 25 or 50 μ M BR for 24 h. CYP2A5 mRNA levels were determined by RT-PCR and normalized to GAPDH mRNA expression. All values represent the mean $\pm$ SEM (n = 6 independent mice) normalized against the control levels. Mean difference is significant from control group at $*P < 0.0001$ (one-way repeated measures ANOVA followed by Dunnett's post-test).

Figure 2. Effect of BR on CYP2A5 protein expression. Mouse primary hepatocytes were treated with 1, 10, 25 or 50 μM BR for 24 h. (A) Western blot analysis of total protein (25 μg total protein/lane) from control and BR-treated mouse primary hepatocytes probed with chicken anti-mouse Cyp2a5 or mouse anti- β -actin antibody. β -actin protein levels are shown as control for protein loading. Each blot represents one of replicates of three samples (each blot showed the same pattern of induction). (B) Densitometric quantification of Western blots. All values represent the mean $\pm$ SEM ($n = 3$ independent mice) normalized against the control levels. Mean difference is significant from control group at $*P < 0.05$ and $**P < 0.001$ (one-way repeated measures ANOVA followed by Dunnett's post-test).

BR induction of CYP2A5 mRNA may not be directly regulated in hepatocytes

Since we hypothesized CYP2A5 is regulated by BR at the transcriptional level, to investigate the mechanism of CYP2A5 induction by BR, luciferase reporter assays were performed using a *Cyp2a5* reporter construct (*Cyp2a5*-3033). The *Cyp2a5*-3033 reporter construct consists of the luciferase gene under the control of a 3033 bp fragment of the *Cyp2a5* 5' untranslated region (*Cyp2a5* 5'-3033/+10). The *Cyp2a5*-3033 and *Renilla* (pRL3-TK) control vector was transfected into primary mouse hepatocytes for 24 hours. The cells were then treated with vehicle or 50 μ M BR for another 24 hours, and reporter activity was measured. BR treatment did not alter luciferase activity (Figure 3).

Figure 3. Effect of 50 μ M BR on *Cyp2a5*-5'-luciferase activities in C57BL/6 mouse primary hepatocytes. Luciferase activities were measured 24 h after transfection. The measured activities were normalized against *Renilla* (pRL-TK) activities. All values represent the mean $\pm$ SEM (n = 5 independent mice).

CYP2A5 induction by BR is mediated by Nrf2

To investigate if CYP2A5 expression is indirectly regulated by BR in hepatocytes, we introduced mouse Nrf2, the transcription factor for CYP2A5 expression, into our reporter assay system. To confirm the involvement of Nrf2 in the regulation of CYP2A5 expression, the *Cyp2a5*-3033 was cotransfected either with pcDNA3.1 or with pcDNA3.1-mNrf2 for 24 hours. Also to investigate if Nrf2 is involved in regulation of BR-induced CYP2A5 expression, *Cyp2a5*-3033 was cotransfected either with pcDNA3.1 or with pcDNA3.1-mNrf2 for 24 hours, and treated with vehicle or 50 μ M BR for another 24 hours. Nrf2 overexpression in the hepatocytes increased the reporter activity in a dose-dependent manner, and overexpression of 0.3 μ g pcDNA3.1-mNrf2 significantly increased the reporter activity approximately by 1.9-fold ($P < 0.05$) relative to control (Figure 4). Furthermore, BR treatment within the Nrf2 overexpressing group had a profound effect on *Cyp2a5* reporter activity. In the Nrf2 overexpressing group, 50 μ M BR treatment significantly increased the reporter activity by 2-fold ($P < 0.001$) relative to the vehicle-treated group (Figure 5).

Figure 4. Nrf2 increases CYP2A5 transcription in primary mouse hepatocytes. Primary mouse hepatocytes were transfected with Nrf2 (0.1 or 0.3 µg) and luciferase activities were measured 24 h after transfection. The measured activities were normalized against *Renilla* (pRL-TK) activities. All values represent the mean $\pm$ SEM (n = 3 independent mice). Mean difference is significant from control group at $*P < 0.05$ (one-way repeated measures ANOVA followed by Dunnett's post-test).

Figure 5. BR increases CYP2A5 transcription in primary mouse hepatocytes in the presence of Nrf2. Primary mouse hepatocytes were transfected with Nrf2 (0.3 μg) and treated with BR (50 μM) for 24 h. Luciferase activities were normalized against *Renilla* (pRL-TK) activities. All values represent the mean $\pm$ SEM (n = 5 independent mice). Mean difference is significant from control group at $*P < 0.0001$ and $**P < 0.001$ (two-way ANOVA followed by Bonferroni post-test).

Transcriptional activation of CYP2A5 by BR involves a StRE in the CYP2A5 promoter

It was previously shown that increased CYP2A5 transcription was due to Nrf2 binding to StRE at position -2467 to -2269 of the *Cyp2a5* 5'-flanking region (Abu-bakar et al., 2007). To confirm this finding, luciferase activity was measured in mouse hepatocytes co-transfected with Nrf2 (0.3 µg) and various truncation constructs (-2603/+10, -2263/+10, -746/+10, and -432/+10) of the *Cyp2a5* promoter. Nrf2 overexpression significantly induced reporter activity in the reporter constructs containing -3033/+10 and -2603/+10 fragments ($P < 0.01$ and $P < 0.001$), but had no effect on the constructs of shorter length (Figure 6).

CYP2A5 induction by BR requires Nrf2

In order to further determine if Nrf2 is required for BR-induced CYP2A5 expression, we used the *Cyp2a5*-3033 with a site-directed mutation of the Nrf2 response element (*Cyp2a5*-ΔNrf2). Mouse hepatocytes were transfected with either the *Cyp2a5*-3033 or the *Cyp2a5*-ΔNrf2 reporter constructs followed by cotransfection with either pcDNA3.1 or pcDNA3.1-mNrf2. Nrf2 overexpression induced the reporter activity as shown previously (Figure 7a). The groups of cells cotransfected with pcDNA3.1-mNrf2 were also treated with 50 µM BR, and the reporter activity of both *Cyp2a5*-3033 and *Cyp2a5*-ΔNrf2 constructs was determined. The BR-mediated increase in CYP2A5 reporter activity was abolished in hepatocytes co-transfected with the *Cyp2a5*-ΔNrf2 construct (Figure 7b).

Figure 6. Effect of Nrf2 cotransfection on *Cyp2a5*-5'-luciferase activities in different reporter constructs transfected in C57BL/6 mouse primary hepatocytes. Luciferase activities were measured 24 h after transfection. The measured activities were normalized against *Renilla* (pR-TK) activities. All values represent the mean $\pm$ SEM (n = 3 independent mice). The effect of Nrf2 on each reporter construct is indicated by fold activity relative to control hepatocytes cotransfected with pcDNA3.1. Mean difference is significant from control group at * $P < 0.01$ and ** $P < 0.001$ (two-way ANOVA followed by Bonferroni post-test).

Figure 7. BR-mediated increased CYP2A5 transcription requires the Nrf2 response element in the CYP2A5 promoter. *Cyp2a5*-5'-luciferase activities were assessed primary mouse hepatocytes 24 h after (A) Nrf2 cotransfection or (B) Nrf2 cotransfection followed by 50 μM BR. The measured activities were normalized against *Renilla* (pRL-TK) activities. All values represent the mean $\pm$ SEM (n = 4 independent mice). Mean difference is significant from the control group at * $P < 0.001$ and ** $P < 0.01$ (two-way ANOVA followed by Bonferroni post-test). wt, wild-type *Cyp2a5*-5'-luciferase construct; ΔNrf2, *Cyp2a5*-5'-luciferase construct with mutated Nrf2 response elements.

BR activates caspase-3 cleavage in Hepa 1-6 and mouse primary hepatocytes

In order to investigate BR toxicity in mouse hepatocytes, caspase-3 cleavage, a marker of apoptosis, was analyzed after Hepa 1-6 cells and mouse primary hepatocytes were treated with increasing doses of BR (0, 1, 10, 25, 50 μ M). Western blot analysis was performed to detect caspase-3 cleavage following the treatments. Caspase-3 cleavage was detected with 10, 25, and 50 μ M BR treatments in the primary hepatocytes, but only with 25 and 50 μ M BR treatments in Hepa 1-6 (Figure 8). In addition, the greatest degree of caspase-3 cleavage was observed at the highest-dose of BR (50 μ M) in both cell types (Figure 8).

Figure 8. Effect of BR on caspase-3 cleavage in (A) Mouse primary hepatocytes and (B) Hepa 1-6 cells. Both mouse primary hepatocytes and Hepa 1-6 cells were treated with 1, 10, 25 or 50 μM BR for 24 h. Western blot analysis of total protein (40 μg total protein/lane) from control and BR-treated cells were probed with rabbit anti-mouse caspase-3 or mouse anti- β -actin antibody. β -actin protein levels are shown as control for protein loading. Each blot represents one of two blots of three samples (each blot showed the same pattern of induction).

Overexpressed CYP2A5 is functional in Hepa 1-6

To optimize the overexpression of CYP2A5 and POR proteins in Hepa 1-6, CYP2A5-V5 and POR-V5 expression plasmids were transfected into Hepa 1-6 cells in various combinations. Western blotting was used to determine the optimal amount of each expression plasmid for transfection. Figure 9a shows the last step of transfection optimization; 2.0 µg of CYP2A5-V5 plasmid and 0.4 µg of POR-V5 plasmid was required to have maximum expression in Hepa 1-6. To test if overexpressed CYP2A5-V5 proteins are functional, a 7-ethoxycoumarin deethylation assay was performed. Overexpression of CYP2A5-V5 alone significantly increased 7-ethoxycoumarin deethylation by 2-fold ($P < 0.001$) relative to control (Figure 9b). Furthermore, cotransfection of POR-V5 in addition to CYP2A5-V5 transfection significantly increased (3-fold; $P < 0.0001$) the activity of CYP2A5-V5 protein in Hepa 1-6 (Figure 9c).

CYP2A5 overexpression partially blocks BR-induced caspase-3 activation in Hepa 1-6 cells

To test the hypothesis that CYP2A5 plays a cytoprotective role against BR toxicity, the effect of CYP2A5-V5 and POR-V5 overexpression in BR-induced caspase-3 cleavage was examined. Briefly, CYP2A5-V5 and POR-V5 expression plasmids were transfected into Hepa 1-6 for 24 hours, and the cells were treated with 50 µM BR for another 24 hours. The total protein lysate from control and BR-treated cells were analyzed via Western blotting with anti-caspase-3 antibody. As previously shown, 50 µM BR treatment activated caspase-3 cleavage by 13-fold relative to control cells transfected with empty vector (pcDNA3.1) ($P < 0.01$) (Figure 10b). However, overexpression of

CYP2A5-V5 and POR-V5 reduced caspase-3 cleavage by 40% to a level of 9-fold ($P < 0.01$) relative to control (Figure 10b).

Figure 9. Optimization of transfection conditions for CYP2A5 overexpression in Hepa 1-6 cells. (A) Western blot showing the optimization of amount of expression vector required to overexpress CYP2A5 and POR protein in Hepa1-6 cells. (B) Western blot analysis of CYP2A5/POR protein. (C) CYP2A5 activity assessed by 7-ethoxycoumarin deethylation assay in Hepa1-6 cells overexpressing pcDNA3.1, control empty vector; POR, oxidoreductase alone; 2A5, CYP2A5 alone; 2A5/POR, co-transfection of both CYP2A5 and POR. All values represent the mean $\pm$ SEM ($n = 5$ independent experiments). Mean difference is significant from control group at $*P < 0.0001$ (one-way repeated measures ANOVA followed by Dunnett's post-test).

Figure 10. Effect of CYP2A5 and POR overexpression on BR-induced caspase-3 cleavage in Hepa 1-6 cells. Hepa 1-6 cells were transfected with CYP2A5 and POR for 24 h and treated with 50 μM BR for another 24 h. (A) Western blot analysis of total protein (40 μg total protein/lane) from control and BR-treated cells were probed with rabbit anti-mouse caspase-3 or mouse anti- β -actin antibody. β -actin protein levels are shown as control for protein loading. Each blot represents one of two blots consisting of four replicates (each blot showed the same pattern of induction). (B) Densitometric quantification of Western blots. All values represent the mean $\pm$ SEM ($n = 4$ independent experiments) normalized against the control levels. Mean differences are significant from control group at $*P < 0.001$ (pcDNA3.1 compared to untreated control) and $**P < 0.01$ (CYP2A5/POR compared to pcDNA3.1 both treated; interaction) (two-way ANOVA followed by Bonferroni post-test).

CYP2A5 overexpressing microsomes have increased BR degradation rate

In order to further investigate the mechanisms underlying the cytoprotective role of CYP2A5 against BR toxicity, we determined whether overexpressed CYP2A5-V5 metabolizes BR in Hepa 1-6 cells, using a BR disappearance assay as previously described (Abu-Bakar et al., 2011). Hepa 1-6 cells were transfected with pcDNA3.1 or CYP2A5-V5/POR-V5 expression plasmids, and microsomal fractions were prepared by ultracentrifugation. Proteins expressed by transfection of CYP2A5-V5/POR-V5 plasmids were localized only in microsomal protein as demonstrated in Figure 11a. BR (10 μ M) was incubated with either microsomes from cells transfected with either pcDNA3.1 or CYP2A5-V5/POR-V5, and the degradation rate was measured by spectrophotometry. CYP2A5-V5/POR-V5 overexpression significantly increased the BR clearance rate by 6.4-fold ($P < 0.003$) relative to control (Figure 11b).

Figure 11. (A) Representative Western blot showing overexpression of CYP2A5 and POR is localized in the microsomal fraction of Hepa 1-6 cells. (B) The rate of BR degradation is increased in microsomes from Hepa 1-6 cells overexpressing 2A5/POR. Microsomes from Hepa 1-6 cells transfected with CYP2A5 and POR were incubated 10 μ M BR and the degradation rate was measured by spectrophotometry. All values represent the mean $\pm$ SEM ($n = 3$ independent experiments). Mean difference is significant from control group at $*P < 0.003$ (Student's t -test).

DISCUSSION

CYP2A5 plays an important role in detoxification of various xenobiotics and drugs. It is also implicated in protection against oxidative stress, which arises from various pathological states. Recently, BR has been identified as an endogenous substrate and inducer of CYP2A5 (Abu-Bakar et al., 2011). Abu-Bakar et al. (2011) demonstrated that CYP2A5 is an inducible BR oxidase that it converts BR into its precursor, biliverdin, which can be recovered back to BR by biliverdin reductase (Abu-Bakar et al., 2011). They suggested this system prevents the wasting of BR from glucuronidation by UGT1A1 and accumulation of BR in pro-oxidant levels (Abu-Bakar et al., 2011). Hence, the question that remains is to what extent BR metabolism by CYP2A5 can prevent the cytotoxic effects of excessive BR and how BR regulates CYP2A5. Therefore, the objective of this study is to investigate the role of CYP2A5 in bilirubin (BR) toxicity and the influence of BR on the regulatory mechanisms of *Cyp2a5* gene expression. Our findings demonstrate that excessive BR induces CYP2A5 transcription resulting in increased mRNA and protein expression and that this induction requires Nrf2 activation in primary mouse hepatocytes. Furthermore, Hepa 1-6 cells overexpressing CYP2A5 protein are partially protected from BR toxicity through direct clearance of excessive BR.

CYP2A5 is known for its unique regulation. CYP2A5 is upregulated following exposure to chemical hepatotoxins and during pathophysiological conditions, in which the levels of most CYPs are either unchanged or downregulated (Juvonen et al., 1985; Camus et al., 1996; Kojo et al., 1998; Kirby et al., 2011). In the present study, BR at high-dose (25 and 50 μ M) significantly induced CYP2A5 mRNA and protein expression

in primary mouse hepatocytes in dose-dependent manner. Our result showing CYP2A5 mRNA induction by BR is in contrast to what was reported by Abu-Bakar et al. (2011) where CYP2A5 mRNA induction was not observed. This difference in CYP2A5 mRNA expression appears to be due to mouse strain differences. In the present study, primary hepatocytes were obtained from C57BL/6 mice whereas Abu-Bakar et al. (2011) used DBA/2J mice (Abu-Bakar et al., 2011). While both strains are widely used in drug metabolism studies, strain characteristics might explain differences in responsiveness to certain inducing agents. For example, differences in glutathione S-transferase activity (GST) may alter net uptake of BR by hepatocytes (Wolkoff et al., 1978, 2012; Higgins and Hayes, 2011). Furthermore, it has been reported that DBA/2 mice lack functional AhR (Poland and Glover, 1975; Okey et al., 1989), which is known to directly regulate the Nrf2 antioxidant pathway and upregulate xenobiotic detoxifying enzymes that are involved in oxidative stress response, cell cycle control, and apoptosis (Nebert et al., 2000; Miao et al., 2005). For example, 3-methylcholanthrene (3-MC) significantly induces microsomal GSTA1/2 protein expression in C57BL/6 mice and has little to no effect in DBA/2O mice (Higgins et al., 2008). Furthermore, the AhR agonist, β -naphthoflavone (β -NF) increases GST activity significantly more in C57BL/6 mice than DBA/2 mice, due to the non-functional AhR in DBA/2 mice (Prochaska and Talalay, 1988; Mclellan et al., 1991). These reports suggest that DBA/2J mice may express relatively lower GSTA1/2 levels due to lack of functional AhR, which in turn may decrease BR net uptake by hepatocytes compared to C57BL/6 mice for the same dose of BR treatment. Therefore it is possible that BR accumulation in hepatocytes was not high enough to drive transcription of CYP2A5 in DBA/2J mice. Moreover, it could be simply

due to the shorter length (6 h) of BR treatment that Abu-Bakar et al. (2011) used in their study. It is also important to note that semi-quantitative Northern blot analysis was used in their study, whereas real-time RT-PCR has been used in the present study. RT-PCR is a newer technique that is more reliable for quantitative analysis and has higher sensitivity and specificity (Bustin et al., 2005).

Our results, also show that BR induces CYP2A5 protein in a dose-dependent manner in agreement with findings by Abu-Bakar et al. (Abu-Bakar et al., 2011). Abu-Bakar et al. (2011) showed that co-treatment of cycloheximide (CHX) with BR, a protein synthesis inhibitor, resulted in an increased half-life of the CYP2A5 protein compared to cells treated only with CHX (Abu-Bakar et al., 2011) indicating that increased CYP2A5 protein levels are likely due to post-translational mechanism involving protein stabilization by BR. However, our findings that BR increases CYP2A5 mRNA and protein in a dose-dependent manner suggest that CYP2A5 induction may be due to either transcriptional or post-transcriptional mechanisms.

To investigate the underlying mechanism of CYP2A5 induction by BR, we performed a reporter assay using a luciferase reporter plasmid containing the *Cyp2a5* 5'-untranslated promoter region from -3033 to +10 bp. To date, a number of transcription factors and their response elements have been identified for CYP2A5 that play important roles in various cellular functions such as the stress and immune responses, cell cycle activation and cellular proliferation, and glucose and xenobiotic metabolism (Cai et al., 2002; Maglich et al., 2002; Abu-Bakar et al., 2004; Ulvila et al., 2004; Abu-bakar et al., 2007; Arpiainen et al., 2007, 2008). However, given that effects of high BR has been shown to be closely related to oxidative stress in various organelles, among the other

transcription factors, Nrf2 appears to be involved in BR-mediated induction of CYP2A5. Nrf2 regulates the expression of genes involved in the glutathione synthesis, ROS clearance and xenobiotic metabolism (Itoh et al., 1999; Kamisako et al., 2000). Furthermore, recently the binding site for Nrf2, the stress responsive element (StRE) has been identified in the CYP2A5 promoter region, and the parent compound of BR, heme has been shown to regulate *Cyp2a5* via Nrf2 activation (Abu-bakar et al., 2007; Lämsä et al., 2012). Nrf2 has been also shown to regulate both constitutive and inducible expression of *Cyp2a5* in response to oxidative stress (Abu-Bakar et al., 2004; Dhakshinamoorthy and Porter, 2004; Purdom-dickinson et al., 2007; Lämsä et al., 2010). In the present study, to investigate the involvement of Nrf2 in the regulation of *Cyp2a5* expression by BR, primary mouse hepatocytes were cotransfected with the CYP2A5 reporter construct and an Nrf2 expression vector. Nrf2 overexpression significantly induced *Cyp2a5* reporter activity, and this increase was significantly augmented with BR treatment. BR did not increase CYP2A5 transcription in the absence of Nrf2 overexpression. Moreover, this BR-mediated increase in transcriptional activity in the presence of Nrf2 overexpression was abolished following site-directed mutation of StRE in the luciferase reporter construct. Hence, our results demonstrate Nrf2 is indeed required for CYP2A5 transcriptional activation by BR.

BR is shown to be toxic to many cell types and intracellular organelles at high concentrations ($> 20 \mu\text{M}$), and prolonged exposure and accumulation of BR can lead to cell damage or death (Roy-Chowdhury et al., 2007; McDonagh, 2010). Although the molecular mechanism underlying the cytotoxic effects of BR is unclear, previous reports show BR directly damages cell membranes at various levels, including mitochondria and

ER membrane, disrupting their functions (Mayor et al., 1986; Silva et al., 2001; Brites and Brito, 2012). Furthermore, these cytotoxic effects of BR are shown to be mainly due to production of ROS resulting in protein oxidation, and lipid peroxidation (Brito et al., 2004, 2008a, 2008b; Kumar et al., 2008; Brites and Brito, 2012). Previous reports have demonstrated that BR can cause apoptosis via both intrinsic and extrinsic signaling pathways (Seubert et al., 2002; Vaz et al., 2011; Brites and Brito, 2012). BR also induces ER stress by disrupting intracellular calcium (iCa^{2+}) homeostasis and increasing ROS production resulting in activation of caspase-3 (Gao et al., 2011). Furthermore, DNA oxidation by BR-induced oxidative stress can also trigger apoptosis (Deganuto et al., 2010). Our results support previous findings by demonstrating that BR induces caspase-3 activation in both Hepa 1-6 and primary mouse hepatocytes.

In order to investigate the cytoprotective role of CYP2A5 against BR toxicity, we transiently overexpressed CYP2A5 protein in Hepa 1-6 cells, a cell line that lacks constitutive expression of CYP2A5. Our results demonstrate for the first time that transient overexpression of CYP2A5 partially protects against BR-induced apoptosis by decreasing BR-induced caspase-3 cleavage. It remains to be shown which apoptotic pathway is involved in BR-induced caspase-3 cleavage. In the extrinsic pathway, apoptosis is initiated by the engagement of a transmembrane death receptor (i.e. Fas) by an extracellular ligand, which leads to the assembly of death-inducing signaling complex (DISC) (Riedl and Salvesen, 2007). DISC then activates the initiator caspase-8, which leads to subsequent activation of caspase-3/7 (Riedl and Salvesen, 2007). Previous studies have shown that caspase-8 activity was significantly induced by BR treatment in Hepa 1c1c7 and rat neurons, suggesting BR-induced apoptosis is mediated by the

extrinsic apoptosis pathway (Seubert et al., 2002; Vaz et al., 2011). However, caspase-8 induction appears to be cell-type specific, hence it remains unclear whether BR acts as a ligand for the death receptor or whether caspase-8 activity is increased indirectly by BR via oxidative stress in cellular organelles (Keshavan et al., 2004).

In the intrinsic mitochondrial pathway, apoptosis is initiated by stimuli that lead to the permeabilization of the outer mitochondrial membrane and the release of cytochrome c, which forms the apoptosome with apoptotic protease activating factor 1 (Apaf1) (Riedl and Salvesen, 2007; Ow et al., 2008). The apoptosome then activates caspase-9, and finally activates caspase-3/7 (Riedl and Salvesen, 2007; Ow et al., 2008). Previous reports have shown that BR treatment also induces cytochrome c release from mitochondria and activation of caspase-9 in various cell types (Seubert et al., 2002; Keshavan et al., 2004; Oakes and Bend, 2005). These effects by BR are mainly due to disruption of the mitochondrial membrane and production of ROS (Seubert et al., 2002; Keshavan et al., 2004; Oakes and Bend, 2005).

BR has been also shown to induce apoptosis via ER stress. BR treatment in synaptosomes increases iCa^{2+} and ROS production significantly (Brito et al., 2006). Increased iCa^{2+} disrupts intracellular homeostasis and produces more ROS, leading to ER stress (Brito et al., 2006; Brites and Brito, 2012). This can then lead to activation of caspase-2/12 and the dissociation of an ER luminal chaperone, the immunoglobulin heavy chain binding protein (Bip), which also initiates apoptosis (Brites and Brito, 2012). Barateiro et al. (2012) demonstrated that BR treatment in oligodendrocyte precursor cells induced iCa^{2+} overload and production of ROS, as well as activation of caspase-2 and the ER stress marker glucose-regulated protein 78 (Barateiro et al., 2012). However, BR-

induced apoptosis via ER stress has not been demonstrated in liver tissue and requires further investigation. Taken together, the exact underlying mechanism of BR-induced apoptosis in liver needs further investigation.

Finally, to determine whether the cytoprotective role of CYP2A5 overexpression in BR toxicity is due to direct clearance of BR, we have modified the BR degradation assay previously established (Abu-Bakar et al., 2005). Microsomes from Hepa 1-6 overexpressing CYP2A5 protein were isolated and used to assess BR degradation *in vitro*. We confirmed that transient transfection of CYP2A5 protein is localized to the microsomal fraction of Hepa 1-6 cells, and that degradation of BR by microsomes from these cells was significantly greater than by microsomes from cells transfected with the empty vector. This suggests that CYP2A5 overexpression protected against BR-induced apoptosis through BR oxidation into less toxic metabolites such as biliverdin as previously described (Abu-Bakar et al., 2011).

In conclusion, this thesis research examined the role of CYP2A5 in BR metabolism by investigating *Cyp2a5* gene regulation by BR, and the cytoprotective role of CYP2A5 in BR toxicity. Our results demonstrate that BR induces CYP2A5 expression at the mRNA and protein levels by increasing CYP2A5 transcription via a mechanism that involves Nrf2 activation. Furthermore, our results suggest that induced CYP2A5 plays a cytoprotective role against BR toxicity by directly lowering the cellular levels of BR and by inhibiting caspase-3 activation. Previous studies have indicated that other enzymes involved in bilirubin metabolism such as UGT1A1 are also regulated by Nrf2 thereby reducing BR levels. For example, a recent study by Kundu et al. (2011) reported that a flavone glycoside, carlinoside, upregulates Nrf2 gene expression and increases its nuclear

translocation and stimulated UGT1A1 promoter activity, which results in a significant increase of BR glucuronidation (Kundu et al., 2011). Therefore, this study suggests Nrf2-mediated CYP2A5 induction in response to BR may provide an additional mechanism to adaptive response of our body against BR toxicity.

However, the question still remains why BR treatment alone did not alter luciferase activity in primary mouse hepatocytes. It is likely that the transfected *Cyp2a5* 5'-3033 reporter construct may not have been able to reach the nucleus while BR activates Nrf2 translocation into nucleus. Hence, whether BR actually activates Nrf2 translocation in primary mouse hepatocytes needs to be investigated. Furthermore, recent studies report that constitutive androstane receptor (CAR) also increases BR clearance (Huang et al., 2003, 2004). Therefore, given that previous studies have suggested CAR binding sites exist in CYP2A5 promoter, it would be compelling to investigate whether BR clearance by CYP2A5 can be also mediated by CAR.

SUMMARY AND CONCLUSIONS

This study investigated the mechanisms for gene regulation and a cytoprotective role of CYP2A5 in response to BR treatment in liver. Our results demonstrate that BR induces CYP2A5 expression at the mRNA and protein levels by increasing CYP2A5 transcription via a mechanism that involves Nrf2 activation. Furthermore, our results suggest that induced CYP2A5 plays a cytoprotective role against BR toxicity by directly lowering the cellular levels of BR and thereby inhibiting caspase-3 activation.

In the present study, BR at high dose significantly induced CYP2A5 mRNA and protein expression in primary hepatocytes in dose-dependent manner. This result showing CYP2A5 mRNA induction by BR is in contrast to what was previously reported by Abu-Bakar et al. (Abu-Bakar et al., 2011). This difference in CYP2A5 mRNA expression appears to be due to the following reasons (1) in our study, primary hepatocytes were obtained from C57BC/6 mice whereas Abu-Bakar et al. (2011) used DBA/2J mice. (2) The BR treatment length was shorter (6 h) in the study by Abu-Bakar et al. (2011) compared to our 24 h treatment. (3) Abu-Bakar et al. (2011) used semi-quantitative Northern blot analysis for CYP2A5 mRNA quantification whereas real-time RT-PCR has been used in the present study.

Our result also demonstrates that the induction of CYP2A5 mRNA and protein expression in primary hepatocytes is mediated by Nrf2 activation. However, whether BR treatment actually causes Nrf2 translocation in primary mouse hepatocytes needs to be further investigated. Moreover, Abu-Bakar et al. (2011) showed that co-treatment of CHX with BR resulted in an increased half-life of CYP2A5 protein compared to cells

treated only with CHX, suggesting that post-translational mechanism is also involved in the regulation of CYP2A5 by BR (Abu-Bakar et al., 2011). Furthermore, previous studies have demonstrated that the CAR also increases BR clearance (Huang et al., 2003, 2004). Previous studies have also suggested that the binding sites for CAR exist in CYP2A5 promoter (Cai et al., 2002; Maglich et al., 2002; Rosenfeld et al., 2003; Foretz et al., 2005) and we have shown that CAR is required for phenobarbital induction of CYP2A5 (Kirby et al., 2011). This suggests that CYP2A5 induction by BR is due to both transcriptional and post-transcriptional mechanisms, and CAR may also be involved.

Lastly, in order to investigate the cytoprotective role of CYP2A5 against BR toxicity, we examined the influence of transiently overexpressed CYP2A5 on BR-mediated apoptosis. Our results demonstrate for the first time that transient overexpression of CYP2A5 partially protects against BR-induced apoptosis by decreasing BR-induced caspase-3 cleavage. However the exact underlying mechanism by which CYP2A5 protects against BR-induced apoptosis in mouse liver remains to be further investigated as previous reports show that BR induces apoptosis via various mechanisms (Seubert et al., 2002; Gao et al., 2011; Vaz et al., 2011; Brites and Brito, 2012).

We also confirmed that transient transfection of CYP2A5 protein is localized to the microsomal fraction of Hepa 1-6 cells, and that degradation of BR by microsomes from transfected cells was significantly greater than by microsomes from cells transfected with the empty vector. This suggests that CYP2A5 overexpression protects against BR-induced apoptosis via BR oxidation into less toxic metabolites such as biliverdin as previously demonstrated (Abu-Bakar et al., 2011). Therefore, this study suggests that

Nrf2-mediated CYP2A5 induction in response to BR may provide an additional mechanism for the adaptive response against BR toxicity.

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APPENDIX I

CELL CULTURE – PRIMARY HEPATOCYTES

0.1 M EGTA, pH 7.4

- Dissolve 0.95 g EGTA in 1 mL of 5N NaOH
- Add 2.5 mL of 10X Hanks' Buffered Salt Solution (HBSS) and 17 mL of MilliQ H₂O
- Adjust pH to 7.4 with 1N HCl and bring to final volume of 25 mL with MilliQ H₂O
- The solution was sterilized by filtration through a 0.45 µm pore filter

1.0 M HEPES, pH 7.4

- Purchased from Invitrogen (Burlington, ON)

Geneticin 50 mg/mL

- Purchased from Invitrogen (Burlington, ON)

7.5% BSA

- Dissolve 0.75 g of BSA (fraction V) in 8 mL MilliQ H₂O
- Bring to final volume of 10 mL with MilliQ H₂O

Insulin-Transferrin-Selenium (ITS)

- Purchased as lyophilized powder from Roche Diagnostics (Mississauga, ON)
- The powder contains of 25 mg insulin, 25 mg transferrin, 25 µg sodium selenite
- The powder was reconstituted in 5 mL MilliQ H₂O to give 1000X stock solution

10 mM Dexamethasone

- Dissolve 3.925 mg dexamethasone in 1 mL ethanol

William's E Media

- Dissolve entire contents of William's E powder purchased from Sigma-Aldrich (Oakville, ON) in 1 L MilliQ H₂O
- Add 2.2 g NaHCO₃ and pH to 7.37 – 7.39

Blanching Solution

- 1.0 M HEPES, pH 7.4	1 mL
- 0.1 M EGTA, pH 7.4	1 mL
- 10X HBSS	10 mL
- MilliQ H ₂ O	88 mL

Collagenase Medium

- William's Media E	60 mL
- 1.0 M HEPES, pH 7.4	0.6 mL
- 7.5% BSA	64 µL
- Collagenase Type III	28.6 mg

Attachment Medium

- William's Media E	444.5 mL
- ITS	500 uL
- FBS (heat-inactivated)	50 mL
- 10 mM dexamethasone	2.5 uL
- 1.0 M HEPES, pH 7.4	5 mL
- Geneticin	75 µL

Serum-Free Medium

- William's Media E	494.5 mL
- ITS	500 µL
- 1.0 M HEPES, pH 7.4	5 mL

CELL CUTURE – HEPA 1-6

Phosphate-buffered saline (PBS), pH 7.4

- NaCl 8 g
- KCl 0.2 g
- Na₂HPO₄ 7H₂O 2.68 g
- KH₂PO₄ 0.24 g
- MilliQ H₂O 900 mL
- pH to 7.4 and bring final volume to 1 L with MilliQ H₂O, and autoclave

Dulbecco's Modified Eagle's Medium (DMEM)

- Purchased from Sigma-Aldrich (Oakville, ON)

Penicillin/Streptomycin

- 100X stock solution was purchased from Sigma-Aldrich (Oakville, ON)
- The stock contains 10,000 units of penicillin and 10 mg streptomycin/mL

Complete media

- FBS 50 mL
- Penicillin/Streptomycin 5 mL
- DMEM 445 mL

BILIRUBIN STOCK PREPARATION

** Bilirubin is light sensitive, so must work in the dark room

** Eppendorf microcentrifuge tube must be light protective amber colored tube

0.1 N NaOH PBS, pH 7.4

- 1 N NaOH 1 mL
- PBS, pH 7.4 9 mL

5 mM Bilirubin

- Bilirubin of mixed isomers was purchased from Sigma-Aldrich (Oakville, ON)
- Dissolve 29.23 mg of bilirubin in 10 mL 0.1 N NaOH PBS, pH 7.4
- Dilute 5 mM stock bilirubin into 2.5 mM, 1.0 mM, 0.1 mM stock

WESTERN BLOTS

2.0 M Tris-HCl, pH 6.8

- Tris Base 12.59 g
- MilliQ H₂O 30 mL
- pH to 6.8 with 12 N HCl and adjust to final volume of 50 mL with MilliQ H₂O
- when adjusting pH, cool it down with ice as 12 N HCl is being added

1.5 M Tris-HCl, pH 8.8

- Tris Base 27.26 g
- MilliQ H₂O 80 mL
- pH to 8.8 with 6 N HCl and adjust to final volume of 150 mL with MilliQ H₂O

1.0 M Tris-HCl, pH 7.5

- Tris Base 12.0 g
- MilliQ H₂O 60 mL
- pH to 7.5 with 6 N HCl and adjust to final volume of 100 mL with MilliQ H₂O

0.5 M Tris-HCl, pH 6.8

- Tris Base 6.0 g
- MilliQ H₂O 60 mL
- pH to 6.8 with 6 N HCl and adjust to final volume of 100 mL with MilliQ H₂O

10% SDS

- SDS 10 g
- MilliQ H₂O 90 mL
- Adjust to final volume of 100 mL with MilliQ H₂O

1% Bromophenol blue

- bromophenol blue 100 mg
- MilliQ H₂O 10 mL

2X Laemmli Buffer

- 2.0 M Tris-HCl, pH 6.8 1.250 mL
- β -mercaptoethanol 2 mL
- MilliQ H₂O 10 mL
- SDS 800 mg
- Glycerol 4 mL
- 1% bromophenol blue 80 μ L
- Adjust final volume to 20 mL with MilliQ H₂O (~2.5 mL)
- β -mercaptoethanol is light sensitive, so aliquot in amber microcentrifuge tubes and keep it at -20 °C

5X Laemmli Buffer (order of addition is important)

- 2.0 M Tris-HCl, pH 6.8 3.125 mL
- β -mercaptoethanol 5 mL
- SDS 2 g (dissolve completely before proceed)
- Glycerol 10 mL (add slowly)
- 1% bromophenol blue 200 μ L
- Adjust final volume to 20 mL with MilliQ H₂O (~1.8 mL)
- β -mercaptoethanol is light sensitive, so aliquot in amber microcentrifuge tubes and keep it at -20 °C

5X Running Buffer, pH 8.3

- Tris Base	15.0 g	30 g	45 g	60 g
- Glycine	72.0 g	144 g	216 g	288 g
- SDS	5.0 g	10 g	15 g	20 g
- MilliQ H ₂ O	final volume			
	1 L	2 L	3 L	4 L

Transfer Buffer, pH 8.3

- Tris Base	3.03 g	6.06 g	9.09 g	12.12 g
- Glycine	14.4 g	28.8 g	43.2 g	57.6 g

- Methanol	200 mL	400 mL	600 mL	800 mL
- MilliQ H ₂ O	<u>final volume</u>			
	1 L	2 L	3 L	4 L
* Do not adjust pH. Should be ~8.1-8.4; reuse 3x				

TBS (Tris buffered saline), pH 7.6 (recipe for ECL+)

- Tris Base	2.4 g	9.6 g
- NaCl	8.0 g	32.0 g
- MilliQ H ₂ O	<u>800 mL</u>	<u>3.2 L</u>
- Final volume	1 L	4 L

* pH to 7.6 with 6 N HCl and adjust to final volume

TTBS, 0.1 % Tween 20 (recipe for ECL+)

- TBS, pH 7.6	999 mL
- Tween 20	1 mL

Cell Lysis Buffer (100 mL stock)

- 1.0 M Tris, pH 7.5	5 mL
- 10% Glycerol	10 mL
- 0.5 M EDTA	1 mL
- 2.5 M NaCl ₂	6 mL
- 0.5% NP-40	500 µL
- <u>MilliQ H₂O</u>	<u>77.5 mL</u>
- Take 10 mL of stock and add one Complete mini tablet	

NUCLEAR FRACTION EXTRACTION BUFFERS

1.0 M HEPES-KOH, pH 7.9

- 1.0 M HEPES, pH 7.4 45 mL
- pH to 7.9 with KOH pellets

1.0 M KCl

- Dissolve 37.275 mg KCl in 50 mL MilliQ H₂O
- The solution was sterilized by filtration through a 0.45 µm pore filter

1.0 M MgCl₂

- Dissolve 10.165 mg MgCl₂ in 50 mL MilliQ H₂O
- The solution was sterilized by filtration through a 0.45 µm pore filter

2.5 M NaCl₂

- Dissolve 7.305 mg NaCl₂ in 50 mL MilliQ H₂O

NP-40 Substitute

- Purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

0.1 M Dithiothreitol (DTT)

- Dissolve 77.125 mg DTT in 5 mL MilliQ H₂O
- DTT is light sensitive; aliquot in amber centrifuge tubes and store at -20°C

1.0 M EDTA, pH 8.0

- Dissolve 18.6 g Na₂•EDTA•2H₂O in approximately 80 mL of MilliQ H₂O
- Add 2.2 g NaOH pellets and once dissolved, adjust pH to 8.0 with 10N NaOH
- ** EDTA does not dissolve completely until the pH reaches ~8
- Adjust final volume to 100 mL with MilliQ H₂O

Buffer A (50 mL)

- 1.0 M HEPES-KOH, pH 7.9 500 μ L
- 1.0 M KCl 500 μ L
- 1.0 M MgCl₂ 75 μ L
- NP-40 200 μ L
- MilliQ H₂O 48.725 mL
- Take 10 mL of the stock, add 50 μ L 0.1 M DTT and one Complete mini tablet

Buffer B

- 1.0 M HEPES-KOH, pH 7.9 1 mL
- Glycerol 12.5 mL
- 1.0 M MgCl₂ 75 μ L
- 2.5 M NaCl₂ 8.4 mL
- 0.5 M EDTA 20 μ L
- MilliQ H₂O 28.005 mL
- Take 10 mL of the stock, add 50 μ L 0.1 M DTT and one Complete mini tablet

MICROSOME EXTRACTION BUFFER

Homogenization Buffer, pH 7.4

- Tris Base 1.2114 g
- Sucrose 20 g
- MilliQ H₂O 80 mL
- pH to 7.4 with 6N HCl and adjust volume to 100 mL

Microsome Storage Buffer, pH 7.4

- Tris Base 1.2114 g
- Glycerol 10 mL
- MilliQ H₂O 30 mL
- pH to 7.4 with 6N HCl and adjust volume to 50 mL

REAGENTS FOR BILIRUBIN DISAPPEARANCE ASSAY

1.0 M Tris-HCl, pH 8.2

- Tris Base 12.0 g
- MilliQ H₂O 60 mL
- pH to 8.2 with 6 N HCl and adjust to final volume of 100 mL with MilliQ H₂O

1.3 M KCl

- Dissolve 9.6915 g KCl in 10 mL MilliQ H₂O

2 mM Bilirubin (in DMSO)

- Dissolve 1.1692 mg of bilirubin in 2 mL DMSO
- ** Bilirubin is light sensitive, so must work in the dark room
- ** Eppendorf microcentrifuge tube must be light protective amber colored tube

TRANSFORMATION OF BACTERIA (to produce more expression vectors)

LB Broth

- NaCl₂ 5 g
- Tryptone 5 g
- Yeast extract 2.5 g
- MilliQ H₂O 450 mL
- pH to 7.0 with 5N NaOH, adjust final volume to 500 mL
- Autoclave

LB Agar (500 mL/20 plates)

- NaCl₂ 5 g
- Tryptone 5 g
- Yeast extract 2.5 g
- MilliQ H₂O 450 mL
- pH to 7.0 with 5N NaOH
- add 10 g bacto-agar
- ** agar will not dissolve until autoclaved
- Autoclave, cool to < 50°C, add 500 µL of ampicillin (50 mg/mL)

APPENDIX II – SOURCES OF MATERIALS

6-well cell culture plate	Corning Inc, Corning, NY
7-ethoxycoumarin	Sigma-Aldrich, Oakville, ON
7-hydroxycoumarin	Sigma-Aldrich, Oakville, ON
24-well cell culture plate	Corning Inc, Corning, NY
96-well microplates (black, white, clear)	Corning Inc, Corning, NY
100 mm cell culture dish	Corning Inc, Corning, NY
Acrylamide	Bio-Rad Laboratories, Mississauga, ON
Amersham Hybond ECL membrane	GE Healthcare, Oakville, ON
Ampicillin	Sigma-Aldrich, Oakville, ON
Anti-mouse β -actin Ab	Sigma-Aldrich, Oakville, ON
Anti-mouse cleaved caspase-3 Ab	Cell Signaling, Whitby, ON
Bilirubin	Sigma-Aldrich, Oakville, ON
Bio-Rad protein assay reagent	Bio-Rad Laboratories, Mississauga, ON
Bottle Top Vacuum filter	Corning Inc, Corning, NY
BSA	Sigma-Aldrich, Oakville, ON
C57BL/6 male mice	Charles River Canada, St Constant, QC
Cell culture flasks	Corning Inc, Corning, NY
Chicken anti-mouse Cyp2a5 Ab	Dr. Juvonen, Kuopio, Finland
Chloroform	Fisher Scientific, Ottawa, ON
Conical tubes	Fisher Scientific, Ottawa, ON
Coumarin	Sigma-Aldrich, Oakville, ON

<i>Cyp2a5</i> 5'-3033/+10	Dr. Jukka Hakkola, Univ. of Oulu, Finland
CYP2A5-V5 expression vector	Dr. Masahiko Negishi, NIEHS, USA
Dexamethasone	Sigma-Aldrich, Oakville, ON
DMEM	Sigma-Aldrich, Oakville, ON
DMSO	Sigma-Aldrich, Oakville, ON
DNase	Promega, Madison, WI
dNTP mix	Invitrogen, Burlington, ON
DTT	Sigma-Aldrich, Oakville, ON
Dual-Luciferase Reporter Assay System	Promega, Madison, WI
ECL Plus	Amersham Biosciences, Baie d'Urfe, QC
EDTA	Sigma-Aldrich, Oakville, ON
EGTA	Sigma-Aldrich, Oakville, ON
FastStart SYBR Green Master Mix	Roche Diagnostics, Mississauga, ON
FBS	Sigma-Aldrich, Oakville, ON
Geneticin	Invitrogen, Burlington, ON
Glass dounce homogenizer	Fisher Scientific, Ottawa, ON
Glycerol	Fisher Scientific, Nepean, ON
HBSS	Invitrogen, Burlington, ON
Hepa 1-6 cell line	ATCC, Manassa, VA
HEPES	Sigma-Aldrich, Oakville, ON
HRP-conjugated goat anti-chicken IgG Ab	Sigma-Aldrich, Oakville, ON
HRP-conjugated goat anti-mouse IgG Ab	Sigma-Aldrich, Oakville, ON
HRP-conjugated goat anti-rabbit IgG Ab	Sigma-Aldrich, Oakville, ON

ImageQuant TL	GE Health Sciences, Piscataway, NJ
Isopropanol	Fisher Scientific, Ottawa, ON
ITS	Roche Diagnostics, Laval, QC
Lipofectamine 2000	Invitrogen, Burlington, ON
Lightcycler capillaries	Roche Diagnostics, Mississauga, ON
Lunar reverse phase C18 column	Phenomenex, Torrance, CA
Magnesium chloride	Sigma-Aldrich, Oakville, ON
Methanol	Fisher Scientific, Nepean, ON
M-MLV RT	Promega, Madison, WI
Mouse collagenase type I	Sigma-Aldrich, Oakville, ON
NADPH	Sigma-Aldrich, Oakville, ON
NanoDrop ND-1000	Thermoscientific, Ottawa, ON
NP-40 Alternative	EMD Millipore, Billerica, MA
Nrf2 HA-tagged expression vector	Dr. Ah-Ng Tony Kong, Univ. of NJ, USA
Nuclease-free water	Ambion, Austin, TX
pcDNA3.1/V5-His TOPO TA Exp. Kit	Invitrogen, Burlington, ON
Pentobarbital	MTC Pharmaceuticals, Cambridge, ON
Penicillin/Streptomycin	Invitrogen, Burlington, ON
POR-V5 expression vector	Dr. Jim Squires, Univ. of Guelph, ON
Potassium chloride	Fisher Scientific, Nepean, ON
Precision plus molecular weight marker	Bio-Rad Laboratories, Mississauga, ON
Complete mini, protease inhibitor tablet	Roche Diagnostics, Mississauga, ON
Random primers	Invitrogen, Burlington, ON

<i>Renilla</i> pRL-TK vector	Promega, Madison, WI
RNase inhibitor	Promega, Madison, WI
Roche Light Cycler	Roche Diagnostics, Indianapolis, IN
SDS	Fisher Scientific, Nepean, ON
Sodium chloride	Fisher Scientific, Nepean, ON
Spectra 100 UV-Vis detector	Spectra-Physics, San Jose, CA
Spectra Physics HPLC system	Spectra-Physics, San Jose, CA
TEMED	Invitrogen, Burlington, ON
Trichloroacetic acid	Sigma-Aldrich, Oakville, ON
Tris base	Fisher Scientific, Nepean, ON
Trizol reagent	Invitrogen, Burlington, ON
TrypLE	Invitrogen, Burlington, ON
Tryptone	Sigma-Aldrich, Oakville, ON
Tygon tubes	Fisher Scientific, Ottawa, ON
Typhoon 9410 Scanner	GE Health Sciences, Piscataway, NJ
Tween-20	Sigma-Aldrich, Oakville, ON
Whatman filter paper	Fisher Scientific, Nepean, ON
William's Medium E	Sigma-Aldrich, Oakville, ON
Yeast extract	Sigma-Aldrich, Oakville, ON