

Fig. 3. Standard Curve for the Real-time PCR Amplification of Human NR1 Splice Variants

A plot of *C_t* values versus the logarithm of 10³, 10⁴, 10⁵, 10⁶, and 10⁷ copies of standard plasmids containing the specific cDNA sequence for each splice variant is indicated.

Table 2. Expression of C-terminal NR1 Splice Variants mRNA in Normal and Alzheimer Patients Hippocampus

	Normal	Alzheimer
NR1-1	0.47 (47%)	0.32 (32%)
NR1-2	0.07 (6%)	0.09 (9%)
NR1-3	0.30 (30%)	0.37 (37%)
NR1-4	0.17 (17%)	0.22 (22%)

All data presented as the C-terminal NR1 splice variant: GAPDH mRNA ratio. The relative amounts of the individual splice variants related to the total sum are given in parentheses as percentages.

Table 3. Expression of N-terminal NR1 Splice Variants mRNA in Normal and Alzheimer Patients Hippocampus

	Normal	Alzheimer
NR1-a	0.63 (84%)	0.31 (39%)
NR1-b	0.12 (16%)	0.49 (61%)

All data presented as the N-terminal NR1 splice variant: GAPDH mRNA ratio. The relative amounts of the individual splice variants related to the total sum are given in parentheses as percentages.

ternal control and target cDNA, or the extended time requirement.²⁵⁾ However, these disadvantages can be overlooked as real-time PCR allows for increased sensitivity and low variability.²⁶⁾ In this study, we utilized a SYBR Green I dye, a nonspecific DNA binding dye, to quantitatively assess the levels of gene expression for NR1 splice variant transcripts in human tissue samples. We demonstrated that fluorescent detection with SYBR Green I dye in real-time RT-PCR applications can be used to quantify PCR products of NR1 splice variant with high sensitivity over a wide linear range. This procedure can be easily used with a high-throughput format, such as a 96-well microtiter plate, allowing several hundred reactions to be processed on a daily basis with the nonspecific nature of the dye being the only drawback, which precludes the use of multiplex reactions. Thus, the use of SYBR Green I dye for detection offers a method for routine quantification of human NR1 splice variant.

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Original Article

Immunochemical method to detect proteins that undergo selective modification by 1,2-naphthoquinone derived from naphthalene through metabolic activation

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ABSTRACT — Naphthalene undergoes biotransformation by a variety of enzymes to yield 1,2-naphthoquinone (1,2-NQ), a reactive metabolite that binds covalently to proteins. Because this covalent modification is thought to account for naphthalene toxicity, a procedure to detect 1,2-NQ bound to macromolecules is required. In this study, we prepared a polyclonal antibody against 1,2-NQ and examined the specificities of the antibody for various aromatic structures and for the regiochemistry of the quinone functionality. Western blot analysis revealed that the antibody prepared against 1,2-NQ recognized the naphthalene moiety with the *ortho*-dicarbonyl group, but not with the *para*-dicarbonyl group; in addition, little cross-reactivity of *ortho*-quinones with different numbers of aromatic rings ($n = 1, 3, 4, 5, 6$) was seen. Dot blot and Western blot analyses with the polyclonal antibody enabled quantitative determination of the formation of protein-bound 1,2-NQ during the metabolic activation of naphthalene. The present method can be expected to be applicable for the identification of the molecular targets of 1,2-NQ derived from naphthalene in cells and tissues.

Key words: Naphthalene, Metabolic activation, Covalent modification, Immunochemical detection

INTRODUCTION

Naphthalene is an important industrial compound and is also ubiquitous in the environment (Fraser *et al.*, 1998; Preuss *et al.*, 2003; Lu and Zhu, 2007). The most important pathways by which the general public is exposed to naphthalene are emissions from fuel combustion (Fraser *et al.*, 1998), cigarette smoke (Ding *et al.*, 2005), and insect repellents (Daisy *et al.*, 2002). One cigarette contains 0.3-4.0 μg of naphthalene in the mainstream smoke and 7.8-46.0 μg in the sidestream smoke. The average daily naphthalene intake from air was calculated to be 1.127 $\mu\text{g}/\text{kg}$ per day for a 70-kg adult, and the corresponding value for a 10-kg child is 4.5 $\mu\text{g}/\text{kg}$ per day (Preuss *et al.*, 2003). Naphthalene levels in food are generally low unless the food has been smoked or grilled. Estimates for daily dietary intake of naphthalene range from 0.0407 to 0.237 $\mu\text{g}/\text{kg}$ per day for a 70-kg adult and from 0.204 to 0.940 $\mu\text{g}/\text{kg}$ per day for a 10-kg child (US Environmental Protection Agency, 2002). Naphthalene is categorized as a Group 2B carcinogen by the International

Agency for Research on Cancer.

Several lines of evidence suggest that naphthalene toxicity *in vivo* and *in vitro* is attributable to its metabolic activation to form highly reactive metabolites (Warren *et al.*, 1982; Buckpitt and Warren, 1983; O'Brien *et al.*, 1985; Troester *et al.*, 2002; Kokel *et al.*, 2006; Fukami *et al.*, 2008). For example, the metabolic activation of naphthalene to produce 1,2-naphthoquinone (1,2-NQ) is shown in Fig. 1. Naphthalene is initially metabolized by cytochrome P-450 isozymes, yielding naphthalene-1,2-oxide (Wilson *et al.*, 1996; Cho *et al.*, 2006; Fukami *et al.*, 2008). Naphthalene-1,2-oxide can undergo hydrolysis by epoxide hydrolase (van Bladeren *et al.*, 1984), resulting in 1,2-dihydroxy-1,2-dihydronaphthalene (naphthalene-1,2-dihydrodiol), which is then converted to 1,2-dihydroxynaphthalene by aldo-keto reductase isozymes (Penning *et al.*, 1999; Palackal *et al.*, 2002). This catechol metabolite undergoes auto-oxidation by superoxide generated from NADPH-dependent microsomal electron transfer to produce 1,2-NQ by a process described by Hiramatsu *et al.* (1990).

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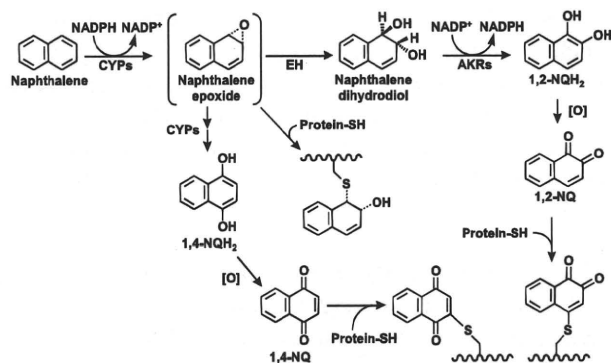


Fig. 1. Metabolism of naphthalene. NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NADP⁺, nicotinamide adenine dinucleotide phosphate, oxidized form; CYPs, cytochrome P450 isozymes; EH, epoxide hydrolase; AKRs, aldo-keto reductase isozymes; 1,2-NQH₂, 1,2-dihydroxynaphthalene; 1,2-NQ, 1,2-naphthoquinone.

1,2-NQ has been proposed to be a toxic metabolite of naphthalene because of its ability to covalently bind to nucleophilic amino acid residues such as cysteine and histidine in cellular proteins (Smithgall *et al.*, 1988; Zheng and Hammock, 1996; Zheng *et al.*, 1997; Penning *et al.*, 1999; Munday *et al.*, 2007; Iwamoto *et al.*, 2007), as shown in Fig. 1. For example, our previous findings suggest that formation of covalent bonds between 1,2-NQ and nucleophilic centers on protein tyrosine phosphatase 1B and on cAMP response element-binding protein (CREB) results in persistent activation of epidermal growth factor receptor (Iwamoto *et al.*, 2007), which leads to tracheal contraction in guinea pigs (Kikuno *et al.*, 2006) and disruption of CREB-mediated transcriptional activity (Endo *et al.*, 2007), respectively.

Western blot analysis with a polyclonal antibody that recognizes 1,2-NQ is a convenient procedure for detecting this reactive species bound to macromolecules without the need for radioisotopes. In previous work, we prepared an antibody that detects 1,2-NQ-protein conjugates (Kikuno *et al.*, 2006; Endo *et al.*, 2007; Iwamoto *et al.*, 2007), but relatively little information was obtained on the antibody's structure specificity. In this study, we addressed this issue by performing immunoblot analyses with a variety of aromatic hydrocarbons. Then we monitored protein-bound 1,2-NQ derived from naphthalene during (1) incubation of naphthalene in a 9,000 × g supernatant of mouse liver in the presence of NADP⁺ and NADPH (S9 mix) and (2) exposure of primary mouse hepatocytes to naphthalene.

MATERIALS AND METHODS

Materials

Chemicals were obtained as follows: 1,2-NQ, 1,4-NQ, 2-methylnaphthalene, acenaphthoquinone, dibenz[*a,c*]anthracene, dibenz[*a,h*]anthracene, pentacene, and 1-naphthalene sulfonic acid sodium salt from Tokyo Chemical Industry Co. (Tokyo, Japan); 1-methylnaphthalene, 1-ethylnaphthalene, 1,2-NQ-4-sulfonic acid sodium salt, dimethyl sulfoxide, 2-anilino-1,4-NQ, 2-chloro-3-pyrrolidino-1,4-NQ, 1,2-dihydroxynaphthalene, 5,8-dihydroxy-1,4-NQ, 5-hydroxy-1,4-NQ, benz[*a*]anthracene, benz[*b*]anthracene, benzo[*e*]pyrene, naphtha[2,3-*a*]pyrene, beta-estradiol, and protease inhibitor cocktail from Sigma-Aldrich Co. (St. Louis, MO, USA); benzene, naphthalene, 1-naphthol, 9,10-phenanthraquinone, benzo[*a*]pyrene, 3-methylcholanthrene, 2-propanol, sodium azide, glycine, and bovine serum albumin from Nacalai Tesque, Inc. (Kyoto, Japan); 1,4-NQ-2-sulfonic acid potassium salt from Eastman Kodak Co. (Rochester, NY, USA); 1,4-phenanthraquinone, 1,4-chrysenequinone, benz[*a*]anthracene-7,12-dione, 1,4-benz[*c*]phenanthraquinone, 5,6-benz[*c*]phenanthraquinone, benzo[*a*]pyrene[6,12]quinone, benzo[*a*]pyrene[7,8]quinone, benzo[*a*]pyrene[7,10]quinone, and benzo[*a*]pyrene[11,12]quinone from Chiron AS (Trondheim, Norway); chrysene, phenanthrene, pyrene, and anthracene from GL Sciences, Inc. (Tokyo, Japan); nicotinamide adenine dinucleotide phosphate, reduced form, from Oriental Yeast Co. (Tokyo, Japan); oxidized nicotinamide adenine dinucleotide phosphate from Kohjin Co. (Tokyo, Japan); 1,4-anthraquinone, 9,10-anthraquinone, catechol, perylene, sucrose, nonfat dry milk, and other chemical reagents from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were of the highest purity available.

Preparation of anti-1,2-NQ

1,2-NQ bound to keyhole limpet hemocyanin (1,2-NQ-KLH) and the polyclonal antibody to 1,2-NQ were prepared as reported previously (Iwamoto *et al.*, 2007). The immunoglobulin G (IgG) fraction of anti-1,2-NQ was purified by protein A-Sepharose CL-4B column chromatography.

Preparation of sorbitol dehydrogenase-1,2-NQ adduct

Rat sorbitol dehydrogenase (SDH) was expressed as His-tagged fusion protein in *Escherichia coli* BL21 cells and purified on Ni-NTA agarose (Qiagen, Valencia, CA, USA). The SDH was allowed to react with 1 mM dithiothreitol for 30 min at 4°C. The reduced proteins (3 ml)

Immunochemical detection of 1,2-naphthoquinone

were loaded on a Econo-Pac 10DG column (Bio-Rad Laboratories, Richmond, CA, USA), and the column was eluted with 3 ml of 50 mM potassium phosphate buffer -0.1 mM EDTA (pH 7.0). To the protein solution containing 0.6 mg of reduced SDH, 1,2-NQ (0.06 mg) dissolved in 0.1 ml of dimethyl sulfoxide was added under nitrogen. After stirring for 20 min, the mixture was loaded on an Econo-Pac 10DG column, and the column was eluted with 3 ml of 50 mM potassium phosphate buffer-0.1 mM EDTA (pH 7.0). The 1,2-NQ bound to SDH (1,2-NQ-SDH) was stored at -80°C before use.

Titration test of antiserum against 1,2-NQ

Ninety-six-well Maxi Sorp plates (Nunc, Roskilde, Denmark) were coated with 50 µl/well of coating buffer containing 1 µg/ml of the appropriate antigen in 0.05 M carbonate buffer (pH 9.6) and incubated in a moist environment for 30 min at room temperature. Coated plates were washed with phosphate-buffered saline containing 0.05% Tween 20 (TPBS). Phosphate-buffered saline containing 1% bovine serum albumin was added to the wells at 0.1 ml/well. After 30 min incubation at room temperature, the plates were washed with TPBS three times. The wells were probed with 50 µl/well of the appropriate antiserum which was diluted with TPBS for 30 min. After plates were washed with TPBS three times, 50 µl/well of alkaline phosphatase conjugated goat anti rabbit IgG (Dako corp., Glostrup, Denmark) at a dilution of 1:5,000 was added to each well and incubated for 30 min at room temperature. After further washing with TPBS three times, 0.1 ml/well of *p*-nitrophenyl phosphate (PNPP) buffer (pH 9.8) containing 1 mg/ml of PNPP, 0.92 M diethanolamine, and 0.5 mM magnesium chloride was added and incubated at room temperature for 30 min and read in a plate reader (Titertek Multiskan; Flow Laboratories Inc., McLean, VA, USA) at 405 and 630 nm.

Enzyme preparation

ICR male mice (6 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were anesthetized by intraperitoneal injection of 50 mg kg⁻¹ pentobarbital (Nembutal injection; Abbott Laboratories, Abbott Park, IL, USA). After the liver was perfused with cold saline, it was homogenized with 4 volumes of buffer containing 0.25 M sucrose, 0.1 mM EDTA, 5 mM Tris-HCl (pH 7.4), and 1% protease inhibitor cocktail. The homogenate was centrifuged at 600 × *g* for 10 min at 4°C, and the supernatant was further centrifuged at 9,000 × *g* for 10 min at 4°C. The resulting supernatant, referred to hereafter as "the enzyme preparation," was adjusted to a volume of 3 ml with 50 mM potassium phosphate

buffer (pH 7.4). Then nonprotein sulfhydryl compounds such as glutathione were removed from the supernatant by means of Econo-Pac 10DG columns. Protein content was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The enzyme preparation was stored in 50 mM potassium phosphate buffer (pH 7.4) at -80°C before use.

Metabolism of polycyclic aromatic hydrocarbons

The reaction mixture (250 µl) for metabolism of polycyclic aromatic hydrocarbons contained 50 mM potassium phosphate buffer (pH 7.4), 100 µM polycyclic aromatic hydrocarbons, 1 mM NADP⁺, 1 mM NADPH, and 9,000 × *g* supernatant of mouse liver (8 mg/ml). Reactions were performed at 37°C for 1 hr and terminated by the addition of SDS-PAGE loading buffer as described below.

Immunoblot analysis

Samples were normalized to a protein content of 20 µg and then mixed with half the volume of SDS-PAGE loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 24% glycerol, 6% SDS, 15 mM 2-mercaptoethanol, and 0.015% bromophenol blue. The mixtures were heated at 95°C for 5 min and then applied to SDS-polyacrylamide gels (12% acryl amide) and subjected to electrophoresis and Western blot analysis according to the method of Kyhse-Anderson (1984).

For Dot blot analysis, samples were adjusted to a protein content of 0.1 µg/µl with water. A drop of sample (2 µl) was placed on a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and allowed to dry for 30 min. The membrane was blocked with the blocking solution at 4°C for 1 hr and probed with anti-1,2-NQ (0.5 µg/ml) for 1 hr. Antibody on the membrane was detected with secondary anti-rabbit IgG antibody coupled to horseradish peroxidase (Cell Signaling Technology, Inc., Beverly, MA, USA). Finally, the proteins were visualized by means of an enhanced chemiluminescence system (Chemi-Lumi One; Nacalai Tesque, Inc., Kyoto, Japan) and recorded on X-ray films (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Preparation of primary mouse hepatocytes

C57BL/6J mice (6 weeks old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). Primary mouse hepatocytes were isolated as described previously (Shinkai *et al.*, 2009). Hepatocytes were cultured at 37°C in a humidified atmosphere of 5% CO₂ in William's medium E containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Two days after isolation,

hepatocytes were cultured for 24 hr in William's medium E without fetal bovine serum. Then the hepatocytes were exposed to 250-750 μ M naphthalene for 1 hr. All animal experimental and surgical procedures were strict-

ly performed as per the Institutional Animal Care and Use Committee guidelines.

RESULTS AND DISCUSSION

Previous studies (Smithgall *et al.*, 1988; Zheng and Hammock, 1996; Iwamoto *et al.*, 2007) have shown that cysteine residues of cellular proteins undergo modification by 1,2-NQ. Immunoblotting has been used to identify the resulting protein adducts by detection of the hapten moiety of protein adducts formed by exposure of cellular proteins to electrophiles. To identify proteins modified by the 1,2-NQ metabolite of naphthalene, we took advantage of immunoblotting by using a polyclonal antibody that recognized the 1,2-NQ moiety of proteins modified with 1,2-NQ. First, we prepared a 1,2-NQ-KLH adduct, and then we immunized rabbits with the adduct and combined the resulting antisera. Using a checkerboard titration, we tested the combined sera against the 1,2-NQ-KLH adduct or a 1,2-NQ-SDH adduct after every bleeding until no increase in the titer was observed. The antisera raised

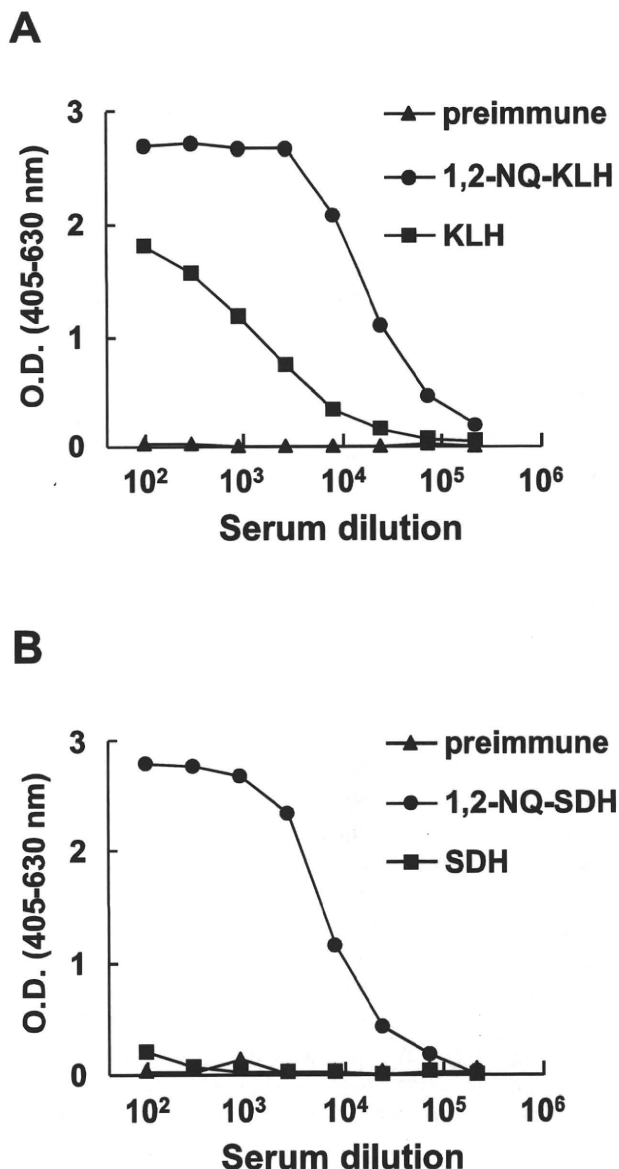


Fig. 2. Titration curves of rabbit antiserum raised against 1,2-NQ. The titer of the serum obtained from rabbits was determined by measuring the binding of serial dilution of antiserum (1/100 to 1/243,000) to plates coated with 1,2-NQ-modified protein (●), or native protein (■). 1,2-NQ-modified proteins were KLH (A) or SDH (B). For a negative control, rabbit preimmune IgG (▲) was substituted for the primary antibody.

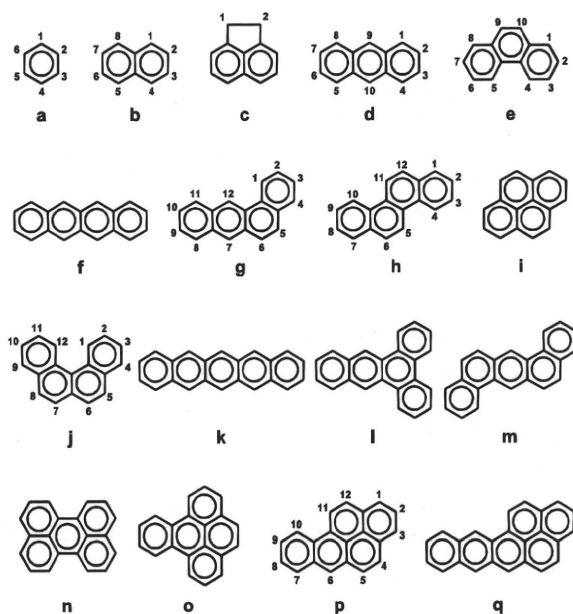


Fig. 3. Structures of aromatic hydrocarbons. (a) benzene, (b) naphthalene, (c) acenaphthalene, (d) anthracene, (e) phenanthrene, (f) benz[b]anthracene, (g) benz[a]anthracene, (h) chrysene, (i) pyrene, (j) benz[c]phenanthrene, (k) pentacene, (l) dibenzo[a,c]anthracene, (m) dibenz[a,h]anthracene, (n) perylene, (o) benzo[e]pyrene, (p) benzo[a]pyrene, (q) naphtho[2,3-a]pyrene.

Immunochemical detection of 1,2-naphthoquinone

Table 1. Cross-reactivity of the polyclonal antibody against 1,2-NQ toward aromatic hydrocarbons

Compound	Structure	Substituent	Reactivity to anti-1,2-NQ	
			without metabolism	with metabolism
Benzene	a	None	No	No
Catechol	a	1, 2 : -OH	No	No
Naphthalene	b	None	No	Yes
1-Naphthol	b	1 : -OH	No	No
1-Naphthalene sulfonic acid sodium salt	b	1 : -SO ₃ Na	No	No
1-Methylnaphthalene	b	1 : -CH ₃	No	No
1-Ethyl-naphthalene	b	1 : -C ₂ H ₅	No	No
2-Methylnaphthalene	b	2 : -CH ₃	No	No
1,2-Dihydroxynaphthalene	b	1, 2 : -OH	No	Yes
1,2-Naphthoquinone	b	1, 2 : =O	Yes	Yes
Acenaphthoquinone	c	1, 2 : =O	No	No
Anthracene	d	None	No	No
1,4-Anthraquinone	d	1, 4 : =O	No	No
9,10-Anthraquinone	d	9, 10 : =O	No	No
Phenanthrene	e	None	No	No
1,4-Phenanthraquinone	e	1, 4 : =O	No	No
9,10-Phenanthraquinone	e	9, 10 : =O	No	No
Benz[b]anthracene	f	None	No	No
Benz[a]anthracene	g	None	No	No
Benz[a]anthracene-7,12-dione	g	7, 12 : =O	No	No
Chrysene	h	None	No	No
1,4-Chrysenequinone	h	1, 4 : =O	No	No
Pyrene	i	None	No	No
1,4-Benz[c]phenanthraquinone	j	1, 4 : =O	No	No
5,6-Benz[c]phenanthraquinone	j	5, 6 : =O	No	No
Pentacene	k	None	No	No
Dibenz[a,c]anthracene	l	None	No	No
Dibenz[a,h]anthracene	m	None	No	No
Perylene	n	None	No	No
Benzo[e]pyrene	o	None	No	No
Benzo[a]pyrene	p	None	No	No
Benzo[a]pyrene[6,12]quinone	p	6, 12 : =O	No	No
Benzo[a]pyrene[7,8]quinone	p	7, 8 : =O	No	No
Benzo[a]pyrene[7,10]quinone	p	7, 10 : =O	No	No
Benzo[a]pyrene[11,12]quinone	p	11, 12 : =O	No	No
Naphtho[2,3-a]pyrene	q	None	No	No

The reaction mixture (250 μ l) containing 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADP⁺, 1 mM NADPH, 100 μ M aromatic hydrocarbons, and enzyme preparation (8 mg/ml) was incubated for 1 hr at 37°C (with metabolism). When the incubation was carried out without metabolism, no NADP⁺ or NADPH was present. The structures of the aromatic hydrocarbons are shown in Fig. 3, and the substituents are shown here.

against 1,2-NQ-KLH showed a higher titer than KLH (Fig. 2A), which suggests that the antisera had low affinity for KLH. Zheng and Hammock (1996) previously reported the preparation of a polyclonal antibody against 1,2-NQ, but the titer of the present antibody against 1,2-NQ was markedly higher than that of the antibody pre-

pared by Zheng and Hammock. In addition, the antisera raised a titer against 1,2-NQ-SDH, but not against SDH. These results suggest that the antisera had high affinity toward the hapten moiety (Fig. 2B).

Table 1 shows the cross-reactivity of the polyclonal antibody against 1,2-NQ toward aromatic hydrocarbons

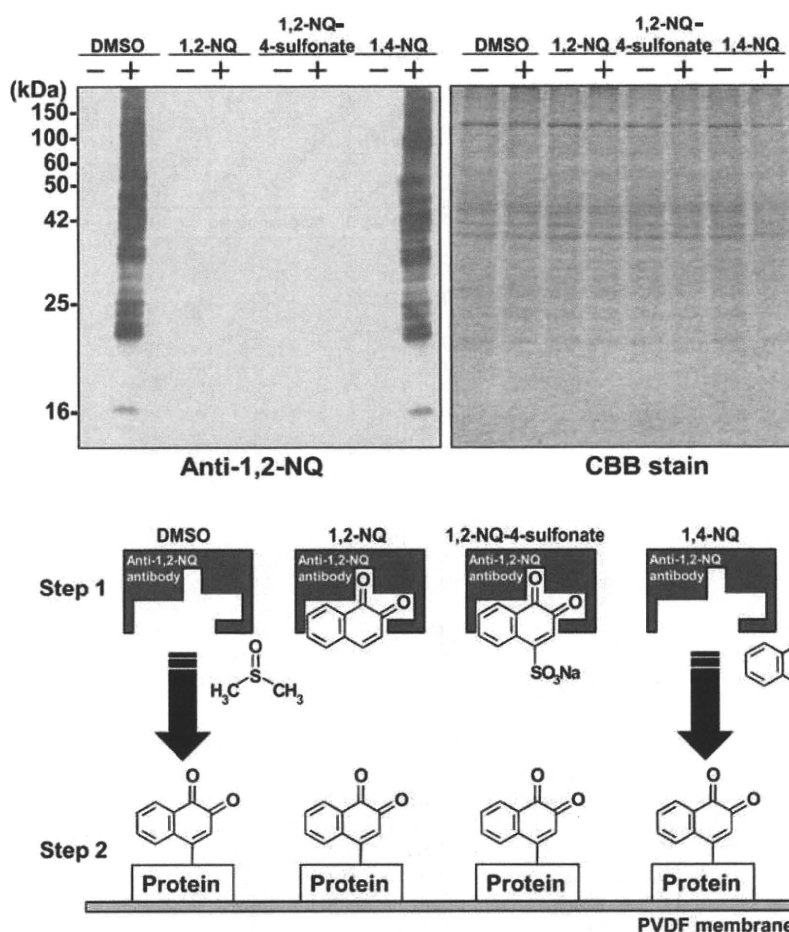


Fig. 4. Regiochemical recognition of NQ by the antibody against 1,2-NQ. Anti-1,2-NQ (50 $\mu\text{g/ml}$) was pre-incubated with dimethyl sulfoxide (DMSO), 1,2-NQ, 1,2-NQ-4-sulfonate, or 1,4-NQ (each 500 μM) for 1 hr at 37°C. Then tris-buffered saline Tween-20 containing 0.1% bovine serum albumin was mixed with the incubation mixture to bring the final concentration to 0.5 μg of anti-1,2-NQ/ml. For detection of proteins that cross-reacted with the antibody against 1,2-NQ, the 9,000 \times g supernatant of mouse liver (8 mg/ml) was allowed to react in the absence (-) or presence (+) of 100 μM 1,2-NQ for 1 hr at 37°C. These samples were subjected to SDS-PAGE and then transferred to a poly(vinylidene fluoride) (PVDF) membrane, which was analyzed with the pre-incubated anti-1,2-NQ antibody. Total amount of protein loaded per lane was 10 μg for immunoblot analysis and CBB stain.

(Fig. 3) and their quinones ($n = 1-6$, where n is the number of rings) on Western blotting. Among the 37 chemicals examined, 1,2-NQ was the only chemical active in the absence of metabolic activation. There was no cross-reactivity of *ortho*-quinones with different numbers of benzene rings, such as 9,10-phenanthraquinone ($n = 3$), 5,6-benz[*c*]phenanthraquinone ($n = 4$), benzo[*a*]pyrene-11,12-quinone, and benzo[*a*]pyrene-7,8-quinone ($n = 4$), toward anti-1,2-NQ. After metabolic activation by the S9 mix, naphthalene and 1,2-dihydroxynaphthalene exhibited immunopositive reactivity. These results indicate that the antibody specifically recognized the naphthalene moiety

of NQs produced from naphthalene. Because both 1,2-NQ and 1,4-NQ are reportedly formed during metabolism of naphthalene by hepatic enzyme preparation (Preuss *et al.*, 2003; Fukami *et al.*, 2008), we examined the regiochemical selectivity of the antibody for NQ. When anti-1,2-NQ was incubated in the absence and presence of 1,2-NQ and related compounds, pretreatment with 1,2-NQ and 1,2-NQ-4-sulfonate in the anti-1,2-NQ solution completely blocked detection of the hepatic proteins modified by 1,2-NQ, whereas pretreatment with 1,4-NQ had no inhibitory action (Fig. 4). Similar results to 1,4-NQ were also observed with 1,4-NQ-2-sulfonate, 5-hydroxy-1,4-NQ,

Immunochemical detection of 1,2-naphthoquinone

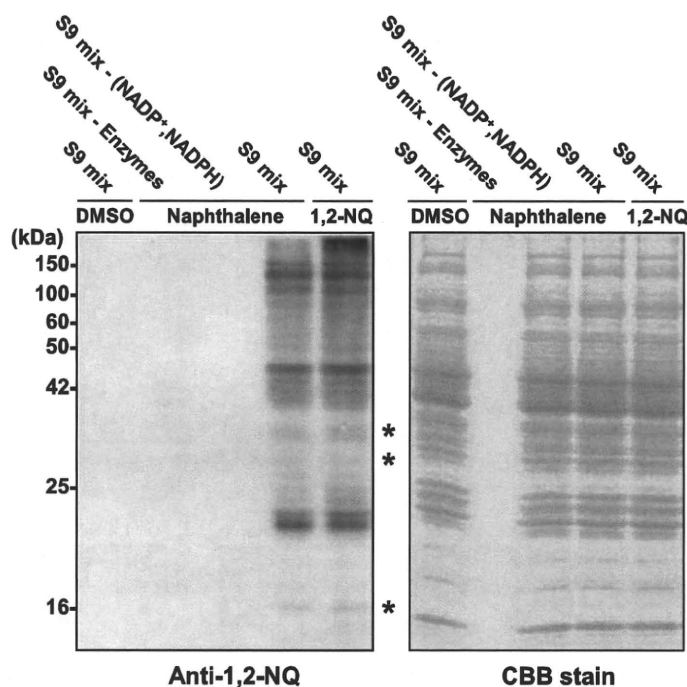


Fig. 5. Immunochemical detection of the hepatic protein of mouse modified by 1,2-NQ during metabolism of naphthalene by mouse hepatic enzyme preparation in the presence of pyridine nucleotides. Naphthalene or 1,2-NQ (100 μ M) was incubated with the reaction mixture for 1 hr at 37°C. Each reaction mixture was described as follows: S9 mix consisted of 9,000 \times g supernatant of mouse liver (8 mg/ml), 1 mM NADP⁺, 1 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4); S9 mix - Enzymes consisted of 1 mM NADP⁺, 1 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4); S9 - (NADP⁺,NADPH) consisted of 9,000 \times g supernatant of mouse liver (8 mg/ml) and 50 mM potassium phosphate buffer (pH 7.4). As a control experiment, DMSO was incubated under the same conditions. Each lane was loaded with 10 μ g of protein sample for immunoblot analysis and 20 μ g of protein sample for CBB stain. Asterisks indicate dominant proteins (14, 28, and 32 kDa) modified by 1,2-NQ during exposure of primary mouse hepatocytes to naphthalene (see Fig. 7).

5,8-dihydroxy-1,4-NQ, 2-anilino-1,4-NQ, and 2-chloro-3-pyrrolidino-1,4-NQ (data not shown). Taken together, these results suggest that the antibody recognized the naphthalene ring with the dicarbonyl group at the *ortho*-position, but not at the *para*-position. Although an epoxide intermediate derived from naphthalene binds to protein (Zheng *et al.*, 1997; Troester *et al.*, 2002) (see Fig. 1), it seems likely that the antibody against 1,2-NQ prepared in our laboratory did not cross-react with the reactive species, because of the absence of a 1,2-dicarbonyl group.

As shown in Fig. 5, the modified proteins detected by the antibody against 1,2-NQ during metabolism of naphthalene by S9 mix were almost the same as the proteins detected after incubation with 1,2-NQ. However, upon omission of either the enzyme preparation of mouse liver or the pyridine nucleotides (NADP⁺ and NADPH) from the reaction mixture, the anti-1,2-NQ reactive bands disappeared entirely, which supports the notion that protein

arylation was due to covalent binding of 1,2-NQ derived from naphthalene. To determine the rate of metabolism of naphthalene to 1,2-NQ *in vitro*, we carried out quantitative evaluation by means of Dot blot and Western blot analyses (Fig. 6A). The response for calibration curves of 1,2-NQ bound to mouse hepatic proteins between the blot area and the concentration of 1,2-NQ used was linear over the range 20 to 100 μ M ($r = 0.99$; Figs. 6B and C). From the results of these experiments, we estimated that approximately 40% of naphthalene was metabolized to 1,2-NQ, which, in turn, was rapidly bound to the tissue proteins in the enzyme preparation. Furthermore, we explored metabolic activation of naphthalene to 1,2-NQ in cells. When primary mouse hepatocytes were exposed to naphthalene, cellular proteins modified by 1,2-NQ were detected in a concentration-dependent manner (Fig. 7). Among cellular proteins modified by 1,2-NQ, 14-, 28-, and 32-kDa protein were identified as major targets for 1,2-NQ. These modified protein were also detected dur-

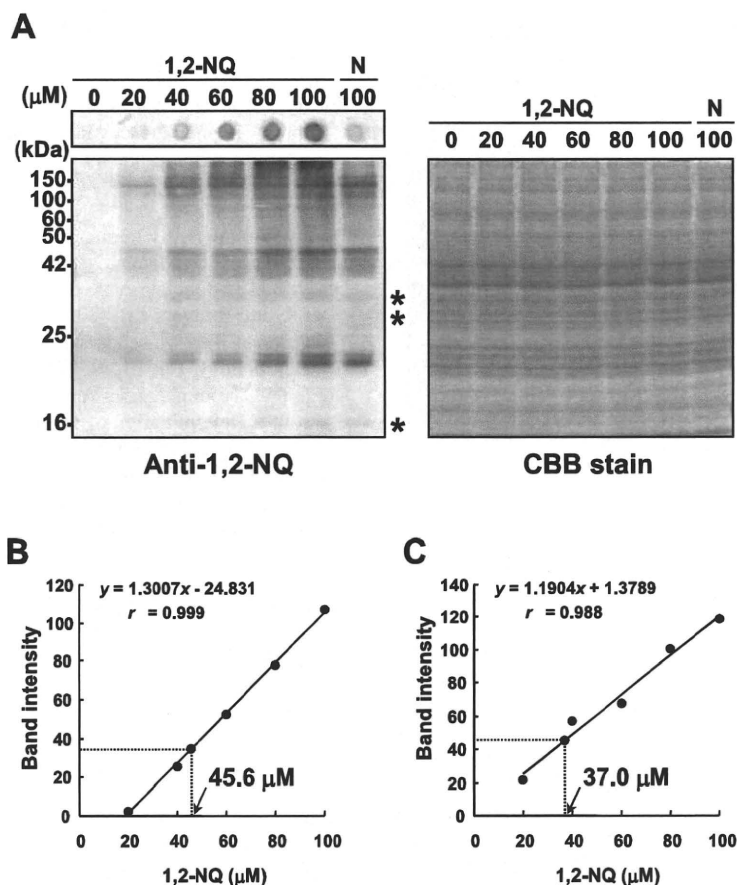


Fig. 6. Immunochemical quantitation of 1,2-NQ bound to $9,000 \times g$ supernatant protein from mouse liver. **A**, Dot blot analysis (upper) and Western blot analysis (lower). **B**, Calibration curves of 1,2-NQ bound to protein as determined by Dot blot analysis. Two μl of sample spot (0.2 μg of protein) was applied to a nitrocellulose membrane. **C**, Calibration curves of 1,2-NQ bound to protein as determined by Western blot analysis. For metabolic activation, naphthalene (N, 100 μM) was incubated with S9 mix (8 mg/ml) for 1 hr at 37°C in 50 mM potassium phosphate buffer (pH 7.4). 1,2-NQ (20–100 μM) was mixed with each protein preparation, and then immunoblot analyses were carried out with antibody against 1,2-NQ as described in Materials and Methods. Total amount of protein loaded per lane was 10 μg for immunoblot analysis and 20 μg for CBB stain. Asterisks indicate dominant proteins (14, 28, and 32 kDa) modified by 1,2-NQ during exposure of primary mouse hepatocytes to naphthalene (see Fig. 7).

ing incubation of hepatic enzyme preparation with 1,2-NQ (see Figs. 5 and 6).

In the present study, we developed an immunochemical procedure for specific determination of 1,2-NQ (as its protein-bound form) formed from naphthalene through metabolic activation by xenobiotic enzymes (Fig. 1). Although 1,2-NQ and 1,4-NQ are known to be highly reactive for the formation of protein adducts, the results of this study suggest that formation of 1,2-NQ is a major pathway for metabolism of naphthalene in mouse liver and that 1,2-NQ binds irreversibly to a huge number of tissue proteins. There are papers by Slaughter *et al.* (1993) that indicate 1,2-quinones are the most important

quinone metabolites of aromatic compounds because formation of 1,4-quinones requires an additional epoxidation of naphthol or another precursor. In addition, they showed that quinones are responsible for most of the protein conjugates, correcting an earlier proposal that Brodie made (Brodie *et al.*, 1971). The quantitative data for 1,2-NQ bound to proteins obtained by Dot blot analysis were similar to the data obtained by Western blot analysis (Figs. 6B and C). Thus, dot blot analysis would be suitable for high-throughput detection of 1,2-NQ derived from naphthalene. Because the antibody against 1,2-NQ prepared in our laboratory selectively recognized the NQ moiety with the *ortho*-dicarbonyl group but not with the *para*-dicarbo-

Immunochemical detection of 1,2-naphthoquinone

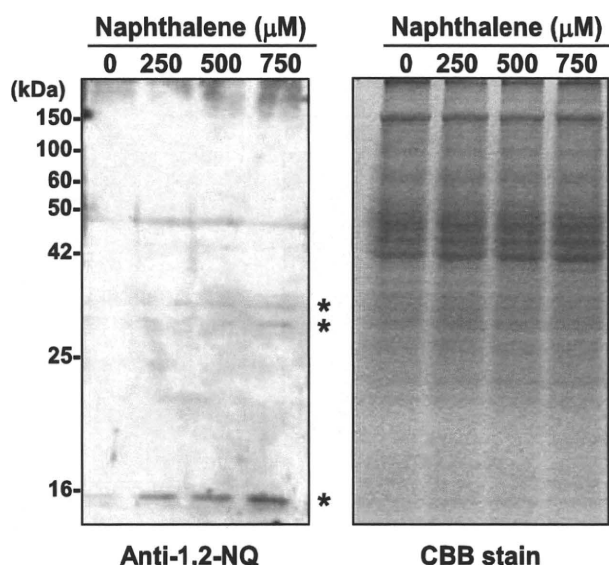


Fig. 7. Cellular proteins modified by 1,2-NQ during exposure of primary mouse hepatocytes to naphthalene. Mouse primary hepatocytes were exposed to 250-750 μM naphthalene (The final concentration of DMSO was 5% in cell culture media) at 37°C for 1 hr. Exposed cells were lysed with 2% SDS solution, and the whole cell lysates (10 $\mu\text{g}/\text{lane}$) were loaded onto the SDS-polyacrylamide gel. Western blot analysis with anti-1,2-NQ antibody was performed. The asterisks indicate the dominant proteins (14, 28, and 32 kDa) modified by 1,2-NQ.

nyl group, the antibody could be used to identify proteins selectively modified by 1,2-NQ following exposure of cultured cells and experimental animals to naphthalene by proteomics analysis without the necessity for radiolabeled compounds as reported by others (Warren *et al.*, 1982).

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Initial Response and Cellular Protection through the Keap1/Nrf2 System during the Exposure of Primary Mouse Hepatocytes to 1,2-Naphthoquinone

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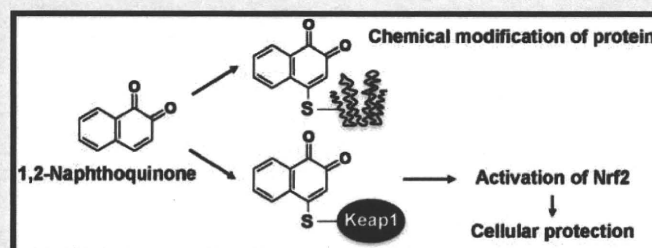
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ABSTRACT: Quinones are reactive chemical species that cause cellular damage by modifying protein thiols and/or catalyzing the reduction of oxygen to reactive oxygen species, thereby promoting oxidative stress. Transcription factor Nrf2 plays a crucial role in cellular defense against electrophilic modification and oxidative stress. In studies using 1,2-naphthoquinone (1,2-NQ) as a model quinone, we found that Keap1, the negative regulator of Nrf2, was readily arylated at its reactive thiols by 1,2-NQ. Exposure of primary mouse hepatocytes to 1,2-NQ resulted in the activation of Nrf2 and the upregulation of some of Nrf2's downstream genes. This interaction was further investigated in hepatocytes from Nrf2 knockout mice in which the proteins responsible for the metabolism and excretion of 1,2-NQ are minimally expressed. The chemical modification of cellular proteins by 1,2-NQ was enhanced by Nrf2 deletion, resulting in increased toxicity. However, deletion of the negative regulatory protein, Keap1, drastically reduced the covalent binding by 1,2-NQ and its cellular toxicity. Experiments with chemicals that inhibit the biotransformation and extracellular excretion of 1,2-NQ suggest that 1,2-NQ undergoes detoxification and excretion into the extracellular space predominantly by two-electron reduction and subsequent glucuronidation by NAD(P)H:quinone oxidoreductase 1 and uridine 5'-diphosphate-glucuronosyltransferases, followed by multidrug resistance-associated protein-dependent excretion. These findings suggest that the Keap1/Nrf2 system is essential for the prevention of cell damage resulting from exposure to 1,2-NQ.



INTRODUCTION

Quinones are an important class of naturally occurring compounds that are found in plants, fungi, and bacteria, primarily as important components of the electron-transport chains involved in cellular respiration and photosynthesis. Some of these quinones exhibit electrophilic characteristics as well as redox properties, resulting in unexpected alterations to proteins through S-alkylation (arylation) and oxidative stress in the body.

1,2-Naphthoquinone (1,2-NQ) is a reactive organic compound produced from naphthalene through metabolic activation in the body, combustion of fuels, or photooxidation of atmospheric polynuclear aromatic hydrocarbons.^{1–4} 1,2-NQ has two chemical characteristics associated with oxidative stress.⁵ The first is its ability to covalently bond with macromolecules such as proteins^{6,7} and DNA,⁸ and the second is its ability to redox cycle and generate reactive oxygen species (ROS).⁹ We previously reported that exposure of guinea pig tracheal rings to 1,2-NQ caused a concentration-dependent tracheal contraction through the phosphorylation of epidermal growth factor receptor

(EGFR).¹⁰ Subsequently, using human epithelial A431 cells, we demonstrated that the increased phosphorylation of EGFR by 1,2-NQ was due, at least partially, to covalent modification of Cys121 and inactivation of protein tyrosine phosphatase 1B (PTP1B), which negatively regulates EGFR activation.⁶ We also found that *ortho*-quinones such as 1,2-NQ interact with thiol compounds to produce ROS.^{11,12} The S-alkyl derivative of 1,2-NQ also exhibits redox activity,¹³ suggesting that glutathione adducts of *ortho*-quinones may also promote oxidative stress-dependent cellular toxicity.

Several lines of evidence indicate that the Keap1/Nrf2 system plays a crucial role in protecting cells from electrophiles and prooxidants, i.e., substances that generate ROS through electron transfer reactions.^{14,15} Under normal conditions, the transcription factor Nrf2 is degraded by the ubiquitin/proteasome system, through which minimal steady-state levels are maintained in cells.

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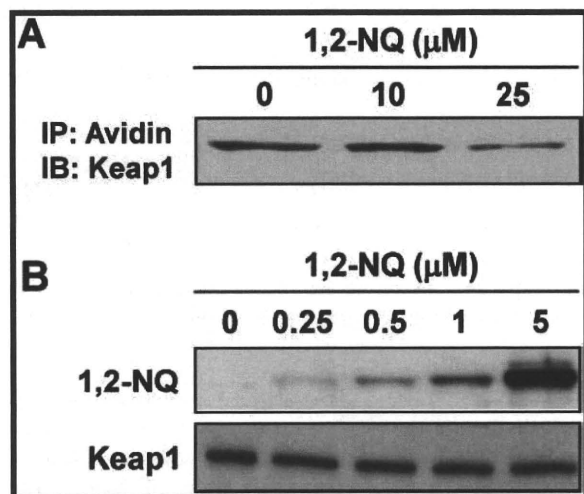


Figure 1. Chemical modification of mouse Keap1 by 1,2-NQ. (A) Covalent attachment of 1,2-NQ to cellular Keap1. Primary mouse hepatocytes were exposed to 1,2-NQ (10 or 25 μM) for 1 h, and the cell lysates were analyzed by using the BPM-labeling assay. (B) Concentration-dependent covalent attachment of 1,2-NQ to recombinant mouse Keap1. Recombinant mouse Keap1 (0.5 μg) was incubated with 1,2-NQ (0.25–5 μM) at 25 $^{\circ}\text{C}$ for 30 min in 20 mM Tris-HCl (pH 8.5). The reaction mixture was then analyzed by Western blot with the indicated antibodies.

When reactive chemicals such as quinones enter cells, Keap1's reactive thiols are modified, either by alkylation or by oxidation to the sulfenic acid. Keap1 is the adaptor of a Cul3 ubiquitin ligase complex that marks Nrf2 for proteasomal degradation by ubiquitination, and the inactivation of Keap1 allows the translocation of Nrf2 into the nucleus. This results in downstream activation of the genes responsible for antioxidant, phase II xenobiotic metabolizing enzymes such as glutathione S-transferases (GSTs),¹⁶ NAD(P)H:quinone oxidoreductase 1 (NQO1),¹⁶ aldo-keto reductases (AKRs),¹⁷ and uridine 5'-diphosphate-glucuronosyltransferases (UGTs),¹⁸ and phase III transporters such as multidrug resistance-associated proteins (MRPs).¹⁹

From previous reports^{5,20,21} and our recent findings,^{12,22} we postulate that 1,2-NQ unbound minimally from cellular proteins is biotransformed to a GSH adduct (1,2-NQ-SG) and/or a monoglucuronide (1,2-NQHG) that does not have the ability to redox cycle, with the polar metabolites then being excreted into the extracellular space through MRPs. With a higher concentration of authentic 1,2-NQ-SG, we found that there is no covalent binding of the GSH adduct to cellular proteins (Miura, T., et al, personal communication), suggesting that it is difficult for 1,2-NQ-SG to bind to cellular proteins. Because Keap1's reactive thiols are a target for electrophiles, and because GSTs, NQO1, AKRs, UGTs, and MRPs are cooperatively regulated by Nrf2, we hypothesize that 1,2-NQ modifies Keap1, leading to Nrf2 activation and decreased 1,2-NQ-induced cellular toxicity. To address these issues, we examined the role of the Keap1/Nrf2 system in protection against 1,2-NQ with primary hepatocytes from wild-type, Nrf2 knockout, and conditional Keap1 knockout mice.

EXPERIMENTAL PROCEDURES

Materials. 1,2-NQ was obtained from Tokyo Chemical Industry (Tokyo, Japan). Biotin-PEAC₅-maleimide (BPM) was obtained from

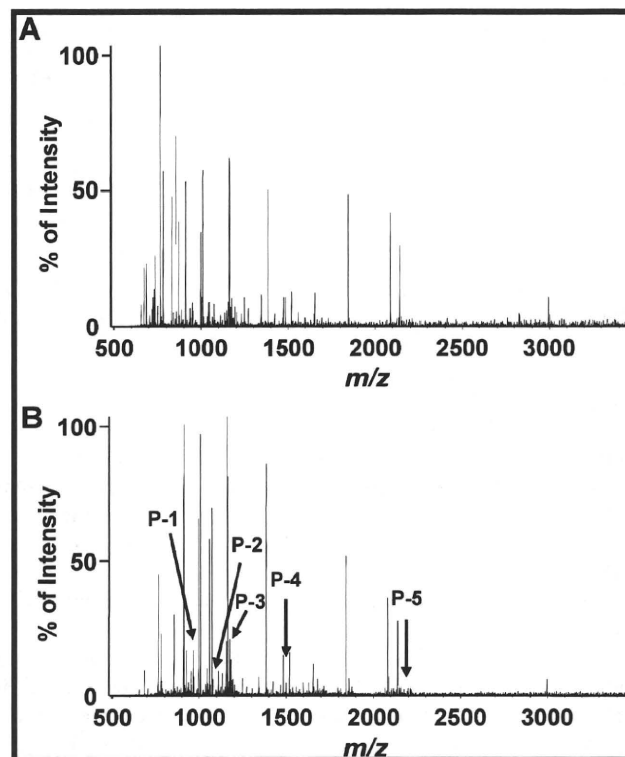


Figure 2. MALDI-TOF/MS analysis of 1,2-NQ-modified cysteine residues in mouse Keap1. Recombinant mouse Keap1 (10 μg) was incubated at 25 $^{\circ}\text{C}$ for 30 min in the absence (A) or presence (B) of 1,2-NQ (10 μM) in a total volume of 20 μL of 20 mM Tris-HCl (pH 8.5). After the reaction, the Keap1 proteins were digested with trypsin and analyzed using MALDI-TOF/MS. Compared with the calculated mass of the unmodified peptides, modified peptides P-1 to P-5, showed an increased mass of 156 Da, corresponding to the addition of a single equivalent of 1,2-NQ.

Dojindo Laboratories (Kumamoto, Japan). Bovine serum albumin (BSA) and polyethylene glycol (PEG) were purchased from Nacal Tesque (Kyoto, Japan). MKS71 was purchased from BIOMOL International (Plymouth Meeting, PA). Anti-Nrf2, antiglutamate cysteine ligase modifier subunit (GCLM), and GCL catalytic subunit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GSTA1 and anti-GSTMI were obtained from Oxford Biomedical Research (Oxford, MI). Diclofenac sodium salt, dicumarol, catalase (CAT), and antiactin were purchased from Sigma (St. Louis, MO). Anti-NQO1 was obtained from Abcam (Cambridge Science Park, UK). L-Buthionine-(S,R)-sulfoximine (BSO) and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2',7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Invitrogen (Carlsbad, CA). All other reagents and chemicals used were of the highest grade available. Anti-GSTA4 and anti-UGT1A were kindly provided by Dr. Akira Hiratsuka (Tokyo University of Pharmacy and Life Sciences, Japan) and Dr. Shinichi Ikushiro (Toyama Prefectural University, Faculty of Engineering Biotechnology Research Center, Japan), respectively. The polyclonal antibody to 1,2-NQ was prepared as reported previously.^{6,23}

Preparation of Polyethylene Glycol-Modified Catalase (PEG-CAT). PEG was dissolved in 1,4-dioxane to 0.05 M in a final volume of 200 mL. 1,1'-Carbonyldiimidazole was then added to the dioxane solution to a final concentration of 0.5 M. After incubation at 25 $^{\circ}\text{C}$ for 2 h, the reaction mixture was dialyzed with Spectra/Por membranes MWCO 2000 (Funakoshi, Tokyo, Japan) against 5,000 mL ($\times 4$) of water at 4 $^{\circ}\text{C}$. After 36 h of dialysis, the activated PEG solution

Table 1. Target Sites of 1,2-NQ Modification in Mouse Keap1^a

peak	position	peptide sequence	calculated MS	observed MS
P-1	273–279	CHALTPR+1,2-NQ	953.4	953.5
P-2	255–261	YDCPQRR+1,2-NQ	1093.4	1093.7
P-3	288–296	CEILQADAR+1,2-NQ	1174.5	1174.6
P-4	484–494	LNSAECYPER+1,2-NQ	1500.6	1500.7
P-5	151–169	CVLHVMNGAVMYQIDSVVR+1,2-NQ	2290.1	2290.3

^a Amino acid sequences of the tryptic peptides containing 1,2-NQ-modified mouse Keap1. Position corresponds to amino acid sequences of wild-type mouse Keap1. Peak number corresponds to the number of the peak in Figure 2 (arrows). Mouse Keap1 (0.5 mg/mL) was incubated with 10 μ M 1,2-NQ at 25°C for 30 min in 20 mM Tris-HCl (pH 8.5).

was lyophilized. The resulting powder was stored at 4 °C. Activated PEG (50 mM), CAT (0.1 mM), and 20 mL of sodium borate buffer (0.1 M, pH 8.5) in a total volume of 100 mL was incubated at 4 °C for 36 h, and then 2-aminoethanol (100 mM) was added to mask the unreacted group of the activated PEG bound to the CAT. The mixture was further incubated for 6 h. PEG-modified CAT was isolated by using an Amicon YM300 ultrafiltration system (Millipore, Billerica, MA). The concentrated solution was lyophilized, and the resulting powder was stored at –20 °C.

Preparation of Keap1. The mouse Keap1 expression construct was prepared as described.²⁴ A plasmid containing mouse Keap1 in the pET21a vector was used as a template for site-directed mutagenesis. The recombinant mouse Keap1 was expressed as a C-terminal His-tagged fusion protein in BL21(DE3)pLysS *E. coli* cells (Promega, Madison, WI) and purified using a ProBond nickel-chelating resin (Invitrogen) as described previously.²⁴

Detection of Keap1 Modified by 1,2-NQ. The BPM-labeling assay was used to detect cellular models.²⁵ Primary mouse hepatocytes were exposed to 1,2-NQ (10 or 25 μ M) for 1 h, washed twice with PBS, and then lysed on ice for 30 min with 200 μ L of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.1% SDS, 0.5% deoxycholic acid, and 1% NP-40) containing 1% protease inhibitor cocktail (Sigma). The cell lysates were then centrifuged at 13,000g, and the supernatant was incubated with 20 μ M BPM for 30 min on ice. The resulting lysates were incubated with 50 μ L of equilibrated avidin-agarose (Sigma) for 12 h at 4 °C. The agarose beads were washed with 1 mL of ice-cold RIPA buffer four times and boiled for 5 min with SDS loading buffer. The eluted proteins were detected by immunoblotting as described below. In the cell-free model, recombinant mouse Keap1 (0.5 μ g) was incubated with 1,2-NQ (0.25–5 μ M) at 25 °C for 30 min in 20 mM Tris-HCl (pH 8.5). The reaction was terminated by the addition of SDS–PAGE loading buffer, and the resulting protein was subjected to immunoblotting as described below.

Matrix-Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF/MS). Wild-type mouse Keap1 (10 μ g) was incubated with or without 1,2-NQ (10 μ M) at 25 °C for 30 min in a buffer containing 20 mM Tris-HCl (pH 8.5) and then dialyzed into 20 mM Tris-HCl (pH 8.5) using an Econo-Pac 10 DG column (Bio-Rad Laboratories, Hercules, CA) to remove free 1,2-NQ. The trypsin-digested mouse Keap1 was mixed with dithiothreitol and trifluoroacetic acid. The peptides were mixed with α -cyano-4-hydroxycinnamic acid (2.5 mg/mL) containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature. The analyses were performed using a Shimadzu AXIMA-TOF² mass spectrometer (Shimadzu, Kyoto, Japan) with a nitrogen laser. All analyses were performed in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies.

Preparation of Primary Mouse Hepatocytes. Primary mouse hepatocytes were isolated from wild-type mice (Nrf2^{+/+}) and Nrf2-deficient mice (Nrf2^{-/-}) using the C57BL/6J background, and from

Alb-Cre::Keap1^{+/+} mice and liver-targeted conditional Keap1-deficient mice (Alb-Cre::Keap1^{lox/-})²⁶ as described.²⁷ The primary hepatocytes were seeded at a density of 8×10^4 cells/cm² and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in William's Medium E containing 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine, penicillin (100 U/mL), and streptomycin (100 μ g/mL). Two days after isolation, the primary mouse hepatocytes were cultured in serum-free medium for 24 h and then exposed to DMSO (0.1%) or 1,2-NQ in serum-free medium. Animal experiments were performed humanely with the approval of the Institutional Animal Experiment Committee of the University of Tsukuba and in accordance with the University of Tsukuba's Regulation for Animal Experiments and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Detection of Intracellular Oxidants. To evaluate the generation of intracellular oxidants, primary mouse hepatocytes were seeded in glass-bottomed culture dishes (MatTek, Ashland, MA). The hepatocytes were pretreated with 20 μ M H₂DCFDA for 1 h before incubation with 10 μ M 1,2-NQ for 20 min at 37 °C in a humidified atmosphere of 5% CO₂. Fluorescence was observed under a fluorescence microscope (DMIRE-2; Leica Microsystems, Wetzlar, Germany).

Western Blot Analysis. Total cell lysates were prepared by solubilization with SDS sample buffer (50 mM Tris-HCl at pH 6.8, 2% SDS, and 10% glycerol). Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL), with BSA as the standard. Samples were normalized to equal protein content and then mixed with half the volume of SDS–PAGE loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 24% glycerol, 6% SDS, 15 mM 2-mercaptoethanol, and 0.015% bromophenol blue. The mixtures were heated at 95 °C for 10 min and then applied to SDS–polyacrylamide gels. The proteins were separated using SDS–PAGE as described²⁸ and then electro-transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories) at 2 mA/cm² for 60 min as described.²⁹ After blocking with 5% skim milk, the membranes were allowed to react with primary antibodies. Secondary antibodies (antirabbit IgG, antimouse IgG, or antigoat IgG) coupled to horseradish peroxidase were used to detect the primary antibodies on the membranes. The proteins for examination were detected using the ECL system (Nacalai Tesque) and then exposed to X-ray films (Konica Minolta Health Care, Tokyo, Japan).

Cellular Viability. The 3-(4,5-dimethylthiazol-2-yl)-2,5-triphenyl tetrazolium bromide (MTT) assay was used to estimate cell viability as described.³⁰ Briefly, primary mouse hepatocytes were exposed to 1,2-NQ for 24 h in 96-well plates and then treated with 5 mg/mL MTT (1/20 volume) for 4 h at 37 °C. After removing the medium, DMSO (100 μ L/well) was added to dissolve the formazan. Absorbance at 540 nm was read using an iMark microplate reader (Bio-Rad Laboratories).

Statistical Analysis. All data are expressed as the means \pm SD from at least three independent experiments. Statistical significance was considered at $P < 0.05$ or $P < 0.01$.

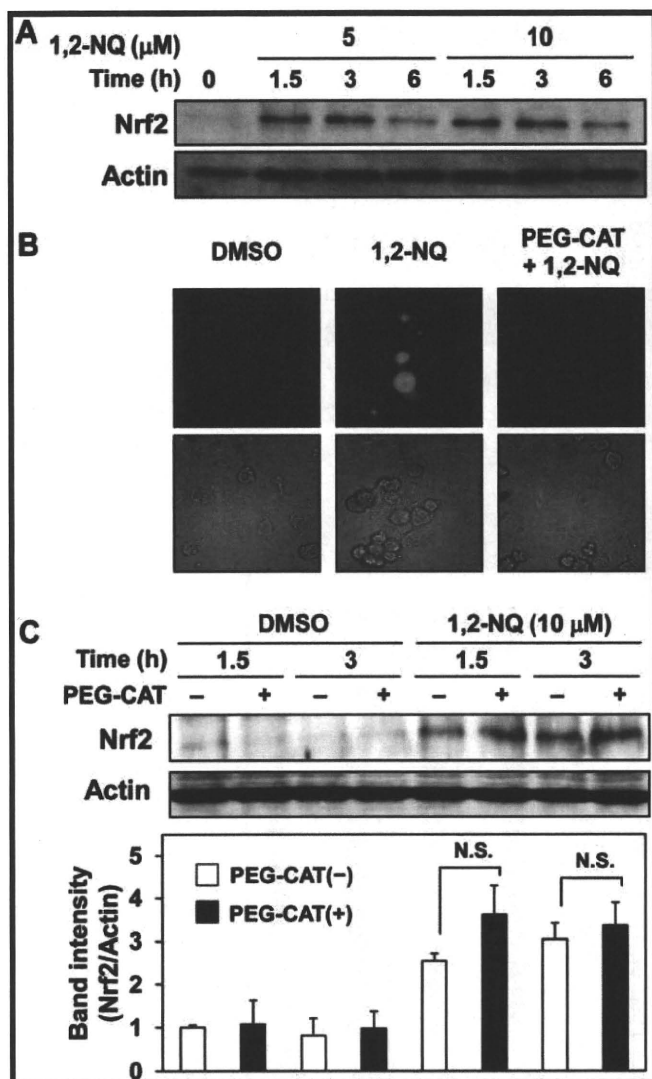


Figure 3. Involvement of hydrogen peroxide in Nrf2 activation during the exposure of primary mouse hepatocytes to 1,2-NQ. (A) Accumulation of Nrf2 by 1,2-NQ. Primary mouse hepatocytes were exposed to 1,2-NQ at the indicated concentrations for 1.5, 3, or 6 h, and total cell lysates (20 μ g) were analyzed by Western blot with the indicated antibodies. Actin was used as the internal control. (B) Detection of intercellular oxidants during exposure to 1,2-NQ. Cells were pretreated with 20 μ M H₂DCFDA for 1 h before incubation with DMSO or 10 μ M 1,2-NQ for 20 min. To scavenge intercellular hydrogen peroxide, hepatocytes were pretreated with PEG-CAT (1,000 U/mL) for 1 h. The panels represent intercellular oxidant generation (top) and bright field (bottom). (C) Effect of PEG-CAT on Nrf2 activation by 1,2-NQ. Cells were pretreated with PEG-CAT (1,000 U/mL) for 1 h before the incubation with DMSO or 10 μ M 1,2-NQ for 1.5 or 3 h. Total cell lysates (20 μ g) were analyzed by Western blot with the indicated antibodies (top). Actin was used as the internal control. The relative intensity of each band was quantified (bottom). Each value represents the mean \pm SD of three independent experiments. N.S., not significant.

RESULTS

Modification of Keap1. BPM-labeling assay was used to evaluate whether 1,2-NQ modified Keap1 in primary mouse hepatocytes. The band intensity for BPM bound to Keap1 decreased in the presence of 1,2-NQ, suggesting that 1,2-NQ was covalently bound to Keap1 within the cells (Figure 1A). When recombinant mouse Keap1 was incubated with 1,2-NQ

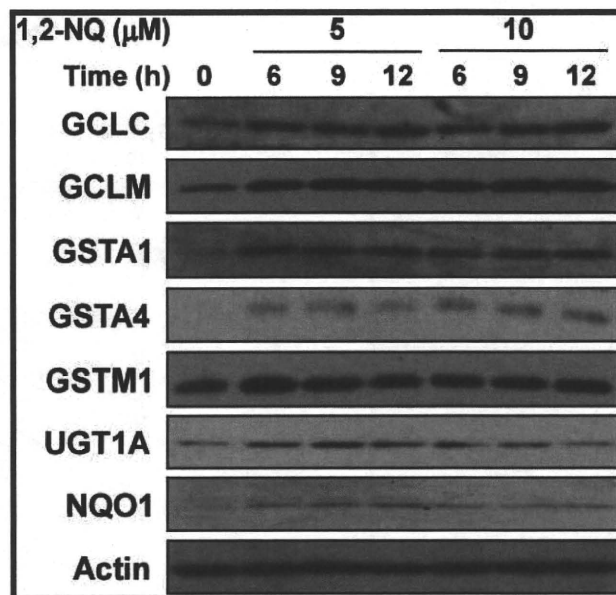


Figure 4. Upregulation of Nrf2 downstream gene products during the exposure of primary mouse hepatocytes to 1,2-NQ. Primary mouse hepatocytes were exposed to 1,2-NQ at the indicated concentrations for 6, 9, or 12 h, and total cell lysates (20 μ g) were analyzed by Western blot with the indicated antibodies. Actin was used as the internal control.

(0.25–5 μ M) at physiological pH, concentration-dependent covalent attachment of 1,2-NQ to Keap1 was seen (Figure 1B). Trypsin was used to digest Keap1 modified by 1,2-NQ, and the fragments obtained were analyzed by using MALDI-TOF/MS. The results (Figure 2 and Table 1) suggested that 1,2-NQ modified mouse Keap1 at Cys151, Cys257, Cys273, Cys288, and Cys489.

Accumulation of Nrf2. Chemical modification of Keap1 through its reactive thiols and/or the oxidative stress associated with the production of ROS are key factors in the activation of Nrf2.^{31,32} Exposure of primary hepatocytes from Nrf2^{+/+} mice to 1,2-NQ resulted in Nrf2 accumulation in the cells (Figure 3A). At concentrations of 5 and 10 μ M 1,2-NQ, accumulation of Nrf2 peaked at 3 h and had declined by 6 h. Under these conditions, intracellular oxidants were produced (Figure 3B). However, pretreatment with PEG-CAT to scavenge intracellular hydrogen peroxide did not significantly affect 1,2-NQ-induced Nrf2 accumulation (Figure 3C). This suggests that 1,2-NQ activates Nrf2 in primary mouse hepatocytes through the chemical modification of Keap1 rather than through the generation of ROS. During 1,2-NQ exposure, the downstream proteins GCLC, GCLM, GSTA1, GSTA4, GSTM1, UGT1A, and NQO1 were upregulated (Figure 4).

Nrf2-Regulated Proteins and the Cellular Toxicity of 1,2-NQ. There are two key metabolic pathways associated with the detoxification of *ortho*-quinones. One is conjugation with GSH, which is an abundant nucleophilic peptide that occurs in the absence and presence of GSTs, and the other is two-electron reduction by AKRs and NQO1 followed by glucuronide formation catalyzed by UGTs,^{5,22} with the resulting polar metabolites then being excreted from cells by MRPs.³³ Western blot analyses of primary hepatocytes isolated from Nrf2^{+/+}, Nrf2^{-/-}, Alb-Cre::Keap1^{+/+}, and Alb-Cre::Keap1^{lox/-} mice demonstrated that Nrf2 deletion markedly repressed the expression of certain phase II xenobiotic metabolizing enzymes and phase III xenobiotic transporters, while Keap1 deletion resulted in the

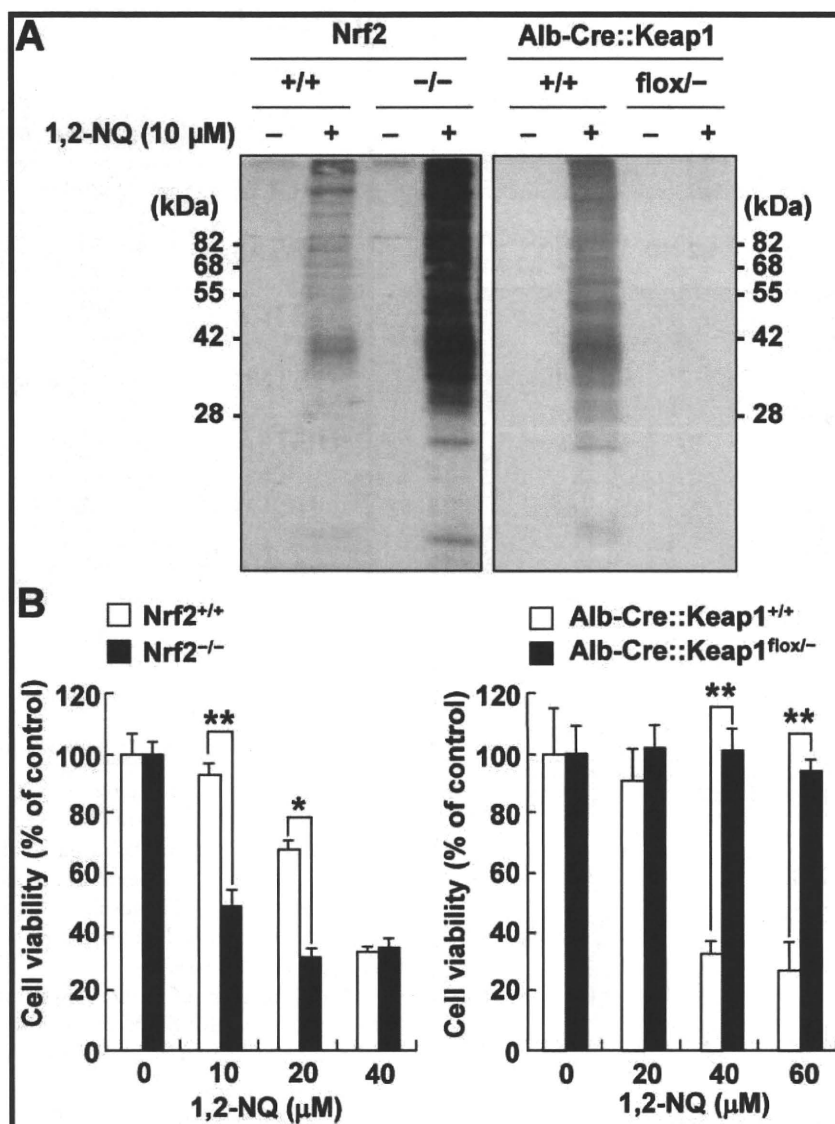


Figure 5. Chemical modification of cellular proteins and cytotoxicity during 1,2-NQ exposure in primary mouse hepatocytes. (A) Primary hepatocytes isolated from Nrf2^{+/+}, Nrf2^{-/-}, Alb-Cre::Keap1^{+/+}, or Alb-Cre::Keap1^{flox/-} mice were exposed to 10 μ M 1,2-NQ for 1 h, and total cell lysates (20 μ g) were analyzed by Western blot with anti-1,2-NQ antibody. (B) Primary hepatocytes isolated from Nrf2^{+/+}, Nrf2^{-/-}, Alb-Cre::Keap1^{+/+}, or Alb-Cre::Keap1^{flox/-} mice were exposed to 1,2-NQ (10–60 μ M) for 24 h, and then an MTT assay was performed. Each value represents the mean \pm SD of four independent experiments. *, $P < 0.05$. **, $P < 0.01$.

overexpression of the same enzymes and transporters (data not shown). Covalent modification of cellular proteins and 1,2-NQ-induced cytotoxicity are dependent on Nrf2 activity. As expected, Nrf2 deletion in primary mouse hepatocytes resulted in an increase in the covalent binding of 1,2-NQ to cellular proteins (Figure 5A) and an increase in the cytotoxicity of 1,2-NQ (Figure 5B), while permanent activation of Nrf2 in Keap1-deficient cells blocked protein arylation by 1,2-NQ (Figure 5A) and reduced cellular toxicity (Figure 5B).

Effects of Inhibitors on Cellular Toxicity. To explore the metabolic pathways involved in the detoxification and excretion of 1,2-NQ, we used inhibitors to GSH, NQO1, UGTs, and MRPs. Pretreatment with BSO (1 mM) decreased cellular GSH content by approximately 50% of the control (data not shown), but the cellular toxicity of 1,2-NQ was unchanged (Figure 6A). Pretreatment with the NQO1 inhibitor, dicumarol, at 50 μ M decreased cellular NQO1 activity by approximately 55% of the control (data not shown), but the cellular toxicity of 1,2-NQ also

was not significantly affected (Figure 6B). However, pretreatment with diclofenac to suppress the activity of UGTs³⁴ and pretreatment with MK571 to inhibit the activity of MRPs³⁵ significantly increased 1,2-NQ cytotoxicity (Figure 6C and D, respectively).

DISCUSSION

The present study demonstrates that 1,2-NQ, like other electrophiles such as natural chemoprevention agents³⁶ and 8-nitroguanosine-3',5'-cyclic monophosphate,³⁷ forms covalent bonds with the reactive thiols in recombinant mouse Keap1 (Figure 1B). Such a modification of Keap1 by 1,2-NQ was also observed in the cells after the exposure of primary mouse hepatocytes (Figure 1A). We also demonstrated that when primary mouse hepatocytes are exposed to 1,2-NQ, the Keap1-associated transcription factor, Nrf2, is activated (Figure 3), resulting in an increase in the levels of downstream gene products

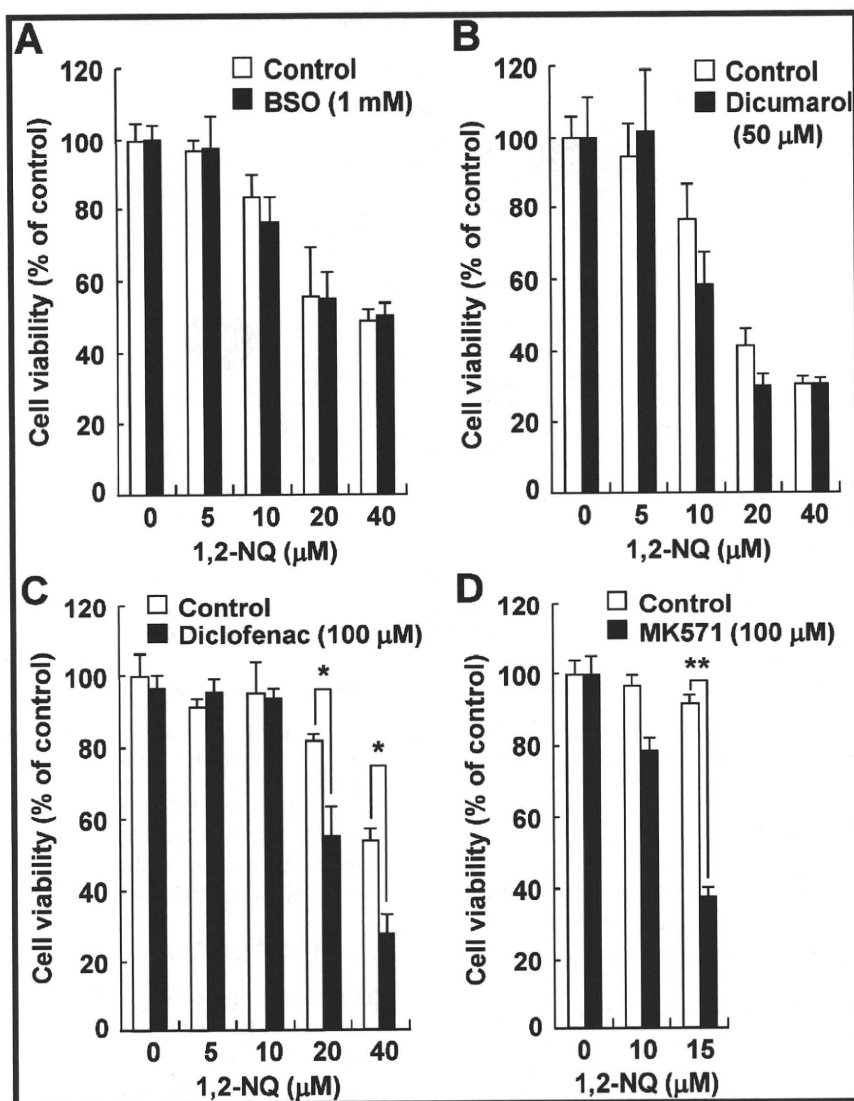


Figure 6. Effects of a variety of inhibitors on cytotoxicity during the exposure of primary mouse hepatocytes to 1,2-NQ. (A) Primary hepatocytes from wild-type mice were treated with 1 mM BSO for 6 h prior to exposure to 1,2-NQ (0–40 μM) for 24 h, and an MTT assay was performed. (B) The cells were treated with 50 μM dicumarol for 6 h prior to exposure to 1,2-NQ (0–40 μM) for 24 h, and an MTT assay was performed. (C) The cells were treated with 100 μM diclofenac for 1 h prior to the exposure to 1,2-NQ (0–40 μM) for 24 h, and an MTT assay was performed. (D) The cells were treated with 100 μM MK571 for 1 h prior to the exposure to 1,2-NQ (0–15 μM) for 24 h, and an MTT assay was performed. Each value represents the mean \pm SD of three independent experiments. *, $P < 0.05$. **, $P < 0.01$.

(Figure 4). Consistent with the notion that Nrf2 action is responsible for the detoxification of 1,2-NQ, the activation of Nrf2 was shown to be associated with both a decrease in the arylation of cellular proteins and a decrease in cellular toxicity (Figure 6).

In our preliminary examination, we found that mouse Keap1 undergoes chemical modification by 1,2-NQ mainly through Cys151.³⁸ The present MALDI-TOF/MS study suggested that, in addition to the modification at Cys151, Keap1 is also modified at Cys257, Cys273, Cys288, and Cys489 by 1,2-NQ. Cys151, Cys273, and Cys288 of Keap1 are highly reactive thiols, whereas Cys257 and Cys489 undergo covalent modification by a variety of electrophiles; this results in the activation of Nrf2.³⁹ Pretreatment with PEG-CAT did not affect Nrf2 activation caused by 1,2-NQ exposure (Figure 3), suggesting that ROS such as hydrogen peroxide, which are produced during the redox cycling of 1,2-NQ, are not predominantly associated with the activation of Nrf2.

Figure 7 summarizes the biotransformation reactions of 1,2-NQ as they relate to its cellular toxicity. We reported previously that in A431 cells, 1,2-NQ inactivates PTP1B, a negative regulator of the transactivation of EGFR, through covalent bonding at Cys121, thereby activating EGFR.⁶ It is likely that the arylation of proteins by 1,2-NQ not only affects electrophilic signaling but also disrupts cellular protein activities⁷ resulting in cytotoxic effects. Indeed, the rates of protein modification by 1,2-NQ in primary mouse hepatocytes correlate with cytotoxicity (Miura, T., et al., unpublished observation). In other words, decreased protein modification by 1,2-NQ is associated with a reduction in the cytotoxicity of 1,2-NQ. In support of this contention, we found substantially reduced levels of arylated proteins and reduced cytotoxicity after exposure to 1,2-NQ in primary hepatocytes from liver-targeted conditional Keap1-deficient mice (Figure 5), which exhibit constitutive Nrf2 activation and upregulation of phase II xenobiotic metabolizing enzymes

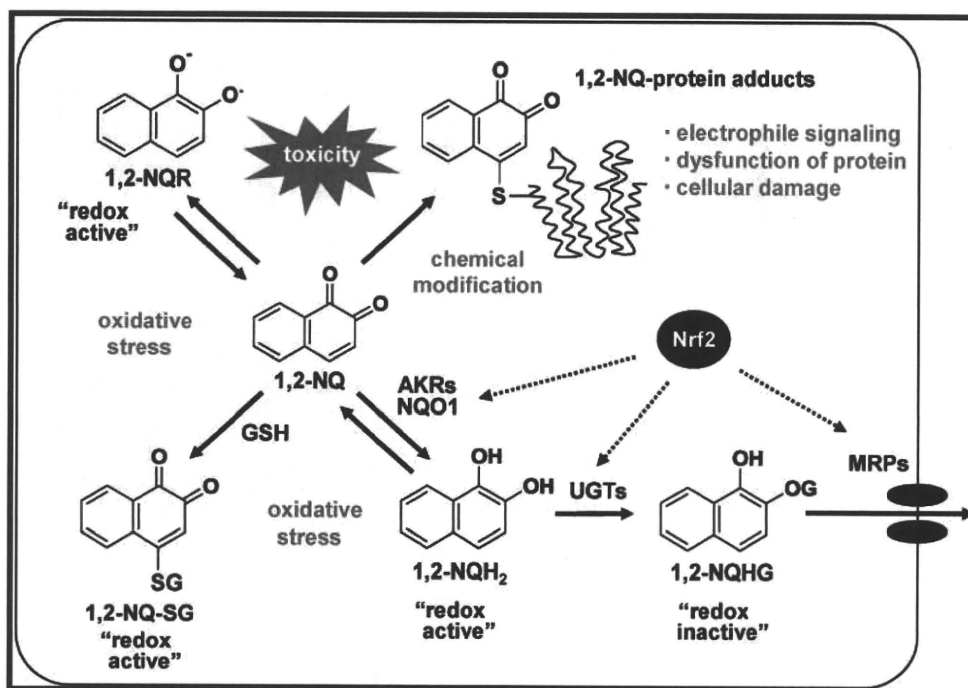


Figure 7. Role of Nrf2 in the metabolic activation associated with oxidative stress and detoxification of 1,2-NQ. 1,2-NQR, the semiquinone radical of 1,2-NQ; 1,2-NQ-SG, the GSH adduct of 1,2-NQ; 1,2-NQH₂, 1,2-dihydroxynaphthalene; 1,2-NQHG, the monoglucuronide of 1,2-NQH₂.

and phase III transporters^{40,41} responsible for the detoxification and excretion of 1,2-NQ.

Quinones undergo one-electron reduction by cytochrome P450 reductase, producing semiquinone radicals that react with molecular oxygen to generate superoxide,⁴² which in turn disproportionates to hydrogen peroxide and oxygen. Hydrogen peroxide can be converted to a hydroxyl radical in the presence of transition metals such as copper and iron. Induction of oxidative stress during the exposure of cells to quinones is, at least partially, attributable to ROS generation.²⁰ The two-electron reduction of ortho-quinone to its hydroquinone is not a detoxification because the disproportionation reaction between quinone and hydroquinone produces a semiquinone radical that initiates oxygen reduction, generating ROS such as hydrogen peroxide.¹² In support of this notion, pretreatment with dicumarol to diminish NQO1 activity did not affect the cytotoxicity of 1,2-NQ (Figure 6B). Conjugation of 1,2-NQ by GSH to yield the 1,2-NQ-SG adduct (Figure 7) is an important pathway to reduce levels of electrophiles that would otherwise be toxic. However, pretreatment with BSO to block GSH synthesis did not enhance the cytotoxicity of 1,2-NQ in primary mouse hepatocytes (Figure 6A). In preliminary studies, BSO treatment did not affect the levels of covalently modified cellular proteins following the exposure of primary mouse hepatocytes to 1,2-NQ (5–40 μM) (Miura, T., et al., unpublished observations). Although the conjugation of quinones with GSH is generally associated with detoxification and excretion, the conjugated quinones retain the ability to redox cycle with the concomitant production of ROS.^{43,44} Taken together, a reasonable explanation for the present observations is that it is not GSH conjugation of 1,2-NQ but rather UGT-catalyzed glucuronide formation from 1,2-NQH₂ that is the ultimate detoxification pathway for 1,2-NQ in primary mouse hepatocytes. This explanation is in agreement with the recent report of detoxification of menadione in HEK 293 cells.⁴⁵

We suggest that the Keap1/Nrf2 system plays a central role in the biotransformation and subsequent excretion of xenobiotic quinones by decreasing their steady-state concentration in cells, thereby reducing their toxicity.

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■ ABBREVIATIONS

1,2-NQ, 1,2-naphthoquinone; ROS, reactive oxygen species; EGFR, epidermal growth factor receptor; PTP1B, protein tyrosine phosphatase 1B; GSTs, glutathione S-transferases; NQO1, NAD(P)H:quinone oxidoreductase 1; AKRs, aldo-keto reductases; UGTs, uridine 5'-diphosphate-glucuronosyltransferases; MRPs, multidrug resistance-associated proteins; 1,2-NQ-SG, GSH adduct of 1,2-NQ; 1,2-NQH₂, 1,2-dihydroxy naphthalene; 1,2-NQHG, monoglucuronide of 1,2-NQH₂; BPM, biotin-PEAC₅-maleimide; BSA, bovine serum albumin; PEG, polyethylene glycol; GCLM, glutamate cysteine ligase modifier subunit; GCLC, GCL catalytic subunit; CAT, catalase; BSO, L-buthionine-(S,R)-sulfoximine; DMSO, dimethyl sulfoxide; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; PEG-CAT, PEG-modified CAT; MALDI-TOF/MS, matrix-assisted laser desorption time-of-flight mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-triphenyl tetrazolium bromide.