

Original Contribution

# An approach to evaluate two-electron reduction of 9,10-phenanthraquinone and redox activity of the hydroquinone associated with oxidative stress

Keiko Taguchi <sup>a</sup>, Sayako Fujii <sup>b</sup>, Shigeru Yamano <sup>c</sup>, Arthur K. Cho <sup>d</sup>, Shinji Kamisuki <sup>e</sup>, Yumi Nakai <sup>f</sup>, Fumio Sugawara <sup>e</sup>, John R. Froines <sup>d</sup>, Yoshito Kumagai <sup>a,d,\*</sup>

<sup>a</sup> Doctoral Programs in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

<sup>b</sup> Master's Program in Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

<sup>c</sup> Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

<sup>d</sup> Southern California Particle Center, Institute of the Environment, University of California, Los Angeles, Los Angeles, CA 90095, USA

<sup>e</sup> Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda, Chiba 278-8510, Japan

<sup>f</sup> Analytical Instruments Division, JEOL Ltd., 3-1-2 Musashino, Akishima, Tokyo 196-8558, Japan

Received 19 February 2007; revised 11 May 2007; accepted 11 May 2007

Available online 24 May 2007

## Abstract

Quinones are widely used as medicines or redox agents. The chemical properties are based on the reactions against an electron donor. 9,10-Phenanthraquinone (PQ), which is a quinone contaminated in airborne particulate matters, forms redox cycling, not Michael addition, with electron donors. Redox cycling of PQ contributes to its toxicity, following generation of reactive oxygen species (ROS). Detoxification of quinones is generally thought to be two-electron reduction forming hydroquinones. However, a hydroquinone of PQ, 9,10-dihydroxyphenanthrene (PQH<sub>2</sub>), has been never detected itself, because it is quite unstable. In this paper, we succeeded in detecting PQH<sub>2</sub> as its stable derivative, 9,10-diacetoxyphenanthrene (DAP). However, higher concentrations of PQ (>4 μM) form disproportionately with PQH<sub>2</sub>, producing the 9,10-phenanthraquinone radical (PQ<sup>•-</sup>) which is a one-electron reducing product of PQ. In cellular experiments using DAP as a precursor of PQH<sub>2</sub>, it was shown that PQH<sub>2</sub> plays a critical role in the oxidative protein damage and cellular toxicity of PQ, showing that two-electron reduction of PQ can also initiate redox cycling to cause oxidative stress-dependent cytotoxicity.

© 2007 Elsevier Inc. All rights reserved.

**Keywords:** Redox cycling; Two-electron reduction; 9,10-Phenanthraquinone; Disproportionation

**Abbreviations:** AKR, aldo-keto reductase; AZQ, 2,5-ethyl(carboethoxyamino)3,6-diaziridinyl-1,4-benzoquinone; DAP, 9,10-diacetoxyphenanthrene; DEP, diesel exhaust particles; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; ESI-MS, electrospray ionization-mass spectrometry; ESR, electron spin resonance; Hepes, *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HPLC, high-performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO, NAD(P)H:quinone oxidoreductase; PAGE, polyacrylamide gel electrophoresis; PAH, polycyclic aromatic hydrocarbon; P450R, NADPH-cytochrome P450 reductase; PM<sub>2.5</sub>, ambient particulate matter; PQ, 9,10-phenanthraquinone; PQ<sup>•-</sup>, 9,10-phenanthraquinone radical; PQH<sub>2</sub>, 9,10-dihydroxyphenanthrene; ROS, reactive oxygen species; SOD, superoxide dismutase; TrxR1, thioredoxin reductase 1.

\* Corresponding author. Doctoral Programs in Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Fax: +81 29 853 3133.

E-mail address: [yk-em-tu@md.tsukuba.ac.jp](mailto:yk-em-tu@md.tsukuba.ac.jp) (Y. Kumagai).

## Introduction

Ambient particulate matter (PM<sub>2.5</sub>) and diesel exhaust particles (DEP) are air pollutants that induce a variety of adverse health effects including cardiovascular diseases [1], allergies [2], and cancers [3]. Recently, oxidative stress has been suggested to be a key biological event in the toxicity of the particulates [4,5]. Organic extracts from DEP, which contain high levels of polycyclic aromatic hydrocarbons (PAHs) and their derivatives, are capable of producing reactive oxygen species (ROS) [6–8]. We have found that 9,10-phenanthraquinone (PQ), a PAH-quinone found in DEP [9] produces superoxide [8,10]. This quinone is toxic to human pulmonary epithelial A549 cells at micromolar concentrations [11] and exhibits both aerobic and anaerobic toxicity in yeast [12]. The

multiple facets of its chemical and toxicological properties have led us to examine the details of its interaction with cellular components [13–15]. One aspect of its biochemistry that needs to be elucidated is its biotransformation by cellular systems since its toxicity involves both redox and additional reactions of its *o*-quinone structure.

In this context, there are two reduced PQ species of biological importance, its semiquinone radical (9,10-phenanthraquinone;  $PQ^{\bullet-}$ ) and its hydroquinone (9,10-dihydroxyphenanthrene;  $PQH_2$ ).  $PQ^{\bullet-}$  can promote redox cycling by transferring electrons from a source such as NADPH to oxygen to generate ROS [8,10]. We have previously reported two possible pathways for  $PQ^{\bullet-}$  formation: an enzymatic reaction catalyzed by flavoenzymes such as NADPH-cytochrome P450 reductase (P450R) or neuronal nitric oxide synthase [8,16] and a nonenzymatic reaction with proximal protein thiols [10]. Formation of hydroquinones has generally been considered to protect cells against quinone-induced oxidative stress [17–19]. However, Bucker et al. [20] found that  $PQH_2$  was somewhat more mutagenic than PQ itself in the rec assay with *Bacillus subtilis* strains. In addition, the two-electron reduction of 2-methyl-1,4-naphthoquinone, mitomycin C, and AZQ, which are derivatives of benzoquinone by purified NAD(P)H:quinone oxidoreductase 1 (NQO1) [21,22] and aldo-keto reductase 1C9 (AKR1C9) [23], has been shown to induce DNA damage and cytotoxicity. Taken together, these reports suggest that if the two-electron reducing products of quinones readily form redox cycling, thereby producing ROS, two-electron reduction could also be a deleterious pathway.

We have recently found that exposure of human pulmonary carcinoma A549 cells to PQ causes a marked oxidative stress as evaluated by oxidative modification of the cellular proteins [11]. Interestingly, the antioxidant protein, Cu,Zn-superoxide dismutase (Cu,Zn-SOD), was downregulated by PQ under these conditions. Because hydroquinones such as  $PQH_2$  react easily with superoxide to form quinones [24], the reduced levels of Cu,Zn-SOD during PQ exposure may have promoted oxidative stress in the A549 cells by enhancing a redox cycling of  $PQH_2$  to PQ. To our knowledge, there are no reports describing the direct two-electron reduction of PQ, in part because of the labile nature of  $PQH_2$ . To address this issue, we developed a convenient assay to convert  $PQH_2$  to its diacetoxy derivative 9,10-diacetoxyphenanthrene (DAP) with high-performance liquid chromatography (HPLC). Using this procedure, we examined the characteristics of PQ to  $PQH_2$  conversion by crude enzyme preparations and a purified preparation containing an aldo-keto reductase 1C (AKR1C) isozyme. The results provide evidence for the first time that NADPH-dependent enzymes such as the AKR1C isozyme mediate the two-electron reduction of PQ and promote redox cycling with  $PQ^{\bullet-}$  as the intermediate.

## Materials and methods

### Materials

PQ and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan); Indomethacin, ciba-

cron blue, ethoxyquin, quercetin, monoclonal anti-dinitrophenyl (DNP) antibody, and DMEM medium containing 4.5 g/L of D-glucose were from Sigma-Aldrich Co. (St. Louis, MO); acetic anhydride was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); dicumarol was from Aldrich Chemical Co. (Milwaukee, WI); 5,5'-dimethyl-1-pyrroline *N*-oxide (DMPO) was from Labotech Co. (Tokyo, Japan); ECL Western blotting detection reagent and Blue Sepharose 6 fast flow and streptavidin-horseradish peroxidase (HRP) conjugate were from Amersham Biosciences Co. (Piscataway, NJ); GlutaMAX-I supplement was from Invitrogen Co. (Carlsbad, CA); biotinylated anti-mouse IgE was from Technopharm Biotechnol. (Villejuif Cedex, France); and anti-thioredoxin reductase 1 (TrxR1) antibody was from Upstate USA, Inc. (Charlottesville, VA). An AKR1C isozyme was purified from liver cytosol of rabbits by the method of Yamano et al. [25]. The enzyme was classified into the AKR1C subfamily because its sequence showed more than 60% identity to members of the AKR1C. Polyclonal antibodies against Cu,Zn-SOD were prepared as described by Shimojo et al. [26]. Antibody against P450R was raised by the method described previously [27]. Antibodies against AKR7A2 and NQO1 were gifts from Dr. John D. Hayes (University of Dundee, UK) and Dr. Nobuyuki Koga (Nakamura Gakuen University, Japan), respectively. All other chemicals used were obtained from commercial sources and were of the highest grade available.

### Synthesis of DAP

PQ (100 mg in 15 ml of tetrahydrofuran) was mixed with Zn dust (50 mg), and then 0.5 ml of acetic anhydride, 1 drop of water, and 3 drops of triethylamine were added. The mixture was stirred at room temperature for 20 min; an additional 50 mg of Zn dust, 1 drop of water, and 3 drops of triethylamine were added; and then the mixture was heated to reflux for 1 h. After cooling, the mixture was extracted with chloroform (40 ml). The chloroform layer was washed with water, saturated with  $\text{NaHCO}_3$ , and then dried over  $\text{MgSO}_4$ . The solvent was removed in vacuo to leave a pale yellow solid (132 mg). The product was recrystallized in benzene and a white solid (45 mg) was obtained. Electrospray ionization-mass and NMR spectrometries (ESI-MS; positive-ion mode) were performed:  $[M+\text{Na}]^+$   $m/z=316.7$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz, ppm)  $\delta$ 8.68 (br d, 2H, ArH), 7.88 (br d, 2H, ArH), 7.65 (m, 4H, ArH), 2.49 (s, 6H,  $\text{COCH}_3$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz, ppm)  $\delta$ 168.2, 135.8, 129.7, 127.3, 127.1, 126.5, 123.0, 122.0, 20.5.

### Enzyme preparation

Wistar male rats (6 weeks) and ICR male mice (6–8 weeks) were used. Liver and other tissues were homogenized in 9 vol of 5 mM Tris-HCl (pH 7.5)–0.25 M sucrose–0.1 mM EDTA. The homogenate was centrifuged at 600g for 10 min and the supernatants were centrifuged at 9000g for 10 min. The pellet (mitochondrial fraction) was suspended in the homogenizing buffer and stored at  $-70^\circ\text{C}$  until use. The supernatants from the mitochondrial isolation were centrifuged at 105,000g for 60 min.

The pellets obtained were resuspended in 0.1 M potassium pyrophosphate (pH 7.4) and again centrifuged at 105,000g for 60 min. The resulting pellets, the microsomal fraction, were again resuspended in homogenizing buffer and stored at  $-70^{\circ}\text{C}$  until use. Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard [28].

For separation of the enzymes responsible for the two-electron reduction of PQ to PQH<sub>2</sub> by Blue Sepharose column chromatography, mice were given feed including 0.5% ethoxyquin (CLEA Japan, Inc., Tokyo, Japan), an inducer for AKR isozymes for 2 weeks [29]. The animals were euthanized with diethylether and the tissues were removed and homogenized in 5 vol of 0.25 M sucrose. The homogenate was centrifuged at 9000g for 20 min. The resulting supernatant (342 mg of protein) was centrifuged at 105,000g for 60 min to obtain the cytosol. Solid ammonium sulfate was added to the lung cytosol of mice to make a 40% saturated solution, followed by centrifugation at 18,000g for 20 min. The supernatants obtained were mixed with solid ammonium sulfate to make a 90% saturated solution. After the mixture was centrifuged, the precipitates were dissolved in 10 ml of 50 mM Tris-HCl (pH 7.2)–0.25 M sucrose (Buffer A) and dialyzed for 6 h against 1500 ml of 50 mM Tris-HCl (pH 7.2). The dialyzed sample (170 mg of protein) was applied onto a Blue Sepharose column (2.5 × 1.5 cm, i.d.), which had been equilibrated with Buffer A at a flow rate of 0.15 ml/min. The column was washed with Buffer A (fraction B-I, No. 4–9), followed by Buffer A–0.5 M KCl (fraction B-II, No. 25–32) and Buffer A–0.5 M KCl–10 mM NADH (fraction B-III, No. 52–64). NADH was removed from fraction B-III by dialysis against buffer A–0.5 M KCl for 3 h (500 ml × 2).

#### Two-electron reduction of PQ

The incubation mixture (0.75 ml) consisted of 5 μM PQ, 0.2 mM NADPH, enzyme preparation (21.24 μg of protein), and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA unless otherwise noted. Reactions were initiated by the addition of the enzyme preparation and allowed to proceed at 25°C for 5 min. To terminate the reaction, a portion (0.5 ml) of the incubation mixture was transferred to a centrifuge tube containing acetic anhydride (20 μl) and heated at 80°C for 5 min. Then the mixtures were mixed with trichloroacetic acid (170 μl, final concentration, 2.5%) and centrifuged at 14,000g for 5 min. The supernatants (40 μl) were subjected to HPLC analysis. Separation of DAP from PQ was carried out on a YMC-Pack ODS-AM (250 × 4.6 mm, i.d., 5 μm particle size, YMC Co. Ltd., Kyoto, Japan) with a Shimadzu LC-10AT pump and SPD-10A UV-VIS detector (Kyoto, Japan). Elution was accomplished with acetonitrile/1% CH<sub>3</sub>COOH (3:2, v/v) at a flow rate of 1 ml/min. Detection was performed at 255 nm. Peak height was determined by a Chromatocorder 11 (System Instruments Co. Ltd., Tokyo, Japan).

#### NADPH consumption

NADPH consumption was determined by the decrease in absorbance at 340 nm, based on the oxidation of NADPH to

NADP<sup>+</sup>, using an extinction coefficient of 6220 M<sup>-1</sup> cm<sup>-1</sup> in a Shimadzu UV-1600 double-beam spectrometer (Kyoto, Japan). The reaction mixture (1.5 ml) contained liver cytosol (42.48 μg of protein), 5 μM PQ, 0.2 mM NADPH, and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA in the absence or presence of SOD (100 U).

#### High-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC/ESI-MS)

Full-scanning and selected ion-monitoring data were collected with HPLC/ESI-MS. The standard sample and the reaction mixture were injected onto a Merck Chromolith SpeedROD RP-18e column (50 × 4.6 mm i.d.; Merck KgaA, Darmstadt, German) at room temperature. The eluent was a solution of acetonitrile containing 0.1% aqueous formic acid (40% for 5 min; 40–100% for 20 min; 100% for 5 min) delivered in a Hewlett Packard Agilent 1100 chromatograph with a flow rate of 0.2 ml/min. Nitrogen was used as the nebulizing and drying gas. ESI spectra were recorded in the positive-ion mode using an Esquire 3000 plus ion-trap instrument (Bruker Daltonik, Bremen, Germany) in the standard mass range ( $m/z$  50–3000) and normal scan resolution (13,000  $m/z/s$ ) with the standard electrospray ion source mounted. For data acquisition, esquireNT 4.0 software (esquireControl V6.08, Build No. 25.0) was used. Data were processed with DataAnalysis V2.0 software (Build No. 99).

#### Production of superoxide

Generation of superoxide was determined by measuring SOD-inhibitable reduction of acetylated cytochrome *c* at 550 nm as described previously [10]. The reaction mixture (1.5 ml) contained 5 μM PQ, 0.2 mM NADPH, rat liver cytosol (42.48 μg of protein), 25 μM acetylated cytochrome *c*, and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA in the absence or presence of SOD from bovine erythrocytes (100 U).

#### Measurement of superoxide and PQ<sup>•-</sup> by electron spin resonance (ESR)

ESR studies were performed at 25°C by using a JES-FA200 spectrometer (JEOL Co. Ltd., Tokyo, Japan) as described previously [8]. Superoxide generated during the reaction of two-electron reduction of PQ by AKR1C isozyme was identified as its DMPO adduct. The reaction mixture (0.3 ml) for measurement of superoxide contained 5 μM PQ (in DMSO, final concentration of 1%), 0.2 mM NADPH, AKR1C isozyme (0–11.9 mU), 3.3% DMSO, 0.897 M DMPO, and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA. For measurement of PQ<sup>•-</sup>, the reaction mixture (0.3 ml) contained 0.1 mM PQ (in DMSO, final concentration of 1%), 0.2 mM NADPH, 1.19 mU AKR1C isozyme, and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA. The spectrometer settings are indicated in the figure legends.

#### Western blot analysis

Samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were boiled after treatment with SDS sample buffer

(62.5 mM Tris-HCl, pH 6.8, 8% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue). Proteins, separated by SDS-PAGE (12% acrylamide gel), were electro-transferred onto PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA) at 2 mA/cm<sup>2</sup> for 60 min, according to the method of Kyhse-Anderson [30]. After blocking with 5% skim milk, the membranes were allowed to react with primary antibodies. Secondary antibodies (anti-rabbit IgG) coupled to horseradish peroxidase were used to detect primary antibodies on the membrane. The proteins examined were detected with an ECL system (Amersham Biosciences Corp., Piscataway, NJ) and exposed to X-ray films (Fuji Photo Film Co. Ltd., Tokyo, Japan).

#### Assays of cytotoxicity

A549 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in DMEM medium con-

taining 4.5 g/L of D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX-I supplement, and 10% (v/v) heat-inactivated fetal bovine serum at 37°C under 95% air plus 5% CO<sub>2</sub>.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [31] was used to estimate the cytotoxicity of PQ or DAP. Proliferating A549 cells were exposed to PQ or DAP (1–20 µM) for 12 h in 96-well microtiter plates and then treated with 5 mg/ml MTT (1/20 (v/v)) for 4 h at 37°C. After removal of the medium, DMSO (100 µl/well) was added to dissolve cells. Absorbance at 540 nm was measured by an ImmunoMini NJ-2300 plate reader (Nippon InterMed, Tokyo, Japan).

#### Determination of oxidatively modified proteins

After the treatment of A549 cells with PQ or DAP (10 µM), total cellular proteins were solubilized in SDS

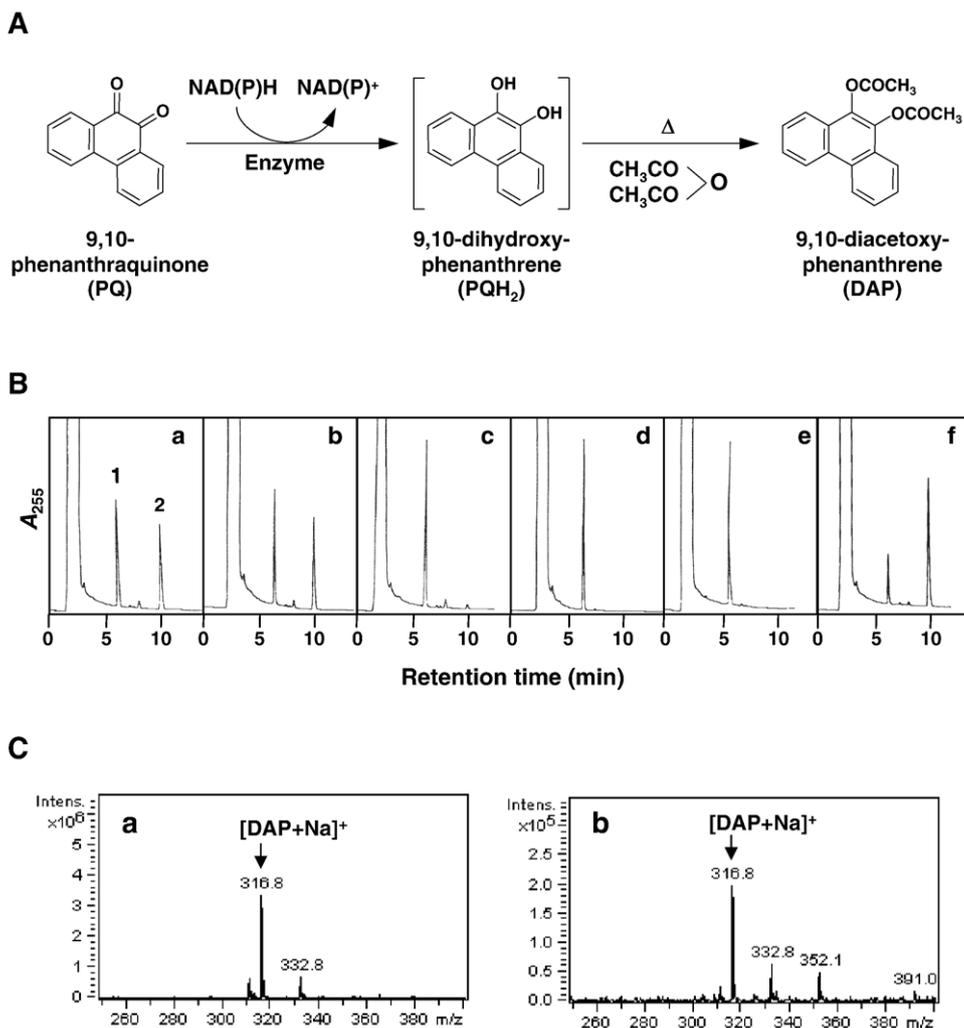


Fig. 1. Assay for two-electron reduction of PQ. (A) Enzymatic two-electron reduction of PQ to PQH<sub>2</sub> which is identified as its diacetoxy derivative. (B) High-performance liquid chromatograms of enzymatic reaction mixtures of PQ. Peak 1, PQ (retention time, 6.8 min); peak 2, DAP (retention time, 10.6 min). a, complete system; b, +NADH/–NADPH; c, –cytosol; d, –NADPH; e, –acetic anhydride; f, –oxygen. Rat liver cytosol (21.24 µg of protein) was incubated with 5 µM PQ, 0.2 mM NADPH, and 0.1 M HEPES (pH 7.6)–0.1 mM EDTA at 25°C for 5 min. Determination of DAP was performed under conditions described under Materials and methods. (C) Positive-ion electrospray mass spectra of authentic DAP (a) and the diacetoxy derivative from enzymatic reducing product of PQ (b).

sample buffer (50 mM Tris-HCl (pH 6.8)–10% glycerol–2% SDS). Cellular proteins were reacted with DNPH to derivatize carbonyl groups and then subjected to SDS-PAGE (20  $\mu$ g of protein) followed by western blotting with anti-DNP, biotinylated anti-IgE antibodies, and streptavidin HRP conjugate [32].

#### Determination of $LD_{50}$ value

Values obtained from MTT assay were analyzed by a nonlinear regression program using PRISM version 3.0 (GraphPad Software, Inc., San Diego) to calculate  $LD_{50} \pm SE$  values.

## Results

#### Identification of $PQH_2$ as its diacetoxy derivative

Fig. 1A shows the reaction sequence in the derivatization of the two-electron reduction product of PQ for HPLC analysis. Incubation of PQ (peak 1; retention time, 6.8 min) with cytosol from rat liver in the presence of NAD(P)H resulted in a product indicated as peak 2 with a retention time of 10.6 min (Figs. 1B, a and b), which has a mass spectrum identical to authentic DAP (Fig. 1C, b). No DAP was seen when either cytosol or NADPH was omitted from the incubation mixture (Figs. 1B, c and d). When acetic anhydride was not added to the reaction mixture, DAP was not detected (Fig. 1B, e). Under anaerobic conditions, the peak of DAP increased (1.8-fold) compared to aerobic conditions (Fig. 1B, f), suggesting that  $PQH_2$  is highly sensitive to molecular oxygen. Addition of a superoxide generating system (0.5 mM xanthine + 0.4  $\mu$ M xanthine oxidase) to an incubation mixture of PQ and cytosol decreased the DAP peak (43% of control), and increased the PQ peak (data not shown), indicating that  $PQH_2$  is oxidized to PQ by superoxide. The ESI mass spectra of both compounds which were eluted at 18.7 min under HPLC conditions with a Merck Chromolith SpeedROD RP-18e column gave a molecular ion peak at  $m/z$  of 316.8, based on a monosodium salt ion,  $[M+Na]^+$ . These results demonstrate that PQ underwent a two-electron reduction to yield  $PQH_2$  enzymatically and that  $PQH_2$  was, in turn, converted to its diacetoxy derivative DAP with acetic anhydride. The standard curve generated from the peak area and the injected DAP quantity was linear ( $r^2=0.999$ ) from 1 to 7.5  $\mu$ M and similar results were observed with PQ (data not shown). Although the production of  $PQH_2$  as determined by DAP and PQ remaining was dependent on the enzyme preparation added, the total amounts of  $PQH_2$  formation and PQ remaining were almost the same (Fig. 2A), supporting that  $PQH_2$  formed from PQ was successfully converted into DAP without further metabolism. In rat liver,  $PQH_2$  formation was much higher in cytosol than in either microsomes (10.3%) or mitochondria (16.7%; percentage of  $PQH_2$  formation in liver cytosol). The  $PQH_2$  formation was the highest in cytosol from liver, followed by cytosol from brain (78%), kidney (73%), and lung (72%), and was minimal in the cytosol from testis (28%), heart (18%), and

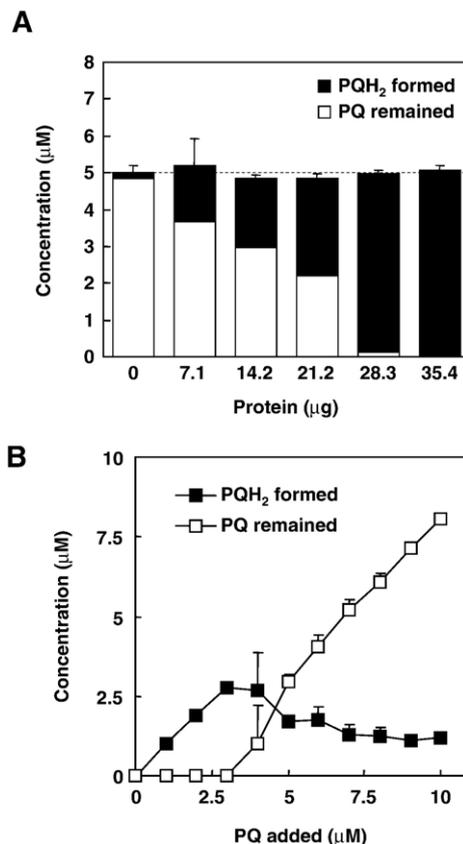


Fig. 2. (A) Material balance study during the reaction of PQ with rat liver cytosol. (B) Concentration-dependent  $PQH_2$  formation during the reaction of PQ with rat liver cytosol. Rat liver cytosol (21.24  $\mu$ g of protein) was incubated with 5  $\mu$ M PQ, 0.2 mM NADPH, and 0.1 M Hepes (pH 7.6)–0.1 mM EDTA at 25°C for 5 min. Determination of DAP was performed under conditions described under Materials and methods. Each value is the mean  $\pm$  SD of three determinations.

spleen (14%; percentage of  $PQH_2$  formation in liver cytosol). When PQ was incubated with rat liver cytosol in the presence of NADPH,  $PQH_2$  formation reached a plateau and then declined at PQ concentrations of greater than 4  $\mu$ M (Fig. 2B). This suggests that a disproportionation reaction of quinones and their hydroquinone to yield the semiquinone radicals as shown by Kalyanaraman et al. [33], at least in part, occurs because this unstable intermediate undergoes readily oxidation by molecular oxygen to produce PQ.

#### Generation of ROS during reduction of PQ by rat liver cytosol

When PQ (7.5 nmol) was incubated with rat liver cytosol in the presence of an excess amount of NADPH (300 nmol) at 25°C for 5 min,  $PQH_2$  (2.8 nmol) and superoxide (18.0 nmol) were produced, while excess consumption of NADPH (50.2 nmol) was detected. In the presence of SOD (100 U), PQ added was completely converted to  $PQH_2$  and NADPH consumption was decreased by 18.3%. Unlike SOD, catalase (100 or 1000 U) and mannitol (100 or 500 mM), which are scavengers for  $H_2O_2$  and  $\cdot OH$ , respectively, had no effect on  $PQH_2$  formation (data not shown).

### Characterization of enzyme(s) responsible for PQH<sub>2</sub> formation

As PQ-dependent NADPH consumption [22,23,29,34–38] and oxygen uptake [39,40] have shown that a number of enzymes could participate in the reduction of PQ, the effects of typical inhibitors for quinone reductases on PQH<sub>2</sub> formation by rat liver cytosol were examined. Dicumarol (<0.01 μM) and cibacron blue (<0.1 μM) were used as inhibitors of NQO1 [38,41], quercetin (<0.1 μM) for NQO2 and AKR1C isozyme [25,42], and indomethacin (<0.1 μM) for AKR1C9 [38] inhibitors decreased PQH<sub>2</sub> formation in a concentration-dependent manner (data not shown). Thus, these observations suggested that multiple NADPH-dependent enzymes participate in the two-electron reduction of PQ in this preparation.

To further investigate the role of superoxide in the PQ-dependent consumption of NADPH, a fractionation study was performed. NQO1, an isozyme of the AKR superfamily, AKR7A2, and TrxR1 are tightly bound by cibacron blue resin [42,43], so this property was used on the lung cytosol from ethoxyquin-treated mice. Ethoxyquin is an inducer of AKR enzymes [29], so animals treated with this compound would be expected to have high PQH<sub>2</sub> formation. Thus, lung cytosol was subjected to Blue Sepharose column chromatography and the fractions collected were monitored for protein content and PQH<sub>2</sub> formation (Fig. 3A). As shown in Fig. 3A, there were at least three peaks, unbound fraction (B-I), KCl-eluted fraction (B-II), and KCl/NADH-eluted fraction (B-III), that exhibited PQH<sub>2</sub> formation. Each fraction contained a variety of proteins on SDS-PAGE (Fig. 3B). As shown in Fig. 3C, fraction B-I, which was the void volume, contained cytochrome P450 reductase (P450R), a microsomal protein contaminant that catalyzes the one-electron reduction of PQ [11] and Cu,Zn-SOD. Fraction B-III contained both AKR7A2 and NQO1 (Fig. 3C). But PQH<sub>2</sub> production by B-III was less than that by B-I (Fig. 3D). These observations suggested that the presence of Cu,Zn-SOD in fraction B-I may affect the observed PQH<sub>2</sub> levels because of its sensitivity to superoxide which could be generated from the reaction of PQH<sub>2</sub> with molecular oxygen. As expected, exogenous addition of Cu,Zn-SOD to an incubation mixture of PQ with B-II and B-III in the presence of NADPH caused an approximately 20-fold enhancement of PQH<sub>2</sub> formation, while no alteration of the product formation by B-I was detected (Fig. 3D). Thus, these results indicated that Cu,Zn-SOD blocks the superoxide-dependent oxidation of PQH<sub>2</sub> produced from PQ.

### Two-electron reduction of PQ by AKR1C isozyme accompanied by redox cycling

The experiments with specific inhibitors for AKR isozymes (data not shown) suggested that enzymes belonging to the AKR superfamily could catalyze reduction of PQ to PQH<sub>2</sub> in rat liver. Consistent with this notion, PQH<sub>2</sub> formation by a cytosol fraction of rat liver containing an AKR1C isozyme was approximately 11-fold of that by lung cytosol from ethoxyquin-treated mice (1317 nmol/mg/5 min vs 118 nmol/mg/5 min). Further studies of PQ reduction were

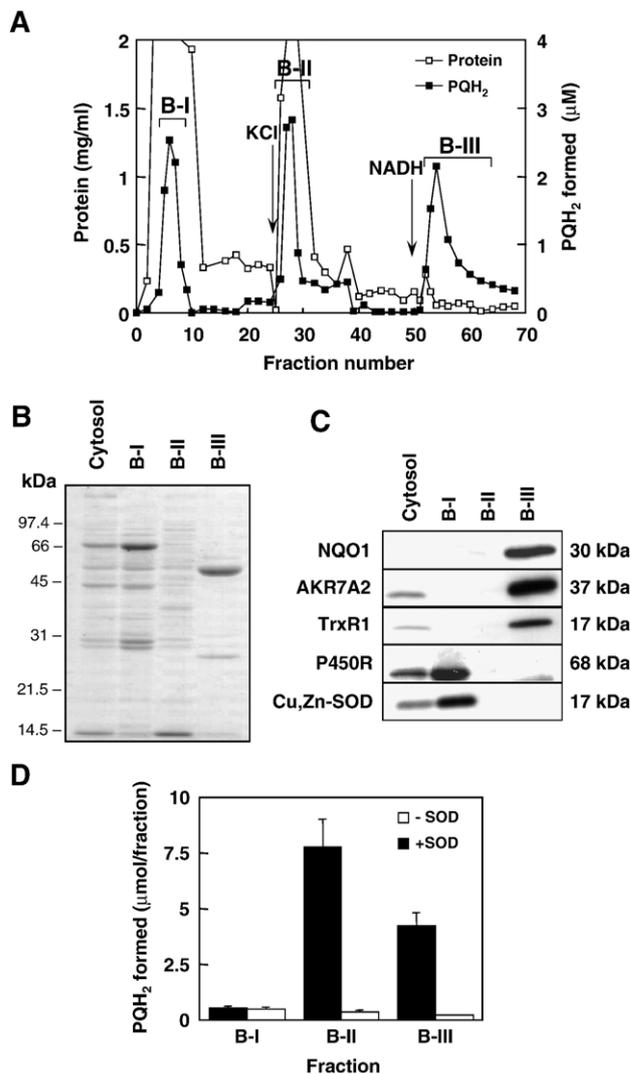


Fig. 3. Separation of two-electron reductases of PQ by Blue Sepharose column chromatography. (A) Lung cytosol from ethoxyquin-treated mice precipitating 90% ammonium sulfate saturation was dissolved in 50 mM Tris-HCl (pH 7.2)–0.25 M sucrose and then dialyzed against the same buffer. The dialyzed sample (170 mg of protein) was applied to the affinity column (2.5 × 1.5 cm, i.d.) and then eluted under the conditions as described under Materials and methods. Finally, fraction B-I (passed through fraction, No. 4–9), fraction B-II (0.5 M KCl-eluted fraction, No. 25–32), and fraction B-III (0.5 M KCl–10 mM NADH-eluted fraction, No. 52–64) were collected. (B) Each fraction (10 μg/well) was subjected to SDS-PAGE. (C) Western blot analysis was performed under the conditions as described under Materials and methods. (D) PQH<sub>2</sub> formation in each fraction in the absence or presence of SOD from bovine erythrocytes (100 U). Each value is the mean ± SD of three determinations.

conducted with a highly purified preparation of rabbit liver AKR1C isozyme [25]. This preparation is a homogeneous protein but its identity has not been established. During the two-electron reduction of PQ by AKR1C isozyme, excess NADPH was consumed (9200 nmol/mg/5 min in the absence of SOD). The role of oxygen in NADPH consumption was further examined under anaerobic conditions. Incubation of PQ with the AKR1C isozyme (2.98 mU) at 25°C for 5 min under anaerobic conditions resulted in 2.5-fold higher levels

of PQH<sub>2</sub> than those obtained under aerobic conditions, suggesting that PQH<sub>2</sub> rapidly undergoes autoxidation to PQ as confirmed by HPLC (see Fig. 1B, f). This notion was further supported by a purified AKR1C isozyme concentration-dependent oxygen consumption as determined with an oxygen electrode during the two-electron reduction of PQ (5 μM) by AKR1C isozyme in the presence of 0.2 mM NADPH (data not shown). To demonstrate that a semiquinone radical species (PQ<sup>•-</sup>) was an intermediate in the superoxide-dependent formation of PQ and PQH<sub>2</sub>, an ESR study determining the presence of radicals was conducted. Generation of superoxide was determined by its adduct, DMPO-OOH, during the two-electron reduction of PQ under aerobic conditions (Fig. 4A). Addition of SOD (100 U/mL) quenched generation of superoxide (data not shown). In Fig. 4B, an AKR1C isozyme concentration-dependent generation of superoxide was quantified. An equivalent incubation conducted in the absence of oxygen resulted in the appearance of PQ<sup>•-</sup> (*g*-value=2.00395) as identified in Valavanidis et al. [43] (Fig. 4C).

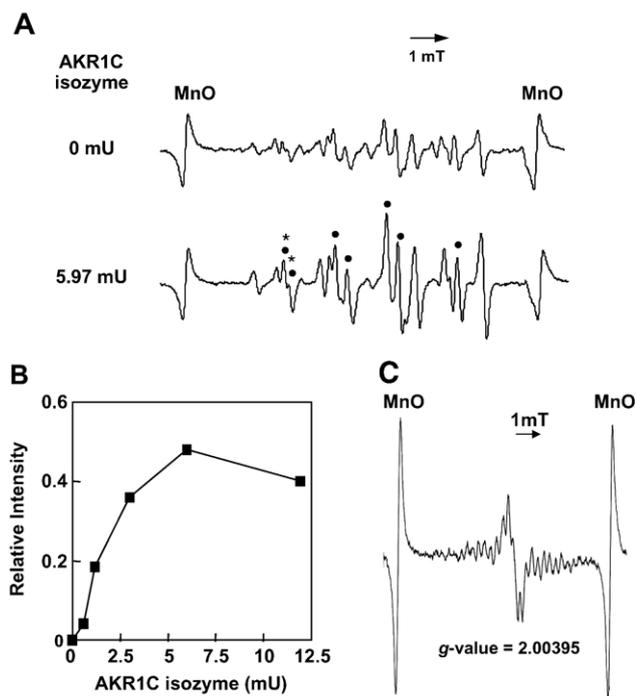


Fig. 4. Generation of superoxide (A, B) and semiquinone radical (C) by two-electron reduction of PQ. (A) ESR spectra. AKR1C isozyme (0 or 5.97 mU) was reacted with 5 μM PQ, 0.2 mM NADPH, 3.3% DMSO, 0.897 M DMPO, and 0.1 M HEPES (pH 7.6)–0.1 mM EDTA. The instrument settings were as follows: modulation width, 100 kHz–0.1 mT; sweep time, 4 min; time constant, 0.3 s; microwave power, 7.99 mW; microwave frequency, 9417 MHz; magnetic field, 335.4±5 mT. Closed circle indicates DMPO-OOH adduct. (B) Enzyme concentration-dependent superoxide generation. The area (\*) was quantified. (C) AKR1C isozyme (1.19 mU) was reacted with 0.2 mM NADPH, 0.1 mM PQ, and 0.1 M HEPES (pH 7.6)–0.1 mM EDTA. PQ was dissolved in DMSO (final concentration of 1%). The instrument settings were as follows: modulation width, 100 kHz–0.4 mT; sweep time, 10 s; time constant, 0.01 s; microwave power, 7.99 mW; microwave frequency, 9417 MHz; magnetic field, 335.4±5 mT.

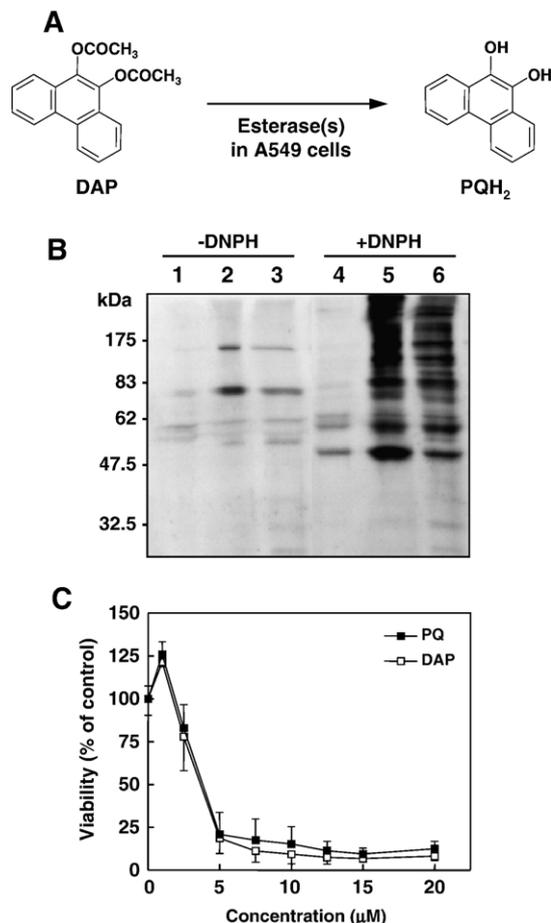


Fig. 5. Oxidative damage by PQH<sub>2</sub> generated by hydrolysis of DAP by esterases in A549 cells. (A) Hydrolysis of DAP by esterases in A549 cells. (B) Accumulation of oxidative modified cellular proteins following treatment with PQ or DAP. Each value is the mean ± SD of eight determinations. Lane 1, control/–DNP, lane 2, 10 μM PQ/–DNP, lane 3, 10 μM DAP/–DNP, lane 4, control/+DNP, lane 5, 10 μM PQ/+DNP, lane 6, 10 μM DAP/+DNP. (C) Cytotoxicity of PQ or DAP in A549 cells.

#### Oxidative protein modification and cellular toxicity caused by PQH<sub>2</sub>

It is well recognized that esters readily undergo hydrolysis by esterase in cells. When DAP was incubated with A549 cell lysate, decreased DAP levels accompanied by PQ appearance were observed (data not shown), suggesting that esterase in the cell is(are) capable of hydrolyzing DAP to PQH<sub>2</sub> (Fig. 5A) which, in turn, is autoxidized to PQ. To investigate whether PQH<sub>2</sub> itself causes protein oxidation and cellular toxicity, A549 cells were exposed to PQ and DAP separately. Both PQ and DAP caused a marked protein oxidation of equal degree as shown in Fig. 5B. Cytotoxicity of DAP, as evaluated by the MTT assay, was identical with that of PQ (LD<sub>50</sub>=2.76±1.03 μM) (Fig. 5C).

#### Discussion

Almost 20 years ago, Kalyanaram et al. [33] established an ESR study of *o*-semiquinones formed during the enzymatic

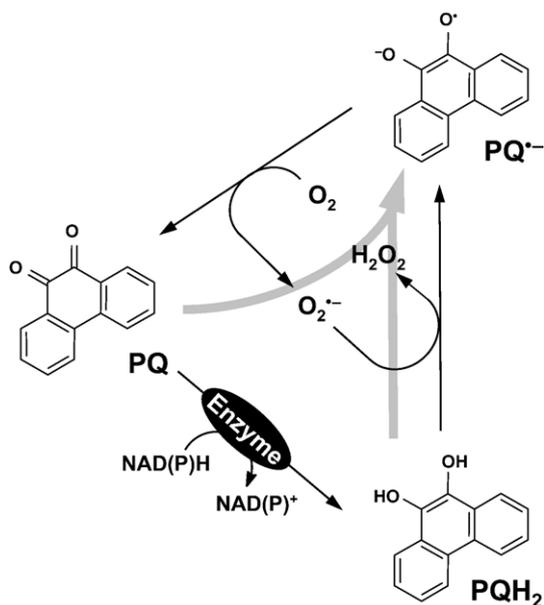


Fig. 6. Summary of PQ redox cycling through two-electron reduction.

and autoxidation of catechol by using spin-stabilizing metal ions,  $Zn^{2+}$  and/or  $Mg^{2+}$ . This method is useful for characterizing semiquinones of catechol estrogen [44], epinephrine [45], and catecholamine [33]. We previously showed that  $PQ^{\bullet-}$  produced enzymatically or nonenzymatically by one-electron reduction of PQ can be a redox cycle intermediate to propagate PQ toxicity [8,10]. It has not yet been shown how another reducing reaction, two-electron reduction, is responsible for detoxification and intoxication of PQ. To address this question, we employed a HPLC assay to detect the diacetoxy derivative of  $PQH_2$  formed during enzymatic reduction of PQ.

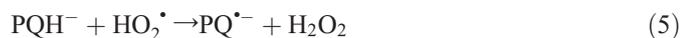
Reduction of PQ by crude and purified enzyme preparations has been extensively studied by monitoring NADPH consumption [22,23,29,34–38]. Studies with a purified two-electron reductase and PQ-dependent NADPH consumption have proposed that this quinone is converted to  $PQH_2$  with excess NADPH consumption associated with redox cycling [37]. Although a consensus from the observations indicates that this redox cycling is associated with two-electron reduction of PQ, direct identification of  $PQH_2$  has not been reported. It was demonstrated for the first time that  $PQH_2$  is a product of an AKR1C isozyme-mediated reduction of PQ. When crude enzyme preparations were incubated with PQ in the presence of NADPH,  $PQH_2$  production and superoxide generation were observed while excess NADPH was consumed. However, either  $N_2$  bubbling or exogenous addition of Cu,Zn-SOD decreased the autoxidation of  $PQH_2$  to PQ and decreased NADPH consumption, suggesting that the  $PQH_2$  formed is redox cycling with both molecular oxygen and superoxide. Hydroquinones, however, are thought not to react with oxygen due to having a high  $pK_a$ . The consistent material balance (Fig. 2A) observed at varying protein concentrations indicates that  $PQH_2$  is the only product formed. Although Cu,Zn-SOD is present in the enzyme preparations examined, the exogenous addition of Cu,Zn-SOD to an incubation mixture

effectively blocked autoxidation of  $PQH_2$  and NADPH consumption, suggesting that superoxide continuously generated during the reaction participates in the oxidation of  $PQH_2$  to PQ.

Enzymes catalyzing  $PQH_2$  formation were found in a variety of tissues including lung and were mainly localized in cytosol. Experiments with inhibitors for NQO1, NQO2, and AKR isozymes indicated that these enzymes contribute to the two-electron reduction of PQ (data not shown). Because pyridine nucleotide-requiring proteins such as NQO1 and AKR isozymes are tightly bound to Blue Sepharose resin [46,47], we attempted to separate these proteins from the crude enzyme preparation of mouse lung. Blue Sepharose column chromatography revealed that multiple proteins are associated with  $PQH_2$  formation in mouse lung cytosol. Western blot analysis of the catalytically active fractions showed that P450R, which catalyzes the one-electron reduction of PQ to  $PQ^{\bullet-}$  and generates superoxide [11], and Cu,Zn-SOD were contained in fraction B-I. Fraction B-III contained AKR7A2, which is an effective catalyst for PQ reduction [48], and NQO1, a two-electron reductase of quinones [17]. However,  $PQH_2$  levels were much higher in incubation mixtures of B-I than of B-III (Fig. 4C), suggesting that extensive autoxidation of the  $PQH_2$  formed by AKR7A2 and NQO1 occurs in the absence of Cu,Zn-SOD. Consistent with this notion, exogenous addition of Cu,Zn-SOD to an incubation mixture containing B-III resulted in a markedly increased  $PQH_2$  level (approximately 20-fold) whereas additional Cu,Zn-SOD did not affect  $PQH_2$  levels in B-I. These observations indicate that  $PQH_2$  is a redox-active chemical whose cycling is blocked by Cu,Zn-SOD.

Further characterization of the reactions during the two-electron reduction of PQ utilized a purified preparation of an AKR1C isozyme obtained from rabbit liver [25]. As expected, PQ was efficiently reduced to  $PQH_2$  (Eq. (1)) by this enzyme but with excess NADPH consumption. Formation of  $PQH_2$  under anaerobic conditions was 2.5-fold greater than that under aerobic conditions, indicating that formation of  $PQH_2$  is dependent on molecular oxygen. Produced  $PQH_2$  is known to interact with PQ through a disproportionation reaction to yield the semiquinone radicals (Eq. (2)) [49]. This notion is supported by a result that more than 4  $\mu M$  PQ added decreased  $PQH_2$  formation, with increasing PQ remained (Fig. 2B). This phenomenon indicates that PQ reacts with its own two-electron reducing form,  $PQH_2$ , to disproportionate to  $PQ^{\bullet-}$ .  $PQ^{\bullet-}$  also reacts rapidly with molecular oxygen to yield PQ and superoxide (Eq. (3)). The generation of superoxide was confirmed by the presence of its DMPO-OOH adduct using ESR techniques (Fig. 5A). Since the autoxidation of  $PQH_2$  was blocked by Cu,Zn-SOD, superoxide derived from molecular oxygen (Eq. (3)) is thought to be responsible for autoxidation of  $PQH_2$ . Since superoxide has a  $pK_a$  of approximately 4, it is postulated that the hydroquinone spontaneously deprotonates or partially interacts with superoxide (Eq. (4)) [24], after which it would be readily oxidized to form the semiquinone radical (Eq. (5)) (Fig. 6).





The semiquinone radical disproportionates readily to quinone and hydroquinone (Eq. (2)) [37]. Superoxide formed during the reduction of PQ is chemically (Eq. (6)) and/or enzymatically (Eqs. (7) and (8)) disproportionated to hydrogen peroxide. By eliminating superoxide, Cu,Zn-SOD terminates the autoxidation of PQH<sub>2</sub> (see Fig. 3D).



We have recently reported that exposure of A549 cells to PQ caused a marked oxidation of cellular proteins evaluated by protein carbonyl formation, together with selective down-regulation of Cu,Zn-SOD [11]. Palackal et al. [50] reported previously that AKR1C isozymes are highly expressed in A549 cells, and in preliminary experiments, we found that A549 cell lysate is capable of producing PQH<sub>2</sub> from PQ (S. Fujii et al., unpublished observation). Moreover, experiments with DAP as a precursor of PQH<sub>2</sub> indicated strongly that PQH<sub>2</sub> itself plays a major role in the oxidative protein modification. Taken together, the oxidative protein damage in A549 cells caused by PQ could be explained by the initial two-electron reduction of PQ to PQH<sub>2</sub> followed by oxidation of the latter to the semiquinone by oxygen to generate superoxide. The superoxide can then act to propagate the redox cycling process by overwhelming the inherent SOD.

Cadenas [19] previously classified hydroquinones, the two-electron reduction products of quinones: (i) redox-stable hydroquinones (e.g., 2-methyl-1,4-naphthoquinone) [17], (ii) redox-labile hydroquinones (e.g., 2-methyl-3-glutathionyl-1,4-naphthoquinone) [51], and (iii) alkylating hydroquinones (e.g., AZQ) [52]. Redox-labile hydroquinones (ii) are autoxidized to undergo redox cycling, whereas redox-stable hydroquinones (i) are conjugated with sulfate and glucuronate, resulting in their detoxification. Alkylating hydroquinones (iii) yield alkylation products (e.g., DNA-quinone) by bioreductive alkylation, leading to toxicity. Autoxidation of redox-labile hydroquinones (ii) can be inhibited (ii-a) or stimulated by SOD (ii-b), by stabilizing redox unstable hydroquinones or generating hydrogen peroxide, respectively [19]. From the present findings, we conclude that PQ is classified as (ii-a).

In conclusion, this is the first report to identify PQH<sub>2</sub> as the product of a NADPH-dependent two-electron reduction of PQ. The direct assay of PQH<sub>2</sub> allowed us to determine the stoichiometric relationship between NADPH consumption and

PQ reduction. Although previous studies based on NADPH consumption have demonstrated that PQ is a good substrate for NQO1 [34], AKR1C9 [38,53], AKR1C12 [54], AKR1C13 [54], AKR7A1 [55], AKR7A2 [48], AKR7A3 [55], and AKR7A5 [56], they were missing the mechanistic details of the redox cycling of PQ to PQH<sub>2</sub> that are associated with oxidative stress by these enzymes. The present study suggests that reduction of PQ to PQH<sub>2</sub> by AKR isozymes or NQO1 can also cause oxidative protein damage due to PQ exposure.

## Acknowledgments

We thank Dr. John D. Hayes of the Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, UK, for providing antibody against AKR7A2 isozyme; Dr. Nobuyuki Koga of the Faculty of Nutritional Sciences, Nakamura Gakuen University, Japan, for donation of antibody against NQO1; and Dr. Nobuhiro Shimojo of the Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, Japan, for his encouragement, and M.S. Rie Koizumi and M.S. Yuya Nishihara of the Master's Program in Environmental Sciences, University of Tsukuba, for their excellent contribution to this study. We are especially grateful to Ms. F. Miyamasu for grammatical correction in the preparation of the manuscript. This research was supported in part by a Grant-in-Aid Nos. 15390184, 18659167, and 18406003 (Y.K.) for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan. K.T. was awarded an Ishidu Shun Memorial Scholarship. Although the research described in this article has been funded in part by the United States Environmental Protection Agency through Grant R827352-01-0 to UCLA, it has not been subjected to the agency's required peer and policy review and therefore does not necessarily reflect the views of the agency and no official endorsement should be inferred.

## References

- [1] Sakakibara, M.; Minami, M.; Endo, T.; Hirafuji, M.; Murakami, S.; Mori, Y.; Sagai, M. Biological effects of diesel exhaust particles (DEP) on isolated cardiac muscle of guinea pigs. *Res. Commun. Mol. Pathol. Pharmacol.* **86**:99–110; 1994.
- [2] Diaz-Sanchez, D. The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. *Allergy* **52**:52–56; discussion 57–58; 1997.
- [3] McClellan, R. O. Health effects of exposure to diesel exhaust particles. *Annu. Rev. Pharmacol. Toxicol.* **27**:279–300; 1987.
- [4] Xiao, G. G.; Wang, M.; Li, N.; Loo, J. A.; Nel, A. E. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. *J. Biol. Chem.* **278**:50781–50790; 2003.
- [5] Hirano, S.; Furuyama, A.; Koike, E.; Kobayashi, T. Oxidative-stress potency of organic extracts of diesel exhaust and urban fine particles in rat heart microvessel endothelial cells. *Toxicology* **187**:161–170; 2003.
- [6] Schuetzle, D. Sampling of vehicle emissions for chemical analysis and biological testing. *Environ. Health Perspect.* **47**:65–80; 1983.
- [7] Li, N.; Venkatesan, M. I.; Miguel, A.; Kaplan, R.; Gujuluva, C.; Alam, J.; Nel, A. Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J. Immunol.* **165**:3393–3401; 2000.

- [8] Kumagai, Y.; Arimoto, T.; Shinyashiki, M.; Shimojo, N.; Nakai, Y.; Yoshikawa, T.; Sagai, M. Generation of reactive oxygen species during interaction of diesel exhaust particle components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. *Free Radic. Biol. Med.* **22**:479–487; 1997.
- [9] Cho, A. K.; Di Stefano, E.; You, Y.; Rodriguez, C. E.; Schmitz, D. A.; Kumagai, Y.; Miguel, A. H.; Eiguren-Fernandez, A.; Kobayashi, T.; Avol, E.; Froines, J. Determination of four quinones in diesel exhaust particles, SRM 1649a, and atmospheric PM<sub>2.5</sub>. *Aerosol Sci. Tech.* **38**:1–14; 2004.
- [10] Kumagai, Y.; Koide, S.; Taguchi, K.; Endo, A.; Nakai, Y.; Yoshikawa, T.; Shimojo, N. Oxidation of proximal protein sulfhydryls by phenanthraquinone, a component of diesel exhaust particles. *Chem. Res. Toxicol.* **15**:483–489; 2002.
- [11] Sugimoto, R.; Kumagai, Y.; Nakai, Y.; Ishii, T. 9,10-Phenanthraquinone in diesel exhaust particles downregulates Cu,Zn-SOD and HO-1 in human pulmonary epithelial cells: intracellular iron scavenger 1,10-phenanthroline affords protection against apoptosis. *Free Radic. Biol. Med.* **38**:388–395; 2005.
- [12] Rodriguez, C. E.; Shinyashiki, M.; Froines, J.; Yu, R. C.; Fukuto, J. M.; Cho, A. K. An examination of quinone toxicity using the yeast *Saccharomyces cerevisiae* model system. *Toxicology* **201**:185–196; 2004.
- [13] Rodriguez, C. E.; Fukuto, J. M.; Taguchi, K.; Froines, J.; Cho, A. K. The interactions of 9,10-phenanthrenequinone with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a potential site for toxic actions. *Chem. Biol. Interact.* **155**:97–110; 2005.
- [14] Hiyoshi, K.; Takano, H.; Inoue, K.; Ichinose, T.; Yanagisawa, R.; Tomura, S.; Cho, A. K.; Froines, J. R.; Kumagai, Y. Effects of a single intratracheal administration of phenanthraquinone on murine lung. *J. Appl. Toxicol.* **25**:47–51; 2005.
- [15] Kumagai, Y.; Hayashi, T.; Miyauchi, T.; Endo, A.; Iguchi, A.; Kiriya-Sakai, M.; Sakai, S.; Yuki, K.; Kikushima, M.; Shimojo, N. Phenanthraquinone inhibits eNOS activity and suppresses vasorelaxation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281**:R25–R30; 2001.
- [16] Kumagai, Y.; Nakajima, H.; Midorikawa, K.; Homma-Takeda, S.; Shimojo, N. Inhibition of nitric oxide formation by neuronal nitric oxide synthase by quinones: nitric oxide synthase as a quinone reductase. *Chem. Res. Toxicol.* **11**:608–613; 1998.
- [17] Lind, C.; Hochstein, P.; Ernster, L. DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch. Biochem. Biophys.* **216**:178–185; 1982.
- [18] Cadenas, E.; Hochstein, P.; Ernster, L. Pro- and antioxidant functions of quinones and quinone reductases in mammalian cells. *Adv. Enzymol. Relat. Areas Mol. Biol.* **65**:97–146; 1992.
- [19] Cadenas, E. Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem. Pharmacol.* **49**:127–140; 1995.
- [20] Buckner, M.; Glatt, H. R.; Platt, K. L.; Avnir, D.; Ittah, Y.; Blum, J.; Oesch, F. Mutagenicity of phenanthrene and phenanthrene K-region derivatives. *Mutat. Res.* **66**:337–348; 1979.
- [21] Siegel, D.; Gibson, N. W.; Preusch, P. C.; Ross, D. Metabolism of diaziquone by NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase): role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. *Cancer Res.* **50**:7293–7300; 1990.
- [22] Jarabak, R.; Jarabak, J. Effect of ascorbate on the DT-diaphorase-mediated redox cycling of 2-methyl-1,4-naphthoquinone. *Arch. Biochem. Biophys.* **318**:418–423; 1995.
- [23] Klein, J.; Post, K.; Seidel, A.; Frank, H.; Oesch, F.; Platt, K. L. Quinone reduction and redox cycling catalysed by purified rat liver dihydrodiol/3 alpha-hydroxysteroid dehydrogenase. *Biochem. Pharmacol.* **44**:341–349; 1992.
- [24] Hiramatsu, M.; Kumagai, Y.; Unger, S. E.; Cho, A. K. Metabolism of methylenedioxymethamphetamine: formation of dihydroxymethamphetamine and a quinone identified as its glutathione adduct. *J. Pharmacol. Exp. Ther.* **254**:521–527; 1990.
- [25] Yamano, S.; Ichinose, F.; Todaka, T.; Toki, S. Purification and characterization of two major forms of naloxone reductase from rabbit liver cytosol, new members of aldo-keto reductase superfamily. *Biol. Pharm. Bull.* **22**:1038–1046; 1999.
- [26] Shimojo, N.; Kumagai, Y.; Homma-Takeda, S.; Shinyashiki, M.; Takasawa, N.; Kushida, K. Isozyme selective induction of mouse pulmonary superoxide dismutase by the exposure to mercury vapor. *Environ. Toxicol. Pharmacol.* **2**:35–37; 1996.
- [27] Kumagai, Y.; Tsurutani, Y.; Shinyashiki, M.; Homma-Takeda, S.; Nakai, Y.; Yoshikawa, T.; Shimojo, N. Bioactivation of lapachol responsible for DNAS scission by NADPH-cytochrome P450 reductase. *Environ. Toxicol. Pharm.* **3**:245–250; 1997.
- [28] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254; 1976.
- [29] Ellis, E. M.; Judah, D. J.; Neal, G. E.; O'Connor, T.; Hayes, J. D. Regulation of carbonyl-reducing enzymes in rat liver by chemoprotectors. *Cancer Res.* **56**:2758–2766; 1996.
- [30] Kyhse-Andersen, J. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10**:203–209; 1984.
- [31] Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**:271–277; 1986.
- [32] Levine, R. L.; Williams, J. A.; Stadtman, E. R.; Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233**:346–357; 1994.
- [33] Kalyanaraman, B.; Felix, C. C.; Sealy, R. C. Peroxidative oxidation of catecholamines. A kinetic electron spin resonance investigation using the spin stabilization approach. *J. Biol. Chem.* **259**:7584–7589; 1984.
- [34] Chesis, P. L.; Levin, D. E.; Smith, M. T.; Ernster, L.; Ames, B. N. Mutagenicity of quinones: pathways of metabolic activation and detoxification. *Proc. Natl. Acad. Sci. USA* **81**:1696–1700; 1984.
- [35] Wermuth, B.; Platts, K. L.; Seidel, A.; Oesch, F. Carbonyl reductase provides the enzymatic basis of quinone detoxication in man. *Biochem. Pharmacol.* **35**:1277–1282; 1986.
- [36] Jarabak, J.; Harvey, R. G. Studies on three reductases which have polycyclic aromatic hydrocarbon quinones as substrates. *Arch. Biochem. Biophys.* **303**:394–401; 1993.
- [37] Jarabak, R.; Harvey, R. G.; Jarabak, J. Redox cycling of polycyclic aromatic hydrocarbon o-quinones: metal ion-catalyzed oxidation of catechols bypasses inhibition by superoxide dismutase. *Chem. Biol. Interact.* **115**:201–213; 1998.
- [38] Del Bello, B.; Maellaro, E.; Sugherini, L.; Santucci, A.; Comperti, M.; Casini, A. F. Purification of NADPH-dependent dehydroascorbate reductase from rat liver and its identification with 3 alpha-hydroxysteroid dehydrogenase. *Biochem. J.* **304** (Pt 2):385–390; 1994.
- [39] Munday, R. Concerted action of DT-diaphorase and superoxide dismutase in preventing redox cycling of naphthoquinones: an evaluation. *Free Radic. Res.* **35**:145–158; 2001.
- [40] Thor, H.; Smith, M. T.; Hartzell, P.; Bellomo, G.; Jewell, S. A.; Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J. Biol. Chem.* **257**:12419–12425; 1982.
- [41] Prochaska, H. J. Purification and crystallization of rat liver NAD(P)H:(quinone-acceptor) oxidoreductase by cibacron blue affinity chromatography: identification of a new and potent inhibitor. *Arch. Biochem. Biophys.* **267**:529–538; 1988.
- [42] Wu, K.; Knox, R.; Sun, X. Z.; Joseph, P.; Jaiswal, A. K.; Zhang, D.; Deng, P. S.; Chen, S. Catalytic properties of NAD(P)H:quinone oxidoreductase-2 (NQO2), a dihydronicotinamide riboside dependent oxidoreductase. *Arch. Biochem. Biophys.* **347**:221–228; 1997.
- [43] Valavanidis, A.; Fiotakis, K.; Bakeas, E.; Vlahogianni, T. Electron paramagnetic resonance study of the generation of reactive oxygen species catalysed by transition metals and quinoid redox cycling by inhalable ambient particulate matter. *Redox Rep.* **10**:37–51; 2005.
- [44] Kalyanaraman, B.; Felix, C. C.; Sealy, R. C. Semiquinone anion radicals of catechol(amine)s, catechol estrogens, and their metal ion complexes. *Environ. Health Perspect.* **64**:185–198; 1985.
- [45] Kalyanaraman, B.; Felix, C. C.; Sealy, R. C. Electron spin resonance-spin stabilization of semiquinones produced during oxidation of epinephrine and its analogues. *J. Biol. Chem.* **259**:354–358; 1984.
- [46] Kelly, V. P.; Ireland, L. S.; Ellis, E. M.; Hayes, J. D. Purification from rat

- liver of a novel constitutively expressed member of the aldo-keto reductase 7 family that is widely distributed in extrahepatic tissues. *Biochem. J.* **348** (Pt 2):389–400; 2000.
- [47] Sharkis, D. H.; Swenson, R. P. Purification by cibacron blue F3GA dye affinity chromatography and comparison of NAD(P)H:quinone reductase (E.C.1.6.99.2) from rat liver cytosol and microsomes. *Biochem. Biophys. Res. Commun.* **161**:434–441; 1989.
- [48] O'Connor, T.; Ireland, L. S.; Harrison, D. J.; Hayes, J. D. Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. *Biochem. J.* **343** (Pt 2):487–504; 1999.
- [49] O'Brien, P. J. Molecular mechanisms of quinone cytotoxicity. *Chem. Biol. Interact.* **80**:1–41; 1991.
- [50] Palackal, N. T.; Lee, S. H.; Harvey, R. G.; Blair, I. A.; Penning, T. M. Activation of polycyclic aromatic hydrocarbon trans-dihydrodiol proximate carcinogens by human aldo-keto reductase (AKR1C) enzymes and their functional overexpression in human lung carcinoma (A549) cells. *J. Biol. Chem.* **277**:24799–24808; 2002.
- [51] Buffinton, G. D.; Ollinger, K.; Brunmark, A.; Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates. Effect of substituents on autoxidation rates. *Biochem. J.* **257**:561–571; 1989.
- [52] Gibson, N. W.; Hartley, J. A.; Butler, J.; Siegel, D.; Ross, D. Relationship between DT-diaphorase-mediated metabolism of a series of aziridinybenzoquinones and DNA damage and cytotoxicity. *Mol. Pharmacol.* **42**:531–536; 1992.
- [53] Penning, T. M.; Mukharji, I.; Barrows, S.; Talalay, P. Purification and properties of a 3 alpha-hydroxysteroid . of rat liver cytosol and its inhibition by anti-inflammatory drugs. *Biochem. J.* **222**:601–611; 1984.
- [54] Ikeda, S.; Okuda-Ashitaka, E.; Masu, Y.; Suzuki, T.; Watanabe, K.; Nakao, M.; Shingu, K.; Ito, S. Cloning and characterization of two novel aldo-keto reductases (AKR1C12 and AKR1C13) from mouse stomach. *FEBS Lett.* **459**:433–437; 1999.
- [55] Knight, L. P.; Primiano, T.; Groopman, J. D.; Kensler, T. W.; Sutter, T. R. cDNA cloning, expression and activity of a second human aflatoxin B1-metabolizing member of the aldo-keto reductase superfamily. *AKR7A3. Carcinogenesis* **20**:1215–1223; 1999.
- [56] Hinshelwood, A.; McGarvie, G.; Ellis, E. Characterisation of a novel mouse liver aldo-keto reductase AKR7A5. *FEBS Lett.* **523**:213–218; 2002.