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Redox Cycling of 1,2-Naphthoquinone by Thioredoxin1 through Cys32 and Cys35 Causes Inhibition of Its Catalytic Activity and Activation of ASK1/p38 Signaling

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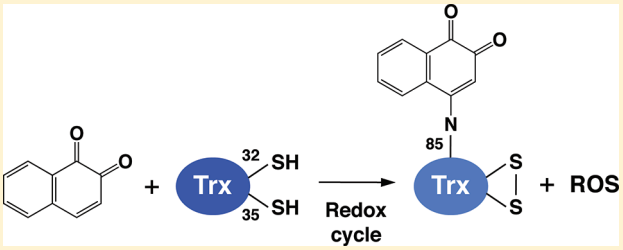
Supporting Information

ABSTRACT: 1,2-Naphthoquinone (1,2-NQ) is an atmospheric chemical capable of (1) redox cycling with electron donors and (2) covalent modification of nucleophilic groups on proteins. In the present study, we investigated its interaction with the redox protein, thioredoxin1 (Trx1), which led to oxidative stress-dependent cell damage. In experiments with purified wild-type Trx1 and its double mutant (32S/35S Trx1), we found that incubation of Trx1 with 1,2-NQ resulted in a redox cycling reaction, generating superoxide and hydrogen peroxide involving Cys32 and Cys35 and an arylation reaction resulting in covalent modification of Lys85 together with a loss of Trx activity. A significant fraction of the lost Trx1 activity following interaction with 1,2-NQ was restored by dithiothreitol. Exposure of RAW264.7 cells to 1,2-NQ generated reactive oxygen species (ROS) and caused a decrease in Trx activity. Trx is a negative regulator of apoptosis signal-regulating kinase 1 (ASK1), and under the conditions of the experiment, 1,2-NQ activated ASK1 and p38, leading to PARP cleavage and apoptotic cell death that were blocked by pretreatment with polyethylene glycol-catalase. These results suggest that Trx1 readily undergoes oxidative modification by 1,2-NQ through the proximal thiols Cys32 and Cys35. It seems likely that ROS production concomitant with decline in cellular Trx activity plays a role in the activation of ASK1/p38 signaling to promote apoptotic cell death cause by 1,2-NQ exposure.



INTRODUCTION

1,2-Naphthoquinone (1,2-NQ) is found at significant concentrations in vehicle exhaust particles and in ambient air samples.[1] Several lines of evidence suggest that quinoid compounds such as 1,2-NQ exhibit two chemical properties[2,3] of toxicological interest. One is their electron transfer abilities, transferring electrons from reducing agents to oxygen to generate reactive oxygen species (ROS), as shown by the electron transfer from dithiothreitol (DTT) to oxygen by 1,2-NQ.[4,5] The second property is their electrophilic character, which makes possible covalent attachment to protein nucleophiles (e.g., cysteine, histidine, and lysine), resulting in the disruption of key cellular proteins especially those containing active site nucleophiles. For example, sensor proteins with reactive thiols (thiolate ions) such as protein tyrosine phosphatase 1B (PTP1B), cAMP response element−binding protein, and kelch-like ECH-associated protein 1 (Keap1) undergo *S*-arylation by 1,2-NQ, thereby altering their functions.[6−8] Thus, it is likely that 1,2-NQ could affect other cellular sensor proteins through redox cycling and/or covalent binding.

Thioredoxins (Trxs) are a family of small pleiotropic proteins that are ubiquitously expressed and evolutionarily conserved from prokaryotes to mammals.[9] Thioredoxin1 (Trx1) is a cytoplasmic protein and is a signaling intermediate in addition to its intrinsic antioxidant activity.[10,11] This redox protein has two redox-active cysteine residues in its conserved active-site sequence, −Cys32-Gly-Pro-Cys35−, which undergo reversible oxidation/reduction reactions at the active site and at additionally conserved cysteine residues (at positions 62, 69, and 73).[12] This redox protein has been implicated in the stimulation of cell proliferation, regulation of transcription of cell survival genes, and inhibition of apoptosis.[13,14] Of interest to our studies with 1,2-NQ was the fact that Trx1 negatively regulates apoptosis signal-regulating kinase 1 (ASK1) kinase activity by directly binding to it at its resting state. When cells are subjected to oxidative stress, the active sites Cys32 and Cys35 of Trx1 undergo reversible oxidation to form a disulfide bond with each other, thereby releasing and activating ASK1.[15] The activated ASK1, in turn, promotes activation of the JNK and p38 MAP kinase pathways which contribute to apoptosis.

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On the basis of these observations, we hypothesized that 1,2-NQ could interact with the vicinal Cys32 and Cys35 thiols of Trx1 to disrupt cellular redox balance and cause substantial apoptosis. To address such a possibility, we investigated the contributions of 1,2-NQ to (1) the redox cycling of recombinant Trx1 via Cys32 and Cys35, leading to ROS generation, and/or (2) covalent modification of Trx1 by 1,2-NQ, resulting in loss of enzyme activity. We also examined the effects of 1,2-NQ on Trx1 activity, ASK1/p38 signaling and apoptotic cell death in mouse RAW264.7 cells.

EXPERIMENTAL PROCEDURES

Materials. 1,2-NQ and 1,2-dihydroxynaphthalene (1,2-NQH_2) were obtained from Tokyo Chemical Industries Co. (Tokyo, Japan). Commercially available 1,2-NQ with greater than 90% purity (based on HPLC analysis) was dissolved in DMSO in a black tube immediately before use. 1,2-NQ was stable in DMSO, but slowly decayed in the phosphate buffered incubation mixture at 25 °C over 2 h. Dimethyl sulfoxide (DMSO), DTT, sodium dodecyl sulfate (SDS), and 3,5-dimethoxy-4-hydroxycinnamic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Nacalai Tesque (Kyoto, Japan). *Escherichia coli* BL21 cells and sequence grade modified trypsin were purchased from Promega (Madison, WI). 2′,7′-Dichlorofluorescein diacetate (H_2DCFDA) was obtained from Invitrogen (Carlsbad, CA). α-Cyano-4-hydroxycinnamic acid was purchased from Bruker Daltonics Japan (Tokyo, Japan). Anti-actin antibody was obtained from Sigma-Aldrich (St Louis, MO). Anti-p38, anti-phosho-p38, and anti-PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ASK1 and anti-phosho-ASK1 antibodies were kindly provided by Dr. Ichijo (University of Tokyo, Japan). Acetylated cytochrome *c* was synthesized according to the method of Shinyashiki et al.[16] 1,2-Diacetoxynaphthalene (DAN) was prepared as previously described.[17] Polyclonal antibody against 1,2-NQ was prepared as reported previously.[18] Polyethylene glycol-catalase (PEG-CAT) was prepared as previously described.[19] All other reagents used were of the highest purity obtainable.

Immunoblot Analysis. Cells were extracted with RIPA lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl−0.5% deoxycholate−0.1% SDS, 1 mM EGTA, 0.4 mM EDTA, and protease inhibitor cocktail. Each sample was normalized to a protein content of 20 μg then mixed with half the volume of SDS−polyacrylamide gel electrophoresis (SDS−PAGE) loading buffer consisting 62.5 mM Tris-HCl (pH 6.8), 24% glycerol, 6% SDS, 15% 2-mercaptoethanol, and 0.015% bromophenol blue. The samples were heated for 1 min at 95 °C and were separated by SDS−PAGE. Electrophoresed proteins were transferred onto PVDF membranes (Bio-Rad). Blots were blocked for 1 h with 5% skim milk in TTBS (20 mM Tris (pH 7.5), 150 mM NaCl, and 1% Tween 20), washed briefly, and incubated with the indicated primary antibodies. HRP-conjugated goat antirabbit IgG was used as the secondary antibody. Protein bands on the membrane were detected using chemiluminescence reagents as per the manufacturer's instructions (Nacalai Tesque) and exposed on high-performance chemiluminescence film (GE Healthcare Bio-Sciences Corp.). Representative blots are shown from three independent experiments. Protein concentration was determined with the Bio-Rad Protein assay kit (Bio-Rad, Richmond, CA) or the BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL), with bovine serum albumin as a standard.

Purification of Wild Type of Human Trx1 (C32/C35 Trx1) and Its Double Mutant (32S/35S Trx1). The plasmids of pQE30-Trx1 and pQE30-Trx1(C32S/C35S) were kindly provided by Dr. Junji Yodoi (Institute for Virus Research, Kyoto University, Japan). All steps during purification were performed at 4 °C. *E. coli* BL21 cells transformed with the expression vector were suspended in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 10 mM 2-mercaptoethanol, and 10% glycerol, and the mixture was sonicated and centrifuged at 37,000*g* for 1 h. The supernatant was then applied to a Ni-IDA ProBond (Invitrogen) column (4 × 1 cm i.d.), which had been equilibrated with equilibration buffer (50 mM Tris-HCl (pH 7.5)−0.1 mM NaCl−10 mM 2-mercaptoethanol). The affinity column was extensively washed with the equilibration buffer, and then Trx1 and its mutants were eluted with the buffer containing 100 mM imidazole. The purity of C32/C35 Trx1 and 32S/35S Trx1 as assessed by SDS−PAGE was >90%. Thiol groups oxidized during purification were reduced by incubation with 10 mM DTT for 1 h, and DTT was removed by an Econo-Pac 10 DG column (Bio-Rad, Richmond, CA). The purified proteins were stored in 50 mM potassium phosphate buffer (pH 7) at −80 °C before use.

Trx Activity in Cell-Free and Cellular Systems. Trx1 activity was measured with the previously reported insulin disulfide reduction assay.[20] For cell-free experiments, recombinant human Trx1 was preincubated with 1,2-NQ or hydrogen peroxide (H_2O_2) at different concentrations in 50 mM potassium phosphate buffer (pH 7.5) (final volume 3 mL) for 30 min at 25 °C. To remove unbound 1,2-NQ, the reaction mixture was loaded onto an Econo-Pac 10 DG column. Then, Trx1 (10 μg) was incubated in a final reaction volume of 100 μL containing 100 mM HEPES (pH 7.6)−3 mM EDTA, 1 mM NADPH, insulin (2.5 mg/mL), and 0.2 μM yeast Trx reductase (American Diagnostica Inc., Greenwich, CT) for 20 min at 37 °C. The reaction was terminated by the addition of 1 mL of 6 M guanidine hydrochloride and 20 μL of 10 mM DTNB. Free thiols generated from insulin reduction were determined by the formation of dinitrobenzene thiol, measured at 412 nm, and the enzyme activity was measured as the increase in absorbance at 2 min.

For cellular experiments, cells were exposed to 1,2-NQ (10 or 20 μM) for 30 min, washed with PBS, and lysed in RIPA buffer. The cell lysates was subjected to an Econo-Pac 10 DG column to remove cellular GSH. The resulting cell lysate (25 μg) was incubated in a final reaction volume of 50 μL containing 85 mM HEPES (pH 7.6), 0.3 mM insulin, 660 μM NADPH, 2.5 mM EDTA, and 5 μM yeast Trx reductase for 20 min at 25 °C. A separate incubation in the absence of the yeast Trx reductase was conducted as a control. Then, 250 μL of a 200 mM Tris-HCl (pH 8) solution that was 1 mM in DTNB, 240 μM in NADPH, and 6 M in guanidine hydrochloride was added to the incubation mixture, and absorbance changes were measured at 412 nm. Trx activity for each lysate sample was obtained as the absorbance at 412 nm subtracted from that of the corresponding control.

Sulfhydryl Content. The free thiol content in C32/C35 Trx1 and 32S/35S Trx1 after treatment with 1,2-NQ were determined by the method of Ellman with modificaions.[21] Thus, C32/C35 Trx1 or 32S/35S Trx1 (200 μM, final concentration) was treated with 5 or 50 μM 1,2-NQ in a total volume of 200 μL containing 50 mM potassium phosphate buffer (pH 7.5) for 10 min at 25 °C. After reaction, 1.3 mL of a mixture of 1 mM DTNB and 8 M guanidine in 0.2 M Tris-HCl (pH 8) was added to the incubation mixture (final volume 1.5 mL). The absorbance was determined at 412 nm against a reference cuvette containing the same amount of DTNB without protein, and the number of free thiols was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.

Measurement of Superoxide and Hydrogen Peroxide. Superoxide was determined by measuring the reduction of acetylated cytochrome *c* at 550 nm as described previously.[4] Briefly, the incubation mixture (1.5 mL) consisted of 10 μM 1,2-NQ, 30 μM C32/C35 Trx1, 50 μM acetylated cytochrome *c*, and 50 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by addition of the quinone to a sample cuvette.

Hydrogen peroxide was determined according to the method of Hildebrandt and Roots.[22] The incubation mixture (1 mL) contained 5 μM 1,2-NQ, 40 μM C32/C35 Trx1 or 32S/35S Trx1, and 50 mM potassium phosphate buffer (pH 7.5). The reaction was performed at 25 °C for different time periods and terminated by the addition of 1 mL of 2.5% trichloroacetic acid. After the reaction mixture was centrifuged at 14,000*g* for 5 min, the supernatant (0.2 mL) was mixed with 1 mL of water, 0.24 mL of 10 mM ferrous ammonium sulfate, and 0.12 mL of 2.5 M potassium thiocyanate. Then, the absorption of each sample at 480 nm was measured. H_2O_2 was used as the calibration standard.

Matrix-Assisted Laser Desorption Ionization–Time-of-Flight–Mass Spectrometry (MALDI-TOF/MS). C32/C35 Trx1 was treated with 10 mM DTT to obtain the fully reduced form. After desalting by an Econo-Pac 10 DG column to remove excess DTT, the protein (20 μM) was incubated with and without 5 μM 1,2-NQ for 10 min at 25 °C in 50 mM Tris-HCl (pH 7.5) and then subjected to an Econo-Pac 10 DG column to remove free 1,2-NQ. The native and 1,2-NQ-modified Trx1 were mixed with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid containing 75% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature. For tryptic digestion peptide analysis, iodoacetamide was added to the protein to a final concentration of 2 mM to block residual cysteine residues. This reaction was allowed to proceed in the dark at room temperature for 1 h. Peptides of the Trx1 samples following digestion with mass spectrometry grade modified trypsin were mixed with a saturated solution of α-cyano-4-hydroxycinnamic acid containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature. The analyses were performed using an AXIMA-TOF^2 spectrometer (Shimadzu, Kyoto, Japan) with a nitrogen laser. All analyses were in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies.

Cell Culture. RAW264.7 cells (ATCC, Manassas, VA) were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM Gluta Max-1, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Apoptotic cell death was assayed using Annexin V stain.

Detection of Intracellular Oxidants. Cells were incubated with 10 μM H_2DCFDA for 30 min and then treated with 1,2-NQ or H_2O_2 for an additional 30 min. ROS generation was detected by using fluorescence microscopy (Leica DM IRE2, Leica Microsystems) and a plate reader (Varioskan, Thermo electron corporation, Waltham, MA) as reported previously.[8]

Cell Viability. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to estimate cell viability, as previously described.[23]

Data Analysis. All data were expressed as the means ± SE from at least three independent experiments. Statistical significance was considered at $p < 0.05$.

RESULTS

Redox Cycling of 1,2-NQ through Cys32 and Cys35 in Trx1. In previous studies, we found that *ortho*-quinones such as 9,10-phenanthraquinone (9,10-PQ) and 1,2-NQ readily react with vicinal thiol compounds such as DTT, resulting in oxidation of this thiol and consumption of oxygen through redox cycling.[4,5] Because Trx1 also has vicinal thiols at Cys32 and Cys35 that are essential for its catalytic activity, we hypothesized that the 1,2-NQ-mediated redox cycling with Trx1 through the proximal thiols could suppress Trx1 activity. To address this hypothesis, we prepared C32/C35 Trx1 and 32S/35S Trx1. When 1,2-NQ (1 and 10 nmol) was incubated with 40 nmol of C32/C35 Trx1 at 25 °C for 10 min, the thiol content decreased in a concentration-dependent fashion (Figure 1A). After incubation with 1 nmol of 1,2-NQ, a loss of ~17 equivalents of thiols occurred with C32/C35 Trx1, which was 17 times the equivalents of 1,2-NQ added, indicating that extensive redox cycling by 1,2-NQ with C32/C35 Trx1 took place. In contrast, incubation of 32S/35S Trx1 with 1 or 10 nmol of 1,2-NQ caused a reduction in thiols approximately equivalent to the added 1,2-NQ, suggesting that direct reaction of 1,2-NQ with a Cys residue of the protein had occurred without oxidation (Figure 1A). These results indicate that the redox cycling of Trx1 with 1,2-NQ is taking place through Cys32 and Cys35 but not with the serine analogue.

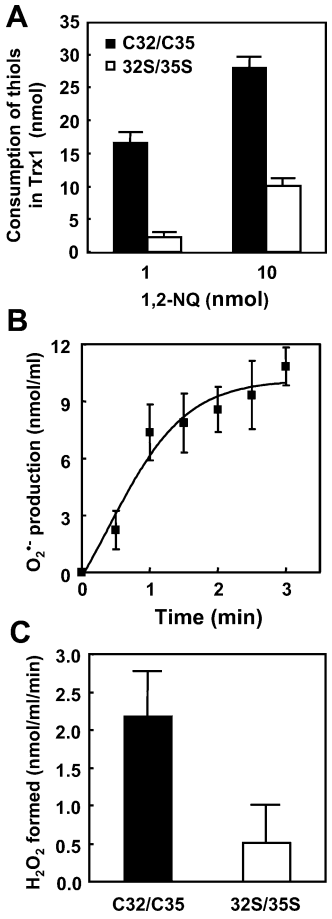

Figure 1. Redox cycling of 1,2-NQ with recombinant Trx1 and its double mutant. (A) C32/C35 Trx1 or 32S/35S Trx1 (200 μM) was incubated with 5 or 50 μM 1,2-NQ in 50 mM potassium phosphate buffer (pH 7.5) at 25 °C for 10 min. (B) 1,2-NQ (5 μM) was incubated with 20 μM C32/C35 Trx1, 50 μM acetylated cytochrome *c* in 50 mM KPi (pH 7.5) in a final volume of 1.5 mL at 25 °C under aerobic conditions. (C) H_2O_2 generation during the reaction of 1,2-NQ with C32/C35 Trx1 or 32S/35S Trx1. The incubation mixture (1 mL) contained 5 μM 1,2-NQ, 40 μM Trx1, or C32S/C35S, and 50 mM potassium phosphate buffer (pH 7.5). Reactions were performed at 25 °C for 10 min. Data are the means ± SD of three independent experiments.

To confirm that 1,2-NQ can undergo a two-electron reduction by C32/C35 Trx1 alone to produce 1,2-NQH_2, we measured its formation as its diacetyl derivative (DAN) by UPLC-MS/MS analysis as previously described with slight modifications.[17] Incubation of 1,2-NQ with recombinant Trx1 in the absence of NAD(P)H under 5% oxygen conditions resulted in the consumption of 1,2-NQ and the formation of a product with the same retention time and mass spectrum as those of authentic DAN (Table S1 and Figures S1 and S2, Supporting Information), suggesting that Trx1 reduces 1,2-NQ to the hydroquinone which then disproportionates with 1,2-NQ to generate semiquinones. The semiquinone, in turn, reduces oxygen to superoxide.[24] Consistent with this possibility, the reaction of 1,2-NQ with a 4-fold excess of C32/C35 Trx1 caused a time-dependent generation of superoxide (Figure 1B). Under these conditions, the production of H_2O_2 at a rate of 2.2 nmol/mL/min was observed as well with C32/C35 Trx1 in the presence of 1,2-NQ, whereas interaction of 32S/35S mutant Trx1 with 1,2-NQ produced a minimal amount of H_2O_2

(Figure 1C). The observed plateau likely reflects the depletion of reduced Trx1.

Reversible Oxidative Inactivation of C32/C35 Trx1 by 1,2-NQ. H_2O_2 (100 μM) or 1,2-NQ (5 μM) caused a reduction in C32/C35 Trx1 catalytic activity which was partially reversible with the addition of DTT, suggesting that oxidative modification of the thiols was involved (Figure 2A).

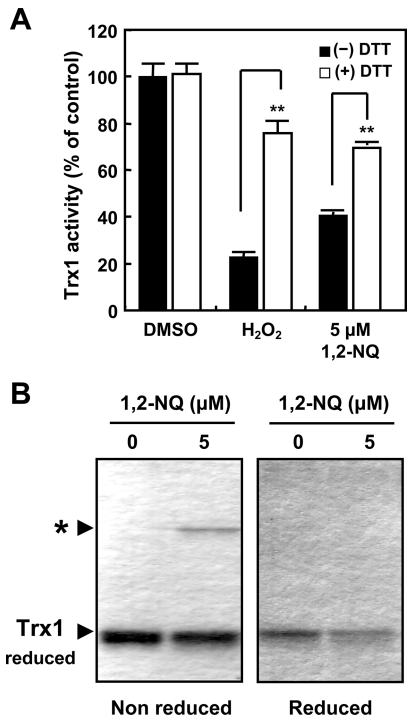

Figure 2. Reduction of the enzyme activity and dimerization during the interaction of Trx1 and its double mutant with 1,2-NQ. (A) After incubation of C32/C35 Trx1 or 32S/35S Trx1 (200 μM) with either 1,2-NQ or H_2O_2, each reaction mixture was incubated with (open bars) or without (closed bars) DTT for 1 h. Then, Trx1 activity was determined under the conditions as described in Experimental Procedures. Data are the means ± SD of three independent experiments and are expressed as a percentage of the initial activity. **, $p < 0.01$. (B) Dimerization of the Trx1 subunit during interaction of C32/C35 Trx1 with 1,2-NQ. Reactions were carried out as described above. The asterisk indicates the dimer of Trx1 on nonreduced SDS−PAGE.

The lack of complete recovery could reflect the oxidation of thiols to sulfinic or sulfonic states not reducible by DTT or, in the case of 1,2-NQ, covalent attachment to the enzyme. SDS−PAGE results (Figure 2B) showed that a dimerization of the Trx1 molecule occurred in the absence of 2-mercaptoethanol (nonreducing conditions) but not in its presence (reducing conditions), further supporting the notion that ROS generated during the interaction of C32/C35 Trx1 with 1,2-NQ promotes intra- and intermolecular disulfide formation.

Covalent Modification of Trx1 by 1,2-NQ. Immunoblot analysis with a specific antibody against 1,2-NQ showed that native Trx1 underwent arylation by 1,2-NQ (Figure 3A). This result was confirmed by MALDI-TOF/MS analysis. As shown in Figure 3B, when native Trx1 (0.25 mg/mL), with a peak of m/z 13012.6 Da, was incubated with 5 μM 1,2-NQ in 50 mM potassium phosphate buffer (pH 7.5) for 10 min at 25 °C, a modified Trx subunit (m/z 13166.5) corresponding to the addition of one equivalent of 1,2-NQ per mole was observed

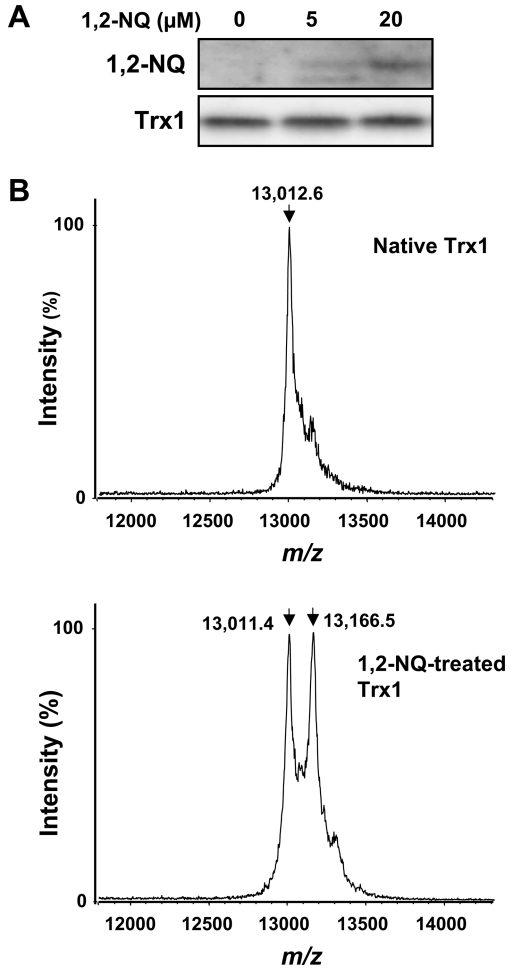

Figure 3. 1,2-NQ covalently binds to Trx1. (A) Recombinant Trx1 was incubated with 1,2-NQ (5 or 20 μM) in 50 mM KPi (pH 7.5) at 25 °C for 30 min and then analyzed by Western blot analysis using the indicated antibodies. (B) MALDI-TOF/MS analysis of Trx1. Recombinant Trx1 (0.25 mg/mL) was incubated with or without 1,2-NQ (5 μM) in 50 mM KPi (pH 7.5) for 10 min at 25 °C.

(Figure 3B). Next, native and 1,2-NQ treated Trx1 were digested with trypsin, and the peptides obtained were analyzed by MALDI-TOF/MS. Peptide mass mapping by MALDI-TOF/MS analysis of the tryptic digest fragments from the native Trx1 identified the peptides of the protein sequence (Figure 4 and Table 1). The observed peptide P-1 and P-2 showed an increased mass of +156 Da, corresponding to the addition of a single equivalent of 1,2-NQ. The peptide sequence and mass of these peptides are P-1 (GQKVGEFSGANK m/z 1377.5) and P-2 (KGQKVGEFSGANK m/z 1505.6) (Figure 4 and Table 1). It should be noted that no peptides containing Cys62 or Cys69 were detected (Table 1).

To identify the 1,2-NQ binding sites, the fragment modified by 1,2-NQ (P-1) was further analyzed by determining the MS/MS spectrum of the $[M+H]^+$ at m/z = 1377.5 and $[M+H]+$ at m/z = 1505.6. The spectrum of the 1,2-NQ-modified fragment (P-1, GQKVGEFSGANK) is shown in Figure 5A and Table 2. In the MS/MS analysis, the singly charged C-terminal product ions (y5, y8, and y9) were observed. Fragment ions (y10, b3, b4, b5, b6, and b10) were observed to have increased by 156 Da, suggesting that the 1,2-NQ-modification site in the sequence is Lys85. The other one (P-2, KGQKVGEFSGANK) is shown in Figure 5B and Table 3.

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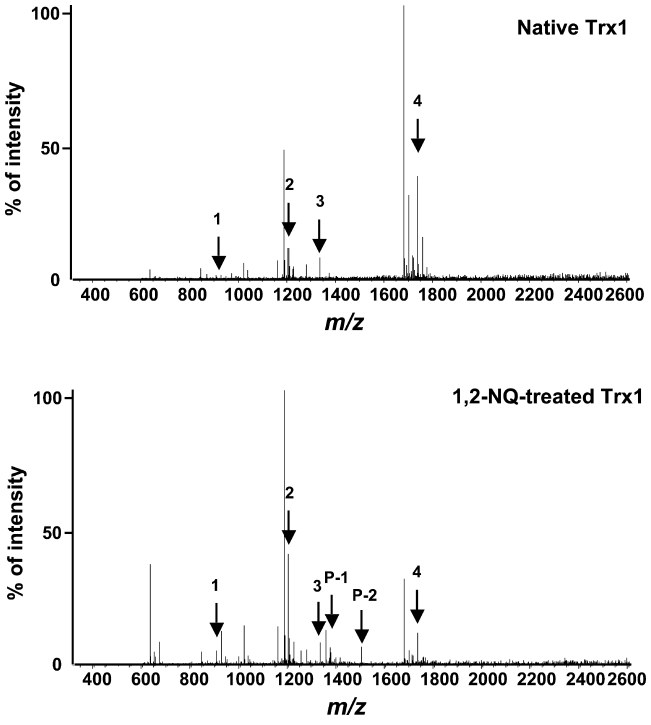

Figure 4. Mass spectrometry analysis of peptides from the Trx1−1,2-NQ adduct digested with trypsin. Native Trx1 (top) and 5 μM 1,2-NQ-treated Trx1 (bottom) were digested with trypsin, and MALDI-TOF/MS analysis was performed under the conditions as described in Experimental Procedures.

Table 1. Peptides Identified by MALDI-TOF/MS Derived from Tryptic-Digested Trx1[a]

peak no.	position	peptide sequence	calcd mass	MS
1	86−94	VGEFSGANK	908.4	908.5
2	73−81	C(CAM)MPTFQFFK	1205.5	1205.4
3	21-Sep	TAFQEALDAAGDK	1336.6	1336.4
4	22−36	LVVVDFSATWC(CAM) GPC(CAM)K	1738.8	1738.7
P-1	83−94 + 1,2-NQ	GQK*VGEFSGANK+1,2-NQ	1377.6	1377.5
P-2	82−94 + 1,2-NQ	KGQK*VGEFSGANK+1,2-NQ	1505.7	1505.6

[a]The amino acid sequences of peptides containing 1,2-NQ-modified human Trx1 are shown. Position corresponds to the portion of the amino acid sequence of human Trx1. Peak no. corresponds to the number of the peak in Figure 4. Human Trx1 was treated with 1,2-NQ (5 μM) for 10 min at 25°C in 50 mM Tris-HCl (pH 7.5). Native Trx1 and 1,2-NQ treated Trx1 were digested with trypsin and analyzed by MALDI-TOF/MS as described in Experimental Procedures. CAM, carbamidomethyl.

1,2-NQ Mediated Reduction of Trx Activity and Activation of ASK1/p38 Signaling in RAW264.7 Cells. Exposure of RAW264.7 cells to 1,2-NQ for 10 min caused a generation of oxidants in the cells (Figure 6A). Under these conditions, intracellular Trx activity also decreased (Figure 6B). Ichijo and his associates have demonstrated that Trx1 acts as a negative regulator of the ASK1/p38 signal transduction pathway which is associated with the induction of apoptotic cell death and that ROS, including hydrogen peroxide, induce dissociation of Trx1 from ASK1, thereby causing ASK1/p38 signal-dependent apoptosis.[25] This report suggests that

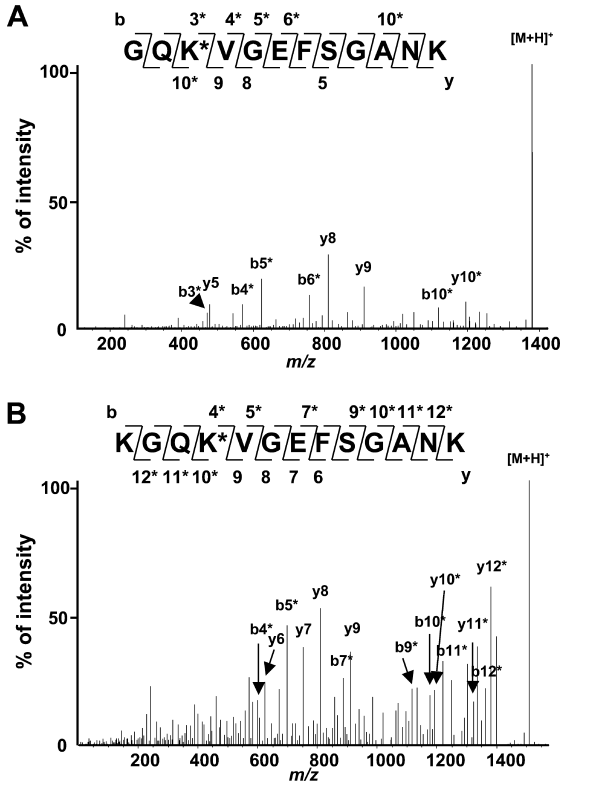

Figure 5. MALDI-TOF/MS/MS analysis of Trx1 bound to 1,2-NQ. The spectrum of the $[M + H]^+$ ion at m/z from the 1,2-NQ-modified fragment (P-1) (A) with the sequence GQKVGEFSGANK and (P-2) (B) with the sequence KGQKVGEFSGANK. The corresponding data are shown in Tables 2 and 3.

Table 2. MS/MS Spectra of P-1[a]

assignment	calcd mass	obsd mass	analyte modifiers
b3*	472.2	472.3	Lys (+1,2-NQ)
b4*	569.2	569.3	Lys (+1,2-NQ)
b5*	626.3	626.5	Lys (+1,2-NQ)
b6*	755.3	755.4	Lys (+1,2-NQ)
b10*	1117.5	1117.6	Lys (+1,2-NQ)
y5	476.2	476.3	
y8	809.4	809.4	
y9	908.4	908.3	
y10*	1192.5	1192.6	Lys (+1,2-NQ)

[a]Native Trx1 and 1,2-NQ treated Trx1 were digested with trypsin and analyzed by MALDI-TOF-TOF as described in Experimental Procedures.

reduction of cellular Trx1 activity caused by 1,2-NQ exposure could also affect the ASK1/p38 signal transduction pathway and thus induce apoptotic cell damage. To test such a possibility, we determined the phosphorylation of ASK1/p38 and the induction of apoptosis following exposure of RAW264.7 cells to 1,2-NQ. As expected, 1,2-NQ caused activation of ASK1 and p38 (Figure 7A) with concomitant apoptosis (Figure 7B). Consistent with these results, 1,2-NQ induced cleavage of PARP, a marker of apoptosis (Figure 8A).

Effect of PEG-CAT on 1,2-NQ-Mediated Apoptosis. To examine whether 1,2-NQ-induced ROS generation is involved in apoptosis, we pretreated with PEG-CAT to scavenge intracellular hydrogen peroxide since PEG-CAT (1000 U/mL) entirely abolishes cellular oxidants in RAW264.7 cells.[19] As

Table 3. MS/MS Spectra of P-2[a]

assignment	calcd mass	obsd mass	analyte modifiers
b4*	598.3	598.4	Lys (+1,2-NQ)
b5*	697.3	697.3	Lys (+1,2-NQ)
b7*	883.4	883.5	Lys (+1,2-NQ)
b9*	1117.5	1117.6	Lys (+1,2-NQ)
b10*	1174.5	1174.6	Lys (+1,2-NQ)
b11*	1245.6	1245.7	Lys (+1,2-NQ)
b12*	1359.6	1359.7	Lys (+1,2-NQ)
y6	623.3	623.2	
y7	752.3	752.2	
y8	809.4	809.5	
y9	908.4	908.3	
y10*	1192.5	1192.4	Lys (+1,2-NQ)
y11*	1320.6	1320.5	Lys (+1,2-NQ)
y12*	1377.6	1377.7	Lys (+1,2-NQ)

[a]Native Trx1 and 1,2-NQ treated Trx1 were digested with trypsin and analyzed by MALDI-TOF-TOF as described in Experimental Procedures.

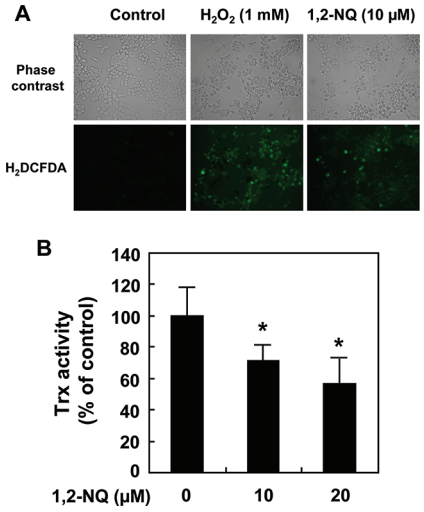

Figure 6. 1,2-NQ produces oxidants and suppresses Trx activity in RAW264.7 cells. (A) The cells were exposed to DMSO (0.1%), 1 mM H_2O_2, or 10 μM 1,2-NQ for 10 min. Cellular oxidant was determined under the conditions described in Experimental Procedures. (B) The cells were treated with indicated concentrations of 1,2-NQ for 1 h. Cellular Trx activity was measured under the conditions described in Experimental Procedures.

shown in Figure 8, PEG-CAT suppressed not only the cleavage of PARP but also the cytotoxicity induced by 1,2-NQ.

DISCUSSION

The present study indicates that, in addition to covalent bond based inactivation, 1,2-NQ can also inactivate purified Trx1 by the production of ROS such as superoxide and hydrogen peroxide. The pK_a value for Cys32 in Trx1 has been determined to be 6.35.[26] Moreover, as the reduction potential of Trx1 is relatively low (E° = −230 mV),[27] it readily participates in redox cycling reactions with 1,2-NQ to form a disulfide bridge as well as undergoing an arylation reaction. We reported previously that the redox-active quinone 9,10-PQ can effectively catalyze the transfer of electrons from DTT to oxygen, generating superoxide.[4] Like DTT, the cysteine residues, Cys32/Cys35, of Trx1 are able to interact with quinones such as 1,2-NQ, resulting in a redox cycling of 1,2-NQ in which catalytic ROS generation and thiol oxidation occur. The postulated reactions of 1,2-NQ with Trx1 including redox cycling are shown in Scheme 1.

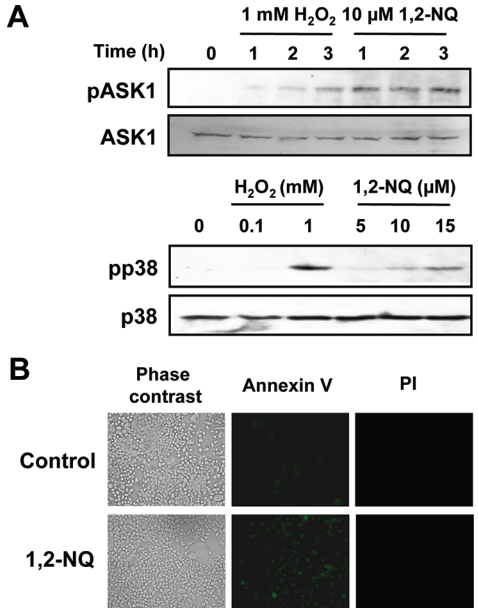

Figure 7. Activation of ASK1/p38 signaling and apoptotic cell death during exposure of RAW264.7 cells to 1,2-NQ. (A) The cells were exposed to the indicated concentrations of 1,2-NQ or H_2O_2 for 1, 2, or 3 h (top) or 1 h (bottom), and then total cell lysates were subjected to Western blot analysis using the indicated antibodies. (B) The cells were exposed to 1,2-NQ (10 μM) for 4 h and then observed by a phase contrast inverted microscope, followed by staining with Annexin V or propidium iodide (PI).

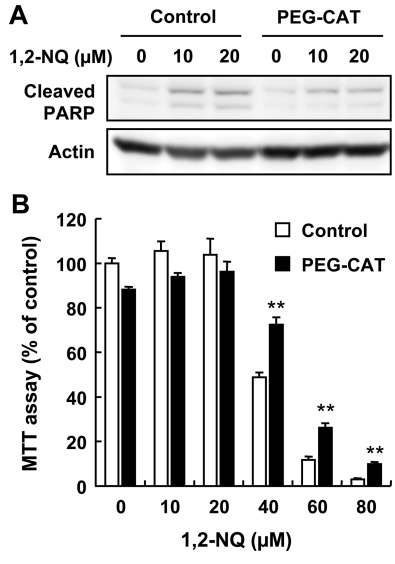

Figure 8. Effect of PEG-CAT on 1,2-NQ-mediated apoptotic cell death in RAW264.7 cells. (A) The cells treated with PEG-CAT (1000 U/mL) for 1 h before exposure to 1,2-NQ (10 or 20 μM) for 24 h, and then total cell lysates were subjected to Western blot analysis using the indicated antibodies. (B) The cells treated with PEG-CAT (1000 U/mL) for 1 h before exposure to 1,2-NQ (10, 20, 40, 60, or 80 μM) for 24 h, and then the MTT assay was performed. Each value represents the mean ± SE of five determinations. $^{**}P < 0.01$ compared with the control.

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Scheme 1

$$\mathrm{Trx(Cys32\text{-}S^{-})(Cys35\text{-}SH) + 1,2\text{-}NQ \longrightarrow Trx(Cys32\text{-}S\text{-}S\text{-}Cys35) + 1,2\text{-}NQH_2} \quad (1)$$

$$\mathrm{1,2\text{-}NQ + 1,2\text{-}NQH_2 \longrightarrow 2 \times 1,2\text{-}NQ^{\bullet -} + 2H^{+}} \quad (2)$$

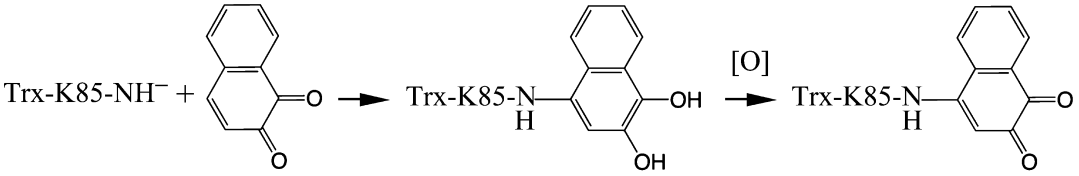
$$\mathrm{1,2\text{-}NQ^{\bullet -} + O_2 \longrightarrow 1,2\text{-}NQ + O_2^{\bullet -}} \quad (3)$$

$$\mathrm{1,2\text{-}NQH_2 + O_2^{\bullet -} \longrightarrow 1,2\text{-}NQ^{\bullet -} + H_2O_2} \quad (4)$$

$$\mathrm{2O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2} \quad (5)$$

$$\mathrm{Trx(Cys32\text{-}S^{-})(Cys35\text{-}SH) + H_2O_2 \longrightarrow Trx(Cys32\text{-}SOH)(Cys35\text{-}SH) + OH^{-}} \quad (6)$$

$$\mathrm{Trx(Cys32\text{-}SOH)(Cys35\text{-}SH) \longrightarrow Trx(Cys32\text{-}S\text{-}S\text{-}Cys35) + H_2O} \quad (7)$$



(8)

$$\mathrm{1,2\text{-}NQ + H^{+} + NAD(P)H \longrightarrow 1,2\text{-}NQH_2 + NAD(P)^{+}} \quad (9)$$

The conserved catalytic site of the Trx family of proteins contains two cysteine residues Cys32 and Cys35, the former one is a thiolate anion at physiological pH.[26] We have found that Trx1 can react with 1,2-NQ to form 1,2-NQH_2 (eq 1, Scheme 1). In this reaction, it is likely that a thiol is arylated by 1,2-NQ and that the thio ether is then cleaved by the second thiolate to form the cyclic disulfide and hydroquinone. The formed 1,2-NQH_2 interacts with 1,2-NQ via a disproportionation reaction to produce semiquinone radical anions (1,2-$NQ^{\bullet -}$) (eq 2, Scheme 1) that react readily with molecular oxygen to yield 1,2-NQ and superoxide (eq 3, Scheme 1). Because superoxide has a pK_a of approximately 4, we speculate that 1,2-NQH_2 also spontaneously deprotonates or partially reacts with superoxide, leading to the formation of 1,2-$NQ^{\bullet -}$ and hydrogen peroxide (eq 4, Scheme 1). Alternatively, the generated superoxide can also undergo a disproportion reaction to yield hydrogen peroxide and molecular oxygen (eq 5, Scheme 1). The produced hydrogen peroxide, generated either by eq 4 or eq 5, oxidizes Trx(Cys32-S^{-})(Cys35-SH), resulting in conversion of the Cys32 thiolate anion into the sulfenic acid (eq 6, Scheme 1),[28] which could ring close to the disulfide (eq 7, Scheme 1). On the basis of the above-mentioned reactions, we suggest that the 1,2-NQ-mediated decline of Trx activity results from the redox cycle-mediated formation of S−S bonds on Trx1 because its catalytic activity is lost. In this context, oxidative stress-induced dimerization of Trx1 through Cys73 has been associated with the reduction of its catalytic activity.[29] We therefore speculate that intermolecular disulfide formation by Cys73 may be involved in the dimerization of Trx1 during redox cycling with 1,2-NQ (Figure 2B). In addition, however, immunoprecipitation experiments following the exposure of A549 cells to 1,2-NQ (Iwamoto, N., et al., unpublished observations) indicated that the quinone binds to Trx1 in cellular milieu so that covalent attachment is also possible. With regard to the covalent attachment of Trx1, we found that 1,2-NQ covalently modifies Lys85 (eq 8, Scheme 1) but not Cys32 and Cys35 on Trx1 (Figure 5 and Table 1). Further study is required to elucidate whether this modification could be involved in the decreased Trx activity by 1,2-NQ.

Protein nucleophiles such as cysteine thiolates, lysine amines, histidine imidazoles, and protein *N*-terminal amines are potential targets for reactive electrophiles such as 1,2-NQ that possess α,β-unsaturated carbonyl groups.[30,31] Uchida and his associates showed that 15-deoxy-Delta12,14-prostaglandin-J_2 and 4-hydroxy-2-nonenal can modify Trx1 through reactive thiols.[32,33] In the case of Trx1 with 1,2-NQ, however, this quinone covalently bound to Trx1 through Lys85 presumably without arylation of its reactive thiol groups (Figures 3−5). Numerous cellular proteins readily undergo covalent attachment by 1,2-NQ,[8,18,18] whereas Trx1, because of its efficient electron donating capacity, promotes Cys32/Cys35-dependent redox cycling by 1,2-NQ. In preliminary observations, we found that Trx1, modified by 1,2-NQ through Lys85, is still redox active, capable of superoxide production when DTT is added to the incubation mixture as an electron donor (Iwamoto, N., et al., unpublished observations). This observation suggests that the 1,2-dihydroxynaphtalene-Lys85 adduct of Trx, generated by Michael addition of 1,2-NQ to Trx, rapidly undergoes autoxidation to yield 1,2-NQ-Lys85 in the protein (eq 8, Scheme 1) and that the quinone moiety is indeed able to interact with the vicinal thiol of DTT. Thus, covalent attachment of 1,2-NQ to Trx1 has resulted in a second redox

center, capable of electron transfer from DTT to oxygen. Thus, the intracellular inactivation of Trx by 1,2-NQ could proceed through oxidation of the vicinal thiols to disulfides. The oxidation could proceed directly by a 1,2-NQ−Trx reaction (eq 1, Scheme 1) or indirectly by hydrogen peroxide generated by reduced forms of 1,2-NQ (eqs 4 and 5, Scheme 1).

In the present study, we also found that exposure of Raw264.7 cells to 1,2-NQ caused ROS generation, decrease in Trx activity, and activation of ASK1/p38 signaling, leading to an induction of apoptosis (Figures 6 and 7). There are multiple possibilities for the oxidative stress associated with redox cycling (e.g., CYP reductase-dependent one-electron reduction, aldo-keto reductase-dependent two-electron reduction, protein interaction with protein proximal thiols, and two-electron reduction) in cellular systems.[4,24,34,35] In these systems, 1,2-NQ can undergo a two-electron reduction to form 1,2-NQH_2 using NAD(P)H, which is facilitated by 1,2-NQ reductases such as aldo-keto reductases, NAD(P)H:quinone oxidoreductase 1, and glyceraldehyde-3-phosphate dehydrogenase[36] (eq 9, Scheme 1). Taken together, we suggest that the diminished Trx activity during exposure of RAW264.7 cells to 1,2-NQ is due to the interaction of Trx1 with 1,2-NQ through intramolecular-disulfide formation between Cys32 and Cys35 and/or NAD(P)H-dependent enzyme-mediated reduction of 1,2-NQ, resulting in ROS generation which contribute to the decline of cellular Trx activity and apoptosis caused by this quinone. Consistent with this notion, pretreatment with PEG-CAT to scavenge hydrogen peroxide suppressed PARP cleavage and cytotoxicity during 1,2-NQ exposure (Figure 8).

In earlier work, we have reported that *S*-arylation of reactive thiols in PTP1B[7] and Keap1[8] by 1,2-NQ activates negatively regulated proteins such as epidermal growth factor receptor and Nrf2. The present study now shows that interaction of 1,2-NQ with Trx1 through oxidative modification of its vicinal thiol also leads to the activation of ASK1, a negatively regulated protein. It is thus an atmospheric electrophile that affects cellular redox signal transduction pathways through covalent- and oxidative-modification of sensor proteins. Since there are a variety of Trx family proteins with a −Cys-X-X-Cys− active-site motif in cells,[37] it seems likely that these redox active proteins are also molecular targets for 1,2-NQ, undergoing oxidative modification by redox cycling, leading to the disruption of these functions.

■ ASSOCIATED CONTENT

Supporting Information

UPLC-MS/MS analysis of authentic DAN and reaction mixtures of Trx1 with 1,2-NQ. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ASK1, apoptosis signal-regulating kinase 1; C32/C35 Trx1, wild type of human Trx1; CBB, coomassie brilliant blue; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 1,2-NQ, 1,2-naphthoquinone; 1,2-NQH_2, 1,2-dihydroxynaphthalene; 9,10-PQ, 9,10-phenanthraquinone; PTP1B, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; Trx1, thioredoxin1; 32S/35S Trx1, double mutant of human Trx1

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