Nuclear Factor (Erythroid 2-like) Factor 2 (Nrf2) as Cellular Protector in Bile Acid and Retinoid Toxicities

by

Kah Poh (Hendrick) Tan

A thesis submitted in conformity with the requirements for the degree of Doctor in Philosophy

Graduate Department of Pharmacology and Toxicology

University of Toronto

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ABSTRACT
Exposure to toxic bile acids (BA) and retinoic acids (RA) is implicated in toxicities related to excessive oxidative stress. This thesis examined roles and mechanisms of the oxidative stress-responsive nuclear factor (erythroid 2-like) factor 2 (Nrf2) in adaptive cell defense against BA and RA toxicities. Using liver cells and mouse models, many antioxidant proteins known to be Nrf2 target genes, particularly the rate-limiting enzyme for glutathione (GSH) biosynthesis, i.e., glutamate-cysteine ligase subunits (GCLM/GCLC), were induced by BA [lithocholic acid (LCA)] or RA (all-trans, 9-cis and 13-cis) treatment. Evidence for increased Nrf2 transactivation by LCA and all-trans-RA was exemplified in HepG2 by: (1) reduced constitutive and inducible expression of GCLM/GCLC upon Nrf2 silencing via small-interfering RNA; (2) increased inducible expression of GCLM/GCLC genes by Nrf2 overexpression, but overexpression of dominant-negative Nrf2 decreased it; (3) increased nuclear accumulation of Nrf2 as signature event of receptor activation; (4) enhanced Nrf2-dependent antioxidant-response-element (ARE) reporter activity as indicative of increased Nrf2 transactivation; and (5) increased Nrf2 occupancy to AREs of GCLM and GCLC. Additionally, in BA-treated HepG2 cells, we observed concomitant increases of many ATP-binding cassette (ABC) transporters (MRPs 1-5, MDR1 and BCRP) in parallel with increased cellular efflux. Nrf2 silencing in HepG2 cells decreased constitutive and inducible expression of MRP2, MRP3 and ABCG2. However, Nrf2-silenced mouse hepatoma cells, Hepa1c1c7, and Nrf2−/− mice had decreased constitutive and/or inducible expression of Mrps 1-4, suggesting species differences in Nrf2-dependent regulation of hepatic ABC transporters. Protection by Nrf2 against BA and RA toxicities was confirmed by observations that Nrf2 silencing increased cell susceptibility to BA- and RA-induced cell death. Moreover, Nrf2−/− mice suffered more
severe liver injury than the wildtype. Increased GSH and efflux activity following increased GCLM/GCLC and ABC transporters, respectively, can mitigate LCA toxicity. Activation of MEK1-ERK1/2 MAPK was shown to primarily mediate Nrf2 transactivation and LCA-induced expression of antioxidant proteins and Nrf2-dependent and -independent ABC transporters. In conclusion, Nrf2 activation by BA and RA led to coordinated induction of antioxidant and ABC proteins, thereby counteracting resultant oxidative cytotoxicity. The potential of targeting Nrf2 in management of BA and RA toxicities merits further investigation.
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ABBREVIATIONS

ABC, ATP-binding cassette; AhR, aryl hydrocarbon receptor; ARE, antioxidant response element; ALP, alkaline phosphatase; ALT, alanine aminotransferase; atRA, all-trans-retinoic acid; BA, bile acid; BSEP, bile-salt export pump; BCRP, breast cancer resistance protein; BDL, bile duct ligation; BSO, buthionine sulfoximine; BHQ, tert-butylhydroquinone; BRCA1, breast cancer 1 (early onset); CA, cholic acid, CAR, constitutive androstane receptor; 9cRA, 9-cis-retinoic acid; 13cRA, 13-cis-retinoic acid; CDCA, chenodeoxycholic acid; ChIP, chromatin immunoprecipitation; CspA, cyclosporin A; CYP3A, cytochrome P450 3A; CYP3A4, cytochrome P450 3A4; CYP7A1, cytochrome P450 7A1; CYP26A1, cytochrome P450 26A1; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid, DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; FRL, ferritin light subunit; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modulatory subunit; GPx1, glutathione peroxidase 1; GSH, glutathione; GSTA, glutathione s-transferase A; GSTP1, glutathione s-transferase P1; HO1, heme oxygenase 1; 4-HNE, 4-hydroxynonenal; JNK, c-Jun N-terminal kinase; LA, α-lipoic acid; LCA, lithocholic acid; LDH, lactate dehydrogenase; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; MDR1, multidrug resistance protein 1; MPT, membrane permeability transition; MRP, multidrug resistance-associated protein; NAC, N-acetyl-L-cysteine; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor (erythroid-2 like) factor 2; NTCP, Na(+)−dependent taurocholate cotransporting polypeptide; PGH, prostaglandin H synthase; PI 3-kinase, phosphatidylinositol 3-kinase; PXR, pregnane X receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; ROS, reactive oxygen species; siRNA, small-interfering RNA; TRx1, thioredoxin reductase 1; UDCA, ursodeoxycholic acid.
CHAPTER 1.0
INTRODUCTION & LITERATURE REVIEW
1.1 General Introduction & Scope of Thesis

Environmental stressors are physical or chemical stimuli from the surrounding environment that can impact the growth, development, reproduction, or physiology of organisms. Throughout the history of evolution, the survival of an organism relies heavily on their ability to adapt to the changing environment, particularly when confronting these environmental stressors. This adaptability, which functions at cellular levels as the cellular adaptive response, is instrumental to counteract or lessen hazardous effects of the stressors entering and/or accumulating in the cell. Without an effective and well-coordinated cellular adaptive system in place, these stressors lead to disturbed homeostasis and pathology. In light of this, compelling evidence has revealed that the nuclear-receptor transcription factors including the pregnane X receptor (PXR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR), liver X receptor (LXR), and vitamin D receptor (VDR), are chief regulators of this cellular adaptive response. These transcription factors are activated principally via ligand-mediated mechanisms by a variety of exogenous and endogenous stressors (i.e., xenobiotics and endobiotics, respectively) to induce a myriad of genes. The resultant gene activation collectively establishes a complex, yet concerted cell defense and detoxification system, thereby preventing the deleterious effects of insurgent stressors. In toxicology, this coordinated detoxification system, which involves activation/deactivation, conjugation, and cellular efflux transport of stressor xenobiotics, is specifically referred to as the phase I, II, and III, respectively, of xenobiotic biotransformation systems.

Along with the preceding ligand-activated nuclear receptors, there is a distinct class of nuclear receptors which are activated by specific stress signals or stress-mediated signaling kinase cascades arising from the effects of insurgent stressors. They also appear to play a vital role in alleviating cellular stress and enhancing cell survival. Among the notable few, the tonicity-responsive enhancer-binding protein (TonEBP or NFAT5) is activated by hyperosmotic stress, NF-κB by inflammatory stimuli, and the Nuclear factor (erythroid 2-like) factor 2 (Nrf2) by electrophilic and oxidative stress. In particular, because of its increasingly important role in xenobiotic detoxification, the oxidative stress-responsive Nrf2 was the primary focus in my research. Nrf2 is gaining considerable interest in worldwide research nowadays with over 500 publications in PubMed's listing for 2007 alone. The growing popularity of Nrf2 may owe to the fact that it provides a direct molecular candidate for uncovering the role of oxidative stress in
Various human diseases. Oxidative stress, consequent to excess intracellular accumulation of reactive oxygen species (ROS), oxidative and nitrite radicals and/or electrophiles, is the most recognized etiopathological factor of almost all degenerative diseases to date. In drug therapy, undesirable oxidative stress is a well-documented contributor to toxicity and adverse drug reactions. With generation of the Nrf2 gene knockout/disrupted mouse models from Kan’s and Yamamoto’s groups back in the late 1990s, the \textit{in vivo} roles of Nrf2 in regulating critical adaptive responses against environmental and oxidative toxicants have since been deciphered. Nrf2 is now known as a master transcriptional activator for a wide array of genes involved in cytoprotective, antioxidative and anti-apoptotic defense, and is readily turned on by numerous natural chemopreventive agents. Hence, the potential of Nrf2 as a molecular target in chemoprevention and in targeting oxidants-induced pathologies has been promising and is currently under intense research.

In my thesis, the role of Nrf2 was further investigated for its possible protection against endogenous toxic bile acid in the liver and colon. We found that activation of Nrf2 and its target antioxidative genes was an adaptive cellular response of liver and colonic cells to toxic bile acids. Silencing of Nrf2 via RNA interference (RNAi) \textit{in vitro} in human cells and \textit{in vivo} Nrf2 disruption in mice demonstrated a heightened sensitivity to toxic bile acid challenge. By serendipity, I have also discovered the most biologically active vitamin A derivatives, namely retinoids or retinoic acids, which are classically known to activate retinoid receptors, to be potent Nrf2 inducers as well. Retinoic acid treatments led to increased production of a reactive lipid peroxide metabolite namely, 4-hydroxynonenal. This reactive compound is one of the major players causing oxidative injury and cell death, and has been reported to activate Nrf2. As knockdown of Nrf2 has led to reduced cell resistance to retinoid toxicity, the protective role of Nrf2 in retinoid toxicity is proposed. Complementary to this, my studies also extended a search into the molecular regulations of Nrf2 in some important ABC efflux transporters. Human hepatic MRP2, MRP3 and BCRP (ABCG2) activated by bile acids and other Nrf2 activators appeared to depend on Nrf2. These transporters add to the list of cytoprotective genes orchestrated by this transcription factor against detrimental oxidative stress.
1.2 History and Components of Nrf2-Keap1-ARE Transcription Pathway

1.2.1 Nrf2

The discovery of Nrf2 originates from studies of β-globin gene expression with the characterization of the locus control region as having critical regulatory properties (Tuan et al., 1985; Forrester et al., 1986). This regulatory region contains a tandem AP1-NFE2 (activating protein 1 and nuclear factor erythroid 2) motif, originally termed as the DNase hypersensitive site 2, which has a strong enhancer activity (Moi & Kan, 1990; Ikuta & Kan, 1991). Subsequent work to clone the transcription factors that bind to this AP1-NFE2 site have identified several members belonging to the cap ‘n’ collar (CNC) subfamily of the basic leucine zipper (bZIP) transcription factors; among the first identified were p45-NFE2 (Chan et al., 1993), Nrf1 (Chan et al., 1993) and Nrf2 (Moi et al., 1994). Nrf3 (Kobayashi et al., 1999) and two distantly related proteins, i.e., Bach1 (Oyake et al., 1996) and Bach2 (Muto et al., 1998), are other CNC family proteins discovered and characterized later. These proteins function as heterodimeric transcription factors by dimerizing with other bZIP proteins such as small Mafs (sMafs)(Igarashi et al., 1994). Targeted gene disruption of Nrf1 in mice resulted in embryonic lethality with severe anemia and liver abnormality being suggested to be the primary culprits (Chan et al., 1998). Nevertheless, Nrf2 disrupted mice were normal, fertile, and did not show a phenotype of developmental deficits (Chan et al., 1996; Itoh et al., 1997), suggesting that Nrf2 is not essential for murine development and survival.

1.2.2 Keap1

Detailed analysis of Nrf2 activity and structure across various species has identified six evolutionarily conserved domains, named Neh (Nrf2-ECH<chicken Nrf2>homologous domain)(Itoh et al., 1997). A summary of roles and functions that has been described for these conserved domains is depicted in Figure 1. The domain in the N terminus, Neh2, was discovered as having a negative regulatory role for the trans-activating activity of Nrf2 (Itoh et al., 1999). Deletion of Neh2 was found to remarkably increase Nrf2's transactivation activity, which hinted that the Neh2 may contain a critical interaction site to which the negative regulator of Nrf2 binds. Using a yeast two-hybridization system and Neh2 as a bait, Keap1 (Kelch-like ECH-associating protein 1), a zinc metalloprotein, was identified to be the major protein, represented 80% of the
Figure 1. Structural topology of Nrf2 and function of its conserved domains. There are six highly conserved domains (Neh1 to Neh6) in Nrf2. Neh2, the most highly conserved domain among species, is located in the N-terminus which was found to interact with Keap1. At C-terminus where Neh1 and Neh3 and a basic leucine zipper structure reside, dimerization with small Mafs and binding to ARE occur. Neh4 and Neh5 were identified to interact with co-activator CBP/p300 during transcriptional activation in the nucleus. Nuclear export signals which may shuttle nuclear Nrf2 to the cytoplasmic proteosomal degradation system have recently been identified to reside in the leucine zipper and Neh5 domain.
independent clones isolated, interacting with the Neh2 domain. Based on Keap1 cDNA sequences, the primary structure of murine Keap1 was predicted to be composed of 624 amino acids and there is ~95% homology between human and mouse (Itoh et al., 1999). Molecular dissecting analysis suggests that Keap1 consists of five domains: the N-terminal region (NTR), the BTB/POZ (Bric-a-brac, tramtrack, broad-complex/poxvirus zinc finger), the intervening region (IVR), the double glycine repeat (DGR) or Kelch domain, and the C-terminal region (CTR)(Itoh et al., 2004A). The BTB/POZ domain is involved in protein homodimerization and heterodimerization, making homomeric and heteromeric multimers of Keap1 (Yoshida et al., 1999). In addition, both BTB and IVR domains are involved in proteosome-dependent Nrf2 degradation (Kobayashi, et al., 2004). The DGR or Kelch domain binds to the Neh2 domain of Nrf2, anchoring Nrf2 onto actin cytoskeleton (Itoh et al., 1999; Kang et al., 2004). From crystal structure analysis of the Kelch domain of human Keap1, it was revealed to have six structurally similar β-propeller blades (Li et al., 2004B). These inter- and intra-blades, which are tied firmly by hydrogen bonds, are believed to construct the complex structure of Keap1-Nrf2 and its anchorage to actin. The C-terminal region of Keap1 was also shown to bind to the Neh2 domain of Nrf2 (Tong et al., 2006A). A “two-site molecular recognition model” has thus been proposed for Keap1-Nrf2 complex whereby the two motifs, namely DLG and ETGE, in the Neh2 of Nrf2 independently associate with the Keap1-DC (DGR and CTR)(Tong et al., 2006B). This double tethering of Nrf2 with Keap1 is thought to contribute to the overall stability of Keap1-Nrf2 complex.

1.2.3 ARE

The antioxidant responsive element or ARE was first identified to be a DNA consensus motif (gene enhancer) on the 5′-flanking region of the rat glutathione s-transferase Ya (GST Ya) whose function was executed upon exposure to electrophilic and planar aromatic compounds and phenolic antioxidants (Friling et al., 1990; Rushmore et al., 1990). Further sequence analysis by means of gene reporter assays delineated that the core ARE is essentially represented by 5′-RGTGACnnnGC-3′ or 3′-YCACTGnnnCG-5′ (Rushmore et al., 1991). In addition to being antioxidant-inducible, this ARE could as well contribute to the basal (constitutive) expression of this rat GST isoform. At that time, the transcription factor(s) responsible to interact with this consensus motif remained unknown. Nevertheless, the high similarity between this ARE sequence and the NFE2-
AP1 motif, which was found to bind Nrf2 and other AP-1 family of transcription factors, as well as the TRE [phorbol-12-O-tetradecanoate-13-acetate (TPA)-responsive element] type-Maf recognition element (T-MARE) has been noted (Kataoka et al., 1994; Xie et al., 1995; Prestera et al., 1993).

The first demonstration that ARE as the cognate enhancer of Nrf2 came from the work of Venugopal and Jaiswal (1996). They identified a cis-element resembling the documented ARE sequence in 5′-flanking regulatory region of the human NQO1 [NAD(P)H oxidoreductase 1] can physically bind Nrf1 and Nrf2 which corresponded with an increased transactivation activity and NQO1 gene induction. Further evidence was subsequently provided by Itoh and coworkers (1997) who observed an impaired constitutive and butylated hydroxyanisole (BHA)-induced expression of the phase II enzymes GSTs Ya and Yb in Nrf2-disrupted mice. Along with this, binding of Nrf2-MafK heterodimer to ARE of the GST Ya gene that was identified earlier (Rushmore et al., 1990) has also been convincingly shown. Questions remain as to whether the ARE sequences between different species are similar and whether there exists an indispensable nucleotide sequence in the core ARE of all ARE-regulated genes. It was found that an active ARE located in the promoter of human glutamate cysteine ligase modulatory subunit (GCLM) gene has a variant ARE sequence (Erickson et al., 2002). These findings called upon a revision of the core ARE to 5′-RTKAYnnnGCR-3′.

1.2.4 Nrf2 transcription complex

Upon activation, cytosolic Nrf2 liberates itself from physical entrapment and/or negative regulation of Keap1 and accumulates in the cell nucleus to bind to its cognate enhancer ARE, thereby inducing a battery of target cytoprotective genes. The small Maf (sMaf) proteins consisting of MafK and MafG are the most common heterodimer partner recruited by Nrf2 to the transcription initiation complex (Itoh et al., 1997). The exact role of sMaf in Nrf2 transactivation activity on ARE remains controversial, with studies showing their participation as positive (Itoh et al., 1997) as well as negative regulators (Venugopal & Jaiswal, 1996). Other bZIP proteins, such as AP-1 (JunD, c-Jun/c-Fos, FRA-1), ATF4 and PMF-1, were suggested mainly by the gene-promoter analysis to also interact/partner with Nrf2, affecting ARE-regulated genes (Jaiswal, 2004). Similar to sMaf proteins, whether these stress-responsive proteins participate in Nrf2 transactivation as co-activators or co-repressors have been controversial with mixed
reports. Other than this, the cyclic AMP responsive element binding protein (CBP)/p300 is a histone acetyltransferase-derived co-activator protein identified to be part of the transcription complex of Nrf2 (Katoh et al., 2001). It is noteworthy that the AP-1 proteins, sMafs, and other bZip proteins can also bind to ARE independent of Nrf2 (Venugopal & Jaiswal, 1996; Tsuji, 2005; Yang et al., 2006), although the nucleotide sequence of cis-acting element may preferentially select certain sets/combinations of these proteins (Yamamoto et al., 2006). The versatility of ARE to interact with multiple transcription factors enables upregulation of ARE-mediated genes in response to various signals and inducers of cellular stress. Whether there exist hierarchical, temporal and spatial, and cell- and/or species-specific regulation of the ARE-mediated genes by various transcription factors, further investigation is warranted.

1.3 Mechanisms of Nrf2 transcription regulation

1.3.1 Role of Keap1-dependent negative regulation

Over the last few years the mechanisms by which the Nrf2 transcription system is orchestrated via collaboration with multiple interacting proteins during unstressed/homeostatic state and during oxidative stress are continually being uncovered. Keap1 appears to play a key regulatory role in Nrf2 activity by physically entrapping Nrf2 inactive in cytoplasmic actin filaments (Kang et al., 1994; Itoh et al., 1999). The cytoplasmic entrapment of Nrf2 and formation of Keap1-Nrf2 anchorage complex renders low basal expressions of ARE-regulated genes in cells under normal physiological conditions. During oxidative and/or electrophilic stress, increased Nrf2 accumulation in the nucleus represents a signature, pre-requisite event of Nrf2-ARE transcription activation (Itoh et al., 1995; Kensler et al., 2006).

Although there is much debate as to how Nrf2 is released from Keap1’s entrapment or negative regulation that leads to its translocation to the nucleus, studies have in consensus shown that Keap1 is the major repressor of Nrf2 through multiple regulatory mechanisms. From in vitro overexpression studies using ARE reporter assays, Keap1 coexpression in cells markedly mitigated Nrf2 transactivation activity (Itoh et al., 1999). The repressive activity of Keap1 can be reversed to a great extent by antioxidant treatments, suggesting that the Keap1-Nrf2 complex may be destabilized by changes in cellular redox state. Moreover, Keap1 knockout mice had a very high constitutive expression of ARE-regulated genes with a persistent accumulation of nuclear Nrf2
Such characteristics were shown to be absent in Keap1:Nrf2 double knockout mice. Recent studies found that Nrf2 hyperactivity in a small cell lung carcinoma cell line (NCI-H1184), 50% of lung cancer cell lines, and about 40% the cancer biopsies from non-small cell lung carcinoma tumor samples could be attributed to somatic mutations in Keap1 (Padmanabhan et al., 2006, Singh et al., 2006). For instance, G364C mutation in Keap1, which potentially affects the interaction with Nrf2, drastically upregulated Nrf2 target genes, NQO1, and heme oxygenase (HO-1) by 15-20 fold in NCI-H1184 cells when compared with other lung carcinoma cells harboring wildtype (unmutated) Keap1 (Padmanabhan et al., 2006). Taken together, both in vitro and in vivo studies unequivocally show that Keap1 is a specific negative regulator of Nrf2.

**Electrophilic cysteine sensors of Keap1**

The observation that Keap1 contains high density of cysteine residues suggests that it may be a sensor for oxidative/electrophilic inducers. These cysteine residues are known to be vulnerable to sulfhydryl modification by reactive radicals and electrophiles. When the modification occurs in critical sites, it may potentially trigger significant structural and functional changes in macromolecules. In a purified Keap1-Nrf2 interacting system, the most reactive cysteine residues of Keap1 (C257, C273, C288, C297) can be irreversibly modified by a thiol modifier, dexamethasone mesylate (Dinkova-Kostova et al., 2002). Subsequent studies testing electrophilic and antioxidant chemopreventive agents further confirmed that multiple cysteine sites of Keap1 can be modified in similar but not entirely uniform manner (Hong et al., 2005A & 200B). It has been suggested that the sites at which sulfhydryl adduction or modification takes place may differ among various classes of oxidative/electrophilic inducers as well as across different species (Dinkova-Kostova et al., 2005). Meanwhile, the order of inducer potency in driving Nrf2-Keap1-ARE transactivation has been shown to be in highly correlated with the order of their reactivity as Michael acceptors or affinity for sulfhydryl groups (Dinkova-Kostova et al., 2001; Kensler et al., 2007). Despite this, the direct evidence or in vivo demonstration of changes in Keap1’s structural conformation upon cysteine modification which leads to Nrf2 release from Keap1 or activation of Nrf2-ARE transcription has not been conclusively established.
Keap1-dependent and -independent Nrf2 degradation

The negative regulatory role of Keap1 is more recognized for its role in regulating Nrf2 homeostasis through promoting Nrf2 degradation. The observations that Nrf2 protein had a short half-life (20 min) and was expressed at low levels despite abundance of its mRNA transcripts during unstressed state suggest a rapid degradation mechanism occurring to Nrf2 in homeostatic conditions (Itoh et al., 2003; McMahon et al., 2003; Nguyen et al., 2003). This rapid turnover of Nrf2 protein results from a facilitated ubiquitin-proteosomal degradation system (Sekhar et al., 2002; Nguyen et al., 2003; Stewart et al., 2003) in which Keap1 appears to play an important role. Using genetically engineered Nrf2-LacZ knock-in mice, the redox-sensitive Neh2 domain of Nrf2 was shown to participate in the proteosome-dependent degradation of Nrf2 (Itoh et al., 2003). Keap1 interacts with this Neh2 domain of Nrf2 to enhance proteosome-dependent degradation (McMahon et al., 2003). Likewise, prolonged Nrf2 half-life or stabilization of Nrf2 protein upon stress inducer exposure is thought to result from disrupted interaction between Keap1 and the Neh2 domain (Nguyen et al., 2003; Stewart et al., 2003).

Zhang and Hannink (2003) reported that the two cysteines (C²⁷³ amd C²⁸⁸) in the IVR domain of Keap1 are required for Keap1-mediated Nrf2 ubiquitylation and degradation; however, the essential role of these cysteines in Nrf2 ubiquitylation has not been agreed upon by others (Kobayashi et al., 2004; Kobayashi et al., 2006). The involvement of IVR as well as BTB domains of Keap1 in Nrf2 degradation has been shown to be crucial in recruiting ubiquitin-proteosome factors (Kobayashi et al., 2004). Cullin 3 (Cul3), a subunit of the E3 ligase complex, was found to interact with the IVR domain of Keap1 to promote ubiquitylation of Nrf2 in a cooperative ubiquitin-proteosome system known as the Cul3-Roc1 complex. These findings suggest that Keap1 functions as an adaptor, whereas Nrf2 is a substrate, in the Cul3-based E3 ligase system. The formation of homodimer Keap1 is necessary for its function as substrate adaptor (McMahon et al., 2006), an observation in line with an earlier report showing that homodimerization of Keap1 at BTB/POZ is needed for sequestering Nrf2 in cytoplasm (Zipper & Mulcahy, 2002).

Additionally, another mode of proteosomal degradation pathway which is Keap1-independent appears to also operate in controlling Nrf2 turnover (Itoh et al., 2003). This degradation pathway of Nrf2 was thought to be less efficient, and functions through
recruitment of particularly nuclear Nrf2 presumably after participation in the transcription complex (Itoh et al., 2004A). Overall, accumulating evidence to date underpins the importance of the Keap1-mediated proteosome-dependent degradation pathway in regulating Nrf2 protein turnover, especially during unstressed/homeostatic state. It is unknown whether this pathway is also important in Nrf2 turnover during oxidative stress state.

1.3.2 Role of stress-mediated cell signaling kinases

Additional regulatory mechanisms, such as stress-mediated cell signaling kinase cascades, may collaborate with Keap1 in regulation of Nrf2 and ARE-mediated genes. It has been postulated that inhibition of Keap1-dependent ubiquitylation/degradation may stabilize Nrf2 protein and that additional post-translational modifications to the Nrf2-Keap1 complex is necessary for a complete dissociation of Nrf2 from Keap1, and hence Nrf2 translocation to the nucleus (Shen et al., 2005; Kensler et al., 2007). Although largely unclear, multiple stress-related signaling pathways, such as the mitogen-activated protein kinase (MAPK), p38 MAPK, phosphotidylinositol 3-kinase (PI3K), c-Jun-N-terminal kinase (JNK), endoplasmic reticulum (ER)-resident kinase (PERK), and protein kinase C (PKC) have been documented to mediate Nrf2 activation and/or regulation of ARE-responsive genes. The involvement of these cell signaling pathways in activating the Nrf2-Keap1-ARE transcription machinery is, however, inconsistent and varies among studies, presumably factors such as different cell system/types, species, and/or Nrf2 inducers used in experiments may have distinct modulatory roles in the interaction.

Protein kinase C (PKC)

PKC is the only signaling kinase to directly phosphorylate Nrf2 in vitro and in vivo. The phosphorylation site of Nrf2 by PKC is at Ser40 which is phylogenetically conserved and located in the Neh2 domain of Nrf2 to which Keap1 binds (Huang et al., 2002). It has been shown that modification at Ser40 is required to disrupt Nrf2 association with Keap1, which corresponded to an increased ARE-mediated activation (Huang et al., 2002). These observations were confirmed in another study, although Nrf2 phosphorylation was found to be dispensable for Nrf2 stabilization/accumulation in the nucleus and subsequent ARE-mediated NQO1 gene expression (Bloom & Jaiswal, 2003). In agreement, phosphorylation of Nrf2 by PKC did not modulate affinity of Nrf2/MafK
complex binding to the ARE of NQO1 in vitro (Huang et al., 2002). These studies imply that PKC phosphorylation of Nrf2 may be an important mechanism to disrupt cytoplasmic Nrf2-Keap1 interaction, but this process is not mandatory for nuclear Nrf2 stabilization or target gene transactivation.

ERK1/2 MAPK
Participation of the MAPK cascade pathway in Nrf2-ARE-mediated transactivation has been reported even much earlier than that of PKC (Yu et al., 2000; Kong et al., 2001; Zipper & Mulcahy, 2000). The extracellular signaling-related kinases 1/2 (ERK1/2), a downstream, multi-target kinase effector of MAPK, is capable of phosphorylating a variety of transcription factors important in preserving cell survival during cellular stress (Junttila et al., 2008). Using in vitro kinase assays, purified recombinant Nrf2 was found to be a substrate of ERK (Zipper & Mulcahy, 2000; Xu et al., 2006). However, by means of site-directed mutagenesis approaches, Zipper and Mulcahy (2003) reported that mutation at all conserved consensus phosphorylation sites of Nrf2 did not reduce its ability to respond to pyrrolidine dithiocarbamate (PDTC) treatment and to execute ARE-mediated transactivation. Hence, these findings suggest the possibility that Nrf2 is a direct phosphorylation target of ERK1/2 MAPK, but, similar to that reported for PKC, the consequent phosphorylation may not be essential for Nrf2 activation and induction of ARE-regulated genes. The precise mechanism through which the MAPK pathway activates Nrf2 transcription machinery has yet to be elucidated. Studies, however, have shown that the co-activator CBP/p300 of Nrf2 transcription complex can be directly phosphorylated by the MAPK cascade which increases Nrf2-ARE transactivation activity (Shen et al., 2004A). Forced ectopic expression of CBP/p300 can also dose-dependently upregulate the Nrf2 target gene thromboxane A2 synthase in lung cells (Yaekashiwa & Wang, 2003). Therefore, targeting CBP/p300 may account for a potential mechanism by which MAPK enhances Nrf2 transactivation.

PI3K-AKT
Nrf2 activation and enhanced ARE-regulated genes by various Nrf2 activators including chemopreventive agents and heavy metals in a number of cell models has often been associated with activation of the PI3K and its downstream AKT (PI3K-AKT), another ubiquitous cell-survival signaling kinase (Martin et al., 2004; Kang et al., 2007; Li et al.,
Similar to ERK MAPK, the unified mechanism of how PI3K activates Nrf2 transcription is still unknown. Nevertheless, PI3K mediating nuclear translocation of Nrf2 in rat-derived cells has been suggested to involve cytoskeletal modifications which may destabilize the Keap1-Nrf2 complex (Kang et al., 2002). It was shown that BHQ-induced PI3K activation could trigger rearrangement and depolymerization of actin microfilaments and cause Nrf2-actin complex to translocate into nuclear/perinuclear compartments. Whether such translocation of Nrf2-actin complex will directly result in enhanced Nrf2 transcription and induction of ARE-regulated genes has not been shown. Recently, the crosstalk between PI3K-AKT and ERK MAPK pathways has been described to stimulate Nrf2-ARE transcription machinery in hyperoxia-exposed pulmonary epithelial cells (Papaiahgari et al., 2006). These observations support a hypothesis that ERK MAPK may also be a downstream target of PI3K-AKT, which may explain the inhibition of either pathway has equally attenuated the Nrf2-ARE transactivation in many experiments.

**p38 MAPK**

On the other hand, the role of p38 MAPK in Nrf2-ARE transcription has encountered conflicting results. Activation of p38 MAPK is common in various cell systems exposed to oxidative stress. Studies using mainly selective pharmacological inhibitor reagents of p38 MAPK reported a positive regulation of this pathway in Nrf2-ARE transcription (Alam et al., 2000; Bologun et al., 2003). On the contrary, the negative regulatory role of p38 MAPK in Nrf2 and ARE-mediated gene activation was noted in studies employing ectopic expression of dominant positive p38 MAPK isoforms (Keum et al., 2006). Nrf2 was also found to be a substrate of p38 MAPK in vitro and the resultant phosphorylation could instead promote association of Nrf2 with Keap1. The opposing effects were reasoned by Keum and colleagues to result from the possible unspecific effects of the pharmacological inhibitors being used which were thought to also interfere with the other kinase pathways involved in Nrf2 activation. Considering that p38 MAPK is known to counterbalance or oppose effects of ERK activation (Xia et al., 1995; Park et al., 2003), it is likely that p38 MAPK is a mediator in the negative feedback loop against positive regulation of ERK MAPK.

**JNK**
Involvement of the JNK pathway in enhancing Nrf2-ARE transcription has been questionable with studies reporting positive regulation (Xu et al., 2006A) or no interaction (Alam et al., 2000). The direct interaction of JNK with the Nrf2-ARE transcription has not been shown, although JNK was able to phosphorylate recombinant Nrf2 in \textit{in vitro} settings (Xu et al., 2006A). In view of the present knowledge, JNK activation is closely associated with cell death and apoptosis induction, with contrasting effects to ERK MAPK activation (Xia et al., 1995; Junttila et al., 2008). Thus, the enhancement of Nrf2 transcription by JNK would indeed contravene the conventional role of Nrf2 as an anti-apoptotic factor.

**PERK**

Cellular stresses, such as triggered by hypoxia, glucose deprivation, and agents causing disturbed calcium homeostasis and protein glycosylation, are able to invoke stress to endoplasmic reticulum (ER) and activate PERK. This kinase has been shown to phosphorylate and physically associate with Nrf2 (Cullinan et al., 2003). The PERK-dependent phosphorylation leads to disruption of Nrf2-Keap1 complex, nuclear Nrf2 accumulation, and enhanced transcription of ARE-regulated genes and cell survival (Cullinan et al., 2003; Cullinan & Diehl, 2004; Liu et al., 2005).

In summary, the above major cell signaling pathways remain a fascinating topic to be explored with respect to their divergent modulatory roles in Nrf2-ARE transcription and their implications in general health and disease. Particularly, the declined Nrf2 transactivation activity observed in aged rodents (Suh et al., 2004) may be a result of decreased ERK MAPK activity reported to follow with aging, which may be implicated in less tolerance to oxidative stress in this population group (Ikeyama et al., 2002).

**1.3.3 Other modes of regulation**

**Positive regulators**

Growing evidence suggests more complex regulatory mechanisms may be involved in the Nrf2-Keap1-ARE transcription machinery. At least four activator proteins having positive regulation towards Nrf2-ARE functional axis have been recently discovered. BRCA1, a well-studied tumor suppressor with its functional mutations linked to increased risks of breast, ovarian and prostate cancers, was found to be an activator of Nrf2 (Bae et al., 2006). Impaired BRCA1 activity, which negatively impacts Nrf2, may promote
carcinogenesis by decreasing cell resistance against oxidative stress. Further, the DJ-1/PARK7, a cancer- and Parkinson’s disease-associated protein implicated in cytoprotection against toxic stresses, was found to increase ARE-mediated NQO1 by stabilizing Nrf2 via disrupting Nrf2-Keap1 complex and subsequent Nrf2 degradation in transformed and primary cell lines of both human and mouse species (Clements et al., 2006). In a genome-wide screening analysis aiming at identifying activators of ARE, Liu et al. (2007) reported that sequestosome 1 (SQSTM1) and dipeptidylpeptidase 3 (DPP3) in primary mouse cortical neurons and IMR-32 neuroblastoma cells can promote Nrf2-ARE transactivation through activation of PKC, PI3K and ERK MAPK signaling. These data indicate possible involvement of intermediate proteins as mediators of the stress-responsive kinases in Nrf2-ARE transactivation. In addition to CBP/p300, another histone acetyltransferase, namely monocytic leukemia zinc-finger protein (MOZ or MYST3) was recently shown to act as a coactivator for the Nrf2/MafK heterodimer complex in rat cells, thereby inducing expression of the Nrf2 target gene, glutathione transferase P (Gstp) (Ohta et al., 2007). The increase of this coactivator during early stage of chemical hepatocarcinogenesis may serve a potential contributor factor for the increased Nrf2-mediated pathway reported in some cancerous cells.

**Negative regulators**

A multitude of mechanisms has been discovered to take part in negative regulation of the Nrf2-ARE transcription machinery. The role of Bach1, a bZip protein, in competing with Nrf2 for sMaf transcription factors and for binding to enhancer ARE has been widely recognized to result in repression of Nrf2-mediated transcription and of Nrf2 target gene induction (Sun et al., 2004; Dhakshinamoorthy et al., 2005). The observations that Bach1 is recruited simultaneously by Nrf2 activators in a dynamic exchange with Nrf2 for the Maf transcription factor network imply that Bach1 may be a negative feedback regulator in controlling Nrf2-ARE mediated transactivation. Other alternative regulatory pathways of Bach1, including reduced protein stability (Shan et al., 2006) and inactivation of Bach1 (Reichard et al., 2007), upon oxidative inducer exposure have been suggested to induce HO- gene via the Nrf2-ARE transcription pathway. Similar to Bach1, other bZIP proteins such as Nrf3 (Sankaranaryanan & Jaiswal, 2004) and p65 isoform of Nrf1 (Wang et al., 2007A) may also act as negative regulators/inhibitors of Nrf2 transcription by competing with Nrf2 for sMaf and/or ARE binding. Meanwhile,
activating transcription factor 3 (ATF3) has been shown to displace coactivator of Nrf2, namely CBP, as a mechanism in its repressive effects on Nrf2 transactivational activity (Brown et al., 2008).

Most recently, retinoic acid receptors (RARs), particularly RARα, which are activated by retinoic acids, were identified to be potent inhibitors of Nrf2 transcription and ARE-mediated gene transactivation (Wang et al., 2007B). When mice were fed a diet deficient in vitamin A, Nrf2 target gene expression was increased in their intestine and such induction was abrogated with replenishment of dietary vitamin A or peritoneal injection with retinoic acids. The mechanism whereby such inhibition is orchestrated remains obscure, although physical association of RARα with Nrf2 has been noted corresponding to the inhibitory action. This report appears to shed light on the potential detrimental effects of dietary vitamin A/carotenoids in lung cancer prevention in previous clinical trials (Omenn et al., 1996). At high concentrations, retinoid acids are capable of producing peroxyl radicals and prooxidative effects (Samokyszyn & Marnett, 1987; Freyaldenhoven et al., 1998). Therefore, negation of Nrf2 activation by retinoic acids may potentially disable the cell to respond adaptively to resultant oxidative stress. However, the inhibitory effect of retinoic acids appears to contradict the reported antioxidative/prooxidative effects from many vitamin A and carotenoid studies in which induction of Nrf2-target antioxidant and phase II enzymes was evident (Burton & Ingold, 1984; Ben-Dor et al., 2005). It has been shown that RAR/RXR activation, which forms a transcription complex with their cognate enhancers, was sensitive to cellular redox state (Demary et al., 2001). The activation was shown to be enhanced by cellular reducing states, but an oxidative state inhibits it. Taken together, these findings suggest that the interaction of RAR with Nrf2 may be influenced by cellular redox balances.

A distinct set of kinase signaling has also been implicated in negative regulation of Nrf2 transcription. It has recently been demonstrated that the tyrosine 568 of Nrf2 is critically involved in its CRM1-mediated nuclear export control which is a coordinated mechanism responsible for Nrf2 dissociation from the nuclear transcription complex, removal of nuclear Nrf2 residence, and subsequent Nrf2 degradation through cytoplasmic proteosome pathways (Jain & Jaiswal, 2006). This tyrosine residue is subject to phosphorylation by the Fyn kinase via regulation of GSK-3β (Jain & Jaiswal, 2007). These interesting observations of Fyn kinase-mediated Nrf2 deactivation and degradation have been shown to occur with exposure to high dose UVB radiation which
resulted in Nrf2 deactivation and suppression of ARE-mediated protective genes (Kannan & Jaiswal, 2006). The interaction and interplay of various regulatory mechanisms/pathways that modulate the Nrf2-Keap1-ARE transcription machinery is depicted schematically in Figure 2.

1.4 Inducers and Activators of Nrf2

1.4.1 Categorization of chemical inducers

Composed by a high cysteine/sulfhydryl residue content, Keap1, which regulates Nrf2 activity, is structurally designed to respond to electrophiles, reactive oxygen species (superoxide, peroxide, and hydroxyl radicals), reactive nitrogen species, and other reactive byproducts such as 4-hydroxynonenal (4-HNE). Hence, chemical inducers capable of modifying cysteine residues are likely also activators/inducers of Nrf2. Following Nrf2 activation, induction of phase II and antioxidative enzymes, particularly GST and NAD(P)H oxidoreductase 1 (NQO1), and elevated GSH levels are characteristic cellular events. Based on known structural-chemical activity, chemical inducers capable of inducing this phase II system are grouped into ten chemically distinct categories (Prestera et al., 1993; Dinkova-Kostova et al., 2005): (1) oxidizable diphenols, phenylenediamines, and quinones; (2) Michael acceptors; (3) isothiocyanates; (4) thiocarbamates; (5) trivalent arsenicals; (6) dithiolethiones; (7) hydroperoxides; (8) vicinal dimercaptans; (9) heavy metals; and (10) polyenes.

The notion that endowment of high sulfhydryl groups is a vital feature of an oxidative stress sensor is supported by a number of observations. First, the inducer potency of the phase II system is positively correlated with the electron withdrawing power of the substitutes at the para position within the methyl cinnamate analogs (Spencer et al., 1991) and with the rate of reactivity with sulfhydryl reagents within the phenolic Michael reaction acceptors (Dinkova-Kostova et al., 2001). Second, the order of inducer potency in the class of heavy metals is highly aligned with the order of their affinity for sulfhydryl residues with Hg\(^{2+}\) being the most potent, followed by Cd\(^{2+}\) and Zn\(^{2+}\) (Talalay et al., 1988).

1.4.2 Chemopreventive agents

Chemopreventive agents are usually referred to as a class of plant-derived compounds known as phytochemicals which are able to activate the Nrf2 transcription machinery and
Figure 2. Schematic illustration of the Nrf2-Keap1-ARE transcription network. Nrf2 is tightly regulated by the ubiquitin-proteosome degradation pathway mediated by Keap1. Various cellular stressors/stimuli including cell signaling kinases and regulatory proteins can influence Keap1-mediated Nrf2 degradation, stabilization and nuclear export/import of Nrf2, and Nrf2-ARE mediated gene activation.
induce cytoprotective genes (Surh, 2003). Induction of these protective genes is a well documented anti-carcinogenic effect dedicated to these chemopreventive agents. It may be terminologically inappropriate to attribute this Nrf2-activating effect to their “antioxidative” properties. Antioxidants are molecules with potential to accept donor electrons from strong oxidants which can in turn become reactive oxidative compounds or “prooxidants” at high concentrations. The fact that chemopreventive agents can activate the oxidative stress-responsive Keap1-Nrf2 system literally suggests an involvement of their prooxidative effects. In light of this, studies have shown that these chemopreventive agents require supraphysiological concentrations (≥ 10⁻⁵ M) to exert their effects on Nrf2 transactivation (Keum et al., 2006; Kang et al., 2007). At such high concentrations, it is likely that the prooxidative effect may have been implicated. Consistent to this notion, the potency of flavonoids to induce ARE-mediated gene expression correlates well with their ability to induce cellular oxidative stress and redox cycling (Lee-Hilz et al., 2006).

It is intriguing how a prooxidative effect, which by general understanding may have caused oxidative damage, may turn out to be chemoprotective. Emerging evidence has shown that exposure to mild to intermediate stresses, as may have been imposed by these prooxidative chemopreventive agents, may indeed confer an adaptation advantage by invoking enhanced cellular protective and tolerance mechanisms. For example, prior exposure to non-lethal, moderately toxic 4-HNE, a reactive lipid peroxidation-derived aldehyde, was shown to facilitate a pre-emptive adaptive response to future greater oxidative stress attack (Chen et al., 2005A). Nrf2 was found to be a key mediator in such adaptation mechanism. The chemopreventive agents identified to be Nrf2 activators include curcumin which is found in turmeric leaves (Kang et al., 2007), sulforaphane/isothiocyanate analogs in broccoli (Thimmulappa et al., 2002), lycopene and carotenoids in tomatoes/carrots (Ben-Dor et al., 2005), epigallocatechin-3-gallate in green tea (Na & Surh, 2008), garlic organosulfurs (Chen et al., 2004A), quercetin (Tanigawa et al., 2007), and resveratrol in grapes/red wine (Kode et al., 2008).

1.4.3 Bifunctional inducers
Bifunctional inducers are a group of polycyclic aromatic compounds that can transactivate genes via xenobiotic responsive element (XRE) and ARE by activating both aryl hydrocarbon receptor (AhR) and Nrf2, respectively. AhR is a basic-helix-loop-helix
transcriptional factor which can be activated by numerous polycyclic and halogenated aromatic hydrocarbons and endogenous ligands such as bilirubin to upregulate genes involved in specific immunologic, toxic, and adaptive responses (Denison & Nagy, 2003). There are two classes of bifunctional inducers: metabolizable and poorly/non-metabolizable. The metabolizable inducers, e.g., β-naphthoflavone, α-naphthoflavone, and many polyaromatic hydrocarbons (PAH) are AhR agonists that can upregulate phase I enzymes e.g., aryl hydrocarbon hydroxylases (AHH): CYP1A1 and CYP1A2. This increased AHH activity subsequently metabolizes these inducers into electrophilic metabolites which can target Nrf2 to induce phase II enzymes as a means of cellular detoxification process (Prochaska & Talalay, 1988; Talalay et al., 2003). The poorly/non-metabolizable inducers, particularly 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can activate both AhR and Nrf2 and induce phase I and II enzymes with mechanism thought to result from a mutual interaction between the AhR/XRE and Nrf2/ARE transcription pathways (Talalay et al., 2003; Ma et al., 2004; Radjendirane & Jaiswal, 1999). Although TCDD exposure can lead to excessive oxidative stress (Stohs, 1990; Dalton et al., 2002), the direct evidence showing TCDD can activate the Nrf2/ARE pathway through resultant oxidative stress has not been reported. Nevertheless, with the identification of active XREs in the promoter region of Nrf2 gene recently, transcriptional upregulation of Nrf2 and hence Nrf2 transactivation activity through activation of AhR is now considered as a major mechanism by which TCDD activates the Nrf2 transcription pathway (Miao et al., 2005).

1.4.4 Signaling molecules

4-hydroxynonenal

The reactive α, β-unsaturated aldehyde 4-hydroxynonenal (4-HNE) is a major end product of ROS-induced lipid peroxidation on ω-6 polyunsaturated fatty acids (arachidonic acid, linoleic acid, etc), which composes a significant part of the phospholipid membrane, or on their peroxides. A major pathway of 4-HNE formation is initiated with an attack of arachidonate by hydroxyl radicals (OH•) generated through Fe2+-mediated Fenton reaction, which creates lipid free radicals. The radicals then undergo addition of oxygen, rearrangements, and cleavage of double-bond carbon bridges to form 4-HNE (Uchida, 2003). Levels of HNE produced at the membranes can achieve 10 µM – 5 mM following exposure to oxidative stressors including environmental
pollutants like nitrogen dioxide, ozone, and diesel exhaust (Uchida, 2003, Hamilton et al., 2004). This highly reactive metabolite has been implicated as the damaging culprit, directly altering function of membrane-associated complexes and causing oxidative damage of macromolecules and consequently cell death (Choudhary et al., 2002, Iles & Liu, 2005). However, studies have also shown that 4-HNE is a potent signaling molecule, which can trigger adaptive responses including the Nrf2 and AP-1 transcription machineries through activating various stress-related signaling pathways involving ERK MAPK, JNK and/or PI3K (Liu et al., 2001; Usatyuk & Natarajan, 2004; Chen et al., 2005A&200B). The reactivity of 4-HNE toward Nrf2 may in part be due to its alkylating and Michael acceptor's properties which can target nucleophilic centers and sulfhydryl groups to form HNE-protein adducts (Iles & Liu, 2005). In fact, exposure to moderate to intermediate levels of 4-HNE that are sufficient to activate Nrf2 without causing extensive cytotoxicity has been shown to elicit an advantageous adaptation-promoting mechanism which pre-conditions cell tolerance/resistance towards future oxidative stress (Chen et al., 2005A). Moreover, moderate levels of 4-HNE were able to induce uncoupling of mitochondria through the uncoupling proteins located within the mitochondrial inner membrane which in turn decreases mitochondrial ROS production (Echtay et al., 2003). Thus, 4-HNE is now regarded as having a dichotomous role determined by its cellular concentration. Exposure to high amounts of 4-HNE is extremely toxic to the cells and causes irreversible cell death; whereas a low to moderate amount may instead become a beneficial biological signal that facilitates adaptive responses and cell survival during oxidative stress. This characteristic is in concordance with its differential effects on cell cycle signaling events which are also concentration-dependent (Awasthi et al., 2005).

15-Deoxy-Δ12,14-prostaglandin J2
15-Deoxy-Δ12,14-prostaglandin J2 (15-d-PGJ2) is a well-defined member of the J2 series of the cyclopentenone prostaglandins (PGs) derived from the ω6 polyunsaturated fatty acid arachidonic acid. It is an endogenous ligand of peroxisome proliferator-activated receptor γ (PPARγ), exerting cytoprotective, anti-inflammatory, anti-apoptotic, and anti-proliferative activities in a cell type- and concentration-specific manner (Straus & Glass, 2001). Owing to its electrophilic α, β-unsaturated carbonyl group in the cyclopentenone ring, 15-d-PGJ2 can behave as a Michael acceptor, forming covalent adducts with
sulfhydryl residues (Kim & Surh, 2006). As such, growing evidence has demonstrated that 15-d-PGJ$_2$ is able to activate the Nrf2 transcription machinery to induce HO-1, peroxiredoxin-1, and glutamate cysteine ligase (GCL) subunits (Itoh et al., 2004B; Zhang et al., 2004B; Chen et al., 2006A). In addition, 15-d-PGJ$_2$ is a signaling molecule targeting multiple redox-sensitive transcription factors. For instance, p53, an oxidative stress-responsive and tumor suppressive transcription factor, was induced by this PG, whereas the activity of other redox-sensitive transcription factors such as AP-1, NF-$\kappa$B, STAT, and hypoxia inducible factor-1 was either suppressed or inhibited (Kim & Surh, 2006).

**8-nitro-cGMP**

The biological role of nitric oxide through the second messenger cyclic GMP (cGMP) has been well documented (Feelisch, 2007). 8-nitro-cyclic GMP (8-nitro-cGMP) is a newly discovered cellular signal produced intracellularly by direct chemical modification of NO on cGMP (Sawa et al., 2007). This modification takes place more promptly during increased NO production corresponding to an oxidative state. 8-nitro-cGMP undergoes redox recycling to produce ROS and reacts with sulfhydryl groups of GSH and other proteins such as Keap1 to form thiol-cGMP adducts, accompanied by the release of nitrite (NO$_2^-$)(Feelisch, 2007). This so-called protein “s-guanylation” by 8-nitro-cCMP has been shown to potentially modify highly nucleophilic cysteine sulfhydryls of Keap1, suggesting that this nitrated cGMP has a redox-sensor signaling capacity of modulating the Nrf2-Keap1-ARE transcription machinery. 8-nitro-cGMP may as well act as the second messenger for the activation of Nrf2 by inflammatory signals, because proinflammatory stimulation by interferon-$\gamma$ or endotoxin lipopolysaccharide can induce iNOS and hence large amounts of NO in the cell which facilitates formation of this nitrated cGMP (Sawa et al., 2007).

**1.4.5 Stressors beyond oxidative stress**

Recent research has extended the classical role of Nrf2 transcription machinery in protection of oxidative stressors to encompass other environmental stressors not conventionally associated with production of oxidative stress. Endoplasmic reticulum (ER) stress, through activation of the cell survival signaling PERK (please see 1.3.2), has been shown to activate Nrf2 and induce cytoprotective genes such as HO-1 (Cullinan et al.,
Although increases in ROS or oxidative stress in parallel with ER stress were observed, treatment with the antioxidant N-acetyl-L-cysteine (NAC) did not block nuclear Nrf2 translocation by an ER stress inducer. This suggests that the Nrf2-ARE transactivation regulation by ER stress is not entirely dependent on oxidative stress or changes in cellular redox.

Biomechanical forces such as shear stresses developed by laminar blood/fluid flow on endothelial and chondrocyte cells have also been implicated in Nrf2 activation and increased ARE-regulated genes (Chen et al., 2003; Hosoya et al., 2005; Healy et al., 2005; Warabi et al., 2007; Dai et al., 2007). The underlying mechanisms of this redox regulatory process have been proposed to operate through a collective yet coordinated event involving activation of PI3K/AKT pathway, cyclooxygenase 2-induced 15-d-PGJ₂, and/or xanthine oxidase and flavoprotein-induced superoxide production and lipid peroxidation. Despite this, further increased mechanical stress which would trigger apoptosis can in turn suppress Nrf2 transcriptional activity through a mechanism believed to involve a negative feedback loop occurring between PI3K and JNK pathways (Healy et al., 2005). Similarly, shear stress from oscillatory flow on endothelial cells which is associated with pro-atherogenic effects can inhibit Nrf2 binding to its ARE (Hosoya et al., 2005). The observation that different modes of biomechanical stress give rise to differing effects on Nrf2 activation is intriguing. This implies existence of modifier mechanisms at work in the cellular dynamic network in which turning on the Nrf2-Keap1-ARE transcription system during cellular stress is part of a cellular decision in favor of cell survival.

Other non-chemical stressors with oxidative stress inducing effects such as UVA and B rays (Hirota et al., 2005; Kannan & Jaiswal, 2006), hyperoxia (Cho et al., 2002), oxidative CYP2E1 (Gong & Cederbaum, 2006), oxidized low-density lipoproteins (Bea et al., 2003), ω-3 polyunsaturated fatty acids (Gao et al., 2007), and pro-inflammatory stimuli (Rangasamy et al., 2002; Morito et al., 2003) were also found to activate Nrf2. The notion that Nrf2 activation can occur upon all circumstances of oxidative stress is being challenged. Recent studies show that high doses of UVB rays (Kannan & Jaiswal, 2006) and chemopreventive epigallocatechin-3-gallate (>100 µM)(Kweon et al., 2006) could instead result in deactivation of Nrf2 and decreased expression of Nrf2 target genes. These observations again highlight the influence of cell-type and treatment conditions, as
well as possible modifier systems at play in the negative feedback loop, in the regulation of Nrf2 transactivation.

1.5 Cytoprotective Genes Regulated by Nrf2

1.5.1 Nrf2-dependent regulation of cytoprotective genes

The induction of cytoprotective genes by Nrf2 in response to stressors represents a classical cellular adaptive response of cell defense. Using high-throughput gene expression screening by means of the cDNA microarray technology to analyze effects of oxidant treatments to mouse models bearing functional and disrupted Nrf2, a wide array of genes directly or indirectly related to oxidative stress defense have been reported to be upregulated in a Nrf2-dependent manner (Thimmulappa et al., 2002; Kwak et al., 2003A; Lee et al., 2003; Rangasamy et al., 2004; Cho et al., 2005). Treatment protocols, including type of treatment, extent of exposure, and target tissues are among the factors influencing regulation of these genes. Many of the activated genes participate in an integral network of cell defense to enhance cell survival. This network of genes can be functionally grouped into distinct classes, including: (1) antioxidative enzymes; (2) phase I, II and III xenobiotic detoxification systems; (3) transcription factor; (4) proteosome-related proteins; (5) chaperone proteins; (6) NADPH-generating enzymes (detail in the following sections and please see Figure 3). A number of genes from classes 1 – 4 have been found in separate studies to be transcriptionally regulated by Nrf2 with identification of active AREs on the gene promoter region (Table 1). The regulation by Nrf2 in these genes usually includes both basal/constitutive and stressor-inducible levels, although none of the target genes is completely dependent on Nrf2 for their expression.

Considering that functional impairment of Nrf2 in the knockout mice may result in a weaker constitutive protection against oxidative stress, the affected genes seen in the high throughput screening assays may not necessarily reflect a true Nrf2 dependency, but consequence of a weakened predisposition. For example, genes involved in the regulation of cell-cycle and cell-death pathways may likely be influenced by differential constitutive vulnerability. Therefore, it requires further studies to validate whether the activated genes observed from the above studies are direct Nrf2 transcriptional targets. Also, a more important question is whether the gene activation observed in mice can be extrapolated to that of the humans. For this, parallel analysis using appropriate human
Figure 3. Classes of Nrf2 target genes. Based on high-throughput cDNA microarray and gene-specific promoter analyses, a wide array of genes induced by oxidant stressors have been suggested to be dependent on Nrf2. These collective genes integrate in a collaborative and supportive manner in cytoprotection and chemoprevention.
Table 1. List of Nrf2 target genes with identified functional AREs on gene promoter

<table>
<thead>
<tr>
<th>Gene (species)a</th>
<th>Category</th>
<th>Location (#ARE)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR (M)</td>
<td>Transcription factor</td>
<td>-230 (1)</td>
<td>Shin et al. (2007)</td>
</tr>
<tr>
<td>AKR1B3 (M)</td>
<td>Antioxidative</td>
<td>-1045 to -951 (1)</td>
<td>Nishinaka &amp; Yabe-Nishimura (2005)</td>
</tr>
<tr>
<td>CYP2A5 (M)</td>
<td>Phase I enzyme</td>
<td>-2386 (1)</td>
<td>Abu-Bakar et al. (2007)</td>
</tr>
<tr>
<td>ETS 1 (H)</td>
<td>Transcription factor</td>
<td>-570 to -1000 (1)</td>
<td>Wilson et al. (2005)</td>
</tr>
<tr>
<td>Ferritin H (H)</td>
<td>Antioxidative</td>
<td>-4117 to -4043 (2)</td>
<td>Pietsch et al. (2003)</td>
</tr>
<tr>
<td>GSTA3 (M)</td>
<td>Phase II conjugation</td>
<td>-150 from exon 1 (1)</td>
<td>Jowsey et al. (2003)</td>
</tr>
<tr>
<td>GSTP (R &amp; M)</td>
<td>Phase II conjugation</td>
<td>-61 (R, 1); -59, -915, -937 (M, 3)</td>
<td>Ikeda et al. (2002), Ikeda et al. (2004)</td>
</tr>
<tr>
<td>GCLC (H)</td>
<td>Antioxidative</td>
<td>-3802 to -2752 (2)</td>
<td>Mulcahy et al. (1997), Wild et al. (1999)</td>
</tr>
<tr>
<td>GCLM (H)</td>
<td>Antioxidative</td>
<td>-302 (1)</td>
<td>Erickson et al. (2002)</td>
</tr>
<tr>
<td>GI-GPx (H)</td>
<td>Antioxidative</td>
<td>-133 (1)</td>
<td>Banning et al. (2005)</td>
</tr>
<tr>
<td>HO-1 (M)</td>
<td>Antioxidative</td>
<td>~ (-4k, -10k)(2)</td>
<td>Alam et al. (1999)(2003)</td>
</tr>
<tr>
<td>Keap1/INrf2 (M)</td>
<td>Transcription factor</td>
<td>-46 (1)</td>
<td>Lee et al. (2007)</td>
</tr>
<tr>
<td>MafG (M, H?)</td>
<td>Transcription factor</td>
<td>Ic-promoter (1)</td>
<td>Katsuoka et al. (2005)</td>
</tr>
<tr>
<td>Nrf2 (M)</td>
<td>Transcription factor</td>
<td>-754, -492 (2)</td>
<td>Kwak et al. (2002)</td>
</tr>
<tr>
<td>Prx1 (H)</td>
<td>Antioxidative</td>
<td>-536, -1429 (2)</td>
<td>Kim et al. (2007)</td>
</tr>
<tr>
<td>PSMB5 (M)</td>
<td>Proteosome</td>
<td>-341, -52 (2)</td>
<td>Kwak et al. (2003B)</td>
</tr>
<tr>
<td>Txn (H)</td>
<td>Antioxidative</td>
<td>-452 (1)</td>
<td>Kim et al. (2001)</td>
</tr>
<tr>
<td>TXAS (H)</td>
<td>Other</td>
<td>-86 (1)</td>
<td>Yaekashiwa &amp; Wang (2003)</td>
</tr>
<tr>
<td>TRx1 (H)</td>
<td>Antioxidative</td>
<td>-62 (1)</td>
<td>Sakurai et al. (2005)</td>
</tr>
<tr>
<td>UGT1A1 (H)</td>
<td>Phase II conjugation</td>
<td>-3430 to -3201 (1)</td>
<td>Yueh &amp; Tukey (2007)</td>
</tr>
</tbody>
</table>

*a Functional ARE is based on evidence: (1) dependence of Nrf2 in gene regulation; (2) transactivational activity of ARE by gene promoter analysis; (3) direct physical interaction of Nrf2 on the ARE sequence(s) on electrophoretic mobility shift assays and/or chromatin immunoprecipitation analysis. a Homo sapiens (H); mouse (M); rat (R).

Abbreviations: AKR1B3, aldose reductase 1B3; GI-GPX, gastrointestinal glutathione peroxidase; Prx1, peroxiredoxin 1; TXAS, thromboxane synthase; Txn, thioredoxin; UGT1A1, UDP-glucuronidase 1A1.
cell systems and/or patient tissue samples exposed to stressors of interest may provide much needed insight.

Although many genes induced coordinately by Nrf2 work in concert in cellular defense, there are certain genes which play more specific roles than others in the overall adaptive response and cytoprotection. These critical genes are generally involved in the major detoxification process of, and are highly inducible by exposure to, the respective toxic stressor. For instance, the reactive lipid peroxide byproduct 4-HNE is mainly detoxified by GSH conjugation to form the 4-HNE-GSH conjugate, a process catalyzed by glutathione S-transferases particularly those of the α-subclass (Awasthi et al., 2004). It was found that the GSH biosynthetic enzyme subunits: glutamate cysteine ligase modulatory and catalytic subunits (GCLM and GCLC, respectively) were highly induced by 4-HNE via the Nrf2 transcription pathway. The rapid conjugation of 4-HNE leads to an initial depletion of GSH followed by a gradual recovery and increased synthesis of GSH as a result of increased activity of GCLM and GCLC through transcriptional activation (Dickinson et al., 2002; Liu et al., 1998).

### 1.5.2 Antioxidative proteins

Oxidative stress occurs when there is a shift of cellular redox balance characterized by the excessive production of oxidative/electrophilic radicals which outweighs the cellular radical-scavenging capacity. If left progressing, these oxidative stressors, consisting of ROS, reactive nitrogen species (RNS), reactive electrophiles, and reactive byproducts of lipid peroxidation, are capable of damaging DNA and many other cellular components, causing cell injury. Activation of Nrf2 is now recognized as a key adaptive defense mechanism to cope with the fatal consequences of oxidative stress by mainly bolstering the antioxidative defense system. The most important cellular antioxidant defense system, the thiol system or both GSH and thioredoxin, has been shown to be the major target of Nrf2 regulation. This cellular thiol system dictates the ability of cells to resist oxidative damage and death, and is involved in electrophilic metabolism and detoxification.

**Glutathione (GSH)**

GSH, a tripeptide made of glutamate, cysteine and glycine, is the most abundant antioxidant in the cell. Of the three precursor amino acids, cysteine is the limiting factor
dictating GSH synthesis (Griffith, 1999). Administration of N-acetyl-L-cysteine (NAC), which structurally resembles cysteine with a more chemically-stable and water-soluble property, can replenish GSH store and ameliorate clinical toxicity associated with reduced GSH levels (Atkuri et al., 2007). GSH serves critical functions, including: (1) conjugating and detoxifying electrophiles; (2) maintaining the thiol status of proteins by preventing oxidation of –SH groups; (3) scavenging free radicals; (4) providing a reservoir for cysteine; (5) modulating vital cellular processes, e.g., DNA synthesis, microtubular maintenance, and immune function; (6) mitochondrial protection and apoptosis regulation; and (7) cotransport peptide for efflux function of some ABC transporters (Lu, 1999; Fernandez-Checa & Kaplanowitz, 2005; Kruh & Belinsky, 2003). GSH depletion upon xenobiotic challenge denotes a weakened cell defense and increased sensitivity to toxicity.

Nrf2 occupies a pivotal role in the maintenance of GSH homeostasis by transcriptionally regulating key enzymes particularly GCLM/GCLC and glutathione peroxidase (Gpx) (Wild et al., 1999; Erickson et al., 2002; Banning et al., 2005). The regulation of other GSH enzymes such as GSH synthetase and GSH reductase was also suggested to be Nrf2-dependent (Lee et al., 2003). The heterodimer GCLM and GCLC subunits have an essential role in GSH homeostasis by catalyzing the first-step, rate-limiting enzymatic reaction in GSH biosynthesis. GCLC or the heavy subunit (~73 kDa) is the catalytic engine which adjoins glutamate and cysteine into $\gamma$-glutamylcysteine followed by a final step of adding glycine via the GSH synthetase at the completion of GSH biosynthesis. GCLM is the smaller (~28 kDa) and modulatory subunit which increases efficiency and substrate affinity of GCLC, and desensitizes negative feedback of GSH on GCLC (Griffith, 1999). Targeted disruption of GCLC in mice resulted in failure of GSH synthesis, leading to embryonic lethality (Dalton et al., 2000; Shi et al., 2000). Although GCLM knockout mice were fertile and survived to adulthood, their decreased GCL activity and GSH synthesis sensitized them to oxidant toxicity (Yang et al., 2002). Knockdown of either subunit via small hairpin RNA caused time-dependent apoptotic cell death in primary neuron cultures, suggesting that both GCLM and GCLC are essential for neuronal survival (Diaz-Hernandez et al., 2005). On the other hand, overexpression of GCL subunits increases cell resistance to TNF-induced mitochondrial apoptosis (Botta et al., 2004). In tandem with this observation, the liver of Nrf2 knockout mice which had repressed GCLM/GCLC activity and lower GSH levels were more susceptible to apoptosis.
induction by TNFα as compared to that of the wildtype mice (Morito et al., 2003). Collectively, accumulating data suggest that regulation of these critical GCLM and GCLC subunits maybe imperative in the cytoprotective role of Nrf2.

**Thioredoxin**
The thioredoxin system is comprised of thioredoxin (Txn), mitochondrial Txn-2, thioredoxin reductases (TRx), NADPH, and peroxiredoxins (Prx), having a redox-maintenance role as important as the GSH system. It mediates thiol-dependent thiol-disulfide exchange reactions important in: (1) regulation of reduced intracellular environment; (2) oxidative defense; (3) cellular growth and apoptosis; (4) DNA synthesis and repair; (5) immune function; and (6) modulation of cell signaling MAPK (Kobayashi-Miura et al., 2007; Arner & Holmgren, 2006). Mice with Txn knockout suffered from gestational death (Matsui et al., 1996; Nonn et al., 2003). Prx cooperates with reduced Txn in preserving reduced forms of protein thiols whereas TRx works in recycling of Txn and vitamin C (ascorbate) from their oxidized counterparts (Mustacich & Powis, 2000). Nrf2 as a major transcriptional regulator of Txn, TRx1 and Prx1 has convincingly been demonstrated (Kim et al., 2001; Kim et al., 2007; Sakurai et al., 2005). Particularly, TRx, the selenium-containing antioxidative enzyme, has been increasingly recognized as possessing central roles in cellular oxidative adaptation, protection of oxidative injury, and mediating chemoprevention of dietary selenium (Mustacich & Powis, 2000; Chen et al., 2005A; Kobayashi-Miura et al., 2007).

**NQO1 and HO-1**
NAD(P)H:oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) are the other two well-documented antioxidative enzymes under direct transcriptional control of Nrf2 (Venugopal & Jaiswal, 2996; Alam et al., 2003). NQO1 is a cytosolic flavoprotein catalyzing two-electron reduction of quinones to hydroquinones. This reaction prevents one-electron reaction-mediated ROS formation which results from the redox recycling of semiquinones in the presence of oxygen. NQO1 is highly inducible during oxidative stress; its activity (quinone reductase) has been assayed to assess the inducer potency of antioxidative enzymes (Talalay et al., 2003). Gene knockout of NQO1 in mice or functional mutation of NQO1 in humans leads to increased cancer risks upon benzene exposure (Iskander & Jaiswal, 2005; Vasiliou et al., 2006). Similar to NQO1, HO-1 is a
sensitive indicator of cellular oxidative stress and participates in the rate-limiting step in the breakdown of heme into carbon monoxide (CO), iron and bilirubin. The byproducts biliverdin/bilirubin and CO can exert cytoprotection against oxidative stress and xenobiotic-induced injuries. The therapeutic potential of targeting HO-1 for chemo- and xenobiotic-protection in multiple organs, ischemic heart disease, and inflammatory diseases has recently received considerable investigation (Prawan et al., 2005; Nath, 2006; Abraham et al., 2007).

1.5.3 Phase II conjugation enzymes and phase III transporters

Phase II conjugation enzymes
As the major xenobiotic detoxification process in the cell, the phase II conjugation increases polarity of the detrimental hydrophobic xenobiotics that enter the cell. The polar conjugated metabolites are then excreted outside the cell through an active membrane-bound pump system involving the phase III efflux transporters. Hence, the collaboration between the phase II conjugation enzymes and phase III efflux transporters constitutes the major intracellular foundation of xenobiotic detoxification. The phase II conjugation enzymes particularly GSTs, have long been suggested to be potential Nrf2 target genes with highly transactivational ARE-like motifs identified on the gene promoter (Rushmore et al., 1991). Nrf2 disruption in mice had lower basal and inducible expressions of many GST isoforms in the liver and intestine (Itoh et al., 1997; McMahon et al., 2001; Chanas et al., 2002). Given that most substrates of GSTs are electrophilic in nature, the direct regulation by Nrf2 of these conjugation enzymes is related well to its physiological role as electrophiles scavenger. Subsequent gene promoter analyses revealed that Gstp1 and Gsta3 in rodents have functional AREs that are under Nrf2 transcriptional control (Ikeda et al., 2002 & 2004; Jowsey et al., 2003). Other than GSTs, certain UDP-glucuronyltransferases (UGTs) have also been suggested to be regulated by Nrf2 (Thimmulappa et al., 2002; Cho et al., 2005) with evidence of a direct regulation recently demonstrated for the human UGT1A1 (Yueh & Tukey, 2007).

Phase III transporters
The phase III efflux transporter particularly the ABC transporters are capable of pumping out the phase II conjugates including those conjugated by GST and UGT. The first report of involvement of Nrf2 in regulation of the ABC transporters comes from Hayashi and
colleagues (2003) who found that the constitutive and inducible expression of Abcc1 or Mrp1 was lower in embryonic fibroblasts derived from the Nrf2−/− mice than that from the wildtype. In a very recent study, Maher et al. (2007) further showed that expression of hepatic Mrp2, Mrp3, and Mrp4 in response to oxidative stress was also suppressed in Nrf2−/− mice. These reports, together with other studies showing induction of some ABC transporters with oxidant treatments (Scotto, 2003; Maher et al., 2005), imply that Nrf2 may play a critical role in adaptive regulation of these efflux transporters. Despite this, mouse Mrp2 is the only ABC transporter that has been conclusively proved to contain a functional ARE readily to be transactivated by Nrf2 (Vollrath et al., 2006). The coordinate and collaborative regulation of the ABC transporters and the phase II conjugation enzymes by Nrf2 presents a synchronized cellular detoxification process upon exposure to oxidant/electrophilic xenobiotics. The role of antioxidative proteins, and phase II and phase III systems in counteracting oxidative stress is illustrated in Figure 4.

1.5.4 Transcription factors
Interestingly, most transcription factors identified to be regulated by Nrf2 are those involved in the autoregulatory feedback pathway of its own transcription. With the discovery of two functional AREs in the gene promoter, Nrf2 can autoregulate itself, enhancing its own expression during oxidative stress (Kwak et al., 2002). Because the protein half-life of cytosolic Nrf2 during non-stress conditions is less than 20 minutes owing to the efficient Keap1-mediated proteosome degradation pathway (Itoh et al., 2003), the auto-activation appears to play a positive regulatory loop, promoting persistent receptor activation during oxidative stress. This is particularly important as studies mainly from Yamamoto’s group have suggested that the Nrf2-Keap1 complex, compared with non-stress conditions, remains intact despite a slower rate of degradation during oxidative stress (Watai et al., 2007; McMahon et al., 2007). The nuclear accumulation of Nrf2, or receptor activation, is hence contributed by the newly synthesized Nrf2 proteins that have escaped from the cytosolic gate of Keap1’s repressive effects. It was also found that the degradation of Keap1 is enhanced during oxidative stress, and this may additionally mitigate the repressive action of Keap1 on Nrf2 degradation (Hong et al., 2005B). Despite this, a recent study from the Jaiswal’s
Figure 4. Schematic illustration of the coordinated Nrf2-regulated defense system. Nrf2 activates a battery of antioxidative, NADPH generating, phase II and phase III cytoprotective genes in response to attack of free radicals and electrophiles. Briefly, the antioxidative enzymes (e.g., the GSH system: GCLM, GCLC, GS, GR, GPx; the Txn system: Prx1, TRx1; the NADPH-regenerating system: G6pd, Pgd, Me1; and the antioxidant system: NQO1, HO-1, catalase, SOD1,) work in concert to preserve redox balance by defending oxidative damage. Electrophilic substrates and metabolites are rapidly detoxified by conjugation with GSH catalyzed by GSTs which are then actively pumped out of the cell by the phase III efflux transporters MRPs.
laboratory has shown that Keap1 (INrf2) is also a target of direct transcriptional regulation of Nrf2 (Lee et al., 2007). The self-promoted transcription of Nrf2 during oxidative stress is apparently being counterbalanced by a negative feedback loop which enhances Keap1 expression. Hence, it appears that there exists a balanced and complex autoregulatory loop to control cellular availability of Nrf2 and its activation during time of stress. Meanwhile, the heterodimer partner of Nrf2 in the sMaf family, namely MafG, is also found to be a direct target of Nrf2 with a phylogenetically well-conserved ARE in the gene intronic region (Katsuoka et al., 2005). The interaction between the Nrf2 and AhR signaling pathways has now been considered as reciprocal and bidirectional. Following the earlier discovery of Nrf2 as a direct transcriptional target of AhR (Miao et al., 2005), Shin and coworkers (2007) further located a functional ARE in gene promoter of the mouse AhR; Nrf2 activation by a pharmacological Nrf2 inducer increased the expression of AhR as well as AhR target genes, Cyp1a1 and Cyp1b1. Functionally, Nrf2 signaling was also shown to modulate fibroblast adipogenesis mediated by AhR.

1.5.5 Other systems of Nrf2-regulated genes

Multiple protein subunits in the 26S proteosomal protein complex, which are engaged in the degradation of oxidative damaged or misfolded proteins and cellular regulatory proteins, are also found to depend on Nrf2 for their induction in response to oxidant stressors (Rangasamy et al., 2004; Kwak et al., 2003B). Accumulation of oxidative-damaged proteins in the cell can impede cellular function and lead to apoptosis. Hence, clearance of these dysfunctional proteins is critical in preserving cell survival during oxidative stress. The 26S proteosome complex consists of a 20S core complex and a 19S regulatory subunit. 20S is the proteolytic complex directly degrading oxidized proteins and ubiquitinylating proteins for degradation recognition; whereas the 19S is involved in recognizing and processing substrates for sequential degradation by the catalytic core (Glickman & Ciechanover, 2002). The catalytic subunit PSMB5 of the 20S proteosome complex has been identified to possess two functional AREs in the gene promoter (Kwak et al., 2003B).

Meanwhile, mouse Cyp2a5 represents the first phase I enzyme directly regulated by Nrf2 through a “stress response element” with consensus sequences uncommon to those for ARE (Abu-Bakar et al., 2007). Unlike other proteins regulated by Nrf2 which are known to be cytoprotective, the role of Cyp2a5 in adaptive responses is presently
unclear. Given that AhR is a known transcriptional regulator of Cyp2a5 (Arpiainen et al., 2005) and AhR is a target gene of Nrf2 (Shin et al., 2007), the regulation of this enzyme by Nrf2 may also be accomplished through an indirect activation pathway involving AhR. Although lacking direct regulatory evidence, the NADPH generating enzymes, chaperone proteins and cytoskeletal keratin proteins have also been proposed to be other important categories of Nrf2-dependent proteins (Kensler et al., 2006; Rangasamy et al., 2004; Kwak et al., 2003B).

1.6 Protection Conferred by Nrf2 Activation

1.6.1 Oxidant and electrophile-induced injury and diseases

Since the Nrf2 gene knockout/disrupted mice from both the outbred ICR(CD-1)(Itoh et al., 1997) and inbred (C57/BL6)(Chan et al., 1996) strains became available about a decade ago, the in vivo role of Nrf2 in protection against various oxidant xenobiotics has attracted considerable investigation. Even though the Nrf2-disrupted mice are fertile and grow normally to adulthood, they exhibited a heightened vulnerable phenotype in response to a variety of acute and chronic environmental stresses. Because Nrf2 is ubiquitously expressed in many tissues, the Nrf2-/- mice have been used as a valuable tool for deciphering key determinants of susceptibility to various xenobiotic challenges occurring to multiple organs. An updated list of published reports of enhanced toxicity/susceptibility effects upon exposure to stressors in various organs using the Nrf2-/- mice is provided in Table 2.

Oxidants and electrophilic inducers become the most direct stressors for which Nrf2 exerts significant protection. Nrf2-/- mice suffered from more serious hepatotoxicity and lethality during acetaminophen overdose which is known to produce highly toxic electrophilic species, namely, N-acetyl-p-benzoquinoneimine by the phase I enzymes in the liver (Chan et al., 2001; Enomoto et al., 2001). Neurotoxins such as MPTP, malonate, 3-nitropropionic acid and 6-hydroxydopamine have long been suggested to elicit their toxic actions on neurons through production of free radicals and oxidative stress. This effect was reaffirmed in the Nrf2-/- mice showing higher sensitivity to neurotoxicity caused by the above drugs (Calkin et al., 2005; Burton et al., 2006; Jakel et al., 2007), further corroborating the contributory role of oxidative stress in a wide spectrum of neurodegenerative diseases.
Table 2. Nrf2 as a multi-organ protector against toxicant/stress challenges: A summary of Nrf2 knockout mice studies

<table>
<thead>
<tr>
<th>Organ/Site</th>
<th>Toxicant challenge and worsened disease/disorder outcomes in Nrf2 knockouts</th>
</tr>
</thead>
</table>
| Lung and its local inflammatory immune response | - Butylated hydroxytolune-induced acute lung injury (Chan & Kan, 1999)  
- Bleomycin-induced pulmonary fibrosis (Cho et al., 2004)  
- Hyperoxia-induced pulmonary injury (Cho et al., 2002)  
- Tobacco smoke-induced emphysema (Iizuka et al., 2005; Rangasamy et al., 2004)  
- Elastase-induced lung inflammation and emphysema (Ishii et al., 2005)  
- Ovalalbumin-induced lung-asthmatic response (Rangasamy et al., 2005)  
- Endotoxin-induced pulmonary inflammation, edema and septic shock (Thimmulapa et al., 2006)  
- Carrageenan-induced inflammation in pleural cavity (Itoh et al., 2004B)  
- Diesel particles-induced lung hyperplasia and oxidative stress (Aoki et al., 2001)  
- Spontaneous and benzo(a)pyrene-induced mutations in the guanine phosphoribosyltransferase transgenic mouse model (Aoki et al., 2007) |
| Liver | - Acetaminophen-induced liver injury (Chan et al., 2001; Enomoto et al., 2001)  
- Anti-Fas antibody or TNFα-induced hepatitis and hepatocellular apoptosis (Morito et al., 2003)  
- 2-amino-3-methylimidazo[4,5-f]quinoline-induced hepatocarcinogenesis (Kitamura et al., 2007) |
| Central nervous system/Brain | - 3-nitropropionic acid or malonate-induced neurotoxicity (Calkins et al., 2005; Shih et al., 2005A)  
- Collagenase-induced intracerebral hemorrhage (Wang et al., 2007C)  
- Ischemia-reperfusion or endothelin-1-induced neuronal injury (Shih et al., 2005B; Shah et al., 2007)  
- 1-Methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity (Burton et al., 2006)  
- 6-hydroxydopamine-induced neurotoxicity (Jakel et al., 2007) |
- Dextran sulfate-induced colonic inflammation and preneoplasia (Osburn et al., 2007) |
| Urinary bladder | - Hydroxybutyl-nitrosamine-induced bladder cancer (Iida et al., 2004 & 2007) |
| Skin | - Dimethylbenz[a]anthracene-induced skin tumors (Xu et al., 2006B) |
| Systemic immune system | - Aging-associated systemic autoimmune disorder in female Nrf2 knockouts (Li et al., 2004A; Ma et al., 2006)  
- Lupus-like autoimmune nephritis in Nrf2−/− female mice (Yoh et al., 2001) |
| Red blood cells | - H₂O₂-induced hemolytic anemia (Lee et al., 2004) |
Meanwhile in the lungs, Nrf2 appears to have extremely important roles because it is the site where exposure to the oxygen-rich atmospheric air and volatile environmental pollutants occurs, and hence is constantly exposed to higher levels of ROS. Acute exposure to diesel particles, hyperoxia (>95% oxygen) or butylated hydroxytoluene (BHT) causes more extensive lung injuries characterized by pulmonary damage, hyperplasia and/or accompanying inflammatory edema in Nrf2-/- mice (Cho et al., 2002; Aoki et al., 2001; Chan & Kim, 1999). It is also noteworthy that deficiency of Nrf2 activity due to functional polymorphism of Nrf2 (Marzec et al., 2007) or of AREs in the gene promoter (Wang et al., 2007D) has been detected in human populations. In the case of Nrf2 polymorphism, a polymorphic site(-617 C/A) which resulted in diminished transactivational and binding activity of Nrf2 in vitro, was associated with a significant 6-fold increased risk (OR 6.44; 95% CI 1.34-30.8) of developing acute liver injury.

Using isolated primary cell culture derived from the Nrf2-/- mice, the cellular-defense role of Nrf2 against electrophilic stresses produced by heavy metals arsenic (Wang et al., 2007E) and methylmercury (Toyama et al., 2007), and the toxic herbicide diquat (Osburn et al., 2006) was also reported.

1.6.2 Inflammation and immunity

The involvement of oxidative stress in aggravation of immune disorders such as pulmonary inflammation, injury and emphysema (associated with chronic obstructive pulmonary disorders, COPD) has been widely recognized (Matthay & Zimmerman, 2005). Despite the multi-factorial nature of the inflammatory disorders, absence of Nrf2, and hence weakened oxidative stress defense, prompted the mice to exacerbated inflammatory outcomes upon stimulation by various inflammatory agents including cigarette smoke, elastase, and carrageenan (Thimmulappa et al., 2006; Itoh et al., 2004B; Ishii et al., 2005). The production of 15-d-PGJ2 consequent to cyclooxygenase-2 activation was shown to play a central role in activating Nrf2, thereby resolving inflammation (Itoh et al., 2004B). Bleomycin, a chemotherapeutic agent which can cause lung inflammation and fibrosis in patients, was also found to increase severity of pulmonary injury and fibrosis in Nrf2-/- mice (Cho et al., 2004).

As a major effector of inflammatory responses, the innate immune system is a critical determinant of susceptibility to sepsis. From an endotoxin lipopolysaccharide plus TNFα-induced sepsis mouse model, the direct modulatory effect of Nrf2 on innate
immunity was also evident (Rangasamy et al., 2004). Nrf2-regulated cellular GSH and antioxidative proteins were found to be essential for optimal NF-κB and Myd88-dependent activations, which are the important adaptive pathways for regulation of interferon-related genes.

In addition, the protective role of Nrf2 in autoimmune disorders has been exemplified in the Nrf2−/− mouse model. A high percentage of aged female Nrf2−/− mice (≥60 weeks) were found to develop systemic autoimmune disorders, most notably lupus-like glomerulonephritis (Li et al., 2004A; Ma et al., 2006; Yoh et al., 2001). These findings underscore the crucial role of oxidative stress, and sex as a modifying factor in the etiology of autoimmune diseases.

1.6.3 Chemical carcinogenesis

Chemical carcinogens are reactive agents which attack cellular DNA, leading to DNA damage and mutation, deregulated cell cycle control, genomic instability, and finally carcinogenesis. Induction of the phase II conjugation enzymes and antioxidative proteins is capable of detoxifying chemical carcinogens by facilitating their excretion and by reducing their detrimental nucleophilic attacking effects (Talalay et al., 2003). Therefore, deficiency of Nrf2 activity would be anticipated to have a higher cancer risk upon carcinogen exposure. In this respect, studies have shown that Nrf2−/− mice had a higher vulnerability to chemical carcinogens targeting at the gastrointestinal tracts, urinary bladder, lungs, liver and skin (Ramos-Gomez et al., 2001 & 2003; Osburn et al., 2007; Iida et al., 2004 & 2007; Kitamura, et al., 2007; Aoki et al., 2007).

In contrast to the chemopreventive role of Nrf2, recent evidence has suggested that constitutive hyperactivity of Nrf2 may predispose some lung cancers to a phenotype of increased drug resistance and aggressiveness (Singh et al., 2006; Padmanabhan et al., 2006). This exaggerated Nrf2 activity brought about an increased expression of antioxidative enzyme and certain MRP genes was due to mutation of key amino acids on Keap1 that defects its Nrf2’s repressive effects. These findings point to Nrf2 being a double-edged sword in cancer development. Although increased Nrf2 activity is desired for cancer prevention, when the tumor is established or the event of carcinogenesis has occurred the enhanced Nrf2 activity may in turn promote tumor growth and resistance to treatment.
1.6.4 Nrf2 as disease preventive target

Enhancing Nrf2 activity with prior or co-administration of plant-derived or synthetic chemopreventive agents has been shown to provide protection against toxicities/injuries with exposure to various oxidant and electrophilic stressors in controlled experiments (Kensler et al., 2006). Particularly, the synthetic triterpenoids are the most potent Nrf2 inducers to date with potency equivalent to 100-time that of another synthetic chemopreventive agent Oltipraz shown to have efficacy in vivo against chemical carcinogenesis (Yates et al., 2006 & 2007). The premise of such protection is based on the high potency of these agents to enhance Nrf2-regulated cytoprotective genes, thereby facilitating a better adaptive response to the insurgent stressors. Compared with the wildtype, Nrf2-/- mice often benefit minimally from administration of these agents, suggesting that the protection is conferred through Nrf2 activation. Because Nrf2 transactivation by these chemopreventive agents is literally an intervention of inducing oxidative stress, factors such as timing, dose, and length of treatment with these agents may be critical in determining intervention effectiveness and in preventing additive oxidative stress that may have caused following exposure to the stressors.

Molecular interventions, for example using RNAi technique, represent another approach to increase Nrf2 activity with the advantage of greater specificity. Utilization of gene-specific small-interfering RNA (siRNA) against the Nrf2 repressor Keap1 has successfully created a chemopreventive phenotype in vitro with enhanced levels of antioxidative enzymes (Devling et al., 2005). This approach of creating transient activation of Nrf2 using siRNA has an advantage over continuous Nrf2 activation. Complete gene knockout of Keap1 with constitutive Nrf2 activation leads to postnatal lethality in mice due to starvation as a result of hyperkeratosis-induced occlusion in the lumen of esophagus and forestomach (Wakabayashi et al., 2003). Alternatively, tissue specific enhancement of Nrf2 activity may appear to be another strategy to avoid unwanted adverse effects in the gastrointestinal tract. Using a cre-lox system, hepatic-specific inhibition of Keap1 in mice was shown to increase liver resistance to acetaminophen-induced toxicity (Okayama et al., 2006).

The decreased cellular defense against oxidative stress may be the major cause of intolerance to oxidative damage and resultant degenerative diseases often seen in the elderly population. The Nrf2 transcriptional activity and corresponding GSH synthesis were significantly declined in the liver of aged rats (Suh et al., 2004). Although the
mechanism for this decreased Nrf2 activity remains obscure, primary hepatocytes of the aged rats have reduced ERK and AKT kinase activities (Ikeyama et al., 2002). Because these cell survival signaling pathways are known mediators of Nrf2 activation (Kensler et al., 2006), their repression is likely to effect a compromised Nrf2 activity. Administration of the antioxidant lipoic acid can significantly reverse this repressed Nrf2 activity and increase GSH levels (Suh et al., 2004), suggesting that the elderly may benefit from the enhancement of Nrf2 activity as a means of degenerative disease prevention.

1.7 Multidrug Resistance-Associated Proteins (MRPs)

1.7.1 Localization, importance, and substrate profile

The MRPs(ABCC1-6 or MRP1-6; ABCC10-12 or MRP7-9), together with the p-glycoprotein (MDR1/ABCB1; P-gp) and the breast cancer resistance protein (BCRP or ABCG2), are referred to as a subset of the ABC superfamily of transmembrane efflux transporters capable of extruding a multitude of drugs and xenobiotics, particularly of chemotherapeutics. Hence, their overexpression in some cancer cells is implicated in the multidrug resistant (MDR) phenotype and usually poor prognosis outcome (Kruh & Belinsky, 2003; Scotto, 2003). In humans, the ABC superfamily constitutes one of the largest plasma membrane-borne proteins, consisting of at least 49 genes that can be assigned to a family tree from A to G (Deeley et al., 2006). The majority of these proteins are energy (ATP)-dependent pumps, consisting of two to three membrane spanning domains (MSDs) and at least one nucleotide-binding domain (NBD) containing the so-called “Walker A” and “Walker B” motifs separated by a conserved “C” signature motif which are ATPase catalytic sites for ATP binding and hydrolysis. All MRPs belong to the C family of the ABC transporters (ABCC), with at least 7 members (MRP1-7) identified to have MDR properties. BCRP (ABCG2) is another well-characterized MDR-like efflux transporter that is composed of only one MSD and half the size of most MRPs (Doyle & Ross, 2003). This “half” transporter functions as a homo-dimer or homo-tetramer, and is increasingly being recognized as having a significant role in drug resistance and xenobiotics disposition. The basic topology of ABC transporters which contribute to the multidrug resistant (MDR) phenotype is schematically depicted in Figure 5.

There are distinct distribution and relative expression of each ABC transporter across varied tissues; however, organs/tissues that are directly involved in xenobiotic metabolism and detoxification, such as the liver hepatocytes and intestinal epithelial cells,
Figure 5. Topological representation of ABC transporters. The basic units of ABC transporters are comprised of 2-3 membrane spanning domains (MSD), intra-membrane loops where ATP hydrolysis occurs at the nucleotide binding domain (NBD).
are found to express most, if not all, of these transporters. MDR1, MRP2 and BCRP are
expressed on the apical domain, whereas MRP1 and MRP3-7 are found on the basolateral
domain (Deeley et al., 2006). By immunohistochemistry, however, MRP4 was reported
to be expressed on the apical domain of kidney proximal tubuloacinar and brain
endothelial cells (Leggas et al., 2004; Van Aubel et al., 2002). Membrane localization,
chromosomal location, and major tissue expression of MRPs can be found in Figure 6
and Table 3.

Physiological importance of some MRPs has been demonstrated with their
functional genetic mutations resulting in characteristic inherited diseases in humans.
Functionally defective or absence of MRP2 protein due to key genetic mutations of the
MRP2 gene causes a mild liver disorder known as the Dubin-Johnson syndrome
(Kartenbeck et al., 1996; Paulusma et al., 1996), an autosomal recessive disorder
characterized by conjugated hyperbilirubinemia, increased urinary excretion of
coproporphyrin I, deposits of a black pigment in centrolobular hepatocytes, and
prolonged retention of bromosulfophthalein, a diagnostic dye extruded by MRP2 as a
 glutathione conjugate (Wada et al., 1998). Impaired anionic and glucuronide-conjugated
drug transport and distribution have been noticed in rodent models of the Dubin-
Johnson syndrome such as the Mrp2 knockout mice (Chu et al., 2006) or EHBR and
GY/TR(−) rat strains which have deficient Mrp2 function (Jansen et al., 1985; Kitamura et
al., 1990). The absence of severe liver dysfunction in patients with the Dubin-Johnson
syndrome suggests possible adaptive compensation of defective MRP2 by other MRPs.
Increased expression of MRP3 at the basolateral domain of hepatocytes has been
reported in liver sections from a Dubin-Johnson patient (Konig et al., 1999). Meanwhile,
genetic deficiency of MRP6 has been implicated in pseudoxanthoma elasticum, a rare
autosomal connective tissue disease characterized by dystrophic and mineralized
connective tissues which manifest into baggy skin, loss of vision and calcification of large
blood vessels in humans (Bergen et al., 2000; Ringpfeil et al., 2000). Increased
mineralization of connective tissues was similarly developed in mice with Mrp6 knockout
(Klement et al., 2005). The mechanism by which MRP6 deficiency causes this disorder
remains unidentified. A secondary, indirect mechanism, however, has been proposed
since connective tissues only express very low or undetectable levels of this transporter
(Belinsky & Kruh, 1999). The exact physiological role of virtually all MRPs is still largely
undefined. Studies from targeted gene-knockout mice suggest all MRPs are dispensable
Figure 6. Localization of ABC Transporters in Human Hepatocytes. Subcellular localization of major ABC efflux transporters including major bile salt transporter (BSEP) and bile salt uptake transporter (NTCP) on the canalicular (Apical) and sinusoidal (basolateral) membrane domains of hepatocytes.

Table 3. Chromosomal location, major tissue expression, and malfunction associated disease for ABC efflux transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Major tissue expression</th>
<th>Disease/condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1 (ABCA1)</td>
<td>7q21</td>
<td>Intestine, liver, PBMC, many</td>
<td>Digoxin drug interaction</td>
</tr>
<tr>
<td>MRP1 (ABCC1)</td>
<td>16p13.1</td>
<td>Testis, ovary, placenta, lung, PBMC, brain</td>
<td>-</td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>10q24</td>
<td>Intestine, liver, kidney, breast</td>
<td>Dubin-Johnson syndrome</td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>17q2.3</td>
<td>Intestine, liver, brain</td>
<td>-</td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>13q32</td>
<td>Kidney, intestine, liver, lung</td>
<td>-</td>
</tr>
<tr>
<td>MRP5 (ABCC5)</td>
<td>3q27</td>
<td>Brain, kidney, lung, intestine, gonad, placenta</td>
<td>-</td>
</tr>
<tr>
<td>MRP6 (ABCC6)</td>
<td>16p13.1</td>
<td>Liver, intestine</td>
<td>Pseudoxanthoma elasticum</td>
</tr>
<tr>
<td>MRP7 (ABCC10)</td>
<td>6p21.1</td>
<td>Testis, intestine, gonads, lung, placenta</td>
<td>-</td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>4q22</td>
<td>Intestine, placenta, breast, liver, kidney, brain</td>
<td>-</td>
</tr>
<tr>
<td>BSEP (ABCB11)</td>
<td>2q24</td>
<td>Hepatocytes</td>
<td>Progressive familial intrahepatic cholestasis-2</td>
</tr>
</tbody>
</table>
for normal growth and reproduction, and their roles in xenobiotic metabolism are increasingly being explored. The highly overlapping substrate profile for most MRPs from in vitro ectopic expression studies implies that these transporters may work collaboratively and in complementary manner in their transporter role (Kruh & Belinsky, 2003). Generally all MRPs are cellular efflux pumps of amphipathic anions including steroid and bile salts, and conjugates of glutathione and glucuronide. Nonetheless, individual transporters may have distinctive preference and possible specificity for certain substrates; evidence showed that Mrp3 knockout mice had significant impairment in hepatic sinusoidal transport of morphine-6-glucuronide (Zelcer et al., 2005). Using inside-out membrane vesicles of polarized and non-polarized cell lines with transfected ectopic expression systems, Mrp4 was identified to be an important prostaglandin efflux pump (Reid et al., 2003). As for their more recognized role in resisting chemotherapy, MRPs are known to be high capacity but low affinity cellular pumps of many chemotherapeutics. The low efficiency/affinity of each MRP for its chemotherapeutic substrates suggests that the consequential cancer resistance phenotype is unlikely contributed solely by any individual transporter. It is likely that overexpression of many ABC transporters coupled with increased activity of other concurrent resistant/cell defense factors have collectively contributed to cancer resistance phenotype. In agreement with this postulation, studies have identified simultaneous increases of multiple MRPs as well as of other cell defense proteins in cancer tissue biopsies and cancer cell lines (Singh et al., 2006). The reported endogenous and exogenous substrates of MRPs are summarized in Table 4.

It is imperative to take note that data obtained from the in vitro ectopic expression systems of isolated transporters may not necessarily be reflective of the physiological context of the respective transporter under study. This purified system neglects the existence of hierarchical and complex interactions of various transporters and with other factors that is usually occurring in cellular context. By virtue of this limitation, it is generally accepted to be more superior to uncovering the physiological role of individual transporters from the targeted gene knockout mouse models. However, studies have frequently noted that in these knockout models, disruption of a target transporter is accompanied by an intrinsic compensatory mechanism, for example, increases in another
<table>
<thead>
<tr>
<th>Gene</th>
<th>Endogenous substrates</th>
<th>Exogenous substrates (chemotherapeutics)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1 (ABCA1)</td>
<td>Estrogen glucuronides, endorphin, glutamate, steroids, β-amyloid, platelet-activating factors</td>
<td>Anthracyclines (doxorubicin, daunorubicin, epirubicin), actinomycin, colchicines, etoposide, teniposide, mitomycin C, mitoxantrone, taxenes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine)</td>
</tr>
<tr>
<td>MRP1 (ABCC1)</td>
<td>Estrogen glucuronides, GSH, GSSG, GSH conjugates, leukotriene C4, bilirubin glucuronides, sulfate conjugates, folic acid</td>
<td>Anthracyclines, colchicines, etoposide, heavy metals (arsenite, arsenate, antimonials), vinca alkaloids (vincristine, vinblastine), paclitaxel, methotrexate</td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>Estrogen glucuronides, GSH, GSSG, GSH conjugates, bilirubin glucuronides, bile conjugates, sulfate conjugates</td>
<td>Cisplatin, CPT-11, SN-38, doxorubicin, etoposide, methotrexate, teniposide, vincristine, vinblastine</td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>Bile salts and their conjugates, GSH conjugates, glucuronides conjugates, sulfate conjugates</td>
<td>Methotrexate, etoposide, teniposide, vincristine, cisplatin, doxorubicin,</td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>Bile salts and their conjugates, nucleotide analogs (cAMP, cGMP), prostaglandins (PGE1, PGE2), GSH and its conjugates, glucuronides conjugates, sulfate conjugates</td>
<td>Methotrexate, nucleoside analogs (PMEA), topotecan</td>
</tr>
<tr>
<td>MRP5 (ABCC5)</td>
<td>Glutamate, phosphate conjugates, GSH and its conjugates, nucleotide analogs (cAMP, cGMP)</td>
<td>Doxorubicin, methotrexate, nucleoside analogs (PMEA), potassium antimonyl tartrate, 5-fluorouracil</td>
</tr>
<tr>
<td>MRP6 (ABCC6)</td>
<td>Cyclic nucleotides (cAMP, cGMP), GSH conjugates</td>
<td>Doxorubicin, etoposide, teniposide, cisplatin</td>
</tr>
<tr>
<td>MRP7 (ABCC10)</td>
<td>GSH conjugates, estrogen glucuronides, leukotriene C4</td>
<td>Anthracyclines, vinca alkaloids, cisplatin, taxanes</td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>Heme, porphyrin, folic acid, bilirubin conjugates, estrogen glucuronides, steroids</td>
<td>Anthracyclines, bisantrene, camptothecin, epirubicin, flavopiridol, mitoxantrone, S38, topotecan, doxorubicin, daunorubicin, flavopiridol, methotrexate, topoisomerase I inhibitors</td>
</tr>
<tr>
<td>BSEP (ABCB11)</td>
<td>Bile salts and their conjugates</td>
<td>-</td>
</tr>
</tbody>
</table>
functionally similar transporter(s) (Lam et al., 2005; Nezasa et al., 2006; Tian et al., 2008). This observation underpins the complementary role among various transporters in their efflux function, and the existence of compensatory mechanisms which can become a confounding factor in correctly deciphering the actual physiological role of individual transporters from respective gene knockout models. The difference in biliary excretion of fexofenadine between Mrp2 knockout mice and Mrp2-deficient TR(-) rats in a recent study also implies existence of species differences in xenobiotic/drug substrate transported by orthologous transporters (Tian et al., 2008).

1.7.2 MRPs as adaptive defense proteins against oxidative stress

The energy-fueled efflux activity of MRPs is increasingly appreciated as an important cell defense system given that their active role in facilitating cellular extrusion whilst limiting retention of conjugated and unconjugated endo- and xeno-biotics. The observation that expression of MRPs is highly inducible by various stressors and during stress conditions such as cholestasis and toxicant-induced liver injury (Lin-Lee et al., 2001; Slitt et al., 2003; Slitt et al., 2007) strongly indicates that these transporters are components of an integral network in cellular adaptive response. Recent studies have shown that constitutive as well as inducible levels of MRPs are subject to regulation by a variety of transcription factors that appear to mutually collaborate with one another in accomplishing complementary tasks in cellular defense and homeostasis maintenance.

Several lines of evidence suggest that MRPs play a critical role in cellular defense against oxidative stress. Many MRPs have been found to be particularly inducible by known chemical oxidants, chemotherapeutics, and oxidative stress inducers such as UV light (Lin-Lee et al., 2001; Scotto, 2003). The inducibility of MRPs upon oxidative stress exposure implies a potential adaptive response of cell defense. Moreover, the common substrates of all MRPs, i.e., GSH conjugates and most classes of cancer chemotherapeutics are known to be oxidative stress inducers (Talalay et al., 2003; Chen et al., 2007). Particularly GSH conjugation represents the major route of detoxification of reactive electrophilic compounds, facilitating their excretion via MRPs. Activation of the ABC efflux system, hence, has a role to reduce cellular accumulation of oxidative metabolites and to prevent generation of excessive oxidative stress. There is indeed an intimate functional collaboration between MRPs and the cellular GSH system. A subset of MRPs, such as MRP1, MRP2 and MRP4, can extrude oxidized and reduced forms of
GSH from the cell, assuming a role in cellular GSH homeostasis (Kruh & Belinsky, 2003). Interestingly, the efflux function of these MRP transporters also relies on the presence of GSH which works as a cotransport molecule. Studies have shown that availability of GSH is essential for the efflux function of the above transporters, consistent with the abolishment of their efflux activity upon depletion of GSH with BSO treatment (Ballatori et al., 2005). These findings indicate a coordinate role of cellular GSH system in detoxification, conjugation, and extrusion of reactive metabolites. Moreover, the discovery that Nrf2 is a transcriptional regulator of mouse Mrp1, Mrp2, Mrp3 and Mrp4 provides further evidence that MRPs are part of the cellular defense system against oxidative stress (Hayashi et al. 2003; Maher et al., 2007; Vollrath et al., 2006). To what extent the increased expression of MRPs, or its enhancement of the cellular efflux system, participate in reducing oxidative stress and in protection against oxidative stress-induced pathology remains an interesting subject of future studies.

Because of their role in cellular transport of steroids, cholesterol steroids, and conjugates of lipophilic xenobiotics, expression of many MRPs is regulated by various steroid nuclear receptors/transcription factors (PXR, CAR, FXR, VDR, LXR, and RAR/RXR) having direct roles in physiological handling and metabolism of steroids, cholesterols, bile acids, and xenobiotics (Eloranta & Kullak-Ublick, 2005). Despite inconsistent results, studies of nuclear receptor(s) knockout mice reveal that PXR, CAR, FXR, VDR and LXR are transcriptional regulators of a subset of MRPs such as MRP2, MRP3, and MRP4 (Kullak-Ublick et al., 2004; Eloranta & Kullak-Ublick, 2005; Handschin & Meyer, 2005). An active gene enhancer region which recognizes FXR, PXR, and CAR is found in the 5'-flanking region of rat Mrp2 (Kast et al., 2002). Consensus motifs for VDR binding are reported to exist in the gene promoter of mouse Mrp3 which can be activated via vitamin D3 or bile acid exposure in the GI tract or intestinal cells, but not in the liver (McCarthy et al., 2005). The differential regulation of MRP3 among different tissues suggests potential epigenetic regulation and requirements of tissue-specific factors for VDR regulation. It is noteworthy that the hepatocyte nuclear factor 4α (HNF4α) whose expression is confined in certain tissues has been found to regulate an array of xenobiotic metabolizing proteins, including the nuclear receptor PXR (Jover et al., 2001; Kamiya et al., 2003). Likewise, the role of liver specific HNF4α is pre-requisite for regulation of CYP3A4 by PXR and CAR in the liver (Tirona et al., 2003). In HNF4α knockout mice, sexual dimorphism in a wide spectrum of target gene expression,
including the phase I enzymes and some ABC transporters, has been identified (Holloway et al., 2008; Wiwi et al., 2004). This observation suggests interplay of sex-specific factors and potentially sex hormones in the complexity of gene regulation by xenobiotic nuclear receptors. A summary of nuclear receptors reported to regulate selected ABC transporters is shown in Table 5.

Table 5. Transcriptional Regulation of Selected ABC transporters by Nuclear Factors

<table>
<thead>
<tr>
<th>Transports</th>
<th>Nuclear receptors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>PXR*</td>
<td>Synold et al. (2001), Geick et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>AP-1*</td>
<td>Daschner et al. (1999), Kurz et al. (2001)</td>
</tr>
<tr>
<td>MRP1</td>
<td>Nrfl</td>
<td>Hayashi et al. (2003)</td>
</tr>
<tr>
<td>MRP2</td>
<td>PXR*, CAR*, FXR*</td>
<td>Kast et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Nrfl*</td>
<td>Vollrath et al. (2006), Maher et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>RARα/RXRα*</td>
<td>Denson et al. (2000)</td>
</tr>
<tr>
<td>MRP3</td>
<td>α1-fetoprotein*</td>
<td>Inokuchi et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>VDR*</td>
<td>McCarthy et al. (2005)</td>
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<td>PXR</td>
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<td>Nrf2</td>
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<td>MRP4</td>
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<td>BCRP</td>
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<td>PPARγ*</td>
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<td>BSEP</td>
<td>FXR*</td>
<td>Ananthanarayanan et al. (2001)</td>
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* Active response element(s) on the gene promoter corresponding to the nuclear receptor has been identified. Abbreviations: ERα, estrogen receptor α; HIF1α, hypoxia-inducible factor 1α; PPARγ, peroxisome proliferator-activated receptor-γ.

1.8 Bile Acids

1.8.1 Physiology and toxicology

Primary bile acids, i.e., cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from the precursor cholesterol in the hepatocytes through a complex yet specific hydroxylation pathway involving the phase I enzymes, such as CYP7A, CYP7B, CYP8B, and CYP27 (Hoffman, 1999). Upon conjugation by amino acids (taurine and
glycine) and sometimes further by glucuronide and sulfate, the conjugated bile acids, or bile salts, are excreted out the cell by an energy-dependent ABC efflux transporter system, particularly BSEP (ABCB11) (Noe et al., 2002). Other ABC transporters such as MRP2, MRP3, MRP4 and BCRP not only transport monovalent bile salts, but also sulfated and glucuronidated bile conjugates (Bodo et al., 2003; Rius et al., 2006; Hirohashi et al., 2000; Janvilisri et al., 2005). Bile salts are the main organic solutes in bile, best known as an emulsifier of dietary lipids/phospholipids to facilitate intestinal absorption of lipids and lipid-soluble vitamins. Once excreted into the intestine, bile salts undergo enterohepatic recycling circulation whereby about 90% of them are reclaimed at the intestinal mucosa and re-enter the liver mainly via the organic uptake transporter system, primarily the Na⁺/taurocholate co-transporting peptide (NTCP or SLC10A1) located on the sinusoidal membrane of hepatocytes.

Upon the hydroxylation reaction of intestinal bacteria on primary bile acids, a group of bile-derived byproducts, namely the secondary bile acids, are produced. Among these bile acids, the lithocholic acid (LCA) produced from CDCA is considered to be the most toxic form of bile acids with genotoxic properties (Kawalek, et al., 1983; Kozoni et al., 2000). It has been suggested as an etiopathological factor in colon carcinogenesis, consistent with its mutagenesis-promoting effect in vivo (Kozoni et al., 2000). Because the high fat and low fiber diet may increase secretion of bile acids while delaying intestinal transit time of bile acids in the GI tract, a higher colon cancer risk of this diet reported in many epidemiological studies has long suggested that secondary bile acids are playing a key role (Rao et al., 2001). Administration of LCA via either the oral or intraperitoneal route causes cholestatic liver diseases in rodents (Hofmann, 2004). Being one of the most diagnosed liver diseases, cholestasis results from an impairment of hepatic bile flow in which accumulation of excess bile acids in the liver cells causes liver toxicity and injury. Induction of cholangiocyte inflammation and bile duct infarction is a major mechanism by which LCA causes cholestasis in rodents (Fickert et al., 2006), similar to that caused by bile duct ligation (BDL), a common procedure used in inducing experimental cholestasis. Despite having cholestatic properties, cases of direct involvement of LCA in causing cholestasis in humans are thought to be rare (Hofmann, 2004). It has been suggested that absorption of LCA in the intestine in humans, is limited by an efficient LCA detoxification mechanism consisting of sulfation, hydroxylation and/or apical efflux transporter systems. Other than LCA, deoxycholic acid (DCA) produced from CA is also a
toxic secondary bile acid which can induce cell injury and apoptotic cell death (Qiao et al., 2001). In contrast to LCA and DCA, ursodeoxycholic acid (UDCA), another secondary bile acid produced from CDCA, has apparently opposite effects. Through mechanisms that are still not fully understood, UDCA exerts protection against toxic effects of bile acids which has made it the only approved drug in the management of cholestasis (Paumgartner & Beuers, 2002). The chemical structure of primary and secondary bile acids present in humans is shown in Figure 7a.

1.8.2 Nuclear receptors and bile acid homeostasis

Exposure to excess bile acids is extremely toxic to the cells, causing necrotic and apoptotic cell death (Palmeira & Rolo, 2004). Constantly facing influx of large amounts of bile acids, the liver and intestinal cells are particularly at risk of exposure to excess bile acids, which may explain cholestasis and colon cancer are the common pathologies associated with bile acid toxicity. Hence, the cellular metabolism, extraction, and transport of bile acids in both the liver and intestinal cells are a highly coordinated and efficient system, enabling enterohepatic recycling of bile acids always in check without causing unwanted toxicity. This tightly controlled physiological regulation is a result of mutual effort accomplished by a spectrum of nuclear receptors, such as PXR, CAR, VDR, RXR, and LXR, which are the transcription factors of many important genes involved in bile synthesis, metabolism, as well as transport (Xie et al., 2003; Handschin & Meyer, 2005; Eloranta & Kullak-Ublick, 2005). Following the discovery that bile acids and their intermediate metabolites are potent agonists/ligands for the above steroid nuclear receptors (Kullak-Ublick et al., 2004), bile acids are now regarded as key signaling molecules which self-regulate their own metabolism and transport. This newfound role of bile acid as signaling molecules has revolutionized the traditional image of bile acids simply as a detergent solvent. Bile acid research has since experienced a renaissance with introduction of “bile-ology” as a specific field looking into signaling and other biological roles of bile acids (Chawla et al., 2000). An interactive diagram illustrating the transcriptional regulation of hepatic bile-metabolizing and transporter genes by nuclear receptors and transcription factors is shown in Figure 7b.

The physiological importance of the above-mentioned nuclear receptors in bile acid metabolism has received intensive studies; particularly valuable data have come
Figure 7a. Chemical Structure of Primary and Secondary Bile Salts.
Figure 7b. Transcriptional Regulation of Hepatic Bile Metabolism and Transport. Genes involved in bile acid synthesis (CYP7A1, CYP8B1, CYP27A1), detoxification (sulfotransferase 2A1, SULT2A1; CYP3A4), and transport (BSEP, NTCP, MRPs 2–4) are transcriptionally regulated by the ligand-activated nuclear receptors (FXR, CAR, VDR, PXR), the hepatocyte nuclear factors (HNF1α, HNF4α), short heterodimer partner 1 (SHP), and Nrf2. Coordinated regulation of these genes is critical in maintenance of hepatobiliary bile secretion, and prevention of hepatic bile acid accumulation and resulting toxicity. Note that SHP and HNF1α are positively and negatively, respectively, regulated by FXR. FXR, CAR, PXR and VDR share an oblige heterodimer partner, RXRα, for their activities in gene transactivation. FXR, PXR and VCR are directly activated by subsets of bile acids; whereas CAR can be activated by bilirubin. Lines with arrowheads and perpendicular bars indicate positive (activation) and negative (inhibition) regulations, respectively. Adopted from Eloranta & Kullak-Ublick (2005) and Geier et al. (2007).
from gene knockout as well as transgenic humanized mouse models. Single knockout of any of the above nuclear receptors or double knockout of PXR and CAR did not result in phenotypic evidence of bile deregulation or bile acid toxicity in mice, indicating that none of the above receptors has an indispensable role in bile acid homeostasis. Despite this, impairment in optimal regulation of genes critical in bile acid metabolism and detoxification in these knockout models prompted the mice to be extremely sensitive to suffer from severe liver injuries upon excess bile acid challenges induced either by BDL or LCA administration. Mice with PXR knockout or harboring human PXR had more severe liver pathologies and significant elevation of liver injury markers, including alanine transaminase (ALT), in response to LCA treatment than did wildtype mice (Staudinger et al., 2001; Xie et al., 2001). Using the single and double knockout mouse models of PXR and CAR, it was found that CAR, rather than PXR, was playing more significant roles in LCA protection (Zhang et al., 2004A). Protection by both PXR and CAR was evident in mice undergoing the BDL procedure to induce cholestatic liver injury (Stedman et al., 2005). Prior activation of PXR and CAR with administration of respective receptor agonists, i.e., pregnenolone 16α-carbonitrile (PCN) and 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), was found to be protective against subsequent bile acid challenges, proposing that these receptors may be potential therapeutic targets for the management of cholestasis (Staudinger et al., 2001; Xie et al., 2001). Of many LCA-detoxifying genes under direct regulation of PXR and CAR, sulfotransferases and CYP3A, which carry out sulfation and hydroxylation, respectively, appear to be the major candidate enzymes linked to direct detoxification and hence LCA protection (Stedman et al., 2004; Sonoda et al., 2002; Saini et al., 2004). The physiological role of PXR in bile acid homeostasis may not be as relevant as its protective role in bile acid toxicity, considering that bile acids are weak agonists of PXR and a more noticeable effect of PXR-mediated genes only occurs at toxicological doses of bile acids (Makishima et al., 2002; Staudinger et al., 2001).

FXR is the first steroid nuclear receptor demonstrated to be directly activated by physiological levels of bile acids, particularly CDCA (Eloranta & Kullak-Ublick, 2005). The bile synthetic enzymes, CYP7A1 and CYP8B1, and the major bile salt transporters BSEP and NTCP, are regulated by FXR (Eloranta & Kullak-Ublick, 2005). Despite having a critical role in bile metabolism, FXR null mice were resistant to BDL-induced obstructive cholestasis with protective mechanisms suggested to include reduced bile acid
synthesis and altered expression of hepatic bile salt transporters BSEP, MDR and MRP4 (Stedman et al., 2006). An unclear adaptive mechanism which shows enhanced phase I hydroxylation and renal elimination of bile acids has also been reported to occur with the FXR-/- mice after BDL (Marschall et al., 2006). It is unknown to what extent the seemingly compensatory adaptation mechanism occurring in FXR-/- mice affects the exact role of this nuclear receptor in bile acid protection. Opposing the findings from the BDL model, induction of cholestasis by CA feeding has resulted in significantly higher liver injury markers, aspartate transaminase (AST) and alkaline phosphatase (ALP) in FXR knockouts (Miyata et al., 2005). Moreover, with the identification of PXR as a target gene of FXR (Jung et al., 2006), the promotional role of FXR in bile acid detoxification via the PXR-mediated pathway appears to disagree with the reported beneficial role of FXR inhibition in bile acid protection (Stedman et al., 2006).

The role of VDR as a direct sensor for intestinal bile acids in inducing the detoxifying hydroxylation enzyme CYP3A, and the major intestinal bile transporter MRP3 and ASBT (Makishima et al., 2002; McCarthy et al., 2005; Chen et al., 2006B) suggests that this nuclear receptor possesses a critical role against bile acid toxicity in the GI tract. These findings purport to suggest a possible mechanism underlying the preventive role of dietary vitamin D in colorectal cancer risk which has long been linked to secondary bile acids (Martinez & Willet, 1998; Lin et al., 2005). Because VDR is mainly expressed in the GI mucosa with trace/undetectable levels in the liver, the protective role of VDR may be more regionally defined than PXR and CAR. To date, the in vivo protective role of VDR in bile acid toxicity and colorectal cancer has not been shown despite the availability of VDR knockout mice (Sakai et al., 2001). Recently, protection by LXR and its target adaptive genes against bile acid-induced hepatotoxicity has been demonstrated in female mice (Uppal et al., 2007).

**1.8.3 Bile acid toxicity and oxidative stress**

The mechanism whereby toxic bile acids cause hepatocyte toxicity and cell death has yet to be conclusively understood. In the case of cholestasis, elevated bile acid retention in the liver cells either caused by defects of intracellular bile extraction and export (intrahepatic cholestasis) or external blockage/impairment of bile flow (extrahepatic cholestasis) leads to cirrhosis and liver failure. The detergent-like physicochemical properties of bile acids have long been thought to play a major part in their toxicity.
Hydrophobic bile acids such as CDCA and DCA can solubilize membrane-bound lipids, leading to damage of cell membranes. However, it has been argued that the detergent-like (surfactant) toxicity of bile acids may not be physiologically relevant (Fischer et al., 1996). The observation that both necrosis and mitochondrial-dependent and -independent apoptosis pathways are involved in bile acid-induced cell death suggests an involvement of multiple mechanisms, possibly distinctively different for specific cell type, concentration of bile acids, and predominant individual bile acids or combinations of individual bile acids resulting in toxicity (Dent et al., 2005; Palmeira & Rolo, 2004). Generally, a higher concentration (≥250 µM) of hydrophobic bile acids usually causes necrosis in primary rat hepatocytes, whereas apoptosis is more common at a lower concentration (<100µM) (Palmeira & Rolo, 2004).

Sokol and colleagues (1993, 1995) have made the first observation that links increased oxidative stress to bile acid toxicity. In primary rat hepatocytes and isolated rat mitochondria, treatment with excess bile acids led to increased production of ROS and lipid peroxidation which preceded the incidence of hepatocyte injury and cell death. Pre-incubation with antioxidants such as α-tocopherol (vitamin E) significantly reduced hepatocytes' lipid peroxidation and cell death. These in vitro findings are supported by in vivo evidence of a systemic oxidative stress in rats with cholestasis (Ljubuncic et al., 2000), as well as clinical data in which increased lipid hydroperoxides and decreased GSH were reported in human cholestatic liver samples and in premature infants with total parenteral nutrition-induced cholestasis, respectively (Vendemiale et al., 2002; Mager et al., 2008). These data denote a pivotal role of oxidative stress in pathogenesis of toxic bile acids. The mechanisms underlying increased production of ROS by bile acids may be attributed to the toxicity of mitochondria which is believed to be the major target of bile acid toxicity (Palmeira & Rolo, 2004). Mitochondria are the main producer of cellular ROS. Bile acid-induced toxicity of mitochondria has been reported to involve induction of membrane permeability transition and subsequent release of mitochondrial apoptosis-triggering cytochrome c (Rolo et al., 2003). Loss of cytochrome c results in impaired electron leak at the level of ubiquinone-complex III, giving rise to superoxide radical production. Upon the Fenton's reaction mediated by the ubiquitous Fe²⁺, superoxide radicals can be transformed into highly reactive hydroxyl and then hydroperoxide radicals, the major promoters of lipid peroxidation. In light of this, alleviation of the mitochondrial oxidative stress by enhancing antioxidant glutathione.
defense has been described as a therapeutic mechanism for UDCA in the management of secondary biliary cirrhosis in rats and NAC in humans (Serviddio et al., 2004; Magers et al., 2008).

Another documented pathway of bile acid-induced ROS production has been ascribed for activation of the NADPH oxidase isoforms (Reinehr et al., 2005A). NADPH oxidase is a major group of oxidant-generating enzymes first identified in activated macrophages that is responsible for production of large amounts of ROS as a means of phagocytic activities. Production of ROS and H$_2$O$_2$ via the NADPH oxidase isoforms in non-phagocytic cells has been shown to be a major catalyst of generating oxidative stress for bile salts and the CD95 ligand in which subsequent activation of CD95 (death receptor) and apoptosis result (Reinehr et al., 2005A&2005B). These findings provide important mechanistic insights into mitochondrial-independent apoptosis stimulated by some toxic bile acids. The production of cellular oxidative stress represents an upstream signaling event which regulates and recruits a complex cell signaling pathway including JNK, p38 MAPK and ERK MAPK, leading to simultaneous or preferential activation of cell survival and apoptosis pathways. Until now, the unified conditions that underlie this contrasting – live or death - pathway remain largely unelucidated. Whether there is a key deciding component or particular pathway in dictating the overall cell fate remains a prominent research question.

Although oxidative stress has been implicated in adverse outcomes of bile acid toxicity, it is presently unknown whether strengthening antioxidative defense through activation of the oxidative stress-responsive Nrf2 has a protective role in bile acid-induced cell death. Therefore, the major goal of my PhD research was to elucidate if toxic bile acids are capable of activating Nrf2, thereby inducing cytoprotective genes to counteract the resultant oxidative stress. Particular focus will be on the cellular GSH system and ABC efflux transporters because these systems, known to be regulated by Nrf2, may be integrated as a coordinated detoxification process to protect the liver and intestinal cells against bile acid toxicity and irreversible cell death.

1.9 Retinoids

1.9.1 Compounds with multifaceted properties
Retinoids are referred to as a group of naturally occurring or synthetic vitamin A and its derivatives with a wide spectrum of physiological and therapeutic properties. Retinol, retinal, retinoic acid and plant-derived precursor carotenoids (α- and β-carotenes) are collectively known as the prototypical vitamin A. Among which, retinoic acid (RA) is the most biologically active vitamin A which is converted from dietary retinols by retinol and retinal dehydrogenases (for chemical structure and enzymatic conversion please see Figure 8). Excess vitamin A is mainly stored as retinyl/retinol esters in the liver cells. This essential nutrient is needed in humans at circulating concentration of $10^{-9} – 10^{-7}$ M for epithelial, immune, and mesenchymal cell differentiation, important in the maintenance of embryonal growth, skin and visual health (Ross et al., 2000). By virtue of these biological effects, vitamin A deficiency results in signature embryogenesis deficits, night blinds, and epithelial hyperkeratinosis which may lead to blindness and dermatological diseases. The antioxidative properties of increased dietary vitamin A have been promoted in the 1990s to be beneficial for cardiovascular and cancer disease prevention. Nevertheless this preventive potential was rejected in the later double-blind, randomized clinical trials of vitamin A supplementation. It was found that β-carotene supplementation may put the cigarette smokers at higher risk of fatal myocardial infarction (Omenn et al., 1996).

Based mostly on the premise of differentiation-promoting effects, high dosage of all-trans-retinoic acid (atRA; tretinoin®), is therapeutically effective in the treatment of acute promyelocytic leukemia (APL). The clinical efficacy of atRA and its cis-isomers [9-cis-retinoic acid, 9cRA (alitretinoin®) and 13-cis-retinoic acid, 13cRA (isotretinoin®)] in other forms of cancer such as neuroblastoma, Kaposi’s sarcoma, cutaneous T-cell lymphoma, squamous cell skin cancer and cervical cancer, and premalignant oral leukoplakia and xeroderma pigmentosum is currently being tested in clinical trials (Freemantle et al., 2003). Despite the failure of supplementation trials of retinol and β-carotene in prevention of primary and secondary lung cancer (Lippman et al., 2001), chemopreventive potential of the retinoid derivatives N-(4-hydroxyphenyl) retinamide or fenretinide (4-HPR) and acyclic retinoids (polyprenoid acids) for breast cancer and hepatocellular carcinomas has proved to be promising in pre- and early-clinical trials (Freemantle et al., 2003; Altucci & Gronemeyer, 2001). Other than malignancy, retinoids are also used in the management of various skin diseases such as acne, psoriasis and photo-aging pigmentation (Roeder et al., 2004). The therapeutic action of RA on cancer
Figure 8. **Chemical Conversion of Retinol to Retinoic Acids.** Retinol is oxidized to the biologically active retinoic acid by dehydrogenases. CYP26 is the major detoxifying enzyme catabolizing retinoic acid into its oxo oxidative forms for cellular excretion.
and skin diseases may extend beyond a simple differentiation-enhancing effect, considering that stimulation of cell death via apoptosis is commonly indicative of treatment outcome (Keedwell et al., 2004). Modulation of major cell cycle proteins and activation of p53, a well-characterized tumor suppressor and apoptotic-inducing protein, are some alternative pathways proposed as part of the therapeutic mechanisms of retinoids (Mrass et al., 2004).

Most recognizable physiological and therapeutic effects of retinoic acids are executed through activating their cognate nuclear receptors, namely, RAR and RXR (Lehmann et al., 1992). RAR and RXR consist of three subtypes, i.e., α, β and γ. RARs bind principally to atRA, cis-RAs and most other retinoids; whereas RXRs bind more specifically to 9cRA (Lehmann et al., 1992; Newcomer et al., 2003). To function as transcription factor, RARs heterodimerize with RXRs and bind to the retinoic acid response elements (RARE) with a direct repeat of the core sequence 5’-PGKTCA spaced by 1 (DR1), 2 (DR2), or 5 (DR5) nucleotide(s) (Durand et al., 1992; Bastien & Rochette-Egly, 2004). A more complex interaction is anticipated for RXRs, because these receptors not only form homodimers and bind to DR1, they are also common heterodimer partners for a variety of other nuclear receptors, including PXR, CAR, LXR, FXR and VDR (Bastien & Rochette-Egly, 2004; Kullak-Ublick et al., 2004). Among the important genes regulated by RAR/RXR are those having fundamental roles in the organization of developmental patterns during embryogenesis such as the Homeobox (Hox) family of genes, and those involved in receptor feedback regulation and retinoid catabolism, including RARβ and CYP26 (Ross et al., 2000). Studies have also shown that physiological levels of RA can bind to and activate other orphan nuclear receptors, such as RORβ and PPARδ/β, with unclear physiological roles (Stehlin-Gaon et al., 2003; Shaw et al., 2003). PXR was also reported to be activated by pharmacological levels (>10^{-6} M) of atRA (Wang et al., 2006). Whether such receptor activation is a direct ligand-mediated effect or an indirect effect through secondary activation pathways remains undefined. The multiple target receptors of RA may explain their pleiotropic activity especially the multitude of undesired toxicities occurring during retinoid therapy.

1.9.2 Retinoid toxicity and oxidative stress
Clinical use of retinoids results in frequent adverse drug reactions and teratogenicity that present a major hindrance in its continued therapeutic application (Ross et al., 2000).
During RA chemotherapy, systemic toxicities affecting the general immune system, respiratory tract, GI tract, liver, skin, peripheral and central nervous system, and unspecific metabolic deregulation such as weight gain have been frequently reported. The RA or atRA syndrome, characterized mainly by severe hyperleukocytosis event, is a life-threatening adverse drug reaction during retinoid therapy. This adverse drug reaction has been reported in 15-50% of APL patients undergoing retinoid chemotherapy with fatality in 10% of these cases (Shen et al., 2004B; Ades et al., 2005; de Botton et al., 2003). Hepatotoxicity, determined by an elevation of the liver injury markers, afflicts as much as 15-50% of adult and children patients undergoing atRA or 9cRA therapy (Shen et al., 2004B; Adamson et al., 2001). Liver cirrhosis that often results from a chronic untreated liver injury has also been reported upon a long-term consumption of diets high in vitamin A, such as the polar bear liver, or of vitamin A supplementation (Penniston & Tanumihardjo, 2006). Besides, retinoid use in early pregnancy has been implicated in teratogenicity with a wide range of abnormal organogenesis in the central nervous system, impairment of reproductive and craniofacial development, and even lethality of the fetus (Ross et al., 2000). Studies from the retinoid receptor knockout mice revealed that RXRα and RARγ may be involved in some teratogenic outcome such as the limb and axial defects (Lohnes et al., 1993; Sucov et al., 1995). However, the mechanistic detail on how these receptors participate in the pathogenesis remains unexplored. To date, the information with regard to the mechanisms underlying retinoid toxicities is very scarce. Elucidation of the mechanisms is expected to offer enlightening insights into future drug development aiming at minimizing adverse reaction while preserving therapeutic effectiveness.

Oxidative stress and production of free radicals may take on a crucial role in RA toxicity and teratogenicity. In the presence of prostaglandin H synthase, oxygen molecules, and precursor hydroperoxide radicals and/or polyunsaturated fatty acids, RA can be oxidized into carbon-centered radicals and then reactive peroxyl radicals (Samokyszyn & Marnett, 1987; Marnett, 1990). Peroxyl radicals are recognized as a major mediator of lipid peroxidation (Marnett, 1990). RA was also found to target the mitochondria and nitrite oxide synthase systems to increase ROS production (Personette et al., 2000). At above micromolar levels, RA as well as its precursors, retinol and retinal, increase metal-dependent superoxide radicals, leading to DNA oxidative damage (Murata & Kawanishi, 2000). In addition, production of free radicals may be enhanced by RA in
an indirect manner. Facilitation by RA of the auto-oxidation of linoleic acid, the major unsaturated fatty acid in cell membrane, to peroxide radicals has been demonstrated (Freyaldenhoven et al., 1998). RA is also known to stimulate direct arachidonic acid release from the phospholipid matrix (Levine & Ohuchi, 1978); increased arachidonate is subject to oxidative catalysis by PGH yielding potent enzyme-derived and hydroperoxide-derived oxidants. PGH-mediated oxidation and production of detrimental oxidants have been associated with adverse health effects, including carcinogenesis and teratogenicity (Marnett, 1990). Consistent with these findings, RA treatment leads to increased ROS generation and oxidative injury in cultured neural crest cells, embryonic stem cells, and rat sertoli cells (Davis et al., 1990; Castro-Obregon & Covarrubias, 1996; Conte de Fronta et al., 2006).

Although direct evidence of oxidative stress in causing the RA syndrome is absent, there is an indirect link showing a possible involvement of oxidative stress. Arsenic oxide treatment given to APL patients as an alternative treatment to atRA has resulted in characteristic RA syndrome, suggesting a common toxicological pathway (Camacho et al., 2000). It is known that arsenic is a potent oxidative stress inducer, capable of activating the Nrf2 transcription pathway (He et al., 2006; Lu et al., 2007). In addition, acute respiratory distress in the RA syndrome due to massive infiltration of differentiated APL cells in the alveolar spaces of lungs is associated with increased expression of IL-8, a chemokine possibly involved in the chemotactic transmigration of immune cells (Tsai et al., 2007). IL-8 has been shown to be a target of Nrf2 (Zhang et al., 2005). **Given the association between retinoid toxicity and oxidative stress, the secondary focus of my thesis was examining whether the Nrf2 transcription machinery is also a target of retinoids. This is particularly interesting as a recent study showing that RA, via interaction with RARα, can instead inhibit Nrf2 transactivation (Wang et al., 2007). The suppression of Nrf2-mediated genes, and hence weakened oxidative stress defense, is implied to have a possible role in the negative effects reported from vitamin A supplementation.**
1.10 Overall Hypothesis and Objectives

**Overall Hypothesis**

Nrf2 is activated by exposure to toxic bile acids or retinoids to induce the expression of cytoprotective genes such as antioxidative enzymes and phase III ABC transporters, thereby increasing cell survival against resultant cytotoxicity and cell death.

**Objectives**

I. To determine whether bile acids or retinoic acids can activate Nrf2 target genes and a subset of ABC transporters.

II. To confirm the induction of the above genes is dependent on Nrf2 with evidence of a concurrent activation of Nrf2-ARE transcription system.

III. To examine the *in vitro* protective role of Nrf2 against LCA or retinoid toxicity using siRNA-mediated Nrf2 knockdown liver cells.

IV. To investigate the *in vivo* role of Nrf2 in bile acid-induced liver injury using Nrf2 knockout mice as a model.

V. To determine whether increases in ABC transporters by bile acids result in functional augmentation of cellular efflux activity.

VI. To provide some mechanism through which bile acids and retinoids induce the above cytoprotective genes. Specific attention will be to stress-related cell signaling pathways.
CHAPTER 2.0

ACTIVATION OF NRF2 BY TOXIC BILE ACIDS PROVOKES ADAPTIVE DEFENSE RESPONSES TO ENHANCE CELL SURVIVAL AT THE EMERGENCE OF OXIDATIVE STRESS

Kah Poh Tan, Mingdong Yang, and Shinya Ito


KPT designed and performed all experiments and wrote the manuscript; MY assisted, established and delivered helpful methods in animal experiments, and performed parts of the analysis (total RNA extraction of animal tissues for real-time PCR).
2.1 Abstract

Oxidative stress, causing necrotic and apoptotic cell death, is associated with bile acid toxicity. Using liver (HepG2, Hepa1c1c7, and primary human hepatocytes) and intestinal (C2bbe1, a Caco-2 subclone) cells, we demonstrated that toxic bile acids, such as lithocholic acid (LCA) and chenodeoxycholic acid, induced the NF-E2-related factor 2 (Nrf2) target genes, especially glutamate-cysteine ligase subunits (GCLM and GCLC), the rate-limiting enzyme in glutathione (GSH) biosynthesis, and thioredoxin reductase 1. Nrf2 activation and induction of Nrf2 target genes were also evident in vivo in the liver of CD-1 mice treated 7-8 h or 4 d with LCA. Silencing of Nrf2 via small-interfering RNA suppressed basal and bile acid-induced mRNA levels of the above genes. Consistent with this, overexpression of Nrf2 enhanced, but of dominant-negative Nrf2 attenuated, Nrf2 target gene induction by bile acids. The activation of Nrf2-ARE (antioxidant responsive element) transcription machinery by bile acids was confirmed by increased nuclear accumulation of Nrf2, enhanced ARE-reporter activity, and increased Nrf2 binding to ARE. Importantly, Nrf2 silencing increased cell susceptibility to LCA toxicity, as evidenced by reduced cell viability, and increased necrosis and apoptosis. Concomitant with GCLC/GCLM induction, cellular glutathione (GSH) was significantly increased in bile acid-treated cells. Cotreatment with N-acetyl-L-cysteine, a GSH precursor, ameliorated LCA toxicity, whereas cotreatment with buthionine sulfoximine, a GSH synthesis blocker, exacerbated it. In summary, this study provides molecular evidence linking bile acid toxicity to oxidative stress. Nrf2 is centrally involved in counteracting such oxidative stress by enhancing adaptive antioxidative response, particularly GSH biosynthesis, and hence cell survival.
2.2 Introduction
Exposure to excessive bile acids is toxic to the cells, contributing an etiopathological factor to a number of liver and intestinal diseases such as cholestasis and colorectal cancer (Debruyne et al., 2002; Rao et al., 2001). Among the bile acids, lithocholic acid (LCA), a hydrophobic secondary bile acid produced by colonic microflora on chenodeoxycholic acid (CDCA), is the most toxic bile acid with genotoxic and mutagenesis-enhancing properties (Kawalek et al., 1983; Kozoni et al., 2000). In rodents, it induces intrahepatic cholestasis-like hepatotoxicity (Staudinger et al., 2001), and it promotes chemical-induced colon carcinogenesis (Kozoni et al., 2000). CDCA, the most hydrophobic primary bile acid, is able to cause severe liver injury in species like rhesus monkey, and mild hepatotoxicity in humans; its chronic administration results in increased colonic production of LCA (Hofmann, 2004).

The integrity and coordination of efficient hepatic bile flow and intestinal bile extraction is hence critical for species survival. The liver and intestinal cells achieve this through a concerted network involving the nuclear transcription factors, such as farnesoid X receptor (FXR), vitamin D receptor (VDR), retinoid X receptor (RXR), liver X receptor (LXR), pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR). These receptors regulate bile-metabolizing and -conjugation enzymes, and bile transporters to prevent excessive accumulation of bile acids (Eloranta & Kullak-Ublick, 2005). Bile acids are regarded as signaling molecules which facilitate synchronization of the above regulators in their handling of cellular bile fate. The crosstalk among these receptors is important in maintaining homeostasis of physiological bile extraction, constituting the baseline protection against bile acid toxicity.

Meanwhile, increased cellular production of reactive oxygen species (ROS) and oxidative stress has been implicated in exposure to toxicological concentrations of bile acids. Bile acid-induced oxidative stress results from induction of membrane permeability transition consequent to mitochondrial toxicity and activation of death receptors (CD95), which subsequently lead to apoptosis, via activation of pro-apoptotic effectors caspases, and necrosis (Palmeiro & Rolo, 2004). Whether there exists any regulator to counteract such oxidative stress and progression of bile acid toxicity is presently unknown. Due to its important role as an oxidative stress sensor
and anti-apoptosis factor (Itoh et al., 2004A), we hypothesized that the nuclear factor (erythroid 2-like) factor 2 or Nrf2, may play a central role by enhancing adaptive response and cell survival during exposure to excess bile acids.

Nrf2, a basic leucine zipper transcription factor that binds to antioxidant responsive element (ARE), is a chief regulator for many antioxidative, cytoprotective genes (Kensler et al., 2006). Among Nrf2 target genes, the glutamate cysteine ligase (GCL), composed of modulatory (GCLM) and catalytic (GCLC) subunits, is the rate-limiting enzyme for cellular biosynthesis of glutathione (GSH), an important intracellular antioxidant in preserving redox balances. Emerging studies have shown that Nrf2 is a multi-organ protector against various toxic reactive insults; among others are chemical carcinogens (Ramos-Gomez et al., 2001) and acetaminophen (Chan et al., 2001). Hence, robust Nrf2 activation in the cell maybe a critical adaptive response to overcome oxidative stress-induced disease processes. However, Nrf2 activation is not merely a cellular response to all circumstances of oxidative stress as exposure to some oxidative stress inducers such as high dose UVB ray would in turn result in Nrf2 deactivation (Kannan & Jaiswal, 2006). Presently, it is not known if toxic bile acids could activate Nrf2.

In this study, we combined in vitro and in vivo approaches to demonstrate that Nrf2 is activated by cytotoxic bile acids, thereby inducing genes, such as GCL and hence GSH biosynthesis, to protect the cells against bile acid toxicity.

2.3 Methods

**Cell Culture and Chemicals**

The human hepatoma-derived HepG2 (ATCC; Manassas, VA) and mouse hepatoma-derived Hepa1c1c7 (a gift from Dr. Patricia Harper, The Hospital for Sick Children, Toronto, ON) were maintained in α-MEM with 10% fetal bovine serum (FBS). C2bbe1, a subclone of colon carcinoma Caco-2 which displays a more homogeneous brush-border epithelial-like morphology (ATCC), were maintained in DMEM supplemented with 10% FBS, 1.5 g/L sodium bicarbonate and 10 mg/L holotransferrin. The human primary hepatocytes were purchased from Celprogen (San Pedro, CA) and grown in specially formulated serum-free growth media (Celprogen). Experiments of all secondary cell lines were conducted within 10 cell passages.
Treatments were given at ~80% confluency for all cell lines except for C2bbe1. Because C2bbe1 cells differentiate to mature colonocytes at confluence, treatments to this cell line were given 2-3 d post-confluency. All chemicals were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Test bile acids were dissolved in dimethyl sulfoxide (DMSO)(0.2% v/v). Oligonucleotides were synthesized at the Toronto Centre for Applied Genomics or Integrated DNA Technologies (IDT; Coralville, IA).

**In vivo Mouse Experiments**

The animal care and experimental procedures were approved by the Animal Care Committee at the University of Toronto and the Hospital for Sick Children. To examine whether Nrf2 target genes may have been modulated during acute exposure to LCA prior to the onset of symptomatic liver injury, 9-12 wk CD-1 mice (Charles River; Montreal, Quebec) were injected i.p. a single dose of LCA at 125 mg/kg body wt. dissolved in sterilized DMSO (final amount <1% body wt.). Mice were killed 7-8 h after the treatment. In a separate experiment aiming to investigate changes in similar genes upon extended treatment with LCA, mice were injected the same dose of LCA dissolved in sterilized corn oil (final amount ~2 % body wt.) twice daily for 4 d. This treatment protocol has been used in the past to induce cholestatic liver injury in mice (Staudinger et al., 2001). Mice were killed 16 h after the last dosing. The use of corn oil as a solvent for LCA in the extended treatment protocol was to avoid the possible toxicity with chronic exposure to DMSO. At necropsy, portions of their livers were sampled in RNAlater reagent (Invitrogen, Carlsbad, CA) and neutral-buffered 10% formalin for mRNA and histological analyses, respectively. Nuclear protein extraction of chilled livers was carried out using Nuclear Extraction Kit (Panomics, Fremont, CA). Sera of mice were collected for analysis of liver function/injury markers: total bilirubbin (TBL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and $\gamma$-glutamyl transferase (GGT) using established automated methods (Dept. of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, ON).
**cDNA Synthesis and Quantitative Reverse-Transcription PCR (qRT-PCR)**

Total RNA was isolated with RNeasy Kit (Qiagen; Valencia, CA) and reverse-transcribed into cDNA using random hexamers and Moloney murine leukemia virus (MMLV) or SuperScript II reverse transcriptase (Invitrogen). Aliquots of cDNA equivalent to 100 ng RNA were used for real-time PCR performed on Applied Biosystems (ABI; Foster City, CA) 7500 Real-Time PCR System or Prism 7700 Sequence Detection system with reaction mode set at 50 °C (2 min), 95 °C (20 s), followed by 40 cycles of 95 °C (15 s) and 56 °C or 60 °C (1 min). The primers for ribosomal 18S, β-actin, tata-box binding protein (TBP), GAPDH, GCLM, GCLC and NQO1 were purchased from pre-designed and -optimized Taqman primer-probe sets (Assay-on-demand gene expression probe, ABI), whereas custom-made primers for SYBGreen real-time PCR detection were used for the other gene transcripts (primer sequences available upon request). To assure specificity, primer pairs were designed to span across two neighboring exons and detection of a single peak in dissociation curve analysis. The ΔΔCt method (Livak & Schmittgen, 2001) was employed to quantify the amplification-fold difference between treatment and vehicle-treated control groups, with Ct value of target genes being adjusted to individual housekeeping gene (GAPDH, β-actin, TBP and/or 18S) whichever expression was not affected by treatment protocols. Measurements were done in duplicate or triplicate with variability < 0.5 Ct.

**Immunoblotting**

Whole Cell lysate was prepared in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Roche). 10-30 µg protein was dissolved in 4-12% bis-tris gel (NuPage® Novex gel system, Invitrogen) and transferred onto a nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ). Primary antibodies (working concentration) used were: rabbit polyclonal anti-GCLC Ab-1 (1:2000) (NeoMarkers; Fremont, CA), rabbit antiserum against GCLM (1:3000) (custom-made; Alpha Diagnostic; San Antonio, TX; see below), rabbit polyclonal anti-Nrf2 c-20 (1:750)(Santa Cruz Biotechnology; Santa Cruz, CA), rabbit polyclonal
anti-TRx1 (1:3000)(Abcam; Cambridge, MA), mouse monoclonal anti-β-actin (1:10000) (Sigma) and goat polyclonal anti-lamin B c-20 (1:200) (Santa Cruz). Based on analyses of hydrophilicity, antigenicity, accessibility and sequence homology with other related proteins, an antiserum against a peptide (amino acids 29-45) of human GCLM was raised in rabbits. The immunogenicity and specificity were checked by ELISA, and its ability to detect a ~28 kDa protein (predicted size of GCLM) with reactivity halted after pre-absorption of the antibody in excess immunogen. To ensure equal loading for whole cell lysate and nuclear protein, β-actin and lamin B, respectively, were probed on the same stripped blot membranes after being used for detecting target proteins.

**RNA interference (RNAi)**
A combination of four gene-specific small-interfering RNA (siRNA) against human Nrf2 (NM_006164) was used (Dharmacon SMARTpool® siRNA reagent, Thermo Fisher Scientific, Lafayette, CO; Cat.#: M-003755). Overnight-seeded HepG2 and C2bbE1 cells at ~40% and ~60% confluence, respectively, were transfected for 48 h with 50 nM siRNA against Nrf2 (siNrf2) or equal molar mismatched siRNA controls (siCtr). These siRNAs were earlier complexed with liposome carrier Dharmafect I (Dharmacon) at 0.2 µL/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Under this condition, the transfected cells after 48 h appeared normal morphologically and did not differ from untransfected cells in cell viability and mRNA levels of inflammatory marker IL-6 (not shown). Treatments with bile acids were then followed for 16-18 h. To ensure achieving functional and specific silencing, the mRNA levels of Nrf2, known Nrf2 target genes and homologous subtypes Nrf1 and Nrf3 were compared between siNrf2 and siCtr groups before and after treatments in all experiments.

**Plasmid Constructs**
The expression vectors for Nrf2 (pEF_Nrf2), dominant negative Nrf2 (pEF_DNrf2) and empty vector (pEF) were kindly provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). To make an ARE-reporter construct (pGL3_ARE), a DNA duplex (CGGGGTACCGCCGCACAAAGCGCTGAGTCACGCGGGAGGCAG
ATCTTCC) (core ARE was underlined; -3595/-3625 of hGCLC gene) containing the indispensable ARE motif of GCLC (-3604/-3614) (Mulcahy et al., 1997) with Kpn I/Bgl II at 5’ and 3’ ends, respectively, was constructed by annealing two PAGE-purified complementary oligonucleotides. This insert was ligated to similar restriction enzyme sites of pGL3 luciferase reporter plasmid with SV-40 promoter (Promega; Madison, WI). Similar reporter construct has been successfully used previously (Mulcahy et al., 1997). The responsiveness and robustness of our ARE reporter to Nrf2 transactivation was confirmed by testing of a panel of Nrf2 activators such as tert-butylhydroquinone (BHQ), lipoic acid and diethyl maleate (not shown), as well as cotransfection with Nrf2 and dominant negative Nrf2 expression vectors. To assure specificity, a mutant ARE reporter construct (pGL3_mARE) introducing three point mutations on ARE was cloned by PCR-mediated site-directed mutagenesis using pfu turbo® DNA polymerase (Stratagene) with complementary primers 5’-AGCtaTGAGgCACGGGAGGCGCAG-3’ (underlined sequence is core ARE; lower cases are mutated nucleotides) on the template pGL3_ARE. The PCR condition was 95 °C (5 min) followed by 22 cycles of 95 °C (15 s), 55 °C (30 s), 68 °C (10 min), and a final extension of 68 °C (10 min). The template was then digested by Dpn I and mutant clones were transformed in XL-1 blue competent cells (Stratagene; La Jolla, CA). Successful insertion and mutation introduction were confirmed by sequencing. The cDNA clone of human Na(+) taurocholate co-transporting polypeptide (NTCP) (Origene Technologies, Rockville, MD) was subcloned into the Not I site of pTarget expression vector (Promega), and stably transfected into HepG2 cells. Stable clones transfected with NTCP or empty vector (pTarget) were selected using 500 µg/mL G-418 growth media.

**Transfection, Reporter Assays and Overexpression Studies**

HepG2 cells at ~50% confluence were transiently transfected overnight with 0.1 µg of the firefly luciferase reporter pGL3_ARE or pGL3_mARE, 0.02 µg of the renilla luciferase control reporter pRL-TK with or without cotransfection with 0.2 µg expression vectors using Lipofectamine™ 2000 (Invitrogen) as transfection carrier. Treatments with bile acids were then carried out for another 16-18 h in all experiments, unless otherwise stated. Conjugated bile acid treatments (GCA and
GCDCA) were done on NTCP- transfected HepG2 cells. Luciferase activities of the cell extracts were determined with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity (RLU) was calculated from firefly luciferase values normalized to those of the control Renilla luciferase, and expressed as ratios to vehicle-treated empty pGL3 promoter construct and, if any, cotransfected expression vector. All experiments were done in triplicate and repeated at least twice. For overexpression studies, Hepa1c1c7 cells at 50% confluence in T25 flasks were transfected with 3 µg of Nrf2 or dominant negative Nrf2 expression vector for 24 h, followed by treatments with bile acids for another 20-22 h.

**Quantitative Chromatin Immunoprecipitation (ChIP)**

The assay was performed using the ChIP assay kit (Upstate, Billerica, MA) with slight modifications. After 6 h of treatments, chromatin protein-DNA of HepG2 cells was fixed (cross-linked) in neutral-buffered 1% formaldehyde at room temperature for 10 min. Further fixation was stopped by 125 mM glycine buffer. The DNA was sheared by sonication on ice into fragments of ~ 500 bp in size. An aliquot (1/4) of sample supernatant was saved as input DNA for later PCR analysis. After pre-clearing with protein A agarose beads, supernatants were incubated with a ChIP-graded anti-Nrf2 antibody (1:250; Santa Cruz) in rotation at 4 °C overnight. To control for nonspecific binding of antibody used, an equal amount of the host antibody against an irrelevant protein (rabbit polyclonal anti-CYP1A1) from the same manufacturer (Santa Cruz) was included in a separate batch of control supernatants and followed through the remaining protocols. Antibody-chromatin complexes were collected by salmon sperm DNA/ protein A beads. DNA was released from crosslinked complexes with proteinase K at 65 °C for 4 h followed by 72 °C for 10 min. DNA was then extracted and eluted with 120 µL Tris (pH 8.0) buffer using the DNeasy Kit (Qiagen) and the contaminant RNA was cleaved with RNase A (Invitrogen). For detection of the ARE of GCLM (-56/-66) (Erickson et al., 2002) and of GCLC (-3604/-3614) (Mulcahy et al., 1997) by real-time PCR, the primer sets and Taqman probe (5'-Fam, 3'-Tamra) were designed by PrimerQuest software (IDT) which amplify 5'-region exactly on the core ARE. The primers for detecting GCLM’s ARE were: sense, 5’-CGCGGGATGAGTACCGTG-3’; antisense, 5’-GGGAGAGCTGATTCCAAACTGA-3’;
probe, 5’-ACGAAGCACTTTCTCGGCTACGAT-3’ which amplify a 79 bp product (-33/-112). For probing the ARE of GCLC, the primers used were: sense, 5’-GGACTGAGACTTTGCCCTAAGAAG-3’; antisense, 5’-GCGCAGTTGTGTTGATACAG-3’; probe, 5’-CGCACAAAGCGCTGAGTCAC-3’ which amplify a 160 bp product (-3479/-3609). Quantitation of Nrf2 bound to these AREs after the treatments was carried out on 5% of DNA eluates with qPCR analysis similar to that for the mRNA, except that the Ct value of amplicon from each sample’s input DNA was used as normalization control as described (Beresford & Boss, 2001).

**Cytotoxicity, Necrosis, and Apoptosis**

For cytotoxicity analysis, a non-toxic assay, namely Alamar Blue™ (Biosource, Nivelles, Belgium), was used. This assay uses a fluorometric indicator to measure the chemical reduction of cell medium which correlates directly to the metabolic activity of viable cells. The working assay medium (10% v/v Alamar Blue in α-MEM, 2% FBS, 1% penicillin/streptomycin, 37 °C) was first incubated with cells seeded on 24-well culture plate prior to treatment to obtain baseline/pretreatment values. The measurement was made at excitation/emission/cut-off $\lambda = 540/590/570$ nm after one hour of incubation with the assay medium at 37 °C. Immediately after the measurement, the cells were rinsed with pre-warmed PBS followed by the treatments. At various time points, treatment media were replaced with fresh assay media to allow for a continuous monitoring of cell viability. The fluorescent unit of each treatment and control was expressed as percent change relative to individual baseline/pretreatment value.

To determine necrosis, cellular release of lactate dehydrogenase (LDH) into treatment media was measured with a LDH detection kit (Roche Applied Science, Indianapolis, IN). To control for cell mass and spontaneous release of LDH by viable cells into media, the ratio of LDH activity in the medium to the cells (cell lysate) was determined and then subtracted from those of the vehicle-treated controls. The measurement was made colorimetrically at $\lambda = 490$ nm. The intra-assay variability (% CV) of duplicate determinations was 2.2.
To assess apoptosis, the cellular caspases activity was measured using the rhodamine 110-conjugated substrate Asp-Glu-Val-Asp (Z-DEVD-R110)(Invitrogen-Molecular Probes, Carlsbad, CA). Although classically known to detect caspase-3 activity, recent analysis by the manufacturer showed that this substrate is also a target of multiple caspases such as 6, 7, 8 and 10. The caspases activity of cell lysate was quantitated at excitation/emission $\lambda = 496/520$ nm, and normalized to individual protein concentration measured by Bio-Rad Protein Assay.

**Total Glutathione (GSH) Quantitation**

Cellular GSH was quantitated with a GSH assay kit (Cayman Chemical, Ann Arbor, MI) based on an established GSH recycling enzymatic method (Tietze, 1969). After 24 h treatment with bile acids, HepG2 cells were lysed in ice-cold MES buffer following a quick freeze-thaw cycle and deproteinized by 0.5 g/mL metaphosphoric acid. An aliquot of each sample was saved before deproteinization for determining protein content. The total GSH of deproteinized cell supernatants was measured against a GSSG standard curve according to the manufacturer’s instruction. The measurement unit was expressed as nmol/mg protein/min. The intra-assay variability (%CV) of duplicate determinations for all samples repeated in four experiments was 3.1.

**Statistical Analysis**

Statistical tests were conducted using SigmaStat 3.1 (San Jose, CA) or SPSS10.1 (Chicago, IL). Normality and equal variance tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA (parametric) or one-way ANOVA on ranks (non-parametric). Once statistical significance was attained ($p<0.05$), the Dunnet’s (parametric) or Dunn’s (non-parametric) test comparing between treatment and control groups was initiated. Comparisons between two groups on single variable were accomplished by Student’s independent t-test (parametric) or Mann-Whitney U test (non-parametric). Difference with $p<0.05$ was considered statistically significant.
2.4 Results

*Induction of Nrf2 target genes by bile acids in liver and intestinal cells*

A dose-response increase in mRNA of GCLM and GCLC following LCA and/or CDCA treatment was noted for HepG2 and C2bbe1 cells with a significant >4-fold induction at ≥50 µM LCA and ≥100 µM CDCA (Fig. 9a). Peak inductions of GCL subunit genes occurred at 50-75 µM LCA and 100-150 µM CDCA. Increased bile acid concentrations, i.e., LCA ≥ 100 µM or CDCA ≥ 250 µM, resulted in increased cell death and reduced inductions of GCL genes at 24 h of treatment. Significantly higher GCL gene transcripts, although at a lower magnitude (2-4 fold induction), were also noticed for the primary human hepatocytes treated with both bile acids (Fig. 9a). So were similar treatments given to the mouse hepatoma Hepa1c1c7. In C2bbe1 cells, CDCA treatment (100 µM) caused a modest increase in GCL genes (~2-fold), whereas treatment with deoxycholic acid (DCA) (≥ 100 µM), a secondary bile acid often associated with toxicity and carcinogenesis in colonic cells, resulted in comparable GCL inductions to those of LCA treatment.

Furthermore, mRNA of Nrf2 and a panel of known Nrf2 target genes such as NAD(P)H quinone oxidoreductase 1 (NQO1), thioredoxin reductase 1 (TRx1), ferritin light subunit (FRL), and heme oxygenase I (HO1) were also simultaneously induced ≥2-fold by bile acids in all test cells (Fig. 9b). Particularly, TRx1, an important seleno-enzyme in cellular thiol and redox maintenance, was increased >4-fold in HepG2 and C2bbe1 cells. Note that glutathione s-transferase P1 (GSTP1), which was induced by bile acids in HepG2 and C2bbe1 cells, was not evident in primary hepatocytes (Fig. 9b). Instead, another GST subtype, GSTA1, was increased by bile acids to ~2-fold in primary hepatocytes (not shown). This disparity suggests possible cell type specificity in regulation of GSTs by bile acids.

Increased protein levels corresponding to mRNA induction were also noted (Fig. 9c). The apoptosis marker (caspases activity) and cell viability analyses showed that a mild toxicity began to occur in HepG2 cells at 60 – 80 µM of LCA treatment, followed by a precipitous increase in cell death and caspase activity at >80 µM (Fig. 9d). Notably, the induction of GCL subunits and other antioxidative genes peaked in the range of LCA (60 – 80 µM) during which HepG2 cells began to
experience mild toxicity. These findings suggest that induction of the cytoprotective genes may represent an adaptive cell defense mechanism against LCA toxicity.

**In vivo activation of Nrf2 target genes**

Acute administration (7-8 h) of LCA to mice at a dose known to induce cholestatic liver injury (Staudinger *et al.*, 2001) resulted in Nrf2 accumulation in the nuclei, a signature event of Nrf2 activation (Fig. 10a). This phenomenon coincided with significant inductions of Nrf2 target genes (Fig. 10b; top panel) found to be increased in the *in vitro* studies. Of note, the increase of TRx1 transcripts rose to ~50-fold at acute exposure to LCA, implying a possible critical role of this enzyme in early toxicity of LCA. At this shorter exposure to LCA, however, analysis of serum liver function and cholestatic markers (ALT, AST, GGT and TBL) as well as liver histology did not indicate liver dysfunction or pathological changes.

With prolonged LCA treatment during which symptomatic liver injury (elevated ALT, AST and TBL, and liver necrosis in histological analysis; not shown) already occurred, induction of Nrf2 target genes, such as GCL subunit gene transcripts, was found to sustain compared with those treated acutely with LCA (Fig. 10b; bottom panel). Nqo1 was increased with prolonged treatment, whereas TRx1 induction was reduced. Consistent with the observations from primary human hepatocytes, a class of mouse Gst (Gsta1/2), rather than Gstp1, was found to be induced by LCA, with ~10-fold induction at 4 d of treatment (Fig. 10b; bottom panel). All gene transcripts did not differ between untreated animals and animals treated with vehicle alone.

**Involvement of Nrf2 and activation of Nrf2-ARE transcription machinery**

To examine if Nrf2 participated in the preceding gene activations, we silenced Nrf2 of HepG2 and C2bbe1 cells via siRNA. This resulted in significant reductions of >60% in Nrf2 mRNA and protein without interfering with other homologous Nrf subtypes (Fig. 11a, 11b). Nrf2 silencing significantly decreased the basal levels of known Nrf2 target genes (Fig. 11c, 11d, 11e), an observation similar to those seen in *in vivo* Nrf2 knockout mice (Lee *et al.*, 2005). Also, the induction of GCLM, GCLC
and other Nrf2 target genes by bile acids in HepG2 cells (Fig. 11c, 11e) and C2bbe1 (Fig. 11d) has been mitigated. Comparable reduction in inducible expressions of Gclm occurred with transfection of dominant-negative Nrf2 in Hepa1c1c7 cells, consistent with the enhanced gene induction with Nrf2 overexpression (Fig. 11f). Similar observations were noted for other Nrf2 target genes such as Gclc and Nqo1.

To verify that there was an activation of Nrf2-ARE transcription machinery with exposure to toxic bile acids, we extracted the nuclear proteins of bile-acid treated HepG2 cells over different time points across 24 h. Translocation of cytosolic Nrf2 to nucleus represents the prerequisite event of receptor activation. Nrf2 started to be enriched in cell nuclei within 1-3 h of bile acid treatments and sustained through 24 h, with CDCA-treated cells showed reduced Nrf2 translocation events with longer time of exposure (24 h) (Fig. 12a). Further, various bile acids were found to increase the activity of an ARE-reporter assay in a dose-dependent manner, suggesting that these bile acids were capable of inducing Nrf2 transactivation (Fig. 12b). The magnitude of luciferase activity of the highest concentration of test bile acids was comparable to those of treatments with antioxidants tBHQ (100 µM) and α-lipoic acid (200 µM), denoting that bile acids are equally potent Nrf2 activators. Note that there was an ~8-fold increase in reporter activity with vehicle DMSO treatment compared with that of the empty vector harboring only the SV40 promoter. This suggests the existence of a strong constitutive Nrf2-ARE transactivational activity in HepG2 cells, an observation in line with the persistent oxidative stress observed in many cancerous cell lines (Brown & Bicknell, 2001). HepG2 cells are deficient in conjugated bile acid transporters such as NTCP which leads to its resistance to conjugated bile acid-induced oxidative stress (Kullak-Ublick et al., 1996). Transfection of NTCP expression vector hence restores, to some degree, its sensitivity to conjugated bile acids. In this study, we found that GCDCA, a known cholestatic conjugated bile acid, significantly induced the ARE reporter. This suggests that activation of Nrf2-ARE may be crucial to counteract the toxicity of GCDCA as reported previously (Dent et al., 2005). The potency of bile acids in activating this reporter based upon molarities was: LCA > CDCA ≈ DCA > GCDCA ≥ UDCA > CA > GCA. This order is in consensus with the toxicity profile of these bile acids.
acids, particularly in terms of their ability to produce oxidative stress (Krahenbuhl et al., 1994). Overexpression of Nrf2 further enhanced the reporter activity by bile acids, whereas coexpression of dominant-negative Nrf2 attenuated the activity, and a mutant ARE construct was completely uninducible by bile acids (Fig. 12c). Using the quantitative ChIP assay, we found an increased Nrf2 occupancy to the AREs of both GCL subunits in the native cell context upon 6 h treatment with bile acids (Fig. 12d). Taken together, our data suggest that activation of the Nrf2-ARE machinery underlies induction of Nrf2-target genes by toxicological concentrations of bile acids.

**Protective role of Nrf2 in bile acid toxicity**

To directly investigate the role of Nrf2 in protection against toxic bile acids, we first silenced Nrf2 of HepG2 cells via RNAi upon which the cells were subjected to toxic LCA challenges. Nrf2 knockdown increased cell susceptibility to toxic LCA with a significantly decreased cell viability starting at 4 h of treatment (Fig. 13a). Without LCA challenge, Nrf2-knockdown cells did not differ in cell viability from those treated with siRNA control (siCtr). Significant protective effects of Nrf2 against LCA toxicity were also observed in C2bbe1 cells, and in HepG2 with ≥300 µM CDCA. To investigate which route of cell death was particularly involved in Nrf2’s protection, established markers of necrosis and apoptosis were examined. LCA at 90 µM was used as at this dose both apoptosis and necrosis were found to simultaneously occur. Necrotic event, as determined by LDH released into the culture media, remained constantly higher in Nrf2 knockdown cells than those of siCtr starting from 2 h of LCA treatment (Fig. 13b). Nrf2 silencing alone did not affect the cellular release of LDH. Similarly, in the assessment of apoptosis, Nrf2-knockdown cells exhibited much higher and prolonged elevation of caspases activity than were siRNA control-treated cells upon LCA treatment (Fig. 13c). Silencing of Nrf2 alone did not result in increased basal caspases activity.

**The role of GSH in resisting LCA toxicity**

The increase in GCLM and GCLC, the rate-limiting enzyme in GSH biosynthesis, observed in earlier experiments after LCA (75 µM) or CDCA (100 µM) treatment was accompanied by a significant increase by >4-fold in cellular GSH levels at 24 h (Fig. 13d).
This increase was comparable to treatment with 200 µM α-lipoic acid, a GSH inducer. To determine whether the induced cellular GSH is a protective mechanism against toxic bile acid, we cotreated HepG2 cells with a toxic dose of LCA and a GSH biosynthesis blocker, buthionine sulfoximine (BSO) which inhibits the activity of GCL subunits and blocks GSH biosynthesis. BSO treatment together with toxic LCA decreased cell resistance toward LCA exposure with more apparent effects in late treatment (~24 h) (Fig. 14b). Furthermore, depletion of cellular GSH by pretreatment with BSO prior to LCA challenge markedly decreased cell resistance with a drastic drop in cell viability within 4 h of treatment. Conversely, toxic LCA challenge in the presence of an antioxidant and GSH precursor N-acetyl-L-cysteine (NAC) was found to alleviate the toxicity. This suggests that the basal as well as inducible GSH are important determinants of cellular resistance to LCA. Consistent with these findings, NAC cotreatment significantly reduced the oxidative stress-responsive ARE-reporter activity by LCA, indicating an antioxidative effect (Fig. 14c). BSO cotreatment, on the other hand, further increased the reporter activity, consistent with a heightened oxidative stress (Fig. 14c).

2.5 Discussion

The discovery of bile acids as key signaling molecules in the enterohepatic circulation system reveals a critical role of hepatic and intestinal xenobiotic nuclear receptors in the metabolism and detoxification of bile acids (Chawla et al., 2000). Particularly, the cytotoxic hydrophobic bile acids, CDCA and LCA, have been shown to be ligands and potent inducers of these receptors. LCA, at physiological and non-toxicological concentrations (5-30 µM) can activate FXR (Makishima et al., 1999; Makishima et al., 2002) and VDR (Makishima et al., 2002), indicating their crucial role in physiological handling of this bile acid. The major detoxification routes of LCA, i.e., sulfation by sulfotransferase 2A (SULT2A) and 7α-hydroxylation by CYP3As, are coordinated by VDR (Makishima et al., 2002; Echchgadda et al., 2004). FXR, which activates the hepatic bile salt export pump BSEP (Ananthanarayanan et al., 2001) and downregulates the bile-synthesizing enzyme CYP7A1 (Makishima et al., 1999), works to prevent intracellular accumulation of bile acids.
Interestingly, at higher and toxicological concentrations of LCA (≥ 50 µM) and CDCA (≥ 100 µM) which potentially cause cell injury, PXR (Staudinger et al., 2001; Makishima et al., 2002) and Nrf2, as shown in this study, are found to be activated. The activation of PXR and Nrf2 induces the major hydroxylation enzymes CYP3As and antioxidative genes (Eloranta & Kullak-Ublick, 2005; Kensler et al., 2006), which may represent an important adaptive mechanism of cellular defense against toxic bile acids. Furthermore, we observed that induction of multiple bile salt/conjugate efflux transporters such as ATP-binding cassette (ABC) transporters ABCC2, ABCC3 and ABCG2 by bile acids is dependent on Nrf2 (Chapter 3). Hence, the collective induction of cytoprotective genes by Nrf2 and PXR appears to set off a second line of protection against possible progression of bile acid toxicity toward irreversible cell death.

In this study, we showed for the first time that many bile acids, especially LCA, CDCA and DCA, are capable of activating redox-sensitive Nrf2. We also provided in vivo evidence that LCA is able to activate Nrf2, inducing similar target genes observed in in vitro studies. The induction of Nrf2 target genes by LCA in vivo was found to precede and sustain through biochemically and histologically overt liver injury, suggesting that the collective induction of these antioxidative genes may be an integral part of cell defense against bile acid toxicity and hepatic injury. Previous studies have reported an increased intracellular production of detrimental hydroperoxides in isolated rat hepatocytes with hydrophobic bile acid exposure (Sokol et al., 1995), an observation in consensus with the increased oxidative stress byproducts in the liver of patients with cholestasis (Vendemiale et al., 2002). Since Nrf2 activation is indicative of cellular antioxidative response, our study provides molecular evidence linking mechanism of bile acid toxicity to oxidative stress.

We further showed that induction of hepatic GCL subunits via Nrf2 which provokes GSH biosynthesis can increase hepatocyte resistance and survival during excessive bile acid exposure. The essential role of GSH in hepatic protection against injury and oxidative xenobiotic insults has been well exemplified (Huang et al., 2001; Glosli et al., 2002). In agreement, in vivo knockout of Nrf2 enhances sensitivity of death receptor-induced hepatic apoptosis as a result of decreased GSH levels (Morito et al., 2003). GSH is also known to protect against mitochondrial...
injury, a major mechanism of bile acid toxicity (Palmeira & Rolo, 2004). A fraction of cytosolic GSH which becomes mitochondrial GSH is crucial in the defense of oxidant-induced mitochondrial-mediated cell death (Fernandez-Checa & Kaplowitz, 2005). Additionally, Nrf2 activation has been shown to protect mitochondria by preventing inhibition of mitochondrial complex II upon exposure to oxidative neurotoxins (Calkins et al., 2005). In intestinal mucosa, cellular GSH has an essential role in maintaining epithelial integrity, transport activity, and metabolism of and susceptibility to luminal toxins (Aw, 2005). Overall, our studies, coupled with supportive evidence from recent literature, suggest that protection conferred by hepatic and intestinal Nrf2 against bile acid-induced oxidative stress is, at least partly, achieved by increasing GSH levels.

The simultaneous induction of other Nrf2 target genes may work in concert with GCL subunits to combat bile acid-induced oxidative stress while facilitating adaptive responses. Of particular importance is TRx1, an enzyme engaged in NADPH-dependent catalysis of various redox proteins (Rundlof & Arner, 2004). It has been shown to act as a key adaptation-promoting mediator for prior exposure to 4-hydroxynonenal, a reactive lipid peroxidation-derived molecule, in inducing cellular tolerance to future oxidative stress attack (Chen et al., 2005A). Indeed, intermediate cellular stress has recently been proposed to provide an adaptation advantage by invoking enhanced cellular survival/tolerance mechanisms (Schoemaker et al., 2003; Chen et al., 2005A). Activation of NF-κB as well as Nrf2 has been shown to play an important role in this adaptation process. The drastic induction of TRx1 observed in mice upon acute exposure to toxic LCA in this study may indicate a critical role of this enzyme in adaptation process against LCA toxicity. To address whether and how this process is taking place, future studies are needed.

The precise mechanisms by which toxic bile acids activate Nrf2 remain a subject of future studies. Increased production of ROS from mitochondrial stress has long been accounted for the main source of oxidative stress induced by bile acids (Palmeira & Rolo, 2004). Insurgence of these ROS potentially targets the cysteine oxidative-sensors of Keap1, an actin-anchored cytosolic sequester that facilitates Nrf2 degradation by ubiquitin-proteosome pathway, which leads to liberation and
activation of Nrf2 (Kensler et al., 2006). Also, subsets of both conjugated and unconjugated bile acids have been shown to activate multiple kinase signaling pathways such as PKC, ERK1/2 MAPK, p38 MAPK, JNK, and/or PI-3/AKT (Dent et al., 2005; Debruyne et al., 2002). These signaling pathways have been shown as well to influence the stability of Nrf2-Keap1 complex, and post-transcriptionally regulate the Nrf2 target genes (Kensler et al., 2006).

In summary, we characterized a molecular cell defense event associated with bile acid-provoked oxidative stress. Exposure to cytotoxic bile acids in the liver and intestinal cells was shown here to cause Nrf2 activation, thereby upregulating a battery of cytoprotective genes, particularly GCL subunits, to enhance cell survival at the emergence of oxidative stress.

### 2.6 Significance
This study demonstrates, for the first time, that toxic bile acids can activate Nrf2 transcription machinery in vitro and in vivo, thereby inducing cytoprotective antioxidant proteins. The findings reaffirm earlier reports that oxidative stress is an important mediator of bile acid toxicity. The critical role of hepatic Nrf2 in promoting cell survival against toxic bile acid insults was suggested. The increased GCL biosynthesis consequent to induction of GCLM and GCLC was shown to be an important adaptive defense mechanism directed by Nrf2 to combat bile acid-induced oxidative stress and cell death. The observations that administration of LCA into mice was able to induce their hepatic Nrf2 target genes provide rationale that testing using Nrf2 knockout mice would be able to address the in vivo role of hepatic Nrf2 in bile acid toxicity.
Figure 9. Induction of Nrf2 target genes by bile acids. a, mRNA levels of GCLM (white bar) and GCLC (black bar) in HepG2 and C2bbe1 cells (left panel) after 24 h treatment with increasing doses of LCA or CDCA; [LCA] = 6.25, 12.5, 25, 50, 75 µM; [CDCA] = 25, 50, 75, 100, 150 µM. Right panel: human primary hepatocytes (passages# 4-6) were treated with 75 µM LCA or 100 µM CDCA for 24 h. Y-axis: fold change vs. vehicle-treated controls. *Significantly different (p<0.05) from vehicle-treated control by one-way ANOVA followed by posthoc test for HepG2 and C2bbe1, or ** p< 0.01 by t-test for primary hepatocyte. Mean and SEM (n=3-6). b, mRNA levels of other known Nrf2 target genes after 24 h treatment with bile acids in HepG2, C2bbe1 and primary hepatocytes; [LCA] = 75 µM; [CDCA] = 100 µM. Mean and SEM (n=3-6).c, Representative immunoblots of protein lysate (10 µg for HepG2, 30 µg for C2bbe1) probed for Nrf2 target genes after 24 h treatment with bile acids. [LCA] = 70 µM; [CDCA] = 100 µM. d, Cell viability (by Alamar BlueTM) and apoptotic marker (caspases activity) measured across increasing concentrations of LCA treatment in HepG2. Viability was measured at 24 h while caspases activity was 6 h of LCA treatment. Note that the induction of GCL genes peaked at 60-80 µM of LCA (shown by closed inverted triangles) during which mild cellular toxicity began to occur. Representative results from 4 determinations are shown.
Figure 10. Activation of Nrf2 in mice treated with LCA. a, Representative immunoblots of nuclear fractions (30 µg) probed against Nrf2 in the liver of mice after 7-8 h treatment with cholestatic LCA (125 mg/kg body wt.). Lamin B was used as loading control for nuclear protein, whereas β-actin was probed to show unintentional recruited cytosolic proteins in nuclear fraction preparation. Note that the increased nuclear Nrf2 cannot be explained by inclusion of contaminant cytosolic proteins. b, mRNA levels of Nrf2 target genes upon LCA treatment for 7-8 h (top panel) or for 4 d (bottom panel) in the liver of mice. Because there were differences in the basal gene expression of Nrf2 target genes between sexes, comparisons of all target genes between treated and untreated groups were adjusted for sex. Induction of antioxidative genes by LCA, however, occurred in both sexes. Significantly different from vehicle-treated controls by t-test. * p<0.05; ** p<0.01. Mean and SEM (n=5-9 for 7-8 h treatment; n=3 for 4 d treatment).
**Figure 11. Involvement of Nrf2 in induction of glutamate cysteine ligase subunits by bile acids.**

a, mRNA of all Nrf subtypes after 72 h treatment with siRNA. Y-axis: fold change vs. vehicle-treated cells transfected with control siRNA (siCtr). *Significant difference (p<0.001) between siRNA groups by t-test. Mean and SEM (n=3-5).

b, Representative immunoblots (20 µg protein lysate) of HepG2 after 48 h treatment with siRNA against Nrf2. c, Basal (treated with vehicle DMSO) and inducible (treated with 70 µM LCA or 100 µM CDCA) gene transcripts of GCLM and GCLC in HepG2 (mean and SEM: n=3-5); and d, basal and inducible levels of GCLM and GCLC gene transcripts in C2bbe1 treated with vehicle or 70 µM LCA. *Significant difference (p<0.01) between siCtr and siNrf2 with or without treatment by t-test. Mean and SEM (n=3-5). e, Basal and inducible expressions of other Nrf2 target genes in HepG2. Mean ± SEM. f, mRNA levels of GCLM induced by 70 µM LCA or 100 µM LCA in Hepa1c1c7 transfected with empty vector (pEF), Nrf2 (pEF_Nrf2) or dominant negative Nrf2 (pEF_DNrf2) expression vector. Y-axis: fold change vs. vehicle-treated cells transfected with empty vector pEF. *Significantly different (p<0.05) from LCA-treated cells transfected with pEF control by t-test. Mean and SEM (n=4).
**Figure 12. Bile acids activate Nrf2 transcription machinery.** a, Representative immunoblots of nuclear fraction (10 µg) extracted from HepG2 treated with bile acids (LCA, 70 µM; CDCA, 100 µM) at indicated time-points over 24 h. Lamin B was used as equal loading control for nuclear protein whereas β-actin was probed to show possible contaminant cytosolic proteins in nuclear fraction preparation. b, ARE-reporter (luciferase) activity of HepG2 treated with increasing doses of various bile acids for 16-18 h. Abbreviations (doses): LCA (50, 70, 90 µM); CDCA (50, 100, 150 µM) CA, cholic acid (150, 200, 400 µM); DCA, deoxycholic acid (50, 100, 150 µM); UDCA, ursodeoxycholic acid (50, 100, 200 µM); GCA, glycocholic acid (200, 400, 800 µM); GCDCA, glycochenodeoxycholic acid (100, 200, 400 µM). # GCA and GCDCA were tested in NTCP-transfected HepG2 for 6 h. Y-axis: fold change in the ratio of luciferase activity (relative luciferase unit) (please see methods and materials for detail) from those transfected with basic pGL3 promoter construct and treated with vehicle DMSO. *Significantly different (p<0.05) from DMSO-treated pGL3_ARE by one-way ANOVA followed by posthoc test. Mean and SEM (n= 2-4) c, ARE-reporter (pGL3_ARE) activity with coexpression of Nrf2 or dominant-negative Nrf2, and mutant ARE-reporter (pGL3_mARE) activity in HepG2 treated with bile acids (LCA = 70 µM; CDCA = 100 µM). Y-axis: fold change in the ratio of luciferase activity (relative luciferase unit) from those transfected with basic pGL3 promoter construct and/or respective expression vector, and treated with DMSO. *Significant difference (p<0.05) between vehicle control and bile acids by one-way ANOVA followed by posthoc test. Mean and SEM (n=3-6). d, ChIP analysis examining Nrf2 occupancy to AREs of both GCLM and GCLC genes upon 6 h treatment with bile acids (70 µM LCA; 100 µM CDCA) in HepG2. BHQ (200 µM), known to transcriptionally activate GCLM and GCLC, was included as positive control. Negligible detection from samples incubated with host IgG (anti-CYP1A1) ruled out contribution of non-specific binding from antibody. *Significantly different (p<0.05) from controls (t=0) and vehicle treatment by t-test. Mean and SEM of triplicate determinations of representative experiments.
Figure 13. Nrf2 is a cellular protector against LCA-induced toxicity. a, Cell viability of HepG2 after knockdown of Nrf2 via siRNA prior to treatment with toxic LCA (100 and 120 µM). Viability was measured at indicated time-points over 24 h. Fluorescent values of each LCA-treated siRNA group was first subtracted by the average values of individual siRNA group treated with vehicle DMSO, and expressed as percent change to baseline/pretreatment values. *Significant difference (p<0.01) between siCtr and siNrf2 groups with t-test. Representative results; mean ± SEM of 4 determinations. b, Analysis of LDH release ratio, a marker of necrotic event or cell injury, upon LCA challenge (90 µM) in HepG2 after Nrf2 silencing. Values of LCA treatment were subtracted by the average values of vehicle treatment for each siRNA group. Representative results were presented; mean of duplicate determinations. c, Analysis of caspases activity upon triggered by LCA toxicity (90 µM) in HepG2 knockdown of Nrf2. Representative results of duplicate determinations were shown.
Figure 14. Increased GSH production is a cellular protective mechanism against bile acid toxicity.  

a, Cellular GSH levels of HepG2 after 24 h treatment with 75 µM LCA, 100 µM CDCA or 200 µM lipoic acid (LA; positive control). Buthionine sulfoximine (BSO; 60 µM), a GSH synthesis blocker, was used as negative control. *Significantly different (p<0.05) from DMSO control by one-way ANOVA followed by posthoc test. Mean and SEM (n=4). 

b, Cell viability of HepG2 treated with toxic LCA (100 µM) together with 0.4 mM NAC, or 60 µM BSO, or with overnight pretreatment with 60 µM BSO (preBSO). Fluorescent units were first subtracted by those of respective treatment control (i.e., DMSO, NAC or BSO alone), and expressed as percent change to individual baseline/pretreatment values. *Significantly different from LCA treatment by one-way ANOVA followed by posthoc test (p<0.05). Mean ± SEM of 3 independent experiments with 4 determinations. 

c, ARE-reporter activity in HepG2 treated with LCA with or without 0.4 mM NAC or 60 µM BSO. Tert-butylhydroperoxide (tBHP, 100 µM), a peroxide radical generator, was used to show that increased cellular oxidative stress led to increased ARE reporter activity. Y-axis: fold change in the ratio of luciferase activity (relative luciferase unit) from those transfected with basic pGL3 promoter construct and treated with vehicle DMSO. *Significant difference (p<0.05). Chemical treatments were for 8 h. Mean and SEM (n=3-4).
CHAPTER 3.0

NUCLEAR FACTOR (ERYTHROID 2-RELATED) FACTOR 2 COUNTERACTS BILE ACID-INDUCED HEPATOTOXICITY: INVOLVEMENT OF ATP-BINDING CASSETTE TRANSPORTERS

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[In submission]

KPT designed and performed all experiments, and wrote the manuscript; GAW analyzed liver histology; MY assisted and delivered helpful methods in animal experiments, and performed parts of the analysis (total RNA extraction of animal tissues for real-time PCR); KK was involved in discussion in methodological development and invented a tool for in-silico analysis of ARE localization on the gene promoter.
3.1 Abstract

**Background & Aims:** A subset of multidrug resistance-associated proteins (MRPs), which belong to the ATP-binding cassette (ABC) transporters, are cellular pumps of bile salts and xenobiotics. Their transcription is regulated by several factors, including an oxidative stress-responsive transcriptional factor, Nrf2. We aimed to investigate if Nrf2 ameliorates bile acid toxicity via upregulation of the MRP transporters. **Methods:** Lithocholic acid (LCA)-induced hepatotoxicity was characterized in Nrf2-/- mice. Involvement of stress-related kinases in activation of Nrf2, and functional importance of MRPs in bile acid toxicity were investigated in HepG2 cells. **Results:** Compared with the wildtype, Nrf2-/- mice were significantly more susceptible to LCA-induced hepatotoxicity with severe liver necrosis and elevated liver injury markers. This was associated with inability of LCA-treated Nrf2-/- mice to induce antioxidant enzymes and ABC transporters including MRPs. siRNA-mediated Nrf2-silencing reduced basal and/or inducible expression of Mrp2, Mrp3 and Mrp4 in mouse livers and mouse liver Hepa1c1c7 cells. In human liver HepG2 cells, Nrf2-dependency of basal and LCA-induced expression was observed in MRP2, MRP3, and BCRP. Consistent with these findings, Nrf2 silencing increased cellular retention of methotrexate, an MRP substrate. Blocking of ABC transporter-mediated efflux aggravated LCA-induced oxidative stress and further decreased cell viability. Inhibition of MEK1-ERK1/2 mitogen-activated protein kinase (MAPK) mitigated Nrf2 transactivation and resultant LCA-induced antioxidative enzymes and ABC transporter expression, thereby exacerbating LCA cytotoxicity. **Conclusions:** Nrf2 counteracts LCA-induced hepatotoxicity partly through upregulation of a subset of the ABC transporters which is under the influence of the MAPK pathway.
3.2 Introduction

Belonging to the cap-n-collar, basic leucine zipper family, nuclear factor-erythroid 2 (E2)-related protein 2 (Nrf2) is an oxidative stress-responsive transcription factor which upregulates cytoprotective genes through binding to its cognate enhancer antioxidant response element (ARE). Glutamate cysteine ligase modulatory (GCLM) and catalytic subunits (GCLC), NAD(P)H:quinone oxidoreductase 1 (NQO1) and thioredoxin reductase 1 (TRx1) are among Nrf2 target genes which have essential roles in preserving cellular redox balance and cell survival under oxidative insults (Kensler et al., 2006). Using Nrf2 gene knockout (Nrf2-/-) mice, the protective role of Nrf2 against various oxidant-induced disease processes including cigarette smoke-induced emphysema (Rangasamy et al., 2004), experimental sepsis (Thimmulappa et al., 2006), and chemical carcinogenesis (Ramos-Gomez et al., 2001) has been demonstrated.

We have recently shown that administration of toxic bile acids to cultured human liver cells and mice, which mimicked conditions of cholestatic liver injury, can activate Nrf2 transcription machinery and induce antioxidative genes such as GCLM, GCLC, and TRx1 (Tan et al., 2007). Induction of these antioxidative genes resembles an adaptive cell defense mechanism against bile acid toxicity. These findings provide molecular evidence for an etiopathological role of oxidative stress in cholestasis (Reinehr et al., 2005A; Ljubuncic et al., 2000), and shed light on the potential of targeting Nrf2 in ameliorating cholestatic liver injury.

The plasma membrane-bound efflux transporters such as those of the ATP-binding cassette (ABC) proteins play a crucial part in bile acid transport and detoxification (Kullak-Ublick et al., 2004). Particularly, the bile salt export pump (BSEP or ABCB11) is the major canalicular transporter responsible for bile acid excretion into bile (Noe et al., 2002). Other ABC proteins such as ABCC2 or multidrug resistance-associated protein 2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), and ABCG2 (BCRP; breast cancer resistance protein) are also capable of extruding bile salts, conjugates of bilirubin and steroids, and a wide spectrum of endo- and xeno-biotics (Kullak-Ublick et al., 2004). These transporters serve as an important modality of cell defense limiting cellular accumulation of toxic compounds. Most recently, Maher et al. (2007) demonstrated that oxidative stress increases hepatic mRNA and protein expression of Mrp2, Mrp3, and Mrp4 in mice through the Nrf2-dependent pathway. It is, however, unknown if induction of these ABC
transporters also occurs with exposure to toxic bile acids via Nrf2 activation, and if so, what functional significance it may have.

Meanwhile, Alekusnes et al. (2006) showed that bile duct ligation (BDL) increased hepatic expression of cytoprotective Nrf2 target genes in the wild-type, but not in the Nrf2-/- mice. However, due to unexpectedly lower bile acid accumulation in the liver of the Nrf2-/- mice after BDL, an in vivo protective role of Nrf2 against bile-induced liver injury in this model was not evident.

In the present study, the in vivo protective role of Nrf2 in bile acid-induced hepatic damage was tested using the lithocholic acid (LCA)-induced liver injury model on wildtype (Nrf2+/+) and Nrf2-/- mice. LCA is the most toxic bile acid; when given to the mice in large amounts, it induces cholestatic liver injury (Zhang et al., 2004A; Fickert et al., 2006) through mechanisms involving induction of bile duct infarcts and destructive cholangitis, similar to cholestasis in humans. Our results show accentuated LCA-induced liver injury in the Nrf2-/- mice. We observed LCA-induced hepatic expression and function of a subset of ABC transporters in an Nrf2-dependent manner, and aggravation of LCA-induced oxidative stress if these transporters were blocked. Our findings further suggest that the MEK1-ERK1/2 mitogen activated protein kinase (MAPK) pathway is involved in collective induction of ABC transporters and Nrf2 activation.

### 3.3 Methods

**Cell lines and materials**

Human hepatoma HepG2 (ATCC), mouse hepatoma Hepa1c1c7 and human hepatoma Huh7 (gifts from Dr. P. Harper and Dr J. Matthews, respectively, University of Toronto, Toronto, ON) were maintained in α-MEM with 10% fetal bovine serum. Test chemicals were purchased from Sigma or CalBiochem. Oligonucleotides were synthesized at the Centre for Applied Genomics in Toronto or Integrated DNA Technologies (Coralville, IA).

**Mouse experiments**

The animal care and experimental procedures were approved by the Animal Care Committee at the University of Toronto. Nrf2-/- mice of ICR background (Itoh et al., 1997) (Riken, Japan) were mated with ICR (CD-1) mice (Charles Rivers, Montreal, QC) to generate heterozygous Nrf2+/+ mice (gifts from Dr. P. Wells, University of Toronto,
Toronto, ON, under the permission from Dr. M. Yamamoto, Tohoku University, Sendai, Japan). The heterozygous colonies were back-cross bred to CD-1 mice for >4 generations to produce a congenic strain. The treatment protocols for acute (8 h) and extended (4 d) injection (i.p.) of LCA (125 mg/kg/dose for the acute, and the same dose 2 times a day for the extended protocol) were described elsewhere (Tan et al., 2007). All mice were killed between 900-1200 h to minimize circadian effects. At necropsy, the median lobe of livers was sampled for mRNA analysis and the left lobe fixed in 10% formalin for histological assessment. Liver histology from three representative cross-sections of each sample was assessed by a veterinary pathologist (GAW) in a blinded manner for severity and frequency of necrotic lesions, and categorized according to a semi-quantitative grading system: background normal (grade 0), mild (grade 1), moderate (grade 2), and extensive (grade 3). Wild type untreated mice occasionally have small isolated foci of liver necrosis and inflammation and thus this level was considered normal (grade 0). Sera were analyzed for liver injury markers: alanine aminotransferase (ALT) and alkaline phosphatase (ALP) by established automated methods (Tan et al., 2007). The sample size was calculated from the estimates of ALT variation (~50% of the mean value), 3-fold increase of mean ALT level from 50 U/L in LCA-treated wild type to 150 U/L in LCA-treated Nrf2-/- mice. Assuming $\beta=0.2$, $\alpha=0.05$, 7 mice/group was required. The experiments were conducted with 8-12 mice in each group.

**Quantitative reverse-transcription PCR (qRT-PCR)**

RNA extraction, reverse transcription, qRT-PCR, and the $\Delta\Delta$Ct method to quantify relative fold-difference of gene transcripts calibrated by internal control genes (ribosomal 18S, tata-box binding protein, $\beta$-actin, and/or glyceraldehydes-3-dehydrogenase) between control and treatments were performed (Tan et al., 2007). Primer sequences were listed in supplementary material: Table A.

**Immunoblotting**

Preparation of crude lysate, membrane and nuclear fractions, and immunodetection were done (Tan et al., 2007; Kwok et al., 2005). Primary antibodies (working concentration) used were: mouse monoclonal anti-$\beta$-actin (1:10000)(Sigma); rabbit antiserum against $\alpha$-connexin (1:1000) (a gift from Dr. D. Williams, University of Toronto, Toronto, ON), mouse monoclonal anti-p-glycoprotein (P-gp)(C219)(1:200) (Abcam, Cambridge, MA),

**RNA interference (RNAi), plasmids and luciferase reporter assay**

Four gene-specific small-interfering RNA (siRNA) against human and mouse Nrf2 (siNrf2) or equal molar mismatched control siRNA (siCtr) (Dharmacon SMARTpool® siRNA reagent, Thermo Fisher Scientific, Lafayette, CO) were used to induce Nrf2 silencing (65-80% knockdown in mRNA and protein) (Tan et al., 2007). The dominant negative Nrf2 expression vector (pEF_DNrf2) (a gift from Dr. J. Alam, Ochsner Clinic Foundation, New Orleans, LA), ARE reporter construct (pGL3_ARE) and mutant ARE reporter construct (pGL3_mARE) were transfected into HepG2 and reporter (luciferase) activities were analyzed as described (Tan et al., 2007).

**Cytotoxicity and reactive oxygen species (ROS) analyses**

Cytotoxicity was examined by the Alamar Blue™ assay (Biosource, Nivelle, Belgium) as described (Tan et al., 2007). A cell-permeant probe for oxidative stress with improved cellular retention properties, 5-(and -6)-carboxy-2’, 7’-difluorodihydrofluorescein diacetate (carboxy-\(\text{H}_2\text{DCFDA}\))(Molecular Probes-Invitrogen, Carlsbad, CA) was used to measure ROS. Briefly, HepG2 was loaded with 5 µM carboxy-\(\text{H}_2\text{DCFDA}\) for 30-min, allowed for 1 h recovery in Opti-MEM (Invitrogen) upon which baseline fluorescent values were determined at excitation/emission \(\lambda = 495/525\) nM. Cells were then exposed to treatments and increase in fluorescence from baseline, which correlates with the production of cellular ROS, was recorded.

**Drug accumulation/uptake analysis**

Experiments were carried out on LCA or vehicle-treated HepG2 as described with slight modifications (Kwok et al., 2005). Radiochemical drugs: \(^3\text{H}\)-vinblastine (Amersham; 0.5
µCi/mL), 3H-methotrexate (Moravek Biochemicals, Brea, CA; 0.5 µCi/mL), 3H-
mitoxantrone (Moravek Biochemicals; 0.17 µCi/mL) were spiked in the uptake buffer
containing unlabeled drugs in a total drug concentration of 0.5 µM. The cell-impermeable
14C-Mannitol (Amersham; 0.017 µCi/mL) was added together to monitor for possible
nonspecific contamination. Because LCA treatment caused mild toxicity, viable cell
population prior to uptake experiments was measured by Alamar Blue™ assay to replace
the less sensitive total protein as denominator adjuster. For efflux inhibition experiments,
the wide-spectrum ABC transporter inhibitors cyclosporin A (CspA)(10 µM) and MK571
(15 µM) were used. Preincubation with these inhibitors in the uptake buffer was first
carried out for 45 min before coincubation with labeled drugs in uptake experiments.

Multiplex kinase activation assay
A sensitive bead-based, quantitative multiplex assay using Luminex xMAP technology (x-
plex assay; Bio-Rad, Hercules, CA) and antibodies from Cell Signaling Technology
(Danvers, MA) was used to probe multiple phosphoproteins representing activation of
multiple stress-related kinase pathways: ERK mitogen-activated protein kinase (ERK
MAPK)[p-MEK1(Ser217/Ser221), p-ERK1/2(Thr202/Tyr204, Thr185/Tyr187), p-p38
MAPK(Thr180/Tyr182)], phosphatidylinositol-3-kinase-Akt (PI-3K-Akt) [p-Akt (Ser473)],
and c-Jun N-terminal kinase (JNK/SAP kinase) [p-JNK(Thr183/Tyr185), p-c-Jun(Ser63)].
Activation of protein kinase C (PKC) was analyzed with immunoblots using goat
polyclonal anti-p-PKCα (Ser657) (Santa Cruz). Fluorescent signals of cell lysate (300-400
ng) were measured by Bio-Plex 200 System (Bio-Rad) and adjusted to individual total
protein (Bio-Rad). Sensitivity, specificity and robustness of the assay were checked by
inclusion of positive controls [epidermal growth factor (EGF) and UV-treated cell lysate],
negative control (untreated cell lysate), and lysate of cells treated with specific inhibitors
(see below).

Kinase inhibitor experiments
To effectively inhibit specific kinase activation without causing additive toxicity, HepG2
cells were preincubated with a high dose of small molecule inhibitors for 30-min followed
by a 5-fold lower cotreatment dose given together with LCA. The pre- and co-treatment
doses were: Gö 6976 [PKCα inhibitor; 100 nM, 20 nM], Ly294002 [PI-3K inhibitors; 20
µM, 4 µM], Sp600125 [JNK/SAP kinase inhibitor; 5 µM, 1 µM], and U0126 [MAPK
inhibitor; 25 µM, 5 µM]. Specific inhibition of kinase activation (>60% inhibition) with minimal interference (<20%) with other kinase pathways by the inhibitors was confirmed by analyzing percent decreases in corresponding phosphoproteins relative to positive controls (EGF treatment) with the multiplex kinase activation assay (supplementary material: Table C).

**Statistical analysis**

Results were expressed as means ± SEM or median ± quartile. Comparisons between two groups were accomplished by Student’s independent t-test (parametric) or the Mann-Whitney \( U \) test (non-parametric). Because of apparent sex differences in basal expression of some mouse genes, gene expression levels were standardized within the same sex to those of the untreated Nrf2\(^{+/+}\) mice (constitutive expression: Table 1) or the corn oil-treated Nrf2\(^{+/+}\) mice (LCA-induced expression: Table 2) before aggregating them for the final comparison. \( P \) values less than 0.05 were considered significant.

**3.4 Results**

*Nrf2\(^{-/-}\) mice were vulnerable to LCA-induced liver injury*

Consistent with the increased susceptibility of Nrf2-silenced liver cells to LCA toxicity in vitro (Tan et al., 2007), targeted disruption of Nrf2 sensitized the mice to LCA-induced liver injury. Nrf2\(^{-/-}\) mice upon 4-day LCA treatment had significantly more severe multifocal liver necrosis than did the Nrf2\(^{+/+}\) wild-type (Fig. 15A and 15B: \( P=0.036 \) with the Mann Whitney \( U \) test). Accompanying this, inflammation of bile ducts characterized by inflammatory cells surrounding ducts and present within the lumen, with occasional necrosis of ductal epithelium, was also noted more frequently in Nrf2 knockouts (Fig. 15A). In agreement with the histological assessment, serum ALT was higher in LCA-treated Nrf2\(^{-/-}\) mice than that of the vehicle controls (\( P<0.001 \), Fig. 15C: left), but statistical significance was not reached between the LCA-treated wild-type and Nrf2\(^{-/-}\) mice most likely due to the high attrition rate for blood sampling and resultant insufficient sample sizes. Serum ALP, which is produced by injured bile canaliculi during cholestasis, was also significantly elevated in Nrf2 knockouts receiving LCA, consistent with pathological evidence of inflamed bile ducts (Fig. 15D: right). Taken together, our findings indicate a heightened susceptibility of Nrf2\(^{-/-}\) mice to LCA-induced liver damage.
Involvement of Nrf2 in constitutive and LCA-induced gene expressions of hepatic ABC proteins in mice

Along with the Nrf2-target antioxidant enzymes, constitutive expressions of Mrp2, 3, 4 and 5, Mdr1a and Ntcp were significantly lower in the Nrf2-/- mice than the wildtype (Table 6). LCA treatment increased expression of the majority of the antioxidative and transporter genes over the vehicle control (corn oil) in both genotypes, except for Ntcp (Table 7). However, the magnitude of inducible gene expression upon LCA challenge was significantly blunted in mice with Nrf2 disruption. The observed induction began within 8 h of acute LCA treatment (supplementary material: Table B) before histological and biochemical liver injury became evident (not shown). Conversely, Bsep expression was higher constitutively in the Nrf2-/- mice (Table 6, and the corn oil arm in Table 7), which was further increased with LCA treatment (Table 7). An exploratory subgroup analysis showed sex differences in gene expression (supplementary material: Table F), especially for LCA-induced expression of Mdr1a/1b, which occurred in male mice only.

Basal and inducible expression of MRP2, MRP3 and BCRP in human liver

HepG2 is dependent on Nrf2

Similar to the above findings in mice, 24 h treatment of human hepatoma HepG2 with LCA, chenodeoxycholic acid (CDCA) or Nrf2 activator tert-butylhydroquinone (BHQ) resulted in significant increases in expression of MRP1-5, BCRP and MDR1 (Fig. 16A and 16B). Induction of these transporter genes by LCA was dose-dependent (supplementary material: Figure I), resembling that of LCA-induced Nrf2 target genes. (Tan et al., 2007) Except for MRP1 and BCRP, induction of most other ABC proteins was similarly noted in another human hepatocyte-derived Huh7 and mouse liver Hepa1clc7 cells (supplementary material: Figure II).

To examine if Nrf2 is required for the LCA-induced expression of ABC transporters in HepG2 shown above, endogenous Nrf2 was specifically silenced via siRNA, which resulted in significantly repressed constitutive and inducible expression of the antioxidative enzyme genes (Tan et al., 2007). We concurrently observed significant decreases in basal and LCA-induced gene expression of MRP2, MRP3 and BCRP (Fig. 17A). The disparity of Nrf2 dependency for MRP4 and MRP1 between HepG2 and in vivo mouse liver may result from species difference. To investigate this, we utilized siRNA to silence endogenous Nrf2 in a well-characterized mouse liver model, Hepa1c1c7. Silencing
of Nrf2 led to significantly lower basal Mrp3 and Mrp4, and inducible Mrp1, Mrp2, Mrp3 and Mrp4 in this cell model (Fig. 17B). Similar effects of Nrf2 silencing on the preceding transporters by LCA occurred to treatments with another bile acid CDCA, and the known Nrf2 inducers such as BHQ and α-lipoic acid in HepG2 and Hepa1c1c7. Despite its induction by bile acids in vitro (Fig. 16, and supplementary Fig. II) and significantly attenuated induction in the LCA-treated Nrf2−/− mice, mRNA levels of human MDR1 and mouse Mdr1a/1b were not influenced by Nrf2 silencing in these cell lines (not shown), suggesting that they are not direct Nrf2 target genes.

Activated MEK1-ERK1/2 MAPK is involved in Nrf2 activation, induction of ABC transporters, and enhancement of cell survival in HepG2 exposed to LCA

There was 2 to 4-fold activation of MEK1-ERK1/2 MAPK and JNK/c-Jun pathways in HepG2 at 0.5 – 6 h of LCA treatment (Fig. 18A). A mild increase of p-AKT (~1.5 fold), a major downstream target of PI 3-kinase, was also detected. Greater than 80% inhibition of MEK1-ERK1/2 MAPK with the MEK inhibitor U0126 (supplementary material: Table C) prevented nuclear accumulation of Nrf2, the obligatory event of Nrf2 activation, in LCA-treated HepG2 (Fig. 18B). Consistent with this observation, LCA-induced ARE reporter activity was significantly suppressed by cotreatment with U0126 (Fig. 18C). More importantly, inhibition of MAPK by U0126 significantly reduced LCA-induced expression of Nrf2 target genes such as GCLM, GCLC, TRx1, and ABC transporter genes including those found to be dependent on Nrf2 (MRP2, MRP3, BCRP)(Fig. 18D). Moreover, induction of MDR1, MRP1 and MRP4 by LCA, which were not dependent on Nrf2 (Fig. 17A), was also attenuated by MAPK inhibition (Fig. 18D).

We also found about 20-30% reduction of LCA-induced Nrf2 target genes and ABC transporters, particularly MRP4 (~50% reduced induction) upon ~75% inhibition of the PI3K/AKT pathway with Ly294002 (not shown), suggesting contribution of this signaling pathway, albeit to less degree, to the regulation of antioxidant and transporter genes. Conversely, inhibition of the JNK pathway to ~70% by Sp600125 affected neither Nrf2-ARE mediated transcription, nor the antioxidative and ABC transporter gene expression (not shown). PKC, which was shown to phosphorylate Nrf2 and release Nrf2 from Keap1 (Huang et al., 2002), remained inactivated by LCA treatment (not shown). Blocking the MEK1/ERK1/2 MAPK activation, thereby inhibiting induction of the cytoprotective and
certain transporter genes, was associated with an increased HepG2 sensitivity to LCA toxicity, as shown by increased cell death (Fig. 18E). Treatment with the inhibitor alone did not reduce cell viability (>90% viability relative to vehicle-treated controls).

**Increased cellular efflux is partly directed by Nrf2 and serves an important cell defense response against bile acid toxicity**

We then studied whether the increased ABC transporter expression by LCA would result in increased efflux activity in HepG2 cells. Vinblastine is mainly an MDR1 substrate, although it may also be transported by MRP 1-3 (Deeley et al., 2006; Doyle & Ross, 2002). Methotrexate is a substrate for MRP 1-5 and BCRP, whereas mitoxantrone is a relatively selective substrate for BCRP (Deeley et al., 2006; Doyle & Ross, 2002; Eloranta et al., 2005). Based on relative mRNA abundance, the expression of ABC transporters in HepG2 was: MRP2 > MDR1 ≥ BCRP ≈ MRP1 ≈ MRP3 ≈ MRP5 > MRP4 (supplementary material: Table E).

LCA-pretreated HepG2 accumulated less vinblastine and methotrexate than did vehicle-pretreated cells (Fig. 19A), consistent with the increased expression of the efflux transporters after LCA exposure (Fig. 16A & 16B). Accumulation of mitoxantrone was only modestly reduced by LCA pretreatment (Fig. 19A). The wide-spectrum inhibitors of ABC transporters, CspA and MK571, reversed the LCA-induced effects, confirming that the LCA-induced reduction in drug accumulation is due to ABC transporter upregulation (Fig. 19B). Also, cellular uptake of methotrexate and, to a less extent vinblastine, was increased with presence of the bile acids LCA, CDCA and cholic acid (supplementary material: Figure III), compatible with their properties as ABC transporter substrates. Importantly, silencing of Nrf2 increased methotrexate uptake (Fig. 19C), indicating that the efflux of methotrexate is mainly mediated by Nrf2-dependent ABC transporters, i.e., MRP2, MRP3 and BCRP (Figure 17A). Vinblastine uptake, however, was not significantly affected by Nrf2 knockdown. CspA or MK571 further enhanced LCA-induced cellular production of ROS (Fig. 19D). This increased level of oxidative stress subsequently led to increased cytotoxicity and more cell death (Fig. 19E). Treatment with either inhibitor alone did not differ in cellular ROS level or cell viability from treatment with vehicle DMSO.
3.5 Discussion

In the present study, we have demonstrated in vivo significance of the oxidative stress-responsive Nrf2 in experimental cholestasis by showing the heightened susceptibility of Nrf2−/− mice to bile acid-induced liver damage. Our findings add Nrf2 to a group of nuclear receptors, such as the pregnane X receptor (PXR) (Uppal et al., 2004), the constitutive androstane receptor (CAR) (Zhang et al., 2004A), and the liver X receptor (LXR) (Uppal et al., 2007), as protectors against bile acid toxicity. These steroid nuclear receptors are physiological sensors of bile acids and their metabolites, having critical roles in maintaining bile acid and cholesterol homeostasis (Eloranta et al., 2005; Handschin & Meyer, 2005). Therefore, the protection conferred by Nrf2, which is activated upon occurrence of bile acid-induced oxidative stress, represents a different mode of hepatic protection against toxicity of bile acids.

Activation of Nrf2 at excessive bile acid exposure executes an adaptive stress-responsive mechanism. This makes Nrf2 a therapeutic target more relevant to situations of clinical cholestasis in which advanced bile acid accumulation and liver injury already takes place. It is noteworthy that a major mechanism of ursodeoxycholic acid and N-acetyl-L-cysteine (Mager et al., 2008) in the management of cholestasis is through reversing mitochondrial oxidative stress (Serviddio et al., 2004; Moreira et al., 2007), the main target of bile acid toxicity. Nrf2 activation has been shown to combat mitochondrial stress and resultant apoptosis (Calkins et al., 2001; Tan et al., 2007).

Our findings from Nrf2−/− mice and mouse liver cells that Mrp2, Mrp3 and Mrp4 are Nrf2 regulated genes are in consensus with a recent report (Maher et al., 2007). In order to gain mechanistic insight and to confirm the relevance in human cells, we have examined the effects of bile acids in human hepatocytes in vitro. As our attempt to use primary human hepatocytes yielded inconsistent results (Appendix II), we used the human hepatoma HepG2 which has functional Nrf2, shows robust induction of Nrf2 target genes, and expresses all ABC efflux transporters of interest. The subset of ABC transporter genes regulated by Nrf2 in human HepG2 was not entirely identical to that of mice. Nonetheless, Nrf2-dependency of MRP2 and MRP3 expression was consistently observed in both human and mouse cell lines, in line with the in vivo mouse livers. The ARE on the gene promoter of mouse Mrp2 has been identified (Vollrath et al., 2006). Potential AREs resided on the promoter region of mouse Mrp3 and Mrp4 have recently been proposed (Maher et al., 2007). In-silico analysis of 5′-region (-10 kb) of human
MRP2, MRP3 and BCRP also reveals putative AREs (supplemental material: Table D). Whether these motifs can be directly transactivated by Nrf2 is currently under investigation.

Compared with the wildtype, we found that Nrf2-/- mice had higher constitutive and inducible levels of the major canalicular bile efflux transporter Bsep, and less Na+-taurocholate cotransport protein (Ntcp), the major bile uptake transporter (Table 6). These changes may account, at least in part, for the lower hepatic bile acids in the Nrf2 knockouts reported by Aleksunes et al (2006). Because acute silencing of Nrf2 in vitro did not affect basal and inducible expression of these bile salt transporters (not shown), and because the Nrf2-/- mice retain the full ability to alter expression of these transporters upon LCA exposure (Table 7), Bsep and Ntcp are unlikely to be direct Nrf2-target genes. Changes of these transporters in Nrf2-/- mice likely result from a compensatory mechanism for the repression of important ABC transporters such as Mrp2 and Mrp4 (Kullak-Ublick et al., 2004). However, the seemingly protective changes in Bsep and Ntcp in the liver of Nrf2 knockouts against bile accumulation did not appear to counterbalance the exogenous overload of LCA in our experimental model.

The collective increase in many ABC transporters by LCA pretreatment led to a decrease in vinblastine and methotrexate cellular uptake, consistent with an increased efflux activity. The role of ABC transporters, particularly Mrp2, Mrp3, Mrp4 and BCRP, in cellular efflux of bile salts and their conjugates has been described (Mennone et al., 2006; Bodo et al., 2003; Rius et al., 2006; Hirohashi et al., 2000; Janvilisri et al., 2005). We showed that Nrf2 silencing with suppressed MRP2, MRP3 and BCRP expression reduced cellular efflux of methotrexate. We further showed that blocking of efflux activity by the ABC transporter inhibitors CspA and MK571 was associated with an increased ROS production and decreased cell resistance to LCA toxicity. This is the first demonstration of the functional importance of ABC efflux transporters against bile acid toxicity.

Bile acid treatment of HepG2 also causes Nrf2-mediated induction of the GSH biosynthesis system, thereby increasing cellular GSH levels (Tan et al., 2007). Nrf2-mediated increase of ABC transporters is likely to be aided by coordinated increases in cellular GSH, a cotransport molecule needed for efflux function of the ABC transporters including MRP1, MRP2 and MRP4 (Deeley et al., 2006; Kruh & Belinsky, 2003). Because Nrf2 is centrally involved in coordinating expression of multiple antioxidative and ABC
transporter genes, the results of our study provide an integral view of the Nrf2-mediated detoxification machinery.

Certain bile acids can activate the stress-related kinase signaling pathways including PKC, ERK MAPK, JNK, PI-3K/AKT and p38 MAPK in a cell type-specific manner (Debruyne et al., 2002; Dent et al., 2005; Le et al., 2006). These major signaling pathways were shown to modulate the stability of Keap1-Nrf2 complex, expression of ARE-regulated genes, and/or transcription of Nrf2 itself (Kensler et al., 2006; Huang et al., 2002; Kang et al., 2002; Li et al., 2007). However, the relative importance of these pathways in bile acid-induced toxicity is unknown. Our findings suggest that the MEK1-ERK1/2 MAPK cascade is pivotally involved to counteract LCA toxicity, and enhance cell survival by mediating Nrf2 activation and induction of cytoprotective genes. This is in support of previous reports that ERK MAPK activation by oxidants is required for Nrf2-mediated expression of GCLM and HO1 genes (Keum et al., 2006; Zipper & Mulcahy, 2003). We additionally found that this signaling pathway is also required for LCA-induced expression of TRx1 and an array of ABC efflux transporters including MDR1 whose expression is not affected by Nrf2 silencing. These results suggest that MDR1 expression is independent of direct regulation by Nrf2, but under the influence of the MAPK pathway activated by bile acids, and/or resultant oxidative stress.

Much remains unknown as to how the ERK1/2 MAPK pathway orchestrates Nrf2 activation and increases ARE-responsive genes. Mutation analysis targeting the conserved MAPK phosphorylation sites in the Nrf2 protein failed to inhibit Nrf2 transactivation and its interaction with Keap1 (Zipper & Mulcahy, 2003). However, a recent in vitro study using GST-pull down recombinant Nrf2 proteins reported that Nrf2 is a substrate of ERK2 (Xu et al., 2006A). It has also been shown that the cyclic AMP response element binding protein (CBP), a coactivator member in the Nrf2/Maf heterodimer transcription complex, is a target of MAPK (Katoh et al., 2001). Phosphorylation of CBP by MAPK can enhance the ARE-driven transcriptional activity (Shen et al., 2004). Therefore, it is possible that the MAPK cascade participates in initiating and sustaining Nrf2-ARE transcription drive through this coactivator.

In summary, our study delineates the Nrf2-mediated collective induction of ABC transporters and antioxidative protein genes as a major cell defense system against bile acids. Extending the work by others about Nrf2-regulation of MRPs, our findings show that Nrf2 is affected by the MEK1-ERK1/2 MAPK cell-survival-enhancing signaling
pathway in regulation of a battery of antioxidative genes and the efflux transporters important in extruding bile acids. The potential of enhancing Nrf2 activity as a therapeutic target in the management of cholestasis and treatment of oxidant-induced liver diseases merits further investigation.

### 3.6 Significance

Using the LCA treatment model of cholestatic liver injury tested on mice with or without gene disruption of Nrf2, this study presents the *in vivo* protective role of Nrf2 against bile acid toxicity. These findings are in further support of the *in vitro* observations in Chapter 2. Moreover, the cytoprotective role of ABC efflux transporters, which are in part regulated by Nrf2, against LCA toxicity is also highlighted. Additionally, this study throws light on the mechanistic understanding by elaborating a cell survival-enhancing stress-responsive cell signaling, namely, the MEK1-ERK1/2 MAPK in mediating Nrf2 transactivation and induction of antioxidant and ABC transporter genes by LCA treatment. The potential of Nrf2 as a therapeutic target in management of cholestasis is proposed for further studies.
Table 6. Constitutive Gene Expression of Antioxidant Enzymes and ABC Transporters in Nrf2+/+ and Nrf2−/− mice.

<table>
<thead>
<tr>
<th></th>
<th>Nrf2+/+</th>
<th>Nrf2−/−</th>
<th>P valuea</th>
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</thead>
<tbody>
<tr>
<td><strong>Antioxidative enzymes</strong></td>
<td></td>
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</tr>
<tr>
<td>Gclm</td>
<td>1.00 ± 0.04</td>
<td>0.84 ± 0.04</td>
<td>0.005</td>
</tr>
<tr>
<td>Gclc</td>
<td>1.00 ± 0.06</td>
<td>0.62 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trx1</td>
<td>1.00 ± 0.05</td>
<td>1.01 ± 0.08</td>
<td>0.738</td>
</tr>
<tr>
<td>Nqo1</td>
<td>1.00 ± 0.06</td>
<td>0.29 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gsta1/2</td>
<td>1.00 ± 0.08</td>
<td>0.48 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gsta4</td>
<td>1.00 ± 0.02</td>
<td>0.57 ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gstm1</td>
<td>1.00 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gstp1</td>
<td>1.00 ± 0.05</td>
<td>0.75 ± 0.07</td>
<td>0.003</td>
</tr>
<tr>
<td>Ho-1</td>
<td>1.00 ± 0.06</td>
<td>0.67 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Transporters</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mrp1</td>
<td>1.00 ± 0.04</td>
<td>0.87 ± 0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>Mrp2</td>
<td>1.00 ± 0.08</td>
<td>0.69 ± 0.07</td>
<td>0.005</td>
</tr>
<tr>
<td>Mrp3</td>
<td>1.00 ± 0.07</td>
<td>0.24 ± 0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mrp4</td>
<td>1.00 ± 0.04</td>
<td>0.48 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mrp5</td>
<td>1.00 ± 0.04</td>
<td>1.23 ± 0.05</td>
<td>0.002</td>
</tr>
<tr>
<td>Mdr1a</td>
<td>1.00 ± 0.07</td>
<td>0.72 ± 0.07</td>
<td>0.005</td>
</tr>
<tr>
<td>Mdr1b</td>
<td>1.00 ± 0.06</td>
<td>0.97 ± 0.09</td>
<td>0.616</td>
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<tr>
<td>Bcrp</td>
<td>1.00 ± 0.05</td>
<td>0.89 ± 0.06</td>
<td>0.156</td>
</tr>
<tr>
<td>Bsep</td>
<td>1.00 ± 0.11</td>
<td>1.81 ± 0.13</td>
<td>0.001</td>
</tr>
<tr>
<td>Ntcp</td>
<td>1.00 ± 0.04</td>
<td>0.70 ± 0.04</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fold change in mRNA levels relative to untreated Nrf2+/+ mice with adjustment for sex. 9 wk-old mice; mean ± SEM (n=10-12). a Student’s t-test (parametric data) or Mann-Whitney U test (non-parametric data).
Table 7. Gene expression of antioxidative enzymes and ABC efflux transporters after 4-day treatment with corn oil vehicle or LCA in mice

<table>
<thead>
<tr>
<th>Antioxidative enzymes</th>
<th>Corn oil vehicle</th>
<th>LCA</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Corn oil vehicle</th>
<th>LCA</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nrf2&lt;sup&gt;+/+&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;-/-&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;+/+&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;-/-&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;+/+&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;-/-&lt;/sup&gt;</strong></td>
<td><strong>Nrf2&lt;sup&gt;+/+&lt;/sup&gt;</strong></td>
</tr>
<tr>
<td>Gclm</td>
<td>1.00 ± 0.05</td>
<td>0.82 ± 0.06</td>
<td>0.024</td>
<td>1.96 ± 0.12&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.53 ± 0.10&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.016</td>
</tr>
<tr>
<td>Gclc</td>
<td>1.00 ± 0.08</td>
<td>0.55 ± 0.04</td>
<td>&lt;0.001</td>
<td>2.03 ± 0.13&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.07 ± 0.10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trx1</td>
<td>1.00 ± 0.05</td>
<td>1.09 ± 0.11</td>
<td>0.509</td>
<td>3.74 ± 0.57&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.39 ± 0.14&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nqo1</td>
<td>1.00 ± 0.06</td>
<td>0.28 ± 0.06</td>
<td>&lt;0.001</td>
<td>2.83 ± 0.43&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.48 ± 0.08&lt;sup&gt;**&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ho-1</td>
<td>1.00 ± 0.09</td>
<td>0.79 ± 0.06</td>
<td>0.007</td>
<td>3.35 ± 0.37&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.10 ± 0.15</td>
<td>&lt;0.001</td>
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<tr>
<td>Gsta1/2</td>
<td>1.00 ± 0.11</td>
<td>0.45 ± 0.05</td>
<td>&lt;0.001</td>
<td>8.56 ± 1.44&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.70 ± 0.11&lt;sup&gt;*&lt;/sup&gt;</td>
<td>&lt;0.001</td>
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<tr>
<td>Gsta4</td>
<td>1.00 ± 0.05</td>
<td>0.69 ± 0.04</td>
<td>&lt;0.001</td>
<td>2.65 ± 0.36&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.16 ± 0.10</td>
<td>&lt;0.001</td>
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<td>Gstm1</td>
<td>1.00 ± 0.05</td>
<td>0.37 ± 0.03</td>
<td>&lt;0.001</td>
<td>1.72 ± 0.07&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.57 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
<td>&lt;0.001</td>
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<td>Gstp1</td>
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<td>0.89 ± 0.08</td>
<td>0.239</td>
<td>1.15 ± 0.23</td>
<td>1.24 ± 0.18</td>
<td>0.157</td>
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<td><strong>Transporters</strong></td>
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<tr>
<td>Mrp1</td>
<td>1.00 ± 0.05</td>
<td>1.02 ± 0.10</td>
<td>0.946</td>
<td>2.70 ± 0.31&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.54 ± 0.10&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.002</td>
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<td>Mrp2</td>
<td>1.00 ± 0.03</td>
<td>0.96 ± 0.05</td>
<td>0.474</td>
<td>1.35 ± 0.09&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.89 ± 0.10</td>
<td>0.005</td>
</tr>
<tr>
<td>Mrp3</td>
<td>1.00 ± 0.07</td>
<td>0.17 ± 0.03</td>
<td>&lt;0.001</td>
<td>1.92 ± 0.16&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.33 ± 0.05&lt;sup&gt;**&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mrp4</td>
<td>1.00 ± 0.08</td>
<td>0.55 ± 0.06</td>
<td>&lt;0.001</td>
<td>2.79 ± 0.27&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.82 ± 0.16</td>
<td>&lt;0.001</td>
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<tr>
<td>Mrp5</td>
<td>1.00 ± 0.05</td>
<td>0.99 ± 0.09</td>
<td>0.921</td>
<td>2.23 ± 0.34&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.97 ± 0.07</td>
<td>0.007</td>
</tr>
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<td>Mdr1a</td>
<td>1.00 ± 0.05</td>
<td>0.66 ± 0.12</td>
<td>0.004</td>
<td>14.45 ± 5.19&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.09 ± 0.74</td>
<td>0.017</td>
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<tr>
<td>Mdr1b</td>
<td>1.00 ± 0.07</td>
<td>1.14 ± 0.15</td>
<td>0.389</td>
<td>12.04 ± 6.65&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.60 ± 0.39</td>
<td>0.069</td>
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<td>Bcrp</td>
<td>1.00 ± 0.05</td>
<td>0.78 ± 0.09</td>
<td>0.032</td>
<td>1.47 ± 0.12&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.02 ± 0.10</td>
<td>0.013</td>
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<tr>
<td>Bsep</td>
<td>1.00 ± 0.06</td>
<td>1.73 ± 0.08</td>
<td>0.005</td>
<td>2.13 ± 0.36&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2.73 ± 0.22&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.059</td>
</tr>
<tr>
<td>Ntcp</td>
<td>1.00 ± 0.10</td>
<td>0.71 ± 0.05</td>
<td>&lt;0.001</td>
<td>0.56 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.50 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.064</td>
</tr>
</tbody>
</table>

Fold change in mRNA levels relative to corn oil-treated Nrf2<sup>+/+</sup> mice. LCA treatment (125 mg/kg body wt.) was twice daily for 4 d. Mean ± SEM (n=8-12). <sup>a</sup>Comparison between genotype; or <sup>*</sup>p<0.05, <sup>**</sup>p<0.01 from corn oil-treated Nrf2<sup>+/+</sup> control with Student’s t-test or Mann Whitney U test.
Figure 15. Heightened sensitivity of Nrf2-/- mice to LCA-induced liver injury. (A) Representative hematoxylin and eosin stained liver sections from LCA-treated mice (extended treatment: 125 mg/kg body wt. twice daily for 4 d). Upper panel shows liver histology with arrows pointing to necrotic foci. Arrows on the lower panel locate representative bile duct. (B) Comparison of liver necrosis grade between the Nrf2 +/+ (n=8) and Nrf2-/- mice (n=10) (0: background; 3: severe necrosis; see experimental procedures for grading description). Values are shown as box-and-whisker plots with median (thick horizontal bar), inter-quartile ranges (box), and extreme values (whisker). Non-parametric comparison between the 2 groups was done with the Mann-Whitney U-test (P=0.036). (C) Serum ALT (left) and (D) serum ALP (right) of mice treated 4 d with LCA or vehicle corn oil. The group means were shown as horizontal bars with the actual values in brackets. Statistical analysis was the Mann-Whitney U test.
Figure 16. Induction of ABC transporter genes by bile acids in human liver HepG2. (A) mRNA levels of MRP1-5, BCRP and MDR1 of HepG2 treated 24 h with 75 µM LCA, 150 µM CDCA, or 200 µM tert-butylhydroquinone (BHQ) (Nrf2 activator). Fold change relative to vehicle DMSO-treated control. Mean ± SEM (n=3-5); *p<0.05 from DMSO-treated control by t-test. (B) Representative immunoblots of HepG2 for ABC transporters and Nrf2 target genes GCLC and NQO1 upon 20 h treatment with 70 µM LCA, 100 µM CDCA or 200 µM BHQ. Connexin and β-actin serve as loading control for membrane and cytosol proteins, respectively.
Figure 17. LCA-induction of a subset of ABC transporters in human HepG2 and mouse Hepa1c1c7 requires Nrf2. mRNA levels of MRP1-5, and BCRP upon 48 h knockdown of Nrf2 (siNrf2) via siRNA followed by 18 h treatment with DMSO and 70 µM LCA in HepG2 (A) and Hepa1c1c7 (B) cells. Mean ± SEM (n=3-5). *p<0.05 from DMSO-treated control by t-test.
Figure 18. MEK1-ERK1/2 MAPK mediates Nrf2 activation and induction of ABC transporter genes.

(A) Phosphoproteins were quantitated on HepG2 treated with 75 µM LCA for 0, 0.5, 1, 2, 3, 6, or 18 h. (B) Representative immunoblots of nuclear extracts of HepG2 treated with DMSO or 75 µM LCA for 6 h in the presence or absence of Ly294002 (LY), U0126 (U), or Sp600125 (SP) (see methods for detail). Lamin B1 was used as equal loading control for nuclear protein whereas β-actin was probed to confirm negligible cytoplasmic contamination in nuclear fraction preparation. (C) ARE-reporter activity of HepG2 treated with DMSO or 70 µM LCA with or without kinase inhibitors (see B for concentration) for 8 h. Specificity of Nrf2-driven reporter activity was confirmed by cotransfection with dominant negative Nrf2 (pEF_DNrf2) and by using a mutant ARE reporter (pGL3_mARE). Relative light unit (RLU) is normalized to DMSO-treated pGL3_ARE. (D) mRNA levels of Nrf2 target and ABC transporter genes of HepG2 treated with LCA with or without U0126 (U) for 12 h. (E) Cell viability of HepG2 after 24 h treatment with LCA (70 µM) with or without kinase inhibitors. Data are mean ± SEM. In A, mean of two determinations with <10% intra-assay variability; Western blots confirmed increased p-ERK1/2 and p-JNK (not shown); in C, n=3 and *p<0.05 by t-test; in D, n=3 and p<0.01 between groups for all genes analyzed; in E, n=4 and *p<0.05 from DMSO-treated control by t-test.
Figure 19. Effects of LCA on efflux of ABC transporters in HepG2 is influenced by Nrf2. (A) 3H-vinblastine, 3H-methotrexate and 3H-mitoxantrone accumulation in HepG2 after 18 h pretreatment with 75 µM LCA or vehicle DMSO. Uptake was measured as radiochemical drug accumulation (DPM per viable cell) at each time point, and was normalized to the mean levels of DMSO-treated group at 60 min set at 100%. Data are mean ± SEM (n=3). (B) Accumulation of 3H-vinblastine and 3H-methotrexate (60-min exposure to radiochemical) in LCA or DMSO-treated HepG2 cells with or without ABC transporter inhibitors (10 µM CspA or 15 µM MK571). Percent uptake of DMSO control without inhibitor treatment is shown. Data are expressed as mean ± SEM; *p<0.05 from respective groups treated without inhibitor (C) HepG2 was treated with 50 nM siRNA for 48 h followed by 16 h treatment of 70 µM LCA and then exposure to radiochemicals for 45-min. Uptake is standardized to DMSO-treated siControl. Data are mean ± SEM; n=3, *p<0.05 from the corresponding siControl with or without LCA treatment. (D) ROS production probed by a sensor fluorescent dye carboxy-H2-DCFDA in HepG2 treated with LCA in the presence of ABC transporter inhibitors (10 µM CspA or 15 µM MK571). 100 µM sodium nitroprusside, a nitric oxide (NO) donor, was used as positive control. Fold change vs. baseline t=0. (E) Cell viability of HepG2 treated with 75 µM LCA in the presence of ABC transporter inhibitors (10 µM CspA or 15 µM MK571); percent change relative to vehicle controls. Data are mean ± SEM; n=3-4.
### Supplementary material: Table A. Primer sequence for qRT-PCR

<table>
<thead>
<tr>
<th>Human genes (accession#)</th>
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<td>BSEP (NM_003742)</td>
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<td>GAPDH (NM_002046)</td>
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<td>GCLM (NM_002061)</td>
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<td>GSTA4 (NM_001512)</td>
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<td>GSTM1 (NM_000561)</td>
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<td>Mouse genes (accession#)</td>
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<td>Bsep(NM_021022)</td>
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<td>Gapdh (NM_001001303)</td>
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<td>Gelm (NM_008129)</td>
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<td>Gele (NM_010295)</td>
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<td>Gstm1 (NM_010358)</td>
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<td>Gstp1 (NM_013541)</td>
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<td>Ho-1 (NM_010442)</td>
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<td>Mrp4 (NM_001033336)</td>
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<td>Mrp5 (NM_013790)</td>
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<td>Nqo1 (NM_008706)</td>
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<td>Nrf2 (NM_010902)</td>
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<td>Ntcp (NM_011387)</td>
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<td>Tbp (NM_013684)</td>
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<td>Trx1 (NM_001042523)</td>
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### Supplementary material:

#### Table B. Gene expression of antioxidative and ABC efflux transporters during acute LCA treatment in mice

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<tr>
<th></th>
<th>Nrf2+/+</th>
<th>Nrf2-/-</th>
<th>P value*</th>
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<tr>
<td><strong>Antioxidative enzymes</strong></td>
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<tr>
<td>Gclm</td>
<td>1.96 ± 0.16**</td>
<td>1.00 ± 0.12</td>
<td>&lt;0.001</td>
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<tr>
<td>Gclc</td>
<td>2.31 ± 0.65*</td>
<td>1.20 ± 0.50</td>
<td>&lt;0.001</td>
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<tr>
<td>Trx1</td>
<td>53.76 ± 10.25**</td>
<td>21.29 ± 8.27**</td>
<td>0.007</td>
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<tr>
<td>Nqo1</td>
<td>1.44 ± 0.15**</td>
<td>0.36 ± 0.13**</td>
<td>&lt;0.001</td>
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<td><strong>Transporters</strong></td>
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<td>Mrp1</td>
<td>1.99 ± 0.23**</td>
<td>1.12 ± 0.48</td>
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<td>Mrp2</td>
<td>2.14 ± 0.32**</td>
<td>1.10 ± 0.18</td>
<td>&lt;0.001</td>
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<tr>
<td>Mrp3</td>
<td>2.08 ± 0.34**</td>
<td>0.24 ± 0.05**</td>
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<td>Mrp4</td>
<td>2.04 ± 0.32**</td>
<td>0.64 ± 0.07**</td>
<td>&lt;0.001</td>
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<tr>
<td>Mrp5</td>
<td>1.26 ± 0.04**</td>
<td>0.67 ± 0.14**</td>
<td>&lt;0.001</td>
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<tr>
<td>Mdr1a</td>
<td>1.92 ± 0.33**</td>
<td>1.28 ± 0.25</td>
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<tr>
<td>Mdr1b</td>
<td>1.50 ± 0.08**</td>
<td>1.52 ± 0.14**</td>
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<tr>
<td>Bcrp</td>
<td>1.87 ± 0.17**</td>
<td>0.63 ± 0.13**</td>
<td>&lt;0.001</td>
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<tr>
<td>Bsep</td>
<td>1.70 ± 0.19**</td>
<td>2.11 ± 0.17**</td>
<td>0.009</td>
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<tr>
<td>Ntcp</td>
<td>1.01 ± 0.06</td>
<td>0.70 ± 0.10**</td>
<td>&lt;0.001</td>
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</tbody>
</table>

Fold change in mRNA levels relative to vehicle treatment after adjustment for sex. Treatment of LCA or vehicle was 8 h. Mean ± SEM (n=6-10). * Comparison between genotype receiving corn oil or LCA; *p<0.05, **p<0.01 from vehicle-treated Nrf2+/+ with Student's t-test or Mann Whitney U test.

#### Table C. Effects of specific small molecule inhibitors on inhibition of phosphoproteins

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<th>p-AKT</th>
<th>p-ERK1/2</th>
<th>p-MEK1</th>
<th>p-JNK</th>
<th>p-c-Jun</th>
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<td>EGF</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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<td>DMSO</td>
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<td>13.9</td>
<td>6.6</td>
<td>18.6</td>
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<td>EGF+Go</td>
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<td>85.4</td>
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<td>EGF+SP</td>
<td>92.5</td>
<td>91.8</td>
<td>101.6</td>
<td>27.9</td>
<td>38.4</td>
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<tr>
<td>EGF+U0126</td>
<td>88.4</td>
<td>18.6</td>
<td>14.4</td>
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<td>46.8</td>
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Mean of duplicate determinations; percentage (%) of fluorescent unit to the positive control (EGF treatment) after subtracted from respective baseline values (t=0). Treatment was for 4 h. Phosphoproteins were quantitated by Bioplex® multiplex kinase assay.
Supplementary material:

Table D. Putative ARE (RTGAYnnnGCR) on -10kb of 5’-region of human MRP2, MRP3 and BCRP genes

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<th>ABC genes (Accession#)</th>
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<td>-8853, -2297</td>
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<tr>
<td>hMRP3 (NM_003786)</td>
<td>-628, -4357</td>
</tr>
<tr>
<td>BCRP (NM_004827)</td>
<td>-5373, -5247, -430, -677</td>
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</table>

Perfect-matched direct (bold) and reverse strands (italic).

Table E. Relative mRNA levels of ABC transporters in HepG2 cells

<table>
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<th>Relative expression (%)¹</th>
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<td>MRP2</td>
<td>100</td>
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<tr>
<td>MDR1</td>
<td>55</td>
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<tr>
<td>BCRP</td>
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<td>MRP1</td>
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<td>MRP3</td>
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<td>MRP5</td>
<td>15</td>
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<td>MRP4</td>
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</table>

¹Mean mRNA level estimated from CT values (qRT-PCR) adjusted with calibrator control gene GAPDH on untreated HepG2 (80-90% confluence). Expression of transporters is in relative to that of MRP2 set at 100%.
Supplementary material:

Table F. Influence of Nrf2 knockout in constitutive gene expression of antioxidant and ABC efflux transporter genes in mice

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<th>Untreated (Constitutive)(^a)</th>
<th>Male</th>
<th>Female</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nrf2(^{+/+})</td>
<td>Nrf2(^{-/-})</td>
<td>Nrf2(^{+/+})</td>
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<tr>
<td>Gclm</td>
<td>1.00 ± 0.04</td>
<td>0.81 ± 0.07</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>Gclc</td>
<td>1.00 ± 0.1</td>
<td>0.5 ± 0.0(^*)</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>Trx1</td>
<td>1.00 ± 0.07</td>
<td>1.09 ± 0.13</td>
<td>1.00 ± 0.08</td>
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<tr>
<td>Nqo1</td>
<td>1.00 ± 0.05</td>
<td>0.23 ± 0.05(^*)</td>
<td>1.00 ± 0.08</td>
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<td>Ho-1</td>
<td>1.00 ± 0.09</td>
<td>0.61 ± 0.06(^*)</td>
<td>1.00 ± 0.09</td>
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<td>0.45 ± 0.05(^*)</td>
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<td>0.64 ± 0.03(^*)</td>
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<td>0.37 ± 0.04(^*)</td>
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<td>0.96 ± 0.07</td>
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<td>0.55 ± 0.05(^*)</td>
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<td>0.55 ± 0.05(^*)</td>
<td>1.00 ± 0.06</td>
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<td>Mrp5</td>
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<td>1.00 ± 0.05</td>
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<td>Mdr1a</td>
<td>1.00 ± 0.08</td>
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<td>1.00 ± 0.08</td>
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<td>Mdr1b</td>
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<td>1.06 ± 0.06</td>
<td>1.00 ± 0.04</td>
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<td>Bsep</td>
<td>1.00 ± 0.18</td>
<td>1.89 ± 0.18(^*)</td>
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<td>Ntcp</td>
<td>1.00 ± 0.07</td>
<td>0.65 ± 0.04(^*)</td>
<td>1.00 ± 0.04</td>
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Fold change in mRNA levels relative to \(^a\) untreated Nrf2\(^{+/+}\) mice for respective sex. Constitutive expression was determined in 9-wk mice from two litermate of each genotype. LCA treatment was twice daily for 4 d. Mean ± SEM (n=4-6). \(^*\) p<0.05 between genotype in untreated or LCA-treated group within the same sex by t-test.
Table F. Influence of Nrf2 knockout in constitutive gene expression of antioxidant and ABC efflux transporter genes in mice (continued)

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Nrf2+/+</th>
<th>Female</th>
<th>Nrf2+/+</th>
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</thead>
<tbody>
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<td>4 d LCA-treated (Inducible)a</td>
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<tr>
<td>Nrf2+/+</td>
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<td>2.18 ± 0.09</td>
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Fold change in mRNA levels relative to, a vehicle or corn-oil treated mice with the same sex. Constitutive expression was determined in 9-wk mice from two littermate of each genotype. LCA treatment was twice daily for 4 d. Mean ± SEM (n=4–6). * p<0.05 between genotype in untreated or LCA-treated group within the same sex; # p<0.05 from vehicle-treated Nrf2+/+ mice within the same sex by t-test.
Figure I. HepG2 cells were treated with increasing concentrations of LCA (6.35, 12.5, 25, 50, and 75 µM) or CDCA (12.5, 25, 50, 75, 100, 150 µM) for 24 h. mRNA levels of ABC transporters adjusted against β-actin or GAPDH were measured as fold change relative to vehicle DMSO-treated control. * p<0.05 from DMSO-treated control by one-way ANOVA followed by Dunnet’s posthoc test. Mean ± SEM (n=3-5).

Figure II. mRNA levels of MRP1-5, BCRP, and MDR1 of mouse hepatoma Hepa1c1c7 and human hepatoma Huh7 after 20 h treatment with 70-75 µM LCA. Fold change vs. DMSO-treated control. *p<0.05 by t-test. Mean ± SEM (n=3).

Figure III. Accumulation of 3H-vinblastine and 3H-methotrexate in HepG2 (60-min exposure to radiochemical) as influenced by increasing doses (0, 5, 50 µM) of bile acids. Uptake is expressed as fraction to DMSO-treated group. Mean ± SEM (n=3).
CHAPTER 4.0

NRF2 AS DETERMINANT OF CELLULAR RESISTANCE IN RETINOIC ACID CYTOTOXICITY

Kah Poh Tan, Kazuhiro Kosuge, Mingdong Yang, and Shinya Ito

[In submission]

KPT designed and performed all experiments, and wrote the manuscript; KK contributed intellectually to methodological development; MY assisted and delivered helpful methods in animal experiments, and performed parts of the analysis (total RNA extraction of animal tissues for real-time PCR)
4.1 Abstract
Clinical use of retinoic acids (RA) is hindered by frequent toxicity with mechanisms related to oxidative stress. RA shown to inhibit Nrf2 and expression of its target cytoprotective genes poses a possibility that RA toxicity may result from cellular disability to cope with oxidative stress. We hereby report our in vitro and in vivo observations that all-trans-RA (atRA) and its cis-isomers, at concentrations implicated in toxicity, activated Nrf2, inducing Nrf2 target genes, particularly the rate-limiting enzyme in glutathione biosynthesis: glutamate cysteine ligase subunits (GCLM/GCLC). RA enhanced nuclear accumulation of Nrf2, antioxidant responsive element (ARE)-reporter activity, and Nrf2 occupancy to AREs in chromatin context. Protein adducts of 4-hydroxynonenal (4-HNE), a signaling molecule of lipid peroxidation, were increased by atRA. RNA interference-mediated silencing of Nrf2, not of retinoid X receptors α and β, mitigated basal and atRA-induced GCLM/GCLC gene expression. Inhibition of MEK1/ERK mitogen-activated protein kinases (MAPK) significantly suppressed atRA-induced Nrf2 activation and ARE-regulated gene expression, reducing cell resistance against toxic RA. Nrf2-silenced cells became prone to RA-induced mitochondrial toxicity and apoptosis. In conclusion, toxic RA activates Nrf2, thereby enhancing adaptive response against resultant oxidative cytotoxicity. Concentration-dependent modulation of the Nrf2 transcription pathway by RA is proposed.

Key words: retinoic acid; Nrf2; glutathione; glutamate cysteine ligase; oxidative stress; mitogen activated protein kinase; 4-hydroxynonenal.
4.2 Introduction

Retinoids are a family of polyisoprenoid lipids often referred to as vitamin A and its analogs. Of all retinoids, retinoic acid (RA) is the most biologically-active form of vitamin A, essential in embryonal development, and maintenance of growth and differentiation of epithelial, fibroblast and myelomonocytic cells (Ross et al., 2000; Mark et al., 2006; Bastien & Rochette-Egly, 2004). RA has been used not only for cancer chemoprevention, but also therapeutically for diseases including cancers such as acute promyelocytic leukemia and neuroblastoma. The differentiation-promoting role of RA, which is the most documented mechanism of its therapeutic effects, is known to be mediated through the retinoid receptors comprising retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Ross et al., 2000; Mark et al., 2006; Bastien & Rochette-Egly, 2004).

A major hindrance for therapeutic use of RA is their conspicuous toxicity and teratogenicity (Ross et al., 2000; Fenaux et al., 2007). During retinoid therapy for acute promyelocytic leukemia, systemic toxicities affect multiple organs including lung and liver. This potentially-fatal condition, called the retinoic acid syndrome, occurs in 15-50% of patients (Shen et al., 2004B; Ades et al., 2005; de Botton et al., 2003). Also, RA has been implicated in a wide range of abnormal organogenesis and lethality of the fetus (Ross et al., 2000; Mark et al., 2006) in which RXRα and RARγ were reported to play some role (Lohnes et al., 1993; Sukov et al., 1995). In contrast to the physiological effects of RA \((10^{-9} – 10^{-8} \text{ M})\) which are accomplished primarily by RARs and RXRs (Ross et al., 2000), the mechanism underlying RA toxicities from pharmacological exposure \((10^{-7} – 10^{-5} \text{ M})\) remains largely unidentified. Despite this, therapeutic doses of RA have been shown to induce production of peroxyl radicals and reactive oxygen species, and resultant oxidative stress (Murata & Kawanishi, 2000; Samokyszyn & Marnett, 1987; Freyaldenhoven et al., 1996; Davis et al., 1990; Castro-Obregon & Covarrubias, 1996; Conte da Fronta et al., 2006). Presently it is unclear if RA-induced oxidative stress is a main contributor to its toxicity, and if so, to what extent cellular defense system of oxidative stress defense involves in protection against the resultant cell injury and cell death.

The nuclear factor [erythroid 2-like] factor 2 (Nrf2) is an important oxidative stress-responsive transcription factor, playing a vital role in combating oxidative damage. Upon activation, it releases from cytoplasmic entrapment and negative regulation by
Keap1 and accumulates in nucleus to activate a vast array of cytoprotective genes via binding to its cognate antioxidant responsive element (ARE) (Kensler et al., 2006). Among the target genes, the glutamate cysteine ligase (GCL) consisting of the modulatory (GCLM) and catalytic (GCLC) subunits compose the rate-limiting enzyme for cellular biosynthesis of glutathione (GSH). GSH is the most critical intracellular antioxidant in preserving cellular redox balances by scavenging excess free radicals and electrophiles. Therefore, the Nrf2-ARE pathway is most likely to counteract against RA-induced oxidative stress. Recently, however, Wang et al. showed that, through an unclear RARα-Nrf2 interaction, binding of Nrf2 to the ARE is dose-dependently inhibited by RA at $10^{-9} - 10^{-6}$ M, thereby suppressing expression of Nrf2-target antioxidative genes (Wang et al., 2007B). It is unknown whether such Nrf2 inhibitory effects of RA, which may deprive cellular ability against RA-induced oxidative stress, can represent potential mechanism underlying retinoid toxicity.

In the present study, we examined the effects of pharmacological concentrations of RA on the Nrf2-ARE pathway. Using in vivo mouse model and a liver cell model (HepG2) with demonstrated Nrf2 and RARα activities (Mangelsdorf et al., 1992; Suzui et al., 2004; Tan et al., 2007), we found that exposure to RA at the pharmacological levels activates Nrf2 and induces Nrf2-ARE binding, increasing expression of cytoprotective genes, particularly the GCL subunits, to resist retinoid-induced cell death.

4.3 Methods

Cell Culture and Chemicals

The human hepatoma-derived HepG2, human embryonic kidney cells HEK293 (ATCC), human mammary adenocarcinoma Mcf7 and mouse hepatoma-derived Hepa1c1c7 (Hepa1) (gifts from Dr. Harper, The Hospital for Sick Children, Toronto, ON), and human primary hepatocytes (Celprogen, San Pedro, CA), were maintained as described (Tan et al., 2007). Test retinoids (all-trans-retinoic acid, atRA; 9-cis-retinoic acid, 9cRA; 13-cis-retinoic acid, 13cRA) and other chemicals were purchased from Sigma, unless otherwise indicated. Oligonucleotides were synthesized at the Centre for Applied Genomics in Toronto or Integrated DNA Technologies (Coralville, IA).

Mouse Experiment
The animal care and experimental procedures were approved by the Animal Care Committee at the Hospital for Sick Children. Nine wk old male CD-1 mice (Charles River, Montreal, Quebec) were injected i.p. with atRA (15 mg/kg and 30 mg/kg body wt) (Scott et al., 1994, Rego et al., 2000) dissolved in filter-sterilized corn oil daily for two days. Retinoid preparations were done in dim lighting and made fresh prior to treatment. Mice were killed 16 h after the last injection. Their livers were sampled in RNAlater reagent (Invitrogen) for mRNA analysis. Sera were collected for analysis of liver function/injury markers, i.e., total bilirubbin (TBL), alanine aminotransferase (ALT) and aspartate aminotransferase (ALP) and \( \gamma \)-glutamyl transpeptidase (GGT) using established automated methods (Tan et al., 2007).

**cDNA Synthesis and Quantitative Reverse-Transcription PCR (qRT-PCR)**

RNA extraction, reverse transcription, qRT-PCR, and the \( \Delta \Delta \text{Ct} \) method to quantify relative fold-difference of gene transcripts calibrated by internal control genes [ribosomal 18S, tata-box binding protein (TBP), \( \beta \)-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH)] between control and treatments were performed as described (Tan et al., 2007).

**Immunoblotting**

Preparation of crude cell lysate and nuclear fractions, and subsequent immunodetection steps were carried out (Tan et al., 2007). Primary antibodies (working concentration) used were: rabbit polyclonal anti-GCLC Ab-1 (1:2000) (NeoMarkers), rabbit antiserum against GCLM (1:3000) (20), rabbit polyclonal anti-Nrf2 c-20 (1:750) (Santa Cruz), rabbit polyclonal anti-TR1 (1:3000) (Abcam), mouse monoclonal anti-GST-Pi (1:500) (Dako), mouse monoclonal anti-\( \beta \)-actin (1:10000) (Sigma), goat polyclonal anti-lamin B c-20 (1:200) (Santa Cruz) and goat polyclonal p-PKC\( \alpha \) (1:500) (Santa Cruz).

**RNA Interference (RNAi)**

A combination of four gene-specific small-interfering RNA (siRNA) (50-75 nM) against human Nrf2 (NM_006164), RXR\( \alpha \) (NM_002957) and RXR\( \beta \) (NM_021976) (Thermo Fisher Scientific, Lafayette, CO) was used. Conditions for siRNA transfection, chemical treatment and mRNA quantitation between cells treated with siRNA against target
receptor(s) and equal molar control scrambled siRNA (siCtr) have been described (Tan et al., 2007).

**Plasmid Constructs, Luciferase Reporter Assays, and Overexpression Studies**

The expression vectors for dominant positive Nrf2 (pEF_Nrf2), dominant negative Nrf2 (pEF_DNrf2) and empty vector (pEF) were kindly provided by Dr. Alam (Ochsner Clinic Foundation, New Orleans, LA). The ARE-reporter (pGL3_ARE) or mutant ARE reporter constructs (Tan et al., 2007) was transiently transfected to HepG2 cells with or without Nrf2 expression vectors overnight followed by retinoid treatments for 16-18 h in all experiments, unless otherwise stated. Luciferase activities of the cell extracts were analyzed with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity (RLU) was calculated from firefly luciferase values normalized to those of the cotransfected control Renilla luciferase, and expressed as ratios to vehicle-treated empty pGL3 promoter construct and, if any, cotransfected expression vector(s). For overexpression studies, HEK293 cells at ~60% confluence in 6-well culture plates were transfected with 1.5 µg of Nrf2 dominant positive or negative expression vector for 24 h, followed by treatment with retinoids for 18-20 h.

**Quantitative Chromatin Immunoprecipitation (ChIP)**

The assay was performed using the ChIP assay kit (Upstate) with slight modifications and quantitation of Nrf2 occupancy of documented AREs of GCLC and GCLM genes upon retinoid treatments via Taqman® qPCR were carried out as described (Tan et al., 2007).

**Lipid Hydroperoxides and 4-HNE Protein Adducts**

Lipid hydroperoxides were analyzed by the ferric thiocyanate assay (Mihaljevic et al., 1996). For 4-HNE detection, HepG2 cells were treated with atRA, 4-HNE (positive control) (CalBiochem) or vehicle. Cell lysate was prepared in ice-cold RIPA buffer containing protease inhibitors (Roche), 400 µM EDTA, 20 µM desferoxamine mesylate and 10 µM butylated hydroxyl toluene, and 10 passages through a 26-gauge syringe needle. 100 µg of cell lysate was dissolved through a 4-12% bis-tris gel in MES buffer containing antioxidant cocktails (Invitrogen) and followed through similar protocols for immunoblotting (see above). 4-HNE protein adducts were probed by a rabbit anti-HNE
antiserum (1:1000) (Alpha Diagnostic) with inclusion of a HNE-conjugated bovine serum albumin protein (Alpha Diagnostic) as positive control to ensure antibody specificity.

**Multiplex Kinase Activation Assay**

A sensitive bead-based, quantitative multiplex assay using Luminex xMAP technology (xplex assay; Bio-Rad, Hercules, CA) and antibodies from Cell Signaling Technology (Danvers, MA) was used to probe multiple phosphoproteins representing activation of multiple stress-related kinase pathways. The kinase pathways with corresponding target phosphoproteins assessed were: mitogen-activated protein kinase (MAPK)\[p-MEK1(Ser^{217}/Ser^{221}), \ p-ERK1/2(Thr^{202}/Tyr^{204}, \ \text{Thr}^{185}/\text{Tyr}^{187})\], p-p38 MAPK(Thr^{180}/Tyr^{182})], phosphatidylinositol-3-kinase-Akt (PI-3K-Akt) \[p-Akt (Ser^{473})\], and c-Jun N-terminal kinase (JNK/SAP kinase) \[p-JNK(Thr^{183}/Tyr^{185}), \ p-c-Jun(Ser^{63})\]. Sample preparation was guided by provided kits and manufacturer’s instructions. Fluorescent signals of cell lysate (300-400 µg) were measured with Bio-Plex 200 System (Bio-Rad) and adjusted to individual total protein (Bio-Rad). Sensitivity, specificity and robustness of the assay were checked by inclusion of positive controls [epidermal growth factor (EGF) and UV-treated cell lysates], negative control (untreated cell lysate), and lysate of cells treated with specific small molecule inhibitors.

**Kinase Inhibitor Experiments**

To effectively inhibit specific kinase activation without causing additive toxicity, HepG2 cells were preincubated with small molecule inhibitors for 30-min followed by a 5-fold lower cotreatment concentration given together with atRA. The pre- and co-treatment concentrations were as follows: Gö 6976 [PKCα inhibitor; 100 nM, 20 nM], Ly294002 PI3K inhibitors; 20 µM, 4 µM], Sp600125 [JNK/SAP kinase inhibitor; 5 µM, 1 µM], and U0126 [MAPK inhibitor; 25 µM, 5 µM]. Specific inhibition of kinase activation (>60% inhibition) with minimal interference (<20%) with other kinase pathways by small molecules was confirmed by analyzing percent decreases in corresponding phosphoproteins relative to positive controls (cells treated with epidermal growth factor, EGF) with the multiplex kinase activation assay.

**Cytotoxicity, apoptosis, and GSH Quantitation**
Cytotoxicity was assessed with the Alamar Blue™ assay (Biosource, Nivelle, Belgium). Cellular caspases 3, 6, 7, 8 and 10 which are established indicators of apoptotic events were analyzed using the rhodamine 110-conjugated substrate Asp-Glu-Val-Asp (Molecular Probes). An assay based on GSH recycling enzymatic method was used to measure cellular GSH (25). Protocol of the above assays has been described (Tan et al., 2007).

**Mitochondrial Membrane Potential**
A cell permeable, lipophilic cationic dye, namely 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)(Cayman Chemical, Ann Arbor, MI) was used to examine mitochondrial membrane potential of HepG2 cells exposed to toxic RA. Healthy cells, with high mitochondrial transmembrane potential ($\Delta\psi_m$) forms J-aggregates with this dye, emit red fluorescence (Ex/Em: 560/595 nm). Whereas loss of membrane potential results in higher free JC-1 monomers will emit mainly green fluorescence (Ex/Em: 485/535). The ratio of red to green fluorescence was hence used as an index of mitochondrial membrane potential with a lower ratio indicating loss of membrane potential and potentially mitochondrial apoptosis.

**Statistical Analysis**
Statistical tests were conducted using SigmaStat 3.1 or SPSS10.1. Comparisons between two groups on single parameter were accomplished by Student’s independent t-test (parametric) or Mann-Whitney U test (non-parametric). Difference with $p<0.05$ was considered statistically significant.

**4.4 Results**
**RA induced GCL subunits and other Nrf2 target genes**
In micromolar range of concentrations, a dose-response increase in mRNA of GCLM and GCLC in HepG2 cells was noted for the 3 tested RAs (Fig. 20A). Induction became evident in 4 h of exposure, which was sustained afterwards to various degrees (Fig. 20B). GCLM/GCLC induction was also observed with retinoid treatment in other cell models including human primary hepatocytes (Fig. 20C). Parallel increases in GCL proteins (Fig 20D) were noted at 24 h treatment which coincided with elevated GSH biosynthesis (Fig. 20E). Several redox-sensitive genes known to be targets of Nrf2 regulation, such as
GSTP1, NQO1, HO1 and TRx1, were similarly induced in HepG2 cells (Fig. 20D, 20F), and the other cell lines (not shown).

**In vivo activation of Nrf2 target genes by RA treatments**

To investigate whether Nrf2 activation and increase in its target genes with RA treatments could be observed in vivo, mice were treated with known therapeutic/teratogenic doses of atRA. RA administration caused an increased hepatic nuclear accumulation of Nrf2, a pre-requisite event of Nrf2 transactivation (Fig. 21A), and a concurrent dose-dependent induction of Nrf2 target genes (Fig. 21B). While GSTP1 was the GST activated by retinoids in human HepG2 cells, it was not evident in mice (not shown). Instead, Gsta (subtypes 1 & 2), the Nrf2 target genes in mice (Itoh et al., 1997), were found to be induced in mouse livers (Fig. 21B). Analysis of liver function/injury markers (TBL, ALT, AST and GGT) did not indicate hepatotoxicity upon RA treatment. This implies that Nrf2 activation by toxic retinoids preceded the occurrence of biochemically overt liver injury.

**Involvement of Nrf2 and mRNA stability in increased GCL subunits by RA**

A study using mice with liver-specific knockout of RXRα has reported a lower constitutive expression of GCL subunits (Wu et al., 2004). Because RXR is the obligatory heterodimer partner in target gene transactivation for many nuclear receptors including RAR, the role of RXR in GCL subunit regulation by retinoids was examined in HepG2 cells. HepG2 expressed mainly α and β subtypes of RXR and α and β subtypes of RAR. siRNA-silencing of RXRα or both RXR-α and -β, which blunted atRA-induced expression of RAR target genes such as CYP26A1 and RARβ failed to mitigate basal and inducible mRNA expression of GCL subunits (Supporting fig. I). Instead, a ~2-fold increase of basal GCLM level was noted upon silencing of both RXR subtypes. Conversely, Nrf2 knockdown causing >60% reduction in Nrf2 mRNA and protein (Supporting fig. II, and Tan et al., 2007) significantly reduced basal and inducible expressions of GCL subunits by atRA and the known Nrf2 activator α-lipoic acid (LA) in HepG2 cells (Fig. 22A) and MCF7 (Supporting fig. III). Similar attenuation was also observed with using other cis-isomer RAs (not shown). In consensus, GCLM and NQO1 expression was significantly enhanced by overexpression of Nrf2 in Hek293 cells (Fig. 22B). In addition to transcriptional
activation, we also found that atRA-induced increase of mRNA of GCL subunits was, at least partly, due to increased mRNA stability effects. Using two global transcription inhibitors (actinomycin D and DRB), we consistently found that the t_1/2 of mRNA decay of GCLM and GCLC were ~3- and ~2-fold, respectively, longer upon exposure to atRA compared with those of vehicle treatment (Supporting fig. IV). These findings are in agreement with previous reports in lung and colon cells exposed to oxidative stress inducers (Liu et al., 1998; Song et al., 2005).

**Activation of Nrf2-ARE transcription machinery by RA**

RA exposures caused an increased Nrf2 accumulation in the nuclei within 1-3 h of treatment (Fig. 23A). Further, a dose-response increase in ARE-reporter activity was noticed with treatments of all test RAs (Fig. 23B). Overexpression of Nrf2 further enhanced RA-induced reporter activity whereas coexpression of dominant-negative Nrf2 mitigated the activity (Fig. 23C), and point mutations of the core ARE completely abolished the reporter activation by RA (Fig. 23C). Even in the absence of RA, ARE-reporter activity was robust (Fig. 23B, Fig. 23C), suggesting Nrf2 transactivation is intrinsically active in the HepG2 cells. These data suggest that the increased transactivation activity of RA via ARE is mediated through Nrf2. Moreover, ChIP analyses demonstrated an increased Nrf2 occupancy to core AREs of 5’-GCLM and -GCLC regions in native chromatin context at 3 h of RA exposures (Fig. 23D).

**Increased formation of 4-HNE protein adducts and lipid peroxidation with RA treatment**

atRA (≥ 30 µM) treatment in HepG2 cells produced markedly higher levels of HNE protein adduct (Fig. 24A). Importantly, the detection of increased protein adducts between 40-50 kDa found in this study replicates observations of a previous study using rat hepatocytes treated with 4-HNE and tert-butylhydroperoxide, a hydroperoxide radical producer (27). Increased 4-HNE protein adducts were detected within 6 h of atRA treatment and became more apparent at 16 h (Fig. 24B). In agreement with increased lipid peroxidation, the lipid hydroperoxide level was significantly increased by 2.5-fold in HepG2 cells after 24 h treatment with retinoids and in the livers of mice receiving atRA (Fig. 24C).
**MEK1/ERK MAPK mediates Nrf2 activation and cytoprotective gene induction by RA**

Exposure to atRA in HepG2 cells resulted in simultaneous activation of MEK1/ERK MAPK and JNK pathways at 30-min of treatment. Particularly, both p-ERK1/2 and p-c-Jun were increased by ~3- to 6-fold and sustained through 8 h of treatment (Fig. 25A). We also found a ~2 fold increase of p-AKT, a downstream target of PI3K, at 30-60 min of treatment with levels subsided gradually to baseline with longer exposure. Activation of p38 MAPK by atRA was not evident. In an attempt to examine whether any of the above activated kinase pathways could be instrumental in Nrf2-ARE transactivation, pharmacological inhibition of individual kinase pathways during atRA treatment was conducted. Inhibition of MEK1/ERK MAPK by the MEK1 inhibitor U0126 significantly reduced atRA-induced reporter activity, and this activity was further halted by coexpression of the dominant negative Nrf2 (Fig. 25B). Moreover, ChIP assay showed that the MEK1/ERK MAPK inhibition also reduced atRA-induced Nrf2 occupancy of the ARE (Fig. 25C), which was associated with a significant reduction of atRA-induced Nrf2 target gene transcripts (Fig. 25D). Inhibition of PI3K/AKT signaling pathway by Ly294002 caused moderate reductions of ARE reporter activity and of TRx1 gene (Fig. 25B, Fig. 25D). Blocking of Nrf2 activation and subsequent induction of cytoprotective genes by inhibition of MEK1/ERK MAPK or, to a less degree, PI3K/AKT could sensitize HepG2 cells to RA toxicity (Fig. 25E), suggesting that these kinases have a crucial role in preserving cell survival during cellular stress. In contrast, inhibition of the JNK pathway by Sp600125 did not have significant effects on ARE transactivation by Nrf2 (Fig. 25B).

**The Protective Role of Nrf2 in RA toxicity**

To directly examine the protective role of Nrf2 in retinoid toxicity, Nrf2-knockdown HepG2 cells were treated with toxic concentrations of atRA. Increased cell susceptibility to retinoid toxicity was consistently seen in Nrf2-knockdown cells with more extensive cell death at 70 µM treatment (Fig. 26A). Cells treated with siNrf2 or siCtr alone did not differ with each other in cell viability (not shown). Apoptotic cell death is the major outcome of retinoid toxicity (Pfahl & Piedrafita, 2003). In line with the above cell viability data, silencing of Nrf2 increased vulnerability of HepG2 cells to atRA-induced apoptosis as evidenced by increased caspases activity (Fig. 26B) and DNA fragmentation (Fig. 26C). The mitochondria were found to be a target of toxic RA. Nrf2 knockdown of HepG2
cells exhibited more severe loss of mitochondrial membrane potential which may present a mechanism to retinoid-induced apoptosis (Fig. 26D and Fig. 26F).

**Cellular GSH is critical determinant in resisting RA toxicity**

The increased cellular biosynthesis of GSH (Fig. 20D) consequent to increased GCL subunits may be a critical adaptive mechanism in cell defense against toxic RA. To test this hypothesis, we treated cells with a mildly toxic concentration of atRA along with BSO, a GSH depleter which inhibits GCL subunit activity and GSH biosynthesis. Cotreatment with BSO decreased cell resistance toward RA toxicity (Fig. 26E) with more notable effects at late treatment (~24 h). Depletion of GSH with overnight pretreatment with BSO led to a marked plunge in cell viability during early treatment (<8 h) (Fig. 26E). Treatment of cells with BSO alone did not differ in cell viability with those treated with DMSO vehicle (not shown). Overall, these findings suggest that cellular GSH and inducibility of endogenous GSH are major components of cellular defense against RA toxicity.

4.5 Discussion

In this study, all test RAs at >10^-6 M in vitro, and pharmacological atRA treatment in mice were shown to activate Nrf2 and its target genes crucial for maintaining cellular GSH homeostasis and redox balances. Induction of these cytoprotective genes represents an adaptive defense response critical for cell survival because Nrf2 silencing remarkably increased RA-induced cell death. The mitochondria were found to be particularly vulnerable to toxic RA. Nrf2 silencing enhanced RA-induced mitochondrial toxicity and apoptosis induction. These findings support the mitochondrial protective role of Nrf2 (Calkins et al., 2005).

At submicromolar concentrations (10^-7 – 10^-6 M), RA paradoxically resulted in 30-40% suppression of GCL gene transcripts in HepG2 and Mcf7 cells relative to vehicle treatment (not shown), presumably as a result of the inhibitory effect of RA-activated RAR on Nrf2 (Wang et al., 2007B). RA at these concentrations, however, did not cause cytotoxicity, oxidative stress, or changes in GSH biosynthesis in our cell models (not shown). In contrast, with increasing RA concentrations >10 µM when oxidative stress is provoked, Nrf2 was activated along with induction of GSH and cytoprotective genes. These findings point to possible concentration-dependent biphasic effects of RA on Nrf2.
function. Increased cellular oxidative state can abrogate RAR activation by preventing DNA binding of RAR transcription complex (Demary et al., 2001). Inhibition of RA on Nrf2 target genes in our experiments was unremarkable as compared with that of recently reported (Wang et al., 2007B). Hence, the effects of RA on Nrf2 may be influenced by differential expression of RAR and RXR, and factors dictating susceptibility to oxidative stress among different tissues and cell types. To address this, further studies are needed.

Rodents receiving 10 mg/kg body wt. of RA could achieve ~2.5 – 4.5 µg/mL (8 – 15 µM) in circulation, and ~10 – 20 µg/mL or µg/g (~33 – 67 µM) in the liver and brains (Le Doze et al., 2000; Kalin et al., 1981; Wang et al., 1980). In humans, plasma concentration after intravenous administration of 90 mg/m² of atRA peaked to 8 µg/ml (~25 µM) with mean levels of ~5 µM (Ozpolat et al., 2003). Similarly, 13cRA administration (160 mg/m²/day orally divided into 2 doses) showed peak levels of 2.5 to 5.4 µM with substantial inter-individual variations (Veal et al. 2007). These studies indicate that circulating RA levels above micromolar concentrations are therapeutically achievable in humans. Preferential tissue disposition of RA maybe another important factor determining target sites of retinoid toxicities.

The fact that relatively high concentrations of RA were needed to produce oxidative stress in HepG2 cells may result from a higher tolerance of this cell line toward oxidative insults. We noted that HepG2 cells had a high constitutive oxidative state with a strong intrinsic ARE-reporter activity (Fig. 4C) and 4-HNE production (Fig. 5A). This oxidative stress-resistant phenotype, as was also observed in many cancerous cell lines (Szatrowski & Nathan, 1991), may acquire higher resistance against oxidative toxicity as shown in some apoptosis-resistant cell model (Brown & Bicknell, 2001; Keightley et al., 2004).

4-HNE is a lipid peroxidation-derived reactive metabolite known to activate Nrf2 and induce GCL subunits (Chen et al., 2005A). In this study, we observed increased lipid peroxidation and 4-HNE production upon RA challenge, which coincided with the significant induction of Nrf2 target genes. Thus, the activation of Nrf2 by RA may be in part driven by increased 4-HNE. In line with this observation, RA can enhance PGH-catalyzed production of carbon centered and peroxyl radicals (Samokyszyn & Marnett, 1987; Freyaldenhoven et al., 1996), autoxidation of linoleic acids (Freyaldenhoven et al., 1998), and production of ROS by targeting mitochondria and nitrite oxide synthase systems (Davis et al., 1990; CastroOregon & Covarrubias, 1996; Personett et al., 2000).
These oxygen radicals are main mediators of lipid peroxidation to produce 4-HNE from the ω-6 polyunsaturated acyl groups of membrane phospholipids.

Given that GSH conjugation is the major route for 4-HNE detoxification and elimination from the cell (Awasthi et al., 2004), the simultaneous induction of GSH-biosynthesis (GCL subunits), -conjugation (GSTP1), and -recycling (GPx1) genes with a concurrent increased cellular GSH may be an adaptive defense mechanism against retinoid and 4-HNE toxicity. The importance of these genes in preserving cellular redox, and preventing cell death and apoptosis caused by xenobiotics has been exemplified (Awasthi et al., 2004; Iles & Liu, 2005; Cumming et al., 2001). To further ascertain the protective role of GSH, we show that depletion of cellular GSH and inhibition of GCL activity by BSO markedly increased the cell susceptibility to retinoid toxicity. This observation is in agreement with the present knowledge stressing that cytosolic and mitochondrial GSH is critical in cell defense against apoptotic stimuli and oxidant-induced mitochondrial injury (Griffith, 1999; Fernandez-Checa & Kaplowitz, 2005).

Depending on cell type, stress inducers and presence of modifier factors, stability of Nrf2/Keap1 complex and ARE-regulated genes have been shown to be modulated by activated stress signaling kinase pathways such as MAPK, PI3K and protein kinase C (PKC) (Kensler et al., 2006). We found that MEK1/ERK MAPK is primarily involved in mediating RA-induced Nrf2 transactivation in HepG2 cells. The immediate activation of this MAPK within 30-min of RA treatment denotes that the late events of 4-HNE production or exacerbation of oxidative stress may not be involved in this kinase activation. This is in agreement with observations that activation of MAPK and JNK pathways does not require changes in redox states consequent to extensive oxidative stress (Go et al., 2004). Inhibition of the MEK1/ERK MARK pathway decreased cell resistance to RA-induced cell death, suggesting that Nrf2 is an important target of the cell survival MAPK cascade. The participation of ERK/MAPK activation in regulation of Nrf2/ARE-regulated genes by chemopreventive agents has been reported (Chen et al., 2006A; Shen et al., 2005); the mechanism by which this signaling pathway interacts with Nrf2-ARE gene activation remains unresolved.

The ability of retinoids to activate Nrf2 provides molecular evidence that oxidative stress is potentially involved in the pathogenesis of retinoid toxicity. Our findings are in agreement with the prooxidative activities reported for RA precursors such as β-carotene and vitamin A (Palozza et al., 2006) with which their dietary supplementation has been
linked to a higher risk of lung cancer in smoker population (Omenn et al., 1996). Recent clinical trials that have witnessed adverse drug reactions of arsenic treatment are in close resemblance with those of classical RA syndrome (Camacho et al., 2000). Considering that arsenic-induced oxidative stress and resultant Nrf2 activation are well documented events in arsenic toxicity (He et al., 2006; Lu et al., 2007; Lantz & Hays, 2006), increased oxidative stress may as well be prerequisite to the occurrence of RA syndrome. In a similar context, increased IL-8 found to be involved with pathogenesis of RA syndrome (Tsai et al., 2007), is a reported target of Nrf2 activation (Zhang et al., 2005).

In conclusion, we show that Nrf2 is activated by toxic RAs through activation of MEK1/ERK MAPK which is accompanied by increased oxidative stress involving production of 4-HNE. Nrf2 activation stimulates adaptive cell defense responses by inducing cytoprotective genes, particularly GCL subunits and hence GSH biosynthesis, to counteract RA-induced oxidative cytotoxicity. Future studies are warranted to ascertain the involvement of oxidative stress in the pathogenesis of retinoid toxicity.

4.6 Significance
This study provides molecular evidence that oxidative cytotoxicity contributes to retinoid-induced cell death. Increased lipid peroxidation with production of deleterious reactive compound 4-HNE may play a part in such adverse outcome. Activation of Nrf2, upon emergence of retinoid toxicity and/or production of 4-HNE, induces critical adaptive responses, particularly GCL subunits and GSH biosynthesis, to reverse resultant mitochondrial toxicity and apoptosis cell death. The involvement of MEK1/ERK1/2 MAPK was also shown to direct Nrf2 activation and induction of antioxidant enzyme genes in HepG2 cells. Depending on treatment concentration and possibly cell-type, RAs were found to possess biphasic effects – inhibiting or activating -- Nrf2, suggesting a divergent role of RA in modulating cellular oxidative stress. In terms of retinoid toxicity implicated by oxidative stress, activating Nrf2 and/or increasing GSH levels may serve a potential therapeutic strategy which merits further investigation.
Figure 20. Induction of Nrf2 target genes, particularly GCLM and GCLC, by RA. A. mRNA levels of GCLM and GCLC in HepG2 cells after 24 h treatment with vehicle DMSO, 1, 10, 25 and 50 µM of three test RA (all-trans-retinoic acid, atRA; 9-cis-retinoic acid, 9cRA; 13-cis-retinoic acid, 13cRA). *Significantly different (p<0.05) from DMSO-treated controls by one-way ANOVA followed by posthoc test (see statistical analysis). Mean ± SEM of n = 4-5. B. Time-course changes in mRNA of GCLM and GCLC induced by various RAs (50 µM) in HepG2. C. mRNA of GCL subunits of other cell lines after 24 h treatment with 50 µM atRA: mouse hepatoma (Hepa1), human colon carcinoma (C2bbe1), human mammary adenocarcinoma (Mcf7), human embryonic kidney (Hek293), and primary hepatocytes. Mean ± SEM of n=3-5. D. Representative immunoblots of protein lysate (30 µg) of HepG2 probing proteins of NRF2 target genes after 24 h treatment with retinoids (50 µM). E. Cellular GSH levels of HepG2 upon 24 h treatment with 50 µM RAs. 200 µM tert-butylhydroquinone (BHQ), an Nrf2 activator and GSH booster, and 60 µM buthione sulfoximine (BSO), a GSH synthesis blocker, was used as positive and negative controls, respectively. *Significantly different (p<0.05) from DMSO-treated controls by one-way ANOVA followed by posthoc test. Mean ± SEM (n=4). F. mRNA expressions of other Nrf2 target genes: glutathione s-transferase P1 (GSTP1), NAD(P)H quinone oxidoreductase(NQO1), thioredoxin reductase 1 (TRx1), ferritin light subunit (FRL) and heme oxygenase 1 (HO-1), and GSH recycling enzyme GSH peroxidase 1(Gpx1) after 24 h treatment with retinoids (50 µM). Mean ± SEM of n =3-4.
**Figure 21. Activation of hepatic Nrf2 in mice treated with atRA.** A. Representative immunoblots of nuclear fraction (30 µg) of mouse liver after treatments with vehicle corn oil or atRA. Lamin B was used as equal loading control for nuclear protein whereas β-actin was probed to confirm similar levels of cytosolic contamination in nuclear fraction preparation. B. Expression of Nrf2 target gene transcripts upon atRA treatment in mouse livers. Significant difference between vehicle-treated control and atRA treatment by t-test; *p<0.05, **p<0.01. Mean ± SEM (n=3/group).
Figure 22. Nrf2 participates in RA-induced GCL subunits and cytoprotective genes. A. Basal (DMSO-treated) and inducible (50 µM atRA or 200 µM LA) expressions of GCL subunits in HepG2 cells. *Significant difference (p<0.05) between siCtr and siNrf2 with or without treatment by t-test. Mean ± SEM (n=3-5). B. mRNA levels of Nrf2 target genes of Hek293 cells transfected 24 h with Nrf2 or DNrf2 expression vector followed by atRA (50 µM) treatment. *Significant difference (p<0.05) by t-test Mean ± SEM (n = 2-4).
Figure 23. **RAs activate Nrf2-ARE transcription machinery.**

A. Representative immunoblots of nuclear fraction (10 µg) of HepG2 treated with retinoid (50 µM) at indicated time-points over 24 h. BHQ treatment (200 µM), a known Nrf2 activator, was included in the analysis as positive control. Lamin B, a nuclear envelope structural protein, was probed on similar immunoblots to control for equal loading of nuclear protein whereas β-actin was probed to rule out the observed increase in nuclear Nrf2 was a result of inclusion of contaminant cytoplasmic proteins. B. An ARE-reporter was transfected to HepG2 overnight and the reporter (luciferase) activity in response to 18-20 h treatment with increasing concentrations of RA was measured. *Significantly different (p<0.05) from vehicle-treated pGL3_ARE by t-test. Mean ± SEM (n = 3-4), or mean and range (n=2). C. ARE-reporter activity cotransfected with or without Nrf2 or DNrf2 expression vector, and mutant ARE (mARE)-reporter activity upon treatment with 50 µM RA. *Significantly different (p<0.05) from vehicle-treated control by one-way ANOVA followed by posthoc test. Mean ± SEM (n = 4). D. ChIP analysis examining Nrf2 occupancy to AREs of both GCLM and GCLC genes after 3 h treatment with RA (50 µM) in HepG2. BHQ (200 µM), known to transcriptionally activate GCLM and GCLC, was included as positive control. Negligible detection from samples incubated with host IgG (CYP1A1) ruled out contribution of non-specific binding from antibody. *Significantly different (p<0.05) from controls (t=0) by t-test. Mean ± SEM (n = 3).
Figure 24. Increased formation of 4-HNE protein adducts and lipid peroxidation with RA treatments.
A. HepG2 was treated with increasing doses of atRA for 16 h or 50 µM 4-HNE (positive control) for 4 h. Note that the immunoblot profile of protein adducts by retinoid treatment (30, 50 µM) exactly resembles that of the 4-HNE treatment with two identifiable adducts at molecular weights ~40-50 kDa, and one each at ~70 kDa and at ~150-200 kDa (indicated by asterisks). The common band at ~60 kDa (arrowhead) could be reduced by preabsorption of antibody with excess 4-HNE-modified BSA, implying that it is possibly a protein adduct abundantly present in HepG2 cells (not shown). L is the lane for molecular weight ladder. B. HepG2 was treated with 50 µM atRA and cell harvested at 0-16 h, and similar immunoblotting was conducted. A 4-HNE conjugated BSA was used as immunoblotting positive control and loaded on the right lane (indicated as “a”). β-actin was probed on stripped immunoblots to ensure equal protein loading. C. Measurement of lipid peroxides in lipid fractions of HepG2 after 24 h treatment with atRA and 9cRA (50 µM) and of mouse liver injected i.p. with atRA (see Fig. 2 for details). Significantly different (* p<0.05, **p<0.01) from vehicle control by t-test.
**Figure 25. MEK1/ERK/MAPK primarily mediates Nrf2 activation by RA.**

A. Phosphoproteins representing activation of MAPK (p-MEK1, p-ERK1/2), JNK (p-JNK, p-c-Jun), PI-3K/AKT (p-AKT), and p38 MAPK were quantitated on HepG2 treated with 50 µM atRA for 0, 0.5, 1, 2, 3, 4 and 8 h. Mean of two independent experiments with duplicate determinations. B. ARE-reporter (luciferase) activity of HepG2 treated 8 h with DMSO or 50 µM atRA with or without specific kinase inhibitors: Ly294002 (Ly; PI-3K/AKT inhibitor), U0126 (U; MEK1/MAPK inhibitor), or Sp600125 (Sp; JNK/SAP kinase inhibitor) (Please see experimental procedures for treatment protocols). Specificity for Nrf2-driven reporter activity was confirmed by cotransfection with a dominant negative Nrf2 (pEF_DNrf2). Relative light unit (RLU) is normalized to DMSO-treated pGL3_ARE. Mean ± SEM of three determinations. *p<0.05 from DMSO-treated control by t-test.

C. ChIP analysis examining Nrf2 occupancy to AREs of GCLC upon 6 h treatment with retinoids (50 µM) with or without U0126 in HepG2. Mean ± SEM of three determinations. *p<0.05 from DMSO treatment by t-test. D. mRNA levels of Nrf2 target genes of HepG2 treated with atRA with or without DFO, U0126 (U) or Ly294002 (Ly) for 12 h. Mean ± SEM of three determinations. * p<0.05 from DMSO treatment by t-test.

E. Cell viability of HepG2 upon 24 and 48 h treatments with 50 µM atRA with or without cotreatment with the kinase inhibitor, or the inhibitor alone. Mean ± SEM of four determinations.
A

Viability (% of time 0) vs Time (h)

- siControl
- siNrf2
- atRA 30 µM
- siNrf2 + atRA 70 µM

B

Caspases activity (Arbitrary unit/mg protein)

- siCtrl
- siNrf2
- siCtrl + atRA 70 µM

C

D

Viability (% of time 0) vs Time (h)

- siControl
- siNrf2
- atRA
- atRA + BSO
- atRA + preBSO

E

F

Mitochondrial membrane potential (JC-1 red/green emission ratio)

- DMSO
- atRA

** siNrf2

* atRA + preBSO
Figure 26. The protective role of Nrf2 and GSH in RA toxicity. A. HepG2 cells were treated with 50 nM siRNA against Nrf2 or mismatched control siRNA for 48 h to knockdown Nrf2 as described in the preceding experiments. Upon which, the cells were subjected to mild (30 µM) or strong (70 µM) toxic atRA challenge. Cell viability was measured with Alamar BlueTM assay at 4, 8, 24 and 48 h. Mean ± SEM of four determinations. Cell viability upon Nrf2 knockdown was similar to that treated with scrambled siRNA (not shown). B. Caspases activity of HepG2 cells treated with siCtr or siNrf2 (see above) followed by 8 h treatment with vehicle or 70 µM atRA. Mean ± SEM of four determinations. **p<0.01 from siCtr and atRA treatment by t-test. C. Agarose gel analysis showing incidents of DNA shearing/fragmentation (late-stage apoptosis indicator) with Nrf2-knockdown HepG2 cells treated with 70 µM atRA for 24 h. 20 µg/mL actinomycin D (ActD), an apoptotic inducer, was used as positive control. D. HepG2 cells treated with siCtr or siNrf2 followed by DMSO or 70 µM atRA for 18-20 h. Upon which mitochondrial membrane potential was assessed with JC-1 dye. Representative confocal images were shown. Bar scale is 10 µm. Red fluorescence (A560/595) indicates intact/normal membrane potential whereas that of green (A485/535 nM) showing loss of membrane potential. 2.5 µg/mL staurosporine, a mitochondrial toxin, was used as positive control (not shown). Representative confocal microscopic images of live cells were shown. E. Cell viability of HepG2 cells treated with GSH synthesis blocker (60 µM BSO) and atRA (30 µM), or pretreated with 60 µM BSO overnight before challenging with 30 µM atRA (preBSO). Values were first subtracted with individual treatment control (vehicle or BSO alone), and expressed as percent change to baseline/pretreatment values. Mean ± SEM of four determinations. F. HepG2 cells treated with control siRNA (siCtr) or siRNA against Nrf2 (siNrf2) followed by 16 h treatment with 70 µM atRA. Mitochondrial membrane potential of cells was then analyzed by JC-1 dye (please see methods for details), and the ratio of red (A560/595) to green (A485/535 nM) fluorescent emission was determined. Mean ± SEM of eight determinations (arbitrary unit ratio of red to green). * Significant difference from respective control (treated with vehicle DMSO) by t-test.
Figure 1. Induction of GCL subunits by RA is independent of RXR. A. mRNA of RXRα and RXRβ of HepG2 upon 48 h treatments with siRNA against RXRα (siRXRa) or both RXRα and β (siRXRαβ) compared with those treated with equal molar siRNA controls (siCtr). Mean ± SEM (n=3-4). B. Representative immunoblots of protein lysate (15 µg) of HepG2 probing for RXR after 48 h siRNA treatments. C. Basal (treated with vehicle) and inducible (treated with 50 µM atRA) expression of RXR target genes, i.e., CYP26A1 and RARβ (upper panel) and GCL subunits (lower panel) and after silencing of RXRα or both RXRα and β. Mean ± SEM (n=3 for GCL analysis; triplicate determinations for RXR target genes analysis). Similar results were observed for 9cRA treatment (not shown).
Supporting Figures

Figure II. Silencing of Nrf2 via siRNA in HepG2 and MCF7. mRNA levels of Nrf2 and other homologous Nrf subtypes after 48 h treatment with siRNA against Nrf2. Mean ± SEM (n=3-4). *Significant difference (p<0.05) between siCtr and siNrf2 by t-test.

Figure III. Silencing of Nrf2 in MCF7 reduced basal and inducible mRNA levels of GCLM and GCLC. mRNA levels of GCLM and GCLC upon 48 h treatment with siRNA against Nrf2 followed by 16-18 h treatment with 50 μM atRA. Mean ± SEM (n=3-4). *Significant difference (p<0.05) between siCtr and siNrf2 by t-test.
Figure IV. Contribution of mRNA stability effect in the upregulation of GCL subunits by retinoid treatment. atRA (50 µM)-induced mRNA stability of GCL subunits were analyzed using two global transcription inhibitors, actinomycin D (15 µg/mL; ACT D) and 5, 6-dichlorobenzimidazole riboside (DRB)(100 µM; DRB). A. The mRNA decay half-life (t1/2) for GCLM analyzed by ACT D: DMSO, 5.5 h; atRA, 15.1 h, and DRB: DMSO, 3.8 h; atRA, 9.5 h. B. The mRNA decay t1/2 for GCLC analyzed by ACTD: DMSO, 2.4 h; atRA, 3.2 h, and DRB: DMSO, 2.4 h, atRA, 4.4 h. Mean ± SEM (n=4).
CHAPTER 5.0

SUMMARY, GENERAL DISCUSSION, AND CONCLUSIONS
5.1 Bile Acids and Retinoids as Potent Nrf2 Activators

The negative outcomes of exposure to toxic bile acids (BAs) and retinoic acids (RAs) have been associated with increased oxidative stress. Because these compounds can interact with multiple cellular targets causing diverse effects, it was unclear to what extent oxidative stress contributes to their overall toxicities and, ultimately, irreversible cell death. In addition, whether there is a cellular antioxidant defense system possible for therapeutic or genetic manipulations to prevent and/or ameliorate their toxicities represents a novel question. My research project sought to examine whether the oxidative stress-responsive transcription factor, Nrf2 plays a crucial protective role against toxicities caused by BAs and RAs. And if so, what adaptive defense responses - critical genes that Nrf2 upregulates – through which cytoprotection is conferred. By knowing the mechanism by which cellular adaptive response is orchestrated toward particular toxicants, it will help develop effective treatment strategies and preventive measures in managing their toxicities.

Recent evidence has shown that Nrf2 activation and regulation of ARE-responsive genes does not necessarily require occurrence of cellular oxidative stress or redox changes (Cullinan et al., 2005). Also, not all circumstances of oxidative stress will result in activation of the Keap1-Nrf2-ARE transcription pathway (Kannan & Jaiswal, 2006). Therefore, my experiments began by examining if BAs (LCA and CDCA) and isomeric RAs (all-trans, 9-cis, and 13-cis) could activate Nrf2 and induce its target genes. LCA and CDCA were chosen because they are the most toxic among the primary and secondary bile acids, respectively. In particular, LCA is known to induce cholestatic liver injury in rodents, serving as a standard treatment protocol for experimental cholestasis (Hoffmann, 2004).

We show that bile acids and retinoic acids were able to increase a battery of antioxidant enzymes in a panel of cell lines, particularly hepatoma (HepG2, Hepa1c1c7) and colon adenocarcinoma C2bbe1 cells (Chapters 2-4). Among these enzymes, GCL subunits (GCLM and GCLC) and TRx1 rose above 4-fold in mRNA from the vehicle control at 24 h of treatment. We also discovered concurrent increases of an array of ABC efflux transporter genes (MRPs 1-5, MDR1 and BCRP) with BA treatments (Chapter 3). Particularly, MRP3 and MRP4 were the transporters with the highest induction (>4-fold). Treatment with the Nrf2 inducers, including BHQ and LA, produced similar patterns of gene induction, suggesting a common regulatory pathway (Chapters 2-4).
It was noted that the magnitude of mRNA induction for all these genes varied considerably, depending on the test chemical, length of treatment, doses, and the cell line under investigation. For bile acids, LCA appeared to be more potent than CDCA; on the other hand, atRA and 9cRA were stronger inducers than 13cRA. Some genes, such as GCLC and TRx1, were induced more profoundly at early treatment, e.g., 4 – 12 h, other genes, notably most MRPs, tended to surge at a later time (>12 h). Therefore, factors such as additional modes of regulation, different sets of cell signaling, positive and negative feedback loops, and requirements for specific coactivators/corepressors maybe at work for transactivation of individual genes. To address this, we found that that treatment-induced mRNA stability, a transcriptional independent mode of gene regulation, occurs to GCL subunits (Chapter 4) and MRP4 (Appendix I). Also, activation of the stress-related kinase pathways was also found to have specific roles in regulation of the above cytoprotective genes (details in next section)(Chapters 3&4).

To ensure that the effects observed are not only characteristic to carcinoma or immortalized cell lines, primary human hepatocytes were also tested (Chapters 2&4). Similar effects of RAs and BAs on antioxidant enzyme genes (GCL subunits, NQO1, TRx1) were evident. However, most ABC transporters, except for MRP3, found to be increased in the carcinoma cell lines were either unaffected or declined in expression in the primary hepatocytes (Appendix II). The disparity in treatment responsiveness between antioxidant enzymes and efflux transporters remains unresolved in the present study. The serum-free formulation of culture media for growth of the primary hepatocytes which contains enriched growth factors, cortisol and other steroids may possibly be influencing/confounding factors. Unlike antioxidant enzymes, ABC transporters are subject to regulation by multiple nuclear receptors, including PXR, CAR, VDR, LXR and FXR (Eloranta & Kullak-Ublick, 2005; Handschin & Meyer, 2005; Scotto, 2003), which are readily activated by steroidal hormones.

Utilizing siRNA targeting endogenous Nrf2 and overexpression assays in HepG2 cells, dependency of Nrf2 in upregulation of the antioxidant enzyme genes, including GCL subunits and TRx1, by RAs and BAs was confirmed (Chapters 2&4). Of many ABC transporters analyzed, Nrf2 is required for constitutive expression and BA-induced MRP2, MRP3 and BCRP (Chapter 3). Nevertheless, there were other transporters induced by BAs, such as MRP4, MRP5 and MDR1, which were not reduced by Nrf2 silencing. This suggests presence of additional stress-activated regulatory pathways in regulation of
these transporters in HepG2 cells. BAs or RAs also caused nuclear accumulation of Nrf2, a pre-requisite feature of receptor activation. They also stimulated activity of an ARE reporter construct transfected into HepG2 cells, and increased physical interaction of Nrf2 with the AREs located upstream of GCLM and GCLC genes in the chromatin context (Chapters 2&4).

Accumulation of nuclear Nrf2 in HepG2 cells exposed to BAs or RAs commenced as short as 1-3 h of treatment, implying that the activation of Nrf2 is considerably rapid and is unlikely a secondary event to activation of another transcription factor(s) which may in general require relatively longer time (> 4 h) to occur as seen with AhR (Miao et al., 2005). For instance, the bifunctional AhR ligands, such as 3-MC, can activate AhR to induce target genes (CYP1As) capable of metabolizing the ligands into reactive oxidants which may then activate Nrf2 (Prochaska & Talalay, 1988; Ma et al., 2004). Alternatively, AhR may as well transcriptionally induce Nrf2 (Miao et al., 2005), leading to increased availability of Nrf2 and hence constitutive levels of Nrf2 target genes.

To address physiological relevance of in vitro studies, we administered LCA and atRA at dose regimens commonly used in inducing cholestasis and teratogenicity, respectively (Chapters 2-4). We found comparable Nrf2 activation and induction of antioxidant enzyme genes in the liver of mice. Confirmatory data were also obtained from Nrf2−/− mice of the CD1/ICR strain (Itoh et al., 1997)(Chapter 3). However, a much more complicated regulation of cytoprotective genes was encountered in these animals, where length of treatment, sex, and possibly degree of liver cell injury are factors influencing the expression of cytoprotective genes. Overall, constitutive and LCA-induced gene expression of all antioxidant enzymes identified previously as Nrf2 target genes was reduced in Nrf2−/− mice compared with the wildtype. Further, LCA-induced Mrps 1-5 and Mdr1a/1b in mouse liver were found as well to be in dependence on Nrf2. Among these transporters, Mrp3 and Mrp4 which play a role in sinusoidal bile salt export (Eloranta & Kullak-Ublick, 2005), were significantly lower in both constitutive and inducible levels in the Nrf2 knockouts. These observations are in agreement with a recent report by Maher et al. (2007) using Nrf2−/− mice bred on the C57/BL6 strain (Chan et al., 1996). However, a lower constitutive Mrp4 with my studies was not noted in the former report. In addition, upon 4 d LCA treatment, Mdr1a/1b (p-glycoprotein), which was drastically increased to >10-fold in the wildtype mice, had only moderate inductions (1.5-2 fold) in the Nrf2 knockouts.
Because MRP2, MRP3 and BCRP were evident to be regulated by Nrf2 in human hepatoma HepG2 cells, species difference in regulation of ABC transporters may possibly exist. Given that the liver tissues sampled from mice contain not only hepatocytes, but also multiple cell types, including cholangiocytes and Kupffer cells, this may likely explain the discrepancy observed in the gene expression findings between HepG2 cells and \textit{in vivo} mouse liver. To address this, regulation of ABC transporters was examined in a mouse hepatoma cell line (Hepa1c1c7) with endogenous Nrf2 silenced by siRNA. With the exception of Mdr1a/1b and Mrp5, dependency of Nrf2 on LCA-induced ABC transporters (i.e., Mrps 1-4) observed in Hepa1c1c7 was consistent with those of Nrf2\textsuperscript{-/-} mice. Therefore, these studies appear to suggest species- and possibly cell type-specific regulation of ABC transporters by Nrf2. In summary, from both \textit{in vitro} and \textit{in vivo} studies, we demonstrated that Nrf2 is activated by BAs and RAs. Activation of the Nrf2 transcription machinery triggers an adaptive response which induces a battery of cytoprotective genes, particularly antioxidant enzymes and ABC transporters.

\textbf{5.2 Nrf2 Coordinates Adaptive Defense via Glutathione and ABC Proteins}

Further investigation was carried out to determine if Nrf2 activation is capable of executing biologically significant protection against BA- and RA-induced toxicity and cell death. Using RNAi technique, HepG2 cells with Nrf2 knockdown exhibited significantly decreased cell resistance with increased events of apoptosis and/or necrosis in response to treatments with toxic BAs or RAs (Chapters 2&4). Similar susceptibility was observed in Nrf2-knockdown C2bbe1 cells exposed to toxic LCA (Appendix III). Considering that LCA toxicity has carcinogenesis-promoting properties (Kozoni et al., 2000), these experiments suggest a possible protective role of colonic Nrf2 in colorectal cancer. In agreement, Nrf2\textsuperscript{-/-} mice were shown recently to develop more colonic inflammation and pre-neoplasia lesions induced by dextran sulfate than did the wildtype (Osburn et al., 2007).

The \textit{in vivo} role of Nrf2 in BA toxicity was further examined in Nrf2\textsuperscript{-/-} mice. Acute (8 h) and extended (4 d) administrations of LCA caused induction of multiple antioxidant enzyme and ABC transporters in the wildtype Nrf2\textsuperscript{+/+} mice. Such induction was markedly mitigated in the Nrf2 knockouts which corresponded to a phenotype of increased severity of liver injury and bile duct inflammation compared with that of the wildtype. It is believed that the \textit{in vivo} protective role of Nrf2 against LCA-induced liver injury maybe
accomplished by collaboration with various systems of cell survival proteins. For instance, cholestatic liver injury is manifest into sustained cellular inflammation in which activation of local inflammatory responses by the innate immunity, including the Kupffer cells, plays an important role in pathogenesis (Schoemaker et al., 2003). Nrf2 has been shown to modulate the inflammatory immunity by assisting with adaptive cell-survival mechanisms, such as NF-κB, in ameliorating inflammation-related injury (Rangasamy et al., 2004). Hence, crosstalk between the Nrf2 pathway and other systems of adaptive and anti-inflammatory systems may occur in a reciprocal manner in defense against bile acid-induced oxidative stress and pro-inflammatory signals that trigger cell death cascades.

The activation of apoptosis-initiating caspase enzymes following treatments with toxic RAs and BAs has hinted that these compounds may act as mitochondrial toxins (Chapters 2&4). Indeed, mitochondrial toxicity and its mediated apoptosis has been a well-documented toxicological effect of cholestatic BAs (Rolo & Palmeira, 2004). Declining membrane potential transition (MPT) of the mitochondria is an ultimate outcome of mitochondrial toxicity and possibly indicative of mitochondrial-mediated apoptosis. Using a non-toxic, sensitive fluorescent dye (JC-1) to quantitate mitochondrial MPT changes in live cells, Nrf2-knockdown HepG2 cells were found to experience significantly greater loss of MPT upon toxic atRA challenge. This analysis suggests that mitochondrial toxicity is a major underlying mechanism of RA-induced cell death. Although the role of Nrf2 in BA-induced mitochondrial toxicity was not examined in my studies, there is evidence that Nrf2 protects against mitochondrial injury caused by neurotoxins (Calkins et al., 2005).

In the present study, glutathione (GSH) was found to be particularly targeted by Nrf2 against insurgence of toxic BAs and RAs (Chapters 2&4). Exposure to these compounds resulted in increased cellular levels of GSH by > 3-fold over the vehicle control, which coincided with increased GCL subunits (GCLM and GCLC), the rate-limiting enzyme of GSH biosynthesis regulated by Nrf2. The essential role of these subunits in regulation of basal and inducible GSH has been established in respective gene knockout models (Dalton et al., 2000; Yang et al., 2002). In a series of cytotoxicity analyses, we showed the critical role of GCL subunits and GSH as key determinants in resisting BA and RA toxicities. BSO, which inhibits the activity of GCL subunits and leads to depletion of cellular GSH store, significantly increased cellular oxidative stress and susceptibility to toxic doses of LCA and atRA. By contrast, preserving GSH store and
replenishment by co-treatment with a GSH precursor NAC enhanced cell resistance against LCA toxicity. Other than GCL subunits, Nrf2 also directed increases of myriad enzymes important in GSH recycling and phase II GSH conjugation, such as GPx1 and GSTP1, in response to RA or BA treatment. Coordinate induction of these enzymes with GCL subunits by Nrf2 represents a primary defense mechanism against toxicities by excess BAs and RAs.

Glutathione (GSH) is the most abundant antioxidant molecule in the cell and constitutes the basic foundation of antioxidant defense. It preserves the cellular redox balance by maintaining and restoring reducing state, and conjugates thiol-attacking electrophiles to facilitate their excretion (Fernandez-Checa & Kaplowitz, 2005). GSH, particularly in the mitochondrial compartments, plays an essential anti-apoptotic role against cell death signals such as TNFα (Mari et al., 2008). The protective role of increased GSH in BA toxicity may thus involve strengthening resistance of mitochondria against resultant oxidative injury and induction of MPT, which can lead to apoptosis. It is noteworthy that a protective mechanism for the cholestatic drug UDCA is through promoting GSH via upregulation of GCLC (Mitsuyoshi et al., 1999). A main therapeutic target of UDCA is to reduce mitochondrial oxidative stress (Serviddio et al., 2004). In the present study, UDCA was also shown to moderately increase ARE-reporter activity, indicating an increased Nrf2 transactivation (Chapter 2). Therefore, enhancing cellular GSH by UDCA may operate through Nrf2 activation. It would be of interest to investigate whether UDCA can activate Nrf2 in vivo, and if so, how such alternative pathway of increasing moderate “oxidative stress” may protect the liver from BA toxicity.

Meanwhile, retinoid toxicity was found in the present study to be associated with increased production of 4-HNE (Chapter 4). This reactive aldehyde of lipid peroxidation can cause damage to multiple cellular components. GSH conjugation by GSTs is a major detoxification pathway of 4-HNE (Awasthi et al., 2005). Therefore, simultaneous increases in GCL subunits and GSTs by Nrf2 in various cell lines and mouse liver highlight a collaborative detoxification process against cellular 4-HNE accumulation. Moreover, similar to BA, mitochondrial toxicity has been identified in the present study to be a target of RAs. Hence, enhanced GSH with RA exposure may as well work toward mitochondrial protection.

ABC transporters were another focus in the present study because of their collective induction by BA treatments, and their important roles as active efflux pumps of
bile salts. The observations that efflux function of MRP1, MRP2, and MRP4 requires GSH, and that this function can be further enhanced by increasing GSH availability suggested an intimate collaboration between GSH and these transporters (Kruh & Belinsky, 2003; Deeley et al., 2006). Genetic defects of BSEP and MRP2 led to varying degrees of intrahepatic cholestasis (Strautnieks et al., 1997; Jansen et al., 1999; Wada et al., 1998), and targeted gene disruption of Mrp4 in mice had heightened sensitivity to BDL-induced liver injury (Mennone et al., 2006). Although MRP3 was previously shown to be a major bile salt transporter on the basolateral domains of hepatocytes and enterocytes (Bodo et al., 2003), Mrp3−/− mice did not seem to suffer more liver injury than the wildtype upon BDL procedure (Zelcer et al., 2005).

In the present study, LCA treatment induced an array of ABC transporters (MRPs 1-5, MDR1, and BCRP) in vitro and in vivo (Chapter 3). In HepG2 cells, the increased efflux transporters led to a reduced cellular retention/uptake of vinblastine and methotrexate. Reduced drug uptake as indicative of increased efflux activity was suggested by the observations that this effect was greatly inhibited by the ABC transporter inhibitors: cyclosporin A (CspA) and MK571. We also provided evidence for a cytoprotective role of these efflux transporters by showing inhibition of the efflux activity sensitized HepG2 cells to greater LCA toxicity. In addition, the increased LCA toxicity upon GSH depletion by BSO (Chapter 2) maybe, at least in part, explained by inhibition of the ABC transporters (MRPs 1, 2, and/or 4) requiring GSH for their function (Deeley et al., 2006). Nrf2 knockdown could inhibit cellular efflux of methotrexate, implying that Nrf2-dependent transporters (MRP2, MRP3, BCRP) are mainly involved in methotrexate efflux. It was also noticed that cellular efflux of methotrexate was more sensitive than that of vinblastine to inhibition by BAs such as LCA, CDCA and cholic acid. Hence, Nrf2-regulated ABC efflux transporters, which affect methotrexate transport, may play a more important role in BAs transport. This notion is supported by the bile transport role of MRP2, MRP3 and BCRP (Eloranta & Kullak-Ublick, 2005), and their relatively greater expression among other transporters in HepG2 cells (Chapter 3).

Our findings that Csp A can enhance LCA toxicity are contradicting its role as a mitochondrial MPT blocker and anti-apoptotic agent (Hatano et al., 2000) shown to be protective against toxicities by glycochenodeoxycholic acid and CDCA (Yerushalmi et al., 2001; Rolo et al., 2003). It is noteworthy that the doses of CspA needed for blocking ABC transporters are usually higher (≥10 µM) then those for blocking MPT (0.5-5 µM) (Choi,
2005; Hatana et al., 2000; Yerushalmi et al., 2001). The concentration of CspA used in our studies (10 µM) was able to significantly block cellular efflux transporters. It is possible that increased accumulation of toxic LCA consequent to this inhibition may have preponderant effects over CspA’s role in MPT protection.

To gain insight into the mechanisms of action by which BAs and RAs activate Nrf2 and transactivate ARE-regulated genes, various stress-responsive signaling kinases were analyzed (Chapters 3&4). We found that LCA and atRA activated identical stress kinases. Particularly, phosphoproteins of MEK1-ERK1/2 MAPK and c-Jun/JNK started to rise at 30-min and perpetuated through 6-8 h. Phosphoprotein AKT which is the major downstream kinase of PI3K was only modestly increased from 1.5 – 2 fold within 1 h of treatment. Activation of MEK1-ERK1/2 MAPK and, to a less extent, of PI3K-AKT, by LCA and RAs was shown in the present study to play a key mediator role of Nrf2 activation and induction of its target genes in HepG2 cells (Chapters 3&4). We additionally found that these stress pathways are also required for LCA induction of ABC transporter genes (MDR1, MRP1 and MRP4) which were shown to be independent of Nrf2 regulation. Blocking of MEK1-ERK1 MAPK with U0126, which abolished induction of cytoprotective genes, decreased cell resistance to LCA toxicity. In support of this, the requirements of ERK for Nrf2 translocation to nucleus and for ARE-regulated HO1 and GCLM have been described upon treatment with chemopreventive agents (Zipper & Mulcahy, 2003; Shen et al., 2004). Nonetheless, the precise mechanism by which ERK increases Nrf2 transactivation has not been certain. The cell survival-promoting role of this MAPK pathway is in agreement with a great deal of observations reported elsewhere (Dumaz & Marais, 2005; Juntttila et al., 2008).

Cell-type specific regulation of Nrf2 transactivation by the above stress-responsive kinases appears to occur. For example, both JNK and ERK were found to activate Nrf2 in the prostate cancer PC-3 cells (Xu et al., 2006A), whereas in the bronchial epithelial HBE1 cells, PKC-delta and PI3K, but not ERK, were found to take part in this mechanism (Zhang & Forman, 2008). It was also noted that activation of the above kinases by LCA and atRA occurred within 30 min the treatment and gradually returned to basal levels (t = 0) after ~6-18 h of exposure. Because the induction of cytoprotective genes was evident even at 48 h of treatment, involvement of other mechanisms may be at work to perpetuate gene induction at later periods of treatment. More studies are needed to address this.
Taken together, our data suggest that increased GSH and cellular efflux following induction of GCL subunits and subsets of ABC transporters, respectively, are critical adaptive defense mechanisms coordinated by Nrf2 to counteract BA and RA toxicities. The cell signaling pathway MEK1-ERK1/2 MAPK is primarily involved in Nrf2 activation and induction of antioxidant and ABC transporter genes by LCA and atRA. A simple schematic illustration of the mechanisms leading to activation of Nrf2 and induction of cytoprotective genes by BAs and RAs is depicted in Figure 27.

5.3 Outstanding Questions and Prospective Studies
The following are selective questions raised from the present studies that I think would be of merit for future investigations.

I. Does enhancement of Nrf2 provide preventive and treatment benefits to management of cholestasis?
In rodents, prior activation of PXR and CAR by administering respective agonists (PCN and TCPOBOP, respectively) has been shown to protect the animals from LCA-induced cholestatic liver injury (Staudinger et al., 2001; Xie et al., 2001). Similarly, protection against oxidative damage in various organs has also been noted with administration of chemopreventive agents/ Nrf2 inducers either before or during oxidant challenges (Xu et al., 2006B; Shih et al., 2005B). Hence, it would be of therapeutic importance to study whether such protection also occurs to cholestasis. By comparing effects between Nrf2−/− and wildtype mice, the question of whether chemopreventive agents exert protection via Nrf2 activation would be answered. On the other hand, the demonstration that UDCA enhances antioxidant defense (Serviddio et al., 2004) raises a question whether Nrf2 is a target of this drug in its action against cholestatic liver injury. All these questions will help identify potential therapeutic agents/strategies for the management of cholestasis and other oxidant-mediated diseases in the liver.

II. Does the cytoprotective role of increased ABC transporters by Nrf2 observed in HepG2 cells occur in vivo and in non-transformed normal hepatocytes?
Our data using HepG2 cells suggested that increased ABC transporters and hence cellular efflux is protective against bile acid toxicity. Concerning physiological relevance in
Figure 27. The mechanisms through which LCA and atRA induce survival kinase pathways and Nrf2 transactivation in HepG2. Excess bile acids/retinoids directly or indirectly activates cellular MEK1-ERK1/2 MAPK or, to a less extent, PI-3K/AKT via inducing cellular stress or production of ROS. Excess ROS and activation of the preceding kinase signaling destabilize Keap1/Nrf2 complex, leading to liberation of Nrf2 to the nucleus and/or increased stabilization of nuclear Nrf2 via decreased ubiquitin-proteosome degradation. Activated Nrf2 binds to its enhancer ARE to transactivate a battery of antioxidative genes and ABC transporters (MRP2, MRP3, BCRP) which play a role to combat bile acid-induced cell injury and cell death. MAPK may regulate Nrf2-mediated transcription by modulating Nrf2-interacting coactivators such as CBP, and regulate subsets of ABC transporters through a Nrf2-independent transcription machinery.
complex systems, verification with appropriate in vivo models is necessary. Because bile salts undergo extensive enterohepatic circulation and expression of similar ABC transporters is common in the GI tract and across many tissues, the hepatic protective role of individual transporters would be best addressed using models with hepatocyte-specific knockout. Moreover, a caveat facing the use of HepG2 cells is its transformed phenotype that has distinct differences than that of normal hepatocytes. HepG2 cells are deficient of many nuclear receptors, including CAR, VDR and FXR (unpublished observations), as well as many organic uptake transporters (Kullak-Ublick et al., 1996) that are present in normal hepatocytes. This may make HepG2 cells a model less than perfect for studies of steroid/bile acid detoxification. Nevertheless, the sufficient expression/function of Nrf2 and ABC efflux transporters in HepG2 cells would however allow studying specific interactions between Nrf2 and respective efflux system.

**III. Does MEK1-ERK1/2 MAPK activation of Nrf2 occur in vivo and in non-transformed normal hepatocytes?**

It is known that the stress-responsive kinases are hyperactive and maybe, to some degree, abnormally regulated in many cancer cells (Roberts & Der, 2007). It is unclear whether such abnormality also happens to HepG2 cells. Therefore, the cell signaling data from HepG2 should be further validated in healthy and normal hepatocytes. Additionally, it would be of importance to determine whether such kinase activation is also occurring in vivo.

**IV. How does MEK1-ERK1/2 MAPK orchestrate Nrf2 activation and induction of ARE-regulated genes?**

Although association of the MAPK activation and increased Nrf2 transactivation and ARE-regulated genes was first documented in 2000 (Yu et al., 2000; Zipper & Mucahy, 2000), the exact mechanism on how this interaction works is still largely unclear. It would also be interesting to examine whether the increased cytoprotective genes and hyperactivity of Nrf2 function in some cancer cells could be a result of an over-reacting MAPK pathway.

**V. Does Nrf2 protection against retinoid toxicity and teratogenicity occur in vivo?**
My study showed that administration of atRA at doses commonly used in teratogenicity/chemotherapeutic studies to mice causes nuclear accumulation of Nrf2 in their liver (Chapter 4). The in vivo protective role of Nrf2 activation in these toxicities could appropriately be addressed on Nrf2−/− mice. Considering that free radicals are known contributors to teratogenicity (Parman et al., 1999; Chen et al., 2004B), Nrf2−/− mice would represent a good model for studying the role of oxidative stress and antioxidant defense in the etiology of birth defects.

VI. Does active ARE(s) present in the gene promoter regions of transporters found to be dependent on Nrf2?
Although Maher and colleagues (2007) presented data from ChIP analysis that the gene promoter of mouse Mrp3 and Mrp4 contains potential AREs which can bind Nrf2, it is unclear whether these AREs are capable of gene transactivation. Moreover, there is currently no report showing active AREs present in any human MRP gene. To what extent can rodent data be extrapolated to situations in humans has raised appreciable concerns (Kensler et al., 2006).

VII. Does thioredoxin reductase 1 (TRx1) play a significant protective role in resisting bile acid and retinoid toxicities?
Significance of TRx enzymes and the thioredoxin system in defense against oxidative stress has caught prominent recognition (Arner & Hømgren, 2006). In my study, the main TRx enzyme, TRx1 was shown to be markedly induced by BA and RA in a Nrf2-dependent manner (Chapters 2-4). In mouse studies, acute (8 h) administration of LCA was implicated in >20 fold induction of hepatic TRx1 mRNA, suggesting its possible critical role in early phase of toxicity (Chapters 2&3). TRx1 also plays a key role in adaptive response in 4-HNE signaling and resultant oxidative damage (Chen et al., 2005A). Hence, it is of importance to determine whether TRx1 also occupies as a critical role as GCL subunits in the defense against BA and RA toxicities.

VIII. Do sex-specific factors play a role in gene regulation of Nrf2?
Sex dimorphism in regulation of target genes by nuclear receptors such as PXR and LXR (Uppal et al., 2004 & 2007) has been reported. In the present study, a number of Nrf2 target genes were also found to be differentially regulated between sexes (Chapter 3).
Aged female Nrf2−/− mice have higher risks of developing autoimmune disorders than their wildtype counterparts or the male mice (Li et al., 2004A; Ma et al., 2006). These observations stress the possible influence of sex-specific factors in Nrf2 function and oxidative stress defense.

5.4 Conclusions
This thesis has provided a number of important findings:

1. Toxic bile acids and retinoic acids can activate an array of cytoprotective genes in a panel of liver cells; among which are antioxidant enzymes, such as GCL subunits (GCLM and GCLC) and TRx1, and a subset of ABC transporters, i.e., MRPs 1-5, MDR1 and BCRP. In HepG2 cells, the increased GCL subunits and ABC transporters are implicated in increased GSH and efflux activity, respectively.

2. Constitutive expression and induction of antioxidant proteins (GCL subunits, TRx1, GSTs, HO-1, NQO1, Nrf2, and/or FRL) by bile acids and retinoic acids are dependent on Nrf2. These observations apply to human and mouse liver cells, as well as the in vivo mouse liver. LCA induction of ABC transporters (MRP2, MRP3, BCRP) in HepG2 cells requires Nrf2. In in vivo mouse livers and mouse hepatoma Hepa1c1c7, Nrf2-dependent regulation of Mrp1, Mrp2, Mrp3, and Mrp4 are evident. Species-specific regulation of ABC transporter is suggested.

3. Toxic bile acids and retinoic acids can activate the Nrf2 transcription machinery, as evidenced by increased nuclear accumulation of Nrf2, ARE-enhancer activity, and physical binding of Nrf2 to their cognate ARE enhancer in HepG2 cells.

4. Nrf2 activation in HepG2 cells leads to protection against bile acid and retinoic acid toxicities, preventing resultant oxidative stress-induced apoptotic cell death. In vivo protection by Nrf2 against LCA-induced liver injury is also evident.

5. Basal and inducible GSH are critical resisting factors promoting cell survival during bile acid and retinoid toxicities in HepG2 cells. Increased cellular efflux activity following induction of ABC transporters is also crucial in counteracting bile acid toxicity in HepG2 cells.

6. Bile acids and retinoic acids activate similar stress-responsive kinases such as MEK1-ERK1/2 MAPK, c-Jun/JNK, and PI3K/AKT in HepG2 cells. Activation of MEK1-ERK1/2 MAPK in HepG2 cells primarily mediates Nrf2 activation and induction of
7. Production of increased 4-HNE, a reactive byproduct of lipid peroxidation capable of activating Nrf2, was found with exposure to retinoic acids in HepG2 cells.

8. In HepG2 cells, increased GCLM and GCLC by retinoic acids may also be mediated through increased mRNA stability.

In summary, this project suggests the potential of Nrf2 as a therapeutic target in the management of bile acid and retinoid toxicities.
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LIST OF PUBLICATIONS AND ABSTRACTS

Article published:

- Tan KP, Yang M, and Ito S. Activation of Nrf2 by toxic bile acids provokes adaptive defense responses to enhance cell survival at the emergence of oxidative stress. *Molecular Pharmacology* 72(5): 1380-1390; 2007

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Appendices
Appendix I

Increased mRNA stability contributes to increased MRP4 mRNA upon treatment with antioxidants/prooxidants. DRB (150 µM) was used as global transcription inhibitor. mRNA decay half-life (t1/2) of MRP4 of HepG2 treated with atRA (50 µM) or vehicle DMSO was determined and compared (see methods for details). Mean ± SEM (n = 4).
Primary human hepatocytes were treated with 70 μM LCA, 100 μM CDCA, 50 μM atRA, 75 μM BHQ or vehicle DMSO for 20-22 h and mRNA of ABC transporters were analyzed by real-time RT-PCR. Fold change vs. vehicle control. Mean ± SEM (n = 3-4).

Gene expression of ABC transporters in primary human hepatocytes was variably influenced by bile acids, retinoids and antioxidants.
**Appendix III**

Nrf2-silenced C2bbe1 cells were vulnerable to LCA toxicity. C2bbe1 cells were treated with siRNA against Nrf2 (siNrf2) or mismatched siRNA control (siCtr) for 48h followed by 100 uM LCA treatment over 24 h. Cell viability at baseline (t=0) and at 4, 8, and 24 h were measured using Alamar Blue assay. Mean ± SEM of 4 determinations.
Activation of Nuclear Factor (Erythroid-2 Like) Factor 2 by Toxic Bile Acids Provokes Adaptive Defense Responses to Enhance Cell Survival at the Emergence of Oxidative Stress

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ABSTRACT

Oxidative stress, causing necrotic and apoptotic cell death, is associated with bile acid toxicity. Using liver (HepG2, Hepa1c1c7, and primary human hepatocytes) and intestinal (C2bbe1, a Caco-2 subclone) cells, we demonstrated that toxic bile acids, such as lithocholic acid (LCA) and chenodeoxycholic acid, induced the nuclear factor (erythroid-2 like) factor 2 (Nrf2) target genes, especially the rate-limiting enzyme in glutathione (GSH) biosynthesis [glutamate cysteine ligase modulatory subunit (GCLM) and glutamate cysteine ligase catalytic subunit (GCLC)] and thioredoxin reductase 1. Nrf2 activation and induction of Nrf2 target genes were also evident in vivo in the liver of CD-1 mice treated 7 to 8 h or 4 days with LCA. Silencing of Nrf2 via small-interfering RNA suppressed basal and bile acid-induced mRNA levels of the above-mentioned genes. Consistent with this, overexpression of Nrf2 enhanced, but dominant-negative Nrf2 attenuated, Nrf2 target gene induction by bile acids. The activation of Nrf2-antioxidant responsive element (ARE) transcription machinery by bile acids was confirmed by increased nuclear accumulation of Nrf2, enhanced ARE-reporter activity, and increased Nrf2 binding to ARE. It is noteworthy that Nrf2 silencing increased cell susceptibility to LCA toxicity, as evidenced by reduced cell viability and increased necrosis and apoptosis. Concomitant with GCLC/GCLM induction, cellular GSH was significantly increased in bile acid-treated cells. Cotreatment with N-acetyl-l-cysteine, a GSH precursor, ameliorated LCA toxicity, whereas cotreatment with buthionine sulfoximine, a GSH synthesis blocker, exacerbated it. In summary, this study provides molecular evidence linking bile acid toxicity to oxidative stress. Nrf2 is centrally involved in counteracting such oxidative stress by enhancing adaptive antioxidative response, particularly GSH biosynthesis, and hence cell survival.

Exposure to excessive bile acids is toxic to the cells, contributing an etiopathological factor to a number of liver and intestinal diseases such as cholestasis and colorectal cancer (Rao et al., 2001; Debruyne et al., 2002). Among the bile acids, lithocholic acid (LCA), a hydrophobic secondary bile acid produced by colonic microflora on chenodeoxycholic acid (CDCA), is the most toxic bile acid, with genotoxic and mutagenesis-enhancing properties (Kawalek et al., 1983; Kozoni et al., 2000). In rodents, it induces intrahepatic cholestasis-like hepatotoxicity (Staudinger et al., 2001), and it promotes chemical-induced colon carcinogenesis (Kozoni et al., 2000). CDCA, the most hydrophobic primary bile acid, is able to cause severe liver injury in species such as rhesus monkey, and it causes mild hepatotoxicity in humans; its chronic administration results in increased colonic production of LCA (Hofmann, 2004).

ABBREVIATIONS: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; FXR, farnesoid X receptor; VDR, vitamin D receptor (VDR); PXR, pregnane X receptor; ROS, reactive oxygen species; Nrf2, nuclear factor (erythroid-2 like) factor 2; ARE, antioxidant-responsive element; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modulatory subunit; GSH, glutathione; MEM, minimal essential medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; TBL, total bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PCR, polymerase chain reaction; NOQ1, NAD(P)H quinone oxidoreductase; TRx1, thioredoxin reductase 1; siRNA, small interfering RNA; siNrf2, small interfering against Nrf2; siCtrl, small interfering RNA control; BHQ, tert-butylhydroquinone; NTCP, Na(+)-dependent taurocholate cotransporting polypeptide; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; ChlP, chromatin immunoprecipitation; bp, base pair(s); LDH, lactate dehydrogenase; MES, 2-(N-morpholino)ethanesulfonic acid; ANOVA, analysis of variance; DCA, deoxycholic acid; GST, glutathione transferase; UDCA, ursodeoxycholic acid; CA, cholic acid; BSO, buthionine sulfoximine; ABC, ATP-binding cassette; NAC, N-acetyl-l-cysteine.
The integrity and coordination of efficient hepatic bile flow and intestinal bile extraction are hence critical for species survival. The liver and intestinal cells achieve this through a concerted network involving the nuclear transcription factors, such as farnesoid X receptor (FXR), vitamin D receptor (VDR), retinoid X receptor, liver X receptor, pregnane X receptor (PXR), and/or constitutive androstane receptor. These receptors regulate bile-metabolizing and conjugation enzymes and bile transporters to prevent excessive accumulation of bile acids (Eloranta and Kullak-Ublick, 2005). Bile acids are regarded as signaling molecules that facilitate synchronization of the above-mentioned regulators in their handling of cellular bile fate. The cross-talk among these receptors is important in maintaining homeostasis of physiological bile extraction, constituting the baseline protection against bile acid toxicity.

Meanwhile, increased cellular production of reactive oxygen species (ROS) and oxidative stress has been implicated in exposure to toxicological concentrations of bile acids. Bile acid-induced oxidative stress results from induction of membrane permeability transition consequent to mitochondrial toxicity and activation of death receptors (CD95), which subsequently lead to apoptosis, via activation of proapoptotic effectors caspases, and necrosis (Palmeira and Rolo, 2004). Whether there exists any regulator to counteract such oxidative stress and the progression of bile acid toxicity is presently unknown. Because of its important role as an oxidative stress sensor and antiapoptosis factor, we hypothesized that the nuclear factor (erythroid 2-like) factor 2 (Nrf2) may play a central role by enhancing adaptive response and cell survival during exposure to excess bile acids.

Nrf2, a basic leucine zipper transcription factor that binds to antioxidant responsive element (ARE), is a chief regulator for many antioxidative, cytoprotective genes (Kensler et al., 2007). Among Nrf2 target genes, the glutamate cysteine ligase (GCL), composed of modulatory (GCLM) and catalytic (GCLC) subunits, is the rate-limiting enzyme for cellular biosynthesis of glutathione (GSH), an important intracellular antioxidant in preserving redox balances. Emerging studies have shown that Nrf2 is a multiorgan protector against various toxic reactive insults; among others are chemical carcinogens (Ramos-Gomez et al., 2001) and acetaminophen (Chan et al., 2001). Hence, robust Nrf2 activation in the cell may be a critical adaptive response to overcome oxidative stress-induced disease processes. However, Nrf2 activation is not merely a cellular response to all circumstances of oxidative stress, because exposure to some oxidative stress inducers such as high-dose UVB ray would in turn result in Nrf2 deactivation (Kannan and Jaiswal, 2006). Presently, it is not known whether toxic bile acids could activate Nrf2.

In this study, we combined in vitro and in vivo approaches to demonstrate that Nrf2 is activated by cytotoxic bile acids, thereby inducing genes, such as GCL and hence GSH biosynthesis, to protect the cells against bile acid toxicity.

Materials and Methods

Cell Culture and Chemicals. The human hepatoma-derived HepG2 (American Type Culture Collection, Manassas, VA) and mouse hepatoma-derived Hepa1c1c7 (a gift from Dr. Patricia Harper, The Hospital for Sick Children, Toronto, ON, Canada) were maintained in α-MEM with 10% fetal bovine serum (FBS). C2bbe1, a subclone of colon carcinoma Caco-2 that displays a more homogeneous brush-border epithelial-like morphology (American Type Culture Collection), was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 1.5 g/l sodium bicarbonate, and 10 μg/ml holo-transferrin. The human primary hepatocytes were purchased from Celprogen (San Pedro, CA), and they were grown in specially formulated serum-free growth media (Celprogen). Experiments of all secondary cell lines were conducted within 10 cell passages. Treatments were given at ~80% confluence for all cell lines except for C2bbe1. Because C2bbe1 cells differentiate to mature colonocytes at confluence, treatments to this cell line were given 2 to 3 days after confluence. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Test bile acids were dissolved in dimethyl sulfoxide (DMSO) [0.2% (v/v)]. Oligonucleotides were synthesized at the Toronto Centre for Applied Genomics (Toronto, ON, Canada) or Integrated DNA Technologies, Inc. (Coralville, IA).

In Vivo Mouse Experiments. The animal care and experimental procedures were approved by the Animal Care Committee at the University of Toronto and the Hospital for Sick Children (Toronto, ON, Canada). To examine whether Nrf2 target genes may have been modulated during acute exposure to LCA before the onset of symptomatic liver injury, 9- to 12-week-old CD-1 mice (Charles River Canada, Montreal, QC, Canada) were injected i.p. with a single dose of LCA at 125 mg/kg b.wt. dissolved in sterilized DMSO (final amount, ~1% b.wt.). Mice were killed 7 to 8 h after the treatment. In a separate experiment aiming to investigate changes in similar genes upon extended treatment with LCA, mice were injected the same dose of LCA dissolved in sterilized corn oil (final amount ~2% b.wt.) twice daily for 4 days. This treatment protocol has been used in the past to induce cholestatic liver injury in mice (Staudinger et al., 2001). Mice were killed 16 h after the last dosing. The use of corn oil as a solvent for LCA in the extended treatment protocol was to avoid the possible toxicity with chronic exposure to DMSO. At necropsy, portions of their livers were sampled in RNAlater reagent (Invitrogen, Carlsbad, CA) and neutral-buffered 10% formalin for histopathology and histological analyses, respectively. Nuclear protein extraction of chilled livers was carried out using Nuclear Extraction kit (Panomics, Fremont, CA). Sera of mice were collected for analysis of liver function/innocuity markers: total bilirubin (TBL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase using established automated methods (Department of Pediatric Laboratory Medicine, The Hospital for Sick Children).

cDNA Synthesis and Quantitative Reverse-Transcription Polymerase Chain Reaction. Total RNA was isolated with RNeasy kit (QIAGEN, Valencia, CA) and reverse-transcribed into cDNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Aliquots of cDNA equivalent to 100 ng of RNA were used for real-time PCR performed on Applied Biosystems (Foster City, CA) 7500 Real-Time PCR system or Prism 7700 Sequence Detection system with reaction mode set at 50°C for 2 min, 95°C for 20 s, followed by 40 cycles of 95°C for 15 s and 56 or 60°C for 1 min. The primers for ribosomal 18S, β-actin, tata-box binding protein, glyceraldehyde-3-phosphate dehydrogenase, GCLM, GCLC, and NADP/H quinone oxidoreductase 1 (NQO1) were purchased from predesigned and preoptimized Taqman primer-probe sets (Assay-on-Demand Gene Expression probe; Applied Biosystems), whereas custom-made primers for SYBR Green real-time PCR detection were used for the other gene transcripts (primer sequences available upon request). To ensure specificity, primer pairs were designed to span across two neighboring exons and detection of a single peak in dissociation curve analysis. The ΔΔC method (Livak and Schmittgen, 2001) was used to quantify the amplification-fold difference between treatment and vehicle-treated control groups, with the Ct value of target genes being adjusted to individual housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, β-actin, tata-box binding protein, and/or 18S).
whichever expression was not affected by treatment protocols. Measurements were done in duplicate or triplicate with variability <0.5 C.

**Immunoblotting.** Whole cell lysate was prepared in radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Ten to 30 µg of protein was dissolved in 4 to 12% bis-tris gel (NuPage Novex gel system; Invitrogen) and then transferred onto a nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Primary antibodies (working concentration) used were as follows: rabbit polyclonal anti-GCLM antibody-1 (1:2000) (NeoMarkers, Fremont, CA), rabbit antiserum against GCLM (1:3000) (custom-made; Alpha Diagnostics, San Antonio, TX; see below), rabbit polyclonal anti-Nrf2 c-20 (1:750) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-thioredoxin reductase 1 (TrxR1) (1:3000) (Abcam Inc., Cambridge, MA), mouse monoclonal anti-β-actin (1:10,000) (Sigma-Aldrich), and goat polyclonal anti-lamin B c-20 (1:200) (Santa Cruz Biotechnology, Inc.). Based on analyses of hydrophilicity, antigenicity, accessibility, and sequence homology with other related proteins, an antisera against a peptide (amino acids 29–45) of human GCLM was raised in rabbits. The immunogenicity and specificity were checked by enzyme-linked immunosorbent assay, and its ability to detect an ~28-kDa protein (predicted size of GCLM) with reactivity halted after preabsorption of the antibody in excess immunogen. To ensure equal loading for whole cell lysate and nuclear protein, β-actin and lamin B, respectively, were probed on the same stripped blot membranes after being used for detecting target proteins.

**RNA Interference.** A combo of four gene-specific small-interfering RNAs (siRNA) against human Nrf2 (NM_006164) was used (DHarmaco SMARTpoolsirRNA reagent; DHarmaco RNA Technologies, Lafayette, CO). Overnight-seeded HepG2 and C2bbE1 cells at ~40 and ~60% confluence, respectively, were transfected for 48 h with 50 nM siRNA against Nrf2 (siNrf2) or equal molar mismatched siRNA controls (siCtr). These siRNAs were earlier complexed with liposome carrier Dharmafect I (DHarmaco RNA Technologies) at 0.2 µL/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Under this condition, the transfected cells after 48 h looked normal morphologically, and they did not differ from untransfected cells in cell viability and mRNA levels of inflammatory marker interleukin-6 (data not shown). Treatments with bile acids were then followed for 16 to 18 h. To ensure achieving functional and specific silencing, the mRNA levels of Nrf2, known Nrf2 target genes, and homologous subtypes Nrf1 and Nrf3 were compared between siNrf2 and siCtr groups before and after treatments in all experiments.

**Plasmid Constructs.** The expression vectors for Nrf2 (pEF-Nrf2), dominant-negative Nrf2 (pEF_DNnrf2), and empty vector (pEF) were kindly provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). To make an ARE-reporter construct (pGL3 ARE), a DNA duplex (CGGGGATCCGGGAGGCAGCTGAGTCAGCGGAGGCGATCTCCC) (core ARE is underlined: ~3595/–3625 of hGCLC gene) containing the indispensable ARE motif of GCLC (~3604/–3614) (Mulcay et al., 1997) with KpnI/BglII at 5’ and 3’ ends, respectively, was constructed by annealing two polycrylamide gel electrophoresis-purified complementary oligonucleotides. This insert was ligated to similar restriction enzyme sites of pGL3 luciferase reporter plasmid with simian virus 40 promoter (Promega, Madison, WI). A similar reporter construct has been successfully used previously (Mulcay et al., 1997). The responsiveness and robustness of our ARE reporter to Nrf2 transactivation was confirmed by testing of a panel of Nrf2 activators such as tert-butyldihydroquinone (BHQ), liplid acid, and diethy maleate (data not shown), as well as cotransfection with Nrf2 and dominant-negative Nrf2 expression vectors. To ensure specificity, a mutant ARE reporter construct (pGL3_mARE) introducing three point mutations on ARE was cloned by PCR-mediated site-directed mutagenesis using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with complementary primers 5’-AGCaTGAGCAGGGAGGCGGACG-3’ (underlined sequence is core ARE, lowercase are mutated nucleotides) on the template pGL3 ARE. The PCR condition was 95°C for 5 min followed by 22 cycles of 95°C for 15 s, 55°C for 30 s, 68°C for 10 min, and a final extension of 68°C for 10 min. The template was then digested by DpnI, and mutant clones were transformed in XL-1 blue competent cells (Stratagene). Successful insertion and mutation introduction were confirmed by sequencing. The cDNA clone of human Nais- (alpha)taurocholate cotransporting polypeptide (NTCP) (OriGene Technologies, Rockville, MD) was subcloned into the NotI site of pTarget expression vector (Promega), and it was stably transfected into HepG2. Stable clones transfected with NTCP or empty vector (pTarget) were selected using 500 µg/ml G-418–supplemented growth media.

**Transfection, Reporter Assays, and Overexpression Studies.** HepG2 cells at ~50% confluence were transiently transfected overnight with 0.1 µg of the firefly luciferase reporter pGL3 ARE or pGL3 mARE, 0.02 µg of the Renilla reniformis luciferase control reporter pRL-TK with or without cotransfection with 0.2 µg of expression vectors using Lipofectamine 2000 (Invitrogen) as transfection carrier. Treatments with bile acids were then carried out for another 16 to 18 h in all experiments, unless otherwise stated. Conjugated bile acid treatments (glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA)) were done on NTCP-transfected HepG2. Luciferase activities of the cell extracts were determined with the Dual-Luciferase Reporter Assay system (Promega). Relative luciferase activity (relative light unit) was calculated from firefly luciferase values normalized to those of the control R. reniformis luciferase, and activity is expressed as ratios to vehicle-treated empty pGL3 promoter construct, and, if any, cotransfected expression vector. All experiments were done in triplicate, and they were repeated at least twice. For overexpression studies, Hep1c17 cells at 50% confluence in T25 flasks were transfected with 3 µg of Nrf2 or dominant-negative Nrf2 expression vector for 24 h, followed by treatments with bile acids for another 20 to 22 h.

**Quantitative Chromatin Immunoprecipitation.** The assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) with slight modifications. After 6 h of treatments, chromatin protein-DNA of HepG2 cells was fixed (cross-linked) in neutral-buffered 1% formaldehyde at room temperature for 10 min. Further fixation was stopped by 125 mM glycine buffer. The DNA was sheared by sonication on ice into fragments of ~500 bp. An aliquot (one fourth) of sample supernatant was saved as input DNA for later PCR analysis. After preclearing with protein A agarose beads, supernatants were incubated with a ChIP-graded anti-Nrf2 antibody (1:250; Santa Cruz Biotechnology, Inc.) in rotation at 4°C overnight. To control for non-specific binding of antibody used, an equal amount of the host antibody against an irrelevant protein (rabbit polyclonal anti-CYP1A1) from the same manufacturer (Santa Cruz Biotechnology, Inc.) was included in a separate batch of control supernatants and followed through the remaining protocols. Antibody-chromatin complexes were collected by salmon sperm DNA/protein A beads. DNA was released from cross-linked complexes with proteinase K at 65°C for 4 h followed by 72°C for 10 min. DNA was then extracted and eluted with 120 µl of Tris, pH 8.0, buffer using the DNeasy kit (QIAGEN), and the contaminant RNA was cleaved with RNase A (Invitrogen). For detection of the ARE of GCLC (~56/–66) (Erickson et al., 2002) and of GCLC (~3604/–3614) (Mulcay et al., 1997) by real-time PCR, the primer sets and Taqman probe (5’-carboxfluo- rescein, 3’-5-carboxytetramethylrhodamine) were designed by PrimerQuest software (Integrated DNA Technologies, Inc.), which amplify 5’-region exactly on the core ARE. The primers for detecting the ARE of GCLC were as follows: sense, 5’-C CGGGGATGATGTAAGTGTGA1CGGTG3; antisense, 5’-GGGAGAGCCTGATCCAAACTCTG3; and probe, 5’-ACGGAAGACCTTTTCTCGGATCACG3, which amplify a 79 bp product (~33/–112). For probing the ARE of GCLC, the primers were used sense, 5’-GGGACTGAGTCCTGACGACAG3; antisense, 5’-GGCAGTCTGTGTGATACTG3; and probe, 5’-CCACAAAGGCTGATGTCG3, which amplify a 160-bp product (~3479/–3609). Quantitation of Nrf2 bound to these AREs after the fixation treatments was carried out on 5% of DNA eluates with qPCR anal-
ysis similar to that for the mRNA, except that the C<sub>t</sub> value of amplicon from each sample's input DNA was used as normalization control as described previously (Beresford and Boss, 2001).

Cytotoxicity, Necrosis, and Apoptosis. For cytotoxicity analysis, a nontoxic assay, namely, Alamar Blue (BioSource, Nivelles, Belgium), was used. This assay uses a fluorometric indicator to measure the chemical reduction of cell medium, which correlates directly to the metabolic activity of viable cells. The working assay medium [10% (v/v) Alamar Blue in α-MEM, 2% PBS, and 1% penicillin/streptomycin, 37°C] was first incubated with cells seeded on 24-well culture plate before treatment to obtain baseline/pretreatment values. The measurement was made at excitation/emission/cut-off λ = 540/590/570 nm after 1 h of incubation with the assay medium at 37°C. Immediately after the measurement, the cells were rinsed with prewarmed PBS followed by the treatments. At various time points, treatment media were replaced with fresh assay media to allow for a continuous monitoring of cell viability. The fluorescent unit of each treatment and control was expressed as percentage of change relative to individual baseline/pretreatment value.

To determine necrosis, cellular release of lactate dehydrogenase (LDH) into treatment media was measured with an LDH detection kit (Roche Applied Science). To control for cell mass and spontaneous release of LDH by viable cells into media, the ratio of LDH activity in the medium to the cells (cell lysate) was determined and then subtracted from those of the vehicle-treated controls. The measurement was made colorimetrically at λ = 490 nm. The intra-assay variability of duplicate determinations was 2.2.

To assess apoptosis, the cellular caspases activity was measured using the rhodamine 110-conjugated substrate N-benzyloxycarbonyl-Asp-Glu-Val-Asp (Invitrogen). Although traditionally known to detect caspase-3 activity, recent analysis by the manufacturer showed that this substrate is also a target of multiple caspases such as 6, 7, 8, and 10. The caspases activity of cell lysates was quantitated at excitation/emission λ = 496/520 nm, and it was normalized to individual protein concentration measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Total GSH Quantitation. Cellular GSH was quantitated with a GSH assay kit (Cayman Chemical, Ann Arbor, MI) based on an established GSH recycling enzymatic method (Tietze, 1969). After 24-h treatment with bile acids, HepG2 cells were lysed in ice-cold MES buffer after a quick freeze-thaw cycle and then deproteinized by 0.5 g/ml metaphosphoric acid. An aliquot of each sample was saved before deproteinization for determining protein content. The total GSH of deproteinized cell supernatants was measured against an oxidized glutathione standard curve according to the manufacturer’s instruction. The measurement unit was expressed as nanomoles per milligram of protein per minute. The intra-assay variability of duplicate determinations for all samples repeated in four experiments was 3.1.

Statistical Analysis. Statistical tests were conducted using SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA) or SPSS 10.1 (SPSS Inc., Chicago, IL). Normality and equal variance tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA (parametric) or one-way ANOVA on ranks (nonparametric). Once statistical significance was attained (p < 0.05), the Dunnett’s (parametric) or Dunn’s (nonparametric) test comparing between treatment and control groups was initiated. Comparisons between two groups on single variable were accomplished by Student’s independent t test (parametric) or Mann-Whitney U test (nonparametric). Difference with p < 0.05 was considered statistically significant.

Results

Induction of Nrf2 Genes by Bile Acids in Liver and Intestinal Cells. A dose-response increase in mRNA of GCLM and GCLC after LCA and/or CDCA treatment was noted for HepG2 and C2bbe1 cells, with a significant >4-fold induction at ≥50 μM LCA and ≥100 μM CDCA (Fig. 1a). Peak inductions of GCL subunit genes occurred at 50 to 75 μM LCA and at 100 to 150 μM CDCA. Increased bile acid concentrations, i.e., LCA ≥100 μM or CDCA ≥250 μM, resulted in markedly cell death and reduced inductions of GCL genes at 24 h of treatment (data not shown). Significantly higher GCL gene transcripts, although at a lower magnitude (2–4-fold induction), were also noticed for the primary human hepatocytes treated with both bile acids (Fig. 1a). So were similar treatments given to the mouse hepatoma Hepa1c1c7 (data not shown). In C2bbe1 cells, CDCA treatment (100 μM) caused a modest increase in GCL genes (~2-fold), whereas treatment with deoxycholic acid (DCA) (~100 μM), a secondary bile acid often associated with toxicity and carcinogenesis in colonic cells, resulted in comparable GCL inductions to those of LCA treatment (data not shown).

Furthermore, mRNA of Nrf2 and a panel of known Nrf2 target genes such as NQO1, TRx1, ferritin light subunit, and heme oxygenase 1 were also simultaneously induced ≥2-fold by bile acids in all test cells (Fig. 1b). Particularly, TRx1, an important seleno-enzyme in cellular thiol and redox maintenance, was increased >4-fold in HepG2 and C2bbe1. Note that glutathione transferase P1, which was induced by bile acids in HepG2 and C2bbe1, was not evident in primary hepatocytes (Fig. 1b). Instead, another GST subtype, glutathione transferase A, was increased by bile acids to ~2-fold in primary hepatocytes (data not shown). This disparity suggests possible cell type specificity in regulation of GSTs by bile acids.

Increased protein levels corresponding to mRNA induction were also noted (Fig. 1c). The apoptosis marker (caspases activity) and cell viability analyses showed that a mild toxicity began to occur in HepG2 cells at 60 to 80 μM LCA treatment, followed by a precipitous increase in cell death and caspase activity at >80 μM (Fig. 1d). It is noteworthy that the induction of GCL subunits and other antioxidative genes peaked in the range of LCA (60–80 μM) during which HepG2 began to experience mild toxicity. These findings suggest that induction of the cytoprotective genes may represent an adaptive cell defense mechanism against LCA toxicity.

In Vivo Activation of Nrf2 Target Genes. Acute administration (7–8 h) of LCA to mice at a dose known to induce cholestatic liver injury (Staudinger et al., 2001) resulted in Nrf2 accumulation in the nuclei, a signature event of Nrf2 activation (Fig. 2a). This phenomenon coincided with significant inductions of Nrf2 target genes (Fig. 2b, top) found to be increased in the in vitro studies. It is noteworthy that the increase of Trx1 transcripts rose to ~50-fold at acute exposure to LCA, implying a possible critical role of this enzyme in early toxicity of LCA. At this shorter exposure to LCA, however, analysis of serum liver function and cholestatic markers (ALT, AST, γ-glutamyl transferase, and TBL) as well as liver histology did not indicate liver dysfunction or pathological changes (data not shown).

With prolonged LCA treatment during which symptomatic liver injury (elevated ALT, AST, and TBL, and liver necrosis in histological analysis; data not shown) already occurred, induction of Nrf2 target genes, such as GCL subunit gene transcripts, was found to sustain compared with those treated acutely with LCA (Fig. 2b, bottom). Nqo1 was in-
increased with prolonged treatment, whereas TRx1 induction was subdued. Consistent with the observations from primary human hepatocytes, α class of mouse Gst (Gsta1/2), rather than glutathione transferase P1, was found to be induced by LCA, with ~10-fold induction at 4 days of treatment (Fig. 2b, bottom). Treatment with vehicle alone did not differ in mRNA of genes under study compared with untreated animals (data not shown).

**Involvement of Nrf2 and Activation of Nrf2-ARE Transcription Machinery.** To examine whether Nrf2 participated in the preceding gene activations, we silenced Nrf2 of HepG2 and C2bbe1 via siRNA. This resulted in significant reductions of >60% in Nrf2 mRNA and protein without interfering with other homologous Nrf subtypes (Fig. 3, a and b). Nrf2 silencing significantly decreased the basal levels of known Nrf2 target genes (Fig. 3, c–e), an observation similar to that seen in in vivo Nrf2 knockout mice (Lee et al., 2005). In addition, the induction of GCLM, GCLC, and other Nrf2 target genes by bile acids in HepG2 (Fig. 3, c and e) and C2bbe1 (Fig. 3d) has been mitigated. Comparable reduction in inducible expressions of Gclm occurred with transfection of dominant-negative Nrf2 in Hepa1c1c7 cells, consistent with the enhanced gene induction with Nrf2 overexpression (Fig. 3f). Similar observations were noted for other Nrf2 target genes such as Gclc and Nqo1 (data not shown).

To verify that there was an activation of Nrf2-ARE transcription machinery with exposure to toxic bile acids, we extracted the nuclear proteins of bile-acid-treated HepG2 over different time points across 24 h. Translocation of cytosolic Nrf2 to nucleus represents the prerequisite event of receptor activation. Nrf2 started to be enriched in cell nuclei within 1 to 3 h of bile acid treatments, and it was sustained through 24 h, with CDCA-treated cells showing reduced Nrf2 translocation events with longer time of exposure (24 h) (Fig. 4a). Furthermore, various bile acids were found to increase the activity of an ARE-reporter assay in a dose-dependent manner, suggesting that these bile acids were capable of inducing Nrf2 transactivation (Fig. 4b). The magnitude of luciferase activity of the highest concentration of test bile acids was compatible with those of treatments with antioxi-

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**Fig. 1. Induction of Nrf2 target genes by bile acids.** a, mRNA levels of GCLM (white bar) and GCLC (black bar) in HepG2 and C2bbe1 cells (left) after 24-h treatment with increasing doses of LCA or CDCA: [LCA] = 6.25, 12.5, 25, 50, and 75 μM and [CDCA] = 25, 50, 75, 100, and 150 μM. Right, human primary hepatocytes (passages 4–6) were treated with 75 μM LCA or 100 μM CDCA for 24 h. y-axis, -fold change versus vehicle-treated controls. *, p < 0.05, significantly different from vehicle-treated control by one-way ANOVA followed by post hoc test for HepG2 and C2bbe1 or **, p < 0.01 by t test for primary hepatocyte. Mean and S.E.M. (n = 3–6). b, mRNA levels of other known Nrf2 target genes after 24-h treatment with bile acids in HepG2, C2bbe1, and primary hepatocytes: [LCA] = 75 μM and [CDCA] = 100 μM. Mean and S.E.M. (n = 3–6). c, representative immunoblots of protein lysate (10 μg for HepG2; 30 μg for C2bbe1) probed for Nrf2 target genes after 24-h treatment with bile acids. [LCA] = 70 μM and [CDCA] = 100 μM. d, cell viability (by Alamar Blue) and apoptotic marker (caspases activity) measured across increasing concentrations of LCA treatment in HepG2. Viability was measured at 24 h, whereas caspases activity was measured at 6 h of LCA treatment. Note that the induction of GCL genes peaked at 60 to 80 μM LCA (shown by closed inverted triangles) during which mild cellular toxicity began to occur. Representative results from four determinations are shown.
dants tert-BHQ (100 μM) and α-lipoic acid (200 μM) (data not shown), denoting that bile acids are equally potent Nrf2 activators. Note that there was a ~8-fold increase in reporter activity with vehicle DMSO treatment compared with that of the empty vector harboring only the simian virus 40 promotor. This suggests the existence of a strong constitutive Nrf2-ARE transactivation activity in HepG2 cells, an observation in line with the persistent oxidative stress observed in many cancerous cell lines (Brown and Bicknell, 2001). HepG2 cells are deficient in conjugated bile acid transporters such as NTCP, which leads to its resistance to conjugated bile acid-induced oxidative stress (Kullak-Ublick et al., 1996). Transfection of NTCP expression vector hence restores, to some degree, its sensitivity. In this study, we found that GCDCA, a known cholestatic conjugated bile acid, significantly induced the ARE reporter. This suggests that activation of Nrf2-ARE may be crucial to counteract the toxicity of GCDCA as reported previously (Dent et al., 2005). The potency of bile acids in activating this reporter based upon molarities was LCA > CDCA ≈ DCA > GCDCA ≳ ursodeoxycholic acid (UDCA) > cholic acid (CA) > GCA. This order is in consensus with the toxicity profile of these bile acids, particularly in terms of their ability to produce oxidative stress (Krähenbühl et al., 1994). Overexpression of Nrf2 further enhanced the reporter activity by bile acids, whereas coexpression of dominant-negative Nrf2 attenuated the activity, and a mutant ARE construct was completely uninducible by bile acids (Fig. 4c). Using the quantitative ChIP assay, we found an increased Nrf2 occupancy to the AREs of both GCL subunits in the native cell context upon 6-h treatment with bile acids (Fig. 4d). Taken together, our data suggest that activation of the Nrf2-ARE machinery underlies induction of Nrf2-target genes by toxicological concentrations of bile acids.

Protective Role of Nrf2 in Bile Acid Toxicity. To directly investigate the role of Nrf2 in protection against toxic bile acids, we first silenced Nrf2 of HepG2 via RNA interference upon which the cells were subjected to toxic LCA challenges. Nrf2 knockdown increased cell susceptibility to toxic LCA, with a significantly decreased cell viability starting from 4 h of treatment (Fig. 5a). Without LCA challenge, Nrf2-knockdown cells did not differ in cell viability from those treated with siCtr (data not shown). Significant protective effects of Nrf2 against LCA toxicity was also observed in C2bbe1 cells, and in HepG2 with ≥300 μM CDCA (data not shown). To investigate which route of cell death was particularly involved in protection by Nrf2, established markers of necrosis and apoptosis were examined. LCA at 90 μM was used because at this dose, both apoptosis and necrosis were found to simultaneously occur. Necrotic event, as determined by LDH released into the culture media, remained constantly higher in Nrf2 knockdown cells than those of siCtr starting from 2 h of LCA treatment (Fig. 5b). Nrf2 silencing alone did not affect the cellular release of LDH (data not shown). Likewise, in the assessment of apoptosis, Nrf2-knockdown cells exhibited much higher and prolonged elevation of caspases activity than did siRNA control-treated cells upon LCA treatment (Fig. 5c). Silencing of Nrf2 alone did not result in increased basal caspases activity.

Role of GSH in Resisting LCA Toxicity. The increase in GCLM and GCLC, the rate-limiting enzyme in GSH biosynthesis, observed in earlier experiments after LCA (75 μM) or CDCA (100 μM) treatment was accompanied by a significant increase by >4-fold in cellular GSH levels at 24 h (Fig. 6a). This increase was comparable with treatment with 200 μM α-lipoic acid, a GSH inducer. To determine whether the induced cellular GSH is a protective mechanism against toxic bile acid, we cotreated HepG2 with a toxic dose of LCA and a GSH biosynthesis blocker, buthionine sulfoximine (BSO), which inhibits the activity of GCL subunits and blocks GSH biosynthesis. BSO treatment together with toxic LCA decreased cell resistance toward LCA exposure with more apparent effects in late treatment (~24 h) (Fig. 6b). Furthermore, depletion of cellular GSH by pretreatment with BSO before LCA challenge markedly lifted cell resistance with a drastic drop in cell viability within 4 h of treatment. Conversely, toxic LCA challenge in the presence of an antioxidant and GSH precursor N-acetyl-l-cysteine (NAC) was found to alleviate the toxicity. This suggests that the basal as well as inducible GSH are important determinants of cellular resistance to LCA. Consistent with these findings, NAC co-treatment significantly reduced the oxidative stress-responsive ARE-reporter activity by LCA, indicating an antioxidative response.

Fig. 2. Activation of Nrf2 in mice treated with LCA. a, representative immunobots of nuclear fractions (30 μg) probed against Nrf2 in the liver of mice after 7- to 8-h treatment with cholestatic LCA (125 mg/kg b.wt.). Lamin B was used as loading control for nuclear protein, whereas β-actin was probed to show unintentional contamination of cytosolic proteins in nuclear fraction preparation. Note that the increased nuclear Nrf2 cannot be explained by inclusion of contaminant cytosolic proteins. b, mRNA levels of Nrf2 target genes upon LCA treatment for 7 to 8 h (top) or for 4 days (bottom) in the liver of mice. Because there were differences in the basal gene expression of Nrf2 target genes between sexes, comparisons of all target genes between treated and untreated groups were adjusted for sex. Induction of antioxidative genes by LCA, however, occurred to both sexes. Significantly different from vehicle-treated controls by t test. *, p < 0.05; **, p < 0.01. Mean and S.E.M. (n = 5–9 for 7- to 8-h treatment; n = 3 for 4-day treatment).
tive effect (Fig. 6C). BSO cotreatment, in contrast, further increased the reporter activity, consistent with a heightened oxidative stress (Fig. 6c).

Discussion

The discovery of bile acids as key signaling molecules in the enterohepatic circulation system reveals a critical role of hepatic and intestinal xenobiotic nuclear receptors in the metabolism and detoxification of bile acids (Chawla et al., 2000). Particularly, the cytotoxic hydrophobic bile acids CDCA and LCA have been shown to be ligands and potent inducers of these receptors. LCA, at physiological and non-toxicological concentrations (5–30 μM), can activate FXR (Makishima et al., 1999, 2002) and VDR (Makishima et al., 2002), indicating their crucial role in physiological handling of this bile acid. The major detoxification routes of LCA, i.e., sulfation by sulfotransferase 2A and 7α-hydroxylation by CYP3As, are coordinated by VDR (Makishima et al., 2002; Echchgadda et al., 2004). FXR, which induces the hepatic bile acid Nrf2, is activated by bile acids and stimulates the transcription of genes involved in detoxification, including glutamate cysteine ligase (GCL) and NQO1.

Fig. 3. Involvement of Nrf2 in induction of glutamate cysteine ligase subunits by bile acids. a, mRNA levels of all Nrf subtypes after 72-h treatment with siRNA. y-axis, -fold change versus vehicle-treated cells transfected with siCtr. *, p < 0.01, significant difference between siRNA groups by t test. Mean and S.E.M. (n = 3–5). b, representative immunoblots (20 μg of protein lysate) of HepG2 after 48-h treatment with siRNA against Nrf2. c, basal (treated with vehicle DMSO) and inducible (treated with 70 μM LCA or 100 μM CDCA) gene transcripts of GCLM and GCLC in HepG2 (mean and S.E.M.; n = 3–5). d, basal and inducible levels of GCLM and GCLC gene transcripts in C2bbe1 treated with vehicle or 70 μM LCA. *, p < 0.01, significant difference between siCtr and siNrf2 with or without treatment by t test. Mean and S.E.M. (n = 3–5). e, basal and inducible expressions of other Nrf2 target genes in HepG2. Mean ± S.E.M. f, mRNA levels of GCLM induced by 70 μM LCA or 100 μM LCA in Hepa1c1c7 transfected with empty vector (pEF), Nrf2 (pEF_Nrf2), or dominant-negative Nrf2 (pEF_DNrf2) expression vector. y-axis, -fold change versus vehicle-treated cells transfected with empty vector pEF. *, p < 0.05, significantly different from LCA-treated cells transfected with pEF control by t test. Mean and S.E.M. (n = 4).
salt export pump BSEP (Ananthanarayanan et al., 2001) and down-regulates the bile-synthesizing enzyme CYP7A1 (Makishima et al., 1999), works to prevent intracellular accumulation of bile acids.

Interestingly, at higher and toxicological concentrations of LCA (≥50 μM) and CDCA (≥100 μM), which potentially cause cell injury, PXR (Staudinger et al., 2001; Makishima et al., 2002) and Nrf2, as shown in this study, are found to be activated. The activation of PXR and Nrf2 induces the major hydroxylation enzymes CYP3As and antioxidative genes (Eloranta and Kullak-Ublick, 2005; Kensler et al., 2007), which may represent an important adaptive mechanism of cellular defense against toxic bile acids. Furthermore, we observed that induction of multiple bile salt/conjugate efflux transporters such as ATP-binding cassette (ABC) transporters ABCC2, ABCC3, and ABCG2 by bile acids is dependent on Nrf2 (K. P. Tan, G. Woodland, M. Yang, K. Kosuge, M. Yamamoto, and S. Ito, unpublished data). Hence, the collective induction of cytoprotective genes by Nrf2 and PXR seems to set off a second line of protection against possible progression of bile acid toxicity toward irreversible cell death.

In this study, we showed for the first time that many bile acids, more potently LCA, CDCA, and DCA, are capable of activating redox-sensitive Nrf2. We also provided in vivo evidence that LCA is able to activate Nrf2, inducing similar target genes observed in in vitro studies. Because the induction of Nrf2 target genes by LCA in vivo was found to precede and sustain through biochemically and histologically overt liver injury, the collective induction of these antioxidative genes may be an integral part of cell defense against bile acid toxicity and hepatic injury. Previous studies have reported an increased intracellular production of detrimental hydroperoxides in isolated rat hepatocytes with hydrophobic bile acid exposure (Sokol et al., 1995), an observation in consensus with the increased oxidative stress byproducts in the liver of patients with cholestasis (Vendemiale et al.,

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**Fig. 4.** Bile acids activate Nrf2 transcription machinery. a, representative immunoblots of nuclear fraction (10 μg) extracted from HepG2 treated with bile acids (LCA, 70 μM; CDCA, 100 μM) at indicated time points over 24 h. Lamin B was used as equal loading control for nuclear protein, whereas β-actin was probed to show possible contaminant cytosolic proteins in nuclear fraction preparation. b, ARE-reporter (luciferase) activity of HepG2 treated with increasing doses of various bile acids for 16 to 18 h. Abbreviations (doses): LCA (50, 70, and 90 μM), CDCA (50, 100, and 150 μM), CA (150, 200, and 400 μM), DCA (50, 100, and 150 μM), UDCA (50, 100, and 200 μM), GCA (200, 400, and 800 μM), and GCDCA (100, 200, and 400 μM). #, GCA and GCDCA were tested in NTCP-transfected HepG2 for 6 h. Fold change in the ratio of luciferase activity (relative luciferase unit) (see Materials and Methods for detail) from those transfected with basic pGL3 promoter construct and treated with vehicle DMSO. *, p < 0.05, significantly different from DMSO-treated pGL3_ARE by one-way ANOVA followed by post hoc test. Mean and S.E.M. (n = 2–4). c, ARE-reporter (pGL3_ARE) activity with coexpression of Nrf2 or dominant-negative Nrf2, and mutant ARE-reporter (pGL3_mARE) activity in HepG2 treated with bile acids (70 μM LCA; 100 μM CDCA); y-axis, fold change in the ratio of luciferase activity (relative luciferase unit) from those transfected with basic pGL3 promoter construct and/or respective expression vector, and treated with DMSO. *, p < 0.05, significant difference between vehicle control and bile acids by one-way ANOVA followed by post hoc test. Mean and S.E.M. (n = 3–6). d, ChIP analysis examining Nrf2 occupancy to AREs of both GCLM and GCLC genes upon 6-h treatment with bile acids (70 μM LCA; 100 μM CDCA) in HepG2. BHQ (200 μM), known to transcriptionally activate GCLM and GCLC, was included as positive control. Negligible detection from samples incubated with host IgG (anti-CYP1A1) ruled out contribution of nonspecific binding from antibody. *, p < 0.05, significantly different from controls (t = 0) and vehicle treatment by t test. Mean and S.E.M. of triplicate determinations of representative experiments.
Because Nrf2 activation is indicative of cellular anti-oxidative response, our study provides molecular evidence linking mechanism of bile acid toxicity to oxidative stress.

We further showed that induction of hepatic GCL subunits via Nrf2, which provokes GSH biosynthesis, can increase hepatocyte resistance and survival during excessive bile acid exposure. The essential role of GSH in hepatic protection against injury and oxidative xenobiotic insults has been well exemplified (Huang et al., 2001; Glosli et al., 2002). In agreement, in vivo knockout of Nrf2 enhances sensitivity of death receptor-induced hepatic apoptosis as a result of decreased GSH levels (Morito et al., 2003). GSH is also known to protect against mitochondrial injury, a major mechanism of bile acid toxicity (Palmeira and Rolo, 2004). A fraction of cytosolic GSH that becomes mitochondrial GSH is crucial in the defense of oxidant-induced mitochondrial-mediated cell death (Fernandez-Checa and Kaplowitz, 2005). In addition, Nrf2 activation has been shown to protect mitochondria by preventing inhibition of mitochondrial complex II upon exposure to oxidative neurotoxins (Calkins et al., 2005). In intestinal mucosa, cellular GSH has an essential role in maintaining epithelial integrity, transport activity, and metabolism of and susceptibility to luminal toxins (Aw, 2005). Overall, our study, coupled with supportive evidence from recent literature, suggests that protection conferred by hepatic and intestinal Nrf2 against bile acid-induced oxidative stress is, at least partly, achieved by increasing GSH levels.

The simultaneous induction of other Nrf2 target genes may work in concert with GCL subunits to combat bile acid-induced oxidative stress and facilitate adaptive responses. Of particular importance is TRx1, an enzyme engaged in NADPH-dependent catalysis of various redox proteins (Rundlöf and Arner, 2004). It has been shown to act as a key adaptation-promoting mediator for prior exposure to 4-hydroxynonenal, a reactive lipid peroxidation-derived molecule, in inducing cellular tolerance to future oxidative stress attack (Chen et al., 2005). Indeed, intermediate cellular stress has recently been proposed to provide an adaptation advantage by invoking enhanced cellular survival/tolerance mechanisms (Schoemaker et al., 2003; Chen et al., 2005). Activation of nuclear factor xB as well as Nrf2 has been shown to play an important role in this adaptation process. The drastic induction of TRx1 observed in mice upon acute exposure to toxic LCA in this study may indicate a critical role of this enzyme in adaptation process against LCA toxicity. To address whether and how this process is taking place, future studies are needed.

The precise mechanisms by which toxic bile acids activate Nrf2 remain a subject of future studies. Enormous production of ROS from mitochondrial stress has long been accounted for the main source of oxidative stress induced by bile acids (Palmeira and Rolo, 2004). Insurgence of these ROS potentially targets the cysteine oxidative-sensors of Keap1, an actin-anchored cytosolic sequester that facilitates Nrf2 degradation by ubiquitin-proteosome pathway, which leads to liberation and activation of Nrf2 (Kensler et al., 2007). In addition, subsets of both conjugated and unconjugated bile acids have been shown to activate multiple kinase signaling pathways such as protein kinase C, extracellular signal-regulated kinase 1/2, mitogen-activated protein kinase, p38 mitogen-activated protein kinase, c-Jun NH2-terminal kinase, and/or phosphatidylinositol 3-kinase/AKT (Debruyne et al., 2002; Dent et al., 2005). These signaling pathways have been shown as well to influence the stability of Nrf2-Keap1 complex and to post-transcriptionally regulate the Nrf2 target genes (Kensler et al., 2007).

In summary, we characterized a molecular cell defense event associated with bile acid-provoked oxidative stress. Exposure to cytotoxic bile acids in the liver and intestinal cells was shown here to cause Nrf2 activation, thereby up-regulating a battery of cytoprotective genes, particularly GCL subunits, to enhance cell survival at the emergence of oxidative stress.
Increased GSH production is a cellular protective mechanism against bile acid toxicity. A cellular GSH levels of HepG2 after 24-h treatment with 75 μM LCA, 100 μM CDCA, or 200 μM ipic acid (LA; positive control). BSO (60 μM), a GSH synthesis blocker, was used as negative control. *p < 0.05, significantly different from DMSO control by one-way ANOVA followed by post hoc test. Mean and S.E.M. (n = 4). b, cell viability of HepG2 treated with toxic LCA (100 μM) together with 0.4 mM NAC, or 60 μM BSO, or with overnight pretreatment with 60 μM BSO (pre-BSO). Fluorescent units were first subtracted by those of negative control.

References


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Fig. 6. Increased GSH production is a cellular protective mechanism against bile acid toxicity. a, cellular GSH levels of HepG2 after 24-h treatment with 75 μM LCA, 100 μM CDCA, or 200 μM ipic acid (LA; positive control). BSO (60 μM), a GSH synthesis blocker, was used as negative control. *p < 0.05, significantly different from DMSO control by one-way ANOVA followed by post hoc test. Mean and S.E.M. (n = 4). b, cell viability of HepG2 treated with toxic LCA (100 μM) together with 0.4 mM NAC, or 60 μM BSO, or with overnight pretreatment with 60 μM BSO (pre-BSO). Fluorescent units were first subtracted by those of negative control. c, ARE-reporter activity in way ANOVA followed by post hoc test. Mean and S.E.M. (n = 4–5).


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