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High Sensitivity of Nrf2 Knockout Mice to Acetaminophen Hepatotoxicity Associated with Decreased Expression of ARE-Regulated Drug Metabolizing Enzymes and Antioxidant Genes

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Nrf2, which belongs to the basic leucine zipper (bZip) transcription factor family, has been implicated as a key molecule involved in antioxidant-responsive element (ARE)-mediated gene expression. In order to examine the role of Nrf2 in protection against xenobiotic toxicity, the sensitivity of *nrf2* knockout mice to acetaminophen (N-acetyl-4-aminophenol (APAP)) was analyzed. The saturation of detoxification pathways after high levels of exposure to APAP is known to induce hepatotoxicity. Two factors important in its detoxification are UDP-glucuronosyltransferase (UDP-GT), an ARE-regulated phase-II drug-metabolizing enzyme, and glutathione (GSH), an antioxidant molecule whose synthesis depends on ARE-regulated γ-glutamylcysteine synthetase (γGCS). Two- to 4-month-old male mice were orally administered a single dose of APAP at 0, 150, 300, or 600 mg/kg. Doses of 300 mg/kg APAP or greater caused death in the homozygous knockout mice only, and those that survived showed a greater severity in hepatic damage than the wild-type mice, as demonstrated by increased plasma alanine aminotransferase activity, decreased hepatic nonprotein sulfhydryl (NPSH) content, and centrilobular hepatocellular necrosis. The high sensitivity of Nrf2-deficient mice was confirmed from observations made at 0, 2, 8, and 24 h after dosing with 300 mg/kg APAP; increased anti-APAP immunoreactivity was also noted in their livers at 2 h. Untreated homozygous knockout mice showed both a lower UDP-GT activity and NPSH content, which corresponded to decreased mRNA levels of UDP-GT (Ugt1a6) and the heavy chain of γGCS, respectively. These results show that Nrf2 plays a protective role against APAP hepatotoxicity by regulating both drug metabolizing enzymes and antioxidant genes through the ARE.

Key Words: **Nrf2; knockout mice; acetaminophen; hepatotoxicity; ARE; oxidative stress; electrophile; UDP-glucuronosyltransferase; glutathione; γ-glutamylcysteine synthetase.**

Drug metabolizing enzymes and antioxidant systems represent 2 major defense mechanisms against xenobiotic toxicity. Electrophiles, radicals, and reactive oxygen species (ROS) are often generated as intermediates or by-products of xenobiotic metabolism. These molecules, if not properly eliminated, provoke lipid peroxidation and oxidation of DNA and other cellular components, which results in various acute and chronic tissue injuries, carcinogenesis, and aging (Halliwell *et al*., 1995a,b; Martin *et al*., 1996; Slaga, 1995). Accumulation of these harmful oxidants in the cell is prevented through the actions of small antioxidant molecules such as GSH and vitamins, and antioxidant enzymes such as heme oxygenase 1 (HO-1), catalase, and superoxide dismutase (SOD) (Rautalahti and Huttunen, 1993; Sen, 1995). Drug metabolizing enzymes, such as glutathione *S*-transferase (GST), work in concert with antioxidant systems by metabolizing electrophiles and xenobiotics, and some of the molecules involved in these 2 defense mechanisms are induced simultaneously in response to xenobiotic exposure (Prestera *et al*., 1995). Such concerted gene induction of drug metabolizing and antioxidant defense systems occurs through a common regulatory region called the ARE (antioxidant responsive element) or EpRE (electrophile responsive element) (Buetler *et al*., 1995; Prestera and Talalay, 1995).

While searching for proteins that regulate expression of the β-globin gene during differentiation, the Nrf family of transcription factors was identified. Originally, Nrf2 was isolated in 1994 from a human K562 erythroid cell line and in 1995 from a chicken erythroid cDNA library (HD3) (Itoh *et al*., 1995; Moi *et al*., 1994). The designation Nrf2 (NF-E2 related factor 2) was derived from the known transcription factor p45 subunit of NF-E2 (Nuclear Factor Erythroid 2), with which it shows high sequence homology. Within the family of bZip transcription factors, Nrf2, p45 NF-E2, and their homologues constitute a discrete subfamily whose members share regions of homology with that of the *Drosophila* Cap'n'Collar (CNC) protein (Chan *et al*., 1993; Itoh *et al*., 1995; Kobayashi *et al*.,

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1999; Moi *et al*., 1994). While the expression of p45 is limited to erythroid cells and megakaryocytes, Nrf2 is widely expressed in tissues that include muscle, kidney, lung, intestine, and liver (Itoh *et al*., 1995; Moi *et al*., 1994). Furthermore, elimination of Nrf2 expression does not influence erythropoiesis (Chan *et al*., 1996; Itoh *et al*., 1997; Kuroha *et al*., 1998). Thus, until recently, the precise function of Nrf2 was unknown. The fact that the Nrf2 binding site, namely the NF-E2 binding motif or MARE (Maf-recognition element), is highly similar to the ARE, and that its tissue-specific expression is most similar to that of ARE-regulated genes led to the speculation that Nrf2 is an important regulator of ARE-mediated gene expression (Itoh *et al*., 1997; Venugopal and Jaiswal, 1996).

The genes that were first shown to be controlled by Nrf2 *in vivo* encode drug-metabolizing enzymes such as GST and quinone oxidoreductase 1 (NQO1) (Itoh *et al*., 1997); these genes are inducible by the antioxidant butylated hydroxyanisole (BHA) which acts through the ARE present in their regulatory regions. Dietary administration of 0.7% BHA for 16 to 20 days greatly impaired the induction of NQO1 and 3 classes of GST (Alpha, Mu, and Pi) in both the liver and intestine of homozygous *nrf2* knockout mice compared to their heterozygous littermates (Itoh *et al*., 1997). It was recently demonstrated that detoxification-enzyme genes are not unique in their regulation by Nrf2. For example, Nrf2 was shown to be essential for the induction of HO-1, peroxiredoxin MSP23, and the cystine transporter (X_c^-) by electrophilic agents in mouse peritoneal macrophages (Ishii *et al*., 2000). HO-1 and peroxiredoxin have antioxidative activities, whereas X_c^- increases the intracellular cysteine necessary for GSH synthesis. The involvement of Nrf2 in the regulation of HO-1 and γGCS, an enzyme which catalyzes the rate-limiting step in the synthetic pathway of GSH, has also been reported by other investigators (Alam *et al*., 1999; Moinova and Mulcahy, 1999). The requirement of Nrf2 in the regulation of such genes was further confirmed by the depressed expression of UDP-GT (Ugt1a6), catalase, and SOD1 observed in *nrf2* knockout mice (Chan and Kan, 1999).

The molecular mechanism of Nrf2 activation by reactive electrophiles has partially been clarified (Ishii *et al*., 2000; Itoh *et al*., 1999). Keap1, a homologue of the *Drosophila* actin binding protein Kelch, binds to the N-terminal Neh2 domain of Nrf2, thereby retaining this transcription factor in the cytoplasm (Adams *et al*., 2000; Itoh *et al*., 1999; Xue and Cooley, 1993). Treatment of cells with reactive electrophiles counteracts this sequestration of Nrf2 by Keap1, such that Nrf2 translocates to the nucleus and upregulates ARE-mediated transcription.

Considering its function, Nrf2 is expected to play an important role in the defense mechanisms against xenobiotic toxicity. In fact, the high sensitivity of *nrf2* knockout mice to butylated hydroxytoluene (BHT)-induced pulmonary injury has recently been reported (Chan and Kan, 1999); the mechanism of their high sensitivity was, however, not clearly demonstrated. APAP was used as a chemical model in the present study to examine the mechanism by which Nrf2 plays a protective role against the toxicity of xenobiotics and to elucidate the relationship between Nrf2-regulated gene expression and such protection. APAP is widely used in tablet form for its antipyretic and analgesic properties, and its metabolic pathways are well characterized (Cohen *et al*., 1998). At low doses, the majority of APAP is readily conjugated with glucuronic acid and sulfate and subsequently eliminated. A small portion of APAP undergoes biotransformation by cytochrome P450s to an electrophilic quinoneimine that nonenzymatically reacts with GSH for excretion. Exposure to high doses of APAP saturates the glucuronidation and sulfation pathways and depletes the GSH pool, thus enabling the reactive APAP intermediate to bind to important intracellular macromolecules and result in cytotoxicity. Both the ARE-regulated phase II drug-metabolizing enzyme UDP-GT, and the antioxidant molecule GSH, whose synthesis depends on the ARE-regulated γGCS enzyme, are important in the detoxification of APAP.

In the present study, we demonstrated that *nrf2* knockout mice are highly sensitive to APAP-induced hepatotoxicity as a result of a lowered activity of drug-metabolizing enzymes and antioxidant systems, which represent the major Nrf2-regulated defense mechanisms against xenobiotic toxicity.

MATERIALS AND METHODS

Reagents. APAP, UDP-glucuronic acid, and acetaminophen glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO). Propylene glycol, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), oxidized glutathione (GSSG), and glutathione reductase (GR) from yeast were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were obtained from commercial sources and were reagent grade or better.

Animals. Two- to 4-month-old male homozygous (–/–) and heterozygous (+/–) *nrf2* knockout mice (ICR/129SVJ chimeric mice [Itoh *et al*., 1997]) and their wild-type littermates (+/+) were used in this study. DNA was taken from the tail of each mouse and analyzed by polymerase chain reaction (PCR) to confirm its genotype. The mice were housed in stainless-steel cages in an animal room maintained at 24 ± 2°C and using a 12-h light/dark cycle (light on at 7:00 A.M. and off at 7:00 P.M.). Food (certified diet M, Oriental Yeast Co, Ltd., Tokyo, Japan) and water were made freely available to the mice, except where otherwise stated.

Treatment of animals. In the dose-response study the administration protocol was performed according to Manautou *et al*. (1996). After overnight fasting, APAP (in 50% propylene glycol as vehicle) was administered to the mice, po, between 8:30 and 9:30 A.M. at doses of either 0, 150, 300, or 600 mg/kg, 5 ml/kg being used in each case. Four mice of each genotype were used for each dose, except for the 150-mg/kg dose in which only 3 heterozygous knockout mice were used. Food was withheld until 2 h after dosing, but water was available throughout the experiment. The animals were sacrificed 24 h after dosing.

In the time-course study a dose of 300 mg/kg APAP was administered to the mice and the animals were sacrificed at 0 (after overnight fasting and without dosing), 2, 8, or 24 h after dosing. Other experimental conditions were the same as those used in the dose-response study. An additional 8 homozygous knockout and wild-type mice were sacrificed after overnight fasting and without dosing, for the purpose of metabolic baseline activity analysis.

TABLE 1
Mortality of *nrf2* Knockout and Wild-type Mice Exposed to Acetaminophen

	Genotype		
Dose (mg/kg)	+/+	+/–	–/–
0	0/4[a]	0/4	0/4
150	0/4	0/3	0/4
300	0/4	0/4	1/4
600	0/4	0/4	3/4

Note. Acetaminophen in 50% propylene glycol was administered (po) to overnight-fasted wild-type (+/+) and homo- (–/–) and heterozygous (+/–) *nrf2* knockout mice.

[a] Number of animals that died within 24 h/number of animals used.

Biochemical assays. Following withdrawal of blood from the posterior vena cava of anesthetized animals, plasma alanine transaminase (ALT) activity was determined using an automated biochemical analyzer, CHEM1® (Bayer Corp., Tarrytown, NY). The animals were sacrificed by exanguination, livers were removed, and microsomes were prepared. Microsomal protein concentration was determined by the method of Lowry *et al*. (1951). Cytochrome P450 content was determined according to Omura and Sato (1964). Hepatic UDP-GT activity from detergent-treated microsomes was determined using APAP as the aglycon substrate according to Bock and Bock-Henning (1987) and Manautou *et al*. (1996). NPSH was extracted from samples of each liver using 1 M perchloric acid and measured by the enzymatic cycling method of Tietze (1969) as modified by Griffith (1980).

Histopathology. Samples of each liver were fixed in 10% neutral buffered formalin for 24 h, processed by routine methodology, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Hepatic lesions were graded from – to +++ (–, no lesions; ±, degeneration of centrilobular hepatocytes; +, slight centrilobular necrosis (necrotic hepatocytes present in 1- to 3-cell layers from the central vein); ++, moderate centrilobular necrosis (necrotic hepatocytes present in more than 3-cell layers from the central vein but limited to less than half of the liver section); +++, severe centrilobular necrosis (necrotic areas occupying half or more than half of the liver section)). Histopathological evaluation was performed blind, in which the identity of both the genotype and type of treatment was not revealed to those undertaking the examination.

Immunohistochemistry. Deparaffinized liver sections were placed in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. Sections were heated in 0.1 M citrate buffer, pH 6.0, in a microwave oven for 6 min and rinsed twice, 5 min each wash, with phosphate-buffered saline containing 0.05% Triton X-100 (PBST). After blocking non-specific binding sites with 4% BlockAce (Dai-Nippon Pharmaceutical, Osaka) for 20 min, sections were incubated in a rabbit polyclonal antibody against APAP (Biogenesis, Kingston, NH; 1:250) for 30 min; negative control sections were incubated in PBST only. Sections were rinsed with PBST, incubated in biotinylated goat antibody against rabbit immunoglobulin at a 1:1000 dilution (DAKO Japan, Tokyo) for 30 min, rinsed twice with PBST, and incubated in streptavidin-horseradish peroxidase at a 1:500 dilution (Vector Laboratories, Burlingame, CA) for 30 min. Diaminobenzidine was used as chromogen, followed by counterstaining with hematoxylin. Each step was performed at room temperature, except where otherwise stated.

Determination of γGCS and Ugt1a6 mRNAs. Total cellular RNA was extracted by RNAzolTM B (TEL-TEST, Inc., Friendswood). The RNA samples (20 μg) were subjected to electrophoresis and transferred to Zeta-Probe GT membranes (Bio-Rad Japan, Tokyo). The membranes were probed with [^{32}P]-labelled cDNA against Ugt1a6 and the catalytic heavy and regulatory light chains of γGCS; β-actin cDNA was used as a positive control. We obtained the cDNA probe against each gene by RT-PCR, using mouse liver RNA as a template.

The primer used for each gene was as follows:

γGCS heavy chain:
Forward primer 5′-ATGGGGCTGCTGTCCCATGG-3′
Reverse primer 5′-AGCCTGATGCTCTCCTAGTA-3′
γGCS light chain:
Forward primer 5′-CTGCAGACCGGGAACCAGCT-3′
Reverse primer 5′-AGATCAGAGGTGCCTATAGC-3′
Ugt1a6:
Forward primer 5′-CTTCCTGCAGGGTTTCTCTTCC-3′
Reverse primer 5′-CAACGATGCCATGCTCCCC-3′

The hybridized membranes were processed to autoradiographs and quantitated by MacBas software.

Statistical analysis. Results are expressed as mean ± standard deviation, where available. Statistical analyses of differences between values of the vehicle control group (0 mg/kg) or those before dosing (0 h) from mice of the

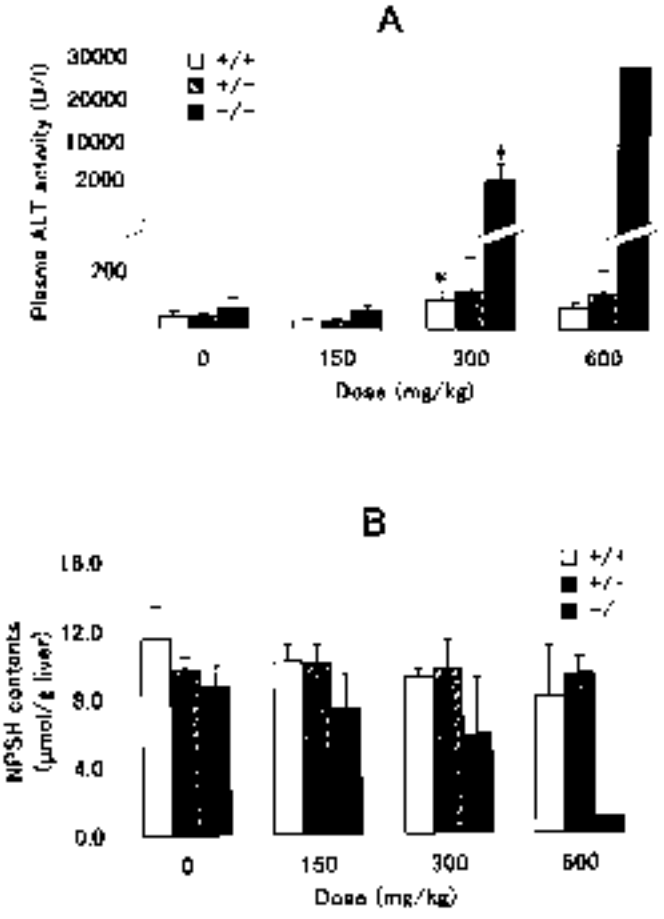

FIG. 1. Plasma ALT activity (A) and hepatic NPSH content (B) of wild type mice (+/+), heterozygous *nrf2* mice (+/–), and homozygous *nrf2* knockout mice (–/–) administered APAP (po) at different doses. Plasma ALT activity was determined from blood samples taken from animals that survived 24 h after dosing. Hepatic NPSH level was measured in the same animals, as described in Materials and Methods. Values are represented as mean ± SD, where $n = 3$ or 4, except for –/– at 600 mg/kg ($n = 1$). *Significantly different from the vehicle control of the same genotype ($p \leq 0.05$).

TABLE 2
Liver Histopathology of *nrf2* Knockout and Wild-type Mice 24 h After Exposure to Acetaminophen at Different Doses

		Genotype		
Dose (mg/kg)	Grade	+/+	+/–	–/–
0	–	4	4	4
150	–	4	3	4
300	–	3	1	0
	±	0	1	0
	+	1	2	2
	++	0	0	0
	+++	0	0	2[a]
600	–	2	1	0
	±	1	0	0
	+	0	3	0
	++	1	0	0
	+++	0	0	4[a]

Note. Acetaminophen in 50% propylene glycol was administered (po) to overnight-fasted wild-type (+/+) and homo- (–/–) and heterozygous (+/–) *nrf2* knockout mice. Number of animals was 4/genotype/dose except for +/– mice at 150 mg/kg ($n = 3$). Livers from all animals were examined by light microscopy and graded from – to +++ for severity of lesions as described in Materials and Methods.

[a] Includes those that died before 24 h.

same genotype were made by Dunnett's multiple comparison test. Comparison between baseline metabolic activities of wild-type and knockout mice were made using the Student's *t*-test. A value of $p \leq 0.05$ was considered significant.

RESULTS

Dose-Response Study

To elucidate the role of Nrf2 in protection against APAP toxicity, we first examined whether the sensitivity of the mice to APAP toxicity was affected by eliminating Nrf2. A previous study showed that 600 mg/kg APAP resulted in hepatic necrosis, with 10% lethality, in adult male CD-1 mice, whereas no hepatotoxicity was observed at a dose of 200 mg/kg (Ginsberg *et al*., 1982). Based on these data, doses of 600, 300, and 150 mg/kg APAP were chosen for the present study. The oral route of administration was selected because Nrf2 is expressed in gastrointestinal tract as well as in liver (Itoh *et al*., 1997). When Nrf2-deficient mice were exposed to APAP, doses of 300 mg/kg and 600 mg/kg resulted in the deaths of 1 and 3 mice, respectively, within 24 h. In contrast, no such lethality was seen in either wild-type or heterozygous knockout mice at any of the doses used (Table 1). These results suggest that *nrf2* knockout mice are more sensitive to APAP toxicity than those expressing Nrf2.

To characterize the APAP toxicity observed in Nrf2-deficient mice, plasma ALT and hepatic NPSH levels were examined in animals that survived for 24 h after APAP treatment. When homozygous knockout mice were treated with 300 and 600 mg/kg APAP, mean plasma ALT activity was markedly increased, while mean hepatic NPSH content showed a decreasing trend compared to the vehicle-treated mice (Figs. 1A and 1B). In contrast, the mean ALT value of wild-type mice showed only minimal increases at 300 and 600 mg/kg (Fig. 1A), except for an animal in which an extremely high ALT activity was observed (this value was eliminated from the statistical analysis). The mean hepatic NPSH of the wild-type mice was only marginally decreased after treatment with 600 mg/kg APAP. These data clearly demonstrate that, although hepatic damage was observed even in the wild-type mice when treated with higher doses of APAP, the extent of the toxicity was considerably more severe in the *nrf2* knockout mice.

To examine the hepatotoxicity induced by APAP in more detail, we performed histopathological examination of the livers from all of the animals, including those that died within 24 h. Administration of 300 mg/kg and 600 mg/kg APAP induced severe (+++) centrilobular hepatocellular necrosis in 2 and 4 of the homozygous knockout mice, respectively (Table

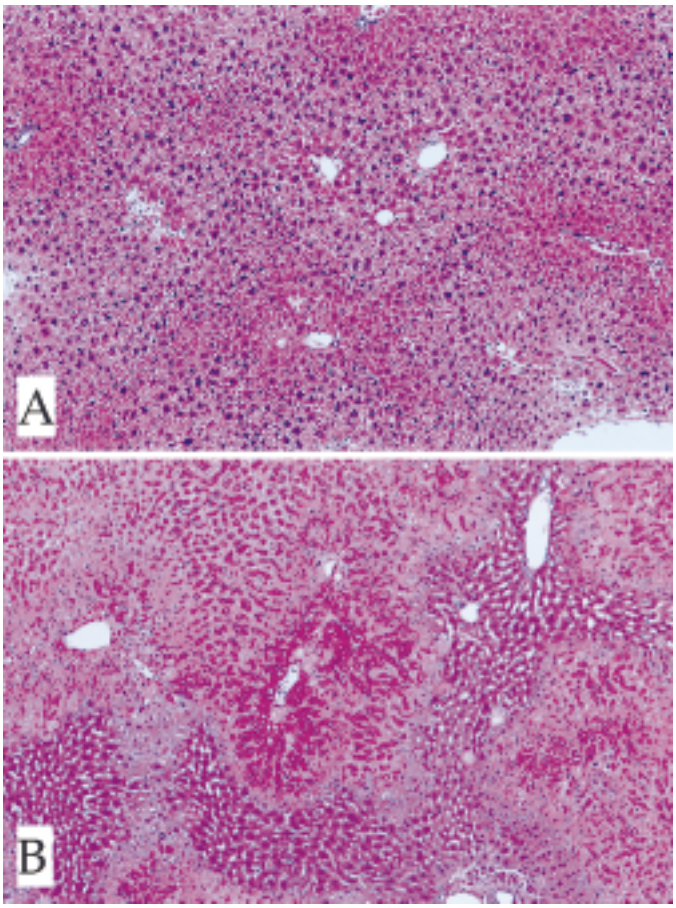

FIG. 2. Photomicrographs of livers from a wild-type mouse (A) and a homozygous *nrf2* knockout mouse (B) 24 h after exposure to 300 mg/kg APAP (po). Slight and severe centrilobular hepatocellular necrosis is present in the wild-type and knockout mouse livers, respectively. H&E. Magnification ×54.

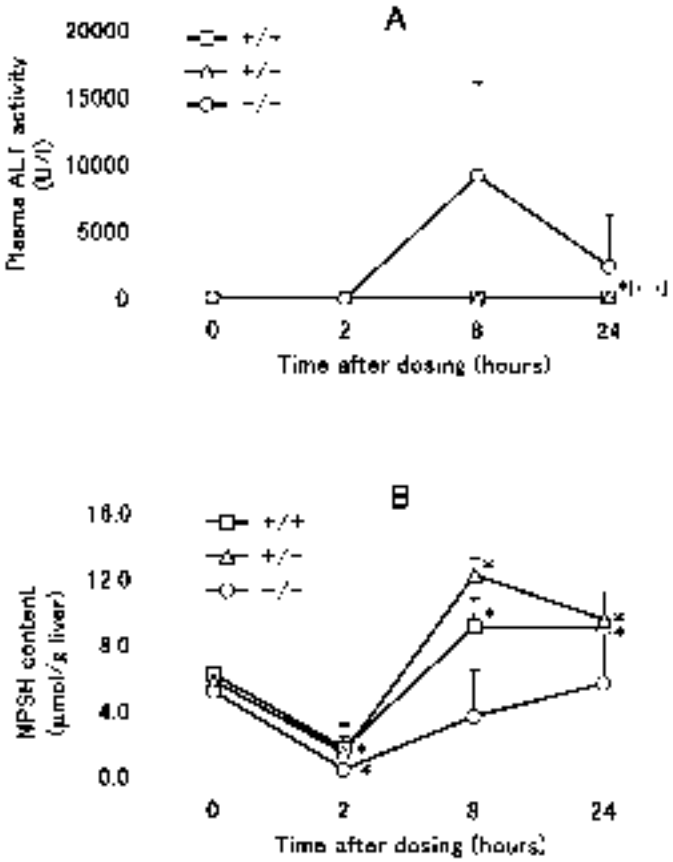

FIG. 3. ALT activity (A) and hepatic NPSH content (B) of wild-type mice (+/+), heterozygous *nrf2* mice (+/–), and homozygous *nrf2* knockout mice (–/–) at different time points after administration of 300 mg/kg APAP (po). Both ALT activity and NPSH content were determined as described in Figure 1. Values are represented as mean ± SD, where $n = 4$, except for –/– 24 h after dosing ($n = 3$). *Significantly different from the pre-dose values (0 h) of the same genotype ($p \leq 0.05$).

2, Fig. 2). The morphological features of the lesion were comparable to those seen in the wild-type mice exposed to 800 mg/kg of APAP (Ginsberg *et al*., 1982). Compared to such marked damage found in the livers of Nrf2-deficient mice, the most severe lesion that occurred in the wild-type mice was only moderate (++) in one of 4 mice treated with 600 mg/kg APAP. These data clearly show that *nrf2* knockout mice are more sensitive to APAP toxicity than wild-type animals.

Time-Course Study

Examination of the changes seen in the levels of toxicity in *nrf2* knockout mice at different time points after APAP exposure allowed further characterization of the high-sensitivity phenotype associated with mice lacking Nrf2. Based on the results of the dose-response study described above, 300 mg/kg was selected as the optimum dose to use in the time-course study. Animals were sacrificed before dosing (0 h) and at 2, 8, and 24 h after dosing. One of the homozygous knockout mice died between 8 and 24 h after receiving the 300 mg/kg APAP dose; however, this dose was not lethal to the other genotypes tested.

Plasma ALT level markedly increased in the homozygous knockout mice at 8 h after dosing, although the change was not statistically significant because of large interindividual differences among animals. On the other hand, wild-type mice showed only a marginal increase after 24 h (Fig. 3A). No significant changes in ALT levels were detected in heterozygous mice at any of the time points tested. These results indicate that hepatic damage occurs earlier in homozygous knockout mice compared to wild-type animals. However, since the only histopathological change detected in the latter was slight hepatocellular necrosis, even after 24 h (Table 2), it is possible that the hepatic injury in the wild-type mice after 8 h was too subtle to be detected by the analysis of ALT.

Hepatic NPSH levels significantly decreased in mice of all genotypes after 2 h (Fig. 3B). Specifically, NPSH content measured 2 h after APAP administration was 29, 25, and 10% of those measured before dosing (after overnight fasting) in the wild-type, heterozygous, and homozygous knockout mice, respectively. The result is consistent with previous findings which showed a decline in hepatic NPSH levels at early time points after APAP exposure (Brady *et al*., 1988). After 8 h, NPSH in the livers of the wild-type and heterozygous knockout mice recovered and even became higher than the pre-dose levels, suggesting complete recovery from the effect of APAP treatment subsequent to over-

TABLE 3
Time-Course Changes of Liver Histopathology in *nrf2* Knockout and Wild-type Mice Exposed to Acetaminophen at 300 mg/kg

		Genotype		
Time after dosing (h)	Grade	+/+	+/–	–/–
0	–	4	4	4
2	–	3	4	0
	±	1	0	4
8	–	4	4	0
	±	0	0	0
	+	0	0	1
	++	0	0	2
	+++	0	0	1
24	–	3	1	0
	±	0	1	0
	+	1	2	2
	++	0	0	0
	+++	0	0	2[a]

Note. Acetaminophen in 50% propylene glycol was administered (po) to overnight-fasted wild-type (+/+) and homo- (–/–) and heterozygous (+/–) *nrf2* knockout mice. Livers from animals killed at different time points were examined by light microscopy and graded from – to +++ for severity of lesions as described in Materials and Methods.

[a] Includes one that died between 8 and 24 h.

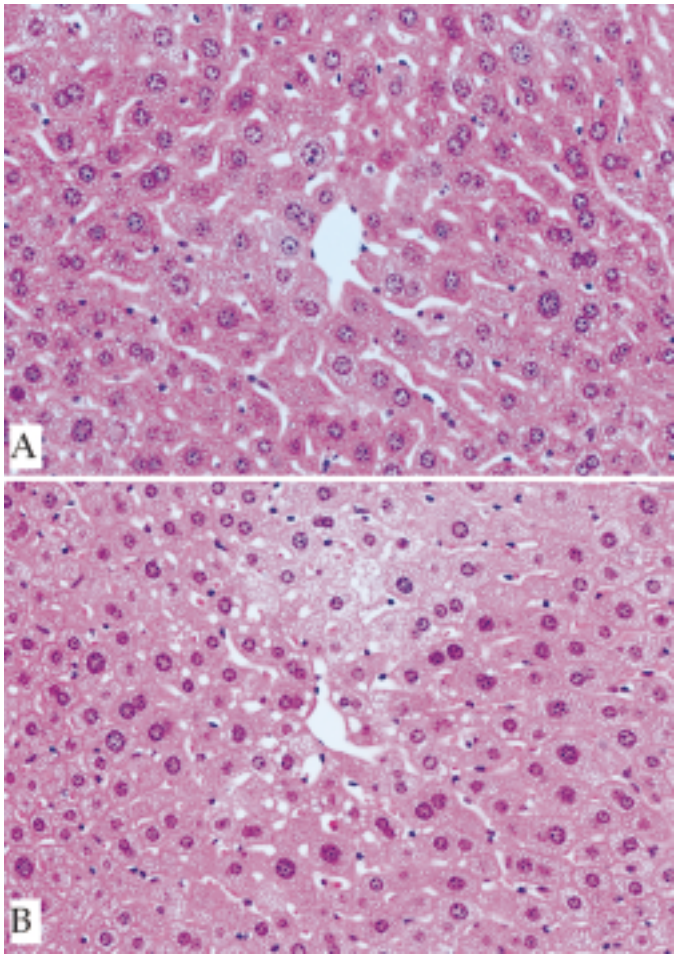

FIG. 4. Photomicrographs of livers from a wild-type mouse (A) and a homozygous *nrf2* knockout mouse (B) 2 h after exposure to 300 mg/kg APAP (po). Slight degeneration of centrilobular hepatocytes with small cytoplasmic vacuoles present in the knockout mouse liver. H&E. Magnification ×152.

night fasting, whereas values remained low in the homozygous knockout mice. Hepatic NPSH content of the latter after 24 h was still at the pre-dose level, indicating that an early decrease in NPSH in mice lacking Nrf2 is followed by slow or insufficient recovery.

Histopathological examination revealed a degeneration in centrilobular hepatocytes with small cytoplasmic vacuoles (grade: ±) in all homozygous knockout mice after 2 h, while most wild type and heterozygous knockout mice showed no abnormality at this time point (Table 3 and Fig. 4). Various degrees of centrilobular hepatocellular necrosis became apparent in all homozygous knockout mice after 8 h, but not in mice of other genotypes. Collectively, these data demonstrate that *nrf2* knockout mice exhibit similar time-course changes of toxicity as those seen in wild-type mice exposed to higher doses of APAP (Brady *et al*., 1988; Cohen, 1998; Mitchell, 1973), thus indicating a shared underlying mechanism of toxicity.

Immunohistochemical Detection of APAP in the Liver

Since the binding of an APAP metabolite to cellular proteins represents an important early event in its hepatotoxicity, the levels of APAP-bound proteins in the livers of wild-type and homozygous knockout mice were examined. Immunohistochemical staining, using an anti-APAP antibody, was performed on mice that had been sacrificed 2 h after dosing with 300 mg/kg APAP. Positive staining for APAP was detected in the centrilobular hepatocytes of homozygous knockout mice, but not in hepatocytes in other regions, nor in livers of wild-type mice (Fig. 5). These results suggest that a greater extent of APAP-protein adduct formation occurs in livers of homozygous knockout mice compared to those of wild-type mice after the same level of APAP exposure.

Baseline Activities of APAP Metabolism

The amount of the reactive APAP metabolite that can bind to cellular proteins depends on both its activation and detoxi-

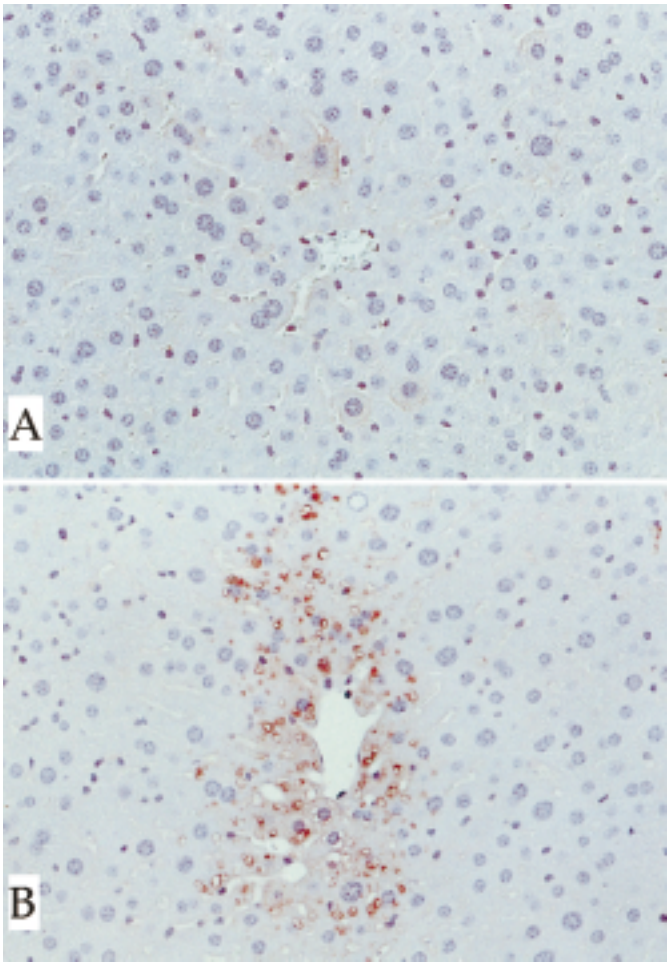

FIG. 5. Immunohistochemical staining for APAP in livers from the same groups as in Figure 4. Negative and positive staining can be seen in centrilobular hepatocytes of wild-type (A) and knockout (B) mouse livers, respectively. Magnification ×152.

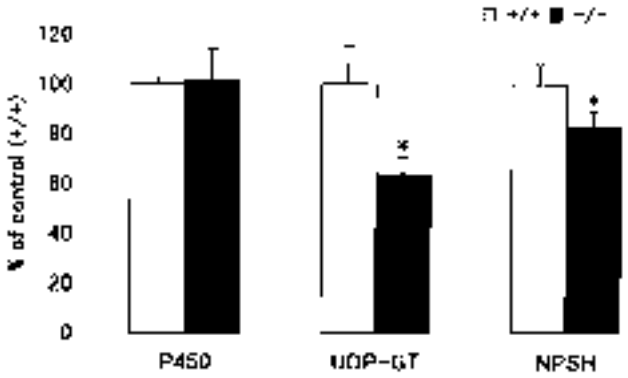

FIG. 6. Comparison of cytochrome P450 content, UDP-GT activity, and NPSH levels in livers from untreated wild-type mice (+/+) and homozygous *nrf2* knockout mice (–/–). P450s and UDP-GT per mg microsomal protein and NPSH per g liver were determined as described in Materials and Methods, and expressed as percentages of control (+/+) mean values (0.59 nmol/mg protein, 3.3 nmol/min/mg protein, and 7.1 μmol/g liver for P450, UDP-GT and NPSH, respectively). Values are represented as mean ± SD, where $n = 8$. *Significantly different from the values of +/+ mice ($p \leq 0.05$).

fication by processes of drug metabolism. To compare the metabolic activity in *nrf2* homozygous knockout mice with that in wild-type mice, cytochrome P450 content, UDP-GT activity, and NPSH level were examined. In the livers of homozygous knockout mice, hepatic UDP-GT activity and NPSH content were reduced to 64 and 82% of that in wild-type mice, respectively (Fig. 6). In contrast, the cytochrome P450 content in the livers of knockout and wild-type mice was similar. These results suggest that Nrf2 affects basal activity of some pathways of APAP metabolism.

One plausible explanation for these differences in metabolic activity is the transcriptional shut-off of drug-metabolizing enzyme and antioxidant genes. To address this question, we measured the mRNA levels of Ugt1a6 and the heavy and light chains of γGCS in the livers of the mice by Northern blot analysis. The mRNA levels of Ugt1a6 and the γGCS heavy chain in the livers of the homozygous knockout mice were lower than were those in the wild-type mice (56 and 64%, respectively: Fig. 7), with a statistical significance of $p \leq 0.05$. On the other hand, the mRNA level of the γGCS light chain was only slightly less than that found in the control mice (88%).

We also analyzed the expression of the above genes 2 h after exposure to APAP to assess whether the induction of these genes affected the sensitivity of the mice to APAP. However, no induction of these genes was detected in mice of either genotype at this time point (data not shown).

DISCUSSION

Nrf2 has been shown to be a key molecule that responds to reactive electrophiles by activating ARE-mediated gene expression. In this study, we showed that the mouse lacking Nrf2 is highly sensitive to APAP. Lower levels of APAP provoke hepatotoxicity in homozygous *nrf2* knockout mice and such high sensitivity appears to be due to an insufficient expression of detoxifying enzymes. As a consequence, APAP-conjugates accumulate in centrilobular hepatocytes, causing their necrosis. These results demonstrate that the *nrf2* gene knockout mouse serves as an important animal model system for the study of acute drug toxicity.

Covalent binding of reactive APAP metabolites to cellular proteins seems to be an important process in the induction of hepatotoxicity (Cohen *et al*., 1998). As many as 23 proteins, some of which are considered critical for cellular functions, have been shown to bind to APAP in mouse liver (Qiu *et al*., 1998). The anti-APAP antibody immunoreactivity in our study was localized in the centrilobular region of the livers of homozygous *nrf2* knockout mice and specifically overlapped with the site of hepatic damage. This observation is in agreement with the previous analysis of Bartolone *et al*. (1989), who demonstrated accumulation of APAP-bound proteins in the centrilobular area after an oral dose of 600 mg/kg APAP. The fact that the anti-APAP antibody only stained the livers of homozygous *nrf2* knockout mice, but not of the wild-type mice, shows that larger amounts of APAP metabolites have accumulated in the former.

The reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), is generated by a cytochrome P450-dependent metabolic pathway (Cohen *et al*., 1998). The relative amount of APAP bio-activated is determined by the activities of other drug metabolizing pathways, such as glucuronidation and sulfation, which detoxify and eliminate APAP. In mice, approximately 60 to 80% of the total APAP is metabolized through the glucuronidation pathway (Hazelton *et al*., 1986; Kim *et al*., 1995; Manautou *et al*., 1996), and most of NAPQI is excreted after GSH conjugation, without causing toxicity. In the homozygous *nrf2* knockout mouse, analyses of these major metabolic parameters revealed general decreases in the levels of detoxification enzyme activities, with no change being observed in that of the cytochrome P450 pathway. This would

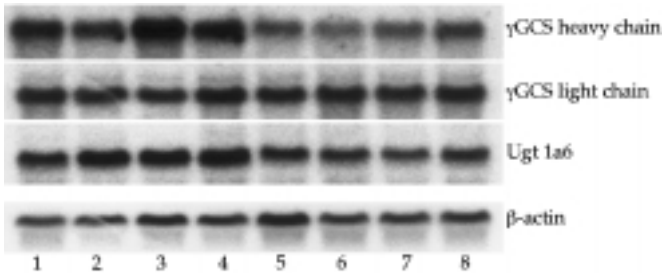

FIG. 7. Expression of γGCS (heavy and light chains) and Ugt1a6 in livers from untreated wild-type mice (+/+, lanes 1–4) and homozygous *nrf2* knockout mice (–/–, lanes 5–8). Total cellular RNA was extracted from each liver, samples (20 μg) were subjected to electrophoresis and transferred to Zeta-Probe GT membrane. The membrane was probed with [^{32}P]-labelled cDNA probes, as indicated in the Figure. β-actin cDNA was used as a control.

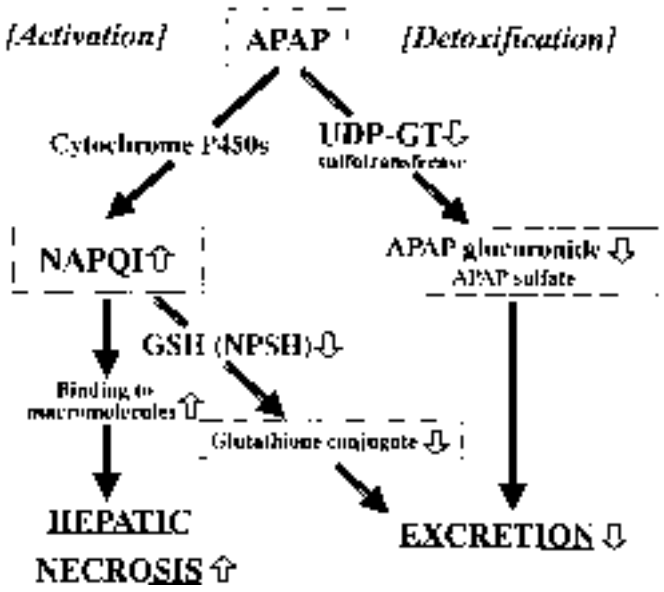

FIG. 8. The effects of knocking out *nrf2* on APAP hepatotoxicity. Nrf2 affects UDP-GT activity and GSH (NPSH) level by controlling the expression of Ugt1a6 and γGCS genes, respectively. In *nrf2* knockout mice, more APAP is biotransformed to the reactive metabolite, NAPQI, due to the low UDP-GT activity. Elimination of NAPQI by conjugation with GSH is also decreased because of the low GSH level. White arrows indicate the changes that occur with loss of Nrf2.

explain the accumulation of the larger amounts of APAP-conjugated proteins in the cell and corresponding severe hepatic damage found in the *nrf2* homozygous knockout mouse (Fig. 8). Sulfation, a minor detoxification pathway of APAP, was not examined in the present study. Whether or not the sulfotransferase gene is ARE-regulated or Nrf2 has any effect on its expression needs to be clarified in future.

A reduction in both UDP-GT activity and NPSH content in the liver of the knockout mouse accompanied decreased expression in the mRNA levels of both Ugt1a6 and the heavy chain of γGCS. Consistent with this observation, Chan and Kan (1999) had reported a decrease in Ugt1a6 mRNA expression in the lung of the homozygous *nrf2* knockout mouse. The low levels of mRNA expression of Ugt1a6 and γGCS heavy chain in untreated homozygous *nrf2* knockout mice suggest a lack of response to natural dietary inducers or to the endogenous oxidative stress that occurs as a result of normal biological activities (Klaunig *et al*., 1995; Martin *et al*., 1996). This observation indicates the importance of Nrf2 in counteracting the oxidative stress generated during normal metabolic processes, in addition to providing protection against xenobiotics.

Oxidative stress appears to be an important factor associated with APAP toxicity. Indeed, metallothionein (MT)-I/II knockout mice were recently shown to be highly sensitive to APAP, perhaps due to the lack of the antioxidant function of MT (Liu *et al*., 1999). In mice, the oxidative stress produced by reactive APAP metabolites may act as a strong inducer of Nrf2-regulated gene expression mediated through the ARE. However, induction of detoxification enzymes and antioxidant systems by xenobiotics might be impaired in the *nrf2* knockout mouse. It is interesting to note that, in the *nrf2* knockout mouse, recovery of NPSH level to normal following APAP exposure was markedly delayed. Two possibilities may explain this result. First, the normal function of hepatocytes might have been lost in the homozygous knockout mice and recovery of NPSH level needed regeneration of hepatocytes. Second, the *nrf2* knockout mice might not have been able to activate the compensatory pathway involved in recovering the NPSH level. We cannot eliminate either possibility, and further roles of Nrf2 in protecting against xenobiotics remain to be elucidated.

In contrast to the unequivocal phenotypic difference between homozygous knockout and wild-type mice, the sensitivity of heterozygous knockout animals to APAP appears to be comparable to that of wild-type mice. Based on the mortality, clinical chemistry, and histopathology data, no differences existed between the heterozygous and wild-type mice following APAP administration. Thus, a single copy of the Nrf2 gene seems to provide sufficient protection against APAP hepatotoxicity.

Our study clearly demonstrates that Nrf2 protects against APAP-induced toxicity by increasing the expression of both drug metabolizing enzymes and antioxidants. Additional studies on the Nrf2-mediated gene regulatory system would provide further insight into the integrated defense mechanisms of biological systems against xenobiotics and xenobiotic-induced oxidative stress.

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