**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 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

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**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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no chemical 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 thioredoxin 1 (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 pH 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-butylenzoquinone (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); non-fat 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⁻¹cm⁻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/ml) were subjected to dot blot analysis as described previously (Miura and Kumagai, 2010).

**Two-dimensional SDS-PAGE**

A431 cells (375 × 10⁶ 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 GluMAX-I supplement (Life Technologies, Carsbad, CA, USA), and 10% (v/v) heat-inactivated fetal bovine serum at 37°C in 5% CO₂. 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/cm² 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 naphthelene-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
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 pI 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 immunochromical 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-NQ.
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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References

Isbell, M.A., Morin, D., Boland, B., Buckpitt, A., Salemi, M. and

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 (pl) indicate the UniProtKB/Swiss-Prot entry. Coverage (%) shows the percentage of the protein sequence covered by identified peptides.

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

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