

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

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor critical for protection against electrophilic and oxidative stress. In a recently engineered mouse with knockdown of kelch-like ECH associated protein 1 (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 and glutathione-S-transferases (Gst), and multidrug resistance-associated proteins. 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.

Key Words: Nrf2; electrophilic stress; oxidative stress; cytoprotection.

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 peroxy-nitrite, 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 peroxy-nitrite to nitrite is carried out by Gpx and Prx; GSH may also be capable of reducing peroxy-nitrite 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 upregulating a battery of cytoprotective genes, such as Nqo1, glutamate-cysteine ligase, catalytic and

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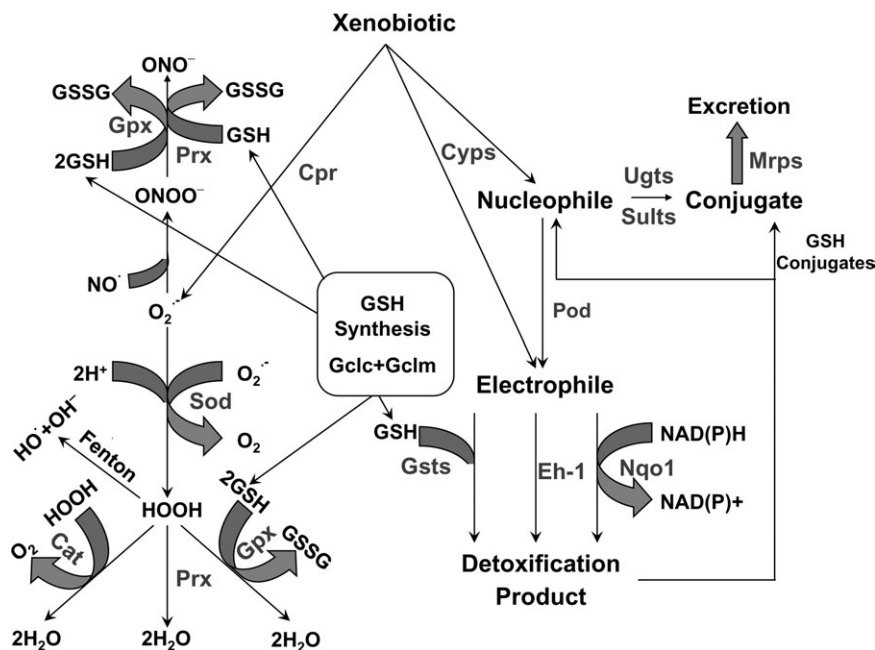


FIG. 1. Illustration of oxidative and electrophilic stress. Oxidative stress is represented on the left side and electrophilic stress on the right side. Xenobiotics can undergo redox cycling, such as in the case of paraquat, which generates superoxide in a reaction catalyzed by cytochrome p-450 reductase (Cpr). Detoxification of superoxide (O_2^-) to hydrogen peroxide (HOOH) is mediated by Sod. HOOH is detoxified to H_2O by Cat, Prx, and 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^- can also react with nitric oxide (NO^\cdot) to form peroxynitrite ($ONOO^-$), which can be detoxified by Gpx, Prx, or GSH. Biotransformation of xenobiotics by Cyp1b1 and Pod can lead to the formation of nucleophiles or electrophiles. Detoxification of electrophilic intermediates by Nqo1 or Eh-1 creates nucleophiles, which can be conjugated with glucuronic acid via Ugts or conjugated with sulfate via Sults and excreted by Mrps. Electrophiles can also be conjugated with GSH in a reaction catalyzed by 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).

modifier subunits (Gclc and Gclm), heme oxygenase-1 (Ho-1), and drug processing genes, such as Gsts, Ugts, and multidrug resistance-associated proteins (Mrps) (Kobayashi and Yamamoto, 2006; Maher *et al.*, 2007; Venugopal and Jaiswal, 1996). 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-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 protein, and binds to antioxidant response elements 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/electrophilic 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 postnatal 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 “knock-down” 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 participate in xenobiotic metabolism and/or are thought to protect against oxidative and electrophilic stress, and compare them 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, Cambridge, 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 mice were used for this study. Mice were euthanized by cervical dislocation, unless otherwise noted. 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 and 5 mg/kg, respectively, *i.p.*). Body temperature was maintained at 37°C 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 preweighed 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 10mM sucrose–250mM Tris buffer (pH 7.5), and microsomal suspensions were prepared by differential centrifugation.

Microsomal protein concentrations were determined by bicinchoninic acid (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-pentoxoresorufin *O*-deethylation, respectively. Cyp2e1 activity was determined by spectrophotometric analysis of 4-nitrophenol hydroxylation. Lastly, Cyp3a11 and 4a14 activities were measured by liquid chromatography-mass spectrometry/mass spectrometry analysis of testosterone 6 β -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). Protein concentrations were determined by the BCA Assay (Pierce Biotechnology). Hepatic GPx and Cat activity in cytosolic fractions were quantified spectrophotometrically, as adapted from previously described methods (Flohe and Gunzler, 1984; Luck, 1963; Tappel, 1978). Superoxide dismutase (Sod) activity was quantified spectrophotometrically utilizing a commercially available kit (Cayman Chemical, Ann Arbor, MI).

GSH quantification. Reduced GSH in livers and bile were 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). Protein concentrations were determined with the BCA Assay Kit from Pierce Biotechnology. Nuclear proteins (40 μ g protein per lane) were electrophoretically resolved using polyacrylamide gels (4% stacking and 10% resolving). Gels were transblotted overnight at 4°C onto polyvinylidene fluoride 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% nonfat milk in PBS-T. Blots were then incubated with primary antibody (1:1000 dilution in 2% nonfat 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% nonfat milk in PBS-T buffer for 1 h at room temperature. Blots were then washed with PBS-T. Protein-antibody complexes were detected using an ECL chemiluminescent kit (Pierce Biotechnology) 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 nonesterified 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 18mM Tris, pH 7.5, 300mM mannitol, 50mM ethylene glycol tetraacetic acid, and 0.1mM phenylmethylsulfonyl fluoride. Five hundred microliters 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 H₂O was added, vortexed, and centrifuged for 5 min at 3000 \times 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). 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), according to previously described methods (Maher *et al.*, 2005; Petrick and Klaassen, 2007). All data were standardized to the internal control glyceraldehyde 3-phosphate dehydrogenase.

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. 2A). 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. 2B). *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), nonesterified 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 GSH was 42% lower in *Nrf2*-null mice and 43% 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). *Nrf2*-null mice had 65% lower and

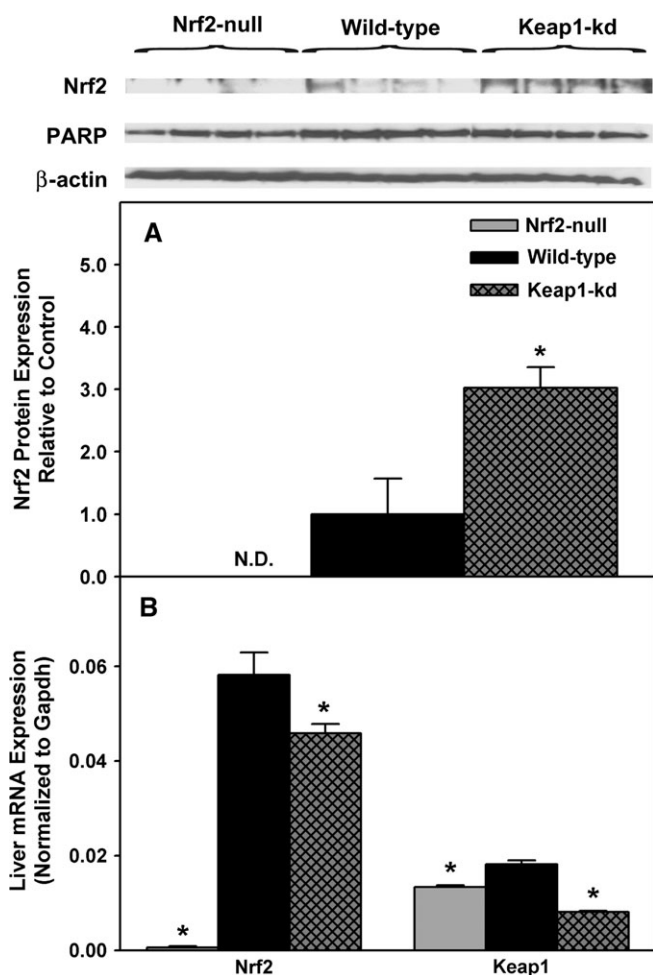


FIG. 2. *Nrf2* protein expression in hepatic nuclear fractions in wild-type, *Nrf2*-null, and *Keap1*-kd mice ($n = 5$) (A). Intensity of protein bands was quantified, and individual blot densities were normalized to wild-type and expressed as mean \pm SEM. Messenger RNA expression of *Nrf2* and *Keap1* in wild-type, *Nrf2*-null, and *Keap1*-kd mice ($n = 5$) (B). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

TABLE 1
Serum, Liver, and Bile Parameters of Hepatic Function in Wild-Type, *Nrf2*-null, and *Keap1*-kd Mice

Analyte (units)	<i>Nrf2</i> -null	Wild-type	<i>Keap1</i> -kd
Serum			
ALTs (IU/l)	20.9 \pm 1.3	19.5 \pm 1.6	22.7 \pm 3.9
Glucose (mg/dl)	176 \pm 6	185 \pm 7	206 \pm 10
Triglycerides (mg/dl)	66.0 \pm 8.9	56.7 \pm 4.9	64.1 \pm 4.0
Cholesterol (mg/dl)	57.0 \pm 1.5	65.8 \pm 2.0	61.4 \pm 5.4
Liver			
Nonesterified fatty acids (mEq/g liver)	0.018 \pm 0.002	0.025 \pm 0.003	0.020 \pm 0.003
Free cholesterol (mg/g liver)	19.0 \pm 4.2	23.0 \pm 4.2	26.3 \pm 3.9
Triglycerides (mg/g liver)	34.2 \pm 1.3	40.2 \pm 7.2	36.9 \pm 10.7
Bile			
Bile flow (ml/min/kg)	106 \pm 5	124 \pm 1	167 \pm 12*
GSH (nmol/min/kg)	371 \pm 24*	644 \pm 51	922 \pm 54*
Bilirubin (mg/min/kg)	7.57 \pm 0.42	7.05 \pm 0.52	6.65 \pm 1.05
Cholesterol (mg/min/kg)	35.5 \pm 0.6	40.1 \pm 1.7	54.3 \pm 3.9*
Phospholipids (mg/min/kg)	37.0 \pm 3.2	35.0 \pm 2.6	54.34 \pm 2.2*

Note. Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

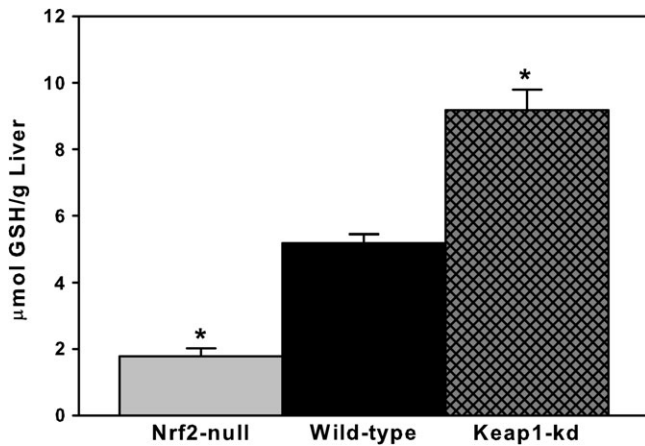


FIG. 3. Hepatic reduced liver GSH content in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

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 Figure 4A. 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). 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 Figure 4B. 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 Figure 4C. Glutaredoxin 1 (Glxr1) and 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. 4D). Messenger RNAs for both Me1 and H6pdh were lower in Nrf2-null mice (62 and

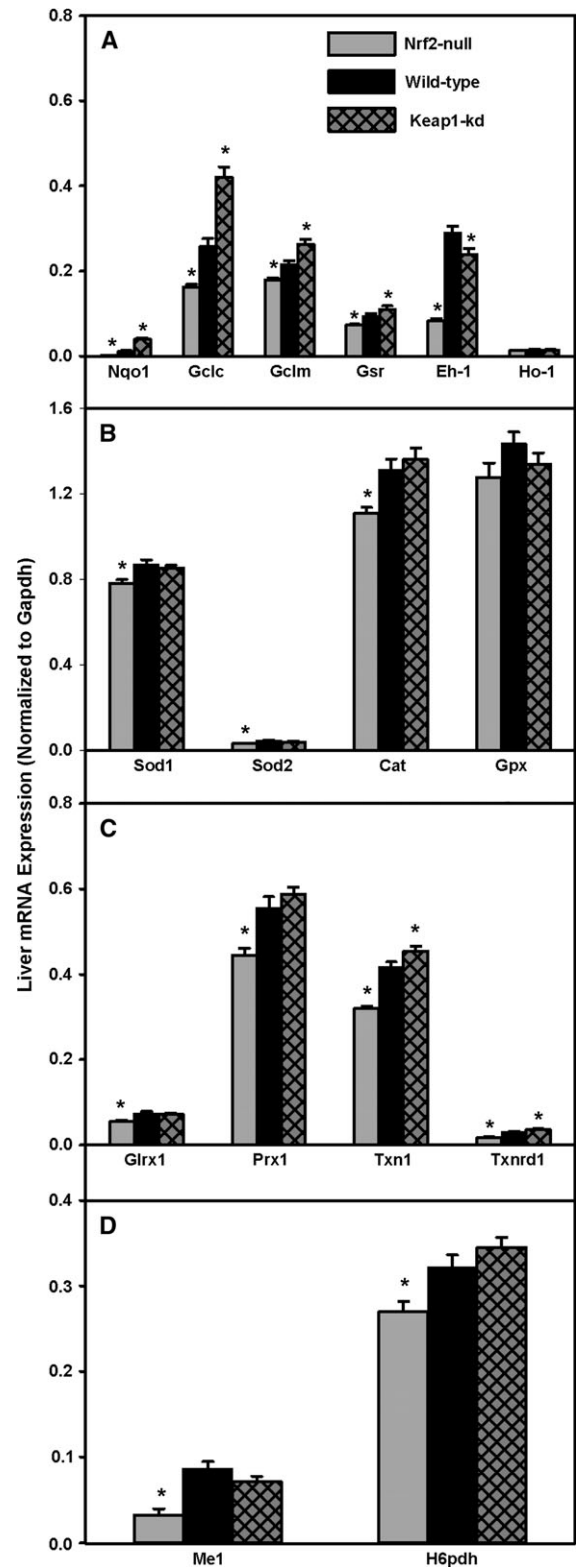


FIG. 4. Messenger RNA expression of prototypical Nrf2 targets (A), superoxide and hydrogen peroxide reducing enzymes (B), redoxins (C), and NADPH generating enzymes (D) in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

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. 5A). 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. 5B). 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- α 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. 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. 6B). 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 Pon3 mRNAs were not different among the three genotypes (data not shown).

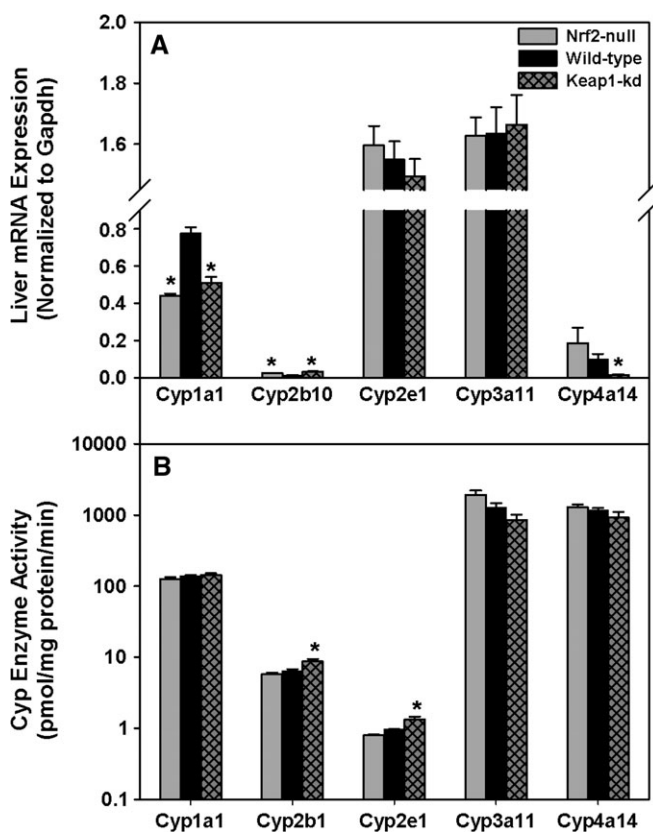


FIG. 5. Messenger RNA expression (A) and enzyme activity (B) of cytochrome p450s in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

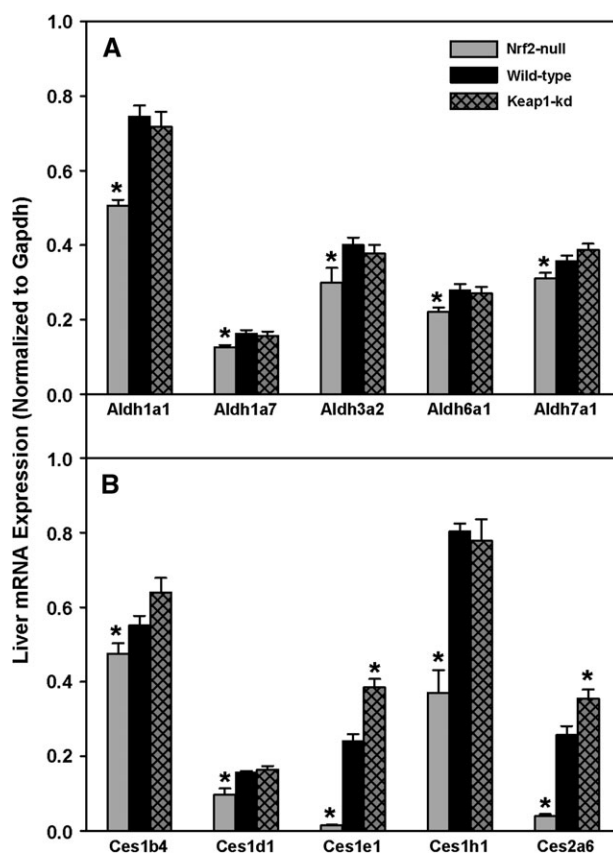


FIG. 6. Messenger RNA expression of Aldhs (A) and carboxylesterases (B) in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

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. 7A). 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, or 2a3 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. 7B). There were no differences in mRNA expression for microsomal Gst1 (mGst1), mGst2, mGst3, Gstm1, or Gstm2 among the three genotypes (data not shown).

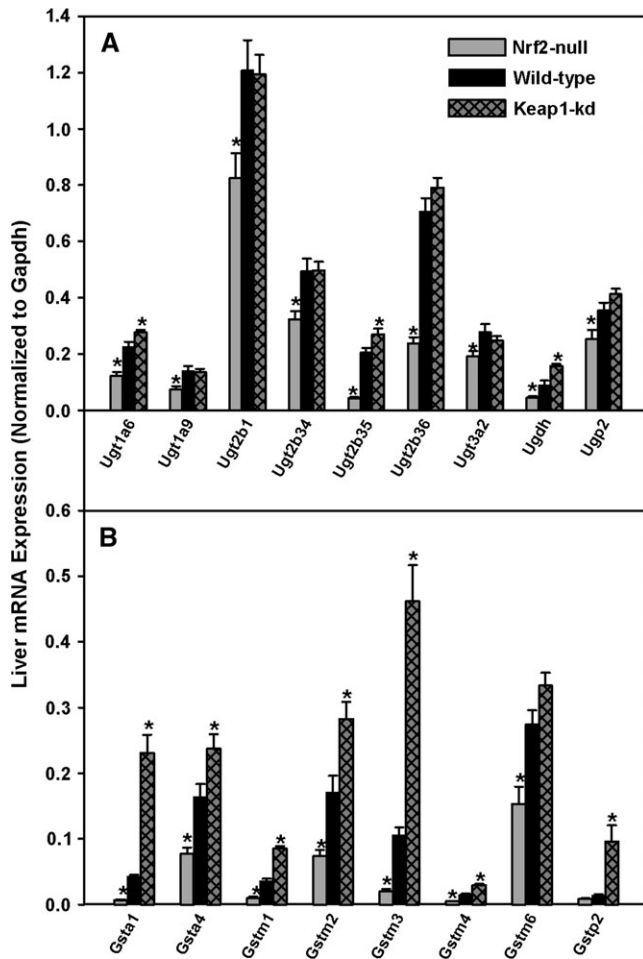


FIG. 7. Messenger RNA expression of UGTs (A) and GSTs (B) in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

Messenger RNAs 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 cosubstrate 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. 8A). 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

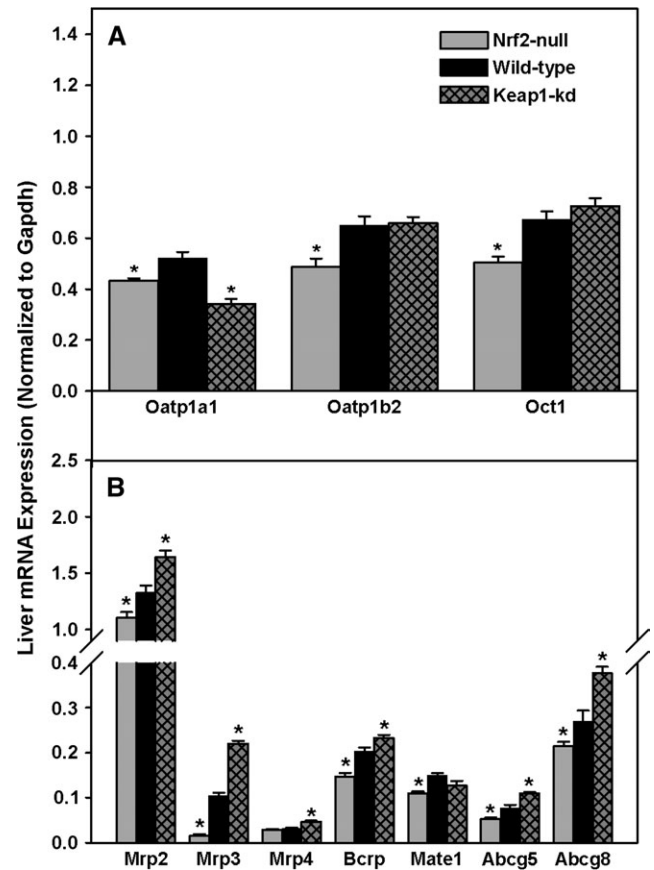


FIG. 8. Messenger RNA expression of uptake (A) and efflux (B) transporters in wild-type, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean \pm SEM. Asterisks (*) indicate a statistically significant difference from wild-type mice ($p \leq 0.05$).

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 cotransporting polypeptide (Ntcp) (data not shown).

Hepatic mRNAs of various efflux transporters were also quantified (Fig. 8B). 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 not differently expressed between 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).

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. For example, the Nrf2 activator oltipraz, which has been used in the identification of possible Nrf2-target genes, also activates the CAR, inducing its target gene Cyp2b10 (Merrell *et al.*, 2008). Therefore, the present study utilized genetic models of Nrf2 under- and overactivation to determine 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 Figure 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). Increased expression of GSH synthetic enzymes (Gclc and Gclm), as discussed in detail below, likely contributes to increased GSH concentrations in liver and biliary excretion of GSH in Keap1-kd mice. There were no differences in total biliary bile acids among genotypes (data not shown). Biliary excretion, possibly as a result of increase bile flow, 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 cosubstrate for Gst-mediated detoxification of reactive electrophiles (Meister and Anderson, 1983). The rate-limiting enzymatic process in GSH synthesis is catalyzed by glutamate-cysteine ligase, 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 (Lee *et al.*, 2007; Moinova and Mulcahy, 1999; Wild *et al.*, 1999) (Fig. 4A). Furthermore, mRNA expression of Gsr, the enzyme responsible for the reduction of oxidized glutathione (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 sulforaphane 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 hydroxyanisole (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, Cat, and Gpx are inducible in response to Nrf2 activators, such as 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 Cat 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. Glx1, 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, that is, 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 and Ogilvie, 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 and Ogilvie, 2008). Prodrug 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, whereas 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 (Landowski *et al.*, 2006; Marsh *et al.*, 2004; 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 cosubstrate 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 cosubstrate 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 and Ogilvie, 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 are 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 (Brangi *et al.*, 1999; Doyle *et al.*, 1998; Litman *et al.*, 2000; Miyake *et al.*, 1999; Ross *et al.*, 1999). 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 with 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. It should be noted that the genes in pattern 3, with the exception of Ho-1, are not known Nrf2-target genes.

Keap1 has been shown *in vitro* to bind and regulate the ubiquitination and subsequent proteasomal degradation of one other protein, phosphoglycerate mutase family member 5 (PGAM5) (Lo and Hannink, 2006). Phosphoglycerate mutases catalyze the conversion of 3-phosphoglycerate to 2-phosphoglycerate, which is an important substrate in glycolysis. Therefore, because Keap1 is capable of binding other proteins, at least *in vitro*, it is possible that some of the genes that are upregulated in Keap1-kd mice could be independent of Nrf2. However, a follow-up study by Lo *et al.* demonstrated *in vitro* that PGAM5 tethers a ternary complex containing both Keap1 and Nrf2 in mitochondria (Lo and Hannink, 2008). Lo and Hannink (2008) also hypothesized that Keap1 may use other proteins, such as PGAM5, to regulate Nrf2 at other subcellular localizations, such as the mitochondria. In addition, PGAM5 has not been shown to transcriptionally regulate any gene. Therefore, it seems unlikely that PGAM5 would be responsible for the upregulation of genes in Keap1-kd mice.

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 activation of Nrf2 in liver results in induction of genes, whose protein products, are more important in the direct detoxification of highly reactive electrophiles

TABLE 2
Categorization of Antioxidant, Phase-I, Phase-II, and Transporter mRNA Expression

Pattern 1			Pattern 2			Pattern 3
Decrease in Nrf2-null mice and increase in Keap1-kd mice			Decrease in Nrf2-null mice and nonsignificant 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	
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

Note. 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 ($p > 0.05$).

formed from xenobiotic exposure than for detoxification of reactive oxygen species.

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