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# Nrf2 the Rescue: Effects of the Antioxidative/Electrophilic Response on the Liver

**Curtis D. Klaassen** and **Scott A. Reisman**
Department of Pharmacology, Toxicology, and Therapeutics University of Kansas Medical Center 3901 Rainbow Blvd. Kansas City, KS 66160

## Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that positively regulates the basal and inducible expression of a large battery of cytoprotective genes. These gene products include proteins that catalyze reduction reactions (NAD(P)H:quinone oxidoreductase 1, Nqo1), conjugation reactions (glutathione-*S*-transferases, Gsts and UDP-glucuronosyltransferases, Ugts), as well as the efflux of potentially toxic xenobiotics and xenobiotic conjugates (multidrug resistance-associated proteins, Mrps). The significance of Nrf2 in the liver has been established, as livers of Nrf2-null mice are more susceptible to various oxidative/electrophilic stress-induced pathologies than wild-type mice. In contrast, both pharmacological and genetic models of hepatic Nrf2 activation are protective against oxidative/electrophilic stress. Furthermore, because certain Nrf2-target genes in the liver could affect the distribution, metabolism, and excretion of xenobiotics, the effects of Nrf2 on the kinetics of drugs and other xenobiotics should also be considered, with a special emphasis on metabolism and excretion. Therefore, this review highlights the research that has contributed to the understanding of the importance of Nrf2 in toxicodynamics and toxicokinetics, especially that which pertains to the liver.

## BACKGROUND

**Historical Perspective—**The first suggestion of the transcriptional regulation of cytoprotective enzymes was described with the idea of monofunctional and bifunctional inducers (Talalay, 1989). Compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polycyclic aromatics, and β-naphthoflavone, are considered bifunctional inducers, in that they increase both phase-I [ i.e. cytochrome p450s (Cyps), NAD(P)H quinone oxidoreductase 1 (Nqo1)] and phase-II [i.e. glutathione-*S*-transferases (Gsts) and UDP-glucuronosyltransferases (Ugts)] enzymes. Monofunctional inducers, such as diphenols, thiocarbamates, 1,2-dithiol-3-thiones, and isothiocyanates, primarily increase phase-II enzymes with the exception of the cytoprotective phase-I enzyme Nqo1 (Talalay, 1989). Descriptions of the cytoprotective nature of the above enzymes are provided later in this review article.



*Corresponding Author:* Curtis D. Klaassen, Ph.D. Department of Pharmacology, Toxicology, and Therapeutics University of Kansas Medical Center 3901 Rainbow Blvd. Kansas City, KS 66160-7417, USA. Phone: (913)588-7500 Fax: (913) 588-7501 cklaasse@kumc.edu.

Monofunctional inducers were recognized to be electrophiles that in some way would lead to the induction of cytoprotective enzymes. In contrast, bifunctional inducers first had to increase phase-I enzymes, generate electrophilic metabolites similar to monofunctional inducers, and then somehow led to the induction of cytoprotective enzymes (Talalay, 1989). Thus, the idea of monofunctional and bifunctional inducers inspired research into what regulatory elements and transcription factors could contribute to the induction of cytoprotective enzymes.

The search for regulatory elements led to the discovery and description of the antioxidant response element (ARE) in the promoter region of rat Gst-Ya by the laboratory of Cecil Pickett in 1990 (Rushmore *et al.*, 1990). This sequence was further characterized in a subsequent manuscript by the same laboratory with point mutation experiments, and the core ARE sequence was found to be TGACNNNGC, where N represents any base. Minor variations of the core ARE have been found in some cytoprotective genes, but for the most part, the core ARE characterized by the Pickett laboratory has endured (Rushmore *et al.*, 1991). In addition, it was also discovered that oxidative stress caused by hydrogen peroxide and phenolic antioxidants that undergo redox cycling induced the expression of the Gst-Ya subunit gene, as well as the NAD(P)H:quinone oxidoreductase 1 (Nqo1) gene through the ARE (Rushmore *et al.*, 1991). Furthermore, gel mobility shift assays performed by the Pickett laboratory suggested that a protein from nuclear extracts binds to the ARE (Rushmore *et al.*, 1990).

The protein that binds to the ARE and initiates transcription of cytoprotective phase-II genes *in vivo* was discovered to be a heterodimer, consisting of nuclear factor erythroid 2-related factor 2 (Nrf2) and a small musculo-aponeurotic factor (Maf) protein (Itoh *et al.*, 1997). Nrf2 was named for its homology to nuclear factor erythroid 2 (NFE2p45), a protein important in erythroid cell-specific gene transcription (Moi *et al.*, 1994). Because of the homology of Nrf2 with NF-E2, it was originally hypothesized that Nrf2 was important in erythropoiesis; however, this was disproven with the engineering of Nrf2-null mice, which develop normally and are not anemic (Chan *et al.*, 1996). A separately engineered colony of Nrf2-null mice confirmed the importance of Nrf2 in the basal and inducible regulation of cytoprotective enzymes, such as Nqo1 and Gsts (Itoh *et al.*, 1997). In response to the phenolic antioxidant butylhydroxyanisole (BHA), the mRNA expression of Nqo1 and Gsts is increased; however, this induction is not observed in Nrf2-null mice, which provided strong evidence that Nrf2 is required for induction of detoxifying enzymes and may be required for protection against xenobiotics (discussed below) (Itoh *et al.*, 1997).

Also in the mid 1990s, a graduate student named Jie Liu joined the laboratory of Curtis Klaassen at the University of Kansas Medical Center. Jie Liu brought with him a number of herbs and herbal ingredients from China. Over the next year, he tested a number of them to determine whether they would protect mice against chemical-induced hepatotoxicity (Liu *et al.*, 1994b). One of these chemicals was oleanolic acid, a natural triterpenoid compound found in many plants. Oleanolic acid is commonly used in Chinese medicine for the treatment of hepatic diseases. The Klaassen laboratory was surprised to find that pretreatment with oleanolic acid protected mice from a rather diverse and exhaustive list of hepatotoxicants (bromobenzene, acetaminophen, carbon tetrachloride, thioacetamide, furosemide, phalloidin, colchicine, cadmium chloride, and lipopolysaccharide) (Liu *et al.*, 1993a; Liu *et al.*, 1993b; Liu *et al.*, 1994a; Liu *et al.*, 1994b; Liu *et al.*, 1995a). Oleanolic acid also protects in other models of injury and disease, such as anaphylactic shock (Zhang and Ma, 1995), and was later shown to protect in a model of myocardial ischemia-reperfusion (Du and Ko, 2006). More recently, in the alloxan-induced diabetic rat model, oleanolic acid had hypoglycemic, hypolipidemic, and antioxidant effects (Gao *et al.*, 2009). Therefore, much data has accumulated showing that oleanolic acid is hepatoprotective.

However, despite an extensive effort to elucidate the molecular mechanism by which oleanolic acid protected from hepatotoxicity (Liu *et al.*, 1995b), nothing concrete was established. It was later realized that oxidative and/or electrophilic stress played a role in the development of hepatotoxicity from the chemicals tested by Liu and Klaassen. Therefore, it was hypothesized that Nrf2 might play a role in the hepatoprotective effects of oleanolic acid. Indeed, almost 15 years after the initial studies, oleanolic acid was shown to be an activator of Nrf2 *in vivo*, thereby contributing to its hepatoprotective effects (Reisman *et al.*, 2009a).

Michael Sporn, a pioneer in the concept and development of chemopreventive chemicals, had been following the work on oleanolic acid and requested a large amount of the natural triterpenoid from Curtis Klaassen and Jie Liu, who kindly obliged. Michael Sporn and his colleagues at Dartmouth College began synthesizing many derivatives of oleanolic acid, and those with the 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) parent structure were found to be the most potent activators of Nrf2 to date (Liby *et al.*, 2005; Yates *et al.*, 2007). Indeed, a derivate of CDDO, namely CDDOMethyl (CDDO-Me), is currently in late phase II stages of clinical development for the treatment of chronic kidney disease by Reata Pharmaceuticals (Irving, TX) (Meyer, 2009), with Nrf2 activation being one of its primary mechanisms of action (Yore *et al.*, 2006; Thimmulappa *et al.*, 2007). Thus, summarized above is how Nrf2 was discovered and identified as a possible drug target for the treatment of disease and how this led to the design and synthesis of a novel compound (CDDO-Me), currently in clinical development.

**Importance of the Liver—**Because the liver is the main organ responsible for the biotransformation and subsequent detoxification of xenobiotics, understanding the transcriptional regulation of the enzymes important for biotransformation has been a central theme of much research in pharmacology and toxicology. Detoxification and subsequent excretion of xenobiotics is a complex process that involves many enzymes, such as those that catalyze reduction and conjugation reactions, as well as efflux transporters that facilitate excretion and elimination of detoxified xenobiotics. As described above, it was serendipitous when a transcription factor was isolated (Moi *et al.*, 1994) and recognized (Itoh *et al.*, 1997) to be capable of positively regulating many of these cytoprotective genes. Moreover, Nrf2 has emerged as a transcription factor that is capable of inducing a large array of detoxification enzymes, biotransformation enzymes, and transporters that aid in the detoxification and elimination of potentially harmful xenobiotics.

**Regulation of Nrf2 activation—**Under homeostatic or non-stressed conditions, Nrf2 is sequestered in the cytosol by the actin binding protein kelch-like ECH associating protein 1 (Keap1) (Itoh *et al.*, 1999), which functions as an adaptor for Cullin 3 (Cul3), an E3-based ligase, that targets Nrf2 for ubiquitination and subsequent proteasomal degradation (Kobayashi *et al.*, 2004). This mechanism of proteasomal degradation of Nrf2 is very efficient, as the half-life of Nrf2 under homeostatic conditions is approximately 20 min, and thus, Nrf2 protein is difficult to detect in unstressed conditions (Itoh *et al.*, 2003; McMahon *et al.*, 2003). However, when oxidative or electrophilic stress becomes more prevalent, the interaction between Nrf2 and Keap1 is disrupted, leading to decreased proteasomal degradation of Nrf2, accumulation of free Nrf2 in the cytosol, and an increase in Nrf2 translocation into the nucleus (Li and Kong, 2009). Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein and binds to antioxidant response elements (ARE). The Nrf2/Maf complex then recruits CREB binding protein and p300 (Zhu and Fahl, 2001), which have been implicated in the recruitment of histone acetyltransferases and RNA polymerases (Vo and Goodman, 2001). The entire complex then initializes transcription of a large battery of cytoprotective genes (Itoh *et al.*, 1997).

**Nrf2 target genes—**Whereas it is difficult to pinpoint which particular genes induced by Nrf2 are most important for its cytoprotective effects, there is no doubt that the coordinated induction of Nrf2-target genes has a dramatic effect on both the dynamics and kinetics of xenobiotics. The effects and activities of some of the major and newly identified Nrf2-target genes will be highlighted here.

**NAD(P)H:quinone oxidoreductase 1 (Nqo1):** Nqo1, regarded as a prototypical Nrf2-target gene, is a cytosolic flavoprotein catalyzing the two-electron reductive metabolism and detoxification of endogenous and exogenous chemicals (Ross, 2004). Nqo1 is critical for cytoprotection against many highly reactive and potentially damaging quinones. As would be expected, Nrf2-null mice have reduced constitutive expression and activity of Nqo1 in liver, forestomach, and small intestine (Li and Jaiswal, 1992; Itoh *et al.*, 1997; McMahon *et al.*, 2001; Ramos-Gomez *et al.*, 2001).

**Heme oxygenase-1 (Ho-1):** Ho-1 catalyzes the first and rate-limiting step in the catabolism of the pro-oxidant heme to carbon monoxide, biliverdin, and free iron. Ho-1 can have both anti-oxidative and anti-inflammatory effects, as biliverdin can be reduced to the antioxidant bilirubin, by biliverdin reductase, and small amounts of carbon monoxide can have anti-inflammatory effects (Wunder and Potter, 2003). Ho-1 mRNA and protein expression of Ho-1 are commonly up-regulated following oxidative stress and cellular injury (Guo *et al.*, 2001) and Nrf2 has been shown to directly regulate *Ho-1* promoter activity (Alam *et al.*, 1999). Analysis of the *Ho-1* promoter identified binding sites resembling AREs (Prestera *et al.*, 1995; Inamdar *et al.*, 1996). Similarly, *in vitro* exposure to diverse toxicants, including diethyl maleate, paraquat, and cadmium chloride, induced Ho-1 mRNA and protein expression in wild-type, but not Nrf2-null peritoneal macrophages (Ishii *et al.*, 2000). However, it should be noted that Ho-1 is not the best Nrf2 target gene. Other mechanisms of transcriptional regulation are known to exist for Ho-1. For example, BTB and CNC homolog 1 (Bach1) is a heme sensing protein that binds to and inhibits Maf proteins, the crucial heterodimer partner for Nrf2 to bind to an ARE (Igarashi *et al.*, 1998; Reichard *et al.*, 2007). In addition, there is evidence to suggest that Bach1 is involved in chromatin remodeling (Yoshida *et al.*, 1999). Therefore, because Ho-1 is regulated by multiple mechanisms in addition to Nrf2, other Nrf2-target genes should be quantified as markers for Nrf2 activation.

**Glutamate-cysteine ligase (Gcl):** GSH (L-γ-glutamyl-cysteinyl-glycine) maintains intracellular redox balance and protects against oxidative insult. Additionally, GSH can detoxify chemicals through direct binding or enzymatic conjugation by glutathione-*S*-transferase (Gst) enzymes. GSH also plays an important role in free radical scavenging. Gcl, also known as γ-glutamyl cysteine synthetase, performs the rate-limiting and ATP-dependent step in GSH synthesis by catalyzing the formation of γglutamylcysteine from glutamine and cysteine. Decreased levels of GSH are observed in livers from Nrf2-null mice (Reisman *et al.*, 2009e). Reduced GSH biosynthesis results from lower amounts of synthetic enzymes, such as Gcl. Gcl is a holoenzyme comprised of two subunits, a modifier subunit (Gclm) and a catalytic subunit (Gclc), both of which contain ARE sequences in their promoters (Seelig *et al.*, 1984; Mulcahy and Gipp, 1995; Mulcahy *et al.*, 1997; Moinova and Mulcahy, 1998).

**Microsomal epoxide hydrolase (Eh-1):** Eh-1 hydrolyzes and inactivates epoxides within the cell. Some information implicates Nrf2 in the regulation of Eh-1 expression. This evidence includes reduced basal mRNA expression of Eh-1 in multiple tissues of Nrf2-null mice (Ramos-Gomez *et al.*, 2001; Crocenzi *et al.*, 2006; Hu *et al.*, 2006). Furthermore, a prototypical Nrf2 activator, oltipraz, induces Eh-1 in wild-type but not Nrf2-null mice,

pointing to a role for Nrf2-mediated control (Ramos-Gomez *et al.*, 2001). However, it should be noted that Eh-1 is not induced in Keap1-knockdown (Keap1-kd) mice (discussed in more detail below), indicating that other regulatory mechanisms may exist for Eh-1 induction (Reisman *et al.*, 2009e).

**Glutathione-S-transferases (Gsts):** Gsts catalyze the nucleophilic attack by reduced glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom, often resulting in detoxification. Gst mRNA and protein expression are decreased in Nrf2-null mice, and Nrf2 is required for Gst induction by BHA and ethoxyquin (Itoh *et al.*, 1997; Hayes *et al.*, 2000; Knight *et al.*, 2008; Reisman *et al.*, 2009e). In contrast, the mRNA expression of many subtypes of Gsts are markedly increased in Keap1-kd mice (Reisman *et al.*, 2009e). Collectively, the above data demonstrate a crucial role for Nrf2 in the regulation of Gsts.

**Sulfiredoxin 1 (Srxn1):** Peroxiredoxin catalyze the reduction of hydrogen peroxide. Srxn1 catalyzes the reduction of the active site of peroxiredoxin, thereby converting this important enzyme from an inactive to an active state. Srxn1 has a functional ARE in its promoter region and can be rapidly induced (20-fold) *in vivo* in the liver by administration of the Nrf2 activator CDDO-trifluoroethylamide (CDDO-TFEA) (Soriano *et al.*, 2009).

**Multidrug resistance-associated proteins (Mrps)—**Mrps are ATP-dependent efflux transporters that export a wide-range of substrates, but especially glutathione, glucuronide, and sulfate conjugates. Four (Mrp2, 3, 4, and 6) of the eight Mrps expressed in mice are expressed in liver (Maher *et al.*, 2005b). Furthermore, Mrp2, 3, 4, and 6 in liver are induced by several Nrf2 activators, namely BHA, oltipraz, and ethoxyquin (Maher *et al.*, 2005a). In a more comprehensive evaluation of the effects of chemical activation of Nrf2 on Mrps, only Mrp2, 3, and 4 were induced in wild-type but not Nrf2-null mice (Maher *et al.*, 2007). In addition, treatment of Hepa-1 cells with *tert*-butyl hydroquinone facilitated Nrf2-binding to mouse promoter regions of Mrp2, 3, and 4 (Maher *et al.*, 2007). However, Keap1-knockdown (Keap1-kd) mice (which have a whole body partial knockdown of Keap1 and discussed further below) have only a modest induction of Mrp2 mRNA expression in liver (Reisman *et al.*, 2009e) and no increase in hepatic Mrp2 protein expression (Reisman *et al.*, 2009c). This observation, coupled with another study that demonstrated the capability of other transcription factors, such as PXR, FXR, and CAR to contribute to Mrp2 regulation, suggests that other regulatory mechanisms might control Mrp2 induction (Kast *et al.*, 2002). In contrast, both Mrp3 and Mrp4 mRNA and protein expression are markedly induced in Keap1-kd mice, suggesting a more prominent role for Nrf2 in their regulation (Okada *et al.*, 2008; Reisman *et al.*, 2009c; Reisman *et al.*, 2009e).

**Bile salt efflux pump (Bsep):** Bsep is almost exclusively expressed in th liver, localizing to the canalicular membrane of hepatocytes and is a major efflux transporter of bile acids from the liver into bile. Recently, it was discovered that human BSEP is a Nrf2 target gene and induced in HepG2 cells by oltipraz (Weerachayaphorn *et al.*, 2009). The involvement of Nrf2 in the regulation of human BSEP was also confirmed by luciferase reporter assays and chromatin immunoprecipitation (Weerachayaphorn *et al.*, 2009). This finding was surprising as Bsep has not been shown to be regulated by Nrf2 in mice, as there is no difference among wild-type, Nrf2-null, and Keap1-kd mice in Bsep mRNA expression (Reisman *et al.*, 2009e), and Bsep is not induced in mice after administration of common Nrf2 activators (Cheng *et al.*, 2007). The lack of Nrf2 regulation of mouse Bsep is hypothesized to be due to variability in the promoter region of BSEP between humans and mice (Weerachayaphorn *et al.*, 2009). Transcriptional regulation of human BSEP by Nrf2 is an important finding, as clinical use of Nrf2 activators may be useful for the treatment of cholestatic liver disease,

not only by decreasing the oxidative stress caused by build-up of bile acids in the liver, but also by mobilizing and excreting toxic bile acids.

**Carboxylesterases (Ces):** Carboxylesterases catalyze the hydrolysis of ester and amide containing endo- and xenobiotics. Ces1e1 and Ces2a6 are regulated in a Nrf2-dependent manner, being lower in Nrf2-null mice and higher in Keap1-kd mice than wild-type mice (Reisman *et al.*, 2009e). In addition, human CES1A1 is induced by the Nrf2 activators, *tert*-butylhydroquinone (tBHQ) and sulforaphane (SFN) in HepG2, Caco-2, and HeLa cells. Furthermore, electrophoretic mobility shift assays and chromatin immunoprecipitation assays demonstrated that Nrf2 binds to an ARE in the CES1A1 gene (Maruichi *et al.*, 2010)

## Nrf2 and Liver Injury

**Higher Susceptibility of Nrf2-null mice to Hepatic Injury—**The potential for Nrf2 to induce many cytoprotective genes, in response to certain stimuli, permits Nrf2 to be extremely diverse in facilitating hepatoprotection from a variety of chemical- and oxidative stress-induced pathologies. Experiments have shown that targeted deletion of Nrf2, as in Nrf2-null mice, leads to enhanced susceptibility to hepatic injury. The first compound selected to illustrate the effects of a loss of Nrf2 was acetaminophen, a well-known and often used hepatotoxicant. Acetaminophen (300 mg/kg, i.p.) administration to Nrf2-null mice decreased non-protein sulfhydryl content in liver, increased serum ALT enzyme activity approximately 10-fold, and caused severe hepatocelluar injury. In contrast, this dose of acetaminophen was relatively non-toxic to wild-type mice (Enomoto *et al.*, 2001). Increased susceptibility of Nrf2-null mice to acetaminophen was also confirmed in another study (Chan *et al.*, 2001).

The acetaminophen study by Enomoto et al. (2001) initiated a series of studies by other laboratories, which also demonstrated the deleterious effects of knocking out Nrf2 in the liver. Pentachlorophenol (PCB) is a ubiquitous environmental pollutant that is known to cause hepatocellular carcinomas. Nrf2-null mice fed PCB had higher hepatic 8-hydroxydeoxyguanosine (8-OH-dG) concentrations, hepatic thiobarbituric-acid-reactive substances (TBARs), centrilobular necrosis, and serum alkaline phosphatase (ALP) activity, as compared to PCB-treated wild-type mice (Umemura *et al.*, 2006). Similar results were generated with arsenic. Compared to wild-type mice, Nrf2-null mice exposed to arsenic had more severe liver damage, arsenic-induced DNA hypomethylation, oxidative DNA damage, and apoptotic cell death (Jiang *et al.*, 2009).

In a chronic model of liver fibrosis utilizing carbon tetrachloride, Nrf2-null mice had aggravated liver fibrosis and an increased inflammatory response, as quantified by hepatic IL-1α, TNFα, and IFNγ mRNA expression, than when carbon tetrachloride was given to wild-type mice (Xu *et al.*, 2008). In another model of chemical-induced liver injury, Nrf2-null mice administered pyrazole, a compound that induces Cyp2e1 and production of superoxide, had increased serum ALTs, centrilobular necrosis, TUNEL staining, nitrotyrosine adduct formation, and TBARs than when pyrazole was administered to wild-type mice (Lu *et al.*, 2008). Thus, it has been repeatedly shown that Nrf2-null mice are highly susceptible to chemical-induced oxidative/electrophilic stress in the liver.

In more clinically-oriented models of hepatic stress, Nrf2-null mice also have more severe hepatic damage than their wild-type counterparts. When fed a high-fat diet, Nrf2-null mice had higher hepatic malondialdehyde (MDA) equivalents than wild-type mice, as well as an absence of induction of genes involved in lipogenic and cholesterologenic pathways (Tanaka *et al.*, 2008). These data suggest that Nrf2 may have more important and complex regulatory roles than simply protection from hepatotoxicants.

Hereditary tyrosinemia type 1 is an autosomal-recessive disease characterized by a genetic inactivation of the enzyme fumarylacetoacetate hydrolase (Fah), which catalyzes the last reaction of the tyrosine catabolism pathway. Nrf2-null mice were crossed with Fah-null mice, which resulted in increased oxidative stress and DNA damage, contributing to accelerated development of hepatocellular carcinomas, as compared to Fah-null mice (Marhenke *et al.*, 2008).

Because the liver is the only organ in the body that is capable of full regeneration, whether Nrf2 plays a role in liver regeneration after two-thirds partial hepatectomy was also investigated (Beyer *et al.*, 2008). Indeed, liver regeneration was significantly impaired in partially hepatectomized Nrf2-null mice, as a result of increased oxidative stress and impaired insulin/insulin growth factor-1 signaling (Beyer *et al.*, 2008).

Therefore, it is apparent that livers of Nrf2-null mice are more susceptible to models of oxidative and electrophilic stress. This is not surprising, as Nrf2 has transcriptional control over many important cytoprotective genes. However, it must be emphasized that the studies described above investigated what occurred in the absence of Nrf2 and should not be confused with what might happen with enhanced Nrf2 activation. Studies demonstrating increased hepatic injury using various models in Nrf2-null mice are further summarized in Table 1.

**Effect of pharmacologic activation of Nrf2 on the liver—**The first method utilized for activation of Nrf2 was pharmacological by the use of small molecules, such as SFN, oltipraz, BHA, the natural triterpenoid oleanolic acid, and the synthetic triterpenoids (CDDO, CDDO-Me, and CDDO-Im) (Hayes *et al.*, 2000; Iida *et al.*, 2004; Liby *et al.*, 2005; Hu *et al.*, 2006). These small molecules activated Nrf2 with various potencies, with the synthetic triterpenoids being the most potent (Yates *et al.*, 2007). However, all have been shown to protect the liver in different models of oxidative and electrophilic stress. For example, oltipraz protected the livers of mice from α-naphthylisothiocyanate (ANIT)-induced cholestasis (Tanaka *et al.*, 2009). Oltipraz also decreased aflatoxin-induced hepatocarcinogenesis in rats (Kensler *et al.*, 1987). In a clinical trial in residents of China exposed to high dietary concentrations of aflatoxin, oltipraz reduced the urinary excretion of the primary oxidative metabolite of aflatoxin B1, namely aflatoxin M1 (Kensler *et al.*, 2000). The Nrf2 activator BHA also protects rodents in a variety of models of oxidative and electrophilic stress. BHA has been shown to protect mice from methylazoxymethanol acetate-induced liver necrosis (Reddy *et al.*, 1982), and acetaminophen hepatotoxicity (Hazelton *et al.*, 1986). BHA also protects rats infected with hepatits B virus from aflatoxin-mediated hepatocarcinogenesis (Wang *et al.*, 1999). Oleanolic acid also activates Nrf2 (Liu *et al.*, 2008; Reisman *et al.*, 2009a), inducing many cytoprotective enzymes, thereby protecting the liver from many hepatotoxicants (Liu *et al.*, 1993a; Liu *et al.*, 1994b; Liu *et al.*, 1995a). The synthetic triterpenoids are also capable of protecting the liver from oxidative and electrophilic stress through activation of Nrf2. For instance, CDDO-Im has been shown to protect the liver from aflatoxin-induced carcinogenesis (Yates *et al.*, 2006), concanavalin A (ConA)-mediated inflammatory liver injury (Osburn *et al.*, 2008), and acetaminophen hepatotoxicity in wild-type but not Nrf2-null mice (Reisman *et al.*, 2009b). Studies of hepatoprotection utilizing chemical activators of Nrf2 are further summarized in Table 2.

More recently, CDDO-Im, a potent Nrf2 activator, was demonstrated to abrogate high-fat diet-induced increases in body weight, adipose mass, and hepatic lipid accumulation in wild-type mice but not in Nrf2-null mice (Shin *et al.*, 2009). Levels of mRNA for fatty acid synthesis enzymes were downregulated after CDDO-Im treatment in the liver of wild-type

mice but not Nrf2-null mice on the high-fat diet. Thus, these data suggest that Nrf2 might also be a novel target for the treatment of obesity.

It is possible to activate Nrf2 through pharmacological means, and such activation does result in hepatoprotection. However, small molecules typically have off-target effects. Oltipraz, for example, also activates the constitutive androstane receptor (CAR) (Merrell *et al.*, 2008), and the synthetic triterpenoids have many other targets, such as NFκB (Yore *et al.*, 2006), STAT3 (Yates *et al.*, 2006), and PPARγ (Place *et al.*, 2003). Therefore, a genetic model of Nrf2 activation through a knockout or knockdown of Keap1, the cytosolic repressor of Nrf2, presents an opportunity to study what occurs within the liver when only Nrf2 is activated.

**Effect of genetic activation of Nrf2 on the liver—**The first model of genetic activation of Nrf2 engineered was the whole body Keap1-null mouse. These mice had enhanced activation of Nrf2; however, the mice died at weaning from malnutrition due to hyperkeratinization of the esophagus and forestomach (Wakabayashi *et al.*, 2003). This postnatal lethality was abolished when the Keap1-null mice were crossed with Nrf2-null mice, indicating that activation of Nrf2 caused the hyperkeratinzation (Wakabayashi *et al.*, 2003). It is hypothesized that this was due to ARE-driven expression of genes important in squamous cell differentiation that occurs when Nrf2 is over-activated to such an extent as in the Keap1-null mouse.

In order to circumvent the postnatal lethality, a hepatocyte-specific Keap1-null mouse (Keap1-CKO) was engineered, using a floxed Keap1 allele driven by an albumin-Cre promoter (Okawa *et al.*, 2006). The Keap1-CKO mice did not die at weaning, as Keap1 was not knocked out in the esophagus or forestomach. These mice had increased hepatic gene expression of Nrf2 target genes, such as Nqo1, Gclc, and Gsts and were extremely resistant to hepatotoxicity from a rather high dose (700 mg/kg, i.p.) of acetaminophen (Okawa *et al.*, 2006). The Keap1-CKO mice have also been shown to be resistant to hepatic injury from ConA-induced inflammation (Osburn *et al.*, 2008) and methylmercury toxicity (Toyama *et al.*, 2007).

It was later determined that mice homozygous for the floxed Keap1 allele, engineered for the Keap1-CKO mice, have a whole body knockdown phenotype of Keap1, where in the liver, Keap1 mRNA expression is decreased 55-60% (Reisman *et al.*, 2009e). Keap1-kd mice have increased expression of Nrf2 target genes, such as Nqo1, Gclc, and Gsts (Reisman *et al.*, 2009e) and are resistant to acetaminophen hepatotoxicity (Reisman *et al.*, 2009c) and bile duct ligation-induced cholestasis (Okada *et al.*, 2009). Therefore, the Keap1-kd mouse phenotype represents a viable model of whole body genetic activation of Nrf2.

## Effect of Nrf2 on toxicokinetics

**Distribution—**Many drugs and other xenobiotics are concentrated in the liver due to their uptake by transporters. Uptake transporters expressed in the liver include the bile acid transporter $Na^+$-taurocholate cotransporting polypeptide (Ntcp), organic anion-transporting polypeptide 1a1 (Oatp1a1), Oatp1a4, Oatp1b2, organic cation transporter 1 (Oct1), organic anion transporter 2 (Oat2), equilibrative nucleoside transporter 1 (Ent1) and Atp8b1 (Klaassen and Lu, 2008). In general, Nrf2 does not affect the mRNA expression of uptake transporters (Reisman *et al.*, 2009e). The one exception is that of Oatp1a1, which is suppressed by Nrf2 and discussed further below.

Oatps are uptake transporters that mediate the sodium-independent transport of various amphipathic solutes. Oatp1a1 is one of the most abundant Oatps in the rodent liver and is

capable of uptake of various organic anionic compounds (Cheng *et al*., 2005a). Both BHA and ethoxyquin decrease the Oatp1a1 mRNA expression approximately 60-80% (Cheng *et al.*, 2005b). In addition, Keap1-kd mice also have decreased mRNA expression of Oatp1a1 in liver (Reisman *et al.*, 2009e). A decrease in Oatp1a1, upon Nrf2 activation, may slow the uptake of certain hepatotoxicants into the liver, giving the liver more time to adapt to oxidative and electrophilic stress, and thus reducing injury.

**Metabolism—**Due to the number of genes transcriptionally regulated by Nrf2, it is not surprising that Nrf2 has a profound and often beneficial effect on metabolism, especially with respect to phase-II conjugation enzyme reactions. Phase-II enzyme reactions include glucuronidation, sulfation, and conjugation with glutathione. These conjugation reactions markedly increase the hydrophilicity of xenobiotics and thus greatly promote their excretion.

UDP-glucuronosyltransferases (Ugts) catalyze the conjugation of a glucuronosyl group from UDP-glucuronic acid to a variety of substrate molecules, making them more water soluble and readily excreted. In particular, Ugt1a6 has been shown to be a Nrf2-target gene, with Nrf2-null mice having decreased Ugt1a6 mRNA expression and total Ugt enzyme activity in liver (Chan *et al.*, 2001; Enomoto *et al.*, 2001; Reisman *et al.*, 2009c). This decrease in hepatic Ugt enzyme activity decreased the ability of Nrf2-null mice to conjugate acetaminophen with glucuronic acid (Reisman *et al.*, 2009c). In addition, hepatic Ugt1a6 can be induced by Nrf2 activators, namely oltipraz and BHA (Buckley and Klaassen, 2009). However, Ugt1a6 mRNA expression is only slightly increased, and Ugt enzyme activity is not enhanced in Keap1-kd mice, possibly indicating that other regulatory mechanisms independent of Nrf2 influence the induction of Ugts (Reisman *et al.*, 2009c). Collectively, the present data indicate that Ugt expression and enzyme activity can be influenced by Nrf2, and because Ugts metabolize approximately 35% of all drugs biotransformed by phase-II reactions (Evans and Relling, 1999), Nrf2 could have a significant impact on the pharmacokinetics of many drugs.

Sulfotransferases (Sults) catalyze approximately 20-25% of phase-II reactions by transferring a sulfonic acid group from the co-substrate 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Evans and Relling, 1999). Not many studies have examined the role of Nrf2 in the expression of Sults, and the few studies that have been conducted produced mixed results. In a report by Alnouti et al. (Alnouti and Klaassen, 2008), at least one of three Nrf2 activators (BHA, oltipraz, and ethoxyquin) given to mice was capable of inducing hepatic Sults, specifically those in the Sult1 family, which have substrate specificity toward phenolic compounds. However, Sult mRNA expression in liver is generally not altered in Keap1-kd mice (Reisman *et al.*, 2009e). A surprising and unexplainable result in another study demonstrated that the ability of Keap1-kd mice to sulfate acetaminophen is lower than in wild-type mice (Reisman *et al.*, 2009c). It was hypothesized that because Sult activity is increased during periods of oxidative stress (Marshall *et al.*, 2000), the more reduced environment caused by increased expression of cytoprotective genes in livers of Keap1-kd mice resulted in lower Sult activity. The little data that exist on the regulation of Sults by Nrf2 suggest that activation of Nrf2 could affect Sult mRNA expression, enzyme activity, and ultimately, drug disposition.

The phase-II enzymes that are most heavily influenced by Nrf2 are the Gsts, which catalyze the conjugation of nucleophilic GSH with reactive and potentially damaging electrophiles. Therefore, increased Gst activity should facilitate detoxification and faster elimination of electrophiles. The Nrf2 activators BHA, ethyoxyquin, and oltipraz induce most hepatic Gsts (Knight *et al.*, 2008). Other compounds that have been shown to induce Gsts in a Nrf2-dependent manner include various flavonoids (Lee-Hilz *et al.*, 2006) and CDDO-Im (Yates *et al.*, 2006). In addition, Gst (Gsta1, a4, m1, m2, m3, m4, and m6) mRNA expression is

significantly decreased in Nrf2-null mice and markedly increased in Keap1-kd mice (Reisman *et al.*, 2009e). The functional importance of Nrf2 on Gst expression and activity was recently shown in a study examining the biliary excretion of sulfobromophthalein (BSP) in Keap1-kd mice (Reisman *et al.*, 2009d). BSP is a prototypical compound utilized to assess hepatobiliary function. BSP is taken up into the liver via Oatps, conjugated with GSH by Gsts, and effluxed into bile via the multidrug resistance-associated protein 2 (Mrp2). Keap1-kd mice had more than twice the Gst enzyme activity when utilizing BSP as a substrate, and excreted double the BSP-GSH into bile within the first 30 min after administration, as compared to wild-type mice (Reisman *et al.*, 2009d).

**Excretion—**After phase-II conjugation, the liver must eliminate xenobiotics, either into the systemic circulation for eventual elimination into the urine, or into the bile for elimination into the feces. If the conjugate is not eliminated promptly, a new and sometimes more harmful electrophile can be produced, such as with the glucuronidation of diclofenac (Kuehl *et al.*, 2005), sulfation of safrole (Boberg *et al.*, 1983), or the glutathione conjugation of dichloromethane (Dekant and Vamvakas, 1993). Therefore, it was again serendipitous when an important set of efflux transporters, the Mrps, were identified as Nrf2-target genes. Because Mrps efflux glucuronide, sulfate, and glutathione conjugates, it is reasonable to conclude that activation of Nrf2 would enhance the capacity of the liver to efflux potentially harmful xenobiotics.

Such functional importance of Mrp induction via Nrf2 activation was recently evaluated with respect to acetaminophen metabolism and excretion. Keap1-kd mice, despite not having increased acetaminophen-glucuronidation activity, were able to excrete approximately 50% more of the acetaminophen-glucuronide conjugate into blood within 22.5 min after acetaminophen administration (Reisman *et al.*, 2009c). Thus, it is likely that the increased excretion of the acetaminophen-glucuronide can be accounted for by the increased expression of Mrp3 in Keap1-kd mice. Therefore, because Keap1-kd mice have higher expression of Mrp3, they have a higher capacity for excretion of acetaminophen-glucuronide conjugates. More studies investigating the functional importance of Nrf2 with respect to drug and toxicant elimination via Mrps would provide greater insight into the importance of Nrf2 in excretion.

## CONCLUDING REMARKS

Nrf2 has emerged as a transcription factor that is capable of inducing a wide battery of genes that aid in the detoxification and elimination of xenobiotics. Evolutionarily speaking, such a transcription factor as Nrf2 is extremely advantageous in an environment where organisms are constantly bombarded with electrophilic and oxidative stress. In regards to pharmacology and toxicology, Nrf2 has evolved to be an important transcription factor. For pharmacology, Nrf2 must be considered because of the possibility of drug-drug interactions resulting from the induction of one or a combination of drug metabolizing enzymes. For toxicology, Nrf2 must be considered, as most toxicities involve some form of oxidative or electrophilic stress. Nevertheless, the role of Nrf2 in the liver has become well-established and should be considered when investigating xenobiotics.

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**Table 1**

Summary of *in vivo* models of hepatic injury using Nrf2-null mice.

| Reference | Model | Result |
|---|---|---|
| Enomoto *et al.*, 2001 | Acetaminophen | Nrf2-null mice had increased liver injury, decreased Ugt1a6 and Gclc gene expression, and decreased Ugt activity. |
| Chan *et al.*, 2001 | Acetaminophen | Nrf2-null mice had increased liver injury and decreased Ugt1a6, Gclc, and Gst pi mRNA expression. |
| Umemura *et al.*, 2006 | Pentachlorophenol | Nrf2-null had increased liver injury, decrease Nqo1 protein expression, and decreased Ugt activity. |
| Xu *et al.*, 2008 | Acute model of carbon tetrachloride | Nrf2-null mice had increased liver injury, delayed onset of hepatocyte proliferation, and an increased inflammatory response. |
| Xu *et al.*, 2008 | Chronic model of carbon tetrachloride | Nrf2-null mice had an increased and prolonged fibrotic response. |
| Lu *et al.*, 2008 | Pyrazole | Nrf2-null mice had increased liver injury, decreased GSH, and decrease Gclc and Ho-1 mRNA expression. |
| Tanaka *et al.*, 2008 | High Fat Diet | Nrf2-null mice had increased TBARs and increased mRNA expression of genes involved in lipogenic and cholesterologenic pathways. |
| Marhenke *et al.*, 2008 | Tyrosinemia utilizing double Fah/Nrf2-null mouse | Increased mortality and liver injury in Fah/Nrf2-null mice, associated with increased 8-OHdG levels, protein carbonylation, and hepatocarcinogenicity |
| Jiang *et al.*, 2009 | Arsenic | Nrf2-null mice exposed to arsenic had more severe liver damage, arsenic-induced DNA hypomethylation, oxidative DNA damage, and apoptotic cell death. |

**Table 2**

Examples of *in vivo* models of hepatoprotection using Nrf2 chemical activators.

| Reference | Model | Result |
|---|---|---|
| Reddy *et al.*, 1982 | Dietary pretreament with BHA of mice exposed to methylazoxymethanol acetate | BHA decreased mortality and liver necrosis in mice exposed to methylazoxymethanol acetate |
| Hazelton *et al.*, 1986 | Dietary pretreament with BHA of mice exposed to acetaminophen | BHA decreased acetaminophen-induced hepatotoxicity and increased hepatic GSH and Ugt enzyme activity. |
| Kensler *et al.*, 1987 | Pretreament of rats with oltipraz exposed to aflatoxin | Oltipraz-treated rats had decreased hepatocarcinogenicity |
| Liu *et al.*, 1993a | Pretreatment of mice with oleanolic acid exposed to acetaminophen | Oleanolic acid decreased acute liver injury, decreased glutathione conjugate formation, and increased glucuronide conjugate formation |
| Liu *et al.*, 1993b | Pretreatment of mice with oleanolic acid exposed to cadmium | Oleanolic acid decreased liver injury. |
| Liu *et al.*, 1994a | Pretreatment of mice with oleanolic acid exposed to various hepatotoxicants | Oleanolic acid decreased liver injury from carbon tetrachloride, cadmium, and acetaminophen, but not allyl alcohol |
| Liu *et al.*, 1994b | Effect of 10 oleanane-type triterpenoid compounds on carbon tetrachloride, acetaminophen, and cadmium hepatotoxicity | Ursolic acid and oleanolic acid decreased liver injury from carbon tetrachloride, acetaminophen, and cadmium. |
| Liu *et al.*, 1995a | Effect of oleanolic acid on chemical-induced liver injury in mice | Oleanolic acid protected from bromobenzene, acetaminophen, carbon tetracholoride, thioacetamide, furosemide, phalloidin, colchicine, cadmium chloride, and lipopolysaccharide |
| Wang *et al.*, 1999 | BHA pretreatment of rats with hepatitis B virus exposed to aflatoxin | BHA reduced hepatic carcinomas, adenomas and oxidative stress, while increasing Gst and Nqo1 activities. |
| Kensler *et al.*, 2000 | Oltipraz treatment of residents of China exposed to high dietary concentrations of aflatoxin | Reduction in the urinary excretion of the primary oxidative metabolite of aflatoxin B1, namely aflatoxin M1 |
| Yates *et al.*, 2006 | CDDO-Im pretreatment of rats exposed to aflatoxin | CDDO-Im decreased aflatoxin adduct formation, hepatocarcinogenesis, and induced Gst and aflatoxin aldehyde reductase protein expression. |
| Osburn *et al.*, 2008 | CDDO-Im pretreatment of wild-type and Nrf2-null mice administered ConA | CDDO-Im protected wild-type but not Nrf2-null mice from ConA-induced inflammatory hepatic injury. |
| Tanaka *et al.*, 2009 | Oltipraz pre- and post-treated mice administered ANIT | Oltipraz protected mice from ANIT-induced cholestasis. |
| Reisman *et al.*, 2009a | Oleanolic acid pretreatment of wild-type and Nrf2-null mice administered acetaminophen | Oleanolic acid protected wild-type mice more than Nrf2-null mice from acetaminophen-induced hepatotoxicity |
| Reisman *et al.*, 2009b | CDDO-Im pretreatment of | CDDO-Im protected wild-type but |

| Reference | Model | Result |
|---|---|---|
| | wild-type and Nrf2-null mice administered acetaminophen | not Nrf2-null mice from acetaminophen-induced hepatotoxicity |
| Shin *et al*., 2009 | CDDO-Im treatment of wild-type and Nrf2-null mice given a high-fat diet | CDDO-Im reduced increases in body weight, adipose mass, and hepatic lipid accumulation in wild-type mice but not in Nrf2-null mice. |