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Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2

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Abstract

The transcription factor NFE2-related factor 2 (Nrf2) mediates detoxification and antioxidant gene transcription following electrophile exposure and oxidative stress. Mice deficient in Nrf2 (Nrf2-null) are highly susceptible to acetaminophen (APAP) hepatotoxicity, and exhibit lower basal and inducible expression of cytoprotective genes, including NADPH quinone oxidoreductase 1 (Nqo1) and glutamate cysteine ligase (catalytic subunit, or Gclc). Administration of toxic APAP doses to C57BL/6J mice generates electrophilic stress and subsequently increases levels of hepatic Nqo1, Gclc and the efflux multidrug resistance-associated protein transporters 1–4 (Mrp1-4). It was hypothesized that induction of hepatic Mrp1-4 expression following APAP is Nrf2-dependent. Plasma and livers from wild-type (WT) and Nrf2-null mice were collected **4,** 24 and 48 hrs after APAP. As expected, hepatotoxicity was greater in Nrf2-null compared to WT mice. Gene and protein expression of Mrp1-4 and the Nrf2 targets, Nqo1 and Gclc, was measured. Induction of Nqo1 and Gclc mRNA and protein after APAP was dependent on Nrf2 expression. Similarly, APAP treatment increased hepatic Mrp3 and Mrp4 mRNA and protein in WT, but not Nrf2-null mice. Mrp1 was induced in both genotypes after APAP, suggesting that elevated expression of this transporter was independent of Nrf2. Mrp2 was not induced in either genotype at the mRNA or protein levels. These results show that Nrf2 mediates induction of Mrp3 and Mrp4 after APAP, but does not affect Mrp1 or Mrp2. Thus coordinated regulation of detoxification enzymes and transporters by Nrf2 during APAP hepatotoxicity is a mechanism by which hepatocytes may limit intracellular accumulation of potentially toxic chemicals.

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CONFLICT OF INTEREST STATEMENT
The authors do not have interests which conflict with publication of the data in this manuscript.

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Keywords

Nuclear factor-E2-related factor 2; Nrf2; acetaminophen; APAP; hepatotoxicity; multidrug resistance-associated proteins; Mrp3; Mrp4

INTRODUCTION

Acetaminophen (APAP) hepatotoxicity remains the leading cause of drug-induced liver failure in the United States with over 100,000 cases of APAP poisonings reported each year (Ostapowicz *et al.*, 2002; Lee, 2003; 2004). When taken at supratherapeutic doses, APAP causes centrilobular hepatocyte degeneration and necrosis in rodents and humans. In response to injury with APAP and other centrilobular hepatotoxicants, there is a recovery phase in which hepatocytes are stimulated to repopulate the liver lobule (Zieve *et al.*, 1985; Lee *et al.*, 1998; Kofman *et al.*, 2005). During this period, rodents are resistant to a second challenge with a higher dose of the same hepatotoxicant, a phenomenon known as autoprotection (Thakore and Mehendale, 1991). Resistance to a different toxicant (heteroprotection) has also been observed (Chanda *et al.*, 1995; Yim *et al.*, 2006). The mechanism(s) underlying the resilience of proliferating hepatocytes to further toxicity is not completely known. Up-regulation of genes involved in detoxification and disposition may contribute to this resistance by efficiently removing the toxicant itself and/or mediators of oxidative stress generated during cell damage.

APAP-induced hepatotoxicity results in over-expression of efflux membrane transporters including some isoforms of the multidrug resistance-associated protein (Mrp) superfamily. **Mrps belong to the ATP-binding cassette, subfamily c (Abcc)**. Mrps are ATP-dependent plasma membrane transporters that are responsible for removal of xenobiotics, their conjugates and oxidative stress products across the sinusoidal (eg. Mrp1, 3 and 4) and canalicular (eg. Mrp2) hepatocyte membranes. Levels of Mrp2 **(Abcc2)**, Mrp3 **(Abcc3)** and Mrp4 **(Abcc4)** mRNA and protein are elevated in mouse liver during APAP injury (Aleksunes *et al.*, 2005, 2006b). Induction of Mrp3 and Mrp4 is localized primarily to centrilobular hepatocytes (Aleksunes *et al.*, 2006b). Similar increases in Mrp2 and Mrp3 are observed in rats treated with APAP (Ghanem *et al.*, 2005). Elevated expression of MRP4 mRNA and protein is also detected in liver specimens obtained from patients after APAP overdose (Barnes *et al.*, 2007). The regulatory pathway(s) responsible for Mrp2-4 up-regulation by APAP are presently unknown.

Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that is a member of the basic leucine zipper family of transcription factors. In response to oxidative stress and/or chemical exposure, Nrf2 dissociates from the cytoskeletal inhibitory protein Kelch-like ECH-associated protein 1 (Keap1) and translocates to the nucleus (Itoh *et al.*, 1999). After translocation, Nrf2 binds to antioxidant response elements (AREs) in the upstream regions of target genes and subsequently activates gene transcription (Friling *et al.*, 1990; Rushmore *et al.*, 1991; Wasserman and Fahl, 1997). The basal and inducible expression of multiple hepatic genes is regulated via Nrf2. For example, Nrf2 mediates up-regulation of the detoxification enzyme NAD(P)H quinone oxidoreductase 1 (Nqo1), the glutathione (GSH) synthesis enzyme glutamate cysteine ligase (catalytic subunit, Gclc), and the cell stress protein heme oxygenase-1 (Ho-1) (Chan *et al.*, 2001; Ishii *et al.*, 2002; Mathers *et al.*, 2004).

Over-expression of Nrf2 as seen in hepatocyte-specific Keap1 conditional knockout mice protects against APAP injury (Okawa *et al.*, 2006). By contrast, mice deficient in Nrf2 (Nrf2-null) are more susceptible to APAP hepatotoxicity (Chan *et al.*, 2001; Enomoto *et al.*, 2001). Enhanced sensitivity of Nrf2-null mice likely results from alterations in pathways responsible for APAP bioactivation and detoxification, as well as impaired compensatory induction of Nrf2 target genes involved in cellular antioxidant defenses (Enomoto *et al.*, 2001). Nrf2 may have

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a similar role in regulating Mrp transporter expression during liver toxicity. In support of this, Nrf2 is required for the constitutive and inducible expression of Mrp1 in mouse embryo fibroblasts (Hayashi *et al.*, 2003). In addition, treatment of mice with the Nrf2 activator chemicals oltipraz, ethoxyquin and butylated hydroxyanisole induces hepatic levels of Mrp2-4 mRNA (Maher *et al.*, 2005). More recently, functional AREs have been identified in the promoters of mouse Mrp1 and Mrp2 (Hayashi *et al.*, 2003; Vollrath *et al.*, 2006).

Therefore, the purpose of the current study is to determine whether the up-regulation of efflux transporters Mrp1-4 after APAP is dependent upon expression of Nrf2. Coordinated up-regulation of detoxification enzymes and efflux transporters during hepatotoxicity via Nrf2 could be a compensatory mechanism to aid in the removal of potentially toxic mediators and promote hepatocyte recovery and proliferation.

MATERIALS AND METHODS

Chemicals

APAP and propylene glycol were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of reagent grade or better. RNAzol B was purchased from Tel-Test Inc. (Friendswood, TX).

Treatment Regimen

Adult male wild-type (WT) and Nrf2-null mice on a mixed C57BL/6 and AKR background were bred at the University of Connecticut. Following overnight fasting, groups of WT and Nrf2-null mice were treated with 200 or 400 mg APAP/kg or vehicle (50% propylene glycol; 5 ml/kg ip). Plasma and liver samples were collected 4, 24 and 48 hrs later. The 4 hr time point was used to evaluate Nrf2 nuclear translocation (Goldring *et al.*, 2004) while hepatotoxicity, mRNA and protein expression for Nrf2-responsive genes and Mrps were evaluated at 24 and 48 hrs (Aleksunes *et al.*, 2005; 2006b). All animal studies were conducted in accordance with National Institutes of Health standards and the *Guide for the Care and Use of Laboratory Animals*. The University of Connecticut Institutional Animal Care and Use Committee has approved all experimental animal protocols.

Survival

Animal survival was assessed by calculating the ratio of the number of surviving animals to the total number of animals treated with APAP.

Alanine Aminotransferase (ALT) Activity

Plasma ALT activity was determined as a biochemical indicator of hepatocellular necrosis. Infinity ALT Liquid Stable Reagent (Thermotrace, Melbourne, Australia) was used according to the manufacturer's protocol.

Histopathology

Liver samples were fixed in 10% neutral-buffered formalin prior to routine processing and paraffin embedding. Liver sections (5 μm in thickness) were stained with hematoxylin and eosin. Sections were examined by light microscopy for the presence and severity of necrosis and degeneration. Liver injury was assessed using a grading system described previously (Manautou *et al.*, 1994).

Branched DNA Signal Amplification Assay

Total hepatic RNA was extracted using RNAzol B reagent (Tel-Test Inc., Friendswood, TX). Mouse Gclc, Nqo1 and Mrp1-4 mRNA was quantified using the branched DNA (bDNA) signal

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amplification assay (Quantigene High Volume bDNA Signal Amplification Kit, Genospectra, Fremont, CA) (Aleksunes *et al.*, 2005). Data are reported as relative light units (RLU) per 10 μg total RNA.

Preparation of Crude Membrane, Cytosol and Nuclear Fractions

Liver cytosol and plasma membrane preparations were made as described previously (Aleksunes *et al.*, 2006b). Liver nuclear extracts were prepared using the NE-PER® Nuclear Extraction Kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Proteins were electrophoretically resolved using polyacrylamide gels (8–12% resolving, 4% stacking) and transblotted overnight at 4°C onto PVDF-Plus membrane (Micron Separations, Westboro, MA). Immunochemical detection of Gclc, Gclm, Nqo1, Nrf2, Mrp1-4 and β-actin proteins are described in Supporting Information Table 1 and as previously published (Aleksunes *et al.*, 2006b). Protein-antibody complexes were detected using an enhanced chemiluminescent kit (Amersham Life Science, Arlington Heights, IL) and exposed to Fuji Medical X-ray film (Fisher Scientific, Springfield, NJ). Intensity of protein bands was quantified using the Discovery Series Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA).

Double Immunofluorescence

Hepatic cryosections (5 μm) were thaw-mounted onto Superfrost slides (Fisher Scientific, Pittsburgh, PA) and immediately fixed in 4% paraformaldehyde in PBS pH 7.4, for 5 min. Sections were rinsed with PBS and blocked at room temperature for 30 min with 5% donkey serum/PBS with 0.2% Triton X-100 (PBS-T). The sections were then incubated overnight with anti-Nrf2 (H-300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:50 in 5% donkey serum/PBS-T (Slitt *et al.*, 2006). All antibody solutions were filtered through 0.22-μm membrane syringe-driven filter units (Millipore Corp., Bedford, MA). Sections were washed with PBS-T and incubated for 1 hr at room temperature with Alexa488-labeled secondary antibody to rabbit IgG (Invitrogen, Carlsbad, CA) diluted 1:200 and rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA) diluted 1:200 in 5% donkey serum/PBS-T. Sections were air dried and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Images were acquired on a Leica TCS-SP laser scanning confocal microscope (Leica Microsystems Inc., Exton, PA) equipped with Argon, GreNe and UV lasers, which allowed excitation at 488, 543 and 358 nm wavelengths for detection of Alexa488, rhodamine and DAPI, respectively. Each fluorescent channel was acquired sequentially and then merged to create the final image. Liver sections from three mice per group were stained and representative images are shown. Negative control staining was performed by incubating cryosections without primary antibody.

Statistical Analysis

Quantitative results were expressed as means ± SE (n = 3–16 mice). Data were analyzed using unpaired t-test or one-way ANOVA followed by Newman-Keuls multiple range test. Histopathological data were rank ordered prior to analysis of variance analysis. Asterisks (*) represent a statistical difference between control and APAP-treated groups and daggers (†) represent a statistical difference between WT and Nrf2-null mice. Significance was set at $p < 0.05$.

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RESULTS

Plasma ALT activity in WT and Nrf2-null mice after APAP

APAP-induced hepatocellular injury was assessed by plasma ALT levels. At the lower dose of APAP (200 mg/kg), significant elevation of plasma ALT activity at 48 hrs was only detected in Nrf2-null mice (Figure 1). WT mice demonstrated a slight increase in plasma ALT activity at 48 hrs that was not statistically significant. Elevations in ALT occurred in WT and Nrf2-null mice administered 400 mg APAP/kg with a significant difference observed between genotypes at 24 hrs. However, there was no difference in ALT levels at 48 hrs between genotypes at this higher dose of APAP. Of note however, WT mice had a greater survival rate (5 of 8 mice total) compared to Nrf2-null mice (3 of 8 mice total) treated with 400 mg APAP/kg (Table 1).

Liver histopathology in WT and Nrf2-null mice after APAP

Severity of hepatocellular lesions was graded using a scale ranging from 0 to 5 (Table 1). Liver sections with grades higher than 2 are considered to have significant injury. At 24 and 48 hrs after 200 mg APAP/kg, 20% and 100% of Nrf2-null mice had significant centrilobular hepatocyte degeneration and necrosis, respectively, compared to 0% of WT mice. Treatment with 400 mg APAP/kg resulted in significant injury in 62.5% and 100% of WT and Nrf2-null mice, respectively, at 24 hrs. By 48 hrs, 100% of mice given 400 mg APAP/kg in both genotypes exhibited damage. However, as noted above, Nrf2-null mice had reduced survival at this time point compared to WT counterparts, which is reflective of their higher susceptibility to APAP toxicity.

Nuclear Accumulation of Nrf2 in livers from APAP-treated WT mice

Nrf2 protein could not be detected in cytosolic fractions from control or APAP-treated WT mice (data not shown). Similar findings were reported by Goldring *et al.* (2004). Low levels of Nrf2 protein were detected by western blot in nuclear extracts from control WT mice (Figure 2A). Treatment of WT mice with APAP (400 mg/kg) resulted in a 2-fold higher level of nuclear Nrf2 protein by 4 hrs (Figures 2A and 2B), consistent with nuclear translocation. As expected, no nuclear Nrf2 protein was detected in control or APAP-treated Nrf2-null mice. Purity of nuclear extract preparations was confirmed by immunostaining for the cytosolic protein Nqo1. Minimal Nqo1 protein was detected in nuclear extracts from both control and APAP-treated WT mice demonstrating little contamination during cell fractionation (data not shown).

Double immunofluorescence staining was performed on liver sections from WT and Nrf2-null mice obtained at 4 hrs to determine the effects of APAP on Nrf2 subcellular localization. In control WT mice, Nrf2 staining (green) was minimal and diffusely localized to the periphery of hepatocytes, with some nuclear expression observed (Figure 3A). The actin cytoskeleton is stained in red. Nrf2 staining in liver sections from APAP-treated WT mice (400 mg/kg) was strong and localized to the nucleus (blue) (Figure 3B). As expected, no Nrf2 staining was detected in cryosections from control or APAP-treated Nrf2-null mice (Figures 3C and 3D, respectively).

Hepatic mRNA expression of Nqo1 and Gclc in WT and Nrf2-null mice after APAP

mRNA expression of two Nrf2-related genes Nqo1 and Gclc is shown in Figure 4. Similar to previous reports, the constitutive hepatic expression of Nqo1 mRNA was lower in Nrf2-null mice (50% of WT mice) (Ramos-Gomez *et al.*, 2001). APAP treatment (400 mg/kg) increased Nqo1 (3.3-fold) and Gclc (4.1-fold) mRNA expression in livers from WT mice at 24 hrs. By contrast, no differences in Nqo1 or Gclc mRNA levels were observed in APAP-treated Nrf2-null mice. No significant mRNA changes were detected in either genotype treated with 200

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mg/kg. These data confirm that translocation of Nrf2 in APAP-treated WT mice was significant and resulted in increased expression of Nqo1 and Gclc mRNA.

Hepatic protein expression of Nqo1 and Gclc in WT and Nrf2-null mice after APAP

Western blots using anti-Nqo1 and anti-Gclc antibodies were performed to confirm Nrf2 dependency on the basal expression of Nqo1 and Gclc and to investigate the effect of APAP on protein expression. Western blots from APAP (200 mg/kg)-treated mice at 48 hrs are shown in Figure 5A. Analysis was limited to the 48 hr time point since changes in protein expression for Nqo1 and Gclc are not observed at 24 hrs (data not shown). Constitutive expression of Nqo1 and Gclc proteins in livers from Nrf2-null mice was reduced to 30% and 80% of WT controls, respectively. Hepatic Nqo1 and Gclc proteins were significantly increased by approximately 1.3-fold in APAP-treated WT mice (Figure 5B). APAP administration did not alter expression of Nqo1 or Gclc proteins in Nrf2-null mice.

Hepatic mRNA expression of Mrp1-4 in WT and Nrf2-null mice after APAP

Constitutive hepatic expression of Mrp1, Mrp2 and Mrp4 mRNA was similar between WT and Nrf2-null mice. In contrast, basal Mrp3 mRNA expression was 60% lower in Nrf2-null mice. APAP treatment (400 mg/kg) increased Mrp1 mRNA (1.5 to 2.7-fold) in both WT and Nrf2-null mice at 24 and 48 hrs (Figure 6). A similar induction of Mrp1 mRNA (1.7-fold) was observed after the lower dose of APAP (200 mg/kg) in Nrf2-null mice at 48 hrs. There was no change in Mrp2 mRNA expression in either genotype after APAP. Expression of Mrp3 and Mrp4 mRNA was increased 3.4- and 4-fold, respectively, at 48 hrs after APAP (400 mg/kg) in WT, but not Nrf2-null mice.

Hepatic protein expression of Mrp1-4 in WT and Nrf2-null mice after APAP

Western blots from APAP (200 mg/kg)-treated mice at 48 hrs are shown in Figure 7A. Analysis was limited to the 48-hr time point because maximal changes in Mrp protein expression are not seen until this time (Aleksunes *et al.*, 2006b). Constitutive expression of Mrp proteins was similar between Nrf2-null and WT mice with the exception of Mrp3, which was 80% lower in mutant mice. Mrp1 protein was increased 1.8-fold in both WT and Nrf2-null mice treated with APAP (Figure 7B). Higher levels of Mrp3 (1.3-fold) and Mrp4 (4.5-fold) were observed 48 hrs after APAP in WT, but not Nrf2-null mice. A slight decrease in Mrp2 protein expression was observed in APAP-treated Nrf2-null mice (67% of APAP-treated WT mice) (Figure 7B).

DISCUSSION

The purpose of the present study was to determine whether induction of Mrp transporters in response to APAP toxicity is dependent on Nrf2 expression. APAP administration stimulated nuclear accumulation of Nrf2 protein at 4 hrs in WT mice suggesting Nrf2 translocation. By 24 and 48 hrs, Nrf2-null mice experienced greater hepatotoxicity and mortality compared to WT mice as previously reported (Chan *et al.*, 2001; Enomoto *et al.*, 2001). During this period, WT mice given APAP demonstrated increases in the prototypical Nrf2 target genes Gclc and Nqo1 as well as Mrp3 and 4 mRNA and protein expression. By contrast, levels of these enzymes and transporters remained unchanged in APAP-treated Nrf2-null mice suggesting that up-regulation of these genes during hepatotoxicity was dependent upon Nrf2 expression and activation. Mrp1 mRNA and protein were similarly increased in both WT and Nrf2-null mice after APAP suggesting that alternate signaling pathways independent of Nrf2 are responsible for up-regulation of this gene. Induction of Mrp1 mRNA and protein in Nrf2-null mice suggests that enhanced APAP toxicity in these mice did not compromise gene transcription or protein synthesis.

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Previous reports regarding the susceptibility of Nrf2-null mice to APAP toxicity focused on the ability of this transcription factor to influence the initiating events of injury (namely, APAP conjugation and detoxification) (Chan *et al.*, 2001; Enomoto *et al.*, 2001). Reduced expression of Nqo1, Gclc, and Ugt1a6 genes in Nrf2-null mice accounted for their heightened susceptibility to APAP-induced liver injury. Unpublished work from our laboratory demonstrates dramatic up-regulation of Mrp4 protein in proliferating centrilobular hepatocytes in mice showing resistance to APAP hepatotoxicity following pretreatment with subtoxic doses of APAP (autoprotection). Interestingly, inhibition of hepatocyte proliferation using the antimitotic agent colchicine, blocks APAP autoprotection and also prevents induction of Mrp4. Given these findings, the current studies were aimed at determining the signaling pathway responsible for increasing expression of not only Mrp4 but also Mrps 1-3. These results indicate that Nrf2-null mice have impaired up-regulation of detoxification and transport genes after APAP and that this likely influences hepatocyte repair, recovery and potentially, autoprotection.

These data are in line with recent *in vitro* chromatin immunoprecipitation work which identified functional ARE sequences in the upstream regions of mouse Mrp2, Mrp3 and Mrp4 genes that can mediate transcriptional up-regulation in response to treatment with *tert*-butyl hydroquinone (Maher *et al.*, 2007). Of note, Mrp1 was not analyzed in that study. Additional work using Nrf2 activating compounds have similarly pointed to a role for Nrf2 in the coordinate induction of detoxification and Mrp transporter genes (Maher *et al.*, 2005; Vollrath *et al.*, 2006). However, it has remained unknown whether similar Nrf2-mediated regulation of transporters could occur in models of liver injury. Data generated in this study add to the existing literature since they demonstrate a significant role for this transcription factor in regulating transporter expression in an *in vivo* model of chemical-induced hepatotoxicity. Figure 8 depicts the proposed signaling pathways for coordinated induction of Nqo1, Gclc, Mrp3 and Mrp4 during APAP-induced hepatotoxicity. Further research may highlight a similar role for Nrf2 in other models of liver damage (i.e., estrogen, intrahepatic and obstructive cholestasis, fatty liver, additional chemical toxins) in which Mrp genes are up-regulated.

Previous work from our laboratory demonstrated a slight induction of Mrp2 mRNA and protein after APAP in C57BL/6J mice (Aleksunes *et al.*, 2005; 2006b). However, Mrp2 mRNA and protein expression in APAP-treated WT mice (mixed C57BL/6 and AKR background) was unchanged in these studies. Differences in mouse strain between the two studies likely explain this observation.

Contribution of individual Mrp transporters to the disposition of APAP conjugates has been an area of active research. Early work using mutant rats deficient in Mrp2 protein (known as TR^{-}rats) demonstrated a role for Mrp2 in dictating the efflux of APAP-glutathione and APAP-glucuronide conjugates across the canalicular membrane (Xiong *et al.*, 2000; Chen *et al.*, 2003). Similarly, sinusoidal excretion of APAP-glucuronide and APAP-sulfate was reduced in transgenic mice lacking Mrp3 and Mrp4, respectively (Manautou *et al.*, 2005; Zamek-Gliszczynski *et al.*, 2006). Lower expression of Ugt1a6 and Mrp3 proteins in Nrf2-null mice likely decreases the formation and sinusoidal efflux of APAP-glucuronide, permitting greater availability of APAP for bioactivation by cytochrome P450 enzymes. Reduced constitutive expression of Mrp3 mRNA and protein in Nrf2-null mice is an interesting observation and warrants additional investigation.

Besides dictating hepatic efflux of APAP metabolites, Mrp3 and Mrp4 may play additional roles in Nrf2-mediated antioxidant defenses. During oxidative stress and cell injury, there are perturbations in a number of cellular pathways. APAP causes depletion of GSH, formation of reactive oxygen and nitrogen species, lipid peroxidation, covalent adduct formation and destruction of heme-containing enzymes, such as the **cytochrome P450** enzymes (Chiu *et*

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al., 2002; Hinson *et al.*, 2004). Enhanced Nrf2-mediated expression of Mrp3 and Mrp4 genes in response to APAP may serve to reduce the retention of byproducts and mediators of cellular injury as well as biliary constituents within hepatocytes. For example, Mrp4 transports a number of substrates associated with cell stress including cyclic nucleotides (cAMP and cGMP), prostaglandins (PGE_1 and PGE_2), GSH and sulfated bile acids (Chen *et al.*, 2001; Reid *et al.*, 2003; Zelcer *et al.*, 2003a; Bai *et al.*, 2004; Rius *et al.*, 2006). Increased basolateral excretion of these substrates to sinusoidal blood may serve not only as a protective mechanism for efficient elimination of cytotoxicants, but alternatively for paracrine signaling to adjacent hepatocytes and other cell types (including Kupffer and stellate cells) involved in progression of and/or recovery from liver disease. Increased Mrp3 and Mrp4 expression may provide alternate routes for bile acid efflux from hepatocytes, particularly during cellular injury (Zeng *et al.*, 2000; Zelcer *et al.*, 2003b). Recent work demonstrates a heightened susceptibility of Mrp4-null mice to obstructive cholestasis, further supporting a role for this transporter in mitigating bile acid toxicity and oxidative-type cell necrosis (Mennone *et al.*, 2006). At present, the susceptibility of Mrp4-null mice to APAP toxicity has not been tested. Since Mrp4 levels are normally low in mouse liver but significantly increased in response to APAP hepatoxicity, it can be hypothesized that Mrp4-null mice would have reduced adaptation to APAP-induced damage. Coordinated regulation of drug metabolism and transport (namely, Mrp3 and Mrp4) by Nrf2 may be one means for promoting recovery during hepatocellular injury.

This study clearly demonstrates for the first time that induction of Mrp3 and Mrp4 transporters during APAP liver injury is dependent upon expression of Nrf2. Our laboratory has also documented the overexpression of NQO1 and MRP4 proteins in livers from patients following APAP overdose (Aleksunes *et al.*, 2006a; Barnes *et al.*, 2007). These data suggest that Nrf2-mediated transcription is similarly activated in human liver in response to APAP-induced cellular injury and strongly supports the use of Nrf2-null mice to investigate the signaling pathways responsible for regulation of transporter and detoxification genes during drug toxicity. A better understanding of Nrf2 regulation of transporter expression in mouse and human liver in relation to control of detoxification genes may provide insight into adaptive recovery mechanisms and development of resistance to cytotoxic xenobiotics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

APAP
: Acetaminophen

ALT
: alanine aminotransferase

AREs
: antioxidant response elements

Abcc
: ATP-binding cassette, subfamily c

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bDNA
branched DNA signal amplification

DAPI
4′,6-diamidino-2-phenylindole

Gclc
glutamate cysteine ligase catalytic

Gclm
glutamate cysteine ligase modifier

GSH
glutathione

Ho-1
heme oxygenase-1

Keap1
Kelch-like ECH-associated protein 1

Mrps
multidrug resistance-associated proteins

Nqo1
NAD(P)H:quinone oxidoreductase 1

NAPQI
N-acetyl-*p*-benzoquinone imine

Nrf2
nuclear factor-E2-related factor 2

PBS-T
PBS-Triton X-100

RLU
relative light units

Ugt
UDP-glucuronosyltransferase

WT
wild-type

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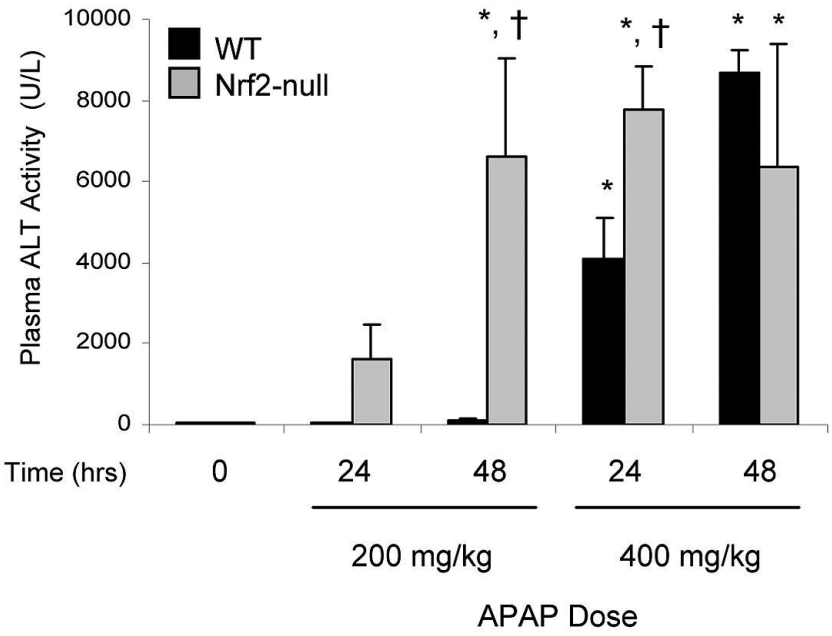

Figure 1. Plasma ALT activity in wild-type and Nrf2-null mice after APAP

Plasma was isolated from WT and Nrf2-null mice 24 and 48 hrs following APAP (200, 400 mg/kg) or vehicle administration. The data are presented as mean plasma ALT activity (U/L) ± SE (n = 3 – 16 animals). Asterisks (*) represent a statistical difference ($p < 0.05$) between pooled 0 hr control and APAP-treated groups and daggers (†) represent a statistical difference ($p < 0.05$) between WT and Nrf2-null mice.

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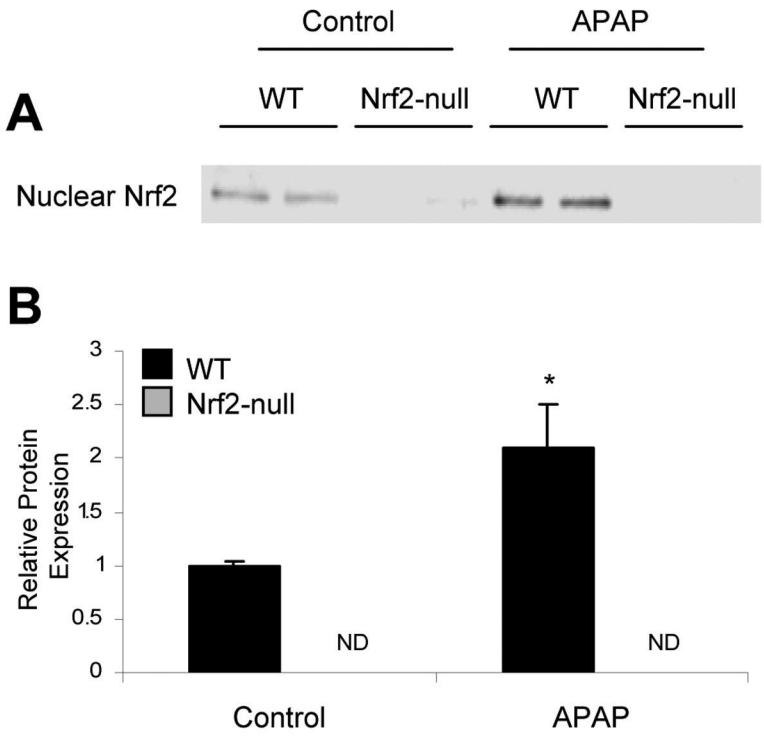

Figure 2. Western blot analysis of Nrf2 in nuclear liver preparations from wild-type and Nrf2-null mice after APAP

An immunoblot for Nrf2 protein was performed using nuclear extracts (60 μg protein/lane) from WT and Nrf2-null mice 4 hrs following treatment with APAP (400 mg/kg) or vehicle. The data are presented as an individual blot (A) and as mean relative protein expression **(normalized to control WT mice)** ± SE *(B)*. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and APAP-treated groups (n = 2 animals). ND, not detected.

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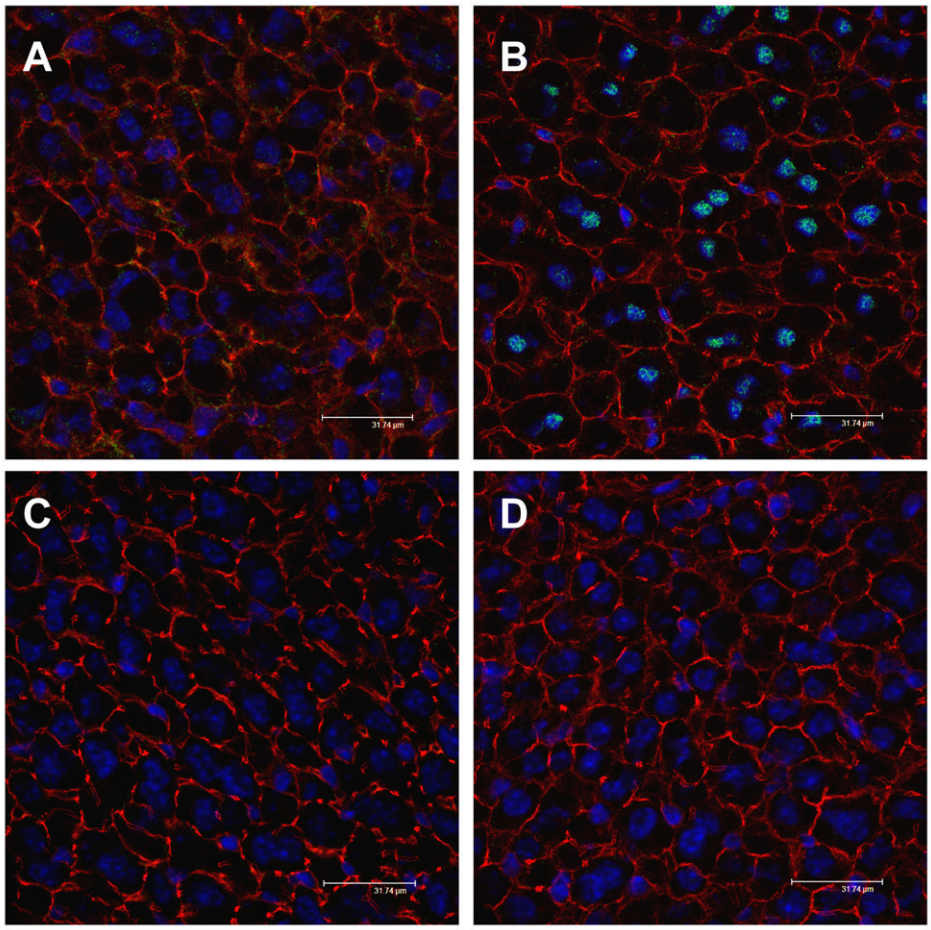

Figure 3. Immunofluorescent localization of Nrf2 in livers from wild-type and Nrf2-null mice after APAP

Indirect immunofluorescence to detect Nrf2 (*green*) and actin (*red*) was conducted on liver cryosections (5 μm) from WT and Nrf2-null mice 4 hrs following treatment with APAP (400 mg/kg) or vehicle. Sections were mounted in Prolong Gold containing DAPI for nuclear staining *(blue)*. Representative images are shown. Panel A: control WT; B: APAP-treated WT; C: control Nrf2-null; D: APAP-treated Nrf2-null. Bar 31.74 μm.

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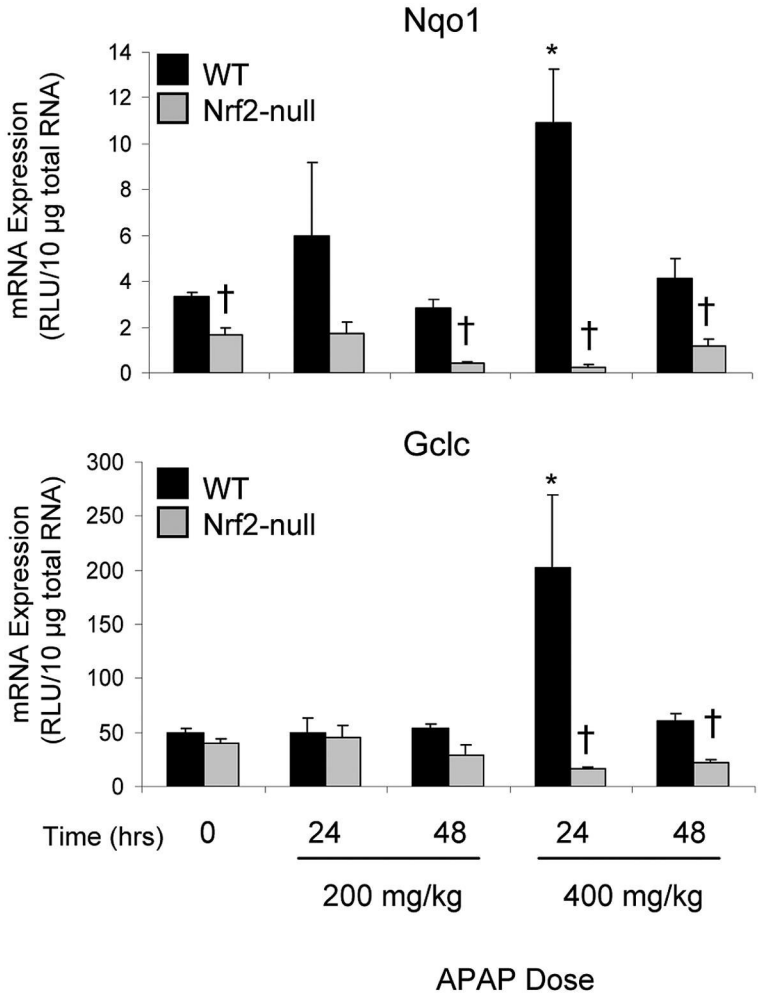

Figure 4. Nqo1 and Gclc mRNA expression in livers from wild-type and Nrf2-null mice after APAP

Total RNA was isolated from livers of WT and Nrf2-null mice 24 and 48 hrs following treatment with APAP (200, 400 mg/kg) or vehicle. RNA was analyzed by the bDNA assay for expression of Nqo1 and Gclc. The data are presented as mean RLU ± SE (n = 3 – 16 animals). Asterisks (*) represent a statistical difference ($p < 0.05$) between pooled 0 hr control and APAP-treated groups and daggers (†) represent a statistical difference ($p < 0.05$) between WT and Nrf2-null mice.

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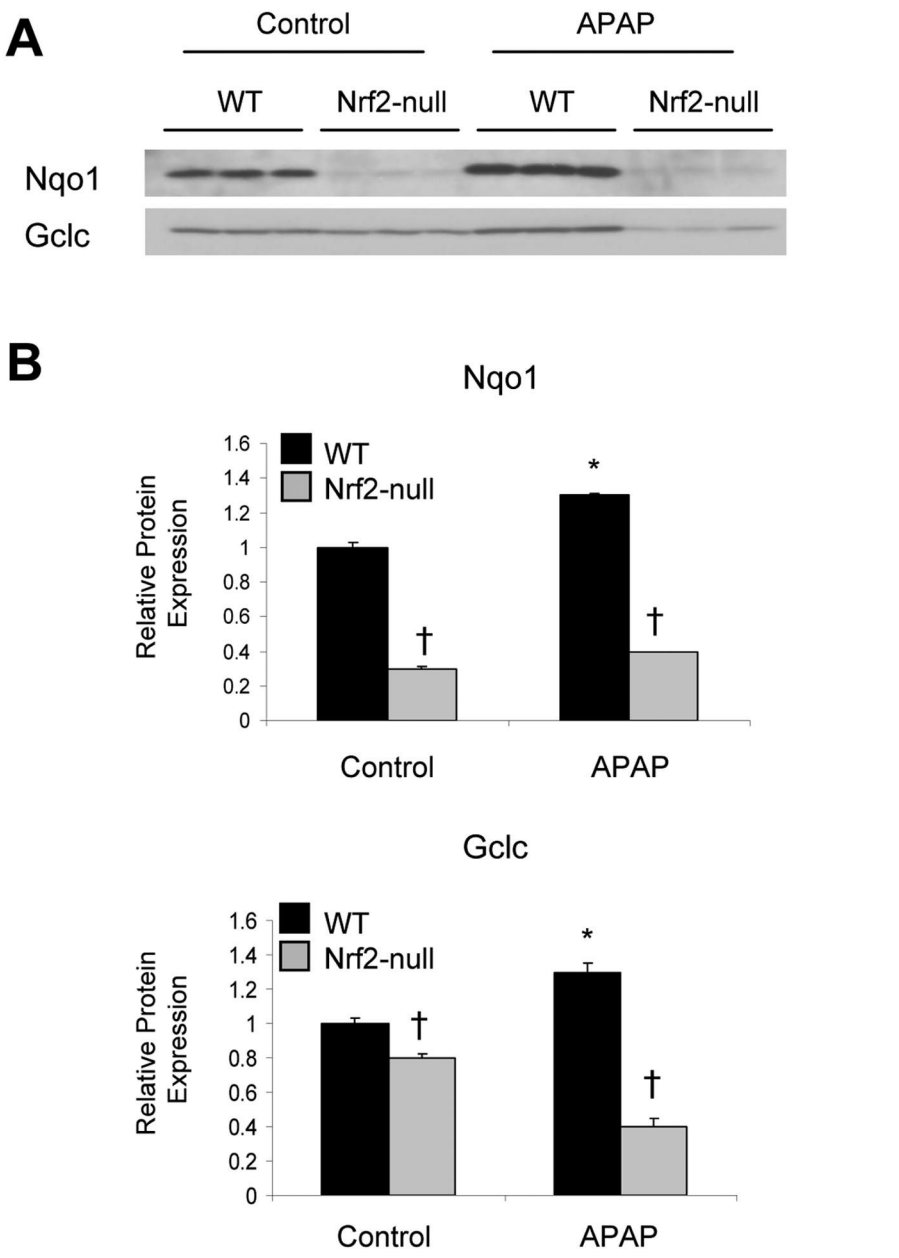

Figure 5. Nqo1 and Gclc protein expression in livers from wild-type and Nrf2-null mice after APAP

Western blots for Nqo1 and Gclc were performed using liver cytosol (50 μg protein/lane) from WT and Nrf2-null mice 48 hrs following treatment with APAP (200 mg/kg) or vehicle. The data are presented as individual blots (A) and as mean relative protein expression **(normalized to control WT mice)** ± SE (n = 3 animals) *(B)*. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and APAP-treated groups and daggers (†) represent a statistical difference ($p < 0.05$) between WT and Nrf2-null mice.

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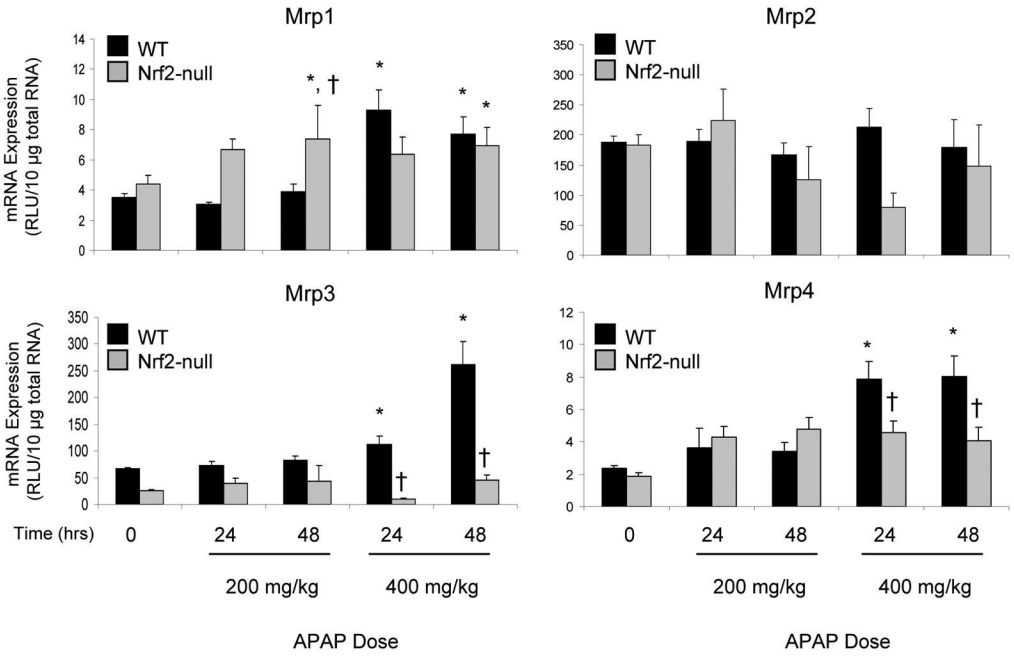

Figure 6. Mrp1-4 mRNA expression in livers from wild-type and Nrf2-null mice after APAP

Total RNA was isolated from livers of WT and Nrf2-null mice 24 and 48 hrs following treatment with APAP (200, 400 mg/kg) or vehicle. RNA was analyzed by the bDNA assay for expression of Mrp1-4. The data are presented as mean RLU ± SE (n = 3 – 16 animals). Asterisks (*) represent a statistical difference ($p < 0.05$) between pooled 0 hr control and APAP-treated groups and daggers (†) represent a statistical difference ($p < 0.05$) between WT and Nrf2-null mice.

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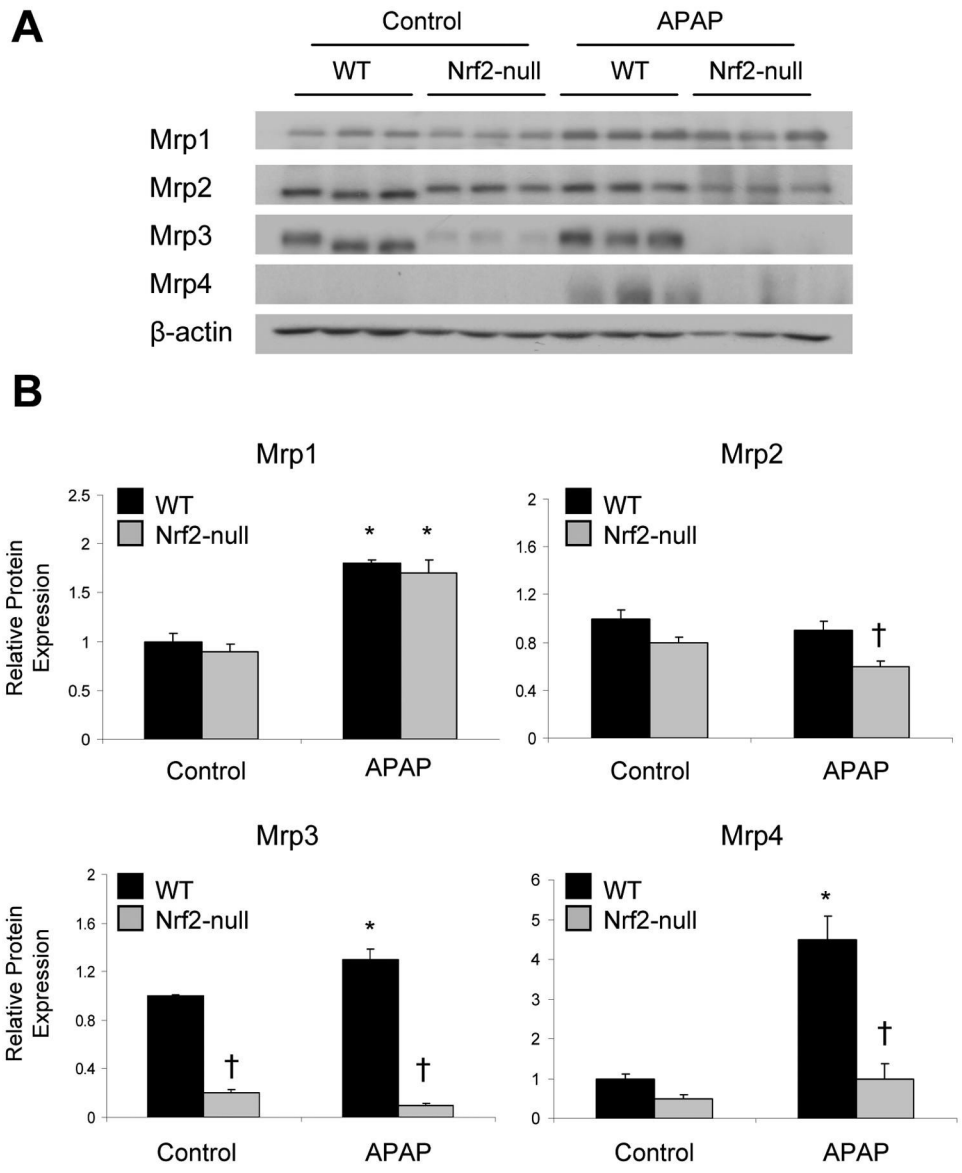

Figure 7. Mrp1-4 protein expression in livers from wild-type and Nrf2-null mice after APAP

Western immunoblots for Mrp1-4 were performed using liver membrane fractions (50 μg protein/lane) from WT and Nrf2-null mice 48 hrs following treatment with APAP (200 mg/kg) or vehicle. The data are presented as individual blots (A) and as mean relative protein expression **(normalized to control WT mice)** ± SE (n = 3 animals) *(B)*. Equal protein loading was confirmed by detection of β-actin. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and APAP-treated groups and daggers (†) represent a statistical difference ($p < 0.05$) between WT and Nrf2-null mice.

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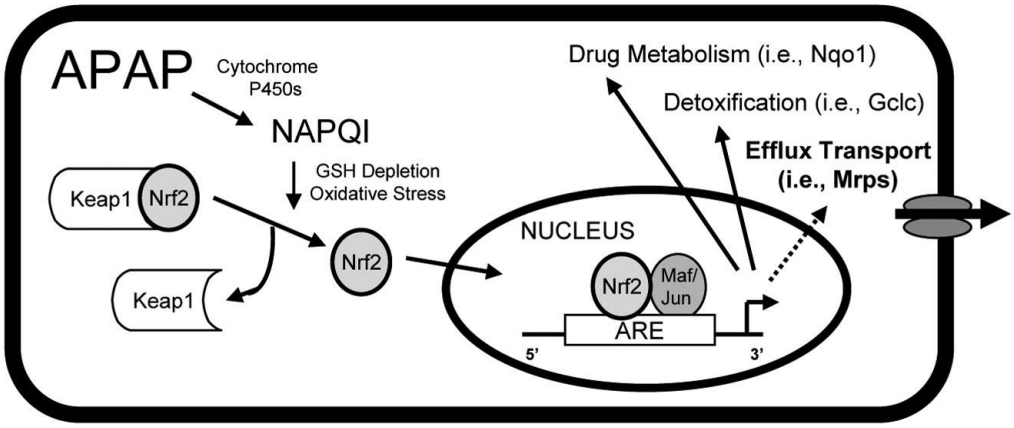

Figure 8. Nrf2-mediated gene transcription during APAP hepatotoxicity

APAP is bioactivated to *N*-acetyl-*p*-benzoquinone imine (NAPQI) by various cytochrome P450 isoforms in the hepatocyte. Generation of NAPQI alters cellular homeostasis by increasing oxidative stress and depleting GSH stores. The net result is release of Nrf2 from Keap1 and translocation of Nrf2 to the nucleus. Once in the nucleus, Nrf2 heterodimerizes with small Maf or Jun proteins, binds ARE sequences in the upstream region of target genes and activates gene transcription. The Nrf2-mediated antioxidant defense in hepatocytes is composed of numerous genes involved in cell stress response, drug metabolism, detoxification, and efflux transport.

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Table 1

Histopathological Analysis of Livers from APAP-Treated WT and Nrf2-null Mice

Livers were removed from WT and Nrf2-null mice 24 and 48 hrs following APAP (200 or 400 mg/kg) or vehicle ip injection and fixed in 10% formalin prior to paraffin embedding and staining with hematoxylin and eosin. Liver slices were evaluated for the severity of degenerative and necrotic changes in the centrilobular regions as previously described (Manautou *et al.*, 1994; Aleksunes *et al.*, 2005). Liver samples with grades greater than 2 are considered to have significant injury. Data were rank ordered prior to statistical analysis. Asterisks (*) represent a statistical difference ($p < 0.05$) from control mice of the same genotype. Daggers (†) represent a statistical difference ($P < 0.05$) from WT mice of the same treatment. Survival data are reported as number of mice/total number of mice (percent alive).

	Histological Grade							
WT	**0**	**1**	**2**	**3**	**4**	**5**	**Percent > 2**	**Survival**
24 hr								
Control	8	0	0	0	0	0	0	8/8 (100%)
200 mg/kg	2	3	0	0	0	0	0	5/5 (100%)
400 mg/kg	1	3	2	1	2	7	62.5*	16/16 (100%)
48 hr								
Control	5	0	0	0	0	0	0	5/5 (100%)
200 mg/kg	0	4	1	0	0	0	0	5/5 (100%)
400 mg/kg	0	0	0	0	1	4	100*	5/8 (62.5%)
Nrf2-null	0	1	2	3	4	5	Percent > 2	Survival
24 hr								
Control	7	0	0	0	0	0	0	7/7 (100%)
200 mg/kg	0	1	3	0	0	1	20*,†	5/5 (100%)
400 mg/kg	0	0	0	2	1	2	100*	5/8 (62.5%)
48 hr								
Control	3	0	0	0	0	0	0	3/3 (100%)
200 mg/kg	0	0	0	0	1	2	100*,†	3/5 (60%)
400 mg/kg	0	0	1	0	0	2	100*	3/8 (37.5%)