PHARMACOLOGIC AND TRANSGENIC ACTIVATION OF NUCLEAR FACTOR-ERYTHROID 2-RELATED FACTOR 2 (NRF2) ALTERS KINETICS AND TOXICODYNAMICS OF XENOBIOTICS

By

Scott A. Reisman

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Submitted to the Graduate Degree Program in Pharmacology, Toxicology, and Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Curtis Klaassen Ph.D. (Chairperson)

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Bryan Copple, Ph.D.

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Grace Guo, Ph.D.

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Greg Reed, Ph.D.

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Glen Andrews, Ph.D.

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The Dissertation Committee for Scott A Reisman certifies that this is the approved version of the following dissertation:

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LIST OF ABBREVIATIONS

AA	acetaminophen
AA-CYS	acetaminophen-cysteine
AA-GLUC	acetaminophen-glucuronide
AA-GSH	acetaminophen-glutathione
AA-NAC	acetaminophen-mercapturate
AA-SULF	acetaminophen-sulfate
Abcg	ATP-binding cassette transporters sub-family g
AhR	aryl hydrocarbon receptor
Aldh	aldehyde dehydrogenase
ALT	alanine transaminase
ARE	antioxidant response element
Bcrp	Breast cancer resistance protein
Bsep	bile salt export pump
BSP	sulfobromophthalein
CAR	constitutive androstane receptor
Cat	catalase
CDDO-Im	2-cyano-3,12 dioxooleana-1,9 diene-28-imidazolide
Ces	carboxylesterase
Cul3	cullin 3
Cyp	cytochrome p450
Cpr	cytochrome p450 reductase
DBSP	disulfobromophthalein
DTNB	5,5'-dithiobis 2-nitrobenzoic acid
Eh-1	epoxide hydrolase-1
Ent1	equilibrative nucleoside transporter 1
Gclc	glutamate cysteine ligase catalytic subunit
Gclm	glutamate-cysteine ligase modifier subunit
Glrx1	glutaredoxin 1
Gpx	glutathione peroxidase;
GSH	glutathione
Gsr	gluathione reductase
Gst	glutathione-*S*-transferase
H6pdh	hexose-6-phosphate dehydrogenase

Ho-1	heme oxygenase-1
Hsp70	70 kDa heat shock protein
Nrf2	nuclear factor-erythroid 2-related factor 2
Kd	knockdown
Keap1	kelch-like ECH associated protein 1
Maf	musculo-aponeurotic fibrosarcoma
Mate1	multidrug and toxin extrusion 1
Me1	malic enzyme 1
Mrp	multidrug resistance-associated protein 1
MT	metallothionein
Mtf-1	metal-responsive-element-binding transcription factor-1
Nqo1	NAD(P)H: quinone oxidoreductase 1
Ntcp	sodium taurocholate co-transporting polypeptide
Oat	organic anion transporter
Oatp	organic anion transporting polypeptide
Oct	organic cation transporter
PAPS	phosphoadenoside 5'-phosphosulfate;
Papps	phosphoadenoside 5'-phosphosulfate synthetase
Parp	polyADP-ribose polymerase
PPARα	peroxisome proliferator-activated receptor-alpha
Prx1	peroxiredoxin 1
PXR	pregnane X receptor;
SAPE	streptavidin-conjugated *R*-phycoerythrin
Sod	superoxide dismutase
SSA	sulfosalicylic acid
Sult	sulfotransferase
Txn1	thioredoxin 1
Txnrd1	thioredoxin reductase 1
Ugt	uridine-diphosphate-glucuronosyltransferases

ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, which, upon translocation into the nucleus, is capable of inducing a variety of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutamate-cysteine ligase, catalytic subunit (Gclc), glutathione-*S*-transferases (Gsts), and multidrug-associated resistance proteins (Mrps). Because Nrf2 can induce many cytoprotective enzymes and transporters, it is a potential target for the prevention of liver injury, for which there are limited treatments. Therefore, it was hypothesized that pharmacologic activation of Nrf2 would protect against acetaminophen (AA) hepatotoxicity, and that genetic ablation and enhancement of Nrf2 activation would alter pharmacokinetics of AA and sulfobromopthalein (BSP). Pharmacokinetic analysis will provide insight into a potentially novel hepatoprotective role for Nrf2.

It was determined whether the natural triterpenoid oleanolic acid and a synthetic derivative 2-cyano-3,12-dioxooleana-1,9-diene-28-imidazolide (CDDO-Im) could protect liver from AA toxicity through activation of Nrf2.

Oleanolic acid increased mRNA expression of the Nrf2 target genes Nqo1, Gclc, and heme oxygenase-1 (Ho-1) in wild-type but not in Nrf2-null mice, and protected against acetaminophen hepatotoxicity in wild-type mice, but to a lesser extent in Nrf2-null mice. The synthetic triterpenoid CDDO-Im also protected the liver from AA-induced injury and induced the Nrf2 target genes Nqo1, Gclc, and Ho-1 in a dose- and time-dependent manner. In contrast, this protection and mRNA induction was ablated in Nrf2-null mice. These studies demonstrate that oleanolic acid and CDDO-Im protect the liver from AA-induced injury by activating the antioxidant transcription factor Nrf2.

A recently engineered mouse with knockdown of Keap1 (Keap1-kd mice), the cytosolic repressor of Nrf2, has a 55% decrease in Keap1 mRNA and a 200% increase in Nrf2 protein in liver. Several experiments with Nrf2-null mice have demonstrated the effects of a lack of Nrf2. However, little is known about the biological effects of more Nrf2 activation. Accordingly, the phenotype of Keap1-kd mice, as well as mRNA expression of detoxifying and antioxidant genes, were compared with Nrf2-null and wild-type mice. The present study revealed three patterns of gene expression, which collectively

suggest that hepatic Nrf2 is more important for the detoxification and elimination of electrophiles rather than reactive oxygen species.

Numerous studies have shown that Nrf2 protects against toxicity, which is frequently attributed to decreased toxicodynamic effects of chemical insults, as exemplified by increased expression of cytoprotective genes. However, the effects of Nrf2 on the kinetics of xenobiotics have not been examined. It was found that Nrf2 increases biliary excretion of BSP by increasing glutathione (GSH) conjugation and biliary excretion of the BSP conjugate. In addition, lack of Nrf2 decreases AA glucuronidation, leading to increased NAPQI formation and hepatotoxicity, whereas activation of Nrf2 enhances detoxification of NAPQI by Nqo1 and elimination of AA glucuronide conjugate via Mrp3. Therefore, the effects of Nrf2 on the kinetics of xenobiotics should also be considered as a means of protection.

Collectively, these studies have shown that liver can be protected by activation of Nrf2, either pharmacologically or genetically, as in the case of genetically-engineered Keap1-kd mice. Nrf2 can also dramatically affect the

kinetics of xenobiotics because of its ability to transcriptionally control genes important in the biotransformation and excretion of chemicals.

GENERAL INTRODUCTION AND BACKGROUND

Biotransformation

Organisms are continually exposed to chemicals, either accidentally as in the case of toxic effects from pesticides or purposefully, such as in the therapeutic use of pharmaceutics. Foreign compounds not produced by organisms are commonly referred to as xenobiotics. Xenobiotics are often lipophilic and must be biotransformed to increase hydrophilicity and allow for excretion into urine or bile. Thus, biotransformation is critical for maintaining homeostasis during xenobiotic exposure, the ultimate goal being to make xenobiotics more water soluble for enhanced excretion (Parkinson, 2008). Biotransformation chemically changes xenobiotics and has many effects, both desirable and undesirable. The pharmacological effects of drugs are often terminated by biotransformation, which can be viewed as desirable for drugs for which one hopes to observe a short-term effect, or undesirable when drugs have to be administered chronically in order to maintain the preferred effect. Also, some non-toxic xenobiotics can be biotransformed to toxic metabolites, which then cause cellular injury. Finally and most importantly for

this dissertation, biotransformation can detoxify potentially harmful xenobiotics, which are typically the parent compound or a reactive metabolite.

The liver is the primary organ where xenobiotics are biotransformed, as it is the organ most exposed to xenobiotics and the most abundant source for biotransformation enzymes. Thus, the liver is paramount for protection of the whole organism against potentially toxic xenobiotics. However, the liver can be overwhelmed by chemicals, which result in liver injury, dysrepair, or liver failure. Increasing the ability of the liver to biotransform and eliminate xenobiotics has become an attractive mechanism for the prevention of liver injury.

Oxidative and electrophilic stress.

Oxidative stress (Fig 0.1, left side) is caused by increased production of reactive oxygen and nitrogen species, such as superoxide, hydroxyl radical, and peroxynitrite, or by depletion of protective antioxidants, such as reduced glutathione (GSH), ascorbate, and α-tocopherol. Superoxide is formed, as in the case of diquat, when redox cycling occurs, and an electron is donated to molecular oxygen. More reactive peroxynitrite and hydroxyl

radicals are generated when superoxide is not adequately detoxified as discussed below. Oxidative stress leads to oxidative damage to critical macromolecules, namely DNA, lipids, and proteins. Detoxification of superoxide occurs enzymatically by first being converted to hydrogen peroxide and then to water by catalase (Cat), glutathione peroxidase (Gpx), or peroxiredoxin. If hydrogen peroxide is not detoxified, it is converted to hydroxyl radical by the Fenton reaction. Hydroxyl radical is almost impossible to detoxify because of its short half-life (10^{-9}s), and thus prevention of its formation by the detoxification of its precursor hydrogen peroxide by Cat, Gpx, or Prx is necessary. Detoxification of peroxynitrite to nitrite is carried out by Gpx and Prx; GSH may also be capable of reducing perxoxynitrite to nitrite (Harwood *et al.*, 2006; Trujillo *et al.*, 2008).

An electrophile is a molecule that contains an electron-deficient atom with a partial or full positive charge that allows it to react by sharing electron pairs with electron-rich atoms (Parkinson, 2008). Electrophilic stress (Fig 0.1, right side) results when electrophiles overwhelm the cell and cause injury by covalently binding to and damaging critical macromolecules. Detoxification of

reactive intermediates or electrophiles occurs by biotransformation, such as conjugation with GSH catalyzed by glutathione-*S*-transferases (Gsts), reduction of quinones by NAD(P)H:quinone oxidoreductase 1 (Nqo1), or hydrolysis of epoxides by epoxide hydrolase-1 (Eh-1). Nucleophilic conjugation mediated by UDP-glucuronosyltransferases (Ugts) or sulfotransferases (Sults) can also prevent the formation of electrophiles by increasing excretion of xenobiotics, limiting their availability for toxification by Cyps or peroxidases (Pods).

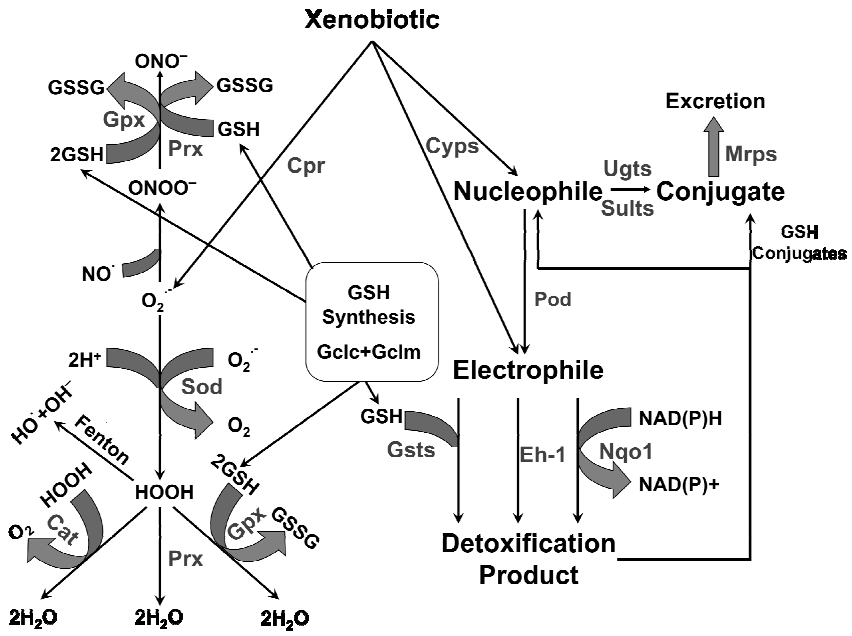


Fig 0.1. Illustration of oxidative and electrophilic stress. Oxidative stress is represented in the left side and electrophilic stress on the right side. Xenobiotics can undergo redox cycling, such as in the case of paraquat, which generates superoxide in a reaction catalyzed by cytochrome p-450 reductase (Cpr). Detoxificaton of superoxide ($O_2^{-\cdot}$) to hydrogen peroxide (HOOH) is mediated by superoxide dismutase (Sod). HOOH is detoxified to H_2O by catalase (Cat), peroxiredoxin (Prx), and glutathione peroxidase (Gpx). GSH is oxidized to glutathione disulfide (GSSG) in the Gpx-catalyzed

reaction. If HOOH is not detoxified, it can undergo a Fenton reaction to generate hydroxyl radical ($OH^{\cdot}$). $O_2^{-\cdot}$ can also react with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^{-}$), which can be detoxified by Gpx, Prx, or glutathione (GSH). Biotransformaton of xenobiotics by cytochrome P-450s (Cyps) and peroxidases (Pod) can lead to the formation of nucleophiles or electrophiles. Detoxification of electrophilic intermediates by NAD(P)H:quinone oxidoreductase 1 (Nqo1) or epoxide hydrolase-1 (Eh-1) creates nucleophiles, which can be conjugated with glucuronic acid via UDP-glucuronosyltransferases (Ugts) or conjugated with sulfate via sulfotransferases (Sults) and excreted by multidrug resistance-associated proteins (Mrps). Electrophiles can also be conjugated with GSH in a reaction catalyzed by glutathione-*S*-transferases (Gsts) and excreted via Mrps. GSH and its synthesis play important roles in both superoxide and electrophile detoxification. The rate limiting enzymes in GSH synthesis are glutamate-cysteine ligase catalytic and modifier subunits (Gclc and Gclm, respectively).

Nuclear factor erythroid 2-related factor 2 (Nrf2)

Over the past decade Nrf2 has emerged as an important transcriptional regulator of antioxidant cellular protection. Upon cellular oxidative/electrophilic insult, Nrf2 initiates a response by up-regulating a battery of cytoprotective and biotransformation genes. Moreover, inductive control over a broad array of genes that facilitate increased detoxification and clearance of reactive metabolites and xenobiotics has earned Nrf2 a crucial role in toxicology and xenobiotic metabolism.

Under physiological conditions, Nrf2 (Fig 0.2) is bound in the cytosol by its repressor Kelch-like ECH associating protein 1 (Keap1). Keap1 functions as an adapter for Cullin 3 (Cul3)-based E3 ligase, a scaffold protein that aids in the ubiquitination and subsequent degradation of Nrf2 (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004a). In fact, Nrf2 has a very rapid turnover, with a half-life of approximately 20 min, and thus Nrf2 is difficult to detect in unstressed conditions (Itoh *et al.*, 2003; McMahon *et al.*, 2003). When an oxidative or electrophilic insult occurs, reactive cysteines of Keap1 are modified, and Keap1 is unable to target Nrf2 for proteasomal degradation

(Dinkova-Kostova *et al.*, 2002). Nrf2 then translocates into the nucleus, heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein, and binds to the antioxidant or electrophilic response element (ARE/EpRE) in the upstream promoter region of a variety of cytoprotective genes, promoting their transcription (Itoh *et al.*, 1997; Venugopal and Jaiswal, 1998; He *et al.*, 2001).

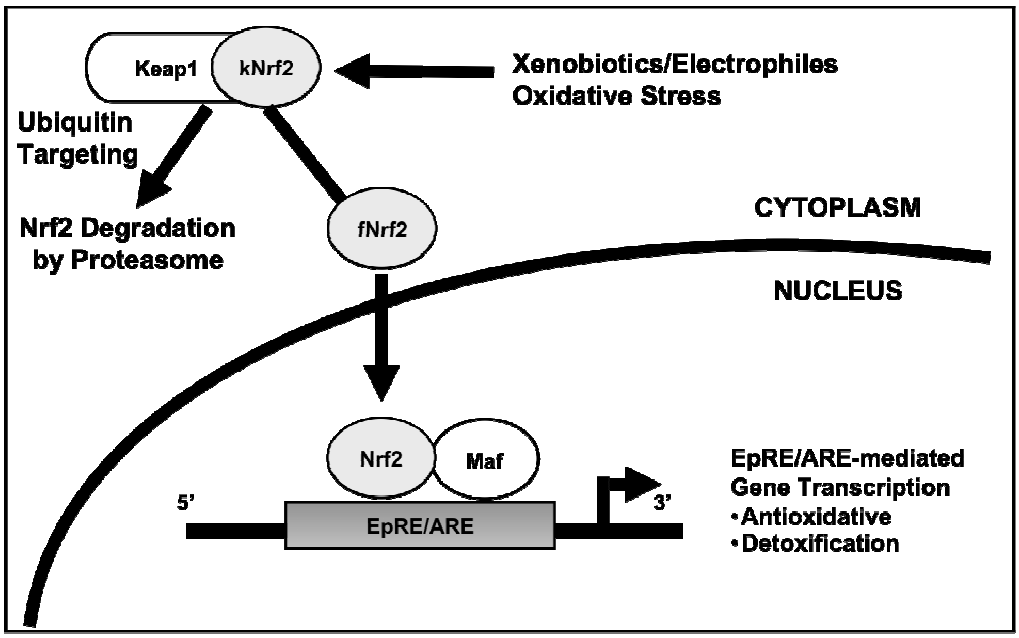


Fig 0.2. Mechanism of Nrf2 activation. Under basal conditions, Nrf2 (kNrf2 or bound Nrf2) is sequestered in the cytosol by Keap1, which acts as a scaffold protein to mediate ubiquitination and proteasomal degradation of Nrf2. Upon exposure to oxidative or electrophilic stress, Keap1 is no longer able to target Nrf2 for proteasomal degradation and free Nrf2 (fNrf2) concentrations increase. Free Nrf2 can then translocate to the nucleus, heterodimerize with a small Maf protein, bind to the electrophilic/antioxidant response element (ARE/EpRE), and initiate gene transcription. ARE-mediated gene transcription increases cellular defenses against oxidative and electrophilic stress.

Genes regulated by Nrf2

The Nrf2-Keap1 pathway regulates a wide variety of important antioxidant enzymes, including but not limited to Nqo1, Gsts, heme oxygenase-1 (Ho-1), and glutamate-cysteine ligase, catalytic subunit (Gclc), Eh-1, and Mrps (Fig 0.3). It was shown through extensive analysis of these genes' regulatory mechanisms that their inducible expression is attained at the transcriptional level through the antioxidant response element (Kobayashi *et al.*, 2004b; Maher *et al.*, 2007). Therefore, the activation of Nrf2 will increase antioxidant genes, such as the ones mentioned above, and protect the organism against stress.

Nqo1. Nqo1 utilizes NAD(P)H as a reducing agent to reduce quinones in a single two-electron step. This two-electron process detoxifies potentially toxic quinones by yielding substrates for conjugation reactions with glutathione, sulfate, or glucuronic acid and promoting excretion. Reactive intermediates detoxified by Nqo1 include the reactive intermediate of acetaminophen metabolism, namely *N*-acetyl-*p*-benzoquinoneimine (NAPQI),

as well as benzo[a]pyrene quinone, and menadione (Jaiswal, 2000; Moffit *et al.*, 2007).

Gclc and Gclm. Glutamate cysteine ligase (Gcl) is a heterodimer which can be dissociated under nondenaturing conditions into a catalytic, or heavy, subunit (Gclc) and a modulatory, or light, subunit (Gclm) (Dickinson *et al.*, 2004). It is the rate limiting enzyme in the synthesis of GSH. GSH is found at millimolar concentrations in most cells, making it the most abundant nonprotein thiol. GSH is essential to antioxidant and electrophile defense, regulation of the cell cycle, and gene expression. The involvement of GSH in the protection of the cell against exposure to toxicants, and in the metabolism of xenobiotic compounds through formation of conjugates, is well established. GSH also interacts with glutaredoxin and protein disulfide isomerases to modulate the tertiary structure of proteins through thiol–disulfide exchange. In addition to the essential role in defense and metabolism, it has become clear in the past few years that GSH contributes to redox signaling mediated by reactive oxygen and nitrogen species (Dickinson *et al.*, 2004).

Gsts. Gsts are a family of detoxification enzymes that catalyze the nucleophilic attack of GSH sulfur to electrophilic substrates, resulting in a detoxified electrophile (Blanchette *et al.*, 2007). Given the fact that Gst substrates include an enormous array of electrophilic xenobiotics, its importance in detoxification cannot be overstated.

Ho-1. Ho-1 is the inducible form of the rate-limiting enzyme in the degradation of heme, converting heme to biliverdin, during which iron is released and carbon monoxide (CO) is emitted. Biliverdin is subsequently converted to bilirubin by biliverdin reductase (Nath, 2006). Bilirubin is an important antioxidant, capable of reducing ROS. Ho-1 also facilitates the combining of free iron with ferritin, reducing free iron concentrations and oxidative stress that results when iron is involved in redox reactions. Furthermore, whereas the clinical toxicity of CO is clearly recognized, much smaller quantities of CO are remarkably cytoprotective, anti-apoptotic, vasorelaxant, and anti-inflammatory (Nath, 2006).

Eh-1. Eh-1 catalyzes the hydrolysis reaction in which potentially toxic electrophilic epoxides are converted to trans-dihydrodiols, which can be

conjugated and excreted from the body. Important substrates for Eh-1 include the epoxides formed in benz[a]pyrene and aflatoxin metabolism that unless detoxified are potent carcinogens (Omiecinski et al., 2000) .

Mrps. Once a xenobiotic has been conjugated by, for example, glucuronic acid, sulfate, or glutathione, it must be effluxed out of the hepatocyte or accumulation and toxicity can occur. One of the most important families of efflux transporters in liver are the Mrps. Mrps are highly inducible, have a wide-variety of substrates, efflux xenobiotics both apically (Mrp2) and basolaterally (Mrp3 and Mrp4), and are induced via Nrf2 activation (Maher *et al.*, 2007).

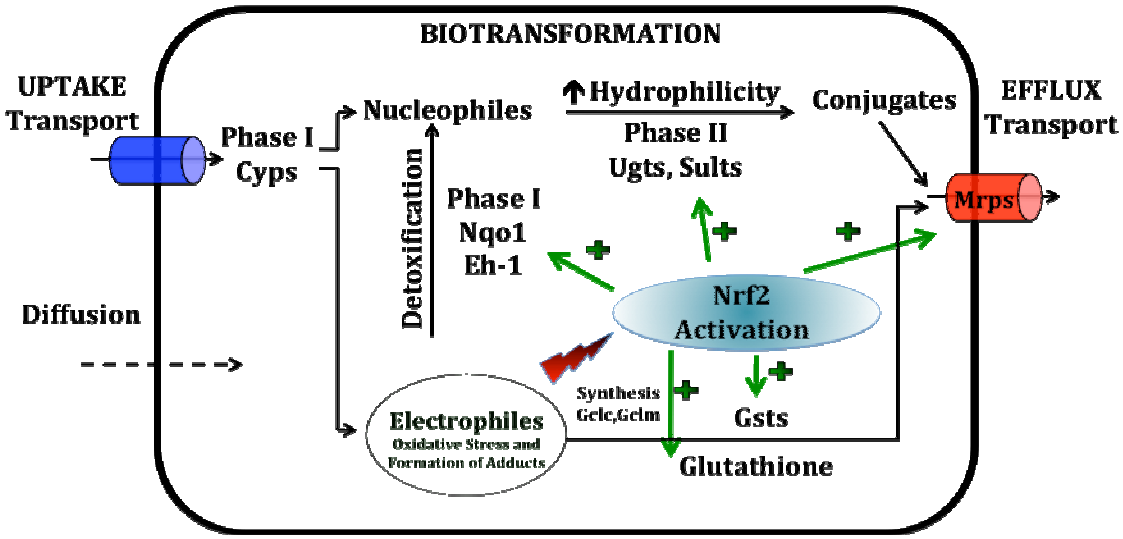


Figure 0.3. Summary of the effects of Nrf2 activaiton on biotransformation in the liver. In response to electrophilic or oxidative stress caused by xenobiotics, Nrf2 induces a variety of cytoprotective enzymes, including Nqo1, Eh-1, Gclc, Gclm, Gsts, Ugts, and Mrps in order to help restore the oxidative balance within the cell and prevent cell death.

Nrf2 Activators

Nrf2-activating chemicals which induce expression of ARE-containing genes have been categorized as cytoprotective agents. These include phenolic antioxidants (β-naphthoflavone, β-NF; butylated hydroxyanisole, BHA; and *tert*-butyl-hydroquinone, tBHQ), synthetic antioxidants (ethoxyquin), derivatives of 1,2-dithiole-3-thiones (oltipraz, 3*H*-1,2-dithiol-3-thione, D3T), phorbol esters (phorbol 12-myristate 13-acetate, PMA), and isothiocyanates (sulforaphane from broccoli, phenethyl isothiocyanate) (Nguyen *et al.*, 2003). These compounds have been used to identify Nrf2 target genes. However, it is frequently overlooked that chemicals have many off-target effects and identifying Nrf2-target genes with absolute certainty is difficult and often impossible using chemicals.

Nrf2-null and Keap1-kd mice.

Nrf2-null mice have lower constitutive expression and an inability to induce many cytoprotective genes, such as Nqo1 and Gclc, upon oxidative/electrophlic insult. Nrf2-null mice are extremely susceptible to chemical models of oxidative and electrophilic stress (Aleksunes and

Manautou, 2007), contributing to increased hepatotoxicity when administered acetaminophen (Enomoto *et al.*, 2001), ethanol (Lamle *et al.*, 2008), pentachlorophenol (Umemura *et al.*, 2006), or a high-fat diet (Tanaka *et al.*, 2008b).

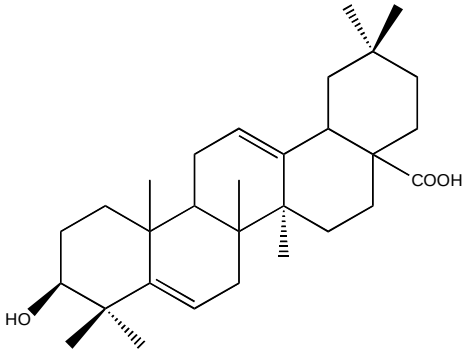
In contrast to Nrf2-null mice, Keap1-null mice were engineered to investigate the effects of increased activation of Nrf2. Unfortunately, even though Keap1-null mice have enhanced activation of Nrf2 and higher constitutive expression of Nrf2-target genes, the mice died at weaning due to malnutrition from hyperkeratosis of the esophagus and forestomach (Wakabayashi *et al.*, 2003). In an attempt to circumvent the post-natal lethality in Keap1-null mice, a hepatocyte-specific Keap1-null mouse was engineered, utilizing an Albumin-Cre loxP system. This mouse is viable, has enhanced activation of Nrf2 and Nrf2-target genes in liver, and decreased susceptibility to acetaminophen hepatotoxicity (Okawa *et al.*, 2006). However, later it was discovered that floxation of the Keap1 allele with loxP sites led to partial disruption of Keap1 expression, creating a whole-body knockdown phenotype (Okada *et al.*, 2008). The Keap1-kd mice is an *in*

vivo model of increased Nrf2 activation without the off-target effects possible with chemical inducers of Nrf2, and should be useful in elucidating accurate effects of Nrf2 activation.

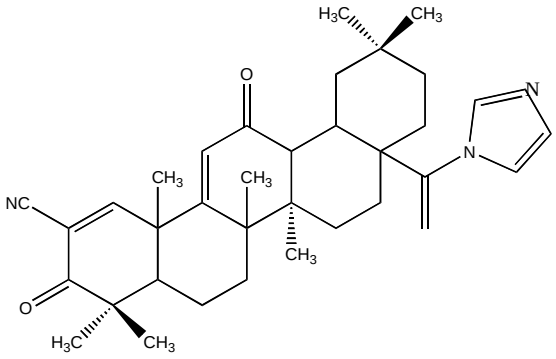
Natural and synthetic triterpenoids

Oleanolic acid (Fig 0.4, upper) is a natural triterpenoid that is a constituent of the leaves of *Olea europaea, Viscum album L*., and other plants, and is the aglycone of many saponins. Oleanolic acid is used in Chinese medicine for the treatment of liver disorders, such as viral hepatitis, and has been shown to protect mice from various hepatotoxicants, including carbon tetrachloride, acetaminophen, bromobenzene, and thioacetamide. In mice pretreated with oleanolic acid, and then exposed to one of the above chemical hepatotoxicants, serum alanine transaminase concentrations and centrilobular necrosis were diminished (Liu *et al.*, 1993a; Liu *et al.*, 1994a; Liu *et al.*, 1994b; Liu *et al.*, 1995b). To partially explain the protective effects of oleanolic acid, a study on toxifying and detoxifying systems was performed. Oleanolic acid did not dramatically affect hepatic Cyp enzyme systems, but did increase hepatic GSH, metallothionein, and Gst activity. However, the

exact mechanism for oleanolic acid-mediated hepatoprotection has not been fully elucidated.



Oleanolic Acid



CDDO-Im

Fig 0.4. Structures of oleanolic acid and CDDO-Im

Although, the limited efficacy of many naturally-occurring substances often precludes clinical use, natural compounds may serve as a critical foundation for drug development. In an effort to make more potent and efficacious compounds with the beneficial effects of oleanolic acid, the Michael Sporn group at Dartmouth College synthesized many derivatives. Among them, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) was 400-times more potent than previous compounds made as inhibitors of production of nitric oxide induced by interferon-γ in mouse macrophages (Honda *et al.*, 1998). In addition, an imidazole group was added (Fig 0.4, lower), making the compound 2- to 3-fold more potent than CDDO (Place *et al.*, 2003).

CDDO-Im was found to increase Ho-1 protein expression *in vivo* (Suh *et al.*, 1999). However, confirmation of activation of Nrf2 *in vivo* was missing from this study. An even more recent study found that if one pretreated mice with CDDO-Im, and then exposed them to a dose of aflatoxin, the mice were protected from liver tumorigenesis (Thimmulappa *et al.*, 2006). It was shown that micromole doses of CDDO-Im induced cytoprotective genes, inhibited

DNA-adduct formation, and dramatically blocked hepatic tumorigenesis. The cytoprotective genes induced are known to have an antioxidant response element capable of binding Nrf2. They were Eh-1, thioredoxin (Trx), Gclc, Gclm, and various isoforms of Gsts (Thimmulappa *et al.*, 2006). Taken collectively, all these results indicate that CDDO-Im is a potent chemopreventitive chemical, possibly acting through the Nrf2-Keap1 pathway and further study of this compound *in vivo* is needed, and of interest is whether CDDO-Im can protect against hepatotoxicants, particularly acetaminophen.

Acetaminophen (AA) biotransformation.

AA is a widely-used and effective antipyretic drug and is often used as a model hepatotoxicant. A therapeutic dose of AA is primarility metabolized by glucuronidation and sulfation in liver (Fig 0.3). However, when these systems become saturated, as in cases of overdose, larger amounts of the reactive intermediate *N*-acetyl-*p*-benzoquinoneimine (NAPQI) are formed via Cyps (Cyp2E1, Cyp3A4/11, and to a lesser extent Cyp1A2). NAPQI is detoxified by GSH, however, when GSH stores are depleted, NAPQI

covalently binds to critical cellular macromolecules (namely proteins), inducing a cascade of reactive intermediates and free radicals that eventually lead to hepatotoxicity (Hinson et al., 2004).

STATEMENT OF PURPOSE

Current therapeutic interventions for treating liver injury are limited; identification of a therapeutic target is a prerequisite for developing new drugs to treat liver injury. Oxidative and electrophilic stresses play key roles in the pathogenesis of liver injury. Nrf2 is a critical transcription factor in antioxidative responses. In quiescent hepatocytes, Nrf2 is sequestered in the cytosol by Keap1, which mediates Nrf2 proteasomal degradation. Under oxidative or electrophilic stress, Keap1 becomes unable to mediate proteasomal degradation of Nrf2, and free Nrf2 accumulates in the cytosol, translocates into the nucleus, and activates gene transcription. The ability of Nrf2 to increase transcription of a large battery of cytoprotective genes is generally recognized. Nevertheless, whether activation of Nrf2 can prevent liver injury has not been thoroughly examined.

The purpose of the first specific aim was to investigate the protective effects against AA hepatotoxicity of the natural triterpenoid oleanolic acid and a synthetic derivative, CDDO-Im, and to determine whether the two xenobiotics mediate such hepatoprotection through Nrf2. Over a decade ago,

our laboratory showed that pretreatment with the natural triterpenoid oleanolic acid protected mice from exposure to mechanistically diverse hepatotoxicants. Mice pretreated with oleanolic acid and then administered bromobenzene, AA, carbon tetrachloride, thioacetamide, furosemide, phalloidin, colchicine, cadmium chloride, or D-galactosamine/lipopolysaccharide had decreased hepatotoxicity. Because electrophilic and/or oxidative stress play a role in the pathogenesis of toxicity from these hepatoxicants, it was hypothesized that oleanolic acid may be activating Nrf2. CDDO-Im was synthesized in an attempt to generate a more potent and effective triterpenoid compound. Because CDDO-Im is structurally similar to oleanolic acid, CDDO-Im was also investigated as a possible Nrf2 activator and alleviator of AA hepatotoxicity.

A considerable amount of knowledge has accumulated regarding the effects of a lack of Nrf2 by performing experiments in the Nrf2-null mouse. However, very little is known about the biological effects of more Nrf2 activation. Keap1-kd mice have increased nuclear accumulation of Nrf2 and present a novel *in vivo* model for investigating the activation of Nrf2 without

the added variable of off-target effects often observed with chemical treatments. The availability of both Nrf2-null mice, which have no Nrf2, and Keap1-kd mice, which have enhanced activation of Nrf2, provide outstanding models to investigate the opposing effects of Nrf2 deficiency and Nrf2 activation in liver. Therefore, the purpose of the second aim was to determine the phenotype of Keap1-kd mice, and compare with Nrf2-null and wild-type mice the mRNAs of genes, which protect against oxidative and electrophilic stress. A fundamental understanding of the mRNA expression of drug metabolizing and detoxification enzymes will provide a framework for future studies using both Nrf2-null mice and Keap1-kd mice.

Numerous studies have shown that Nrf2-null mice have an increased sensitivity to hepatoxicants, and this is frequently attributed to decreased toxicodynamic effects, as exemplified by increased expression of cytoprotective genes, such as Nqo1 and Gsts, protecting against oxidative or electrophilic stress. However, nothing is known about the influence of Nrf2 on the kinetics of xenobiotics. Therefore, the purpose of the third aim was to investigate the effects Nrf2 has on the kinetics of sulfobromopthalein (BSP), a

xenobiotic used to study hepatobiliary function, as well as AA, a well known hepatotoxicant. Both BSP and AA are biotransformed by enzymes under the transciriptional control of Nrf2.

Overall, the data generated from these studies will 1) determine whether the natural triterpenoid oleanolic acid and its synthetic derivative, CDDO-Im, activate Nrf2 and protect against AA hepatotoxicity; 2) characterize the mRNA expression of oxidative stress and drug processing genes among wild-type, Nrf2-null, and Keap1-kd mice to lay a foundation for future studies; and 3) determine how Nrf2 effects the kinetics of BSP and AA.

PART I

Pharmacologic Activation of Nrf2 and Induction of Target Genes by the Natural Triterpenoid Oleanolic Acid and a Synthetic Derivative CDDO-Im Protect the Liver from Acetaminophen Hepatotoxicity

CHAPTER ONE

Oleanolic Acid Activates Nrf2 and Protects Against Acetaminophen Hepatotoxicity

ABSTRACT

Oleanolic acid (OA) is a natural triterpenoid found in some plants, which protects against various hepatotoxicants. In order to determine whether nuclear factor erythroid-2 related factor 2 **(**Nrf2), a transcription factor known to induce various antioxidant and cytoprotective genes, is activated by OA, wild-type and Nrf2-null mice were treated with OA (90 mg/kg, i.p.) once daily for three days. OA increased nuclear accumulation of Nrf2 in wild-type but not Nrf2-null mice, as determined by Western blot and immunofluorescence. OA-treated wild-type mice had increased hepatic mRNA expression of the Nrf2 target genes NAD(P)H:quinone oxidoreductase 1 (Nqo1); glutamate-cysteine ligase, catalytic subunit (Gclc); heme oxygenase-1 (Ho-1); as well as Nrf2 itself. In addition, OA increased protein expression and enzyme activity of the prototypical Nrf2 target gene, Nqo1, in wild-type, but not in Nrf2-null mice. OA protected against acetaminophen (AA) hepatotoxicity in wild-type mice, but less so in Nrf2-null mice. Collectively, the present study demonstrates that OA facilitates Nrf2 nuclear accumulation, causing induction of Nrf2-dependent genes, which contributes

to protection from acetaminophen hepatotoxicity.

INTRODUCTION

Oleanolic acid (OA) is a natural triterpenoid that is a constituent of the leaves of *Olea europaea, Viscum album L*., and other plants, and is an aglycone of many saponins. OA is used in Chinese medicine for the treatment of liver disorders, such as viral hepatitis, and has been shown to protect mice from various hepatotoxicants that cause oxidative and electrophilic stress, including carbon tetrachloride, acetaminophen (AA), bromobenzene, and thioacetamide. In mice pretreated with OA, and then exposed to one of the above chemical hepatotoxicants, serum alanine transaminase concentrations and centrilobular necrosis were diminished (Liu *et al.*, 1993a; Liu *et al.*, 1994a; Liu *et al.*, 1994b; Liu *et al.*, 1995b).

The purpose of the present study was to determine whether OA activates the Nrf2-Keap1 pathway and whether this contributes to protection from AA hepatotoxicity. Nuclear factor erythroid 2-related factor 2 (Nrf2), and its repressor kelch-like ECH-associated protein 1 (Keap1), have been characterized over the past decade as an important endogenous cellular mechanism for coping with oxidative stress (Kensler *et al.*, 2007). Nrf2 is a

transcription factor that binds to antioxidant response elements (AREs) in upstream promoter regions of various cytoprotective enzymes, the induction of which helps restore the intracellular balance between oxidants and antioxidants. Under conditions when oxidative/electrophilic stress is low, Keap1 sequesters Nrf2 in the cytosol, by acting as an adaptor for Cul3-based E3 ligase, to target Nrf2 for proteasomal degradation (Tong *et al.*, 2006). When oxidative/electrophilic stress is increased in the cell, Nrf2 is able to avoid Keap1-mediated proteasomal degradation by Cul3, and translocate into the nucleus (McMahon *et al.*, 2003). Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein and binds to AREs, promoting transcription of various cytoprotective genes (Itoh *et al.*, 1997; Venugopal and Jaiswal, 1998; He *et al.*, 2001).

Nrf2 target genes include, but are not limited to, glutathione-*S*-transferases (Gsts), NAD(P)H:quinone oxidoreductase 1 (Nqo1), UDP-glucuronosyltransferases, glutamate-cysteine ligase catalytic subunit (Gclc), glutamate-cysteine ligase modifier subunit (Gclm), and heme oxygenase-1 (Ho-1). Such enzymes are referred to as the “Nrf2 regulon” and provide

antioxidative protection (Kobayashi and Yamamoto, 2006). Nrf2-null mice have compromised antioxidant protection and are highly susceptible to target-organ injury from AA (Enomoto *et al.*, 2001), benzo[a]pyrene (Ramos-Gomez *et al.*, 2001), diesel exhaust (Aoki *et al.*, 2001), hyperoxia (Cho *et al.*, 2002), hydrogen peroxide (Kraft *et al.*, 2004), and many other oxidative-type pathologies. In contrast, mice with a hepatocyte-specific deletion of Keap1 have marked increases in Nrf2 target genes and protection from AA hepatotoxicity (Okawa *et al.*, 2006).

In wild-type mice, OA induces many cytoprotective genes, such as the Nrf2-dependent genes Nqo1, Gclc, and Ho-1 (3-fold to 5-fold over vehicle) (Liu *et al.*, 2008). However, in this study by Liu et al. nuclear translocation of Nrf2 protein was implied but not actually shown. Furthermore, Nrf2-null mice were not used by Liu et al. to demonstrate definitively whether induction of Nqo1, Gclc, and Ho-1 was caused by Nrf2 activation, as other transcription factors and mechanisms are capable of inducing these genes (Dahl and Mulcahy, 2001; Okey *et al.*, 2005; Ferrandiz and Devesa, 2008; Knight *et al.*, 2008). The purpose of the present study was to investigate whether OA (90

mg/kg, i.p.), administered once daily for three days, as was used previously in hepatoprotection studies (Liu *et al.*, 1995a), activates the Nrf2-Keap1 pathway using wild-type and Nrf2-null mice, and whether activation of Nrf2 is responsible for OA-mediated protection against AA hepatotoxicity.

METHODS

Reagents. OA was a gift from Dr. Jie Liu (NIEHS, Research Triangle Park, NC). Nqo1 and β-actin antibodies were purchased from Abcam (Ab2346, Ab8227, respectively, Cambridge, MA). Nrf2 antibody was purchased from Santa Cruz Biotechnology (sc-30915, Santa Cruz, CA). All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Husbandry. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories, Inc (Wilmington, MA). Nrf2-null mice on a mixed C57BL/6 and AKR background were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) and backcrossed seven generations into C57BL/6 mice to >99% congenicity, as was determined by Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and fed Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) *ad libitum*. The housing facility is an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all

procedures were preapproved in accordance with the Institutional Animal Care and Use Committee guidelines.

Animal Treatment. Wild-type and Nrf2-null mice were dosed with OA (90 mg/kg, i.p.) or vehicle (2% Tween 80, 10 mL/kg, i.p) in the morning, once daily for three days. Livers were removed on the morning of the fourth day, frozen in liquid nitrogen, and stored at -80ºC. For the hepatoprotection study, wild-type and Nrf2-null mice were dosed with OA (90 mg/kg, i.p.) or vehicle (2% Tween 80, 10 mL/kg, i.p) in the morning, once daily for three days. On the fourth day, mice were administered AA (500 mg/kg, i.p.) in saline (pH 8). Blood and livers were collected 8 h later. Consistent cross-sections from the largest lobe of the liver were fixed in 10% zinc formalin for 48 h.

Total RNA Isolation. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Total RNA concentrations were determined spectrophotometrically at 260 nm. The RNA samples were diluted to 500 ng/μL with diethyl pyrocarbonate-treated deionized water.

Branched DNA Signal Amplification (bDNA) Analysis. The mRNA expression of Nqo1, Gclc, and Ho-1 in mouse livers was quantified using the bDNA assay (Quantigene 1.0 bDNA signal amplification kit; Panomics, Inc., Fremont, CA) with modifications. Gene sequences of interest were accessed from GenBank. Target sequences were analyzed using ProbeDesigner software v1.0 (Chiron Diagnostics Corp., Walpole, MA) to design oligonucleotide probe sets (capture, label, and blocker probes). All probes were designed with a melting temperature of 63ºC, enabling hybridization conditions to be held constant (i.e., 53ºC) during each hybridization step. Probe sets for Ho-1 and Nqo1 were previously described (Aleksunes *et al.*, 2005). Probe sets for Gclc and metallothionein-1 (MT) are listed in Table 1.1. Because of sequence homology between MT and MT, the MT probe set may also detect MT. Total RNA was added to each well of a 96-well plate containing 50 μL of each diluted probe set. RNA was allowed to hybridize with the probe sets overnight at 53ºC. Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was quantified with a Synergy 2 Multi-Detection Microplate Reader interfaced with

Gen5 Reader Control and Data Analysis Software (Biotek, Winoosky, VT). Data are presented as relative light units (RLU) normalized to control.

Nrf2 and Nqo1 Protein Expression. Livers were homogenized in sucrose-Tris buffer (0.25 mol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4) and centrifuged at 100,000 g for 60 min at 4℃. The resulting supernatant, containing the cytosolic fraction, was used to assay for Nqo1 protein. Nuclear extracts were prepared with the NE-PER nuclear extraction kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Nuclear extracts were used for Nrf2 immunoblotting. Protein concentrations were determined with BCA Assay Kit from Pierce Biotechnology (Rockford, IL).

Cytosolic and nuclear proteins (40 µg protein/lane) were electrophoretically resolved using polyacrylamide gels (4% stacking and 12% resolving for Nqo1; 4% stacking and 10% resolving for Nrf2). Gels were transblotted overnight at 4ºC onto a nitrocellulose membrane for Nqo1, and a polyvinylidene fluoride membrane for Nrf2. Membranes were then washed with PBS–buffered saline containing 0.05% Tween-20 (PBS-T). Membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS–T.

Blots were then incubated with primary antibody (1:1000 dilution for both Nqo1 and Nrf2, in 2% non-fat milk in PBS-T) for 3 h at room temperature. Blots were then washed in PBS-T and incubated with secondary antibody conjugated with horseradish peroxidase (1:2000 dilution for Nqo1 and Nrf2) in 2% non-fat milk in PBS-T buffer for 1 hr at room temperature. Blots were then washed with PBS-T. Protein-antibody complexes were detected using an enhanced chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film (Denville Scientific, Metuchen, NJ). Intensity of protein bands was quantified using the Discovery Series Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA). Intensity values were normalized to β-actin and expressed as relative protein expression.

***Immunofluorescence*.** Nrf2 was detected as previously described (Slitt *et al.*, 2006). Briefly, cryosections were air-dried at room temperature for 5 min and fixed with 4% paraformaldehyde. The sections were blocked at room temperature for 30 min with 5% donkey serum/phosphate-buffered saline with 0.2% Triton X-100 (PBS-Tx), and then incubated overnight with Nrf2 antibody diluted 1:50 in 5% donkey serum/PBS-Tx. A fluorescein isothiocyanate-

labeled secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:200 was used, along with rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA) diluted 1:200 in 5% donkey serum/PBS-Tx. 4,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) staining was performed to define nuclear regions. Antibody solutions were filtered through 0.22 μm membrane syringe-driven filter units (Osmonics Inc., Minnetonka, MN) prior to use. Frozen liver sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included in the analysis (data not shown). Images were captured on an Olympus BX41 fluorescent microscope with a DP70 camera and DP Controller software (Olympus, Melville, NY).

Nqo1 activity assay. Cytosolic Nqo1 enzyme activity was determined by quantifying the reduction of 2,6-dichlorophenol-indophenol (DCPIP) as described previously (Ernster, 1967; Benson *et al.*, 1980).

Serum alanine transaminase (ALT) concentrations. Serum ALT concentrations were determined as a biochemical indicator of hepatocellular necrosis using Pointe Scientific Liquid ALT Reagent Set (Canton, MI)

according to the manufacturer's protocol.

Histopathology. Liver samples were fixed in 10% neutral-buffered zinc formalin prior to routine processing and paraffin embedding. Liver sections (5 µm) were stained with hematoxylin and eosin. Liver sections were analyzed and assigned one of six scores of severity of necrosis: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). A section was defined as a single viewpoint at low power (x100) magnification.

Statistical Analysis. All data but histological grading were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$). Histopathological data were rank ordered prior to ANOVA analysis, which was followed by Newman-Keuls multiple range test ($p \leq 0.05$).

RESULTS

Nrf2 nuclear translocation in OA-treated wild-type and Nrf2-null mice. OA increased Nrf2 protein 432% in nuclear extracts from livers of wild-type mice (Fig 1.1, upper). Nrf2-null mice treated with vehicle or OA did not have detectable levels of Nrf2 protein in the nuclear fractions. Indirect immunofluorescent analysis revealed minimal Nrf2 staining (green) in frozen sections from wild-type mice (Fig 1.1, lower). The actin cytoskeleton was stained in red. Nrf2 staining of liver sections from OA-treated wild-type mice was strong and localized to the nucleus (blue). No Nrf2 staining was observed in vehicle- or OA-treated Nrf2-null mice.

Nrf2, Nqo1, Gclc, and Ho-1 mRNA expression in OA-treated wild-type and Nrf2-null mice. OA increased mRNA expression of Nrf2 (347%), Nqo1 (1108%), Gclc (521%), and Ho-1 (955%) in wild-type mice (Fig 1.2). Nrf2-null mice treated with OA showed no increase in Nrf2, Nqo1, Gclc, or Ho-1 mRNA expression.

Nqo1 protein expression and enzyme activity in OA-treated wild-type and Nrf2-null mice. Because Nqo1 is considered the prototypical Nrf2-target

gene, Nqo1 protein and enzyme activity were evaluated. Wild-type mice treated with OA had increased Nqo1 protein expression (79%) as determined by western blot (Fig 1.3, upper). Nrf2-null mice had much lower Nqo1 protein expression (-73%) than wild-type mice. Furthermore, OA did not change Nqo1 protein expression in Nrf2-null mice. OA induced Nqo1 enzyme activity 154% in wild-type mice (Fig 1.3, lower). Basal Nqo1 activity is lower in Nrf2-null mice (-47%) than in wild-type mice and is not induced by OA.

OA-mediated protection against AA in wild-type and Nrf2-null mice. OA did not alter serum ALTs or liver histology in wild-type or Nrf2-null mice (data not shown). OA-pretreatment decreased AA-induced hepatotoxicity in wild-type mice, as indicated by an 82% reduction in serum ALTs (Fig 1.4) and reduction in necrosis grade (Table 1.2). AA produced more severe hepatotoxicity in Nrf2-null mice (increases in serum ALTs [250%] and necrosis grade) than in wild-type mice, similar to a previous report of increased sensitivity of Nrf2-null mice (Enomoto *et al.*, 2001). OA-pretreatment of Nrf2-null mice protected against AA hepatotoxicity (reduction in serum ALTs [65%] and necrosis grade), but less so than in wild-type mice.

Of note, one Nrf2-null mouse pretreated with vehicle and challenged with AA died before completion of the study (8 h).

MT mRNA expression in *OA-treated wild-type and Nrf2-null mice*. Because OA produced some protection from AA hepatotoxicity in Nrf2-null mice, mRNA expression of MT, a Nrf2-independent gene (Ohtsuji *et al.*, 2008) that has been shown to protect against AA hepatotoxicity (Liu *et al.*, 1999), was quantified in OA-treated wild-type and Nrf2-null mice (Fig 1.5). OA induced hepatic MT mRNA expression in both wild-type (31-fold) and Nrf2-null mice (22-fold).

Table 1.1. Oligonucleotide probes used for analysis of mouse gene expression by bDNA signal amplification assay.

Gene	Accession #	Target[a]	Function[b]	Probe Sequence
Gclc	**NM_012815**	1548-1566	CE	atggctcggagctggtctgTTTTTctcttggaaagaaagt
		1996-1724	CE	cttaattagcttcaggtagttcagaataTTTTTctcttggaaagaaagt
		1725-1749	CE	tcattagttctccagatgctctcttTTTTTctcttggaaagaaagt
		1848-1870	CE	tcattagttctccagatgctctcttTTTTTctcttggaaagaaagt
		1896-1917	CE	acttcgcttttctaaagcctgaTTTTTctcttggaaagaaagt
		1528-1547	LE	ggccttgctacacccatccaTTTTTaggcataggacccgtgtct
		1567-1588	LE	atgagcgtgtactcctctgcagTTTTTaggcataggacccgtgtct
		1589-1612	LE	ccattgatgatggtgtctatgctcTTTTTaggcataggacccgtgtct
		1656-1678	LE	tcgacttccatgttttcaaggtaTTTTTaggcataggacccgtgtct
		1679-1696	LE	ctgcatcgggtgtccacgTTTTTaggcataggacccgtgtct
		1750-1770	LE	ctctcatccacctggcaacagTTTTTaggcataggacccgtgtct
		1771-1794	LE	agtcaggatggtttgcaataaactTTTTTaggcataggacccgtgtct
		1822-1847	LE	tttcaaaatgaggctatagttgatctTTTTTaggcataggacccgtgtct
		1918-1939	LE	gggtcgcttttacctccactgtTTTTTaggcataggacccgtgtct
		1613-1632	BL	caggaaacacgccttccttc
		1633-1655	BL	ggagttcagaatggggatgagtc
		1795-1821	BL	catcagttattacactgtcttgcttgt
		1871-1895	BL	tccaagtaactctggacattcacac
MT	**NM_013602**	72-92	CE	ggtccattccgagatctggtgTTTTTctcttggaaagaaagt
		170-190	CE	ggagcagcagctcttcttgcaTTTTTctcttggaaagaaagt
		130-147	LE	caggcgcaggagctggtgTTTTTaggcataggacccgtgtct
		191-207	LE	cagcccacgggacagcaTTTTTaggcataggacccgtgtct
		227-244	LE	ggcgcctttgcagacacaTTTTTaggcataggacccgtgtct
		245-262	LE	gcacgtgcacttgtccgcTTTTTaggcataggacccgtgtct
		263-283	LE	ctgttcgtcacatcaggcacaTTTTTaggcataggacccgtgtct
		284-302	LE	tttacacgtggtggcagcgTTTTTaggcataggacccgtgtct
		303-323	LE	cgctgggttggtccgatactaTTTTTaggcataggacccgtgtct
		93-112	BL	ggtggagcaggagcagttgg
		113-129	BL	caagtgcaggagccgcc
		148-169	BL	ggaggtgcacttgcagttcttg
		208-226	BL	gccctgggcacatttggag

[a]Target refers to the sequence of the mRNA transcript as enumerated in the GenBank file.

[b]Function refers to the use of the oligonucleotide probe in the assay (CE, capture extender; LE, label extender; BL, blocker probe).

Table 1.2. Histological analysis of livers from vehicle- and OA-pretreated aild-type and Nrf2-null mice after AA challenge.

	Histological Grade						
Treatment Group	**0**	**1**	**2**	**3**	**4**	**5**	**p≤0.05**
Wild-type VC-AA	0	0	0	2	3	0	
Wild-type OA-AA	0	3	2	0	0	0	*
Nrf2-null VC-AA	0	0	0	0	0	5	*
Nrf2-null OA-AA	0	0	0	1	4	0	†

Grade of liver injury in wild-type and Nrf2-null mice pretreated with OA (90 mg/kg, i.p.) once daily for three days (n=5 for all groups except Nrf2-null mice receiving vehicle, n=4), administered acetaminophen (500 mg/kg, i.p.) on the fourth day, and sacrificed 8-h later. Liver sections were analyzed at low power (x100) with six scores of severity: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). Values are expressed as mean ± S.E.M. Abbreviations: WT, wild-type; null, Nrf2-null. Asterisks (*) indicate a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$). Daggers (†) indicate a statistically significant difference from Nrf2-null mice receiving vehicle ($p \leq 0.05$).

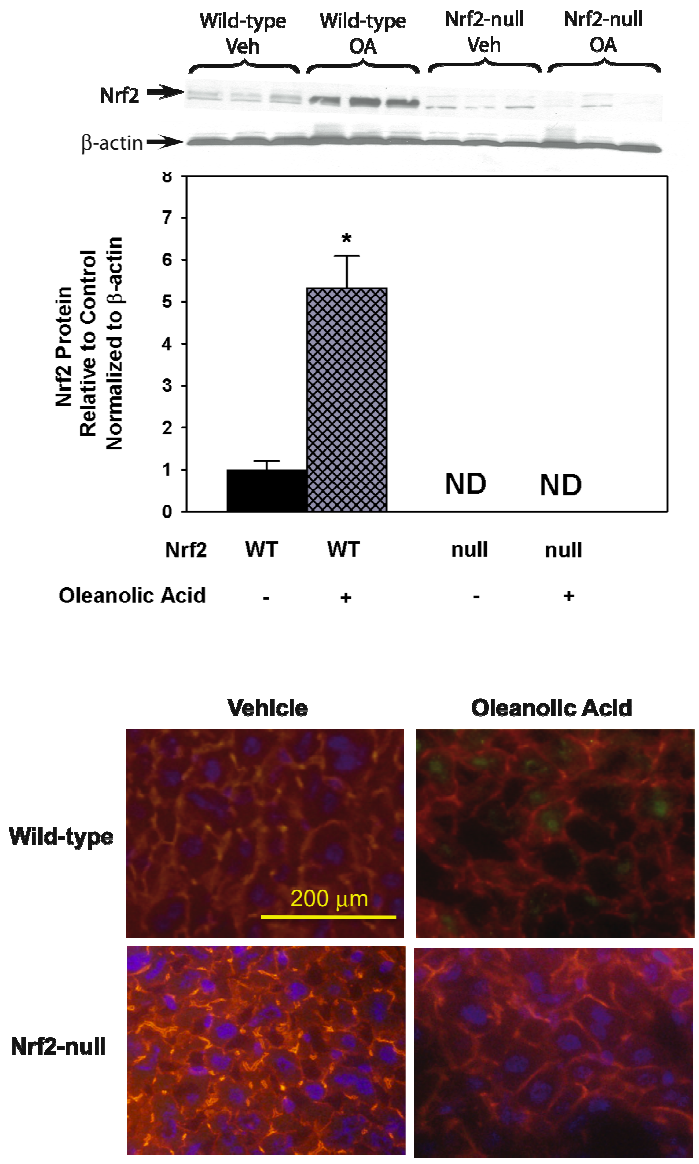


Fig 1.1. *Upper*. Western blot of liver nuclear fractions for Nrf2 after treatment with OA (90 mg/kg, i.p.) once daily for three days. Also shown is the quantification of specific band intensity, normalized to β-actin, and expressed relative to control as mean ± S.E.M. Abbreviations: ND, Not Detected; Veh,

Vehicle; OA, OA; WT, wild-type; null, Nrf2-null. Asterisks (*) indicate a statistically significant difference from wild-type mice treated with vehicle ($p \leq 0.05$). *Lower*. Immunofluorescent localization of Nrf2 in livers from wild-type mice after vehicle or OA. Indirect immunofluorescence to detect Nrf2 (green) and actin (red) was performed on liver cryosections (5 μm) from wild-type mice after OA (90 mg/kg, i.p.) treatment once daily for three days. Sections were mounted in Prolong Gold containing DAPI for nuclear staining (blue). Representative images are shown at high-power magnification (x400). Bar represents 200 μm.

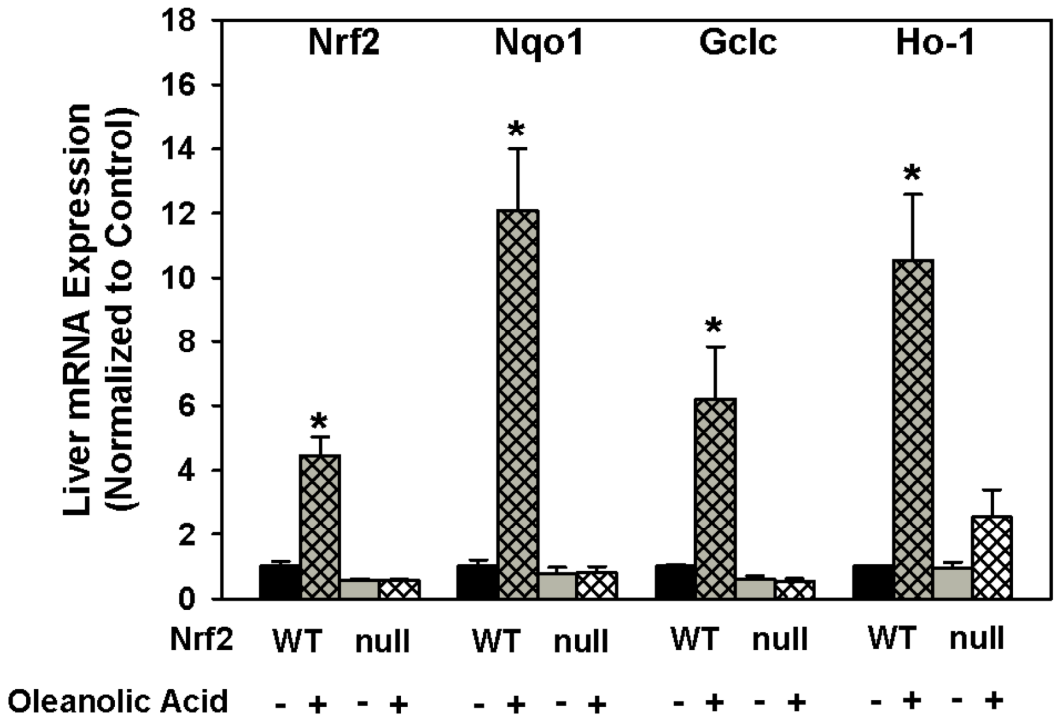


Fig 1.2. Messenger RNA expression of Nqo1, Gclc, and Ho-1 in livers of wild-type and Nrf2-null mice after treatment with OA (90 mg/kg, i.p.) once daily for three days. Messenger RNA was quantified by the bDNA assay. Data is expressed relative to wild-type controls as mean ± S.E.M. Abbreviations: WT, wild-type; null, Nrf2-null. Asterisks (*) indicate a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

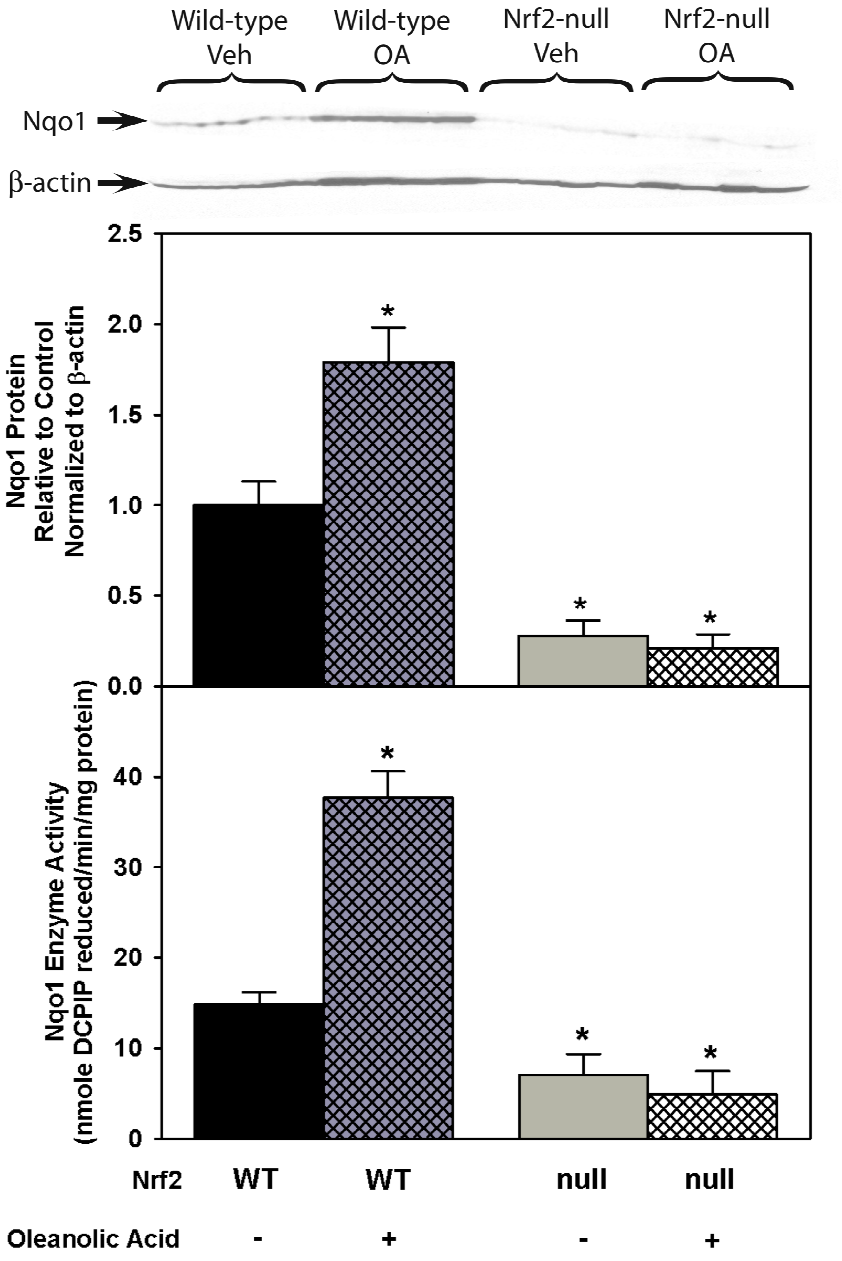


Fig 1.3. *Upper*. Protein expression of Nqo1 determined by western blot in livers of wild-type and Nrf2-null mice after treatment with OA (90 mg/kg, i.p.)

once daily for three days. Also shown is the quantification of specific band intensity, normalized to β-actin, and expressed relative to control as mean ± S.E.M. *Lower*. Nqo1 enzyme activity in livers of wild-type and Nrf2-null mice after treatment with OA (90 mg/kg, i.p.) once daily for three days. Units are in nmole DCPIP reduced per min per mg of protein expressed as mean ± S.E.M. Abbreviations: Veh, Vehicle; OA, OA; WT, wild-type; null, Nrf2-null. Asterisks (*) indicate a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

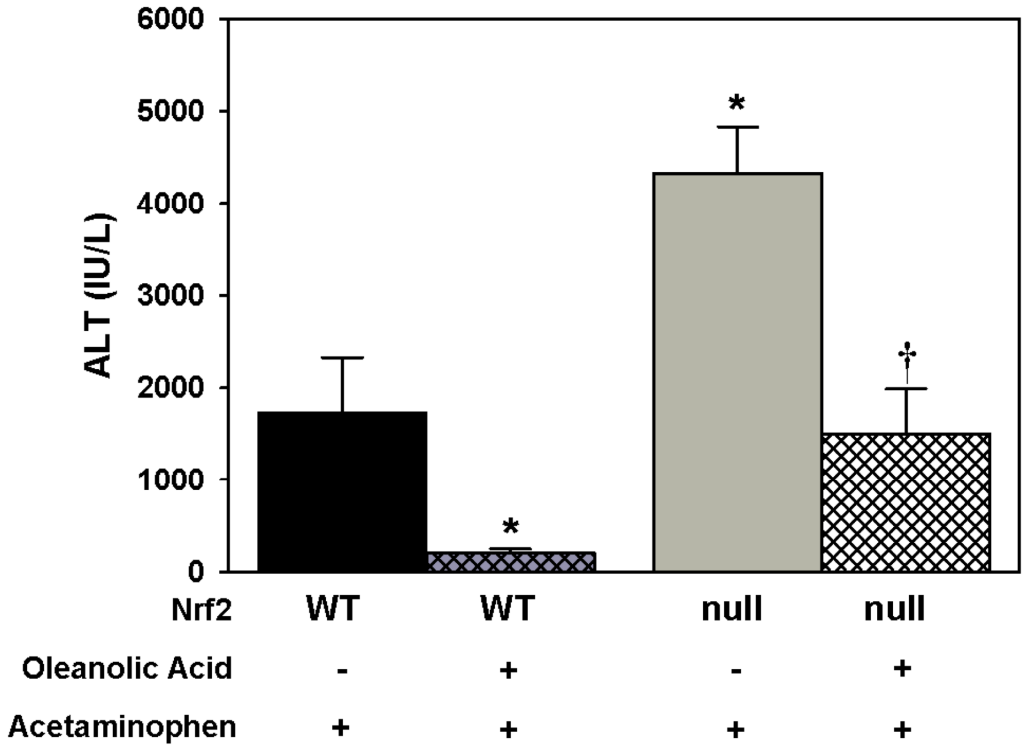


Fig 1.4. Serum alanine transaminase (ALT) levels in wild-type and Nrf2-null mice pretreated with OA (90 mg/kg, i.p.) once daily for three days (n=5 for all groups except Nrf2-null mice receiving vehicle, n=4), administered acetaminophen (500 mg/kg, i.p.) on the fourth day, and sacrificed 8-h later. Units are in International Units/Liter (IU/L) expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$). Daggers (†) indicate a statistically significant difference from Nrf2-null mice receiving vehicle ($p \leq 0.05$).

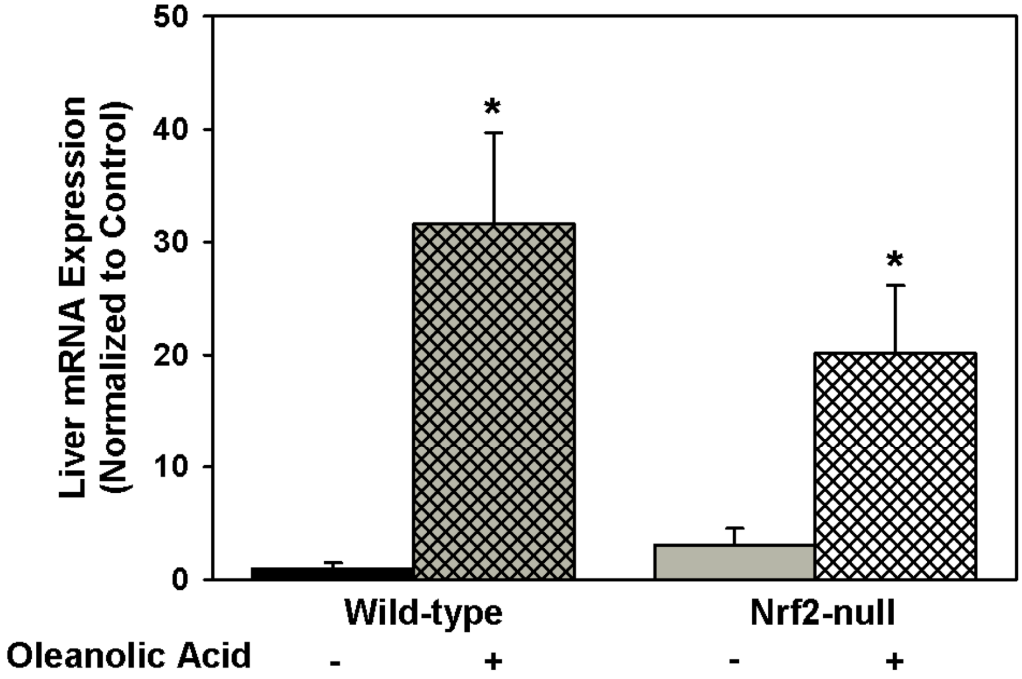


Fig 1.5. Messenger RNA expression of MT from livers of wild-type and Nrf2-null mice after treatment with OA (90 mg/kg, i.p.) once daily for three days. Messenger RNA was quantified by the bDNA assay. Data is expressed relative to wild-type controls as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

DISCUSSION

OA is a natural triterpenoid used for many years in the treatment of hepatitis in China. In mice, OA can protect the liver from various xenobiotics, such as the classic hepatotoxicants carbon tetrachloride, AA, bromobenzene, thioacetamide, and cadmium (Liu *et al.*, 1993a; Liu *et al.*, 1993b; Liu *et al.*, 1994a; Liu *et al.*, 1994b; Liu *et al.*, 1995a). These chemicals exert hepatic damage, in part, by generating hepatocellualar oxidative stress (Marzella *et al.*, 1986).

Nrf2 is a transcription factor capable of inducing the expression of a battery of cytoprotective and antioxidant proteins, including Nqo1, Gclc, and Ho-1. Nqo1 catalyzes the reduction of quinones, protecting cells against redox cycling and oxidative stress (Riley and Workman, 1992). More specifically for this study, Nqo1 can reduce the toxic metabolite of AA back to the parent compound *in vitro* (Moffit *et al.*, 2007). Gclc is the rate-limiting enzyme in the synthesis of glutathione, a major antioxidant molecule in cells, which is critical for the maintenance of cellular redox homeostasis and detoxification of electrophiles (Dalton *et al.*, 2000). Ho-1 catalyzes the

breakdown of heme into bilirubin, carbon monoxide, and iron. Bilirubin is an antioxidant, and its generation following Ho-1 induction reduces cytotoxicity caused by oxidative stress (Clark *et al.*, 2000). In addition, small quantities of carbon monoxide are cytoprotective, anti-apoptotic, vasorelaxant, and anti-inflammatory (Ryter and Otterbein, 2004). Also, hemin-mediated induction of Ho-1 or pretreatment with biliverdin (a bilirubin precursor) protects rats against AA-induced hepatotoxicity (Chiu *et al.*, 2002).

Previous studies have suggested that OA is unable to activate the Nrf2-Keap1 pathway because OA is a natural compound and lacks necessary functional groups (Michael acceptors) possessed by its synthetic derivatives, including CDDO-Im (Dinkova-Kostova *et al.*, 2005; Yates *et al.*, 2007). Nqo1 is a prototypical target gene of Nrf2 activation; therefore, Nqo1 mRNA expression and enzyme activity are used as biomarkers of Nrf2 activation. In previous studies, OA was unable to induce Nqo1 activity *in vitro* in Hepa1c1c7 murine hepatoma cells (Dinkova-Kostova *et al.*, 2005). In addition, a low, single, oral dose of OA (9 mg/kg) failed to induce hepatic Nqo1 mRNA expression in mice (Yates *et al.*, 2007). Thus, in these previous

studies, OA did not activate Nrf2 because both the dose and number of doses of OA were insufficient.

Because it has previously been shown that OA (90 mg/kg, i.p.) given once daily for three days is protective against various hepatotoxicants (Liu *et al.*, 1995a), the present studies were undertaken to establish whether OA is an activator of the Nrf2-Keap1 pathway. OA (90 mg/kg, i.p.), given once daily for three days, increases nuclear accumulation of Nrf2 in wild-type, but not in Nrf2-null mice, as determined by both western blot of nuclear fractions and immunofluorescence. Also, the OA-dosing regimen used in the current studies, increases mRNA of Nrf2, as well as three characteristic Nrf2-target genes (Nqo1, Gclc, and Ho-1) in wild-type, but not in Nrf2-null mice.

In addition to mRNA expression, Nqo1 protein expression and activity were quantified because Nqo1 is a prototypical Nrf2-target gene (Venugopal and Jaiswal, 1996; Dhakshinamoorthy and Jaiswal, 2000; Jaiswal, 2000; Dhakshinamoorthy and Jaiswal, 2001). OA increased protein expression of Nqo1 in wild-type mice (Fig 4). Nrf2-null mice have lower Nqo1 protein expression than their wild-type counterparts, as reported previously

(Aleksunes *et al.*, 2006c). OA was not able to increase Nqo1 protein expression in Nrf2-null mice, which correlates with the mRNA expression data. In addition, OA increased Nqo1 enzyme activity in wild-type, but not Nrf2-null mice.

The functional involvement of Nrf2 in OA-mediated hepatoprotection was investigated using AA as a model hepatotoxicant. AA produced liver injury in both genotypes, but the injury was more severe in Nrf2-null mice. Enhanced susceptibility of Nrf2-null mice to AA is consistent with previous reports (Enomoto *et al.*, 2001). OA-pretreatment of wild-type mice decreased AA hepatotoxicity. OA-pretreatment also decreased AA hepatotoxicity in Nrf2-null mice, although the reduction was less than in the wild-type mice.

Because OA also had some hepatoprotective effects against AA toxicity in Nrf2-null mice, the possibility that OA might induce MT in Nrf2-null mice and produce protection by this mechanism was evaluated. MT is a low-molecular-weight, cysteine-rich, metal-binding protein that is suggested to be capable of trapping reactive oxygen and nitrogen species, as well as electrophiles (Klaassen *et al.*, 1999), which all contribute to the pathogenesis

of AA hepatotoxicity (Michael *et al.*, 1999). MT has at least one ARE in its promoter region and is induced by Nrf1 (a transcription factor in the same family as Nrf2), but not Nrf2 (Ohtsuji *et al.*, 2008). OA induces MT in both mice and rats (Liu *et al.*, 2008), and OA-mediated MT induction protects against cadmium toxicity (Liu *et al.*, 1993b). In addition, MT-null mice are more susceptible than wild-type mice to AA-mediated hepatotoxicity (Liu *et al.*, 1993b). OA induced hepatic MT mRNA expression in both wild-type and Nrf2-null mice (Fig 5), which suggests that that other mechanisms, especially MT induction, in addition to Nrf2 activation might contribute to protection from OA-mediated hepatoprotection from AA.

Other genes that may contribute to Nrf2-independent hepatoprotection by OA include a 70 kDa heat shock protein (Hsp70) and the G protein-coupled bile acid receptor 1 (Gpbar1 or Tgr5). Hsp70 mRNA is induced by OA (Liu *et al.*, 2008) and acts to protect cells by reducing the ability of oxidized proteins to aggregate, permitting them time to refold and return to a functional conformation. Furthermore, Hsp70-null mice are more sensitive to AA hepatotoxicity (Tolson *et al.*, 2006). TGR5 mRNA is also induced by OA

(Sato *et al.*, 2007) and is a bile acid responsive and cell surface G protein-coupled receptor that when activated suppresses macrophage proinflammatory cytokine production, particularly interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor α (TNFα) (Kawamata *et al.*, 2003; Keitel *et al.*, 2008). Induction of IL-1 and TNFα lead to induction of inducible nitric oxide synthase (iNos) (Busse and Mulsch, 1990; Kilbourn *et al.*, 1990). iNos contributes to acetaminophen hepatotoxicity by leading to increased production of nitric oxide and subsequent peroxynitrite formation. Thus, iNos suppression, possibly mediated by OA, may contribute to the observed hepatoprotection from AA in Nrf2-null mice (Bourdi *et al.*, 2002; James *et al.*, 2003). Thus, OA has Nrf2-dependent and Nrf2-independent effects, both of which likely contribute to OA-mediated hepatoprotection from AA.

Collectively, increased nuclear accumulation of Nrf2, increased mRNA expression of Nrf2-target genes, and up-regulation of Nqo1 protein and activity provide strong evidence that OA activates the Nrf2-Keap1 pathway. In addition OA protected from AA hepatotoxicty in wild-type but less so in Nrf2-null mice, suggesting that OA also activates Nrf2-independent

cytoprotective mechanisms. Thus, this study establishes OA as a Nrf2 activator; however, Nrf2-independent mechanisms that contribute to OA-mediated hepatoprotection from AA require further study.

CHAPTER TWO

CDDO-Im Protects from Acetaminophen Hepatotoxcity Through Induction of Nrf2-Dependent Genes

ABSTRACT

CDDO-Im is a synthetic triterpenoid recently shown to induce cytoprotective genes through the Nrf2-Keap1 pathway, an important mechanism for the induction of cytoprotective genes in response to oxidative stress. Upon oxidative or electrophilic insult, the transcription factor Nrf2 translocates to the nucleus, heterodimerizes with small Maf proteins, and binds to antioxidant response elements (AREs) in the upstream promoter regions of various cytoprotective genes. To further elucidate the hepatoprotective effects of CDDO-Im, wild-type and Nrf2-null mice were pretreated with CDDO-Im (1 mg/kg, i.p.) or vehicle (DMSO), and then administered acetaminophen (500 mg/kg, i.p.). Pretreatment of wild-type mice with CDDO-Im reduced liver injury caused by acetaminophen. In contrast, hepatoprotection by CDDO-Im was not observed in Nrf2-null mice. CDDO-Im increased Nrf2 protein expression and Nrf2-ARE binding in wild-type, but not Nrf2–null mice. Furthermore, CDDO-Im increased the mRNA expression of the Nrf2 target genes NAD(P)H: quinone oxidoreductase-1 (Nqo1); glutamate-cysteine ligase, catalytic subunit (Gclc); and heme-oxygenase-1 (Ho-1), in both a

dose- and time-dependent manner. Conversely, CDDO-Im did not induce Nqo1, Gclc, and Ho-1 mRNA expression in Nrf2-null mice. Collectively, the present study shows that CDDO-Im pretreatment induces Nrf2-dependent cytoprotective genes and protects the liver from acetaminophen-induced hepatic injury.

INTRODUCTION

Oxidative stress is an injurious phenomenon that results from an imbalance in the ratio of pro-oxidant species to anti-oxidative defense mechanisms within a cell that ultimately damages three major critical macromolecules in cells: DNA, lipids, and proteins. Oxidative damage can lead to a variety of pathologies, including cancer, Parkinson's disease, atherosclerosis, diabetes, Alzheimer's disease, and even the aging process (Valko *et al.*, 2007). One, or a combination of the following three factors, manifests oxidative stress. The first factor is an increase in the amount of oxidants, which include any number of highly reactive species possessing a single unpaired electron, capable of damaging DNA, lipids, and proteins. In addition to the amount of oxidants, there can be a decrease in the antioxidant status. Antioxidants include free radical scavengers, such as glutathione (GSH), ascorbic acid, and α-tocopherol. There are also detoxifying enzymes that contribute to antioxidant status, such as glutathione-*S*-transferases (Gsts); UDP-glucuronyl transferases (Ugts); NAD(P)H: quinone oxidoreductase 1 (Nqo1); catalytic and modifier subunits of γ-glutamyl

cysteine ligase (Gclc, Gclm), which synthesize GSH; and heme oxygenase-1 (Ho-1), which catabolizes heme into iron, carbon monoxide, and the free radical scavenger bilirubin (Cho *et al.*, 2006). Finally, an inability to repair oxidized DNA, lipids, and proteins also may contribute to oxidative stress. Examples of macromolecule repair include DNA repair by base or nucleotide exclusion, and protein repair by thioredoxin and glutaredoxin.

Over the past decade, the Nrf2-Keap1 pathway has been characterized as an important endogenous mechanism for combating oxidative stress. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor that induces the expression of various cytoprotective enzymes possessing an antioxidant response element (ARE) in the promoter region. Nrf2 activation and subsequent cytoprotective gene induction promotes the restoration of the balance between oxidants and antioxidants after oxidative insult. Under conditions where oxidative stress is low, kelch-like ECH-associated protein-1 (Keap1) sequesters Nrf2 in the cytosol and acts as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2 (Tong *et al.*, 2006). Increased oxidative stress promotes

Nrf2 avoidance of Keap1-mediated proteasomal degradation by Cul3 and subsequent translocation of Nrf2 into the nucleus. Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein and binds to the ARE, promoting transcription of various cytoprotective genes (Itoh *et al.*, 1997). Nrf2-target genes include, but are not limited to Gsts, Nqo1, Ugts, Gclc, Gclm, and Ho-1, collectively being referred to as the "Nrf2 regulon" (Kobayashi and Yamamoto, 2006). Furthermore, Nrf2-null mice are susceptible to injury from acetaminophen (Enomoto *et al.*, 2001), benzo[a]pyrene (Ramos-Gomez *et al.*, 2001), diesel exhaust (Aoki *et al.*, 2001), hyperoxia (Cho *et al.*, 2002), hydrogen peroxide (Kraft *et al.*, 2004), and other types of oxidative stress-induced injury.

Oleanolic acid is a naturally-occurring triterpenoid used in China for the treatment of hepatitis and is protective against chemical-induced liver injury in mice (Liu *et al.*, 1993; 1995a; 1995b). Although, the limited efficacy of many naturally-occurring substances often precludes clinical use, but they may serve as a critical foundation for drug development. In an effort to create a more efficacious and potent triterpenoid, 2-cyano-3,12 dioxooleana-1,9 diene-

28-imidazolide (CDDO-Im) was synthesized (Honda *et al.*, 1998). CDDO-Im has been characterized as being effective in diminishing iNOS production in an *in vivo* model of inflammation (Place *et al.*, 2003). CDDO-Im also activates the Nrf2-Keap1 pathway in both *in vitro* and *in vivo* models (Liby *et al.*, 2005). Furthermore, pretreatment with CDDO-Im is protective against the LPS-inflammatory response and aflatoxin-induced liver tumorigenesis (Thimmulappa *et al.*, 2006; Yates *et al.*, 2006). The ability of CDDO-Im to act as both an anti-inflammatory and antioxidant chemical renders this compound a prime candidate for chemoprevention.

The purpose of the present study was to evaluate the protective effects of CDDO-Im in an acute chemical model of acetaminophen-induced hepatotoxicity. Because oxidative stress is a main contributor to the hepatotoxic effects of acetaminophen (Knight *et al.*, 2001; Jaeschke *et al.*, 2003), and CDDO-Im has been previously shown to activate Nrf2, the hepatoprotective effects of CDDO-Im against acetaminophen hepatotoxicity were evaluated in both wild-type and Nrf2-null mice.

METHODS

Materials. Acetaminophen and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). CDDO-Im was a generous gift from Dr. Michael Sporn (Dartmouth College, Hanover, New Hampshire). β-actin antibody was purchased from Abcam (Ab8227, Cambridge, MA). Nrf2 antibody was purchased from Santa Cruz Biotechnology (sc-30915, Santa Cruz, CA).

Animals and Husbandry. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Nrf2-null mice on a mixed C57BL/6 and AKR background were obtained from Dr. Jefferson Chan (University of California-Irvine, Irvine, CA). Nrf2-null mice were then backcrossed seven generations into C57BL/6 mice and >99% congenicity was determined by Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and had access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) and water *ad libitum*. The housing facility is an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were

preapproved in accordance with Institutional Animal Care and Use Committee guidelines.

Hepatotoxicity Study. Mice were treated with CDDO-Im (1 mg/kg, i.p.) or vehicle (DMSO, 5 mL/kg, i.p.) once daily for three days. On the fourth day, mice were administered acetaminophen (500 mg/kg, i.p.) or vehicle (saline, pH 8, 20 mL/kg, i.p.). Six hours after acetaminophen administration, blood samples were taken for quantification of serum ALTs and liver samples were taken for histopathology. A sample of liver was also frozen in liquid nitrogen and stored at -80ºC.

Serum alanine aminotransferase (ALT) concentrations. Serum ALT concentrations were determined as a biochemical indicator of hepatocellular necrosis using Pointe Scientific Liquid ALT Reagent Set (Canton, MI) according to the manufacturer's protocol.

Histopathology. Samples were taken consistently as cross-sections of the largest lobe of the liver. Liver sections, approximately 5 μm thick, were fixed in 10% neutral-buffered zinc formalin, processed, stained with hematoxylin and eosin, and analyzed by light microscopy for liver injury. Samples were

blinded before analysis. Grade of liver injury was analyzed semiquantitatively with six scores of severity: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). A section was defined as a single viewpoint at low power (x100) magnification.

Nrf2 Protein Expression. Nuclear extracts were prepared with the NE-PER nuclear extraction kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined with the BCA Assay Kit from Pierce Biotechnology (Rockford, IL). Nuclear proteins (60 µg protein/lane) were electrophoretically resolved using polyacrylamide gels (4% stacking and 7.5% resolving). Gels were transblotted overnight at 4ºC onto a polyvinylidene fluoride membrane. Membranes were then washed with PBS–buffered saline containing 0.05% Tween-20 (PBS-T). Membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS–T. Blots were then incubated with primary

antibody (1:1000 dilution, in 2% non-fat milk in PBS-T) for 3 h at room temperature. Blots were then washed in PBS-T and incubated with secondary antibody conjugated with horseradish peroxidase (1:2000 dilution, in 2% non-fat milk in PBS-T) for 1 hr at room temperature. Blots were then washed with PBS-T. Protein-antibody complexes were detected using an enhanced chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film (Denville Scientific, Metuchen, NJ). Intensity of protein bands was quantified using the Discovery Series Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA). Intensity values were expressed as protein expression relative to control. β-actin was utilized as a loading control.

Nrf2-ARE binding. Nrf2-ARE binding was determined using the ELISA-based TransAM™ Nrf2 Kit from Active Motif, according to manufacturer specifications (Carlsbad, CA). Briefly, the kit contains a 96-well plate to which oligonucleotides containing the consensus ARE have been immobilized. Nrf2 from nuclear extracts then binds to the oligonucleotide and is detected through use of an antibody against Nrf2. A secondary antibody

conjugated to horseradish peroxidase is then added and allowed to bind to the primary antibody. The signal is detected at 450 nm using a spectrophotomer. The data is displayed as mean optical density (OD) at 450 nm.

Dose-Response Study. Wild-type mice were dosed (i.p.) with CDDO-Im at 0.1, 1.0, 3.0, or 10.0 mg/kg or vehicle (DMSO, 5 mL/kg). Nrf2-null mice were dosed (i.p.) with CDDO-Im at 1.0 mg/kg or vehicle (DMSO, 5 mL/kg). Livers were removed 6 hrs after dosing, frozen in liquid nitrogen, and stored at -80ºC until RNA isolation.

Time-Course Study. Wild-type mice were dosed with CDDO-Im (1 mg/kg, i.p.) or vehicle (DMSO, 5 mL/kg, i.p.). Livers were removed 6, 12, 18, 24, 48, and 72 hrs after dosing, frozen in liquid nitrogen, and stored at -80ºC until RNA isolation.

Total RNA Isolation. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc.,

Friendswood, TX) according to the manufacturer's protocol. Total RNA concentrations were determined spectrophotometrically at 260 nm. Two

hundred ng/μl RNA solutions were prepared by dilution with diethyl pyrocarbonate-treated deionized water.

Branched DNA Signal Amplification (bDNA) Analysis. Specific mRNAs were quantified using the bDNA assay (1.0 Quantigene bDNA signal amplification kit; Panomics, Inc., Fremont, CA). Gene sequences of interest were accessed from GenBank. Target sequences were analyzed using ProbeDesigner software v1.0 (Bayer Corp., Emeryville, CA) to design oligonucleotide probe sets (capture, label, and blocker probes). All probes were designed with a melting temperature of 63ºC, enabling hybridization conditions to be held constant (i.e., 53ºC) during each hybridization step. Probe sets for Ho-1 and Nqo1 were developed as described previously (Aleksunes *et al.*, 2005). Probe sets for Gclc are listed in Table 1.1. Total RNA was added to each well of a 96-well plate containing 50 μL of each diluted probe set. RNA was allowed to hybridize at 53ºC with the probe sets overnight. Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was quantified with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management

software v5.02. Data are presented as relative light units (RLU) or as normalized to control.

Statistical Analysis. All data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$). ALT values were log transformed before statistical analysis. Histopathological data were rank ordered prior to ANOVA analysis, which was followed by Newman-Keuls multiple range test ($p \leq 0.05$).

RESULTS

CDDO-Im pretreatment lowers serum ALT concentrations and necrosis in livers of wild-type but not Nrf2-null mice after acetaminophen administration. CDDO-Im or vehicle (DMSO) did not increase serum ALT concentrations in wild-type or Nrf2-null mice (data not shown). Wild-type mice administered acetaminophen had elevated serum ALT concentrations (481 IU/L). Acetaminophen administered to CDDO-Im pretreated wild-type mice resulted in less of an increase in serum ALT concentrations (110 IU/L) (Fig 2.1). After acetaminophen administration, Nrf2-null mice had much higher serum ALT concentrations (2103 IU/L) than wild-type mice, as previously reported (Enomoto *et al.*, 2001). Acetaminophen administered to CDDO-Im pretreated Nrf2-null mice resulted in serum ALT concentrations similar to Nrf2-null mice not pretreated with CDDO-Im.

CDDO-Im did not produce histologically observable abnormalities in either wild-type or Nrf2-null mice (data not shown). Grading of liver necrosis after acetaminophen is shown in Table 2.2. Acetaminophen produced moderate hepatic necrosis in wild-type mice, however, CDDO-Im

pretreatment reduced the acetaminophen-induced hepatic necrosis. Acetaminophen produced much more severe hepatotoxicity in the Nrf2-null mice, and CDDO-Im pretreatment did not protect Nrf2-null mice from acetaminophen-induced necrosis.

Effect of CDDO-Im on translocation of Nrf2 to the nucleus and binding to the ARE. CDDO-Im increased Nrf2 protein expression 380% in hepatic nuclear fractions from wild-type mice (Fig 2.2, upper). No Nrf2 protein was detected in vehicle or CDDO-Im-administered Nrf2-null mice. Activated Nrf2 (or Nrf2 capable of binding to the ARE) in hepatic nuclear fractions was determined via ELISA (Fig 2.2, lower). CDDO-Im activated Nrf2 in wild-type mice, whereas no activated Nrf2 was detected in vehicle or CDDO-Im treated Nrf2-null mice.

Dose- and time- dependent effects of CDDO-Im on Ho-1, Nqo1, and Gclc induction in wild-type mice. Wild-type mice were administered various doses of CDDO-Im from 0.1 to 10 mg/kg, i.p., and hepatic Ho-1, Nqo1, and Gclc mRNA expression were quantified (Fig 2.3, upper). CDDO-Im induced Ho-1 mRNA expression at all dosages used, with a 125% increase after 0.1

mg/kg and 765% increase at 10 mg/kg. CDDO-Im did not induce Nqo1 or Gclc at 0.1 mg/kg. However, CDDO-Im did increase mRNA expression of both Nqo1 and Gclc at 1.0 mg/kg (58 and 156%, respectively) with much more induction observed after the highest dose of 10 mg/kg (199 and 408%, respectively).

To determine the time-response effects of CDDO-Im, wild-type mice were administered CDDO-Im (1 mg/kg, i.p.), and livers removed at various times thereafter (6-72 hrs) (Fig 2.3, lower). CDDO-Im induced Ho-1 mRNA expression, which was induced only at 6 hrs (224%). Nqo1 peaked between 18-24 hrs (323%) and returned back to control levels by 48 hrs, whereas Gclc mRNA induction peaked at 12 hrs (272%) and returned to control levels by 48 hrs.

Effect of CDDO-Im and acetaminophen on Ho-1, Nqo1, and Gclc-1 mRNA induction in wild-type and Nrf2-null mice. CDDO-Im or vehicle was administered to wild-type and Nrf2-null mice. CDDO-Im induced Ho-1 (411%), Nqo1 (153%), and Gclc (248%) mRNA expression in wild-type mice (Fig 2.4). Wild-type mice pretreated with CDDO-Im or vehicle and

administered acetaminophen had increased Gclc and Nqo1 mRNA expression compared to wild-type mice receiving CDDO-Im or vehicle only. Interestingly, wild-type mice pretreated with CDDO-Im and then administered acetaminophen had lower Ho-1 mRNA expression than wild-type mice pretreated with vehicle and administered acetaminophen. Furthermore, CDDO-Im pretreatment had no effect on Nqo1 or Gclc mRNA expression when acetaminophen was also administered. Neither CDDO-Im, acetaminophen, nor combination of the two induced Ho-1, Nqo1, or Gclc mRNA expression in Nrf2-null mice.

Table 2.1. Histological analysis of livers from vehicle- and CDDO-Im-pretreated wild-type and Nrf2-null mice after AA challenge.

	Histological Grade						
Treatment Group	**0**	**1**	**2**	**3**	**4**	**5**	**p≤0.05**
Wild-type VC-AA	0	0	1	4	0	0	
Wild-type CDDO-Im-AA	0	2	3	0	0	0	*
Nrf2-null VC-AA	0	0	0	0	3	2	*
Nrf2-null CDDO-Im-AA	0	0	0	2	1	2	

Grade of liver injury was analyzed at low power (x100) with six scores of severity: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). Asterisks (*) indicate a statistically significant difference from DMSO pretreated wild-type mice ($p \leq 0.05$).

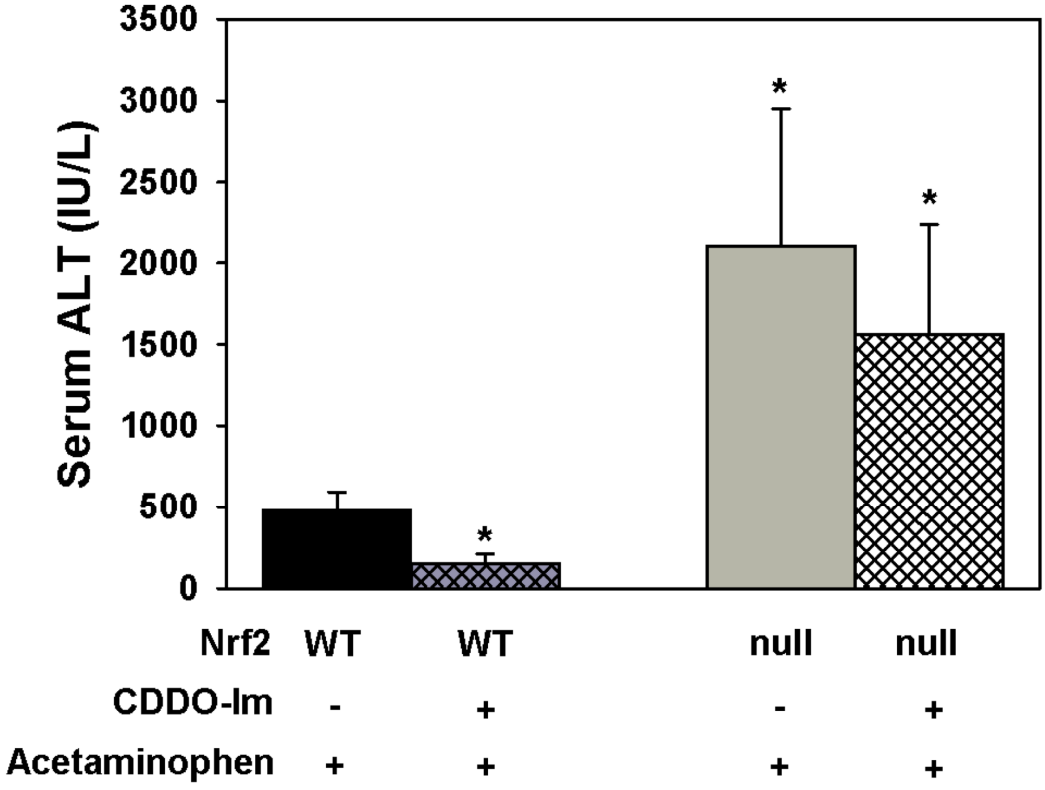


Figure 2.1. Serum alanine transaminase (ALT) levels in wild-type and Nrf2-null mice (n=5) pretreated with vehicle (DMSO) or CDDO-Im and then administered acetaminophen. ALT concentrations are expressed as International Units/Liter. Values are expressed as mean ± S.E.M. ALT values were log transformed before statistical analysis. Asterisks (*) indicate a statistically significant difference from DMSO pretreated wild-type mice ($p \leq 0.05$).

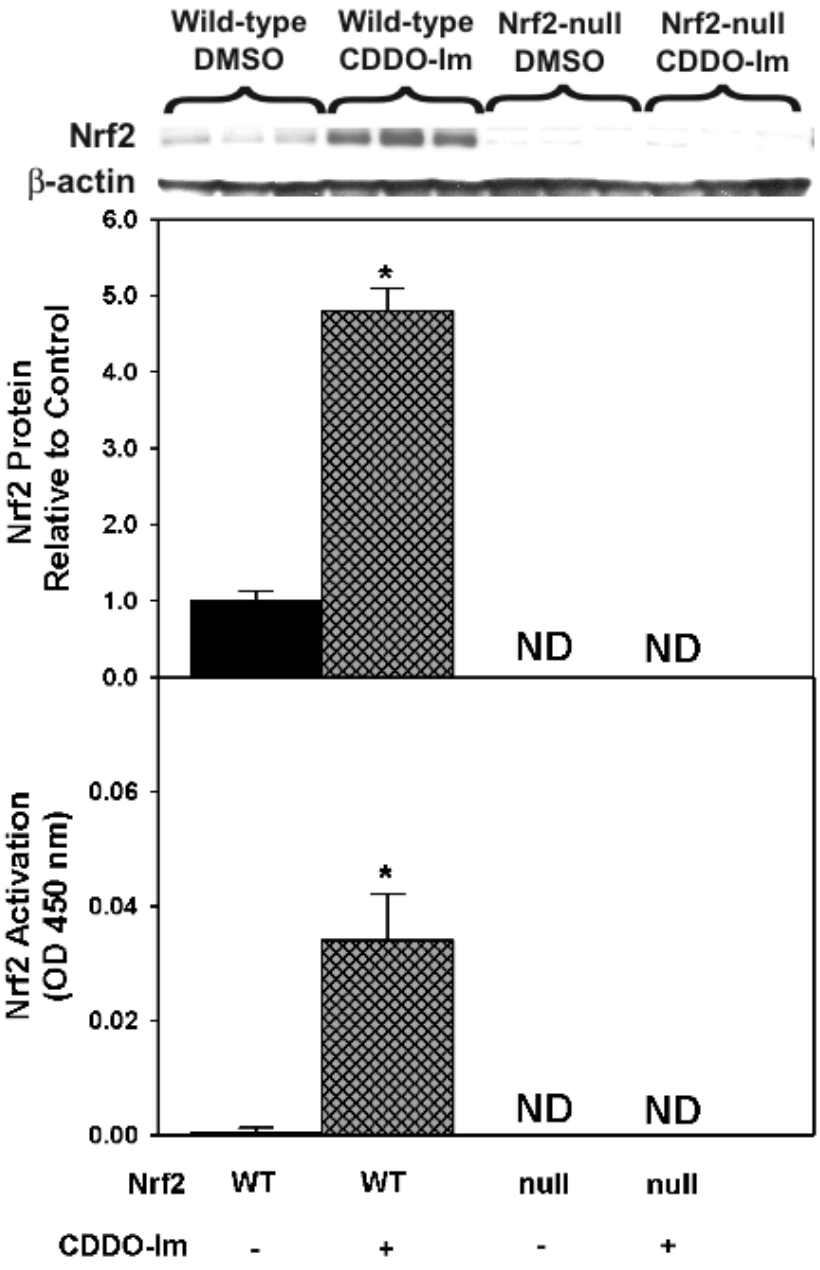


Figure 2.2. *Upper*. Protein expression of Nrf2 determined by western blot in livers of wild-type and Nrf2-null mice after treatment with CDDO-Im (1 mg/kg, i.p.) once daily for three days. Also shown is the quantification of specific

band intensity, expressed relative to control as mean ± S.E.M. *Lower*. Activated Nrf2 (or Nrf2 capable of binding to the ARE) in hepatic nuclear fractions was determined via. Values are expressed as mean optical density (OD) at 450 nm ± S.E.M.

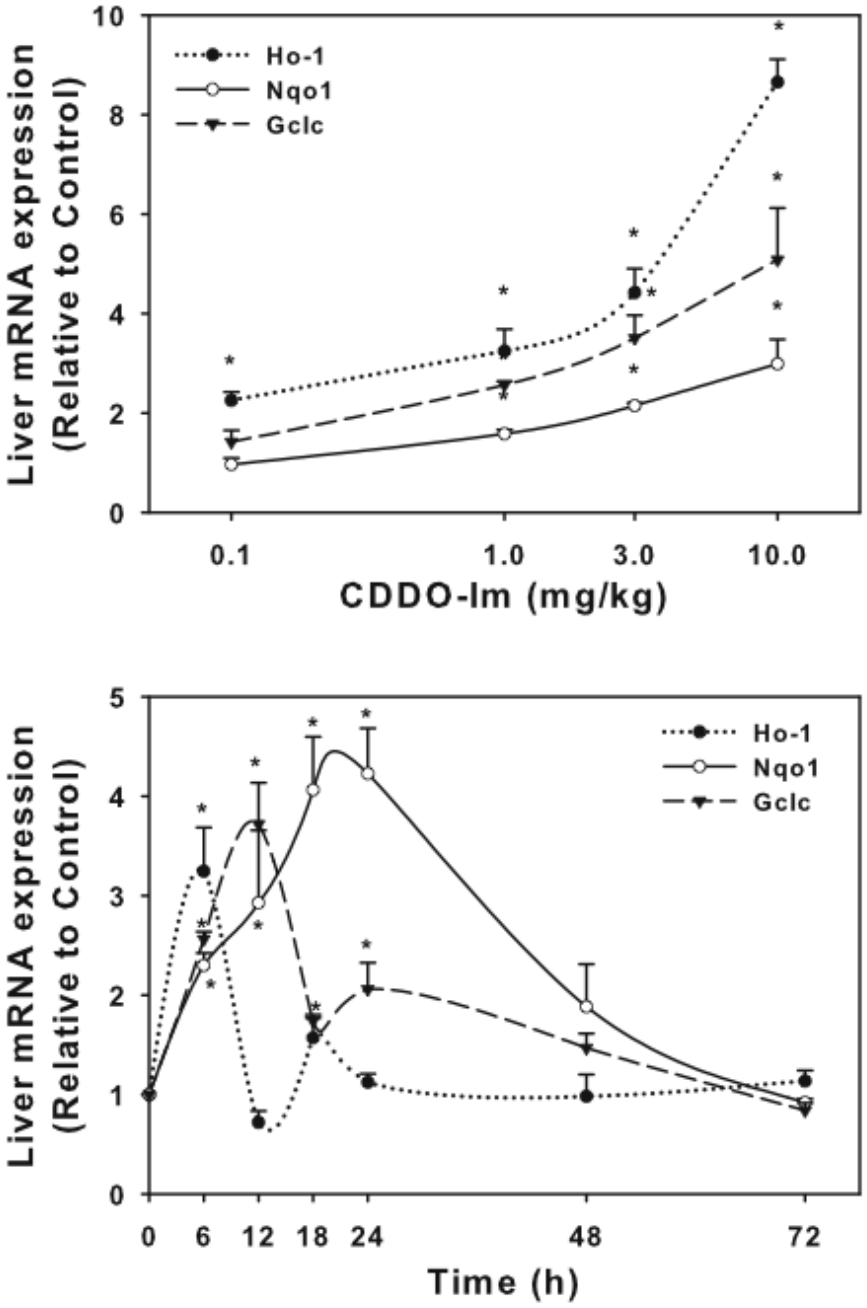


Figure 2.3. *Upper*. Hepatic mRNA expression of Nqo1, Gclc, and Ho-1 in wild-type mice after various doses of CDDO-Im. Livers were removed 6 h

after dosing and mRNA quantified by the bDNA assay. *Lower*. Hepatic mRNA expression of Nqo1, Gclc, and Ho-1 in wild-type mice at different time points after CDDO-Im (1 mg/kg, i.p.) administration. mRNA was quantified by the bDNA assay. Data is expressed as relative to control mean $\pm$ S.E.M. Asterisks (*) indicate statistically significant differences between CDDO-Im treated and control groups ($p \leq 0.05$).

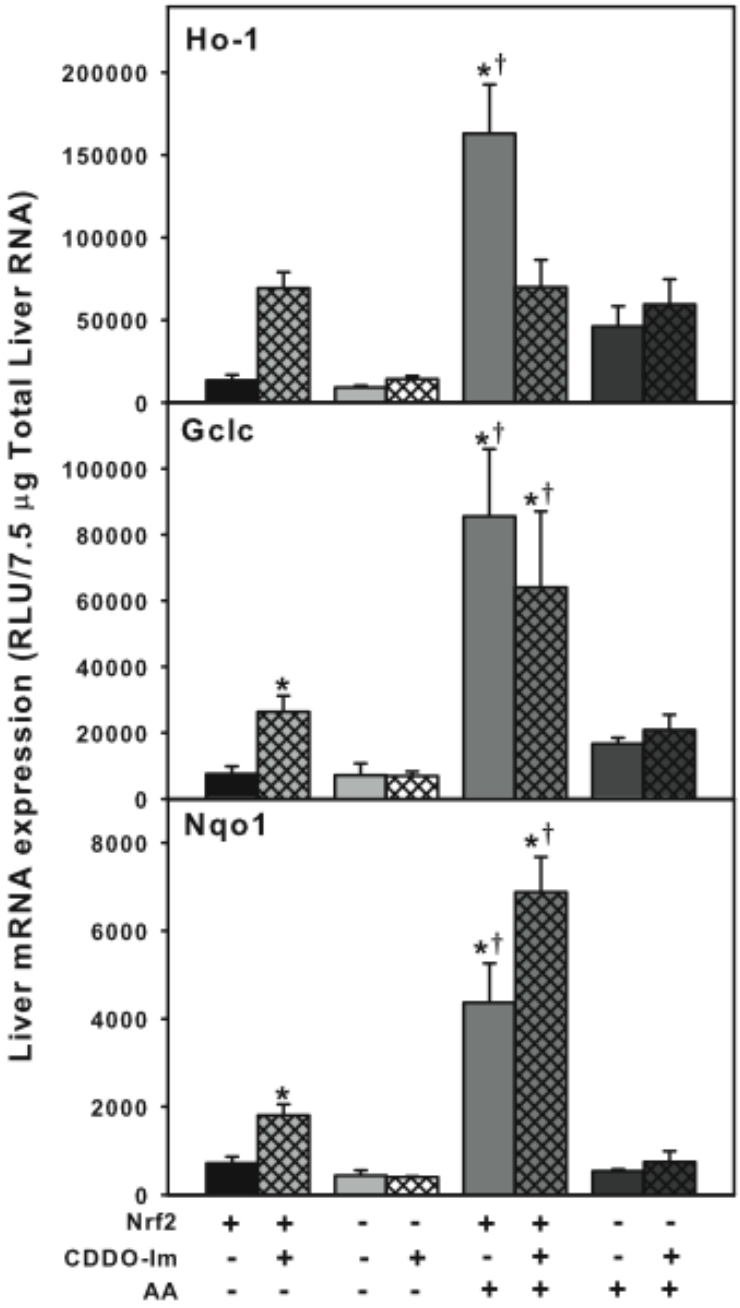


Figure 2.4. Messenger RNA expression of Nqo1, Gclc, and Ho-1 in livers of wild-type and Nrf2-null mice after CDDO-Im (1 mg/kg, i.p.) or DMSO administration. Also, shown is the combined effect of CDDO-Im and

acetaminophen in wild-type and Nrf2-null mice. Messenger RNA was quantified by the bDNA assay. Data is expressed as Relative Light Units (RLU) per 7.5 μg of total RNA. Abbreviations: WT, wild-type; null, Nrf2-null. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant differences between treated and control groups ($p \leq 0.05$). Daggers (†) indicate a statistically significant increase from CDDO-Im treated wild-type mice.

DISCUSSION

CDDO-Im has emerged in recent years as a possible protective compound against oxidative stress. CDDO-Im decreases the inflammatory response in a variety of *in vitro* and *in vivo* models. For example, CDDO-Im decreases iNOS formation *in vivo* in primary mouse macrophages after interferon-g administration (Place *et al.*, 2003). Also, related triterpenoid compounds have been shown to decrease the inflammatory response through direct inactivation of IkBa-kinase, resulting in a decrease in NFkB activation (Shishodia *et al.*, 2006).

CDDO-Im activates the Nrf2-Keap1 pathway (Liby *et al.*, 2005). Activation of the Nrf2-Keap1 pathway leads to a cytoprotective response through the up-regulation of genes containing an antioxidant response element in their promoter regions (Itoh *et al.*, 1997). Cytoprotective genes exert their effects by decreasing the amount of oxidative stress and restoring the balance between oxidants and antioxidants in the cell. Pretreatment with CDDO-Im protects the liver from aflatoxin-induced tumorigenesis (Yates *et al.*, 2006). Furthermore, it has also been shown that CDDO-Im protects from

the LPS-inflammatory response via a Nrf2-dependent mechanism (Thimmulappa *et al.*, 2006).

Because liver is the major site of biotransformation for exogenous chemicals, a model of hepatotoxicity that involves electrophilic stress was utilized. Acetaminophen is primarily metabolized in the liver by sulfotransferases (Sults) and Ugts. High doses of acetaminophen saturate sulfation and glucuronidation detoxification pathways and result in a larger fraction of acetaminophen being biotransformed to the reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI), by cytochrome p450 2e1 (CYP2e1). NAPQI is detoxified by GSH conjugation, however, upon GSH depletion, NAPQI covalently binds to critical macromolecules and produces hepatotoxicity (Parkinson et. al, 2008). NQO1 is not only capable of detoxifying NAPQI (Moffit *et al.*, 2007), but is induced in livers of patients who died from acetaminophen overdose (Aleksunes *et al.*, 2006), suggesting that NQO1 induction may be an adaptive response to acetaminophen hepatotoxicity. Because Nrf2 target genes, such as Nqo1, are involved in detoxification of highly reactive intermediates, a chemical that activates Nrf2

might be a good cytoprotective compound, protecting from the hepatotoxicity of acetaminophen and other chemicals.

The present study was aimed at characterizing the hepatoprotective effects of CDDO-Im against a well-known hepatotoxicant, acetaminophen. CDDO-Im pretreatment protected wild-type mice from acetaminophen-induced hepatic injury, as determined by serum ALTs and histopathology. In contrast, CDDO-Im pretreatment did not protect from acetaminophen hepatotoxicity in Nrf2-null mice.

CDDO-Im facilitated Nrf2 translocation to the nucleus, which correlated with increased Nrf2 binding to consensus AREs (Fig 2.2). Furthermore, the Nrf2-dependent genes Ho-1, Nqo1, and Gclc were induced in both a dose- and time- dependent manner by CDDO-Im. Ho-1 mRNA expression was up-requlated the most by CDDO-Im, followed by Gclc, and then Nqo1. The induction of Ho-1, Nqo1, and Gclc in livers of wild-type mice by CDDO-Im was not observed in Nrf2-null mice, strongly suggesting the induction of cytoprotective genes and subsequent hepatoprotective effects of CDDO-Im are Nrf2-dependent.

Acetaminophen induced Ho-1, Nqo1, and Gclc in wild-type but not Nrf2 –null mice, similar to a previous report (Aleksunes et al., 2008). Pretreatment of wild-type mice with CDDO-Im did not further induce Nrf2 target genes in response to acetaminophen, suggesting that CDDO-Im-mediated hepatoprotection is not related to the adaptive response to acetaminophen injury. Rather, CDDO-Im pretreatment enhanced Nrf2 cytoprotective gene expression prior to acetaminophen exposure, which likely contributed to detoxification of the reactive electrophilic intermediate NAPQI, as well as resulting oxidative stress, thereby protecting against hepatic damage. In conclusion, CDDO-Im pretreatment induces Nrf2-dependent genes, in both a dose- and time-dependent manner, which protects from acetaminophen-induced hepatotoxicity.

PART II

Characterization of Keap1-Knockdown Mice with Enhanced Activation of Nrf2

CHAPTER THREE

Increased Nrf2 Activation in Livers from Keap1-Knockdown Mice Increases Expression of Cytoprotective Genes that Detoxify Electrophiles More Than Those That Detoxify Reactive Oxygen Species

ABSTRACT

Nrf2 is a transcription factor critical for protection against electrophilic and oxidative stress. In a recently engineered mouse with knockdown of Keap1 (Keap1-kd mice), the cytosolic repressor of Nrf2, there is a 55% decrease in Keap1 mRNA and a 200% increase in Nrf2 protein in liver. Experiments with Nrf2-null mice have demonstrated the effects of a lack of Nrf2. However, little is known about the biological effects of more Nrf2 activation. Accordingly, the hepatic phenotype of Keap1-kd mice, as well as the hepatic mRNA expression of cytoprotective genes were compared among wild-type, Nrf2-null, and Keap1-kd mice. Three distinct patterns of hepatic gene expression were identified among wild-type, Nrf2-null, and Keap1-kd mice. The first pattern encompassed genes that were lower in Nrf2-null mice and considerably higher in Keap1-kd mice than wild-type mice, which included genes mainly responsible for the detoxification and elimination of electrophiles, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1) and glutathione-*S*-transferases (Gst), and multidrug resistance-associated proteins (Mrps). The second pattern encompassed genes that were lower in

Nrf2-null mice but not increased in Keap1-kd mice, and included genes, such as epoxide hydrolase-1, UDP-glucuronosyltransferases, aldehyde dehydrogenases, as well as genes important in the detoxification of reactive oxygen species, such as superoxide dismutase 1 and 2, catalase, and peroxiredoxin 1. The third pattern encompassed genes that were not different among wild-type, Nrf2-null, and Keap1-kd mice and included genes such as glutathione peroxidase, microsomal Gsts, and uptake transporters. In conclusion, the present study suggests that increased activation of hepatic Nrf2 is more important for the detoxification and elimination of electrophiles than reactive oxygen species.

INTRODUCTION

Oxidative and electrophilic stresses are implicated in the development of numerous diseases. Oxidative stress is caused by increased production of reactive oxygen and nitrogen species, such as superoxide, hydroxyl radical, and peroxynitrite, or by depletion of protective antioxidants, such as reduced glutathione (GSH), ascorbate, and α-tocopherol. Oxidative stress leads to oxidative damage to critical macromolecules. Superoxide is enzymatically detoxified by first being converted to hydrogen peroxide and then to water by catalase (Cat), GSH peroxidase (Gpx), or peroxiredoxin (Prx) (Fig 1, left side). Detoxification of the hydroxyl radical is not usually possible because of its short half-life (10^{-9}s), and thus prevention of its formation by detoxification of its precursor hydrogen peroxide by Cat, Gpx, or Prx is necessary. Detoxification of peroxynitrite to nitrite is carried out by Gpx and Prx; GSH may also be capable of reducing perxoxynitrite to nitrite (Harwood *et al.*, 2006; Trujillo *et al.*, 2008).

Electrophilic stress is caused by effects of reactive metabolites, such as the cytochrome p-450 (Cyp)-mediated formation of *N*-acetyl-*p*-benzo-

quinoneimine (NAPQI) in acetaminophen hepatotoxicity, or the epoxide formed in benzo[a]pyrene-mediated carcinogenesis. Electrophiles cause injury by covalently binding to and damaging critical macromolecules. Detoxification of reactive intermediates or electrophiles typically occurs through enzymatic processes, such as conjugation with GSH by glutathione-*S*-transferases (Gsts), reduction of quinones by NAD(P)H:quinone oxidoreductase 1 (Nqo1), or hydrolysis of epoxides by epoxide hydrolase-1 (Eh-1) (Fig 1, right side). Nucleophilic conjugation mediated by UDP-glucuronosyltransferases (Ugts) or sulfotransferases (Sults) can also prevent the formation of electrophiles by limiting xenobiotic availability for toxification by Cyps or peroxidases (Pods).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcriptional regulator of antioxidative cellular protection. Upon cellular oxidative/electrophilic insult, Nrf2 initiates a response by up-regulating a battery of cytoprotective genes, such as Nqo1, glutamate-cysteine ligase, catalytic and modifier subunits (Gclc and Gclm), heme oxygenase-1 (Ho-1), and drug processing genes, such Gsts, Ugts, and multidrug resistance-

associated proteins (Mrps) (Venugopal and Jaiswal, 1996; Kobayashi and Yamamoto, 2006; Maher *et al.*, 2007). Moreover, inductive control over a broad array of genes that facilitate increased detoxification and clearance of reactive metabolites and xenobiotics has earned Nrf2 a crucial role in toxicology and xenobiotic metabolism.

Under physiological conditions, Nrf2 is bound in the cytosol by its repressor Kelch-like ECH associating protein 1 (Keap1). Keap1 functions as an adapter for Cullin 3 (Cul3)-based E3 ligase, a scaffold protein that aids in the ubiquitination and subsequent degradation of Nrf2 (Cullinan *et al.*, 2004; Kobayashi *et al.*, 2004). In fact, Nrf2 has a very rapid turnover, with a half-life of approximately 20 min, and thus Nrf2 protein is difficult to detect in unstressed conditions (Itoh *et al.*, 2003; McMahon *et al.*, 2003). When an oxidative or electrophilic insult occurs, reactive cysteines of Keap1 are modified, and Keap1 is unable to target Nrf2 for proteasomal degradation (Dinkova-Kostova *et al.*, 2002). Nrf2 then translocates into the nucleus, heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein, and binds to antioxidant response elements (ARE) in the upstream promoter

region of a variety of cytoprotective genes, promoting their transcription (Itoh *et al.*, 1997).

Nrf2-null mice have lower constitutive expression and an inability to induce cytoprotective genes, such as Nqo1 and Gclc, upon oxidative/electrophlic insult. Nrf2-null mice are extremely susceptible to chemical models of oxidative and electrophilic stress (Aleksunes and Manautou, 2007), contributing to increased hepatotoxicity when administered acetaminophen (Enomoto *et al.*, 2001), ethanol (Lamle *et al.*, 2008), pentacholorphenol (Umemura *et al.*, 2006), or a high-fat diet (Tanaka *et al.*, 2008b).

In contrast to Nrf2-null mice, Keap1-null mice were engineered to investigate the effects of increased activation of Nrf2. Unfortunately, even though Keap1-null mice have enhanced activation of Nrf2 and higher constitutive expression of Nrf2-target genes, the mice died at weaning due to malnutrition from hyperkeratosis of the esophagus and forestomach (Wakabayashi *et al.*, 2003). In an attempt to circumvent the post-natal lethality in Keap1-null mice, a hepatocyte-specific Keap1-null mouse was

engineered, utilizing an Albumin-Cre loxP system. This mouse is viable, has enhanced activation of Nrf2 and Nrf2-target genes in liver, and decreased susceptibility to acetaminophen hepatotoxicity (Okawa *et al.*, 2006). However, later it was discovered that mice homozygous for Keap1 loxP sites (no Albumin-Cre transgene) have decreased or a "knockdown" of Keap1 (Keap1-kd), leading to increased activation of Nrf2 in multiple organs (Okada *et al.*, 2008)**.**

A considerable amount of knowledge has accumulated on the effect of a lack of Nrf2 by doing experiments in Nrf2-null mice. However, very little is known about the biological effects of increased Nrf2 expression. Therefore, the purpose of this study was to determine the hepatic phenotype of Keap1-kd mice, as well as the mRNAs of genes whose protein products are thought to protect against oxidative and electrophilic stress, were compared with Nrf2-null and wild-type mice. The model used in the present study for increased activation of Nrf2 is the Keap1-knockdown (Keap1-kd) mouse. Both mouse models, Nrf2-null and Keap1-kd, have been backcrossed into C57BL/6 mice to >99% congenicity, allowing for comparisons among wild-type, Nrf2-null,

and Keap1-kd mice without the added variable of strain differences.

METHODS

Reagents. Nrf2 antibody was purchased from Santa Cruz Biotechnology (sc-30915, Santa Cruz, CA). β-Actin antibody was purchased from Abcam (Ab8227, Cambrdige, MA). PolyADP-ribose Polymerase (PARP) antibody, originally purchased from BD Pharmingen (cat. # 556362, San Jose, CA), was a gift from Dr. John Robertson (University of Kansas Medical Center, Kansas City, KS). All other chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Husbandry. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Inc (Wilmington, MA). Nrf2-null mice were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) (Chan *et al.*, 1996). Keap1-kd mice were supplied by Dr. Masayuki Yamamoto (Tohoku University, Aoba-ku, Sendai, Japan) (Okada *et al.*, 2008)**.** In an attempt to make a hepatocyte-specific Keap1-null mouse, utilizing a loxP, Alb-Cre system (Okawa *et al.*, 2006), a Keap1-kd mouse, in which Keap1 was decreased throughout the body, was engineered (Okada *et al.*, 2008)**.**

Nrf2-null and Keap1-kd mice were backcrossed into the C57BL/6 background, and >99% congenicity was confirmed by the speed congenics group at Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and had access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) and water *ad libitum*. The housing facility is an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were preapproved in accordance with Institutional Animal Care and Use Committee guidelines.

Bile Collection. Wild-type, Nrf2-null, and Keap1-kd mice (n=5) were anesthetized by injection of ketamine/midazolam (100 mg/kg and 5 mg/kg, respectively, i.p.). Body temperature was maintained at 37℃ by rectal-probe controlled heating pads. Subsequently, the common bile duct was cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing through a high abdominal incision. Bile was collected for 15 min into pre-weighed 0.6-ml microcentrifuge tubes and immersed in ice. The volumes of bile samples were determined gravimetrically, using 1.0 as specific gravity for bile.

Serum and bile enzyme analyses. Glucose, alanine transaminase (ALT), triglycerides, cholesterol, phospholipids, and total bilirubin concentrations were quantified spectrophotometrically using commercially available kits (Pointe Scientific, Canton, MI).

Hepatic enzyme analyses. Livers were collected from wild-type, Nrf2-null, and Keap1-kd mice (n=5) in the morning (prior to 11:00 a.m.) Livers were homogenized in ice-cold 10 mM sucrose- 250 mM Tris buffer (pH 7.5), and microsomal suspensions were prepared by differential centrifugation. Microsomal protein concentrations were determined by BCA Assay (Pierce Biotechnology, Rockford IL). Microsomal cytochrome p450 (Cyp) activities were determined by XenoTech, LLC (Lenexa, KS) using established methods. Briefly, Cyp1a1 and 2b1 activities were quantified by fluorimetric analysis of 7-ethoxyresorufin *O*-deethylation and 7-pentoxyresorufin *O*-deethylation, respectively. Cyp2e1 activity was determined by spectrophotometric analysis of 4-nitrophenol hydroxylation. Lastly, Cyp3a11 and 4a14 activities were measured by LC-MS/MS analysis of testosterone 6β-hydroxylation and lauric acid 12-hydroxylation, respectively.

Cytosolic extracts were prepared with the NE-PER nuclear extraction kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by the BCA Assay (Pierce Biotechnology, Rockford IL). Hepatic glutathione peroxidase (GPx) and catalase (Cat) activity in cytosolic fractions were quantified spectrophotometrically, as adapted from previously described methods (Luck, 1963; Tappel, 1978; Flohe and Gunzler, 1984). Superoxide dismutase (Sod) activity was quantified spectrophotometrically utilizing a commercially available kit (Cayman Chemical, Ann Arbor, MI).

Glutathione (GSH) Qunatification. Reduced GSH in livers was quantified utilizing the GSH –Glo™ Glutathione Assay from Promega (Madison, WI) according to their protocol.

Nrf2 Protein Expression in Hepatic Nuclear Extracts. Nuclear extracts were prepared with the NE-PER nuclear extraction kit according to their protocol (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined with the BCA Assay Kit from Pierce Biotechnology (Rockford, IL). Nuclear proteins (40 µg protein/lane) were electrophoretically resolved using

polyacrylamide gels (4% stacking and 10% resolving). Gels were transblotted overnight at 4ºC onto PVDF membranes. Membranes were then washed with PBS–buffered saline containing 0.05% Tween-20 (PBS-T). Membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS–T. Blots were then incubated with primary antibody (1:1000 dilution in 2% non-fat milk in PBS-T) for 3 h at room temperature. Blots were washed in PBS-T and incubated with species-appropriate secondary antibody conjugated with horseradish peroxidase (1: 2000 dilution) in 2% non-fat milk in PBS-T buffer for 1 hr at room temperature. Blots were then washed with PBS-T. Protein-antibody complexes were detected using an ECL chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film (Denville Scientific, Metuchen, NJ). Equal protein loading was confirmed by β-actin and PARP. Nrf2, β-actin, and PARP proteins migrated the same distance as proteins of approximately 110, 45, and 116 kDa, respectively. Intensity of protein bands was quantified by Discovery Series Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA). Individual blot densities were normalized to that of wild-type mice.

Quantification of hepatic non-esterified fatty acids, free-cholesterol, and triglycerides. Lipids were extracted from livers as described previously (Tanaka *et al.*, 2008b). In brief, 100 mg of liver was homogenized in 1 ml of buffer containing 18 mM Tris, pH 7.5, 300 mM mannitol, 50 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. Five hundred μL of homogenate was mixed with 4 ml of chloroform/methanol (2:1) and incubated overnight at room temperature with occasional shaking. The next day, 1 ml of H_2O was added, vortexed, and centrifuged for 5 min at 3000 g. The lower lipid phase was collected and concentrated by vacuum. The lipid pellets were dissolved in PBS with 0.01% Triton X-100. Liver lipid samples were analyzed spectrophotometrically for free-cholesterol (500 nm), nonesterified fatty acids (550 nm), and triglycerides (500 nm) using commercially available kits (Pointe Scientific, Canton, MI and Wako Diagnostics, Richmond, VA).

Messenger RNA quantification. Mouse liver mRNA expression was determined from liver tissue homogenates prepared as described by the manufacturer's protocol utilizing Quantigene Plex 2.0 technology (Panomics, Fremont, CA). Individual bead-based oligonucleotide probe sets, specific for

each gene examined, were developed by Panomics, Inc. Genes and accession numbers are freely available at http://www.panomics.com. Samples were analyzed using a Bio-Plex 200 System Array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad, Hercules, CA). Assays were performed according to the manufacturer's protocol. The mRNA of Cyp2b10 and Mrp4 was quantified using liver tissue homogenates with Quantigene 1.0 technology (Panomics, Fremont, CA), according to previously described methods (Maher *et al.*, 2005; Petrick and Klaassen, 2007). All data were standardized to the internal control Gapdh.

Statistical Analysis. All data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$).

RESULTS

Nrf2 protein in hepatic nuclear fractions and hepatic mRNA expression of Nrf2 and Keap1. Nrf2 protein in hepatic nuclear fractions of wild-type, Nrf2-null, and Keap1-kd mice was quantified using immunoblotting (Fig 3.1A). As expected, Nrf2 protein was not detected in Nrf2-null mice. However, Nrf2 protein in hepatic nuclear fractions from Keap1-kd mice was 200% higher than wild-type mice. Both β-actin and PARP were used to confirm equal loading.

Nrf2 mRNA expression was not detected in Nrf2-null mice and was 22% lower in Keap1-kd mice than in wild-type mice (Fig 3.1B). Keap1 mRNA expression was lower in Nrf2-null (-24%) and Keap1-kd mice (-55%) than in wild-type mice.

Serum, liver, and bile parameters. Various serum, liver, and bile parameters of hepatic function in the three genotypes of mice were quantified (Table 1). Serum ALT, glucose, triglyceride, and cholesterol concentrations were not different among genotypes, neither were hepatic histology (data not shown), non-esterified fatty acids, free cholesterol, nor triglycerides. Bile flow

tended to be lower in Nrf2-null mice and was higher in Keap1-kd mice. The biliary excretion of total bilirubin was similar in the three genotypes. There were no differences in the biliary excretion of cholesterol and phospholipids between Nrf2-null and wild-type mice. In contrast, the biliary excretion of cholesterol and phospholipids was higher in Keap1-kd mice than wild-type mice.

Hepatic GSH concentrations. Gclc and Gclm are Nrf2-dependent genes important in the *de novo* synthesis of GSH (Moinova and Mulcahy, 1999). Therefore, reduced GSH content was quantified among the three genotypes (Fig 3.2). Nrf2-null mice had 65% lower and Keap1-kd mice had 77% higher GSH concentrations in liver than wild-type mice.

Hepatic mRNA expression of classical oxidative and electrophilic-stress genes. Hepatic mRNA expression of prototypical Nrf2-target genes is depicted in Fig 3.3A. The expression of mRNAs for Nqo1, Gclc, Gclm, and GSH reductase (Gsr) was lower in Nrf2-null mice (-88, -38, -16, and -23%, respectively) and higher in Keap1-kd mice (222, 63, 22, and 17%, respectively). Epoxide hydrolase-1 (Eh-1) was 72% lower in Nrf2-null mice

and 18% lower in Keap1-kd mice. Ho-1 mRNA expression was not different among the three genotypes.

Hepatic mRNA expression of superoxide and hydrogen peroxide metabolizing enzymes is shown in Fig 3.3B. There was less than a 15% decrease in mRNA for Sod1, Sod2, and Cat in Nrf2-null mice, whereas there was no difference between Keap1-kd and wild-type mice. Gpx mRNA was not different among the three genotypes. In addition, there were no differences in Sod, Cat, or Gpx enzyme activities among the three genotypes (data not shown).

Hepatic mRNA expression of redoxins is shown in Fig 3.3C. Glutaredoxin 1 (Glrx1) and peroxiredoxin 1 (Prx1) mRNA were lower in Nrf2-null mice (-27 and -20%, respectively), whereas there were no differences between wild-type mice and Keap1-kd mice. Thioredoxin 1 (Txn1) and thioredoxin reductase 1 (Txnrd1) mRNA were lower in Nrf2-null mice (24 and 41%, respectively) and higher in Keap1-kd mice (9 and 23%, respectively).

Hepatic mRNA of malic enzyme 1 (Me1) and hexose-6-phosphate dehydrogenase (H6pdh), enzymes important in generating nicotinamide

adenine dinucleotide phosphate (NADPH), were quantified (Fig 3.3D). Messenger RNAs for both Me1 and H6pdh were lower in Nrf2-null mice (62 and 16%, respectively), but were unchanged between Keap1-kd and wild-type mice.

Cytochrome P-450 and major transcription factor mRNA expression and Cyp activity of liver microsomes. Hepatic mRNA expression and enzyme activity of major Cyps were quantified (Fig 3.4A). Cyp1a1 mRNA was lower in both Nrf2-null (43%) and Keap1-kd (34%) mice than wild-type mice. Cyp2b10 mRNA was higher in both Nrf2-null (92%) and Keap1-kd (155%) mice than wild-type mice. The amounts of Cyp2e1 and 3a11 mRNAs were not different among the three genotypes. Cyp4a14 mRNA tended to be higher in Nrf2-null and was much lower in Keap1-kd (85%) mice than wild-type mice. Enzyme activities of Cyp1a1, 2b1, 2e1, 3a11, and 4a14 were quantified (Fig 3.4B). There were no differences in Cyp1a1, 3a11, and 4a14 enzyme activity among genotypes. Cyp2b1 and 2e1 enzyme activities were unchanged between Nrf2-null and wild-type mice, but increased 36 and 38%, respectively, in Keap1-kd mice. Also, there were no differences in mRNA

expression of the transcription factors aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor-alpha (PPARα) among genotypes (data not shown). However, constitutive androstane receptor (CAR) mRNA was 59% lower in Nrf2-null mice, whereas there were no differences between Keap1-kd and wild-type mice (data not shown).

Hepatic mRNA expression of the phase-I genes: aldehyde dehydrogenases and carboxylesterases. Hepatic mRNAs of aldehyde dehydrogenase 1a1 (Aldh1a1), 1a7, 3a2, 6a1, and 7a1 were decreased in Nrf2-null mice (14-33%), whereas there were no differences between Keap1-kd and wild-type mice (Fig 3.6A). Aldh1b1, 2, 4a1, 8a1, and 9a1 mRNA were not different among the three genotypes (data not shown).

Hepatic mRNAs of carboxylesterase 1b4 (Ces1b4), 1d1, and 1h1 were lower in Nrf2-null mice (14-46%), whereas there were no differences between Keap1-kd and wild-type mice (Fig 3.5B). Ces1e1 and 2a6 mRNAs were markedly lower in Nrf2-null mice (94 and 85%, respectively) and higher in Keap1-kd mice (38 and 59%, respectively) than wild-type mice. The

expression of paraoxonase 1 (Pon1) and 3 mRNAs were not different among the three genotypes (data not shown).

Hepatic mRNA expression of the phase-II genes: Ugts, Gsts, and Sults. Messenger RNAs for Ugt1a9, 2b1, 2b34, 2b36, 3a2, and the uridine 5'-diphospho-glucuronic acid (UDP-GA) synthesizing enzyme UDP-glucose pyrophosphorylase (Ugp2) were lower in Nrf2-null mice (28-66%), whereas there were no differences between wild-type and Keap1-kd mice (Fig 3.6A). Messenger RNAs of Ugt1a6, 2b35 and the UDP-GA synthesizing enzyme UDP-glucose dehydrogenase (Ugdh) were lower in Nrf2-null mice (45-78%) and higher in Keap1-kd mice (23-75%). There were no differences in mRNA expression of Ugt1a1, 1a5, 2a3, or 2b35 among the three genotypes (data not shown).

Hepatic mRNAs of Gsta1, a4, m1, m2, m3, m4, m6, and p2 were lower in Nrf2-null mice (40-84%) and higher in Keap1-kd mice (45-585%) than in wild-type mice (Fig 3.6B). There were no differences in mRNA expression for microsomal Gst1 (mGst1), mGst2, mGst3, Gstt1, or Gstt2 among the three genotypes (data not shown).

Hepatic mRNAs of hepatic Sults were quantified (Alnouti and Klaassen, 2006). There were no differences among the three genotypes for mRNAs of Sult1a1, 1d1, and the Sult co-substrate 3'-phosphoadenoside 5'-phosphosulfate (PAPS) synthesizing enzyme, PAPS synthetase 1 (Papps1) (data not shown). Sult5a1 mRNA expression was 10% higher in Keap1-kd mice, whereas its expression in Nrf2-null mice did not differ from wild-type mice (data not shown). In addition, Papps2 mRNA was 25% higher in Nrf2-null mice than wild-type mice, whereas its expression in Keap1-kd mice was not different from wild-type mice (data not shown).

Hepatic mRNA expression of uptake and efflux transporters. Hepatic mRNA expression of uptake transporters in livers of the three genotypes of mice was quantified (Fig 3.7A). Hepatic mRNAs of organic anion transporting polypeptide 1a1 (Oatp1a1), 1b2, and organic cation transporter 1 (Oct1) were lower (17-25%) in Nrf2-null mice. Surprisingly, in Keap1-kd mice, Oatp1a1 mRNA was 35% lower, whereas there were no differences in Oatp1b2 and Oct1 mRNA expression between Keap1-kd and wild-type mice. In addition, there were no differences in the mRNA expression among the three

genotypes for organic anion transporter 2 (Oat2), Oatp1a4, 2b1, equilibrative nucleoside transporter 1 (Ent1), or sodium taurocholate co-transporting polypeptide (Ntcp) (data not shown).

Hepatic mRNAs of various efflux transporters were also quantified (Fig 3.7B). The mRNA of Mrp2, Mrp3, breast cancer resistance protein (Bcrp), and the two cholesterol and plant sterol efflux ATP-binding cassette transporters, Abcg5 and Abcg8, were lower in Nrf2-null (17, 85, 27, 29, and 20%, respectively) and higher in Keap1-kd mice (24, 111, 16, 45, and 40%, respectively). Mrp4 mRNA was lowly and similarly expressed in both wild-type and Nrf2-null mice, but increased 55% over wild-type mice in Keap1-kd mice. Messenger RNA of multidrug and toxin extrusion 1 (Mate1) was 26% lower in Nrf2-null mice, whereas there was no difference between wild-type mice and Keap1-kd mice. There were no differences in mRNA among genotypes for multidrug resistance protein 2 (Mdr2), Mrp6, ATP-binding cassette transporter a1 (Abca1), or bile salt export pump (Bsep) (data not shown).

Table 3.1. Serum, liver, and bile parameters of hepatic function in wild-type, Nrf2-null, and Keap1-kd mice.

Analyte (Units)	Nrf2-null	Wild-type	Keap1-kd
Serum			
ALTs (IU/L)	20.9 ± 1.3	19.5 ± 1.6	22.7 ± 3.9
Glucose (mg/dL)	176 ± 6	185 ± 7	206 ± 10
Triglycerides (mg/dL)	66.0 ± 8.9	56.7 ± 4.9	64.1 ± 4.0
Cholesterol (mg/dL)	57.0 ± 1.5	65.8 ± 2.0	61.4 ± 5.4
Liver			
Non-esterified Fatty Acids (mEq/g Liver)	0.018 ± 0.002	0.025 ± 0.003	0.020 ± 0.003
Free Cholesterol (mg/g Liver)	19.0 ± 4.2	23.0 ± 4.2	26.3 ± 3.9
Triglycerides (mg/g Liver)	34.2 ± 1.3	40.2 ± 7.2	36.9 ± 10.7
Bile			
Bile Flow (mL/min/kg)	106 ± 5	124 ± 1	167 ± 12 *
Bilirubin (mg/min/kg)	7.57 ± 0.42	7.05 ± 0.52	6.65 ± 1.05
Cholesterol (mg/min/kg)	35.5 ± 0.6	40.1 ± 1.7	54.3 ± 3.9 *
Phospholipids (mg/min/kg)	37.0 ± 3.2	35.0 ± 2.6	54.34 ± 2.2 *

Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

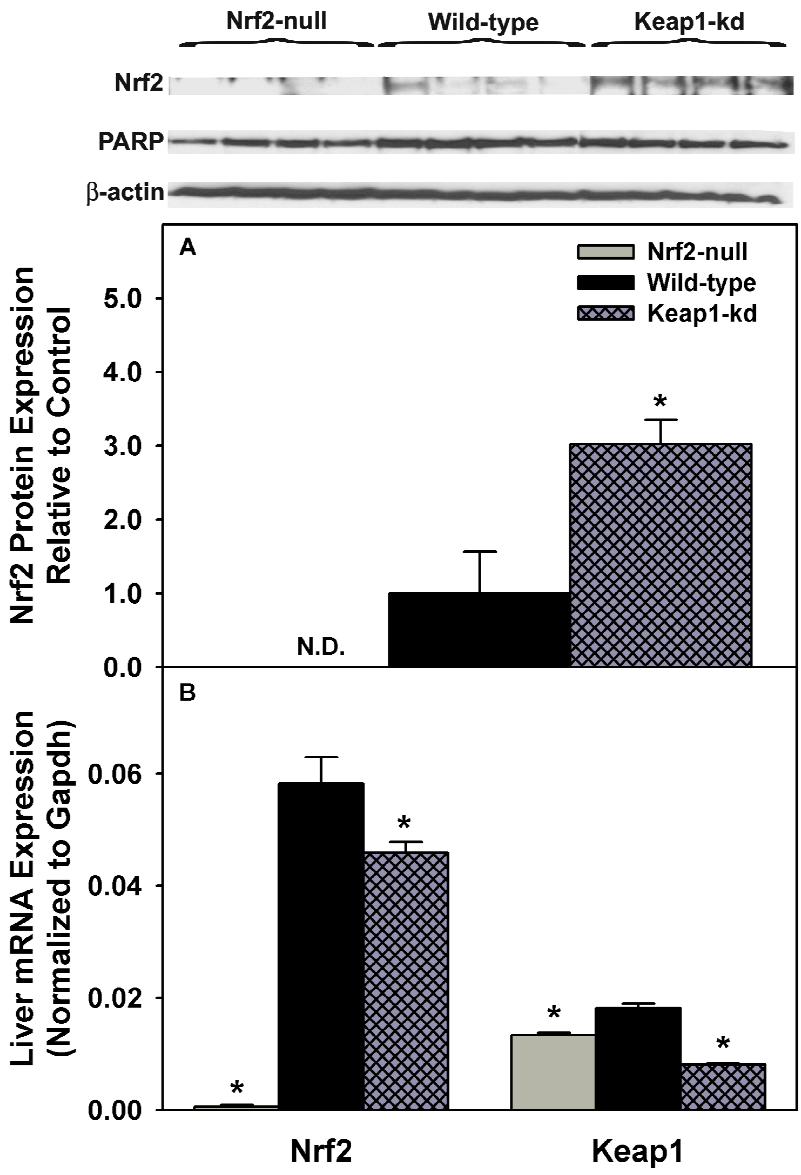


Fig 3.1. Nrf2 protein expression in hepatic nuclear fractions in wild-type, Nrf2-null, and Keap1-kd mice (A). Intensity of protein bands was quantified, and individual blot densities were normalized to wild-type and expressed as mean ± S.E.M. Messenger RNA expression of Nrf2 and Keap1 in wild-type,

Nrf2-null, and Keap1-kd mice (B). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

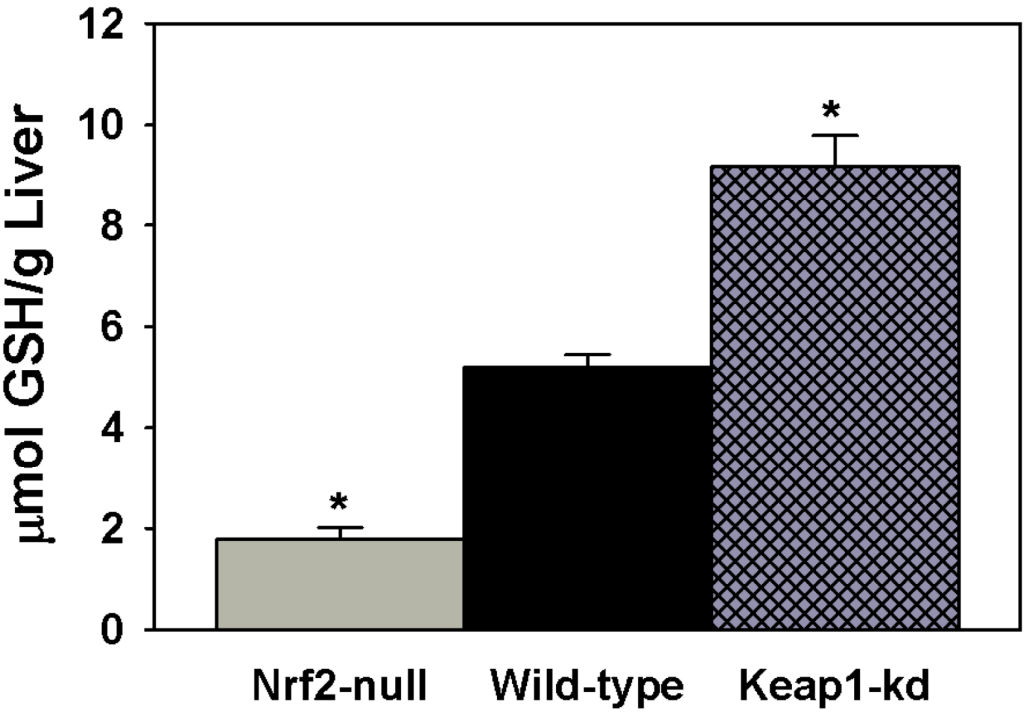


Fig 3.2. Hepatic reduced liver glutathione content in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

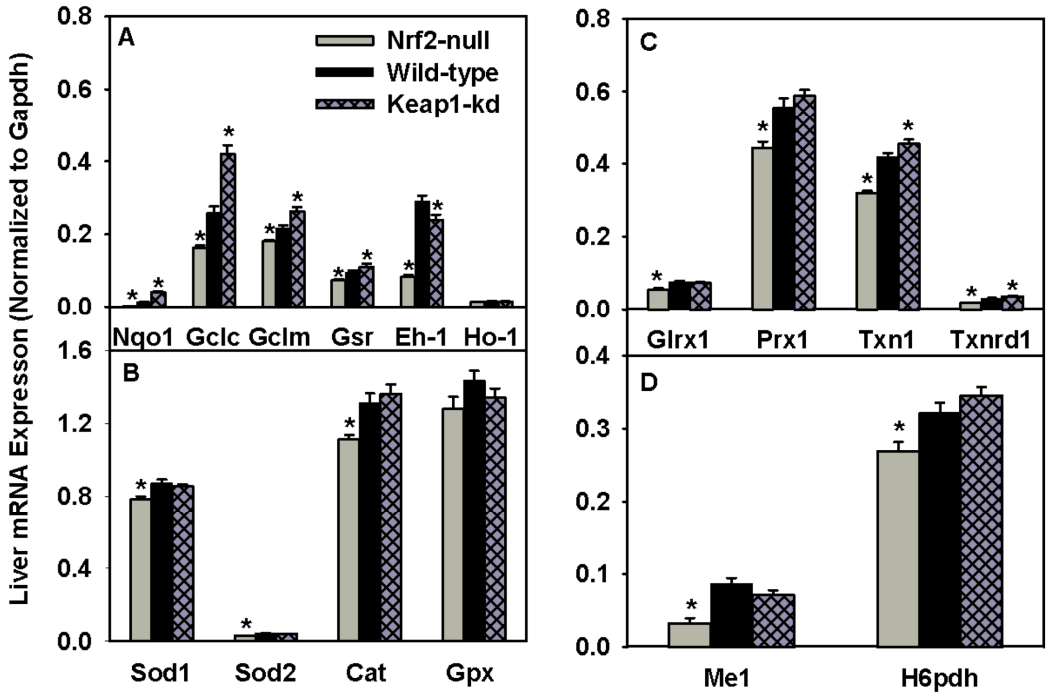


Fig 3.3. Messenger RNA expression of prototypical Nrf2 targets (A), superoxide and hydrogen peroxide reducing enzymes (B), redoxins (C), and NADPH generating enzymes (D) in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

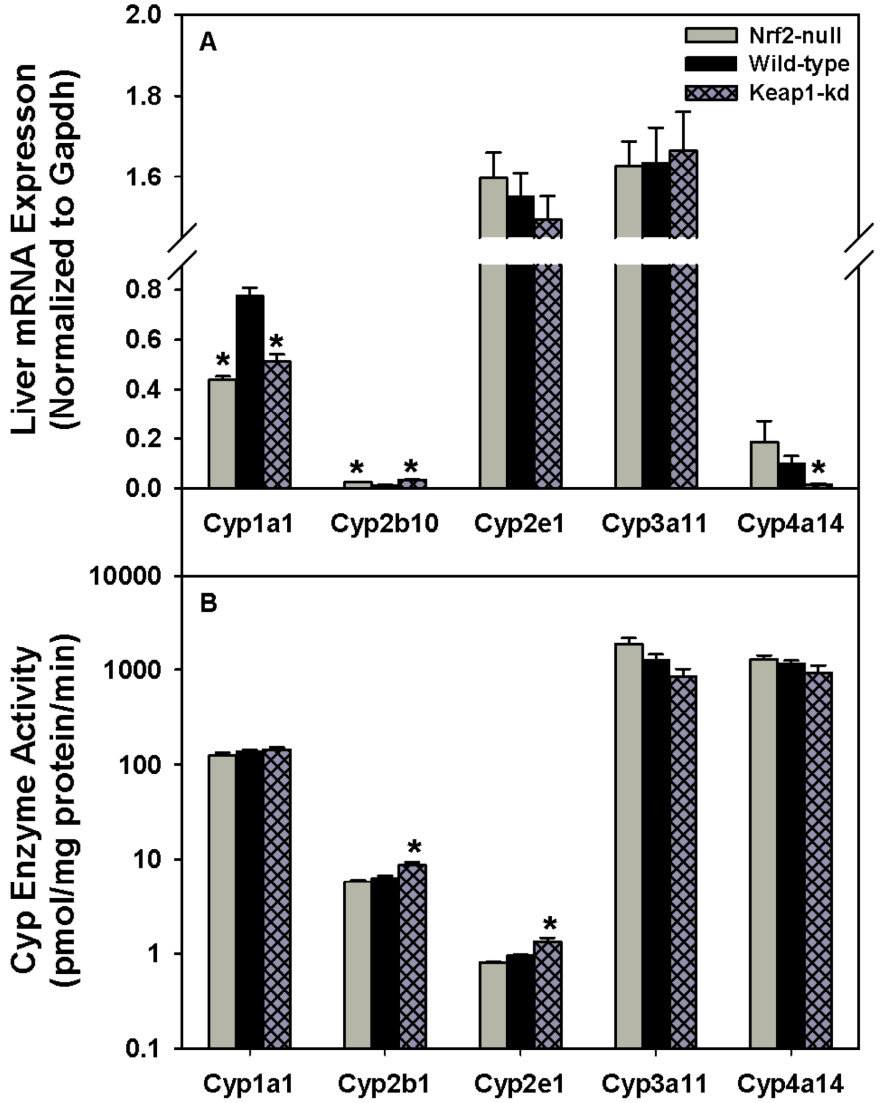


Fig 3.4. Messenger RNA expression (A) and enzyme activity (B) of cytochrome p450s in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

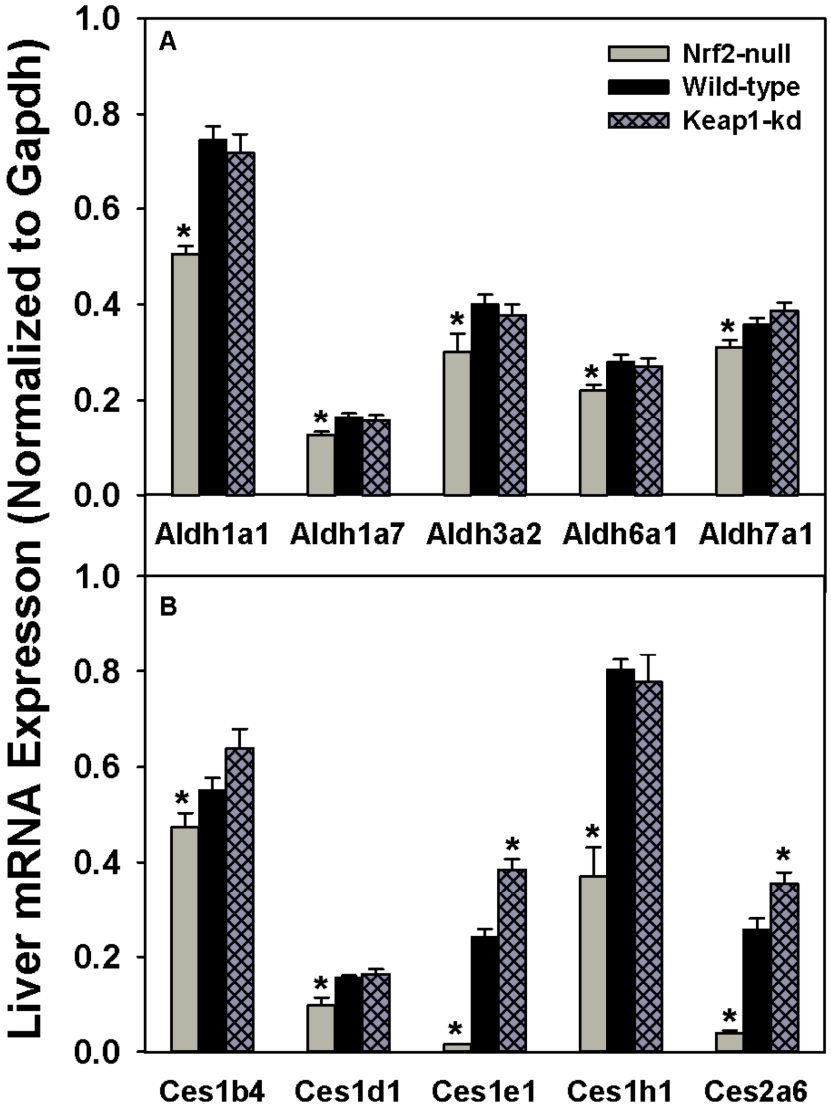


Fig 3.5. Messenger RNA expression of aldehyde dehydrogenases (A) and carboxylesterases (B) in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

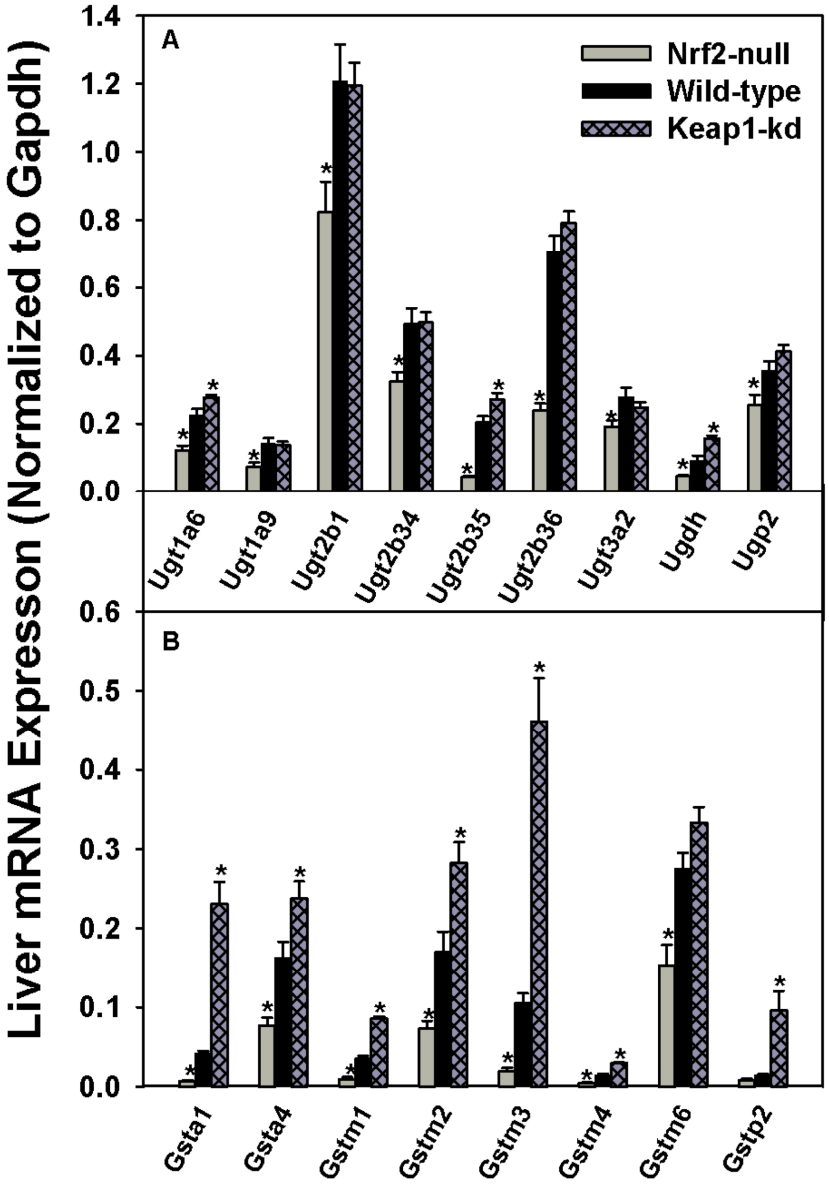


Fig 3.6. Messenger RNA expression of UDP-glucuronosyltransferases (A) and glutathione-*S*-transferases (B) in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

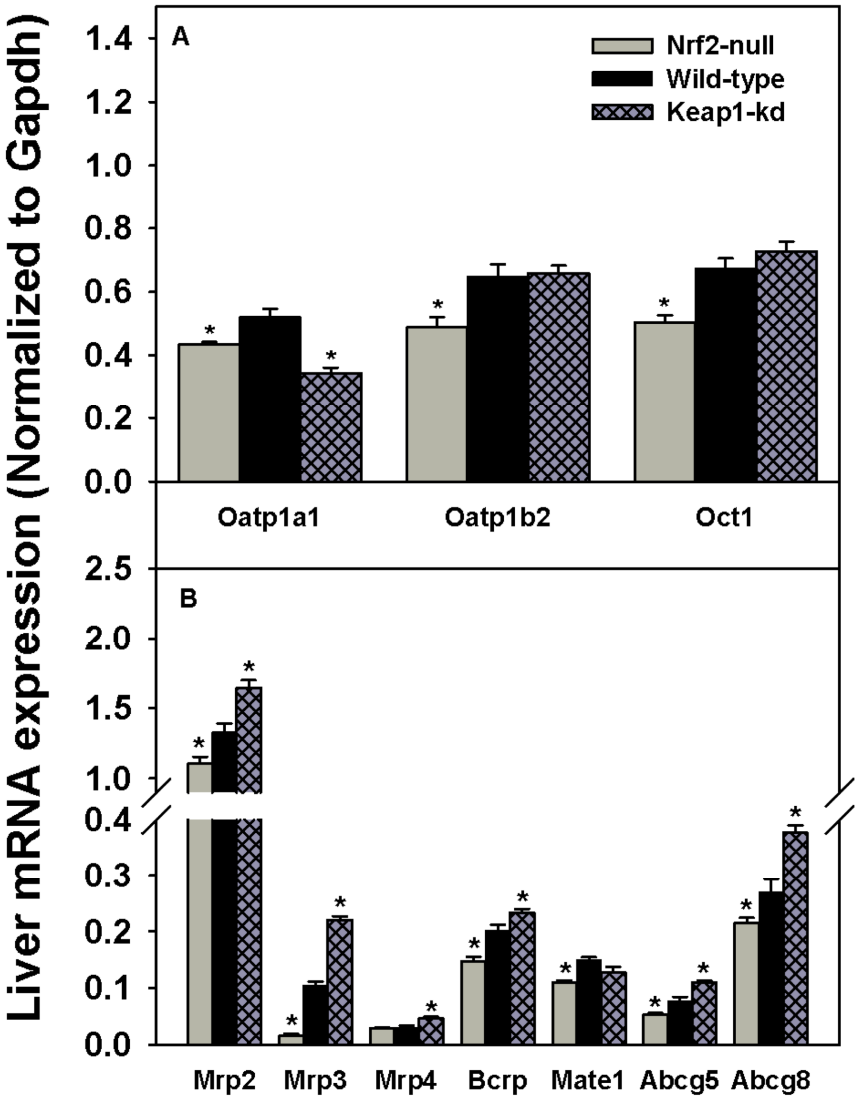


Fig 3.7. Messenger RNA expression of uptake (A) and efflux (B) transporters in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

DISCUSSION

Nrf2 is a transcription factor that upon activation by oxidative/electrophilic insult induces a battery of cytoprotective genes. Nrf2-null mice are highly susceptible to a variety of oxidative/electrophilic stress-induced pathologies because of reduced basal and inducible expression of detoxification enzymes (Aleksunes and Manautou, 2007). In contrast, chemicals that activate Nrf2 protect rodents from pathologies linked to or caused by oxidative/electrophilic stress, leading to the hypothesis that Nrf2 is a target for chemoprevention (Lee and Surh, 2005). However, because chemical compounds typically have many off-target effects, it would be more informative to study activation of Nrf2 in a mouse model of increased Nrf2 activation. Therefore, the present study determined which genes have decreased mRNA expression with lack of Nrf2, and which genes have more mRNA expression when there is increased activation of Nrf2.

Upon activation, Nrf2 translocates into the nucleus, as reflected by the amount of Nrf2 protein in the nucleus in wild-type, Nrf2-null, and Keap1-kd mice. As shown in Fig 2, wild-type mice have very little Nrf2 protein in the

nucleus, similar to previously published results (Kwak *et al.*, 2001; Maher *et al.*, 2007). Low Nrf2 protein in the nucleus in wild-type mice is most likely due to the rapid turnover of Nrf2 under basal or unchallenged conditions (Kobayashi *et al.*, 2004). As expected, no hepatic Nrf2 mRNA or protein was detected in Nrf2-null mice, similar to previous results (Tanaka *et al.*, 2008a). Nrf2-null mice also have lower Keap1 mRNA expression, most likely due to loss of Nrf2 binding to a functional ARE in the promoter region of Keap1 in a proposed negative feedback mechanism (Lee *et al.*, 2007). In contrast, Keap1-kd mice have 55% lower Keap1 mRNA expression, which is likely the mechanism for tripling the amount of Nrf2 protein in the nucleus of Keap1-kd mice. Keap1-kd mice also have a modest decrease in Nrf2 mRNA expression, which suggests a possible negative feedback pathway for Nrf2 mRNA expression when Nrf2 is over-activated.

Most basal hepatic physiological parameters in serum, liver, and bile were not different among wild-type, Nrf2-null, and Keap1-kd mice (Table 1). However, bile flow tended to be lower in Nrf2-null mice and was significantly higher in Keap1-kd mice. Increased bile flow in Keap1-kd mice is due to

increased biliary excretion of GSH, as GSH excretion regulates bile acid-independent bile flow (Ballatori and Truong, 1989). The reason for the increased biliary excretion of GSH in Keap1-kd mice is the higher hepatic GSH concentrations, resulting from the increased expression of GSH synthetic enzymes (Gclc and Gclm) as discussed in detail below. There were no differences in total biliary bile acids among genotypes (data not shown). Biliary excretion but not biliary concentrations of phospholipids and cholesterol were increased in Keap1-kd mice.

Hepatic concentrations of GSH in the three mouse genotypes were proportional to the amount of activated Nrf2. GSH is a prominent cellular antioxidant that protects against oxidative/electrophilic stress by directly scavenging reactive oxygen species or acting as a co-substrate for Gst-mediated detoxification of reactive electrophiles (Meister and Anderson, 1983). The rate-limiting enzymatic process in GSH synthesis is catalyzed by γ-glutamylcysteine synthetase, which is made up of the catalytic and modifier subunits, Gclc and Gclm, respectively. Nrf2-null mice have lower, whereas Keap1-kd mice have higher hepatic mRNA expression of Gclc and Gclm,

genes positively regulated by Nrf2 (Moinova and Mulcahy, 1999; Wild *et al.*, 1999; Lee *et al.*, 2007) (Fig 4A). Furthermore, mRNA expression of Gsr, the enzyme responsible for the reduction of oxidized glutatihione (GSSG) to reduced GSH, is lower in Nrf2-null mice and higher in Keap1-kd mice. Because Gclc, Gclm, and Gsr mRNA expression is lower in Nrf2-null mice and higher in Keap1-kd mice, the amount of GSH in the liver was determined. Nrf2-null mice have less than half, whereas Keap1-kd mice have almost double the hepatic reduced GSH as wild-type mice (Fig 3). Higher GSH concentrations in the Keap1-kd mouse should increase resistance to a variety of oxidative/electrophilic stress-induced pathologies, whereas lower reduced GSH concentrations render the Nrf2-null mice more susceptible to such pathologies.

With few exceptions, typical Nrf2-target genes are lower in Nrf2-null mice and higher in Keap1-kd mice. Nqo1 is a Nrf2-dependent flavoprotein that catalyzes the two-electron reduction and detoxification of electrophilic quinones and its derivatives (Jaiswal, 2000). Nqo1 is a prototypical Nrf2 target gene and is expressed lower in Nrf2-null mice and higher in Keap1-kd

mice (Fig 4A). Eh-1 is induced by sulforophane and 3H-1,2-dithiole-3-thione (D3T) in wild-type but not Nrf2-null mice (Kwak *et al.*, 2001; Thimmulappa *et al.*, 2002). However, Eh-1 mRNA is not increased in Keap1-kd mice, suggesting that other mechanisms aside from Nrf2 nuclear translocation are important for Eh-1 mRNA induction in the liver. Ho-1 catalyzes the breakdown of heme into iron, carbon monoxide, and biliverdin. Biliverdin is then reduced to bilirubin, an antioxidant, via biliverdin reductase. Ho-1 is induced, in mice, by many Nrf2 activators or stressors, such as CDDO-Im (Liby *et al.*, 2005), butylated hyroxyanisole (Keum *et al.*, 2006), hyperoxia (Cho *et al.*, 2002), and arsenite (Gong *et al.*, 2002). However, no difference in Ho-1 expression was observed among wild-type, Nrf2-null, and Keap1-kd mice, a result that is consistent with previous reports (Okawa *et al.*, 2006). The lack of induction of Ho-1 in Keap1-kd mice is attributed to the presence of Bach1, a protein that suppresses the activity of Maf proteins, which are important heterodimers for Nrf2-ARE binding and subsequent Ho-1 gene transcription (Keum *et al.*, 2006).

The superoxide and hydrogen peroxide detoxifying enzymes Sod1, Sod2, catalase, and Gpx are inducible in response to Nrf2 activators, such as 3H-1,2-dithiole-3-thione (D3T) (Zhu *et al.*, 2005), phenolic acids (Yeh and Yen, 2006), and shear stress (Jones *et al.*, 2007). In the present study, a minor decrease in hepatic mRNA expression of Sod1, Sod2, and catalase was observed in Nrf2-null mice (10-30%), whereas there was no increase in Keap1-kd mice. Gpx mRNA expression was not different among the three genotypes (Fig 4B). There were also no differences among the three genotypes in Sod, Cat, or Gpx enzyme activities. The lack of induction of Sods, Cat, and Gpx in Keap1-kd mice suggests that nuclear translocation of Nrf2 does not induce enzymes capable of detoxifying superoxide and related reactive oxygen species in liver.

Redoxins are important for protein repair, as they are responsible for the reduction of protein disulfide bonds that could disrupt proper protein folding and function. Peroxiredoxins reduce hydrogen peroxide to water, protecting against hydroxyl radical formation. Glrx1, Prx1, Txn1, and Txnrd1 have lower hepatic mRNA expression in Nrf2-null mice, whereas only Txn1

and Txnrd1 are induced in Keap1-kd mice (Fig 4C). NADPH is a cofactor for many oxidoreductase reactions that are important in detoxifying reactive oxygen species. Me1 and H6pdh are enzymes that regenerate NADPH after it has been oxidized. Both Me1 and H6pdh are lower in Nrf2-null mice but not increased in Keap1-kd mice (Fig 4D).

Chemicals, such as acetaminophen, benzo[a]pyrene, carbon tetrachloride, and benzene, are activated to reactive intermediates by Cyps, which can result in toxicity. Knowledge of Cyp activity in toxicity studies utilizing Nrf2-null and Keap1-kd mice is critical, because changes in Cyp activity could alter the amount of toxic metabolite generated, leading to an incorrect interpretation of results, i.e. less injury caused by decreased toxic metabolite formation and not increased detoxification of the metabolite. Even though there were differences among genotypes in mRNA expression of Cyp1a1, 2b10, and 4a14, minor differences, if any, were detected in the enzyme activity of major Cyps. Thus, Cyps most likely will not play a role in the interpretation of results from toxicity studies involving Nrf2-null and Keap1-kd mice.

Aldhs catalyze the oxidation of a wide variety of electrophilic aliphatic and aromatic aldehydes to carboxylic acids (Parkinson, 2008). Nrf2-null mice have lower expression of Aldh1a1, 1a7, 3a2, 6a1, and 7a1, whereas Keap1-kd mice were not different from wild-type mice (Fig 6A). The lack of induction of Aldhs in Keap1-kd mice suggests that Aldhs are not induced by Nrf2 in this mouse model.

Carboxylesterases (Cess) catalyze the hydrolysis of ester- and amide-containing chemicals and are important in the activation of prodrugs (Parkinson, 2008). Produrg strategies allow for improvement of oral bioavailability of poorly absorbed drugs. Expression of Ces1b4, 1d1, 1e1, 1h1, and 2a6 were lower in Nrf2-null mice, wheras only Ces1e1 and 2a6 were higher in Keap1-kd mice (Fig 6B). An increase in expression of Cess in Keap1-kd mice suggests that activation of Nrf2 increases expression of Cess and metabolism of xenobiotics by Cess. An increase in Ces1e1 also might increase the amount of prodrug (i.e. the chemotherapeutics floxuridine and gemcitabine) converted to the active form, providing a more efficacious result (Marsh *et al.*, 2004; Landowski *et al.*, 2006; Taketani *et al.*, 2007).

Hepatic mRNA expression of phase-II enzymes is also altered in Nrf2-null and Keap1-kd mice. Ugts catalyze the conjugation of a glucuronosyl group from UDP-GA to a variety of substrate molecules, making them more water soluble and readily excreted. Increased excretion of xenobiotics decreases the amount of compound available for biotransformation to a toxic electrophilic metabolite. In addition, Ugt-catalyzed reactions are responsible for approximately 35% of all drugs metabolized by phase-II enzymes (Evans and Relling, 1999). In general, Ugt mRNA expression is lower in Nrf2-null mice, whereas Keap1-kd mice are not different from wild-type mice, with the exception of a minor increase in mRNA expression of Ugt1a6 and Ugt2b35 (Fig 7A). The availability of the co-substrate UDP-GA is required for the enzyme activity of Ugts. Both Ugdh and Ugp2 mRNA expression were lower in Nrf2-null mice, whereas Ugdh was significantly higher and Ugp2 tended to be higher in Keap1-kd mice.

Sults catalyze approximately 20-25% of phase-II reactions by transferring a sulfonic acid group from the co-substrate PAPS (Evans and Relling, 1999). There are few differences in the amount of the various Sult

mRNAs among wild-type, Nrf2-null, and Keap1-kd mice, suggesting that Nrf2 does not play a major transcriptional role in the expression of Sults.

Gsts catalyze the conjugation of nucleophilic GSH with reactive and potentially damaging electrophiles (Parkinson, 2008). Substrates for Gsts include hydroperoxides of fatty acids, phospholipids, cholesterol, and quinone-containing compounds (Hayes *et al.*, 2005). The expression of many Gsts is dependent on Nrf2, with most Gsts having 41-85% lower expression in Nrf2-null mice and 45-585% higher expression in Keap1-kd mice (Fig 7B). A decrease in hepatic GSH concentration and Gst mRNA expression contributes to Nrf2-null mice being highly susceptible to electrophilic stress. In contrast, Keap1-kd mice have increased hepatic GSH concentrations and Gst mRNA expression and most likely have an increased resistance against damaging electrophiles that can be neutralized via GSH conjugation. The ability to produce and use more GSH appears to be one of the most important benefits of increased hepatic activated Nrf2, as observed in Keap1-kd mice.

Uptake transporters are important for the hepatic uptake and clearance of xenobiotics, an important process in the first-pass effect

(Klaassen and Lu, 2008). In general, mRNA expression of hepatic uptake transporters was similar among genotypes, with the exception of Oatp1a1, 1b2, and Oct1, which exhibit relatively minor differences (<25%) in expression (Fig 8A). Of note, Oatp1a1 expression is lower in Keap1-kd mice, which is consistent with what has been observed upon administration of Nrf2 activators (Cheng *et al.*, 2005). Furthermore, Oatp and Oct1 mRNA are generally not altered by Nrf2 activators or other microsomal enzyme inducers (Cheng *et al.*, 2005). Thus, this data suggests that uptake transporters in the liver are not activated by Nrf2.

Hepatic efflux transporters are important for the elimination and overall clearance of xenobiotics from the liver. Mrps are a group of ATP-dependent transporters that are important in cytoprotection, because Mrps can remove potentially toxic xenobiotics, metabolites, and endogenous substrates from cells (Maher *et al.*, 2007). Mrp2 and Mrp3 mRNA expression is lower in Nrf2-null mice, but higher in Keap1-kd mice. Mrp4 mRNA was lowly expressed and not different between Nrf2-null and wild-type mice, but was increased 55% over wild-type in Keap1-kd mice (Fig 8B). Bcrp is an efflux transporter

for substrates, such as mitoxantrone, anthracyclines, camptothecins, topotecan, and SN-38, the active metabolite of irinotecan (Doyle *et al.*, 1998; Brangi *et al.*, 1999; Miyake *et al.*, 1999; Ross *et al.*, 1999; Litman *et al.*, 2000). Bcrp mRNA expression is 28% lower in Nrf2-null mice and 15% higher in Keap1-kd mice. Mate1 effluxes organic cations, such as metformin and tetraethylammonium, from hepatocytes into bile (Hiasa *et al.*, 2006; Terada *et al.*, 2006). Mate1 mRNA is expressed at a 27% lower level in Nrf2-null mice. There was no difference in Mate1 mRNA expression between Keap1-kd and wild-type mice, similar to a previous report in which Nrf2 activators did not induce Mate1 mRNA expression (Lickteig *et al.*, 2008). Abcg5 and Abcg8, transporters involved in the efflux of cholesterol and potentially toxic plant sterols from the liver into bile, were lower in Nrf2-null mice and higher in Keap1-kd mice. The increase of mRNA expression of efflux transporters may provide Keap1-kd mice the ability to increase the clearance of potentially toxic xenobiotics, thereby decreasing time of exposure and toxicity.

Table 2 categorizes mRNA expression of detoxifying and transporter

genes among wild-type, Nrf2-null, and Keap1-kd mice into three different patterns. The first pattern encompasses genes that have decreased mRNA expression in Nrf2-null mice and increased expression in Keap1-kd mice compared to wild-type mice. Pattern 1 genes include Nqo1, Gsts, and Mrps, which detoxify and eliminate electrophiles. The second pattern consists of genes that have decreased mRNA expression in Nrf2-null mice but no difference between Keap1-kd and wild-type mice. Prominent genes in pattern 2 are the superoxide detoxifying enzymes Sod1, Sod2, Cat, and Prx1, as well as some Ugts and Aldhs. The third pattern includes genes that were not different among wild-type, Nrf2-null, and Keap1-kd mice and includes genes such as some Ugts, Sults, some Aldhs, Gpx, Ho-1, and uptake transporters.

In conclusion, this study has shown that whereas Nrf2-null and Keap1-kd mice have normal livers under standard institutional animal care conditions, baseline defenses against electrophilic stress are lower in Nrf2-null mice and higher in Keap1-kd mice. In addition, classical reactive oxygen species reducing enzymes, such as Cat, Gpx, and Sods, were not induced in livers of Keap1-kd mice, whereas genes, such as Gsts, Nqo1, and Mrps,

important in detoxifying and eliminating electrophiles are markedly increased in Keap1-kd mice. The major advantage Keap1-kd mice have against reactive oxygen and nitrogen species in the liver appears to be an increase in hepatic GSH concentrations. Collectively, these results suggest that hepatic Nrf2 is more important in the direct detoxification of highly reactive electrophilies formed from xenobiotic exposure than for detoxification of reactive oxygen species.

Pattern 1			Pattern 2			Pattern 3
Decrease in Nrf2-null Mice and Increase in Keap1-kd Mice			Decrease in Nrf2-null Mice and Non-Significant Change in Keap1-kd Mice			No Changes Among Genotypes
Gene	**Nrf2-null % change**	**Keap1-kd % change**	**Gene**	**Nrf2-null % change**	**Keap1-kd % change**	**Gene**
Gstp2*	-40	586	Ugp2	-29	16	Ho-1
Gsta1	-84	448	Ces1b4	-14	16	Gpx
Gstm3	-81	336	Ugt2b36	-66	12	Pon1
Nqo1	-88	222	Txn1	-23	9	Pon3
Gstm1	-74	140	Aldh7a1	-13	8	Aldh1b1
Mrp3	-85	111	Oct1	-25	8	Aldh2
Gstm4	-67	102	H6pdh	-16	7	Aldh4a1
Ugdh	-50	75	Prx1	-20	6	Aldh8a1
Gstm2	-57	65	Ces1d1	-38	4	Aldh9a1
Gclc	-37	63	Cat	-15	4	Ugt1a1
Ces1e1	-94	59	Oatp1b2	-25	1	Ugt1a5
Mrp4*	-3	55	Ugt2b34	-35	0	Ugt2a3
Gsta4	-53	45	Ugt2b1	-32	-1	Ugt2b35
Abcg5	-29	45	Glrx1	-27	-1	Gstt1
Abcg8	-20	40	Sod1	-10	-2	Gstt2
Ces2a6	-85	38	Ugt1a9	-47	-3	mGst1
Ugt2b35	-79	32	Ces1h1	-54	-3	mGst2
Mrp2	-17	24	Aldh6a1	-21	-3	mGst3
Ugt1a6	-46	23	Aldh1a1	-32	-4	Sult1a1
Txnrd1	-41	23	Aldh1a7	-23	-5	Sult1d1
Gclm	-16	22	Aldh3a2	-25	-6	Papps1
Gsr	-23	17	Ugt3a2	-31	-11	Oat2
Bcrp	-27	16	Sod2	-32	-14	Oatp1a4
			Mate1	-26	-15	Oatp2b1
			Me1	-62	-18	Ent1
			Eh-1	-72	-18	Ntcp
			Oatp1a1	-17	-35	Mdr2
						Mrp6
						Abca1
						Bsep

Table 3.2. Categorization of Antioxidant, Phase-I, Phase-II, and transporter mRNA expression. Percent change from wild-type in Nrf2-null and Keap1-kd mice are presented. The first pattern represents genes that

had significant decrease in Nrf2-null mice ($p \leq 0.05$) and a significant increase in Keap1-kd mice ($p \leq 0.05$). The second pattern represents genes that had significant decrease in Nrf2-null mice ($p \leq 0.05$) and no change from wild-type in Keap1-kd mice ($p > 0.05$). The third pattern of gene expression represents genes that were not different among wild-type, Nrf2-null, and Keap1-kd mice. *No significant difference between Nrf2-null and wild-type mice ($p > 0.05$).

In conclusion, this study has shown that whereas Nrf2-null and Keap1-kd mice have normal livers under standard institutional animal care conditions, baseline defenses against electrophilic stress are lower in Nrf2-null mice and higher in Keap1-kd mice. In addition, classical reactive oxygen species reducing enzymes, such as Cat, Gpx, and Sods, were not induced in livers of Keap1-kd mice, whereas genes, such as Gsts, Nqo1, and Mrps, important in detoxifying and eliminating electrophiles are markedly increased in Keap1-kd mice. The major advantage Keap1-kd mice have against reactive oxygen and nitrogen species in the liver appears to be an increase in hepatic GSH concentrations. Collectively, these results suggest that hepatic Nrf2 is more important in the direct detoxification of highly reactive electrophilies formed from xenobiotic exposure than for detoxification of reactive oxygen species.

PART III

Enhanced Activation of Nrf2 in Keap1-Knockdown Mice Increases the Biotransformation and Excretion of Sulfobromophthalein and Acetaminophen

CHAPTER FOUR

Nrf2 Activation Enhances Biliary Excretion of Sulfobromophthalein by Inducing Glutathione-*S*-Transferase Activity

ABSTRACT

Sulfobromophthalein (BSP) is used to study hepatobiliary excretory function. BSP is conjugated with glutathione (GSH), whereas its dibrominated analog disulfobromophthalein (DBSP) is not conjugated with GSH prior to biliary excretion. In addition, both BSP and DBSP are transported into hepatocytes via organic anion transporting polypeptides (Oatps) and excreted into bile via multidrug resistance-associated protein 2 (Mrp2). Nrf2 is a transcription factor that under basal conditions is tethered in the cytosol by Keap1. Oxidative stress facilitates Nrf2 nuclear translocation and subsequent induction of cytoprotective genes, including GSH-synthetic enzymes, GSH-*S*-transferases (Gsts), and Mrp transporters. The current study determined whether varying the amount of Nrf2 activation would effect the elimination of BSP and DBSP. Male wild-type (WT), Nrf2-null, and Keap1-knockdown (Keap1-kd) mice were administered BSP or DBSP. Within 30 min, Nrf2-null mice excreted 25%, WT mice 52%, and Keap1-kd mice 80% of the injected BSP. Liver GSH content was not altered by BSP. The biliary excretion of GSH and mRNA expression of major Gsts were directly proportional to the

amount of Nrf2. Moreover, BSP-GSH conjugation activity in the liver of Nrf2-null and Keap1-kd mice was 42 and 237% of WT mice, respectively. In contrast to BSP, there were no differences in biliary excretion or plasma disappearance of DBSP among the three genotypes, indicating that differences in transporter expression among genotypes do not affect BSP or DBSP biliary excretion. Collectively, these results indicate that increased biliary excretion of BSP, and possibly other compounds, is due to Nrf2-induced Gst mRNA expression and enzyme activity.

INTRODUCTION

Sulfobromophthalein (BSP) is a prototypical compound used to assess hepatobiliary transport, biotransformation, and excretory mechanisms. BSP is transported into hepatocytes via the organic anion transporting polypeptide (Oatp) family of uptake transporters (Kanai *et al.*, 1996; Hagenbuch *et al.*, 2000; Cattori *et al.*, 2001; van Montfoort *et al.*, 2002). BSP is conjugated with glutathione (GSH) in hepatocytes by glutathione-*S*-transferases (Gsts) (Brauer and Pessotti, 1949; Krebs and Brauer, 1958; Grodsky *et al.*, 1959; Combes and Stakelum, 1960; Combes and Stakelum, 1961; Combes, 1965; Yalcin *et al.*, 1983; Alin *et al.*, 1985; Tahir *et al.*, 1985) and then excreted from hepatocytes into the bile via the efflux transporter, multidrug resistance-associated protein 2 (Mrp2) (Cui et al., 2001; Tanaka et al., 2003). The dibrominated analog of BSP, disulfobromophthalein (DBSP), is also used to assess hepatobiliary function. Similar to BSP, DBSP is transported into the liver via Oatps and exported by Mrp2 (Johnson and Klaassen, 2002). In contrast to BSP, however, DBSP is not conjugated with GSH before biliary

excretion and therefore, is excreted into bile as the parent compound (Javitt, 1964).

The Nrf2-Keap1 pathway has been characterized over the past decade as an important endogenous cellular mechanism for coping with oxidative stress. Nuclear factor erythroid-2 related factor 2 (Nrf2) is a transcription factor that promotes transcription of a battery of cytoprotective genes via antioxidant response elements (AREs) in promoter regions, thus restoring the intracellular balance between oxidants and antioxidants. Under conditions when oxidative stress is low, Keap1 (kelch-like ECH-associated protein-1) sequesters Nrf2 in the cytosol by acting as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2 (Tong et al., 2006). Upon increased oxidative stress within the cell, Nrf2 circumvents Keap1-mediated proteasomal degradation and translocates into the nucleus (Bloom and Jaiswal, 2003). Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein and promotes transcription of various cytoprotective genes through direct ARE binding (Itoh *et al.*, 1997; Venugopal and Jaiswal, 1998; He *et al.*, 2001). Genes up-regulated by Nrf2

include, but are not limited to, Mrp2, which effluxes BSP and DBSP into bile; glutamate-cysteine ligase catalytic (Gclc) and modifier (Gclm) subunits, which are responsible for the rate limiting step in GSH synthesis; and Gsts (Ramos-Gomez *et al.*, 2001; Solis *et al.*, 2002; Maher *et al.*, 2007).

Because activation of the Nrf2-Keap1 pathway results in induction of Mrp2 as well as genes responsible for both the synthesis and conjugation of GSH, the present study investigated whether changes in the amount of Nrf2 *in vivo* (Nrf2-null, WT, and Keap1-kd mice) would alter the pharmacokinetics of BSP and DBSP. Nrf2-null mice, which have no functional Nrf2 protein, are highly susceptible to tissue injury due to a decreased capability to induce cytoprotective genes upon oxidative stress (Aoki *et al.*, 2001; Enomoto *et al.*, 2001; Ramos-Gomez *et al.*, 2001; Kraft *et al.*, 2004). Keap1-kd mice have decreased functional Keap1 protein and therefore, have increased Nrf2 in the nucleus, resulting in constitutively expressed, higher levels of cytoprotective genes (Okada *et al.*, 2008). The current study investigates the functional importance of Nrf2 on the pharmacokinetics of BSP and DBSP in wild-type, Nrf2-null, and Keap1-kd mice.

METHODS

***Materials*.** Sulfobromophthalein was purchased from Sigma-Aldrich (St. Louis, MO). Disulfobromophthalein was purchased from SERB Laboratories (Paris, France). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

***Animals and husbandry*.** Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Inc (Wilmington, MA). Nrf2-null mice were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) (Chan *et al.*, 1996). Keap1-knockdown (Keap1-kd) mice were graciously supplied by Dr. Masayuki Yamamoto (Tohoku University, Aoba-ku, Sendai, Japan) (Okada *et al.*, 2008).

Both Nrf2-null and Keap1-kd mice were backcrossed into C57BL/6 mice, and >99% congenicity was confirmed by the speed congenics group at Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and had free access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) and water *ad libitum*. The housing facility is an American Animal Associations

Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were preapproved in accordance with Institutional Animal Care and Use Committee guidelines.

Determination of plasma concentration and biliary excretion of BSP and DBSP. Wild type, Nrf2-null, and Keap1-kd mice (n=5, 8 weeks old) were anesthetized by injection of ketamine/midazolam (100 mg/kg and 5 mg/kg, respectively, i.p.). Body temperature was maintained at 37℃ by rectal-probe-controlled heating pads. Subsequently, the right carotid artery was cannulated with PE-10 tubing, and the common bile duct cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing through a high abdominal incision. Depth of anesthesia was monitored by pinching the footpad before and throughout surgery, and if necessary, additional anesthetic drugs were administered during sample collection. Bile samples were collected in 15-min periods into pre-weighed 0.6-ml microcentrifuge tubes for 5 periods. The tubes into which bile was collected were immersed in ice. After the first bile collection, BSP (80 μmol/kg/10 ml) or DBSP (120 μmol/kg/10 ml) was injected via the carotid cannula. Thirty-35 μl of blood were collected into heparinized

tubes at -2, 7.5, 22.5, 37.5, 52.5 min after BSP or DBSP injection. The volumes of bile samples were determined gravimetrically, taking 1.0 as specific gravity. Concentrations of BSP and DBSP in bile and plasma were quantified spectrophotometrically at 580 nm after an appropriate dilution of the samples with 0.1 M sodium hydroxide.

Determination of hepatic GSH depletion by BSP. In WT mice, the peak excretion rate of BSP was within 20 min after injection. Therefore, WT mice were surgically prepared as described above, and after a 15-min bile collection, saline or BSP (80 µmol/kg/10 ml) was injected. During these experiments, bile and plasma samples were collected at the same time points described above. The livers were removed 20 min after saline or BSP administration, frozen in liquid nitrogen, and stored at –80ºC.

Total GSH. Total GSH was quantified in bile and liver using a commercial glutathione assay kit (Sigma, St. Louis, MO). Bile was diluted 60x in 5% 5-sulfosalicylic acid (SSA) solution, whereas liver was homogenized (0.1 g/ml) in 5% SSA and further diluted 10x in 5% SSA. Samples were pre-incubated in assay buffer, glutathione reductase, and 5,5'-dithiobis(2-nitrobenzoic acid)

(DTNB) at room temperature prior to adding NADPH. In the presence of NADPH, the GSH-dependent production of 5-thio-2-nitrobenzoic acid (TNB) was quantified spectrophotometrically at 412nm.

BSP-GSH conjugation. Optimal assay conditions were described previously (Goldstein and Combes, 1966; Klaassen and Plaa, 1967). The BSP-GSH solution was prepared with 0.1 M (pH 8.4) sodium pyrophosphate buffer to achieve a final solution of 226 µM BSP, 21.1 mM GSH, at pH 8.0. All components were incubated at 37℃ and 546 µl of incubation medium (23 µl BSP + 68 µl GSH + 455 µl pyrophosphate buffer) was added to 1-ml cuvettes. Livers were homogenized (0.4 g/ml) in 250 mM sucrose-10 mM Tris buffer (pH 7.4) with protease inhibitors. The cytosolic fraction was separated after two consecutive centrifugations (1000 g for 12 min; 100,000 g for 60 min at 4℃). Cytosols were diluted 50x in sodium pyrophospha te buffer, incubated at 37℃, and 454 µl of each sample was added to the BSP and GSH mixture in 1-ml cuvettes. Glutathione conjugation of BSP was determined spectrophotometrically at 330 nm, and corrected for the amount of BSP per

cuvette, cytosolic dilution factor, protein content of sample, time in min of conjugation reaction, and non-enzymatic conjugation.

Total RNA Isolation. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Total RNA concentrations were determined spectrophotometrically at 260 nm. One mg/ml solutions were prepared from the isolated RNA solutions by diluting with diethyl pyrocarbonate-treated deionized water. Total liver RNA from each group of mice was then pooled and stored at -80℃.

Multiplex suspension array. Livers were collected from naïve, 8-week-old WT, Nrf2-null, and Keap1-kd male mice (n=5). After RNA isolation and dilution to 1 mg/ml, samples were pooled. Mouse liver Gst mRNA expression was determined by a multiplex suspension array assay (Quantigene Plex 1.0, Panomics, Fremont, CA). Individual bead-based oligonucleotide probe sets specific for each mouse Gst isoform were developed by Panomics Inc., using gene accession numbers for mouse Gst isoforms. The accession numbers for Gst mRNA are freely available on the Panomics website at http://www.panomics.com. Gapdh mRNA expression was used as an internal

control for each sample. Samples were analyzed using a Bio-Plex 200 System Array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex Data Manager Software (Bio-Rad, Hercules, CA). Assays were performed according to the manufacturer's protocol (Panomics, Inc., Fremont, CA). Briefly, 10 μg mouse liver total RNA was incubated overnight at 54℃ with X-MAP beads containing oligonucleotide capture probes, label extenders, and blockers. The next day, beads and bound target RNA were washed and subsequently incubated with bDNA amplifier at 46℃ for 1 hr. Samples were then washed and incubated with label (biotin) at 46℃ for 1 hr. Samples were washed and incubated with streptavidin-conjugated *R*-phycoerythrin (SAPE), which binds biotinylated probes, and incubated at room temperature for 30 min. SAPE fluorescence was then detected for each specific mRNA within each sample. All data were standardized to the internal control, Gapdh. Data are expressed as the ratio of Gst mRNA to Gapdh mRNA per 10 μg of total RNA.

Statistical analysis*.* All data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$).

RESULTS

Bile flow and GSH excretion. Bile flow tended to be lower in Nrf2-null mice, whereas it was higher (~30%) in Keap1-kd mice (Fig 4.1A). Nrf2-null mice excreted GSH 30% slower, whereas Keap1-kd mice excreted GSH 30% faster than WT mice (Fig 4.1B).

Plasma concentration and biliary excretion of BSP. Nrf2-null mice had slower plasma disappearance of BSP than WT mice, whereas there was not a statistical difference in the plasma disappearance of BSP between Keap1-kd and WT mice (Fig 4.2A). After BSP injection, Nrf2-null mice continued to have a slower bile flow, whereas Keap1-kd mice had a higher bile flow than WT mice (Fig 4.2B). Nrf2-null mice had slower biliary excretion of BSP and lower cumulative biliary excretion of BSP than WT mice (Fig 4.2C). In contrast, Keap1-kd mice had faster biliary excretion of BSP and higher cumulative biliary excretion of BSP than do WT mice (Fig 4.2D). Thirty min after BSP injection, Nrf2-null mice excreted 25%, WT mice 52%, and Keap1-kd mice 80% of the injected BSP.

Plasma concentration and biliary excretion of DBSP. There were no differences in plasma disappearance, biliary excretion, or cumulative biliary excretion of DBSP in WT, Nrf2-null, and Keap1-kd mice (Figs 4.3A, 4.3C, and 4.3D). However, after DBSP administration, Keap1-kd mice maintained higher bile flow (Figure 4.3B). It should be noted that Keap1-kd mice also had a higher bile flow rate than WT mice before injection of DBSP (Fig 4.1A).

Effect of BSP administration on Hepatic GSH concentration. Twenty min after BSP (80 μmol/kg) administration, GSH concentrations were quantified in WT mice to determine whether GSH depletion after BSP administration might play a role in biliary excretion of BSP. GSH concentrations in liver were not decreased by BSP (Fig 4.4).

BSP-GSH conjugation activity. BSP-GSH conjugation activity was determined in hepatic cytosol fractions. Consistent with the biliary excretion of BSP, conjugation of BSP with GSH is 42% less than in WT mice, whereas Keap1-kd mice have 137% higher conjugation activity than WT mice (Fig 4.5).

Gst mRNA expression. In order to quantify various Gsts that could play a role in GSH conjugation, total liver RNA was pooled (n=5) from naïve WT,

Nrf2-null, and Keap1-kd mice. The mRNA expression of various hepatic Gsts was quantified utilizing multiplex suspension array technology. The mRNA expression of Gsta1, a4, m1, m2, m3, and p2 were 33-77% lower in Nrf2-null mice but 100-1600% higher in Keap1-kd mice (Fig 4.6). The mRNA expression of Gstt1, Gstt2, MGst1, and Mgst3 was not different among the three genotypes (data not shown).

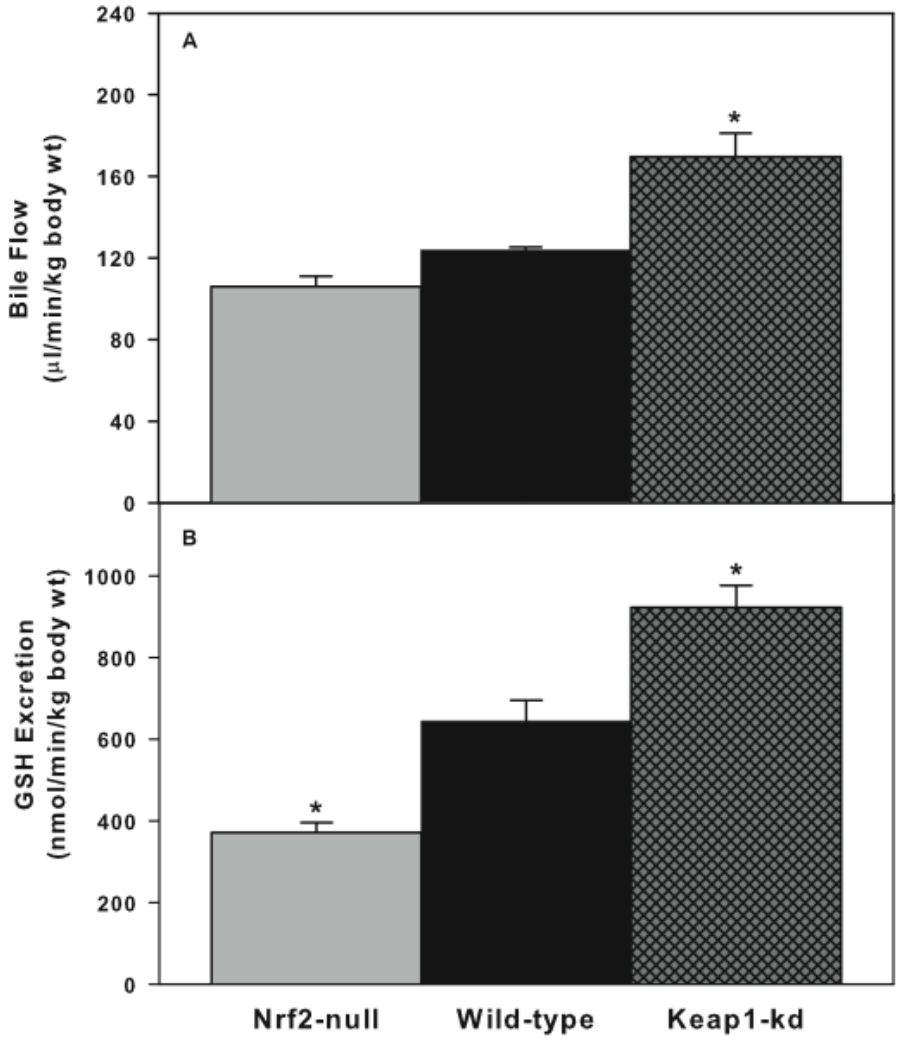


Figure 4.1. Basal bile flow (A) and GSH excretion (B) in wild-type, Nrf2-null, and Keap1-kd mice (n=5). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

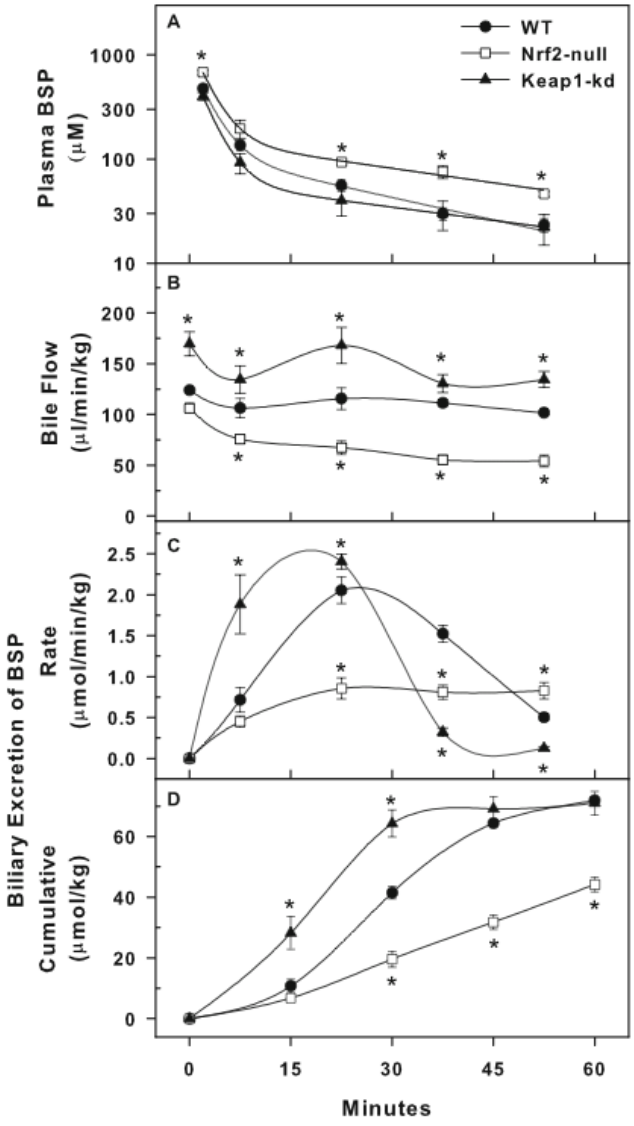


Figure 4.2. Plasma disappearance (A), bile flow (B), biliary excretion (C), and cumulative biliary excretion (D) of BSP (80 μmol/kg) in wild-type, Nrf2-null, and Keap1-kd mice (n=5). Values are expressed as mean ± S.E.M.

Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

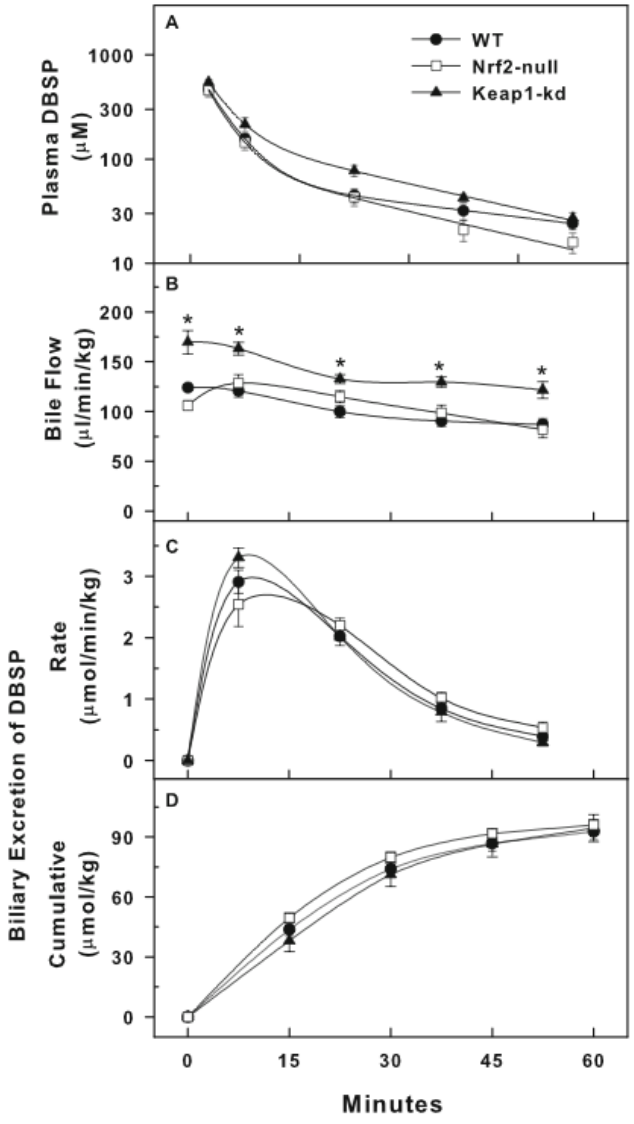


Figure 4.3. Plasma disappearance (A), bile flow (B), biliary excretion (C), and cumulative biliary excretion (D) of DBSP (120 μmol/kg) in wild-type, Nrf2-null, and Keap1-kd mice (n=5). Values are expressed as mean ± S.E.M.

Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

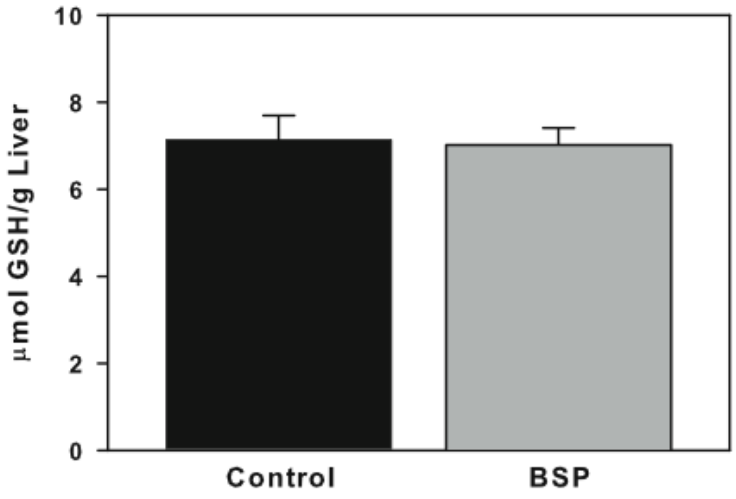


Figure 4.4. Liver GSH concentration in wild-type mice 20 min after intra-vascular injection of saline or BSP (80 μmol/kg, n=5). No differences were observed between treated and untreated groups.

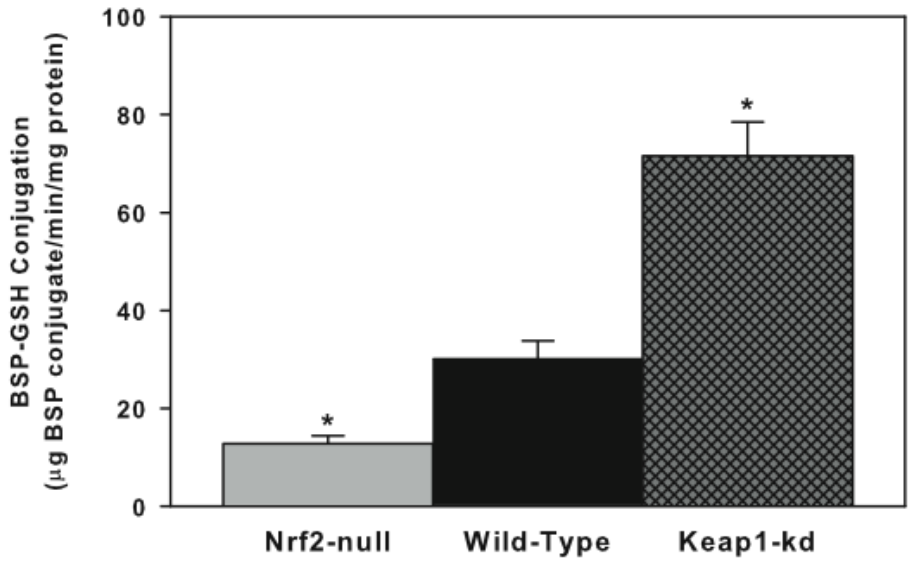


Figure 4.5. BSP-GSH conjugation activity in wild-type, Nrf2-null, and Keap1-kd mice (n=5). Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

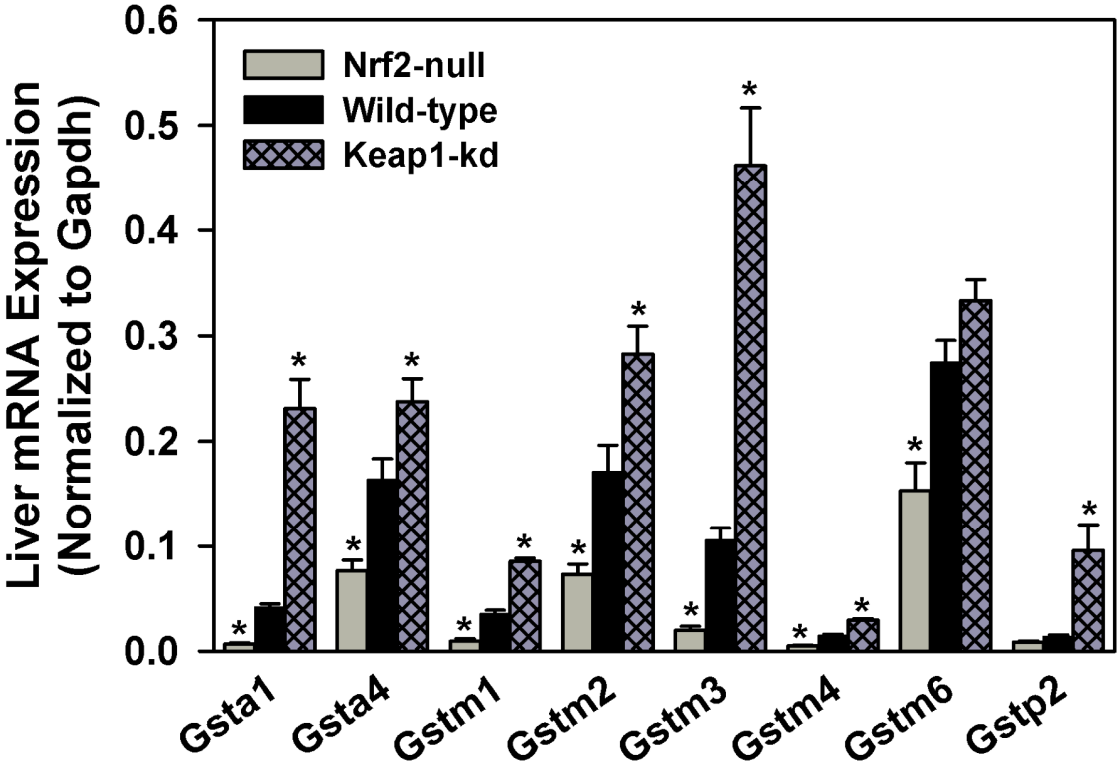


Figure 4.6. Messenger RNA expression of Gsta1, a4, m1, m2, m3, m4, m6, and p2 in wild-type, Nrf2-null, and Keap1-kd mice. Total RNA from livers (n=5) was quantified using multiplex suspension array. The data are expressed as relative light units (RLU) normalized to Gapdh. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

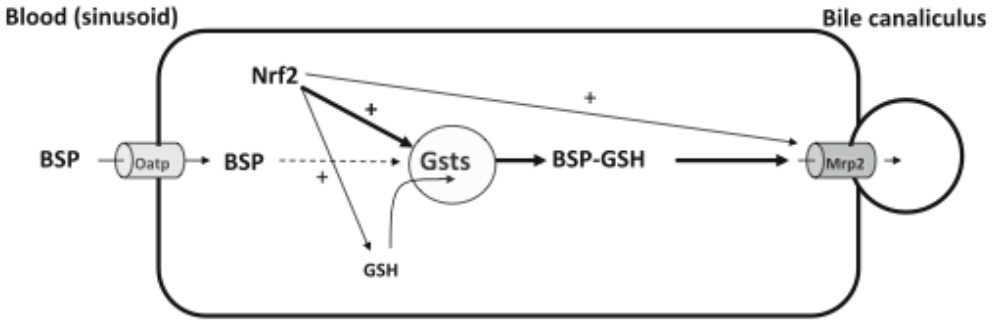


Figure 4.7. Summary of Nrf2-dependent BSP excretion. Enhanced Nrf2 activation increases BSP biliary excretion by increasing the mRNA expression and activity of glutathione-*S*-transferases (Gsts). BSP excretion is not affected by uptake transporters (Oatps), the efflux transporter Mrp2, or GSH content in this model.

DISCUSSION

BSP has been used for decades as a model compound to assess hepatobiliary function both clinically and experimentally. In the present study, BSP was used to elucidate the role of Nrf2 in hepatobiliary function. Nrf2 is a transcription factor that upon activation induces a wide variety of cytoprotective genes, such as GSH-synthesis enzymes, Gsts, and Mrps; all of which play a role in biliary excretion of xenobiotics.

Basal bile flow tends to be lower in Nrf2-null mice, whereas it is higher in Keap1-kd mice. These trends in bile flow correlate with the decreased and increased biliary excretion of GSH in Nrf2-null and Keap1-kd mice, respectively (Fig 4.1B). GSH provides the osmotic force that drives bile acid-independent bile flow (Ballatori and Truong, 1989; Ballatori and Truong, 1992). In addition, there were no differences in the biliary excretion of total bile acids ($p>0.05$, data not shown).

Among wild-type, Nrf2-null, and Keap1-kd mice, Nrf2-null mice have the slowest disappearance of BSP from the plasma, slowest bile flow after BSP, slowest biliary excretion of BSP, and lowest cumulative excretion of

BSP (Fig 4.2). The opposite is true when Nrf2 is more highly activated (Keap1-kd mice), thus there is a direct correlation with the amount of activated Nrf2 and plasma disappearance of BSP, bile flow, and biliary excretion of BSP.

Clearly, Nrf2 influences bile flow and GSH biliary excretion; however, because Nrf2 affects multiple pathways that could affect BSP biliary excretion, additional experiments were performed to elucidate how Nrf2 alters BSP disposition. To investigate the possible role of transporters, the dibrominated analog of BSP, namely DBSP, was used in experiments similar to those performed with BSP. DBSP is transported into hepatocytes and effluxed into bile by the same transporters as BSP, but unlike BSP, DBSP is not conjugated with GSH. Therefore, if BSP biliary excretion differences are due to a transporter-mediated phenomenon rather than a biotransformation phenomenon, then there should be differences in the biliary excretion of DBSP among the three genotypes. However, no such differences in plasma disappearance and biliary excretion of DBSP were observed among the three

genotypes, demonstrating that transporter activity is not responsible for the changes observed in the biliary excretion of BSP (Fig 4.3).

After parallel studies with DBSP failed to show differences in the biliary excretion among the genotypes, BSP metabolism was considered in more detail. Because BSP is conjugated with GSH before biliary excretion, and GSH excretion is a determinant of bile acid-independent flow, experiments were conducted to determine whether the differences in biliary excretion of BSP were due to depletion of GSH after BSP administration. The lack of decrease in GSH concentrations 20 min following BSP administration demonstrates that differences in biliary excretion of BSP are not due to depletion of the co-substrate, GSH (Fig 4.4).

Because the aforementioned studies suggested Nrf2-dependent differences in BSP biliary excretion might be due to GSH conjugation, Gst activity and mRNA expression were quantified. Interestingly, Nrf2-null mice have 42% less and Keap1-kd mice have 137% more enzyme activity to conjugate BSP with GSH, than do WT mice. Furthermore, the mRNA expression of six Gst isoforms, namely Gsta1, a4, m1, m2, m3, and p2 was

33-77% lower in Nrf2-null mice and 100-1600% higher in Keap1-kd mice. The differences in expression of Gsts confirms that the differences in biliary excretion of BSP in WT, Nrf2-null, and Keap1-kd mice are due to differences in activity of liver to conjugate BSP with GSH. The mechanism by which Nrf2 modulates BSP biliary excretion is summarized in Fig 4.7.

Numerous studies have shown that Nrf2 protects against toxicity, which is frequently attributed to decreased toxicodynamic effects, as exemplified by increased expression of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), protecting against oxidative or electrophilic stress (Aleksunes and Manautou, 2007). The present study emphasizes the importance of the Nrf2-Keap1 pathway on the toxicokinetics of chemicals. Whereas activation of Nrf2 protects cells from electrophiles through induction of cytoprotective genes, Nrf2 also increases biliary excretion of potentially toxic xenobiotics, as exemplified by increased GSH conjugation and biliary excretion of BSP, which represents another important defense mechanism.

CHAPTER FIVE

Altered Disposition of Acetaminophen in Nrf2-null and Keap1-Knockdown Mice

ABSTRACT

Acetaminophen (AA) is a widely-used antipyretic drug that causes hepatotoxicity at high doses. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that mitigates electrophilic stress from AA by inducing genes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), multidrug resistance-associated proteins (Mrps), and glutathione (GSH) synthesis enzymes. To determine whether Nrf2 activation alters the biotransformation, excretion, and in turn, the hepatotoxicity of AA, male wild-type, Nrf2-null, and Keap1-knockdown (Keap1-kd) mice (which have increased activation of Nrf2) were administered a single subtoxic dose of AA (50 mg/kg, iv), after which, AA and its metabolites (AA-glucuronide, AA-GLUC; AA-sulfate, AA-SULF; AA-glutathione, AA-GSH) were quantified in plasma, bile, and liver. AA-GLUC concentrations were reduced in plasma and elevated in livers of Nrf2-null mice due to decreased glucuronidation activity and lower expression of the basolateral efflux transporter Mrp3. In contrast, Keap1-kd mice had higher plasma and lower hepatic AA-GLUC concentrations, due to higher Mrp3 expression. Lower glucuronidation activity of Nrf2-null mice increased

the proportion of AA available for sulfation, resulting in elevated AA-SULF concentrations in plasma, bile, and liver. Decreased AA sulfation activity in Keap1-kd mice resulted in lower AA-SULF concentrations. In general, GSH-derived AA conjugates were increased in Nrf2-null mice and decreased in Keap1-kd mice. Furthermore, Nqo1, an enzyme capable of detoxifying the reactive intermediate of AA metabolism, NAPQI, had 85% lower activity in Nrf2-null mice and 415% higher activity in Keap1-kd mice relative to wild-type. In addition, hepatotoxicity 6 h after AA (600 mg/kg, ip) was most severe in Nrf2-null mice and absent in Keap1-kd mice. In conclusion, lack of Nrf2 decreases AA glucuronidation, leading to increased NAPQI formation and hepatotoxicity, whereas activation of Nrf2, as in Keap1-kd mice, enhances detoxification of NAPQI by Nqo1 and elimination of AA-GLUC via Mrp3.

INTRODUCTION

Nuclear factor erthyroid 2-related factor 2 (Nrf2) is a member of the family of basic leucine zipper transcription factors. Under basal conditions, Kelch-like ECH-associated protein 1 (Keap1) sequesters Nrf2 in the cytosol (Itoh *et al.*, 2003; McMahon *et al.*, 2003). During periods of oxidative/electrophilic stress, the Nrf2-Keap1 interaction is disrupted, permitting Nrf2 to translocate to the nucleus. Once in the nucleus, Nrf2 complexes with small musculo-aponeurotic fibrosarcoma (Maf) proteins, and the complex binds to antioxidant response elements (ARE), promoting transcription of a large battery of cytoprotective genes (Itoh *et al.*, 1997; Venugopal and Jaiswal, 1998; He *et al.*, 2001). Nrf2-ARE-dependent genes include the prototypical target gene NAD(P)H: quinone oxidoreductase 1 (Nqo1), and others such as glutamate-cysteine ligase, catalytic and modifiers subunits (Gclc and Gclm), glutathione-*S*-transferases (Gsts), UDP-glucuronosyltransferases (Ugts), multidrug resistance-associated proteins (Mrp2-4), and many others (Venugopal and Jaiswal, 1996; Kobayashi and Yamamoto, 2006; Maher *et al.*, 2007).

Acetaminophen is a widely-used analgesic and antipyretic drug that can produce hepatic injury with acute overdosing. AA is conjugated with glucuronic acid and sulfate, reactions catalyzed by Ugts and sulfotransferases (Sults), respectively (Nelson, 1990). In mice, AA-glucuronide (AA-GLUC) is excreted into bile via Mrp2 (Xiong *et al.*, 2000). However, the basolateral transporter Mrp3 has a higher affinity for AA-GLUC and effluxes AA-GLUC into blood (Manautou *et al.*, 2005). Furthermore, induction of rat Mrp3 by phenobarbital, *trans*-stilbene oxide, diallyl sulfide, or oltipraz increases efflux of AA-GLUC into blood (Gregus *et al.*, 1990; Slitt *et al.*, 2003). AA-sulfate (AA-SULF) is excreted into bile primarily via Mrp2, and to a lesser extent by breast cancer resistance protein (Bcrp) (Zamek-Gliszczynski *et al.*, 2005). AA-SULF can also be effluxed into blood via Mrp3 and Mrp4 (Zamek-Gliszczynski *et al.*, 2006). Some AA is also bioactivated by cytochrome P450s (Cyps) to *N*-acetyl-*p*-benzoquinone imine (NAPQI), which can be reduced back to AA *in vitro* in a redox reaction catalyzed by Nqo1 (Moffit *et al.*, 2007) or scavenged through conjugation with glutathione (GSH) in a reaction that mainly occurs nonenzymatically. The resultant AA-GSH

conjugate is then excreted as AA-GSH or one of its hydrolysis products, AA-cysteinylglycine (AA-Cys/Gly), AA-cysteine (AA-Cys), or the acetylated cysteine product, namely AA-mercapturate (AA-NAC). AA-GSH and AA-NAC are then effluxed into bile via Mrp2 (Chen *et al.*, 2003).

The role of Nrf2 in AA hepatotoxicity has been previously investigated, but mainly with respect to the effects of Nrf2-dependent alterations in gene expression. Enomoto et al. (Enomoto *et al.*, 2001) concluded that Nrf2 protects against acetaminophen-induced liver injury by up-regulating Ugt1a6, Gclc, and Gclm; however, their experiments were performed in Nrf2-null mice, demonstrating increased sensitivity to AA hepatotoxicity due to lower expression of cytoprotective enzymes, not protection from AA hepatotoxicity in a model of Nrf2 activation. Okawa et al (Okawa *et al.*, 2006), also basing conclusions mainly on mRNA expression data, deduced that hepatocyte-specific Keap1-null mice are resistant to AA hepatotoxicity because of increased hepatic Nrf2 activation and higher constitutive mRNA expression of Nqo1, Gclc, and Gsts. Unfortunately, these two studies (Enomoto et al., 2001 and Okawa et al., 2006) relied heavily on mRNA expression data and did not

investigate the effect of Nrf2 on the disposition of AA.

Keap1-knockdown (Keap1-kd) mice, in which Keap1 is expressed at low levels, resulting in increased Nrf2 activation, have recently been characterized (Reisman et al., Chapter Four; (Okada *et al.*, 2008). Keap1-kd mice have increased, whereas Nrf2-null mice have decreased hepatic mRNA expression of Nqo1, Gclc, Gclm, Bcrp, Mrp2, Mrp3, and Mrp4. Nrf2-null mice have decreased hepatic Ugt mRNA expression levels, whereas Keap1-kd mice have Ugt mRNA expression unchanged from wild-type mice, with the exception of increases in Ugt1a6 and Ugt2b35. Also, Sult mRNA expression levels and Cyp enzyme activities generally do not differ among livers from wild-type, Nrf2-null, and Keap1-kd mice (Reisman et al., Chapter Four).

Because Nrf2 can transcriptionally regulate genes important in the biotransformation and excretion of AA, namely Ugts, Gclc, Gclm, Nqo1, and Mrps, the purpose of this study was to investigate the disposition of AA and susceptibility to AA hepatotoxicity in Nrf2-null and Keap1-kd mice. Ugt and Sult activities, as well as protein levels of Bcrp, Mrp2, Mrp3, and Mrp4, were examined in livers from wild-type, Nrf2-null, and Keap1-kd mice to determine

whether changes in mRNA expression, as previously observed, functionally affect AA disposition and susceptibility to AA hepatotoxicity.

METHODS

Reagents. All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO). Mrp3 (M_5II-2), Mrp4 (M_4I-10), and Bcrp (BXP-53) antibodies were provided by Dr. George Scheffer (VU Medical Center, Amsterdam, Netherlands)**.** Mrp2 antibody was provided by Bruno Steiger (University Hospital, Zurich, Switzerland).

Male albino guinea-pig microsomes were purchased from Xenotech, LLC (Lenexa, KS). 3-hydroxy benzo[a]pyrene was purchased from the National Cancer Institute's Chemical Carcinogen Reference Standards Repository operated under contract by Midwest Research Institute (Kansas City, MO).

Animals and Husbandry. Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Inc (Wilmington, MA). Nrf2-null mice were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) (Chan *et al.*, 1996). Keap1-knockdown (Keap1-kd) mice were supplied by Dr. Masayuki Yamamoto (Tohoku University, Aoba-ku, Sendai, Japan) (Okada *et al.*, 2008).

Both Nrf2-null and Keap1-kd mice were backcrossed into C57BL/6

mice, and >99% congenicity was confirmed by the speed congenics group at Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and had access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) and water *ad libitum*. The housing facility is an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were preapproved in accordance with Institutional Animal Care and Use Committee guidelines.

Determination of hepatobiliary disposition of acetaminophen. Wild type, Nrf2-null, and Keap1-kd (n=5) mice were anesthetized by injection of ketamine/midazolam (100 mg/kg and 5 mg/kg, respectively, i.p.). Body temperature was maintained at 37℃ by rectal-probe-con trolled heating pads. Subsequently, the right carotid artery was cannulated with PE-10 tubing, and the common bile duct was cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing through a high abdominal incision. Depth of anesthesia was monitored by pinching the footpad before and throughout surgery, and if necessary, additional anesthetic drugs were administered to

mice. Bile samples were collected in 15-min periods into pre-weighed 0.6 ml microcentrifuge tubes for 5 periods. The bile collection tubes were immersed in ice. After the first bile collection, AA (330 μmol/kg, 20% propylene glycol in saline, 5 mL/kg) was injected into the right femoral vein. Blood (30-35 μl) was collected into heparinized tubes at 2, 7.5, 22.5, 37.5, and 52.5 min after AA injection. Volume of bile was quantified gravimetrically, using 1.0 for specific gravity. Bile and plasma were stored at -80°C. Livers were removed at 1 h, frozen in liquid nitrogen, and stored at -80°C.

Analysis of acetaminophen and its metabolites. Liver, plasma, and bile samples were prepared as described previously (Manautou *et al.*, 2005). Liver samples (1:3 wt/vol) were homogenized in ice-cold HPLC-grade methanol. Homogenates were centrifuged at 1200 g for 30 min at 4ºC, and the supernatents were diluted further (1:2) in 12.5% methanol/1% acetic acid in water (mobile phase A). Bile and plasma samples were diluted (1:1) with ice-cold methanol and then centrifuged at 1200 g for 30 min at 4ºC. Supernatants were further diluted (1:1 for bile and 1:1.5 for plasma) with mobile phase A. Further dilutions in mobile phase A were made where

appropriate to be within the limits of the standard curve. HPLC-UV detection was performed as previously described with modifications (Howie *et al.*, 1977). Mobile phase A consisted of 12.5% methanol/1% acetic acid in water, and mobile phase B consisted of 50% methanol in water. The mobile phase was first maintained at 8% for mobile phase B for 5 min, followed by a linear gradient of 10 min, ending at 25% mobile phase B. Flow rate was kept constant at 1 ml/min, and peaks were detected at 254 nm. The concentrations of AA and its metabolites were calculated using an AA standard curve. Retention times of AA and its metabolites were determined using authentic standards provided by McNeil-PPC, Inc. (Fort Washington, PA).

Hepatic microsomal preparation and *in vitro* biochemical assays. Liver was dounce homogenized in 0.1 M potassium phosphate buffer containing 20% glycerol and 10 mM dithiothreitol (1:10 w/v, pH 7.8). Homogenates were centrifuged at 10,000 g at 4ºC for 20 min, and the supernatant was centrifuged at 100,000 g for 1 h. The cytosolic supernatant was stored at -80°C. The microsomal pellet was resuspended in 0.1 M sodium phosphate

buffer (pH 7.8) and used immediately.

Hepatic bioactivation of AA to NAPQI was determined *in vitro*, using a method described previously with modifications (Manautou *et al.*, 1994). Briefly, 0.1 M sodium phosphate buffer (pH 7.8), 0.83 mM NADP, 15 mM $MgCl_2$, 20 mM glucose-6-phosphate, 4 IU glucose-6-phosphate dehydrogenase, 1 mM *N*-acetylcysteine (NAC, as the trapping agent for NAPQI), and microsomes (1-2 mg protein) were preincubated at 37ºC in glass test tubes in a total volume of 200 μL. After 10 min, 100 μL of AA (20 mM, incubation concentration) in sodium phosphate buffer (pH 7.8) was added and incubated for 20 min at 37ºC. The reaction was stopped after 30 min by addition of 300 μL of ice-cold HPLC-grade methanol. The tubes were then centrifuged at 3200 g for 30 min at 4°C. The supernatant was filtered (45-μm nylon filters) and analyzed by HPLC, as described above. Control incubations were similarly prepared without NAC, AA, or microsomes, and upon HPLC analsys, no AA- NAC peaks were detected. Activity was expressed as nmoles AA-NAC/min/mg protein.

Hepatic Ugt activity was determined using a modified method

(Manautou *et al.*, 1996). Briefly, 0.1 M sodium phosphate buffer (pH 7.8), 10 mM $MgCl_2$, 2.5 mM UDP-glucuronic acid (UDP-GA), 0.5% Brij 58 (microsomal activator), and microsomes (1-2 mg protein) were preincubated for 10 min in glass test tubes in a total reaction volume of 250 μL. After 10 min, 100 μL of AA (5 mM incubation concentration) in 0.1 M sodium phosphate buffer (pH 7.8) was added to the preincubation mixture. The reaction was stopped after 30 min by addition of 300 μL of ice-cold HPLC-grade methanol. The tubes were then centrifuged at 3200 g for 30 min at 4°C. The supernatant was filtered (45 μm nylon filters) and analyzed by HPLC, as described above. Control incubations were similarly prepared without either UDP-GA, AA, or microsomes, and upon HPLC analsys, no AA-GLUC peaks were detected. Activity was expressed as nmoles AA-UDP-GA/min/mg protein.

Hepatic Sult activity was quantified using cytosolic extracts (Mizuma *et al.*, 1984; Liu and Klaassen, 1996). Briefly, cytosols were preincubated at 37°C for 10 min. Then 0.5 mM AA and 0.1 mM 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in 0.1 M sodium phosphate buffer were added, and the mixture was incubated at 37°C. After 20 min, 25 μL of 25% (v/v) $HClO_4$

was added to stop the reaction. Fifty microliters of 0.8 M $BaCl_2$ was added to the incubation mixture, and the mixture was centrifuged at 3000 g for 10 min at 4ºC. The supernatant was transferred to a clean test tube, and 300 μL of ice-cold HPLC-grade methanol was added. The mixture was centrifuged a second time at 3000 g for 10 min at 4ºC. The supernatant was filtered (45-μm nylon filters) and analyzed by HPLC, as described above. Control incubations were similarly prepared without PAPS, acetaminophen, or cytosols, and upon HPLC analysis, no AA-SULF peaks were detected. Activity was expressed as nmoles AA-SULF/min/mg protein.

Cytosolic Nqo1 enzyme activity was determined by quantifying the reduction of 2,6-dichlorophenol-indophenol (DCPIP), as described previously (Ernster, 1967; Benson *et al.*, 1980) and modified (Aleksunes *et al.*, 2006a).

UPD-GA determination. Hepatic concentrations of UDP-GA were determined using the method of formation of benzo[a]pyrene 3-glucuronide, as described previously (Singh *et al.*, 1980). Briefly, approximately 50 mg of liver was boiled for 3 min in 5 mL of water. After cooling, liver was dounce homogenized and centrifuged at 3000 g for 10 min at 4ºC. The supernatant

was further diluted to a final concentration of 5 mg wet weight of liver/ml of water. The reaction mixture (200 μl) contained 20 μmol Tris-HCl buffer, pH 7.6; 1 μmol $MgCl_2$; 0.01% Brij-58 (w/v); 10 nmol 3-hydroxybenzo[a]pyrene in 10 μl of methanol; 50 μl liver tissue homogenate; and 50 μg male albino guinea-pig liver microsomal protein in 0.25 M sucrose. The mixture was incubated for 30 min at 37ºC with mild shaking. Six ml of chloroform/methanol (2:1, v/v) and 0.8 ml of water were added to the mixture, and tubes were shaken. Tubes were centrifuged at 1000 g for 1 min at room temperature, and 200 μl of the supernatant was transferred to an opaque 96-well plate. Fluorescence of the benzo[a]pyrene 3-glucuronide was quantified at 378 excitation/425 emission. Concentrations of UDP-GA in unknown liver samples were calculated using a standard curve of known concentrations of UDP-GA.

Immunofluorescence staining. Immunofluorescence of Mrp2, Mrp3, Mrp4, and Bcrp was performed similarly as described previously (Aleksunes *et al.*, 2006b). Cryostat cut tissue sections (5 μm) were fixed with 4% paraformaldehyde for 5 min. All antibody solutions were filtered through 0.22

μm membrane syringe-driven filter units (Millipore, Billerica, MA). Sections were blocked with 5% goat serum/phosphate-buffered saline with 0.1% Triton X (PBS-Tx) for 1 h and then incubated with Mrp2, Mrp3, Mrp4, or Bcrp primary antibody diluted 1:100 in 5% goat serum/PBS-Tx for 2 h at room temperature. After incubation with primary antibody, the sections were washed three times in PBS-Tx and incubated for 1 h with goat anti-rat IgG Alexa 488 for Mrp3, Mrp4, and Bcrp and with goat anti-rabbit IgG Alexa 488 for Mrp2 (Invitrogen Corporation, Carlsbad, CA) diluted 1:200 in 5% goat serum/PBS-Tx. The sections were air-dried and mounted in Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Corp.). Frozen liver sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included in the analysis (data not shown). Images were captured on an Olympus BX41 fluorescent microscope with a DP70 camera and DP Controller software (Olympus, Center Valley, PA).

Hepatotoxicity Studies. Wild-type, Nrf2-null, and Keap1-kd mice were administered AA (4 mmol/kg, i.p., 20% propylene glycol in saline, 10 mL/kg).

Six h later blood was collected by cardiac puncture under pentobarbital anesthesia, and livers were removed. A portion of the largest lobe of the liver was placed in 10% phosphate-buffered zinc formalin to fix for 24 h. Tissues were processed and sectioned according to standard histological techniques. Tissue sections were scored using a scale from 0 to 5 according to the severity of necrosis as described previously (Manautou *et al.*, 1994). Histopathology scoring was as follows: no injury = grade 0; minimal injury involving single to few hepatocytes = grade 1; mild injury affecting 10–25% of hepatocytes = grade 2; moderate injury affecting 26–40% of hepatocytes = grade 3; marked injury affecting 41–50% of hepatocytes = grade 4; or severe injury affecting more than 50% of hepatocytes = grade 5. Sections with scores higher than 2 are considered to exhibit significant liver injury. Serum ALTs were quantified using a commercially available kit according to manufacturer's protocol (Pointe Scientific, Canton, MI).

Statistical Analysis. All data but histophathological data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$) utilizing SigmaStat Software (Systat Software Inc., San

Jose, CA). N=5 for all groups, and values are expressed as mean ± S.E.M. Histopathological data were rank ordered prior to ANOVA analysis, which was followed by Newman-Keuls multiple range test ($p \leq 0.05$).

RESULTS

Plasma disposition of AA and its conjugates. One h after AA (330 μmol/kg, i.v.) administration, serum ALT concentrations ranged from 35-65 IU/L with no differences among genotypes, indicating that there was no apparent hepatotoxicity at this dose during the 1 h of bile collection (data not shown). Plasma disappearance of AA was decreased in Nrf2-null mice and increased in Keap1-kd mice (Fig 1A). AA-GLUC was the most abundant AA-metabolite detected in plasma. The plasma appearance of AA-GLUC was decreased in Nrf2-null mice and increased in Keap1-kd mice at 7.5 and 22 min (Fig 1B). Plasma AA-GLUC concentrations in Nrf2-null mice did not approach the plasma concentrations of wild-type mice during the 1 h timeframe, reaching a maximum concentration that was only 48% of wild-type mice. Plasma AA-GLUC concentrations were 45-62% higher in Keap1-kd mice for the first 22.5 min, but were comparable to wild-type mice at subsequent time points. Plasma AA-SULF was 61-82% higher in the Nrf2-null mice, whereas it was markedly reduced 85-96% in Keap1-kd mice (Fig 1C). AA-GSH conjugates in plasma were not different among genotypes, and

AA-Cys and AA-NAC conjugates were not detected in plasma (data not shown).

Biliary disposition of AA and its conjugates. Even though only a small portion of AA is excreted non-biotransformed into bile, Nrf2-null mice had higher, and Keap1-kd mice had lower, cumulative biliary excretion of AA (Fig 2A). There were no differences in the biliary excretion of AA-GLUC among genotypes (Fig 2B). Cumulative biliary excretion of AA-SULF was increased 40-43% in Nrf2-null mice at 45 and 60 min, and decreased 35-40% at 30, 45, and 60 min in Keap1-kd mice (Fig 2C). AA-GSH was the most abundant metabolite excreted into bile. Cumulative biliary excretion of AA-GSH was increased 54-76% in Nrf2-null mice (Fig 2D, values at 30 and 45 min tended to be higher than wild-type, $p \leq 0.06$). There was no difference in cumulative biliary excretion of AA-GSH between Keap1-kd and wild-type mice. AA-Cys was not detected in bile, and biliary excretion of AA-NAC was low and not different among genotypes (data not shown).

Acetaminophen and its metabolites in liver. Fig 3 shows the hepatic concentrations of AA and its metabolites in wild-type, Nrf2-null, and Keap1-kd

mice at 1 h after administration. Accumulation of AA in livers of Nrf2-null and wild-type mice was similar after 1 h. However, the hepatic concentration of AA after 1 h was 71% lower in Keap1-kd mice than wild-type mice. Higher liver concentrations of AA-GLUC, AA-SULF, and AA-GSH accumulated in Nrf2-null mice, whereas these conjugates were reduced (AA-GLUC and AA-SULF) or unchanged (AA-GSH) in Keap1-kd mice. There was no difference in hepatic concentrations of AA-NAC between Nrf2-null and wild-type mice; however, Keap1-kd mice had 79% lower hepatic concentrations of AA-NAC. There were no differences in AA-Cys in the liver after 1 hr among the three genotypes (data not shown).

In vitro bioactivation, glucuronidation, and sulfation activity. Hepatic microsomes were used to quantify AA bioactivation and Ugt activity, whereas cytosols were used to determine Sult activity. *In vitro* bioactivation activity of AA was not different among genotypes (Fig 4A). In comparison to wild-type mice, *in vitro* glucuronidation activity of AA was 40% lower in Nrf2-null mice, whereas there was no increase in Keap1-kd mice (Fig 4B). *In vitro* sulfation

activity of AA was unchanged in Nrf2-null mice and 29% lower in Keap1-kd mice than wild-type mice (Fig 4C).

Hepatic UDP-GA determination. UDP-GA concentrations in livers from wild-type, Nrf2-null, and Keap1-kd mice were determined because Ugt enzyme activity depends on availability of UDP-GA (Singh and Schwarz, 1981; Gregus *et al.*, 1983). There were no differences in hepatic UDP-GA concentrations among the three genotypes (Fig 5).

Nqo1 enzyme activity. Enzyme activity of the Nrf2 prototypical target gene Nqo1 was quantified in wild-type, Nrf2-null, and Keap1-kd mice (Fig 6). Nqo1 activity was reduced 85% in Nrf2-null mice but elevated 514% in Keap1-kd mice.

Immunofluorescence of Bcrp, Mrp2, Mrp3, and Mrp4. Hepatic expression of Bcrp, Mrp2, Mrp3, and Mrp4 proteins was assessed in frozen liver sections from wild-type, Nrf2-null, and Keap1-kd mice using indirect immunofluorescence (Fig 7). The immunofluorescence of Bcrp and Mrp2 was consistent with localization to canalicular junctions between adjacent hepatocytes and was similar among all three genotypes. Mrp3 and Mrp4

proteins were localized to basolateral hepatocyte membranes. Immunofluorescence of Mrp3 was difficult to detect on the plasma membrane in Nrf2-null mice, moderately expressed in all hepatocytes in wild-type mice, and enhanced in all hepatocytes in Keap1-kd mice. Staining of Mrp4 was low in livers from wild-type and Nrf2-null mice, whereas Keap1-kd mice had enhanced immunostaining of Mrp4 predominantly on centrilobular hepatocytes.

Hepatotoxic effects of AA. Vehicle-administered wild-type, Nrf2-null, and Keap1-kd mice had no differences in serum ALT concentrations or necrosis grading (ALT values below 60 IU/L) and no apparent histopathological damage (data not shown). AA-admininistered (4 mmol/kg, i.p.) wild-type mice had serum ALT concentrations indicative of hepatic injury (Fig 8). AA-administered Nrf2-null mice had elevated (550%) serum ALT concentrations, whereas Keap1-kd mice had 57% lower serum ALT concentrations (62 IU/L) when compared to wild-type mice. AA administration caused moderate hepatocellular necrosis in wild-type mice, severe hepatocellular necrosis in Nrf2-null mice, and no hepatocellular damage in Keap1-kd mice (Table 1).

***Table 5.1*. Histological analysis of livers from wild-type, Nrf2-null, and Keap1-kd mice after AA challenge.**

	Histological Grade						
Treatment Group	**0**	**1**	**2**	**3**	**4**	**5**	**P≤0.05**
Nrf2-null	0	0	0	0	3	2	*
Wild-type	0	0	3	2	0	0	
Keap1-kd	3	2	0	0	0	0	*

Necrosis grading in wild-type, Nrf2-null, and Keap1-kd mice 6 h after AA (600 mg/kg, i.p.) administration. There were no differences in necrosis grading in vehicle-treated wild-type, Nrf2-null, and Keap1-kd mice with necrosis grades of 0 (data not shown). Histopathology scoring was as follows: no injury = grade 0; minimal injury involving single to few hepatocytes = grade 1; mild injury affecting 10–25% of hepatocytes = grade 2; moderate injury affecting 25–40% of hepatocytes = grade 3; marked injury affecting 40–50% of hepatocytes = grade 4 or severe injury affecting more than 50% of hepatocytes = grade 5. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

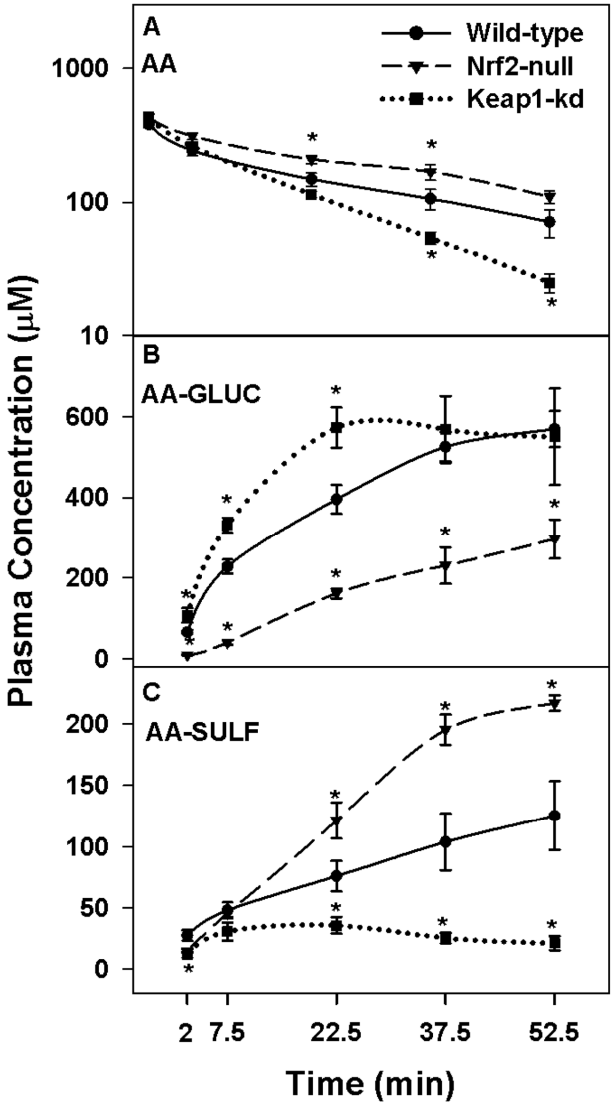


Fig 5.1. Plasma concentrations of AA (A), AA-GLUC (B), and AA-SULF (C) in wild-type, Nrf2-null, and Keap1-kd mice administered AA (330 μmol/kg, i.v.). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

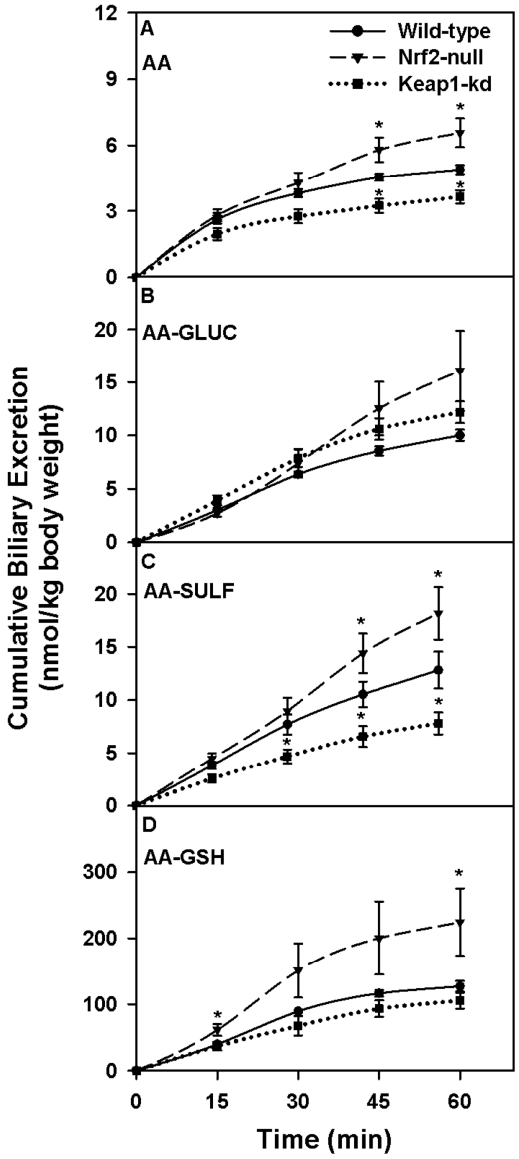


Fig 5.2. Cumulative biliary excretion of AA (A), AA-GLUC (B), AA-SULF (C), and AA-GSH (D) in wild-type, Nrf2-null, and Keap1-kd mice administered AA (330 μmol/kg, i.v.). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

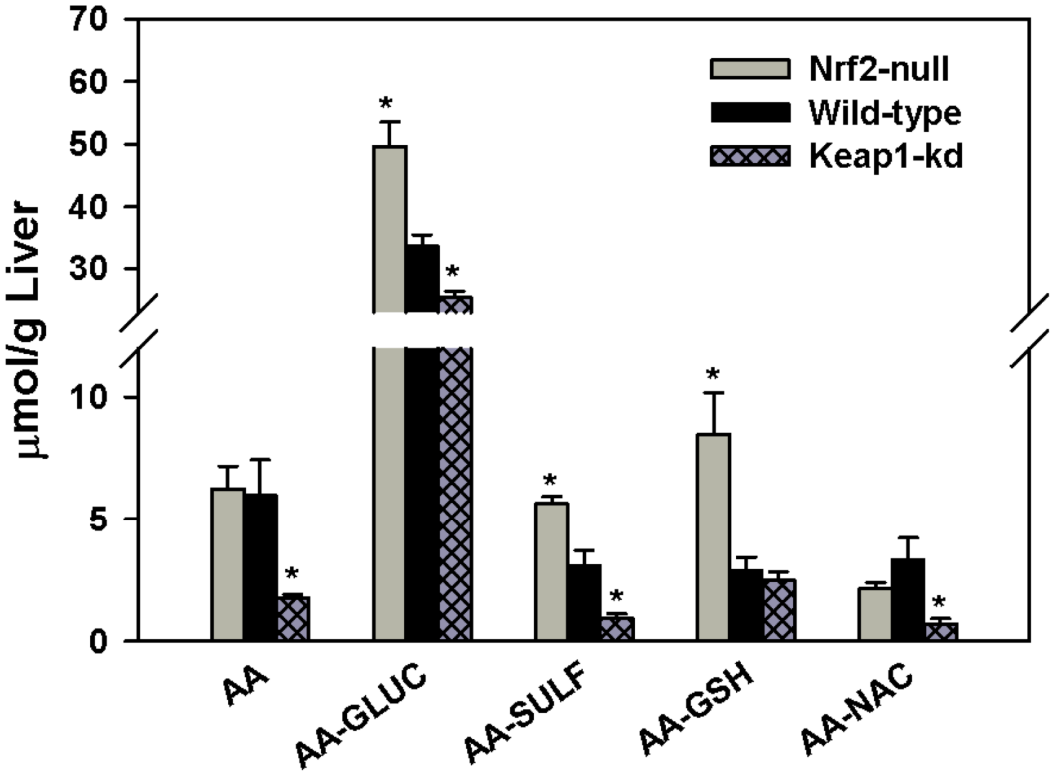


Fig 5.3. Hepatic concentrations remaining 1 h after administration of AA (330 μmol/kg, i.v.) for AA, AA-GLUC, AA-SULF, AA-GSH, and AA-NAC in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

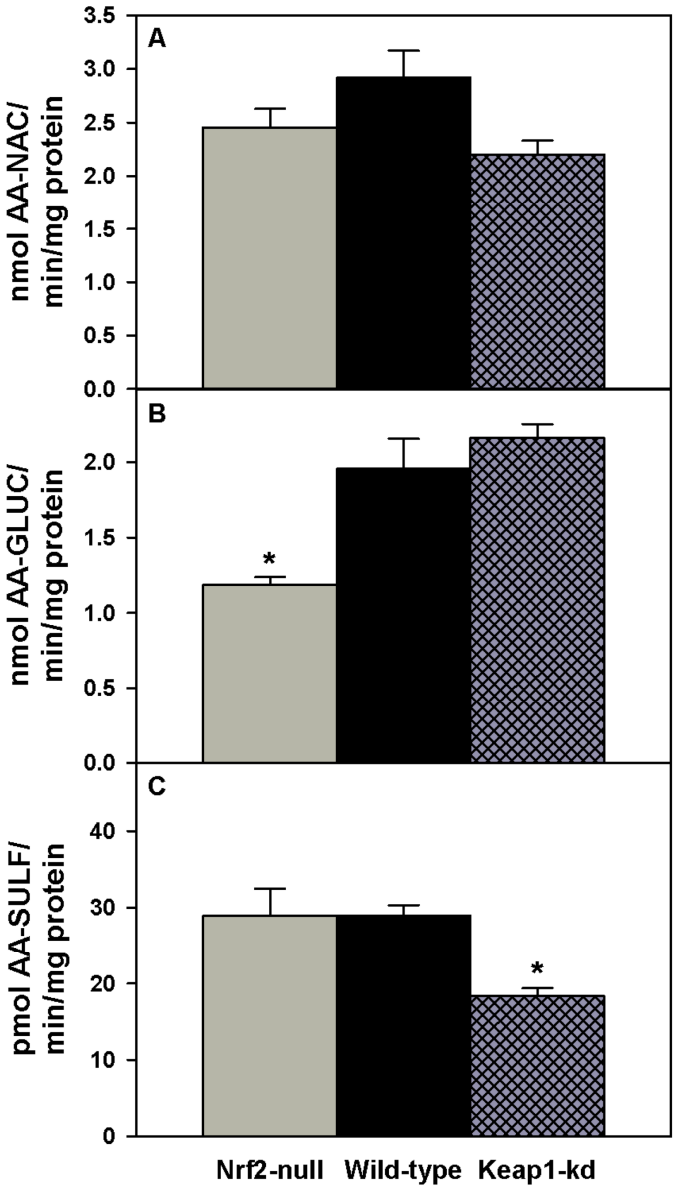


Fig 5.4. *In vitro* enzyme activities of AA bioactivation (A), glucuronidation (B), and sulfation (C). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

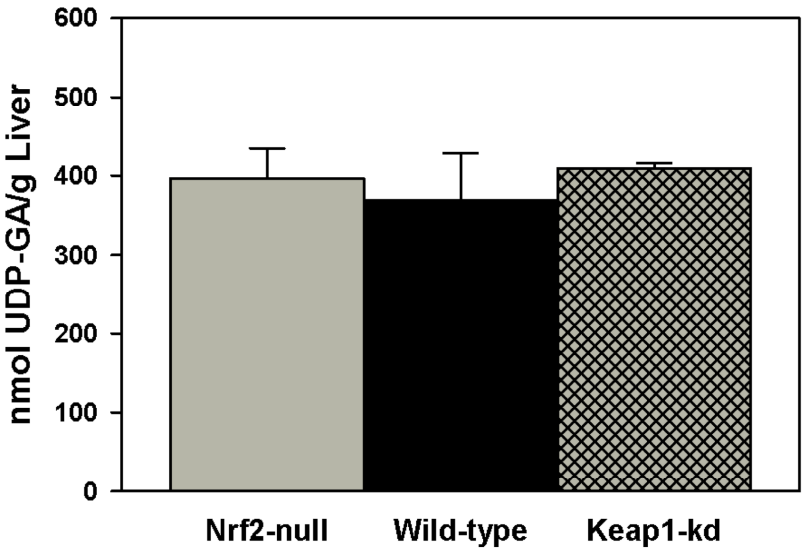


Fig 5.5. Hepatic UDP-GA concentrations in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M.

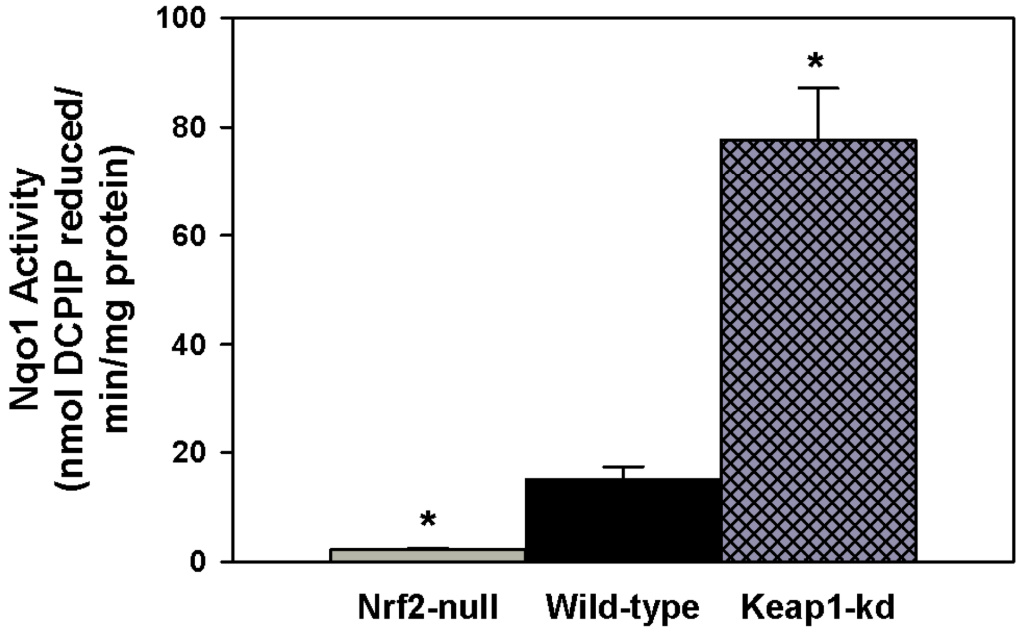


Fig 5.6. Hepatic Nqo1 enzyme activity in wild-type, Nrf2-null, and Keap1-kd mice. Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

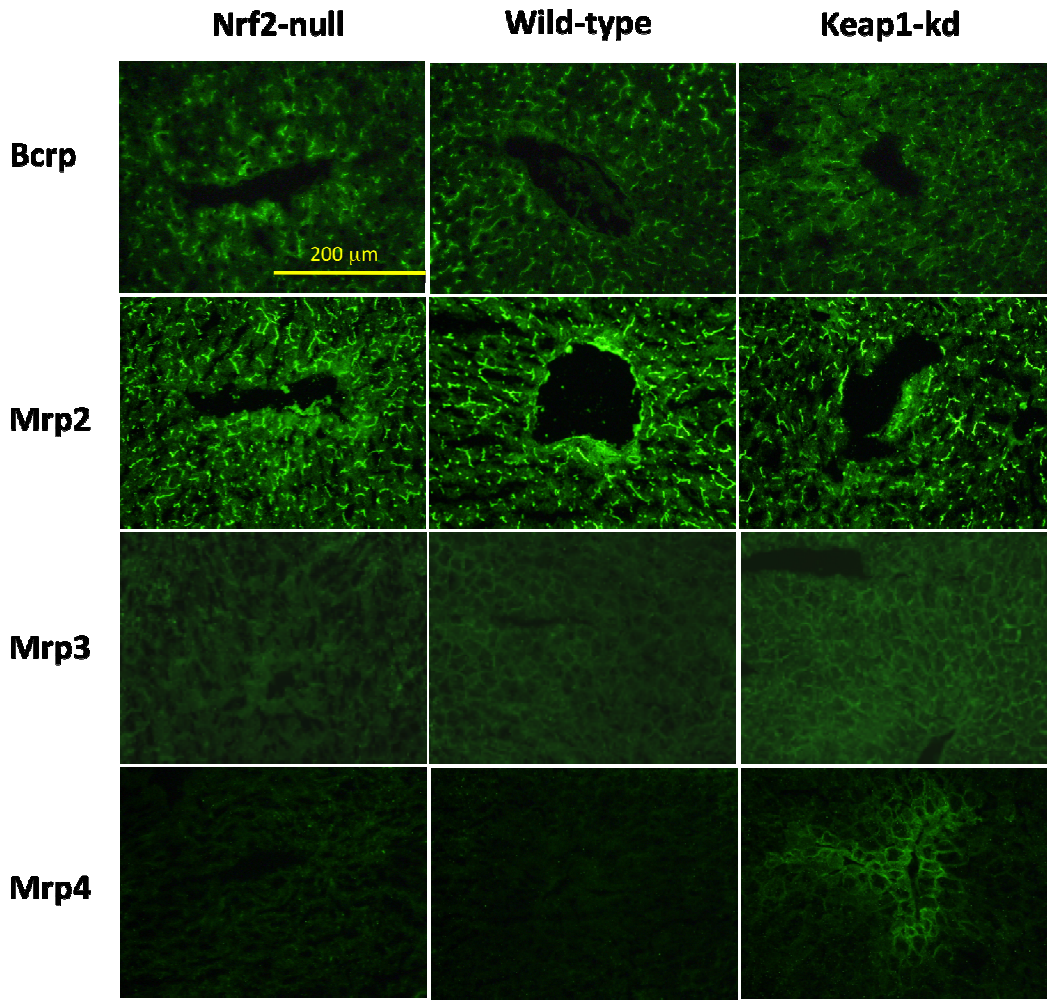


Fig 5.7. Immunofluorescent analysis of Bcrp, Mrp2, Mrp3, and Mrp4 protein in wild-type, Nrf2-null, and Keap1-kd mice. Indirect immunofluorescence against Bcrp, Mrp2, Mrp3, and Mrp4 (green) was performed on liver cryosections. Representative regions are shown. The bar represents 200 μm.

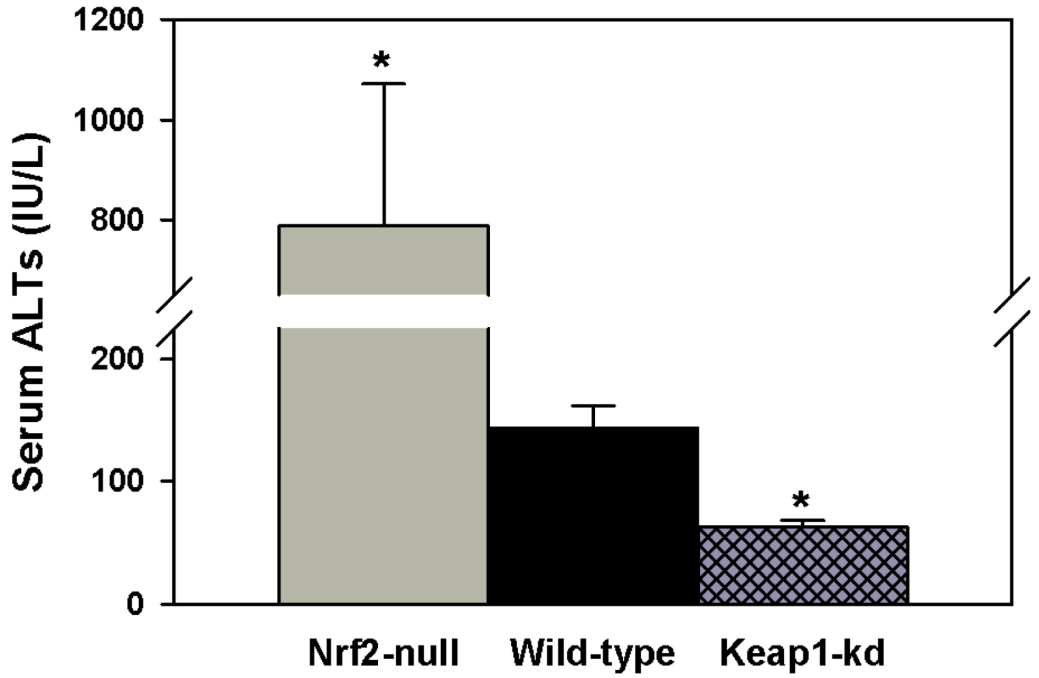


Fig 5.8. Serum ALT concentrations in wild-type, Nrf2-null, and Keap1-kd mice 6 h after AA (600 mg/kg, i.p.) administration. There were no differences in serum ALT concentrations in vehicle-treated wild-type, Nrf2-null, and Keap1-kd mice with values all below 60 IU/L (data not shown). Values are expressed as mean ± S.E.M. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

DISCUSSION

The increased susceptibility of Nrf2-null mice to AA hepatotoxicity has been attributed to decreased Ugt1a6 mRNA expression and reduced GSH concentrations in liver (Chan *et al.*, 2001; Enomoto *et al.*, 2001). In contrast, the increased resistance of hepatocyte-specific Keap1-null mice to AA hepatotoxicity, where there is increased activation of Nrf2, is suggested to be due to increased mRNA expression of Ugt1a6 and the GSH synthesizing enzyme, Gclc (Okawa *et al.*, 2006). These previous studies using Nrf2-null and hepatocyte Keap1-null mice drew conclusions about sensitivity to AA hepatotoxicity mainly on gene expression data. Because the biotransformation of AA is paramount in determining the severity of hepatotoxicity, the effects of differences in Nrf2 activation in *in vivo* mouse models were also determined in the present study. Herein, the pharmacokinetics of AA were investigated in Nrf2-null mice, which lack Nrf2, and Keap1-kd mice, which have enhanced activation of Nrf2.

The concentration of AA in plasma decreased more slowly in Nrf2-null mice and faster in Keap1-kd mice than in wild-type mice. Generally, changes

in plasma disappearance of xenobiotics are due to alterations in uptake into liver, biotransformation, and/or excretion from liver into bile or plasma. Uptake transporters, such as organic anion transporting polypeptides, mediate the entrance of a large number of xenobiotics into hepatocytes (Klaassen and Lu, 2008). However, AA is generally not considered a substrate for uptake transporters, but instead is thought to enter hepatocytes by diffusion, indicating that uptake of AA does not likely play a role in the altered plasma disappearance of AA among wild-type, Nrf2-null, and Keap1-kd mice (McPhail *et al.*, 1993).

In mice, glucuronidation accounts for approximately 50% of AA excreted into urine and bile, significantly influencing the amount of AA available for other pathways of biotransformation, such as sulfation and bioactivation (Gregus *et al.*, 1988). Plasma concentrations of AA-GLUC are lower in Nrf2-null mice and higher in Keap1-kd mice (first 22.5 min) after AA administration (Fig 1B). Reduced efflux of AA-GLUC into plasma of Nrf2-null mice is attributed to lower microsomal AA glucuronidation activity (Fig 4C) and Mrp3 expression (Fig 7). Ugt1a6 mRNA expression is increased in

Keap1-kd mice (Reisman et al., Chapter Four); however, there was no change in AA glucuronidation activity from livers of Keap1-kd mice (Fig 4C). Therefore, increased hepatic efflux of AA-GLUC into plasma in Keap1-kd mice reflects the pronounced up-regulation of Mrp3 mRNA and protein (Fig 7) (Okada *et al.*, 2008). Efflux of AA-GLUC into bile is similar among genotypes, most likely due to comparable amounts of Mrp2 protein among the three genotypes (Fig 7). There is more AA-GLUC remaining in liver 1 h after AA administration in Nrf2-null mice, but less in Keap1-kd mice, which correlates inversely with the plasma appearance of AA-GLUC and Mrp3 protein. Glucuronidation is also influenced by the availability of the co-substrate UDP-GA. However, there were no differences in hepatic UDP-GA concentrations among genotypes, which suggests that differences in AA-GLUC among genotypes are not due to availability of UDP-GA (Singh and Schwarz, 1981; Gregus *et al.*, 1983). Therefore, lack of Nrf2 results in lower AA-GLUC in the plasma and higher amounts of AA-GLUC remaining in the liver, due to reduced AA-glucuronidation activity and Mrp3-mediated efflux from liver to plasma. In contrast, enhanced activation of Nrf2 in Keap1-kd

mice increases AA-GLUC in the plasma and decreases AA-GLUC remaining in the liver 1 h after AA administration, which is attributed to elevated Mrp3 expression.

In mice, sulfation accounts for approximately 5% of the AA excreted into urine and bile, and any change in glucuronidation or bioactivation of AA could influence the quantity of AA available for sulfation (Gregus *et al.*, 1988). Sulfation in rats is limited by the availability of PAPS; however, in mice, sulfation of AA is limited by sulfotransferase activity (Kim *et al.*, 1995; Liu and Klaassen, 1996). AA-SULF concentrations in plasma, liver, and bile are inversely proportional to Nrf2 activation (i.e., increased AA-SULF in Nrf2-null mice and decreased AA-SULF in Keap1-kd mice). Expression of transporters capable of effluxing AA-SULF (Mrp2, Mrp3, Mrp4, and Bcrp) is either similar among all three genotypes or lower in Nrf2-null mice, suggesting that efflux is unlikely to be responsible for the differences, as no change or a decrease in efflux transporter expression should not result in an increase in hepatic efflux of AA-SULF (Nrf2-null mice). In addition, *in vitro* AA-sulfation activity was similar between Nrf2-null and wild-type mice. Taken together, the decrease

in AA-GLUC activity and increase in formation and excretion of AA-SULF in Nrf2-null mice suggests a metabolic shift to sulfation to compensate for the decrease in the amount of AA undergoing glucuronidation. Keap1-kd mice have lower *in vitro* sulfation activity (Fig 4B), resulting in reduced AA-SULF concentrations in plasma, liver, and bile. Phenol sulfotransferases in rat liver have higher activity when in a partially oxidized state, as the fully reduced state gives rise to a ternary complex that impedes binding of the co-substrate PAPS (Marshall *et al.*, 2000). It is possible that the more reduced environment caused by increased expression of cytoprotective genes in Keap1-kd mice liver results in lower Sult activity. Therefore, Nrf2-null mice have increased AA-SULF in plasma, liver, and bile because of decreased glucuronidation activity, which leaves more AA available for sulfation, but Keap1-kd mice have decreased AA-SULF directly due to decreased AA-sulfation activity.

Ultimately, AA hepatotoxicity depends on the quantity of AA bioactivated by Cyps and the ability to detoxify NAPQI through GSH conjugation or Nqo1-mediated quinone reduction. In general, Cyp2e1, 1a1,

and 3a11 enzyme activities are not different among genotypes (Reisman et al., Chapter Four). Therefore, it was not surprising that the *in vitro* ability to generate NAPQI was not different among the three genotypes (Fig 4A). However, Nrf2-null mice generate more AA-GSH *in vivo*, as evident by more AA-GSH excreted into bile and remaining in liver (Fig 2D and 3). The decreased ability to form AA-GLUC in Nrf2-null mice increases the availability of AA for bioactivation to the reactive metabolite NAPQI. Decreased microsomal AA glucuronidation and Nqo1 activity (Fig 6) likely contribute to increased AA-GSH formation in Nrf2-null mice. In turn, increased AA-GSH formation further decreases already reduced concentrations of GSH in Nrf2-null mice (Reisman et al., Chapter Four) and increases sensitivity to AA hepatotoxicity. Keap1-kd mice excrete similar quantities of AA-GSH into bile as wild-type mice (Fig 2). Keap1-kd mice also have similar concentrations of AA-GSH as wild-type mice remaining in their livers 1 h after administration of AA. However, increased Nqo1 enzyme activity (Fig 6) explains the decrease in the combined total of AA-GSH and its breakdown product AA-NAC remaining in the liver after 1 h. The absence of Nrf2 decreases

glucuronidation of AA, and therefore increases the availability of AA for Cyp metabolism and subsequent AA-GSH synthesis. In contrast, enhanced Nrf2 activation does not affect glucuronidation activity but does increase the ability of hepatic Nqo1 to detoxify NAPQI.

Nrf2 plays a crucial role in the pharmacokinetics and metabolism of AA, and, in turn, hepatotoxicity. Decreased ability of Nrf2-null mice to conjugate and excrete the parent compound and to detoxify NAPQI results in increased sensitivity to hepatotoxicity (Fig 8 and Table 1). In contrast, Keap1-kd mice, with increased activation of Nrf2, have an enhanced ability to detoxify and excrete AA, resulting in decreased sensitivity to AA hepatotoxicity. In fact, Keap1-kd mice do not display any hepatotoxicity at a relatively high dose of AA (4 mmol/kg, i.p.).

In conclusion, previous studies have shown that Nrf2 protects against hepatotoxicity, and the proposed explanation has been that the protection is due to an increase in genes that protect against electrophilic stress (Aleksunes and Manautou, 2007). Whereas there is little doubt that the induction of cytoprotective genes, via Nrf2, is important in the prevention of

hepatic injury, the present study emphasizes the importance of the Nrf2-Keap1 pathway on the pharmacokinetics of AA. Collectively, the results presented herein have shown that lack of Nrf2 decreases AA glucuronidation and increases AA available for Cyp-mediated NAPQI formation and hepatotoxicity, whereas activation of Nrf2 enhances the detoxification of NAPQI by Nqo1 and the elimination of AA-GLUC via Mrp3.

GENERAL SUMMARY AND CONCLUSIONS

Mammals are frequently exposed to electrophilic and oxidative stressors and in turn, have developed highly efficient cytoprotective mechanisms (Li and Kong, 2008). When exposed to electrophilic/oxidative stress, cells counter with induction of detoxification enzymes, antioxidants, and efflux transporters, allowing them to reduce an oxidative environment and return to homeostasis. A homeostatic balance between oxidants and antioxidants in cells is required for normal cellular functions and the prevention of diseases, such as cancer and liver injury. A key transcription factor regulating the antioxidative system response is Nrf2. Many studies have demonstrated increased sensitivity to oxidative and electrophilic stress in mice lacking Nrf2 (Aleksunes and Manautou, 2007). However, few have investigated whether there are protective effects when Nrf2 is activated and target genes induced, especially in the liver of whole animals. Therefore, the purpose of this study was to investigate the pharmacologic (triterpenoids) and genetic effects (Keap1-kd mice) of increased Nrf2 on AA-induced liver injury, expression of hepatic drug processing genes, and the pharmacokinetics of

two compounds whose biotransformation occurs primarily in the liver, BSP and AA.

The experiments in Chapter One investigated the mechanism of action of oleanolic acid, a natural triterpenoid compound, shown previously to protect the liver from a wide-variety of chemicals with diverse mechanisms for producing liver toxicity (Liu *et al.*, 1995a). Daily injection of oleanolic acid (3 days) activated Nrf2, indicated by an increase in Nrf2 nuclear accumulation. Oleanolic acid also increased mRNA expression of Nrf2-target genes (Nqo1, Gclc, and Ho-1), as well as protein expression and enzyme activity of the prototypical Nrf2-target gene Nqo1 in wild-type, but not in Nrf2-null mice. Furthermore, oleanolic acid pretreatment protected wild-type mice more than Nrf2-null mice from AA-induced hepatotoxicity.

Oleanolic acid also demonstrated mild hepatoprotection from AA in Nrf2-null mice, indicating a Nrf2-independent mechanism of action. A possible explanation for this is that oleanolic acid induces cytoprotective enzymes in a manner independent of Nrf2. Two such cytoprotective proteins induced by oleanolic acid include metallothionein (MT), a low-molecular-

weight protein rich in cysteine residues, and a 70 kDa heat shock protein (Hsp70) (Liu *et al.*, 2008). Because of the high sulfhydryl content in Mt, it has been suggested to react with and detoxify reactive oxygen species and electrophiles (Klaassen and Cagen, 1981). MT can be induced by Nrf2-independent stress mechanisms, including the transcription factors Nrf1 and the metal-responsive-element-binding transcription factor-1 (Mtf-1) (Laity and Andrews, 2007; Ohtsuji *et al.*, 2008). Also, MT-null mice are more sensitive to AA toxicity (Liu *et al.*, 1999). Hsp70 acts to protect cells by reducing the ability of oxidized proteins to aggregate, permitting them time to refold and return to a functional conformation. Hsp70-null mice are more sensitive to AA hepatotoxicity (Tolson *et al.*, 2006). Collectively, the ability of oleanolic acid to induce cytoprotective genes independent of Nrf2 provides a possible explanation for part of the protection observed from AA hepatoxicity in oleanolic acid-pretreated Nrf2-null mice.

The experiments in Chapter Two investigated whether CDDO-Im, a synthetic triterpenoid, could also activate Nrf2 and protect from AA-hepatotoxicity. Indeed, CDDO-Im induced Nrf2-target genes in both a time-

and dose-dependent manner in wild-type mice, whereas no induction of Nrf2-target genes was detected in Nrf2-null mice. Also, the dramatic hepatoprotection CDDO-Im had in AA-administered wild-type mice was not observed in Nrf2-null mice. To summarize Chapters One and Two, both oleanolic acid and CDDO-Im protected the liver from AA-induced injury, at least partially, by activating the cytoprotective transcription factor Nrf2.

Oleanolic acid has an oral bioavailability of approximate 0.7% (Jeong *et al.*, 2007). Therefore, alternative compounds were synthesized in order to not only take advantage of the positive effects of oleanolic acid, but also to increase its bioavailability. Because CDDO-Im and related synthetic triterpenoids (CDDO and CDDO-Me) have much higher oral bioavailability and efficacy than the natural triterpenoid oleanolic acid, they are currently in Phase II clinical testing (Reata Pharmaceuticals, Inc.). The synthetic triterpenoids are being tested as oral drugs for the treatment of autoimmune, cardiovascular, and renal diseases because of their ability to restore redox homeostasis. This restoration of redox homeostasis inhibits the production of inflammatory cytokines, and reduces inflammation and cellular damage from

pro-oxidant species. Perhaps the synthetic triterpenoids could also be developed for the treatment and prevention of liver diseases, as few therapies for treating hepatotoxicity currently exist.

Because chemicals can have many off-target effects, a genetic model of Nrf2 activation would be extremely useful in determining the specific gene patterns induced by Nrf2. Keap1-kd mice have a 55% decrease in Keap1 mRNA and a 200% increase in Nrf2 protein in liver. Therefore, the purpose of the second aim was to determine the phenotype of Keap1-kd mice and compare the patterns of gene expression among wild-type, Nrf2-null and Keap1-kd mice. A fundamental understanding of the mRNA expression of drug metabolizing and detoxification enzymes will provide a framework for future studies using both Nrf2-null mice and Keap1-kd mice. Indeed, three patterns of gene expression were observed among wild-type, Nrf2-null, and Keap1-kd mice. The first pattern encompassed genes that were lower in Nrf2-null mice and considerably higher in Keap1-kd mice than wild-type mice, and included genes mainly responsible for the detoxification and elimination of electrophiles, such as Nqo1 and Gsts, as well as Mrps. The second pattern

encompassed genes that were lower in Nrf2-null mice, not increased in Keap1-kd mice, and included genes important in the detoxification of reactive oxygen species, such as Sod1, Sod2, catalase, and Prx1. The third pattern encompassed genes that were not different among wild-type, Nrf2-null, and Keap1-kd mice and included genes such as Gpx1, microsomal Gsts, Ho-1, and uptake transporters. In conclusion, the study presented in the third chapter suggests that hepatic Nrf2 is more important for the detoxification and elimination of electrophiles than reactive oxygen species. This study provides a framework and foundation for future studies involving Keap1-kd mice in order to explore the effects of increased activation of Nrf2.

In studies that have investigated the increased sensitivity to the toxicity as a result of a loss of Nrf2 or the increased resistance to toxicity due to activation of Nrf2, none have investigated the effects of Nrf2 on the kinetics of a xenobiotic. The conclusions from many of these studies are often based exclusively on gene expression data. Therefore, the purpose of Chapters Three and Four was to determine whether Nrf2 alters the kinetics of two well-

known and well-studied compounds biotransformed in the liver, namely BSP (Chapter Three) and AA (Chapter Five).

The elimination of BSP was most affected by the activity of Gsts, which is decreased in Nrf2-null mice and increased in Keap1-kd mice. In addition, the elimination of BSP was not effected by differential Nrf2-dependent transporter expression among wild-type, Nrf2-null, and Keap1-kd mice. For the kinetics of AA, a lack of Nrf2 (Nrf2-null mice) decreased AA biotransformation and elimination, leading to increased formation of the toxic intermediate and hepatotoxicity, whereas activation of Nrf2 (Keap1-kd mice) enhanced detoxification of the reactive metabolite of AA and elimination of conjugated AA via efflux transporters. Therefore, it is important to note that the toxicokinetics of a xenobiotic due to increased Nrf2 activation must be investigated not just toxicodynamics. This will allow one to determine whether the elimination of the toxicant is being affected by increased biotransformation and/or efflux transport.

With activation or blocking of any target in pharmacology with a chemical, there are often unintended adverse effects, which are sometimes

overlooked because one is often only interested in and evaluating the desired effects. Indeed, Nrf2 does have and could have more adverse effects, which will be discussed here. Occasionally mentioned in the literature, are the beneficial and adverse effects of Nrf2 activaiton (Hayes and McMahon, 2006). Nrf2 has a beneficial cytoprotective effects in healthy cells under stress, whereas Nrf2 activation can also result in cancer cell persistence and growth. Some human lung, gallbladder, breast, cancers have mutations in Keap1, which result in enhanced activation of Nrf2, which most likely would increase protection of the cancer cells from conventional chemotherapies (Singh *et al.*, 2006; Nioi and Nguyen, 2007; Ohta *et al.*, 2008; Shibata *et al.*, 2008a; Shibata *et al.*, 2008b). In contrast, RNAi-mediated silencing of Nrf2 gene expression in non-small cell lung cancer inhibited tumor growth and increased efficacy of chemotherapy (Singh *et al.*, 2006; Nioi and Nguyen, 2007; Ohta *et al.*, 2008; Shibata *et al.*, 2008a; Shibata *et al.*, 2008b). Therefore, a compound that activates Nrf2 could allow a cancer cell that would normally be eliminated by the body to progress to malignancy.

One of the major detoxifying pathways induced by Nrf2 activation is the

ability to synthesize and use GSH. Compounds that are bioactivated to a toxic metabolite by GSH could have increased toxic effects because of increased reactive metabolite formation, due to activation of Nrf2 and subsequent induction of GSH synthesis genes and Gsts. For example, hexachlorobutadiene, a halogenated alkene used as an industrial solvent, is conjugated with glutathione in the liver and then sent to the kidney for processing to mercapaturic acids (Dekant, 1996). After removal of the glutamic acid and glycine residues by peptidases, b-lyase produces a reactive metabolite, which leads to nephrotoxicity (Dekant, 1996). It is plausible that increased GSH concentrations and Gst activity due to Nrf2 activation could lead to increased nephrotoxicity from hexachlorobutadiene, as well as increased toxicity for other compounds activated by GSH (dichloromethane, dichloroethane, bromobenzene). In contrast, inducing Nrf2 and subsequently Gsts in cancer cells could lead to the ability to more readily activate chemotherapies, such as canfosfamide, to the reactive metabolite that kills cancer cells. Canfosfamide is metabolized into two fragments by Gstp1, a Nrf2-dependent gene. The cytotoxic fragment reacts with RNA, DNA and

protein in the cancer cell leading to apoptosis. The glutathione analogue fragment remains bound to Gstp1 and prevents it from causing chemotherapy resistance. So, canfosfamide not only kills cancer cells directly, but also helps other chemotherapy drugs work more efficiently (McIntyre and Castaner, 2004).

The first two studies showed that Nrf2, when activated pharmacologically, protects from chemical-induced liver injury by up-regulating cytoprotective genes. Further studies in a genetic mouse model of Nrf2 activation showed that hepatic Nrf2 induces genes mainly responsible for the detoxification of electrophiles and not genes that detoxify reactive oxygen species. In addition, the increase of Nrf2-regulated cytoprotective genes resulted in more favorable kinetic profiles of AA and BSP for their detoxification and elimination. Overall, inductive control over a broad array of genes that facilitate increased detoxification and clearance of reactive metabolites and xenobiotics has earned Nrf2 a crucial role in toxicology and xenobiotic metabolism. For toxicology, Nrf2 must be considered as a means of protection and a potential target for treatment after poisoning. For

pharmacology, Nrf2 must be considered in drug development especially for drugs that are biotransformed by Ugts and/or Gsts or transported by Mrps as kinetics could be drastically and unfavorably altered.

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