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

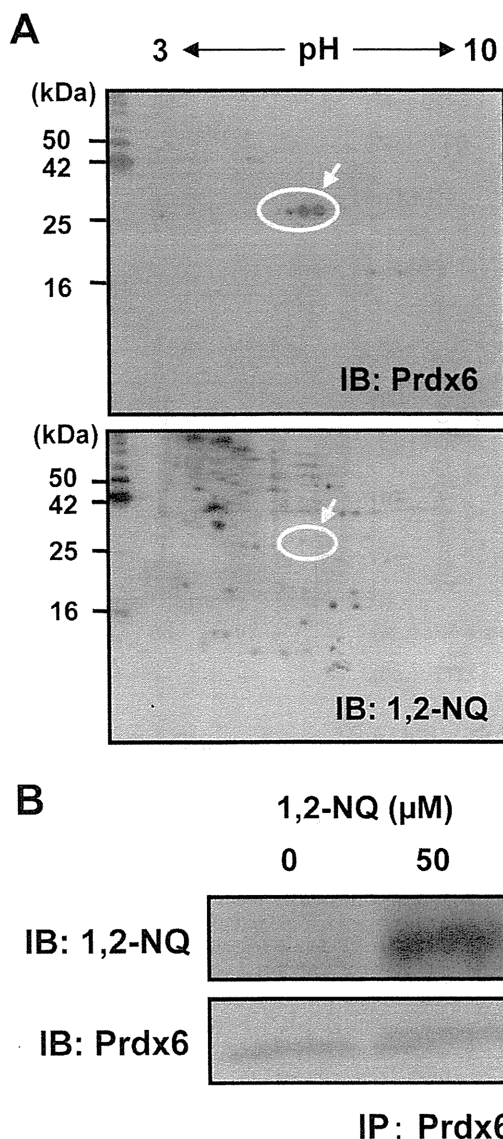

Fig. 2. 2D-SDS/PAGE analysis of cellular proteins bound to 1,2-NQ in A549 cells. A. Cells were exposed to 70 μM 1,2-NQ for 30 min at 37°C. Cell lysate (50 μg) was separated by 2D electrophoresis and subjected to western blot analysis with the indicated antibodies. B. Cells were exposed to 50 μM 1,2-NQ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

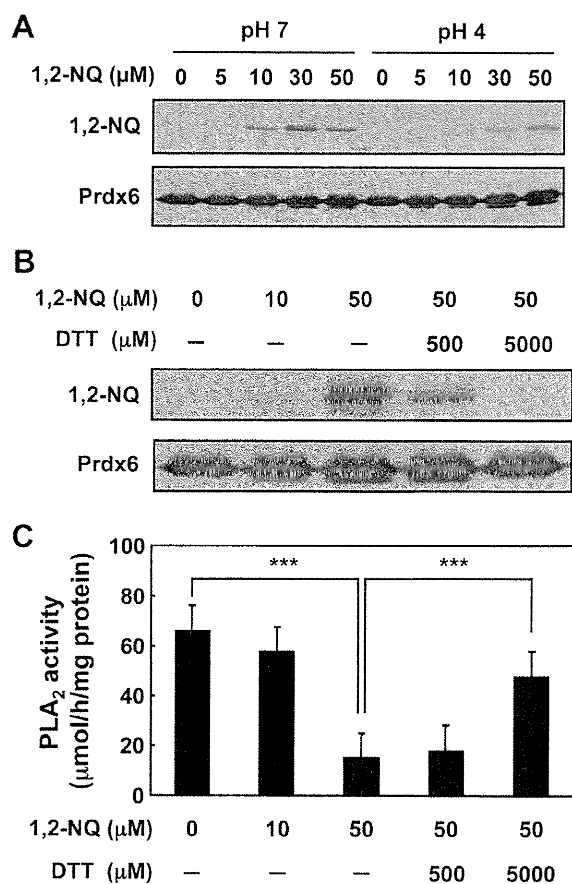

Fig. 3. pH-dependent covalent binding of 1,2-NQ to purified Prdx6, and inhibition of Prdx6-catalyzed PLA_2 activity by 1,2-NQ through covalent modification. A. Human Prdx6 and was incubated with the indicated concentrations of 1,2-NQ for 10 min at 37°C in 50 mM potassium phosphate buffer (pH 7) or 50 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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