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Letter

Peroxiredoxin 6 is a molecular target for 1,2-naphthoquinone, an atmospheric electrophile, in human pulmonary epithelial A549 cells

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ABSTRACT — 1,2-Naphthoquinone (1,2-NQ) is an electrophile found in the atmosphere, which reacts readily with protein nucleophiles to form a stable protein adduct. Peroxiredoxin 6 (Prdx6) is predominantly expressed in lung tissue and functions in antioxidant defense by facilitating the repair of damaged cell membranes via reduction of peroxidized phospholipids. In the present study, human A549 pulmonary epithelial cells were exposed to 1,2-NQ to explore whether 1,2-NQ can bind covalently to Prdx6, thereby disrupting its catalytic activity. Two-dimensional SDS/PAGE followed by western blot analysis with a specific antibody against 1,2-NQ showed that Prdx6 was covalently modified by 1,2-NQ. Using purified human Prdx6, it was found that 1,2-NQ bound covalently to Prdx6 through Cys47, Lys144 and Cys91, resulting in a significant reduction in phospholipase A_2 activity. These results suggest that arylation of Prdx6 by 1,2-NQ may, at least in part, be involved in the cellular toxicity induced by 1,2-NQ.

Key words: 1,2-Napthoquinone, Peroxiredoxin 6, Phospholipases A_2

INTRODUCTION

There are a huge number of electrophiles in the atmosphere that can covalently modify cellular proteins to form stable adducts (Iwamoto *et al.*, 2010). Of these, we have focused on 1,2-naphthoquinone (1,2-NQ) (Cho *et al.*, 2004). Accumulating evidence indicates that 1,2-NQ reacts with protein thiols via the Michael reaction to form 1,2-dihydroxynaphthalene-protein adducts, which are rapidly auto-oxidized to 1,2-NQ-protein adducts (Fig. 1) (Kumagai *et al.*, 2012; Miura *et al.*, 2011a). For example, sensor proteins containing reactive nucleophiles undergo arylation by 1,2-NQ, thereby disrupting their function (Endo *et al.*, 2011, 2007; Iwamoto *et al.*, 2007; Miura *et al.*, 2011a, 2011b; Sumi *et al.*, 2010). In a previous study, we found that several unidentified proteins expressed by human pulmonary epithelial A549 cells are modified after exposure to 1,2-NQ (Miura *et al.*, 2011b). This suggests that the cellular cytotoxicity mediated by 1,2-NQ may be caused by reduction and/or loss of function of essential proteins.

Peroxiredoxin 6 (Prdx6), a bifunctional 25 kDa protein with both glutathione peroxidase and phospholipase A_2 (PLA_2) activity, is the only mammalian 1-Cys member of the Prdx superfamily. It is expressed in all major organs, with particularly high levels being observed in lung tissue (Manevich and Fisher, 2005). Prdx6, when stably overexpressed in cells, protects against oxidative stress, whereas antisense treatment results in oxidative stress and apoptosis. Also, inhibition of Prdx6-catalyzed PLA_2 activity results in alterations in lung surfactant phospholipid synthesis and turnover (Manevich and Fisher, 2005). These observations suggest that 1,2-NQ may modify Prdx6 via reactive nucleophiles, resulting in a decrease in catalytic activity. To address this issue, we conducted experiments using A549 cells, and cell-free studies using purified human Prdx6.

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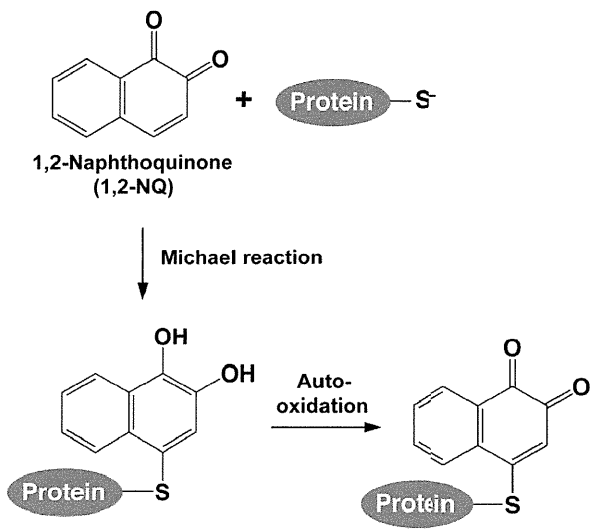

Fig. 1. Covalent modification of 1,2-NQ to protein thiols.

MATERIALS AND METHODS

Materials

1,2-NQ was purchased from Tokyo Kasei Industries, Ltd. (Tokyo, Japan). A specific antibody against 1,2-NQ was prepared as previously described (Iwamoto *et al.*, 2007). All other reagents used were of the highest purity available.

Cell culture

The human lung epithelial cell line, A549 (ATCC, Manassas, VA, USA) was grown in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum, 2 mM Gluta Max-1 and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2.

2-Dimensional SDS-polyacrylamide gel electrophoresis (2D-SDS/PAGE)

Sample preparation and the gel composition used for the second-dimension SDS-PAGE were as outlined in "2-D Electrophoresis PRINCIPLES and METHODS" (GE Healthcare, Bio-Sciences Corp., Piscataway, NJ, USA). Cells were extracted with lysis buffer (6 M urea, 1 M thiourea and 3% CHAPS) and the lysates were sonicated in short bursts to avoid heating. IPG strips (7 cm; pH interval: 3-10) were rehydrated prior to isoelectric focusing (IEF) in a solution containing 8 M urea, 2% CHAPS, a small amount of SDS, 0.3% dithiothreitol (DTT) and 0.5% IPG buffer for 10 hr. The rehydrated strips are then placed onto the cooling plate of an electrofocusing chamber and IEF was performed at a voltage of 3,500 V (Multiphor II, GE healthcare). After IEF, the IPG strips are equilibrated in the presence of SDS, DTT, urea, glycerol and iodoacetamide, and then placed on top of a vertical SDS gel for the second dimension.

Preparation of Prdx6

The expression vector encoding Prdx6 (Prdx6-pET21b) was kindly provided by Dr. Aron B. Fisher, University of Pennsylvania (PA, USA). The plasmid was transformed into *E.coli* BL21 cells for protein expression. Bacterial cultures were grown to 0.6 absorbance units at 600 nm in LB broth at 37°C with shaking at 120 rpm (Taitec, Saitama, Japan). The cultures were then induced by the addition of 1 mM IPTG and grown for an additional 12 hr at 37°C. Prdx6 was purified using Ni-IDA ProBond (Invitrogen, Carlsbad, CA, USA) as described previously (Iwamoto *et al.*, 2007). The purity of the Prdx6 preparation, as assessed by SDS-PAGE, was > 90%. Thiol groups oxidized during purification were reduced by incubation with 10 mM DTT for 1 hr and the DTT was removed on an Econo-Pac 10 DG column. Each enzyme preparation was stored at -80°C in 50 mM potassium phosphate buffer (pH 7.0) before use.

Western blotting

Cells were extracted with RIPA buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1 mM ethyleneglycoltetraacetic acid (EGTA), 0.4 mM ethylenediaminetetraacetic acid (EDTA) and a protease inhibitor cocktail). SDS-PAGE and subsequent immunoblot analysis were performed as reported previously (Miura *et al.*, 2011a). The protein concentrations were determined using the Bio-Rad Protein assay kit (Bio-Rad, Richmond, CA, USA) or the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology Inc., Rockford, IL, USA), incorporating bovine serum albumin as a standard. Western blotting with a specific antibody against 1,2-NQ was used to detect Prdx6 bound to 1,2-NQ according to the method of Miura and Kumagai (2010).

Immunoprecipitation

Cell lysates containing 1 mg of cellular protein from A549 cells exposed to 1,2-NQ were incubated overnight at 4°C with an anti-Prdx6 antibody (Lab Frontier, Seoul, Korea) with constant shaking. Protein A sepharose CL-4B beads (GE Healthcare) were then added to the samples and incubated with rocking for 3 hr at 4°C. The beads were rinsed three times with RIPA buffer and pelleted by centrifugation

at 13,000 *g* for 1 min. The proteins were eluted by boiling the beads in SDS-PAGE loading buffer for 5 min. Proteins were detected by immunoblot analysis as described above.

PLA_2 activity

The enzyme activity was measured at pH 4 (40 mM acetate buffer with 5 mM EDTA) using a liposome-based fluorescence assay (bisbodipy-C11-PC) for rapid screening. The liposomal substrate was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/bisbodipy-C11-PC/phosphatidylglycerol/cholesterol at a molar ratio of 10:0.05:2:3. Total lipid was 0.171 μmol in 250 μl of sodium acetate (50 mM) plus EGTA (1 mM) buffer at pH 4. To stop the reaction, the medium was diluted to 1 ml with assay buffer and the fluorescent product was measured at 490 nm (excitation) and 520 nm (emission). Standard curves constructed using bodipy-C11 fatty acid at pH 4 were linear up to 4 mM, and were used to calculate PLA_2 activity (Kim *et al.*, 1997).

Liquid chromatography-mass spectrometry (LC-MS) analysis

Prdx6 was incubated with 0, 5, or 50 μM 1,2-NQ for 30 min at 25°C in a buffer containing 50 mM potassium phosphate (pH 7.0). Trypsin-digested Prdx6 was mixed with 100 μM DTT and trifluoroacetic acid. To improve the ionization efficiency during mass spectrometry, samples were purified with Zip-tip μC18 (Millipore, Bedford, MA, USA) before MS analysis. Peptides were separated by NanoAQUITY UPLC (Waters, Milford, MA, USA). The MS analyses were performed using an SYNAPT HDMS (Waters) with a NanoLockspray source. All analyses were conducted using the positive ion mode and the instrument was calibrated immediately prior to each series of studies.

Data analysis

All data were expressed as the mean ± S.D. from at least three independent experiments. Data were analyzed using a *t* test and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The results of immunoblot analysis with an anti-Prdx6 and 2D-SDS/PAGE showed that A549 cells expressed Prdx6 and its charge isomers, with a molecular subunit size of 26 kDa and pI values ranging from approximately 5.7 to 6.5 (Fig. 2A). Western blotting with an anti-1,2-NQ antibody indicated that the quinone was covalently bound to Prdx6 in cells under these conditions, while numerous cellular proteins were modified after exposure to 1,2-NQ (Fig. 2A). These results were supported by subsequent immunoprecipitation studies (Fig. 2B).

Recombinant human Prdx6 was then prepared to confirm the covalent attachment of 1,2-NQ to Prdx6. When

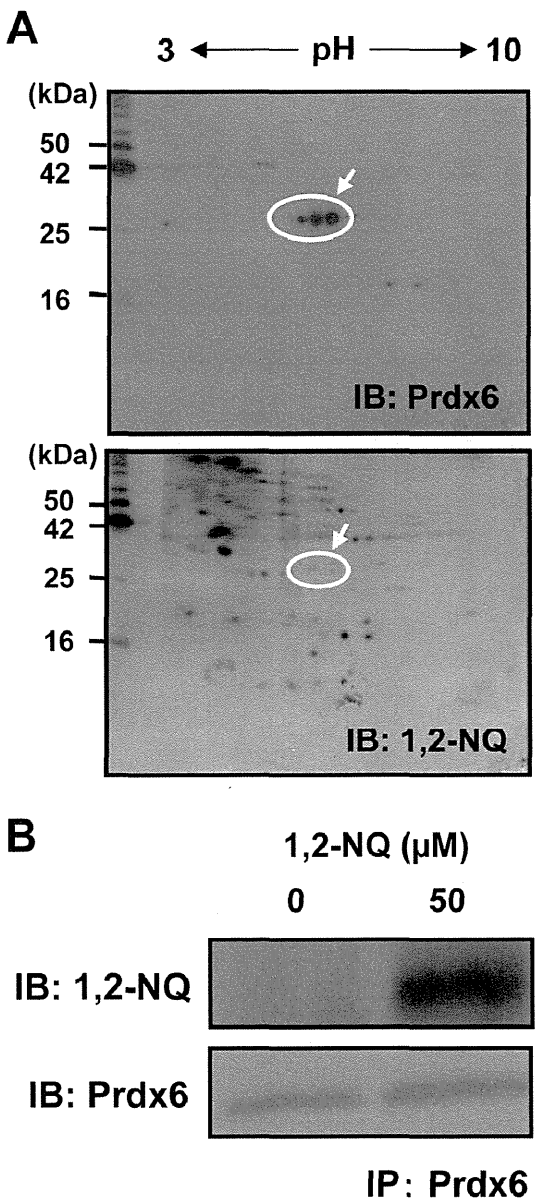

Fig. 2. 2D-SDS/PAGE analysis of cellular proteins bound to 1,2-NQ in A549 cells. A. Cells were exposed to 70 μM 1,2-NQ for 30 min at 37°C. Cell lysate (50 μg) was separated by 2D electrophoresis and subjected to western blot analysis with the indicated antibodies. B. Cells were exposed to 50 μM 1,2-NQ for 30 min at 37°C. Cellular Prdx6 was immunoprecipitated with a Prdx6 antibody. Arrows indicate the Prdx6 bands.

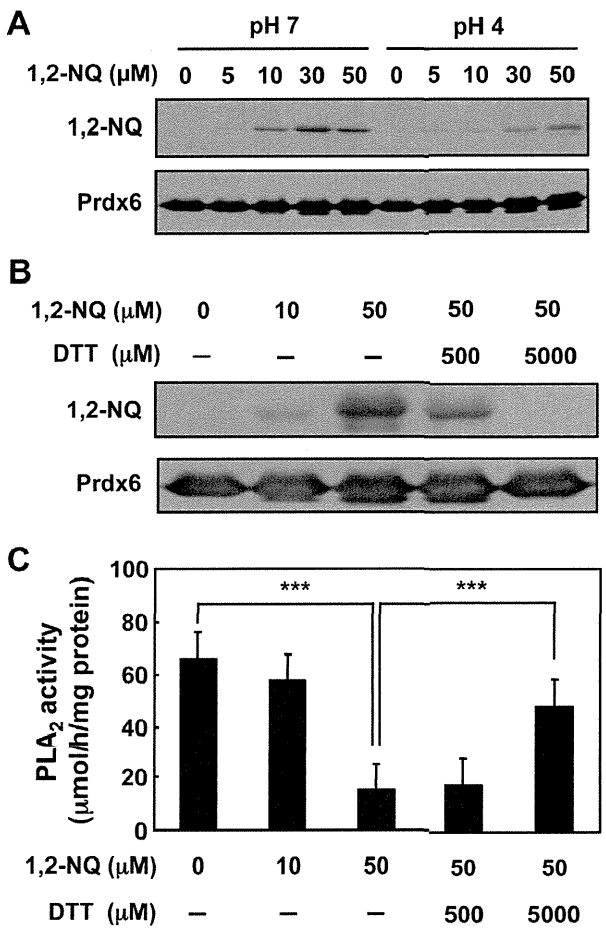

Fig. 3. pH-dependent covalent binding of 1,2-NQ to purified Prdx6, and inhibition of Prdx6-catalyzed PLA_2 activity by 1,2-NQ through covalent modification. A. Human Prdx6 and was incubated with the indicated concentrations of 1,2-NQ for 10 min at 37°C in 50 mM potassium phosphate buffer (pH 7) or 50 mM acetate buffer (pH 4) followed by western blotting with the indicated antibodies. B. Human Prdx6 was incubated with 10 or 50 μM 1,2-NQ for 60 min at 37°C in the absence and presence of DTT followed by western blotting with the indicated antibodies. C. Human Prdx6 was incubated with 10 or 50 μM 1,2-NQ for 60 min at 37°C in the absence and presence of DTT and PLA_2 activity Measured. Data represent the mean ± S.D. of three determinations. ***$P < 0.001$.

purified Prdx6 was incubated with 1,2-NQ at pH 7, Prdx6 was arylated in a concentration dependent manner (Fig. 3A). However, the level of Prdx6 modification induced by 1,2-NQ was markedly reduced under acidic conditions (pH 4) (Fig. 3A), suggesting that reactive nucleophiles play a role in the arylation of 1,2-NQ. Consistent with this, LC-MS analysis identified Cys47, Cys91 and Lys144 as the sites on Prdx6 modified (Table 1). The *S*-arylation sites identified on Prdx6 are in agreement with those reported for quinone methides derived from 2,6-di-*tert*-butyl-4-mrthylphenol (Meier *et al.*, 2007).

Modification of Prdx6 by 1,2-NQ would affect its catalytic activity. Therefore, we measured Prdx6 activity in the absence and presence of 1,2-NQ, using PLA_2 activity as the readout. As shown in Figs. 3B and C, incubation of Prdx6 with 1,2-NQ resulted in a concentration-dependent reduction in PLA_2 activity concomitant with covalent binding. The covalent modification and diminished PLA_2 activity induced by 1,2-NQ was markedly inhibited by treatment with the thiol compound, DTT. These results show that covalent binding of 1,2-NQ to Prdx6 results in a reduction in catalytic activity.

The results of the present study also indicated that, of all the proteins bound to 1,2-NQ, Prdx6 is the target protein for this atmospheric electrophile in A549 cells. His26, Ser32 and Asp140 are reported to be the catalytic sites of Prdx6 responsible for PLA_2 activity. His26 is the site of interfacial binding to the liposomal surface, Ser32 plays a key role in the maintenance of Prdx6 structure, and Asp140 is critical for catalysis (Manevich *et al.*, 2007). However, the results of the LC-MS analysis carried out in the present study showed that 1,2-NQ irreversibly bound to human Prdx6 via Cys47, Cys91 and Lys144. A possible explanation for inhibition of the PLA_2 activity during 1,2-NQ exposure is that 1,2-NQ covalently binds to Lys144, which is in close proximity to Asp140 and, by so doing, affects the environment around the Asp140 residue associated with the PLA_2 activity catalyzed by Prdx6.

We previously reported that intratracheal administration of 1,2-NQ to mice causes antigen-related airway inflammation, characterized by infiltration of eosinophils and lymphocytes around the airways, and an increase in

Table 1. 1,2-NQ-modified human Prdx6 peptides identified by LC-MS

Position	Peptide sequence	Calculated mass (Da)	Observed mass (Da)
42-53	DFTPVCTTELGR+1,2-NQ	1494.79	1494.69
85-97	DINAYNCEEPTEK+1,2-NQ	1681.71	1681.65
143-155	LKLSILYPATTGR+1,2-NQ	1588.91	1588.87

the number of goblet cells within the bronchial epithelium (Inoue *et al.*, 2007). Because PLA_2 activity plays an important role in lung surfactant homeostasis, and is responsible for the bulk of the degradation of internalized phosphatidylcholine and its re-synthesis via the re-acylation pathway, (Schremmer *et al.*, 2007), we speculate that chemical knockdown of Prdx6 by 1,2-NQ is, at least partially, involved in 1,2-NQ-mediated inflammation.

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Letter

Methylglyoxal activates the human transient receptor potential ankyrin 1 channel

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ABSTRACT — Methylglyoxal (MG) is an endogenous carbonyl compound that is produced in large quantity under hyperglycemic conditions, which are believed to contribute to the development of diabetic neuropathy. However, the mechanism by which this occurs and the molecular targets of MG are unclear. In the present study, we investigated the effect of MG on transient receptor potential ankyrin 1 (TRPA1) activation in human TRPA1-expressing HEK293 cells. MG activated TRPA1-expressing HEK293 cells, but failed to activate human capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1)-expressing HEK293 cells or mock-transfected HEK293 cells. MG also induced calcium (Ca^{2+}) influx in a concentration-dependent manner, and the concentration-response curve indicates that the effect of MG has an EC_{50} of 343.1 ± 17.3 μM. Interestingly, the time course in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human TRPA1-expressing HEK293 showed considerable differences in response to MG and cinnamaldehyde. Furthermore, we examined four endogenous carbonyl compounds, including MG, glyceraldehyde, glycolaldehyde, and glyoxal; only MG notably activated TRPA1-expressing HEK293 cells. These results may provide insight into the TRPA1-mediated effects of MG on diabetic neuropathy.

Key words: Methylglyoxal, TRPA1, Diabetic neuropathy

INTRODUCTION

Neuropathic pain is one of the most common complications of diabetes mellitus (Vinik *et al.*, 2000). This pain is characterized by hyperesthesia, dysesthesia, hyperalgesia, paresthesia, and allodynia (Brown and Asbury, 1984), and it is difficult to treat with currently available therapeutic strategies (Jensen *et al.*, 2006). Although diabetic neuropathic pain has often been reported to be associated with abnormal Ca^{2+} homeostasis (Fernyhough and Calcutt, 2010), its underlying mechanisms have not been completely elucidated.

Transient receptor potential ankyrin 1 (TRPA1) is a Ca^{2+}-permeable, nonselective cation channel in a subset of polymodal nociceptive neurons. It is activated by noxious cooling (< 17°C) (Story *et al.*, 2003) and multiple pungent compounds including allyl isothiocyanate (Jordt *et al.*, 2004; Bautista *et al.*, 2005; Macpherson *et al.*, 2005, 2007) and unsaturated aldehydes such as acrolein that are contained in cigarette smoke (Andrè *et al.*, 2008, 2009; Simon and Liedtke, 2008). Functional studies, channel localization, and analysis of TRPA1-deficient mice indicate that the channel is the primary molecular site through which they activate the pain pathway (Kwan *et al.*, 2006; Levine and Alessandri-Haber, 2007). In addition, recent studies demonstrated that TRPA1 contributes to the maintenance and development of diabetic hypersensitivity (Wei *et al.*, 2009).

Methylglyoxal (MG) is an endogenous carbonyl compound physiologically generated as an intermediate of glycolysis. MG accumulates during hyperglycemia (Beisswenger *et al.*, 2001; Lapolla *et al.*, 2003), and it has been suggested that this accumulation may contribute to the development of diabetic neuropathy. Indeed, several investigations have demonstrated that MG induces apoptosis in rat Schwann cells (Fukunaga *et al.*, 2004), adrenaline secretion in the peripheral nervous systems (Davies *et al.*, 1986), and an increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the human neuroblastoma cells (Kuhla *et al.*, 2006). Therefore, we hypothesized that TRPA1-chan-

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nel activation by MG could be a cause of diabetic neuropathic pain.

The purpose of the present study was to examine whether TRPA1 acts as a molecular target for MG. To prove this, we examined the ability of MG to activate human TRPA1 by measuring $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells.

MATERIAL AND METHODS

Chemicals

Cinnamaldehyde, MG, glyceraldehyde, glycolaldehyde, glyoxal, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cloning of human TRPA1

Oligo(dT)-primed cDNA was synthesized from 1 μg of the total RNA isolated from human dorsal root ganglion (Clontech, Mountain View, CA, USA) using the SuperScript™ III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. An aliquot of the cDNA (2 μl) was then subjected to PCR amplification using Pfx DNA polymerase (Invitrogen) using the following primers, 5'-CACCATGAAGTGCAGCCTGAGGAAGA-3' (N-terminal forward primer with CACC sequence) and 5'-CTAAGGCTCAAGATGGTGTGTTTTT-3' (C-terminal reverse primer without a stop codon). The PCR products were then separated on a 0.8% agarose gel and the bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). Purified PCR products were subcloned into the pENTR™/d-TOPO vector (Invitrogen) and named hTRPA1-pENTR/d-TOPO. Six mutations identified by sequencing were corrected using the Quik-Change Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Next, hTRPA1-pENTR/d-TOPO was recombined with the pcDNA5/FRT mammalian expression vector (Invitrogen) using *attL* and *attR* reactions with Gateway™ LR Clonase™ enzyme mix (Invitrogen) and named hTRPA1-pcDNA5/FRT.

Development of human TRPA1-HEK293 stable cell line

HEK293 cells containing the FLP recombination site (Invitrogen) were cotransfected with hTRPA1-pcDNA5/FRT and pOG44 vectors (Invitrogen) using lipofectamine LTX (Invitrogen). Stable clones expressing TRPA1 were then selected using hygromycin B antibiotic selection and colonies were cultured to produce a large stock of TRPA1-expressing cells. TRPA1-protein expression was confirmed following previously published protocols (Ohkawara *et al.*, 2010).

Intracellular Ca^{2+} measurement using FlexStation

Cells were plated at 80-90% confluence on a 96-well plate, poly-D-lysine black-walled, clear-bottomed plates (Griner bio-one, Frickenhausen, Germany) 24 hr before initiating the experiments. The cells were incubated for 1 hr at 37°C in Hank's balanced salt solution (HBSS) buffer (HBSS with 20 mM HEPES buffer, pH 7.4) containing FLIPR® calcium 4 assay reagent (Molecular Devices, Sunnyvale, CA, USA) followed immediately by fluorescence measurement. Fluorescence was measured using FlexStation (excitation at 485 nm and emission at 525 nm, using a 515 nm cutoff) and SoftMax Pro 4.7.1 software (Molecular Devices). The test compounds were prepared in DMSO and added to the HBSS buffer (final DMSO concentration, 0.2%). EC_{50} values were determined using Prism 4 software (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

We first examined the ability of MG to activate TRPA1 using FlexStation-based calcium flux assay. MG caused an increase in $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells (Fig. 1B), but not in human TRPV1-expressing cells (Fig. 1C) or mock-transfected HEK293 cells (Fig. 1D). These results indicate that an increase in $[Ca^{2+}]_i$ by MG was mediated by TRPA1. The TRPA1 agonist cinnamaldehyde also increased $[Ca^{2+}]_i$ to a similar extent (Fig. 1A). Interestingly, the time course in $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells shows marked differences in response to MG and cinnamaldehyde. The initial slope of the fluorescence change for MG is less steep than that for cinnamaldehyde. Furthermore, the maximal intensities induced by MG were approximately 20% higher than those induced by cinnamaldehyde (Figs. 1A and B). These kinetics are consistent with those previously reported for heterologously expressed TRPV1 channels (Tóth *et al.*, 2005). For example, the response to capsaicin is rapid and diminishes after reaching an initial maximum. In contrast, the response to resiniferatoxin is gradual. This response pattern is consistent with the sustained channel opening regulated by resiniferatoxin (Winter *et al.*, 1990; Liu and Simon, 1996), which contributes to channel desensitization and/or potent irritation and cytotoxicity. Although correlations between Ca^{2+} response pattern-mediated TRPA1 and physiological functions have not been reported, TRPA1 activation by MG may be an important mechanism in painful diabetic neuropathy.

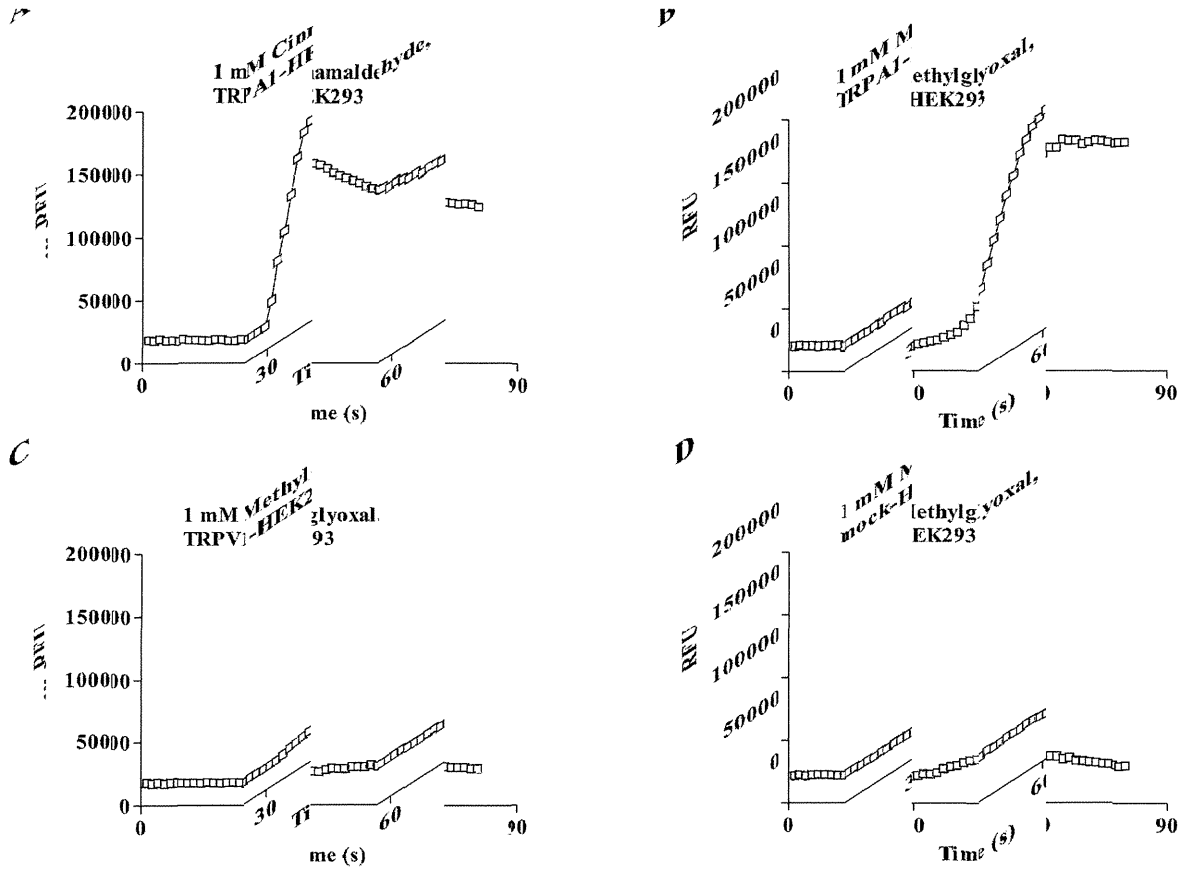

Fig. 1. Effect of MG on $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells. Time course of $[Ca^{2+}]_i$ in human TRPA1- (A,B), human TRPV1-(C) expressing HEK 293 cells, or in mock-transfected(D) HEK293 cells stimulated by 1 mM cinnamaldehyde (A) and 1 mM MG (A-C). Test compounds were added at 30-s time points, and the fluorescence was monitored using a FlexStation. RFU represents the relative fluorescence units of the calcium 4 assay reagent. The fluorescent traces are shown in the averages of three wells.

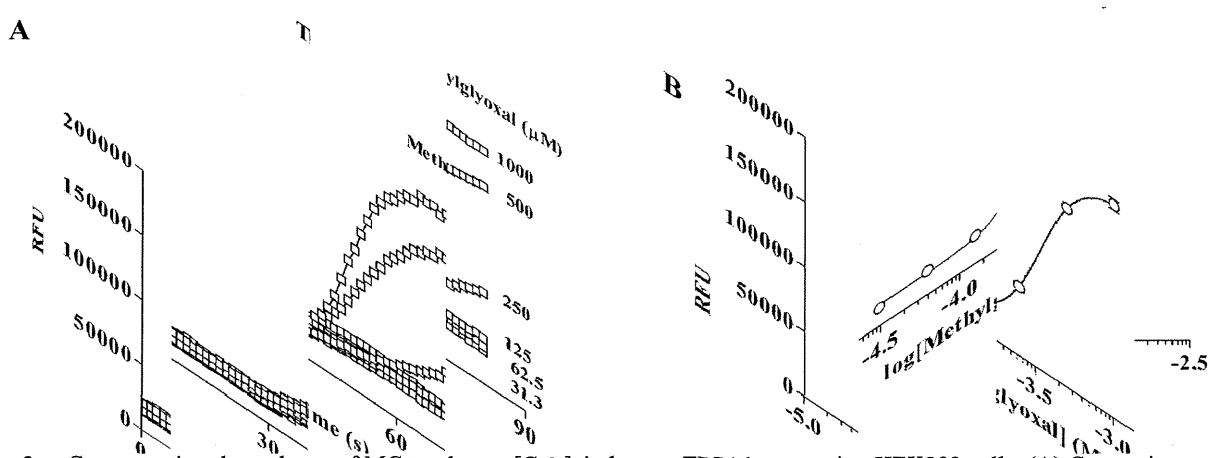

Fig. 2. Concentration-dependence of MG to elevate $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells. (A) Comparison of time courses of fluorescence changes induced by different concentrations of MG. (B) Concentration-response relationships for MG-induced $[Ca^{2+}]_i$ changes in human TRPA1-expressing HEK293 cells. RFU represents the relative fluorescence units of the calcium 4 assay reagent. Data are the mean ± S.E. of at least three separate experiments. Solid lines were fitted to the Hill equation.

Next, we investigated the concentration-dependence of MG on human TRPA1-expressing HEK293 cells. As shown in Fig. 2A, MG induced a concentration-dependent increase in $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells. The concentration-response curve indicates that the effect of MG has an EC_{50} value of 343.1 ± 17.3 μM (mean ± S.E. for three experiments) (Fig. 2B). It should be mentioned that the concentration of MG in diabetic patients is below the EC_{50} value for MG to activate TRPA1 (Beisswenger *et al.*, 2001). However, the intracellular concentration of MG varies widely and is generally higher than that in the plasma. For example, 300 μM of MG was found in Chinese hamster ovary cells (Chaplen *et al.*, 1998). Thus, our results may be physiologically relevant.

In addition to MG, the hyperglycemic conditions may lead to the production of other endogenous carbonyl compounds. To ascertain whether the activation of TRPA1 is confined to MG or widespread, we examined four endogenous carbonyl compounds, including MG, glyceraldehyde, glycolaldehyde, and glyoxal. Of the four endogenous carbonyl compounds, only MG caused notable $[Ca^{2+}]_i$ increases in human TRPA1-expressing HEK293 cells (Fig. 3A). Glyceraldehyde appeared to be a very weak activator (EC_{50}: >1 mM) (Fig. 3B), whereas glycolaldehyde and glyoxal did not have an effect on human TRPA1-expressing HEK293 cells (Figs. 3C, D). This result indicates that only certain endogenous carbonyl compounds have a stimulatory effect on TRPA1.

Several TRPA1 ligands have been shown to activate TRPA1 through the covalent modification of intracellular cysteine and lysine residues in the N-terminal region of the ion channel (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). MG also modifies arginine,

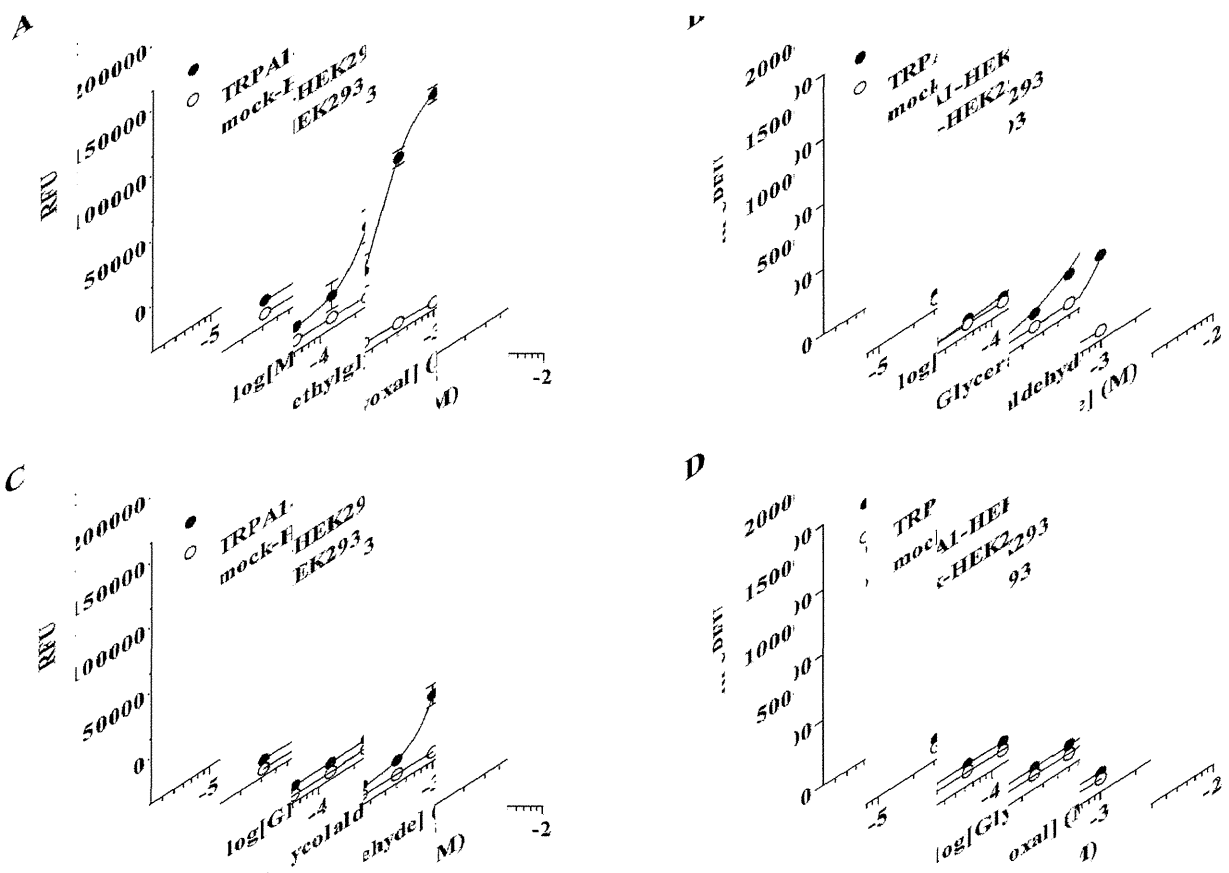

Fig. 3. Concentration-dependence of endogenous carbonyl compounds to elevate $[Ca^{2+}]_i$ in human TRPA1-expressing HEK293 cells and mock-transfected HEK293 cells. Concentration-response relationships for (A) MG-, (B) glyceraldehydes-, (C) glycolaldehyde-, and (D) glyoxal-induced $[Ca^{2+}]_i$ changes in human TRPA1-expressing HEK293 cells and mock-transfected HEK293 cells. RFU represents the relative fluorescence units of the calcium 4 assay reagent. Data are the mean ± S.E. of at least three separate experiments.

lysine, and cysteine residues in proteins (Lo *et al.*, 1994). Therefore, the TRPA1 activation by MG may be caused by the modification of lysine and/or cysteine residues.

In summary, we identified TRPA1 as a novel molecular target of MG; however, its precise mechanism is not yet understood. Further detailed studies regarding the activation of TRPA1 and its molecular mechanism may lead to the development of new therapeutic strategies for painful diabetic neuropathy.

ACKNOWLEDGMENTS

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Original Article

A method for detecting covalent modification of sensor proteins associated with 1,4-naphthoquinone-induced activation of electrophilic signal transduction pathways

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ABSTRACT — While metabolic activation of naphthalene, yielding 1,2-naphthoquinone (1,2-NQ) and 1,4-NQ that can covalently bind to cellular proteins, has been recognized to be associated with its toxicity, the current consensus is that such electrophile-mediated covalent modification of sensor proteins with thiolate ions is also involved in activation of cellular signal transduction pathways for cellular protection against reactive materials. In the present study, we developed an immunochemical assay to detect cellular proteins adducted by 1,4-NQ. Dot blot analysis indicated that the antibody prepared against 1,4-NQ recognized the naphthalene moiety with the *para*-dicarbonyl group, rather than with the *ortho*-dicarbonyl group. Furthermore, little cross-reactivity of *para*-quinones with either a different number of aromatic rings (n = 1) or substituent groups was observed. With this specific antibody against 1,4-NQ, we identified nine target proteins of 1,4-NQ following exposure of human epithelial carcinoma cell line A431 to 1,4-NQ. Among them, heat shock protein 90 (HSP90) and HSP70 are of interest because covalent modification of these chaperones causes activation of heat shock factor-1, which plays a role in the cellular response against electrophiles such as 1,4-NQ. Thus, our method, which does not use radiolabeled compounds, would be applicable for exploring activation of electrophilic signal transduction pathways coupled to covalent modification of sensor proteins during exposure to naphthalene as well as 1,4-NQ.

Key words: Naphthalene, 1,4-Naphthoquinone, Covalent modification, Immunochemical detection, Electrophilic signal transduction

INTRODUCTION

Naphthalene is a ubiquitous environmental chemical contaminant in the atmosphere through fuel combustion (Preuss *et al.*, 2003), cigarette smoke (Ding *et al.*, 2005), and insect repellents (Daisy *et al.*, 2002). This compound has also been categorized as a Group 2B carcinogen by the International Agency for Research on Cancer. It is well recognized that naphthalene toxicity *in vivo* and *in vitro* requires metabolic activation to produce electrophilic metabolites such as 1,2-naphthoquinone (1,2-NQ) and 1,4-NQ (Buckpitt and Warren, 1983; Troester *et al.*, 2002; Warren *et al.*, 1982) (Fig. 1). Because of their electrophilic properties, 1,2-NQ and 1,4-NQ are thought to covalently modify protein thiols to form protein adducts that undergo conformational changes, leading to electrophilic signal transduction (Iwamoto *et al.*, 2007; Miura *et al.*, 2011c), alteration in their functions and cellular damage (Endo *et al.*, 2007; Sumi *et al.*, 2010).

In our previous investigations, we developed an immu-

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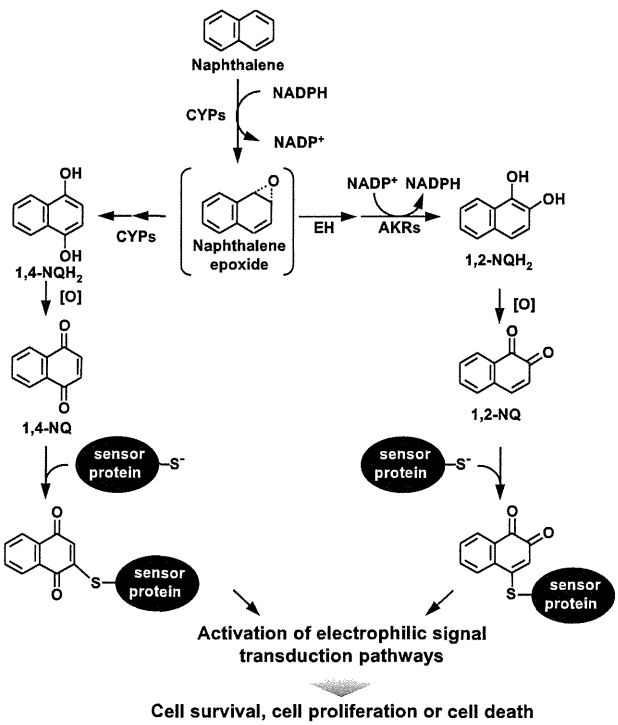

Fig. 1. Postulated cellular responses through covalent modification of sensor proteins during metabolic activation of napthalene to produce 1,2-naphthoquinone and 1,4-naphthoquinone. NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; CYPs, cytochrome P450 isozymes; EH, epoxide hydrolase; AKRs, aldo-keto reductase isozymes; 1,2-NQH_2, 1,2-dihydroxynaphthalene; 1,2-NQ, 1,2-naphthoquinone; 1,4-NQH_2, 1,4-dihydroxynaphthalene; 1,4-NQ, 1,4-naphthoquinone.

nochemical method for detection of proteins modified by 1,2-NQ with a specific antibody against 1,2-NQ, but not 1,4-NQ (Miura and Kumagai, 2010). Using anti-1,2-NQ, it has been demonstrated that protein tyrosine phosphatase 1B (PTP1B) (Iwamoto *et al.*, 2007), cAMP response element-binding protein (Endo *et al.*, 2007), inhibitory κB kinase β (Sumi *et al.*, 2010), Kelch-like ECH-associated protein 1 (Keap1) (Miura *et al.*, 2011c), glyceraldehyde-3-phosphate dehydrogenase (Miura *et al.*, 2011a, 2011b), peroxiredoxin 6 (Takayama *et al.*, 2011) and thioredoxin1 (Shinkai *et al.*, 2012) are molecular targets of 1,2-NQ for covalent modification. More importantly, it has been shown that activations of epidermal growth factor receptor and NF-E2-related factor 2 (Nrf2) are attributable to S-arylation of PTP1B through Cys121 and of Keap1 through Cys151 by 1,2-NQ, respectively (Iwamoto *et al.*, 2007; Kobayashi *et al.*, 2009). These findings suggest that covalent attachment of sensor proteins with reactive thiol groups exhibiting low p*K*a values, such as PTP1B and Keap1, plays a crucial role in the initial response and cellular protection against 1,2-NQ through the activation of electrophilic signal transduction pathways (Kumagai *et al.*, 2012) (Fig. 1).

In the present study, we prepared a polyclonal antibody against 1,4-NQ and assessed the specificity of this antibody by western blot analysis. Identification of sensor proteins modified by 1,4-NQ was performed following exposure of human epithelial carcinoma A431 cells to 1,4-NQ at a low concentration, as sensor proteins with thiolate ions (S^-) readily undergo *S*-arylation by 1,4-NQ under these conditions (Fig. 1).

MATERIALS AND METHODS

Materials

Chemicals were obtained as follows: 1,2-NQ, 1,4-NQ and *tert*-butylbenzoquinone (TBQ) from Tokyo Chemical Industry Co. (Tokyo, Japan); dimethyl sulfoxide (DMSO), 2-anilino-1,4-NQ, 5,8-dihydroxy-1,4-NQ, 5-hydroxy-1,4-NQ, β-lapachone and 1,4-benzoquinone from Sigma-Aldrich Co. (St. Louis, MO, USA); polyclonal goat anti-rabbit immunoglobulins/AP from Dako Co. (Glostrup, Denmark); anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody from Cell Signaling Technology Inc. (Danvers, MA, USA); 2-methyl-1,4-NQ, 2-propanol, sodium azide, glycine and bovine serum albumin from Nacalai Tesque Inc. (Kyoto, Japan); nonfat dry milk, and other chemical reagents from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents used were of the highest purity available. 1,2-NQ and 1,4-NQ were purified on an Ultra Pack column (Yamazen, Co., Osaka, Japan), before use.

Immunogen preparation

The polyclonal antibody against 1,4-NQ was prepared by the method of Miura and Kumagai (2010). Briefly, water-soluble keyhole limpet hemocyanin (KLH; 21 mg) was dissolved in 3.5 ml of 4 M guanidinium chloride containing 5 mg of dithiothreitol (DTT). The mixture was stirred under argon at 25°C in the dark for 2 hr. The reduced KLH (3 ml) was loaded on an Econo-Pac 10 DG column (Bio-Rad Laboratories Inc., Hercules, CA, USA), which had been equilibrated with 0.1 M Tris-HCl, pH 8.5, to remove DTT. Then, 3 ml of the KLH solution (15 mg of protein) was reacted with 5 mM 1,4-NQ under argon at 25°C for 1 hr. The mixture (3 ml) was loaded on an Econo-Pac 10 DG column, which had been equilibrated

with 0.1 M Tris-HCl, pH 8.5, to remove free 1,4-NQ. The resulting 1,4-NQ-KLH immunogen was stored at -80°C.

Thiol concentration

To determine the protein thiol content, KLH or 1,4-NQ-KLH (0.4 mg each) was reacted with 0.5% SDS, 133 mM dithionitrobenzoic acid (DTNB), and 40 mM Tris-HCl (pH 8.2)-4 mM EDTA as reported previously (Kumagai *et al.*, 2002). Detection of 2-nitro-5-thiobenzoic acid, which is formed by the reaction of DTNB with free thiols on KLH, was assessed by following the increase in absorbance at 412 nm for 2 min after the addition of DTNB, using a molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$, in a Shimadzu UV-1800 double-beam spectrometer (Shimadzu Co., Kyoto, Japan).

Purification of antibody specific to 1,4-NQ

Female New Zealand White rabbits (8 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan). Rabbits were immunized by multiple intramuscular inoculations containing a total of 1 mg of immunogen emulsified in complete Freund's adjuvant (Sigma-Aldrich Co.). The rabbits were boosted at multiple subcutaneous sites twice at 2-week intervals with 1 mg of protein per boost emulsified in incomplete Freund's adjuvant. Antisera were collected at the beginning of the week after the second boosting. The rabbits were subsequently boosted twice a month for 4 months and bled at various times thereafter. The immunoglobulin G (IgG) fraction of anti-1,4-NQ was isolated from the serum by Protein A-Sepharose CL-4B column chromatography (Miura and Kumagai, 2010). All animal procedures were approved by the University of Tsukuba Animal Care and Use Committee.

To remove anti-KLH antibody from the IgG fraction of anti-1,4-NQ, affinity chromatography was performed with Affi-Gel 15 (Bio-Rad Laboratories Inc.) covalently coupled to KLH (2 mg of KLH per ml of gel). The IgG fraction of anti-1,4-NQ (1 mg) was loaded on the KLH-Affi-Gel 15 column (4 cm × 0.7 cm i.d.), and was circulated at 4°C for 12 hr. The flow rate was 0.5-0.6 ml/min. The column was washed with TTBS (0.1 M Tris, pH 8, 0.15 M sodium chloride and 0.05% Tween-20) (anti-1,4-NQ fraction, No. 1-3) and 0.1 M glycine-HCl (pH 3) (anti-KLH fraction, No. 13-14), and then the elution was collected up to 1.5 ml per fraction. The anti-1,4-NQ fraction was concentrated by an Ultracel YM-50 centrifugal filter unit (Millipore Co., Billerica, MA, USA). Protein content was determined by the Bradford assay (Bradford, 1976); bovine serum albumin served as the standard.

Titration test of antibodies against 1,4-NQ

Antibody levels against 1,4-NQ were measured by the enzyme-linked immunosorbent assay (ELISA). Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 50 µl/well of coating buffer containing 1 µg/ml of the appropriate antigen in 50 mM carbonate buffer, pH 9.6, and incubated in a moist environment for 30 min at room temperature. Coated plates were washed with phosphate-buffered saline containing 0.05% Tween20 (TPBS). PBS containing 1% bovine serum albumin (0.1 ml/well) was added to the wells and incubated for 30 min. After washing with TPBS three times, the wells were probed with 50 µl/well of the appropriate antiserum, which was diluted with TPBS, for 30 min. Following three washes with TPBS, 50 µl/well of alkaline phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1:5,000 was added to each well and incubated for 30 min at room temperature. After another three washes with TPBS, 0.1 ml/well of p-nitrophenyl phosphate (PNPP) buffer, pH 9.8, containing 1 mg/ml of PNPP, 0.92 M diethanolamine and 0.5 mM magnesium chloride was added and incubated at room temperature for 30 min and read in a plate reader (Titertek Multiskan; Flow Laboratories Inc., McLean, VA, USA) at 405 and 630 nm.

Antigen specificity evaluation of anti-1,4-NQ antibody

Human epithelial carcinoma A431 cell lysate was prepared by sonication in four volumes of buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1 mM EDTA and 1% protease inhibitor cocktail (Sigma-Aldrich Co.). The cell lysate was centrifuged at 600 × *g* for 10 min at 4°C, and the supernatant was further centrifuged at 9,000 × *g* for 10 min at 4°C. The resulting supernatant (0.1 mg/ml), referred to hereafter as "S9", was incubated with each polycyclic aromatic hydrocarbon quinone (10 µM) for 30 min at 25°C in 0.1 M Tris-HCl, pH 7.5. To terminate the reaction the mixture was added to an equal volume of 2 × dot blot sample buffer containing 0.125 M Tris-HCl, pH 6.8, and 4% SDS and heated at 95°C for 5 min. The samples (0.1 µg/µl) were subjected to dot blot analysis as described previously (Miura and Kumagai, 2010).

Two-dimensional SDS-PAGE

A431 cells (375 × 10^4 cells/60 mm dish) were incubated for 24 hr in Dulbecco's modified Eagle's medium containing 4.5 g/l D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM GlutaMAX-I supplement (Life Technologies, Carlsbad, CA, USA), and 10% (v/v) heat-inactivated fetal bovine serum at 37°C in 5% CO_2. Before treatment, cells were serum-starved overnight and then

exposed to DMSO or 1,4-NQ for 1 hr in serum-free medium. Exposed cells were rinsed twice with 1 ml of Dulbecco's phosphate-buffered saline (Wako Pure Chemical Industries Ltd.). Cells were harvested by 0.15 ml of lysis buffer containing 8 M urea, 4% CHAPS and 0.4 M Tris, and sonicated for 5 min in pulses of 30 sec interspersed with 30 sec of cooling in ice-cold water, using Bioruptor (CosmoBio, Tokyo, Japan) with output setting of H (High, 200W). The cell lysates were centrifuged at 13,000 × *g* for 10 min at 4°C. The resulting supernatants (60 μg) were adjusted to 125 μl with 9.8 M urea, 4% CHAPS, 2% IPG buffer and bromophenol blue, and then applied to an Immobiline DryStrip pH 3-10, 7 cm (GE Healthcare, Piscataway, NJ, USA) for 10 hr under silicon oil. Isoelectric focusing was performed as reported previously (Miura *et al.*, 2011b). Proteins separated by isoelectric focusing were further separated by SDS-PAGE according to the method of Laemmli (Laemmli, 1970).

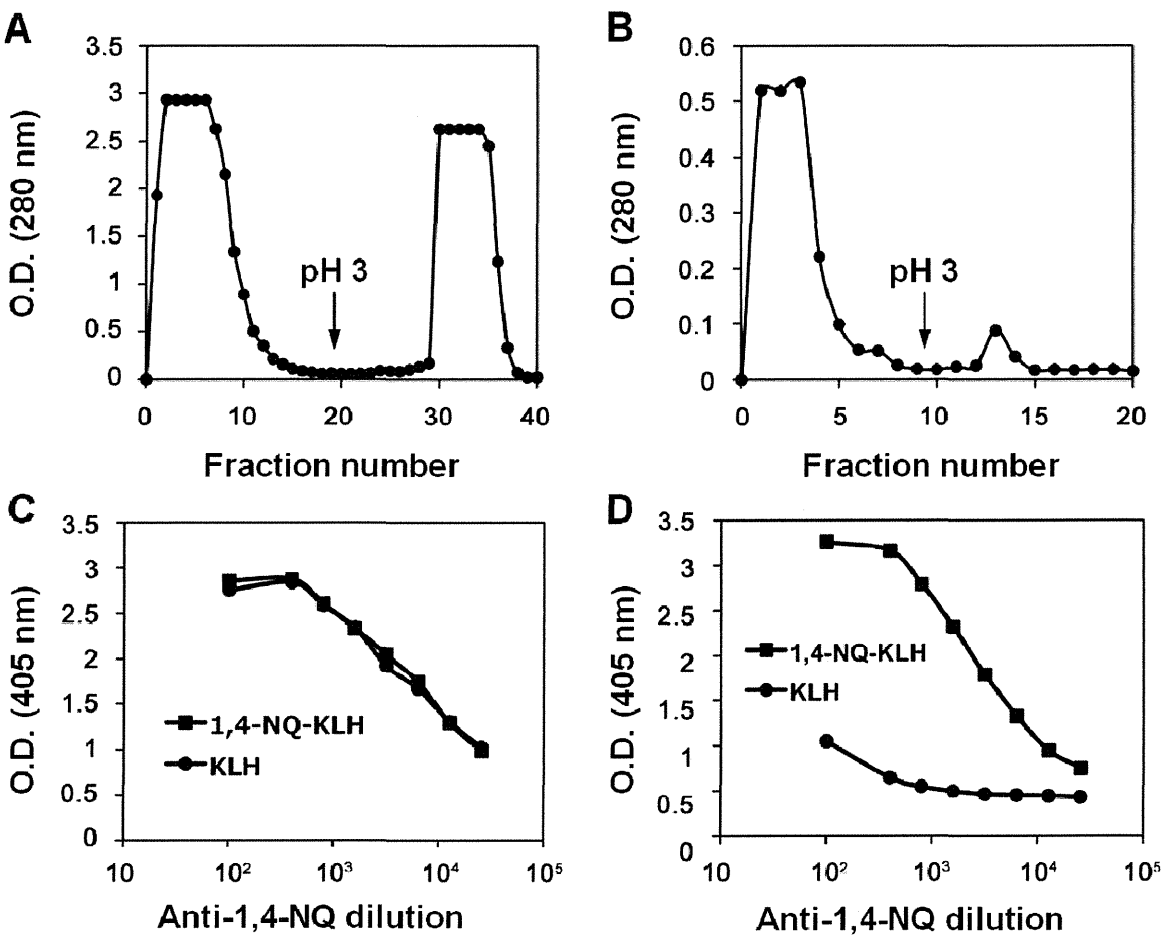

Fig. 2. Titration curves of rabbit polyclonal antibodies against 1,4-NQ (C and D) following purification of anti-1,4-NQ by Protein A-Sepharose CL-4B and KLH-Affi-Gel 15 column chromatographies (A and B). **A.** The antiserum (0.6 g) was loaded on a Protein A-Sepharose CL-4B column (6.6 cm × 1.2 cm i.d.) and washed with 20 mM potassium phosphate buffer (pH 7.2)-1.5 M NaCl and then 0.1 M glycine-HCl (pH 3). Up to 5 ml per fraction of IgG fractions 30-37 were collected. The IgG fractions were neutralized immediately by 0.5 ml of 1 M Tris-HCl (pH 8). **B.** The IgG fraction (1 mg) was loaded on a KLH-Affi-Gel 15 column (4 cm × 0.7 cm i.d.) and circulated at 4°C for 12 hr. Then, the column was washed with TTBS (anti-1,4-NQ fraction, No. 1-3) and 0.1 M glycine-HCl (pH 3) (anti-KLH fraction, No. 13-14). Up to 1.5 ml per fraction were collected. **C.** The titer of the IgG fraction (5 mg/ml) was determined by measuring the binding of serial dilutions of IgG (1/100 to 1/25600) to plates coated with native KLH (•) or 1,4-NQ-KLH (■). **D.** The titer of the anti-1,4-NQ fraction (1 mg/ml) was determined by measuring the binding of serial dilutions of anti-1,4-NQ antibody (1/100 to 1/25600) to plates coated with native KLH (•) or 1,4-NQ-KLH (■).

Immunoblot analysis

Proteins separated by SDS-PAGE were electro-transferred onto hydrophilic poly(vinylidene fluoride) membranes (Pall Co., Port Washington, NY, USA) at 2 mA/cm^2 for 1 hr, according to the method of Kyhse-Andersen (Kyhse-Andersen, 1984). After blocking with 5% skim milk, the membrane-bound proteins were incubated with anti-1,4-NQ antibody. Anti-rabbit IgG, HRP-linked antibody was used to detect primary antibodies on the membrane. Proteins were detected with an ECL system (Nacalai Tesque Inc.) and exposed to X-ray film (Konica Minolta Health Care Co., Tokyo, Japan).

1,4-NQ-modified protein identification by liquid chromatography-mass spectrometry

Proteins were stained by Coomassie brilliant blue followed by in-gel digestion for 4 hr at 37°C by MS grade modified trypsin (Promega Co., Madison, WI, USA). The tryptic digests were subjected to nanoUPLC-MS/MS analysis (Waters Co., Milford, MA, USA) as described previously (Miura *et al.*, 2011b). The resulting data were collected by MassLynx version 4.1 software (Waters Co.). ProteinLynx Global Server Browser version 2.3 software (Waters Co.) and Biopharmlynx version 1.2 software (Waters Co.) were used for baseline subtraction and smoothing, deisotoping, *de novo* peptide sequence identification, and database searches.

RESULTS AND DISCUSSION

For immunoblot analysis with an antibody against 1,4-NQ, we prepared a 1,4-NQ-KLH adduct. Incubation of commercial KLH with and without 1,4-NQ, following reduction by DTT as described in the Materials and Methods, resulted in a drastic consumption of the KLH thiol content (before reaction, 30.9 ± 3.3 μmol of thiol/g of protein; after reaction, 0.08 ± 0.1 μmol of thiol/g of protein), suggesting that KLH was extensively modified by 1,4-NQ. Next, we immunized rabbits with the adduct and combined the resulting antisera. Using a checkerboard titration, we tested the combined sera against the 1,4-NQ-KLH adduct following every bleeding until no enhancement of the titer was observed. However, the IgG fraction obtained by Protein A-Sepharose CL-4B column chromatography indicated that the titer of the antibody against 1,4-NQ-KLH was almost the same as that against KLH itself (Figs. 2A and C). Thus, we further purified the IgG fraction by KLH covalently coupled to Affi-gel 15 to remove specific antibodies against KLH (Fig. 2B). With the final preparation of the IgG fraction, it was revealed that the antisera raised the titer against 1,4-NQ-KLH, but not against KLH (Fig. 2D), suggesting that the antisera had high affinity toward the hapten moiety.

The cross-reactivity of the polyclonal antibody against 1,4-NQ with its related aromatic hydrocarbon quinones was examined by dot blot analysis (Fig. 3). Among the 10 chemicals examined, there was little cross-reactivity with *ortho*-quinone β-lapachone, although a faint blot was detected with 1,2-NQ under these conditions. The antibody prepared showed no recognition of the naphthalene-moiety with substitution groups such as 5-hydroxy-1,4-NQ, 5,8-dihydroxy-1,4-NQ, 2-methyl-1,4-NQ and 2-anillino-1,4-NQ, and of *para*-quinones with a different number of aromatic rings such as 1,4-benzoquinone and TBQ. These results suggest that the antibody recognized the naphthalene ring with the dicarbonyl group at the *para*-position without substitution groups, but not at the *ortho*-position.

Exposure of A431 cells to 1,4-NQ resulted in covalent modification of cellular proteins. Increasing concentrations of 1,4-NQ up to 50 μM or prolonged exposure to this quinone (24 hr) enhanced the covalent binding of 1,4-NQ to the cellular proteins, leading to substantial cell

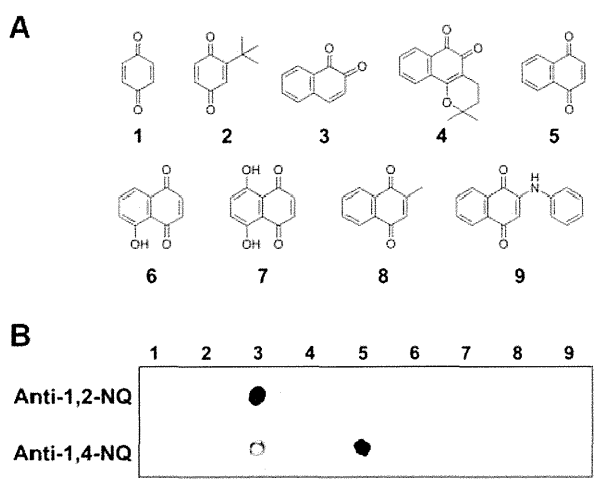

Fig. 3. Cross-reactivity of the polyclonal antibody against 1,4-NQ with aromatic hydrocarbon quinones. **A.** Structures of aromatic hydrocarbon quinones: 1, 1,4-benzoquinone; 2, TBQ; 3, 1,2-NQ; 4, β-lapachone; 5, 1,4-NQ; 6, 5-hydroxy-1,4-NQ; 7, 5,8-dihydroxy-1,4-NQ; 8, 2-methyl-1,4-NQ; 9, 2-anillino-1,4-NQ. **B.** Dot blot analysis. The S9 (0.1 mg/ml) was incubated with each aromatic hydrocarbon quinone (10 μM) for 30 min at 25°C in 0.1 M Tris-HCl, pH 7.5. Anti-1,2-NQ antibody was prepared as reported previously (Miura and Kumagai, 2010).

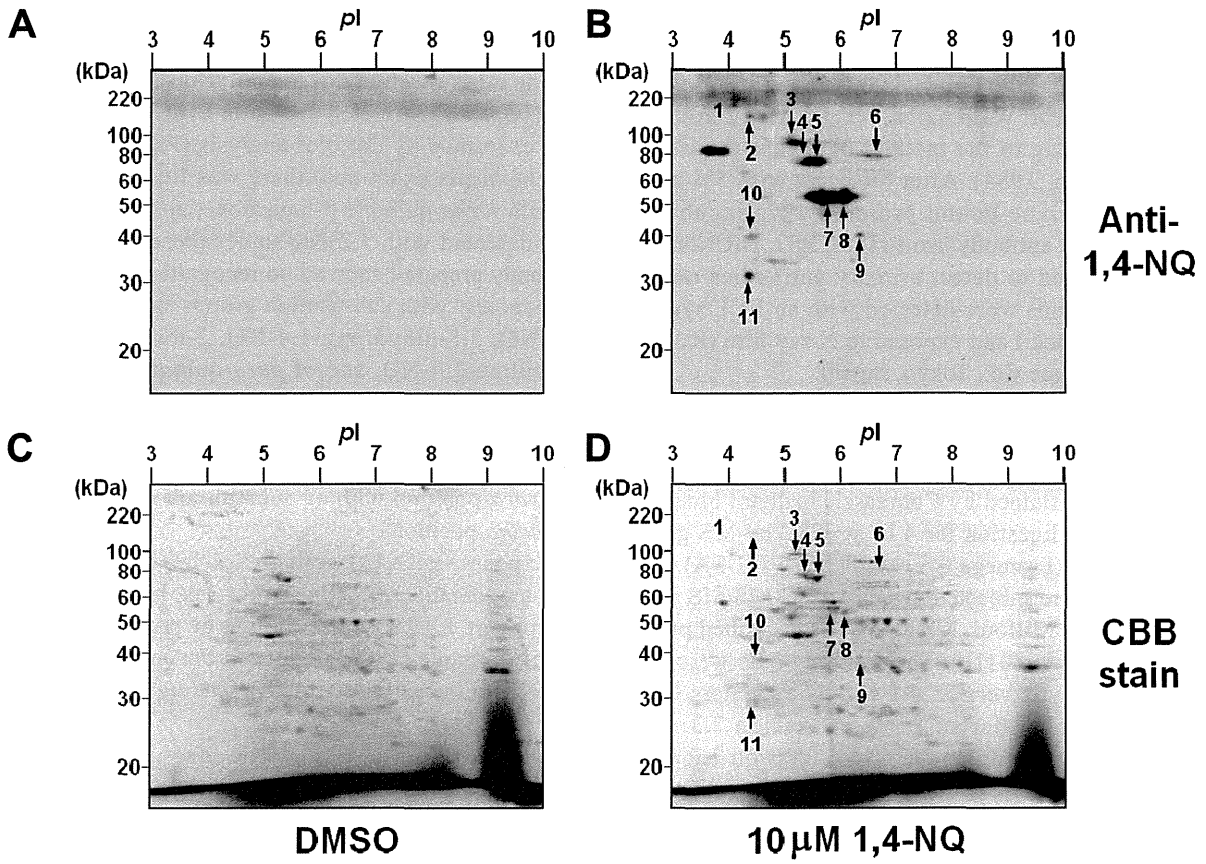

Fig. 4. Two-dimensional SDS-PAGE of cellular proteins from A431 cells exposed to 1,4-NQ. A431 cells were exposed to DMSO (A and C) or 10 μM 1,4-NQ (B and D) for 1 hr. Cell lysates (60 μg) were subjected to isoelectric focusing on Immobiline DryStrip pH 3-10, and then separated by SDS-PAGE. These gels were immunoblotted with anti-1,4-NQ (upper) and stained with CBB stain (lower). Numbered arrows indicate proteins that were subjected to tryptic digestion followed by nanoU-PLC-MS/MS analysis, as shown in Table 1.

death (data not shown). In the present study, to detect significant covalent modification of sensor proteins by 1,4-NQ, A431 cells were exposed to 10 μM 1,4-NQ for 1 hr. Under these conditions, eleven proteins with *p*I values ranging from 4.5 to 6.7 were detected as targets of 1,4-NQ (Fig. 4), indicating that relatively acidic proteins were readily modified by this electrophile. As shown in Table 1, nine cellular proteins were identified. Using radiolabeled compounds, other groups have also found that heat shock protein 90 (HSP90), HSP70, tubulin and actin are modified during exposure to 1,4-NQ or metabolic activation of naphthalene (Isbell *et al.*, 2005; Lame *et al.*, 2003). Lame *et al.* (2003) identified a large number of cellular proteins that were covalently bound to 1,4-NQ; this discrepancy can be explained by the following differences in experimental design: 1) a relatively high concentration of 1,4-NQ (15 μM vs. 10 μM), 2) longer exposure to 1,4-NQ (24 hr vs. 1 hr), and 3) different type of cells used (normal human bronchial epithelial cells vs. human epithelial carcinoma cell line).

In our present study, we developed an immunochemical method for detection of cellular proteins modified by 1,4-NQ, an electrophilic metabolite of naphthalene. The polyclonal antibody prepared could be used to identify proteins selectively modified by 1,4-NQ following exposure of cultured cells and experimental animals to naphthalene by proteomics analysis, without the necessity for radiolabeled compounds as reported by others (Isbell *et al.*, 2005). Among the identified 1,4-NQ protein adducts, HSP90 and HSP70 are negative regulators of heat shock factor-1 (HSF-1), a transcription factor responsible for the cellular response against electrophiles such as 1,4-

Table 1. Summary of 1,4-NQ modified proteins identified in A431 cells

No.	Accession	Description	kDa	*p*I	Coverage (%)
1		Unknown			
2		Unknown			
3	P08238	Heat shock protein HSP 90 beta	83.2	4.77	10.9
4	P11142	Heat shock cognate 71 kDa protein	70.9	5.20	61.3
5	P08107	Heat shock 70 kDa protein 1A 1B	70.0	5.32	61.9
6	P12956	X ray repair cross complementing protein 6	69.8	6.20	32.5
7	Q5SU16	Beta 5 tubulin	49.6	4.59	37.6
8	P31943	Heterogeneous nuclear ribonucleoprotein H	49.2	5.85	49.4
9	Q53SS8	Poly RC binding protein 1	37.5	6.71	31.5
10	P60709	Actin cytoplasmic 1	41.7	5.14	28.5
11	D0PNI1	Tyrosine 3 monooxygenase tryptophan 5 monooxygenase activation protein zeta polypeptide	27.7	4.53	51.4

Numbered proteins, which are shown in Fig. 4, were analyzed by LC-MS/MS following in-gel tryptic digestion. Protein identification was achieved using the ProteinLynx Global Server Browser version 2.3 software (Waters Co.). The accession number, theoretical molecular weight (kDa), and theoretical isoelectric point (*p*I) indicate the UniProtKB/Swiss-Prot entry. Coverage (%) shows the percentage of the protein sequence covered by identified peptides.

NQ. Under basal conditions, HSF-1 exists largely as an inactive monomer in the cytoplasm. Upon exposure to chemicals causing covalent modifications, HSP90 and/or HSP70 are modified and HSF-1 is trimerized and translocated into the nucleus, followed by upregulation of its downstream genes (e.g., HSPs) through activation of the heat shock response element (Akerfelt *et al.*, 2010; Jacobs and Marnett, 2010). Consistent with this, our preliminary study indicated that exposure of A431 cells to 1,4-NQ (5 μM) caused the nuclear translocation of HSF-1 (R. Sha, unpublished observation). While we have recently reported that 1,2-NQ activates the transcription factor Nrf2 coupled to covalent modification of the sensor protein Keap1, which negatively regulates Nrf2 (Miura *et al.*, 2011c), exposure to 1,4-NQ (5 μM) also resulted in activation of Nrf2, as determined by its nuclear accumulation in the cell (R. Sha, unpublished observation). Under these conditions, we did not detect Keap1 as a target protein of 1,4-NQ by the present method. A reasonable explanation for these observations is that HSP90 is an abundant protein in the cell (see Fig. 4, spot 3), whereas Keap1 is minimally expressed in the cell. Although further research is required to improve the detection sensitivity of covalent modifications of sensor proteins such as Keap1, our method would be useful for investigating electrophilic signal transduction pathways in a variety of cell types.

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