Chemical Knockdown of Protein-tyrosine Phosphatase 1B by 1,2-Naphthoquinone through Covalent Modification Causes Persistent Transactivation of Epidermal Growth Factor Receptor*5

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1,2-Naphthoquinone (1,2-NQ), an atmospheric contaminant, causes the contraction of guinea pig trachea through the activation of epidermal growth factor receptor (EGFR) by inhibiting protein-tyrosine phosphatases (PTPs). Phosphorylation of EGFR is negatively regulated by PTPs, but details of the mechanism by which 1,2-NQ inhibits PTPs have not been elucidated. Results described in this report demonstrate that 1,2-NQ forms covalent bonds with PTP1B after exposure to human epithelial A431 cells. In this study, a concentration-dependent phosphorylation of EGFR was found to be coupled to the reduction of PTP activity in the cells. The reduction in PTP activity was due to the irreversible modification of PTP1B, and when PTP1B was overexpressed by the cells, the 1,2-NQ-mediated phosphorylation was suppressed. Studies with purified PTP1B and 1,2-NQ showed that the reduction in enzyme activity was due to a nucleophilic attack by the quinone on the enzyme, to form covalent bonds. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry analysis and mutation experiments revealed that PTP1B inactivation was primarily due to covalent attachment of the quinone to Cys-121 of the enzyme, with binding to His-25 and Cys-215 as well. Collectively, the results show that covalent attachment of 1,2-NQ to PTP1B is at least partially responsible for the reduction of PTP activity, which leads to prolonged transactivation of EGFR in the cells.

Most intracellular signaling cascades are mediated by receptor tyrosine kinases, and the activated signals are transmitted to downstream proteins by sequential phosphorylation. Because protein tyrosine phosphorylation is negatively regulated by protein-tyrosine phosphatases (PTPs),2 these dephosphorylation enzymes play important roles in the signal transduction controlled by receptor tyrosine kinases. PTPs are a diverse family of enzymes that can be divided into several subgroups, including receptor PTPs and non-receptor PTPs. All PTPs contain an essential cysteine residue in the active site motif, HCX3,R(S/T) (1). This sulfhydryl group, as thiolate anion, contributes to the formation of a thiol-phosphate intermediate in the dephosphorylation reaction and is therefore essential for enzyme activity (2). Because of the low pKa of the catalytic cysteine (e.g. ~5.4 for mammalian PTPs) (3), PTP thiols are readily oxidized to their sulfenic acid (-SOH) state by reactive oxygen species (ROS) (4, 5), thereby decreasing dephosphorylation activity. Current consensus is that PTP activity is regulated by endogenous oxidant such as hydrogen peroxide (H2O2) in cells (6, 7). The decreased PTP activity associated with epidermal growth factor receptor (EGFR) activation caused by H2O2 is transient and restored by cellular reduction systems (8). However, if reactive thiols of PTPs undergo covalent modification by exogenous electrophiles, irreversible inactivation of PTPs, resulting in prolonged phosphorylation of EGFR, would occur.

We have been examining the reactivity and toxicity of selected quinones (9–13) in an effort to establish mechanistic pathways that may account for the adverse health effects associated with exposure to mixtures in which they are present. Quinones, including 1,2-NQ of this study, have been found at significant concentrations in vehicle exhaust particles and in ambient air samples (14–16), so their toxic actions have considerable relevance to understanding air pollution toxicity. Quinones are a class of organic compounds that undergo two toxicologically relevant reactions (9, 17, 18). One is as an elec-

2 The abbreviations used are: PTP, protein-tyrosine phosphatase; 1,2-NQ, 1,2-naphthoquinone; PTP1B, protein-tyrosine phosphatase 1B; EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; DTT, dithiothreitol; HRP, horseradish peroxidase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TBS, Tris-buffered saline; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nN, p-nitrophenol; pNPP, p-nitrophenyl phosphate; KLH, keyhole limpet hemocyanin.
trophile, forming covalent bonds with tissue nucleophiles to irreversibly alter key cellular proteins. In the second reaction the quinone acts as an electron transfer agent, transferring electrons from a reducing source such as NADPH to oxygen, generating ROS such as H₂O₂.

We recently reported that 1,2-NQ elicits a contraction of smooth muscle in guinea pig tracheal preparations under conditions that indicated transactivation of EGFR was involved in the contraction process (19). These studies also suggested that 1,2-NQ-induced EGFR activation was caused, at least partially, by covalent modification and/or thiol oxidation of PTP1B, leading to the reduction of its dephosphorylation activity. Of the multiple PTPs, PTP1B is ubiquitously expressed (20) and negatively regulates receptor tyrosine kinases such as EGFR (21), so that inhibition of this PTP would lead to EGFR activation. Although 1,2- and 1,4-NQ and their derivatives are reported to be potent inhibitors for PTP1B (22, 23), their detailed mechanism of action still remains unclear. This report describes results of a study of 1,2-NQ-PTP1B interaction, which demonstrates that 1,2-NQ-mediated covalent modification of PTP1B, accompanied by reduction of its enzyme activity, results in the transactivation of EGFR in human epithelial A431 cells. The results also show that arylation of PTP1B through Cys-121 is the predominant event in the loss of enzyme activity caused by 1,2-NQ.

EXPERIMENTAL PROCEDURES

Materials—1,2-NQ was purchased from Tokyo Kasei Industries, Ltd. (Tokyo, Japan). Naphthalene, Dulbecco’s modified Eagle’s medium, penicillin streptomycin solution, imidazole, p-nitrophenol (pNP), and 3,5-dimethoxy-4-hydroxynaphthalene acid were obtained from Sigma. trans-1,2-Dihydroxy-1,2-dihydroporphalene was kindly donated by Dr. S. Yamano (Fukuoka University, Japan). 5,5’-Dithiobis(2-nitrobenzoic acid) (DTNB) and isopropyl-1-thio-β-D-galactopyranoside were purchased from Nacalai Tesque (Kyoto, Japan). p-Nitrophenyl phosphate (pNPP) was purchased from ICN Biomedicals (Irvine, CA). Escherichia coli BL21 cells and sequence grade modified trypsin were purchased from Promega (Madison, WI). Gluta Max-1, fetal bovine serum, Opti-MEM medium and pcDNA3.1/HisC were obtained from Invitrogen. FuGENE HD transfection reagent was purchased from Roche Applied Science. α-Cyano-4-hydroxycinnamic acid was purchased from Bruker Daltonics Japan (Tokyo, Japan). pET15b was purchased from Novagen (Madison, WI). EGFR-neutralizing antibody LA-1 was purchased from Upstate (Lake Placid, NY). All other reagents used were of the highest purity available.

Preparation of Anti-1,2-NQ—The polyclonal antibody against 1,2-NQ was prepared by a modification of the method of Zheng and Hammock (24). Water-soluble keyhole limpet hemocyanin (KLH, 20 mg) was dissolved in 3.5 ml of 4 M guanidinium chloride, followed by addition of 20 mg of dithiothreitol (DTT) to reduce oxidized thiol groups of the KLH. The mixture was stirred under argon at room temperature in the dark for 2 h. The reduced proteins were loaded to an Econo-Pac 10 DG column (Bio-Rad), which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA to remove DTT. Then the KLH solution (equivalent to 10 mg) was mixed with 1,2-NQ (1 mg dissolved in Me₂SO) under argon at 25 °C for 20 min, and the mixture was dialyzed against the equilibrated buffer described above. The binding efficiency of 1,2-NQ to KLH was measured using a Shimadzu high-performance liquid chromatography system (Kyoto, Japan). An aliquot (40 μl) of the reaction mixture was applied to an AM-type YMC-ODS column (250 × 4.6-mm inner diameter, 5-μm particle size, Yamamura Labs, Kyoto, Japan), at a flow rate of 1 ml/min. Water/acetonitrile (3:2, v/v) was used as the mobile phase, and detection was performed at 255 nm. Under these conditions, the retention time of 1,2-NQ was 7.9 min. Final preparation of KLH-1,2-NQ adduct was 0.33 mg of 1,2-NQ bound per milligram of KLH. Antibodies against 1,2-NQ were raised in male New Zealand White rabbits. The IgG fraction was isolated from the serum obtained according to previously described methods (25).

Cell Culture and Transfection—Human epidermoid carcinoma cell line A431 cells (ATCC, Manassas, VA) were grown at 37 °C in a humidified atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM Gluta Max-1 and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). A431 cells were grown to ~70% confluency on 35-mm plates. Transfection was performed with FuGENE HD transfection reagent according to manufacturer’s instructions. A431 cells were transfected with lacZ (pcDNA/His-lacZ) or PTP1B (pcDNA/His-PTP1B) for 48 h.

Western Blotting—Cells were extracted with radioimmunoprecipitation assay 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 mixture. Each sample was normalized to a protein content of 20 μg then mixed with half the volume of SDS-PAGE loading buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 24% glycerol, 6% SDS, 15% 2-mercaptoethanol, and 0.015% bromphenol blue. The samples were heated at 95 °C for 1 min and were separated by SDS-PAGE (7.5 or 12% acrylamide) (26). Electrophoresed proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad). Blots were blocked for 12 h with 5% skim milk in TTBS (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Tween 20), washed briefly and incubated with the indicated primary antibodies. Anti-EGFR (Upstate, Lake Placid, NY), anti-phospho EGFR that recognized Tyr-1068 (Sigma), anti-PTP1B (see below), and anti-1,2-NQ were used as primary antibodies. HRP-conjugated rabbit anti-sheep that recognized anti-phospho EGFR, HRP-conjugated goat anti-rabbit, and HRP-conjugated horse anti-mouse (Cell Signaling Technology, Beverly, MA) were used as secondary antibodies.

For immunoprecipitation studies, cell lysates were incubated with Affi-gel 10 (Bio-Rad) cross-linked with anti-37-kDa PTP1B IgG (8.2 mg of protein/ml of gel) which was prepared according to the manufacturer’s instructions. The gels were centrifuged at 13,000 × g for 1 min and then washed with 1 ml of ice-cold radioimmunoprecipitation assay lysis buffer three times, and the PTP1B from the cell lysates was eluted with 20 μl of 0.1 M glycine-HCl (pH 3.0) at 25 °C. Proteins were detected by immunoblot analysis as described above. Protein bands on the membrane were detected by using chemiluminescence.
Arylation of PTP1B by 1,2-NQ

reagents and film according to the manufacturer’s instructions (GE Healthcare Bio-Sciences Corp.) and exposed on high-performance chemiluminescence film (GE Healthcare Bio-Sciences Corp.) (27). Data for Western blotting are representative of three or more experiments. Protein concentration was determined with the Bio-Rad Protein assay kit (28) or the BCA protein assay reagent (Pierce), with bovine serum albumin as a standard.

Mutagenesis and Protein Expression—Complementary DNA, corresponding to the 37-kDa form (NH2-terminal 321 residues) of PTP1B, was obtained by PCR, placed downstream of the phage T7 RNA polymerase promoter at the Ndel site of pET15b. A plasmid containing the isolated catalytic domain of human 37-kDa PTP1B in a pET15b vector was used as a template for site-directed mutagenesis. Cys-to-Ser and His-to-Ala mutations were introduced using a PCR-based approach following established procedures (29). The resulting construct was verified by DNA sequencing on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting plasmid was transformed into E. coli BL21 cells for protein expression. The bacterial cultures were grown in LB broth at 37 °C with shaking at 120 rpm (TAITEC, Saitama, Japan) to 0.6 absorbance units at 600 nm. The cultures were then induced by the addition of 0.2 mM isopropyl-1-thio-galactopyranoside at 600 nm. The cultures were then induced by the addition of 0.6 absorbance units at 600 nm. The cultures were then induced by the addition of 0.2 mM isopropyl-1-thio-galactopyranoside and grown for an additional 20 h. The cells were harvested by centrifugation, and the cell lysates were analyzed by electrophoresis and immunoblotting using the protocol outlined above.

Purification of 37-kDa PTP1B and Its Mutants—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. The supernatant was retained following centrifugation at 100,000 × g for 1 h of the sonicated-cell lysates, and then applied to a Ni-IDA ProBond (Invitrogen) column (4 × 1 cm inner diameter), which had been equilibrated with 50 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 10 mM 2-mercaptoethanol solution. The affinity column was extensively washed with the equilibrated buffer, and then 37-kDa PTP1B and its mutants were eluted with the buffer containing 100 mM imidazole. The purity of the PTP1B preparations, as assessed by SDS-PAGE, was >90%. Thiol groups oxidized during purification were reduced by incubation with 20 mM DTT for 1 h, and the DTT was removed by an Econo-Pac 10 DG column. Each enzyme preparation was stored in 50 mM potassium phosphate buffer (pH 7.0) at −80 °C before use. The polyclonal antibody against 37-kDa PTP1B was prepared by the same method used for anti-1,2-NQ.

Dephosphorylation Activity— Cultured cells were treated with different concentrations of 1,2-NQ for 10 min. Then, the medium was removed, and cell radioimmune precipitation assay lysis buffer was added to the culture dish. The cell extracts were scraped from the dish and transferred to microcentrifuge tubes. Supernatants obtained from centrifugation at 13,000 × g for 10 min were used for phosphatase assays.

Phosphatase Assay—Briefly, cell lysate (100 μg) was incubated with 4 mM pNPP and 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, bovine serum albumin (0.1 mg/ml). The reaction was allowed to proceed for 20 min at 37 °C and terminated by addition of 1.8 ml of 1 M NaOH. The pNP formed was determined at 400 nm using a Hitachi U-1500 spectrophotometer (Tokyo, Japan).

A two-stage incubation method was employed to monitor the effect of 1,2-NQ on PTP1B activity. The incubation mixture (0.2 ml) for the first stage consisted of 37-kDa PTP1B (0.1 mg), different concentrations of 1,2-NQ and 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, unless other noted. Reactions were initiated by addition of 1,2-NQ. After incubation at 25 °C for various times, aliquots (1 μl) were transferred to the second incubation mixture (1 ml) to determine PTP1B activity. The second incubation reaction mixture consisted of the aliquot, 4 mM pNPP, 100 mM acetate buffer (pH 5.5), and 0.1 mM EDTA. Incubations were carried out at 25 °C for 10 min and terminated by addition of NaOH to make a final concentration of 1 M. Under these conditions, dephosphorylation of pNPP was linear with time and protein concentration.

Determination of Free SH Groups—PTP1B was incubated at 25 °C with 50 μM 1,2-NQ in a total volume of 200 μl containing 50 mM potassium phosphate buffer (pH 7.5). After 10 min, the reaction was stopped by adding 300 μl of 200 mM Tris-HCl (pH 8.2), 20 mM EDTA. The reaction mixture and control untreated enzymes were mixed with solution containing 20 μl of 10 mM DTNB, 150 μl of 5% SDS, and 830 μl of H2O. After 2 min, the concentration of thionitrobenzoic acid released was determined spectrophotometrically using a molar extinction coefficient of 13,600 at 412 nm (30).

Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)—PTP1B was incubated with 0 or 50 μM 1,2-NQ for 10 min at 25 °C in a buffer containing 50 mM potassium phosphate buffer (pH 7.5) and then dialyzed into 50 mM potassium phosphate buffer (pH 7.0) using an Econo-Pac 10 DG column to remove free 1,2-NQ. The trypsin-digested PTP1B were mixed with DTT and trifluoroacetic acid. To improve the ionization efficiency of mass spectrometry, samples were purified with Zip-tip C4 or C18 (Millipore, Bedford, MA) before MS and MS/MS analysis. Peptides were mixed with α-cyano-4-hydroxycinnamic acid (2.5 mg/ml) containing 50% acetonitrile and 0.1% trifluoroacetic acid and dried on stainless steel targets at room temperature and pressure. The analyses were performed using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics Japan, Ltd., Tokyo) with a nitrogen laser. All analyses were in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies.

Data Analysis—All data were expressed as means ± S.E. from at least three independent experiments. Statistical significance was considered at p < 0.05. Data were fitted by nonlinear regression analysis according to a Michaelis-Menten kinetic model (GraphPad Prism version 4.0, San Diego, CA).

RESULTS

Phosphorylation of EGFR Coupled to Reduction of PTP Activity by 1,2-NQ in the Cellular System—Exposure of A431 cells to 1,2-NQ for 10 min caused a concentration-dependent phosphorylation of EGFR (Fig. 1A). Under these conditions, 1,2-NQ also inhibited cellular PTP activity in a concentration-dependent manner, but the reduction of PTP activity by 1,2-NQ
reached a plateau at 50 μM (Fig. 1A). To examine whether or not 1,2-NQ-induced EGFR phosphorylation is ligand-dependent, we pretreated the cells with a EGFR-neutralizing antibody LA-1 for EGFR prior to exposure of A431 cells to 1,2-NQ. As shown in Fig. 1B, A431 cells subjected to the EGFR-neutralizing antibody LA-1 exhibited a complete inhibition of EGFR-induced EGFR phosphorylation, whereas only a slight suppression of 1,2-NQ-induced EGFR phosphorylation was detected. These results suggested that a ligand-independent EGFR phosphorylation through the inhibition of PTP activity occurs during exposure of A431 cells to 1,2-NQ. In temporal experiments conducted over 720 min, EGFR phosphorylation by EGFR in A431 cells was seen up to 180 min, after which it declined (Fig. 1C). In contrast, EGFR activation caused by 1,2-NQ was persistent even at 720 min but with a biphasic transactivation of EGFR phosphorylation (see Fig. 1C). Because PTP inactivation by 1,2-NQ could be due to either thiolate oxidation or arylation, we examined the effect of ROS-scavenging agents on the transactivation. H$_2$O$_2$-mediated phosphorylation of EGFR was blocked by pretreatment of A431 cells with either catalase conjugated with polyethylene glycol or Trolox, a scavenging agent for ROS, whereas EGFR activation caused by 1,2-NQ was not (supplemental Fig. S1). These results suggest that such a prolonged phosphorylation of EGFR during exposure of A431 cells to 1,2-NQ was not due to thiol oxidation and could be due to covalent binding of this quinone to cellular PTPs.

If PTP1B is responsible for the transactivation of EGFR caused by 1,2-NQ, overexpression of PTP1B in A431 cells should diminish EGFR phosphorylation by overwhelming the 1,2-NQ. Consistent with this notion, 1,2-NQ (20 μM)-induced activation of EGFR was suppressed by increasing PTP1B levels in the cells (Fig. 2A). To identify whether or not PTP1B is one of the molecular targets for irreversible modification by 1,2-NQ, we prepared a specific antibody against 1,2-NQ as described under “Experimental Procedures.” As shown in Fig. 2B, immunoprecipitation with anti-PTP1B, followed by Western blotting analysis with the anti-1,2-NQ, revealed that 1,2-NQ was covalently bound to PTP1B in A431 cells. To explore the mechanistic details of the covalent modification of PTP1B by 1,2-NQ further, we worked with purified 37-kDa PTP1B in subsequent experiments.

Involvement of Covalent Binding of 1,2-NQ in Decreased 37-kDa PTP1B Activity—As shown in Fig. 3A, 37-kDa PTP1B activity was inhibited by 1,2-NQ in a time- and concentration-dependent manner. The apparent first order decay rates were linear with concentration with a slope of 0.014 μM$^{-1}$min$^{-1}$. When fixed concentrations of 1,2-NQ were incubated with PTP1B for 10 min, the reduction of PTP1B activity caused by 1,2-NQ paralleled the increase in covalent modification of this quinone to PTP1B (Fig. 3B). H$_2$O$_2$ is reported to inhibit PTP1B activity through the oxidation of Cys-215 presumably to the corresponding sulfenic acid (7), but the decreased PTP1B activity was readily restored by addition of 4 mM DTT through reduction of the oxidized thiol groups (Fig. 3C). However, DTT had no effect on the loss of PTP1B activity caused by 1,2-NQ (Fig. 3C), supporting the notion that an irreversible or covalent bond-based inactivation rather than thiol oxidation of 1,2-NQ is occurring.

To further characterize the covalent modification of PTP1B by 1,2-NQ, we compared 1,2-NQ with the other naphthalene derivatives shown in Fig. 4A. In earlier studies we observed guinea pig tracheal contraction caused by 1,2-NQ by EGFR phosphorylation, whereas trans-1,2-dihydroxy-1,2-dihydronaphthalene and naphthalene (see Fig. 4A) were without effect on the contractile action and activation of EGFR (19). This suggests an obligatory role of an electrophilic function in the contraction of guinea pig trachea. As shown in Fig. 4B, the extent of covalent binding of 1,2-NQ (5 and 50 μM) increased with a corresponding reduction of 37-kDa PTP1B activity. However, trans-1,2-dihydroxy-1,2-dihydronaphthalene and naphthalene, which are incapable of binding to PTP1B, did not exhibit any inhibitory action on PTP1B (Fig. 4B). These results indicate that the decreased PTP1B activity was dependent on the electrophilic property of 1,2-NQ.
Modification Sites of 1,2-NQ—We then measured the thiol content of 37-kDa PTP1B after reaction with 1,2-NQ. Although there are six equivalents of cysteine residues in 37-kDa PTP1B (Cys-32, Cys-92, Cys-121, Cys-215, Cys-226, and Cys-231), the thiol content of the PTP1B prepared in the present study was 5.41 equivalents of thiol per mol of molecule ($n = 6$).

When we determined the stoichiometry of the interaction of 50 $\mu$M 1,2-NQ with 13 $\mu$M 37-kDa PTP1B, as determined by the consumption of reactive thiols and remaining 1,2-NQ during a 10-min reaction of the protein with 1,2-NQ at 25 °C, it was suggested that approximately two equivalents of thiols in PTP1B underwent chemical modification by 1,2-NQ.

MALDI-TOF MS Analysis of Arylated 37-kDa PTP1B—To determine the modification sites in 1,2-NQ, the native and 1,2-NQ-treated PTP1B were digested with trypsin and then analyzed by MALDI-TOF MS. Peptide mass mapping by MALDI-TOF MS analysis of the tryptic fragments from the native PTP1B accounted for $\sim 70\%$ of the protein sequence (Fig. 5 and Table 1). Compared with the calculated mass of the unmodified peptides, modified peptides P-1 to P-4, showed an increased mass of +156 Da, corresponding to the addition of a single equivalent of 1,2-NQ. The peptides, their sequence and masses are: P-1 (CAQYWPQK, $m/z = 1179.6$), P-2 (HEASDFPCR, $m/z = 1217.6$), P-3 (GSLKCAQYWPQK, $m/z = 1564.6$), and P-4 (ESGSLSPEHGPVVHCSAGIGR, $m/z = 2330.1$) (Fig. 5 and Table 1). These peptides were also detected in the selective ion monitoring analysis of tryptic peptides from the 1,2-NQ-treated PTP1B using electrospray ionization-liquid chromatography-MS (supplemental Fig. S2). No other peptides with 1,2-NQ adducts were detected by selective ion monitoring analysis.
To confirm the 1,2-NQ binding sites, the fragments modified by 1,2-NQ (P-1 and P-2) were further analyzed by MS/MS analysis using MALDI-TOF-TOF MS. The MS/MS spectrum of the [M+H]⁺ at m/z = 1179.5 from the 1,2-NQ-modified fragment (P-1, CAQYWPQK) is shown in Fig. 6A. In the MS/MS analysis, the singly charged C-terminal product ions (y₄, y₅, and y₆) and NH₃ loss fragment ions (y₃₋₁₇ and y₄₋₁₇) were observed. Fragment ions (a₂, b₃, b₄, and b₅) were observed to have increased by 156 Da, suggesting that the 1,2-NQ-modification site in the sequence is Cys-121.

The MS/MS spectrum of the [M+H]⁺ at m/z = 1217.4 from the 1,2-NQ-modified fragment (P-2, HEASDFPCR) is shown in Fig. 6B. The singly charged C-terminal product ions (y₁, y₃, y₄, y₅, y₆, y₇, and y₈) and the NH₃ loss fragment ions (y₃₋₁₇ and y₄₋₁₇) were observed in the MS/MS analysis. Fragment ions (b₂, b₃, b₄, b₅, b₆, b₇, and b₈) were observed to have increased by 156 Da. Furthermore, immonium and related ions (His, 110.1 and 138.1) were shown to increase by 156 Da. These results confirmed that the 1,2-NQ Michael adduct is with His-25. Furthermore, it was confirmed by electrospray ionization-liquid chromatography-MS/MS analysis (supplemental Fig. S3). In this manner, we identified the two target residues as His-25 and Cys-121.

If the covalent modification of Cys-121 and His-25 of 37-kDa PTP1B is involved in the reduction of its dephosphorylation activity, substitution of these nucleophilic amino acids with those that are incapable of reacting with 1,2-NQ should prevent the observed loss of catalytic activity caused by this quinone. To confirm this possibility, mutations of Cys-121 and Cys-215 to Ser (C121S and C215S) and His-25 to Ala (H25A) were performed. Dephosphorylation activities of wild type, C121S, and H25A of 37-kDa PTP1B were 51.7, 48.5, and 51.9 units/mg of protein, respectively, suggesting that these amino acid residues themselves do not affect PTP1B activity. However, substitution of Ser-121 for Cys-121, but not substitution of Ala-25 for His-25, prevented the decrease in PTP1B activity caused by 1,2-NQ (50 μM) (Fig. 7A). Consistent with this observation, covalent binding of 1,2-NQ to the wild-type PTP1B was lower after mutation of Cys-121 to Ser, but only slightly lower after mutation of His-25 to Ala (Fig. 7B). It should also be noted that there was a reduction of covalent binding of 1,2-NQ to PTP1B with substitution of Ser-215 for Cys-215, suggesting that not only Cys-121 and His-25 but also Cys-215 undergo irreversible modification by 1,2-NQ under the conditions.

**DISCUSSION**

Quinones interact with cellular systems by two mechanisms, as electrophiles and as electron transfer agents to yield ROS, with individual quinones exhibiting these mechanisms to varying degrees (17). We reported recently that 1,2-NQ induces potent contraction of guinea pig tracheal rings ex vivo through transactivation of receptor tyrosine kinases, including EGFR (19). In the present study, exposure of A431 cells to 1,2-NQ resulted in a phosphorylation of EGFR coupled to decreased activity, substitution of these nucleophilic amino acids with those that are incapable of reacting with 1,2-NQ should prevent the observed loss of catalytic activity caused by this quinone. To confirm this possibility, mutations of Cys-121 and Cys-215 to Ser (C121S and C215S) and His-25 to Ala (H25A) were performed. Dephosphorylation activities of wild type, C121S, and H25A of 37-kDa PTP1B were 51.7, 48.5, and 51.9 units/mg of protein, respectively, suggesting that these amino acid residues themselves do not affect PTP1B activity. However, substitution of Ser-121 for Cys-121, but not substitution of Ala-25 for His-25, prevented the decrease in PTP1B activity caused by 1,2-NQ (50 μM) (Fig. 7A). Consistent with this observation, covalent binding of 1,2-NQ to the wild-type PTP1B was lower after mutation of Cys-121 to Ser, but only slightly lower after mutation of His-25 to Ala (Fig. 7B). It should also be noted that there was a reduction of covalent binding of 1,2-NQ to PTP1B with substitution of Ser-215 for Cys-215, suggesting that not only Cys-121 and His-25 but also Cys-215 undergo irreversible modification by 1,2-NQ under the conditions.
PTP activity in a concentration-dependent manner. Unlike EGF, however, 1,2-NQ caused a prolonged ligand-independent activation which persisted at least up to 720 min compared with 180 min for EGF (Fig. 1, B and C). These observations suggest that 1,2-NQ irreversibly inactivates PTPs that act as negative regulators for EGFR thereby causing its transactivation. This inactivation could be due to either a Michael addition reaction by the quinone to the protein thiol or to its oxidation by H$_2$O$_2$ generated by quinone-based electron transfer. To address these possibilities, a comparison of the effects of antioxidants on phosphorylation of EGFR in A431 cells by H$_2$O$_2$ and 1,2-NQ were examined. The effect of H$_2$O$_2$, but not that of 1,2-NQ, was effectively diminished by pretreatment with either catalase conjugated with polyethylene glycol or Trolox (supplemental Fig. S1), indicating that a Michael reaction was likely for 1,2-NQ action. It should be noted that ~40% of cellular PTP activity was unaffected by 1,2-NQ even at 50 μM (Fig. 1A), suggesting that not all of the PTPs in A431 cells are sensitive to 1,2-NQ.

Because PTP1B is known to regulate EGFR negatively and has a reactive thiol (Cys-215) at the active center (31), we used PTP1B as a model for PTP modification by 1,2-NQ. As expected, EGFR phosphorylation caused by 1,2-NQ was repressed in cells transfected with human PTP1B cDNA (Fig. 2B), i.e., higher levels of PTP1B reduced the capacity of a given concentration of 1,2-NQ to activate cellular EGFR. Immunoprecipitation with anti-PTP1B and subsequent immunoblot analysis with specific antibody against 1,2-NQ revealed that 1,2-NQ indeed binds to endogenous PTP1B (Fig. 2B). These observations suggest that PTP1B plays a critical role in the 1,2-NQ-mediated activation of EGFR in the cells. Abdelmohsen et al. (32) reported previously that 2-methyl-1,4-NQ (menadione), benzoquinone, and 2,3-dimethoxy-1,4-NQ all phosphorylate EGFR in WB-F344 rat liver epithelial cells and that the activation of EGFR caused by menadione is associated with inhibition of PTP regulating EGFR. Thus, it seems likely that menadione, which is also electrophilic, and 1,2-NQ are bound to PTP1B, thereby causing substantial transactivation of EGFR.

Based on the cellular effects, we focused on the direct reaction between PTP1B and 1,2-NQ. Experiments with purified recombinant 37-kDa PTP1B showed that a mechanism-based inactivation of the PTP1B by 1,2-NQ followed second-order kinetics with a rate constant of 0.014 M$^{-1}$ min$^{-1}$. These results indicate that there is a chemical reaction between 1,2-NQ and 37-kDa PTP1B with a rate constant of 0.014 M$^{-1}$ min$^{-1}$. The pseudo-first-order plots (see Fig. 3A) would then imply that a single modification is responsible for enzyme activation even though more than one residue is modified. Although Rhee et al. (6) showed that H$_2$O$_2$-mediated oxidation of the catalytic site thiol (Cys-215) to sulfinic acid of PTP1B decreased its enzyme
activity, the decrease was completely restored by DTT (7). However, as shown in Fig. 3C, this reducing agent was without effect on the decrease in PTP1B activity caused by 1,2-NQ. Furthermore, a comparison study with trans-1,2-dihydroxy-1,2-dihydronaphthalene and naphthalene without α,β-unsaturated carbonyl group showed that the quinone function is required for binding to PTP1B and for reduction of the enzyme activity. Collectively, these data indicate that 1,2-NQ inactivates PTP1B by covalent attachment by a Michael addition reaction with second order reaction kinetics.

The PTP superfamily is characterized by a highly conserved active site sequence motif referred to as HCX₈XR/S/T (1) in which the environment surrounding the cysteinyl thiol reduces the pKₐ by several orders of magnitude. The pKₐ value of the active thiol (Cys-215) in PTP1B is reported to be 5.4 (3), indicating that the cysteine thiol is highly ionized at physiological pH and likely initiates hydroslysis of phosphotyrosyl esters through a nucleophilic attack to form a cysteine-phosphate intermediate. The enhanced reactivity resulting from the low pKₐ makes the active site cysteine residue target for covalent or oxidative modification. With this reactivity in mind, we determined the stoichiometry of 1,2-NQ bound to 37-kDa PTP1B by mass spectrometric analysis, which is possible in this study, such a similar result was also observed by MALDI-TOF-TOF MS (Fig. 4) and electrospray ionization-liquid chromatography-MS/MS (data not shown) analyses of the P-4 fragment. Nevertheless, our findings, 1) that the P-4 fragment obtained following digestion of 37-kDa PTP1B incubated with 1,2-NQ contained Cys-215 and 2) that mutation of C215S reduced covalent binding of 1,2-NQ to PTP1B, suggest that Cys-215 is also a target for 1,2-NQ. A reasonable explanation for our inability to detect Cys-215 attachment is that the high energy used in MS/MS analysis may have cleaved the C–S bond of the Cys-215 or the sulfur atom packs against the side chain of Tyr-124, which in turn forms a hydrogen bond with His-214, the residue preceding the active site Cys-215. Attachment of the 1,2-NQ structure to Cys-215 would disrupt this delicate packing arrangement, thereby perturbing the active site. Although we could not detect covalent modification of Cys-215 by 1,2-NQ binding in MS/MS analysis in the present study, such a similar result was also observed by MALDI-TOF-TOF MS (Fig. 4) and electrospray ionization-liquid chromatography-MS/MS (data not shown) analyses of the P-4 fragment.

In this study, we also found that persistent transactivation of EGFR coupled to reduction of PTP activity during exposure of A431 cells to 1,2-NQ was biphasic (Fig. 1C). In this work, we also found that persistent transactivation of EGFR coupled to reduction of PTP activity during exposure of A431 cells to 1,2-NQ was biphasic (Fig. 1C), i.e. following 1,2-NQ exposure EGFR activation reached a maximal level at 30 min then declined to a minimum at 180 min followed by an increase over the next several hours. We speculate that not only PTP1B but other PTPs also undergo covalent modification at Cys-215 and/or Cys-215, leading to the accumulation of phosphorylated EGFR even at 720 min after 1,2-NQ exposure. As shown in Fig. 7, substitution of Ser-121 for Cys-215 in 37-kDa PTP1B is a highly nucleophilic group available for covalent attachment by 1,2-NQ, thereby causing substantial reduction of dephosphorylation activity.

Hansen et al. (33) reported previously that Cys-121, a non-active site cysteine residue, is the site of allosteric inhibition of PTP1B activity. This cysteine residue is only 8.1 Å from the catalytic Cys-215 (34) (distance between Cα atoms), and the sulfur atom packs against the side chain of Tyr-124, which in turn forms a hydrogen bond with His-214, the residue preceding the active site Cys-215. Attachment of the 1,2-NQ structure to Cys-121 would disrupt this delicate packing arrangement, thereby perturbing the active site. Although we could not detect covalent modification of Cys-215 by 1,2-NQ binding in MS/MS analysis in the present study, such a similar result was also observed by MALDI-TOF-TOF MS (Fig. 4) and electrospray ionization-liquid chromatography-MS/MS (data not shown) analyses of the P-4 fragment. Nevertheless, our findings, 1) that the P-4 fragment obtained following digestion of 37-kDa PTP1B incubated with 1,2-NQ contained Cys-215 and 2) that mutation of C215S reduced covalent binding of 1,2-NQ to PTP1B, suggest that Cys-215 is also a target for 1,2-NQ. A reasonable explanation for our inability to detect Cys-215 attachment is that the high energy used in MS/MS analysis may have cleaved the C–S bond of the Cys-215/1,2-NQ complex. There are a number of PTPs in which Cys-121 (90%) and Cys-215 (100%) are highly conserved (34).

In this study, we also found that persistent transactivation of EGFR coupled to reduction of PTP activity during exposure of A431 cells to 1,2-NQ was biphasic (Fig. 1C), i.e. following 1,2-NQ exposure EGFR activation reached a maximal level at 30 min then declined to a minimum at 180 min followed by an increase over the next several hours. We speculate that not only PTP1B but other PTPs also undergo covalent modification at Cys-121 and/or Cys-215 by 1,2-NQ, leading to the accumulation of phosphorylated EGFR even at 720 min after 1,2-NQ exposure. As shown in Fig. 7, substitution of Ser-121 for Cys-215 caused a complete abolishment of decreased PTP1B activity caused by 1,2-NQ at 5 μM. At 50 μM 1,2-NQ, however, about only 50% of the loss of PTP1B caused by 1,2-NQ was restored by mutation of Cys-121. Taken together, we suggest that the specificity of thiol modification in PTP1B during reaction with

### Table 1: Peptides identified by MALDI-TOF MS from PTP1B

| Position | Peptide sequence | Calculated mass | MS | Peak no. |
|----------|------------------|-----------------|----|----------|
| 13–24    | SGWSAATIQDTR    | 1366.7          |    | 9        |
| 25–33    | GNASDPFPCR      | 1061.4          |    | 6        |
| 25–33    | HEASDPFPCR+1.2-NQ| 1217.4          |    |         |
| 48–56    | DVSPFDHRSR      | 1059.5          |    | 5        |
| 59–73    | LQHENDYDINASLR  | 1772.9          |    | 14       |
| 74–79    | WEEQ    | 763.3           |    | 1        |
| 80–103   | SYLTQGIPFLTNCGFPMVEMVWQQK | 2864.4 | 2862.8 | 17     |
| 106–112  | GVVMLNR | 788.4           |    | 3        |
| 117–128  | GSLCKAQWYPQK+1.2-NQ | 1564.7 | 1564.6 | 3-4     |
| 121–128  | CAQQWYPQK      | 1023.5          |    | 4        |
| 121–128  | CAQWYPQK+1.2-NQ | 1179.5          |    |         |
| 151–156  | SYTVVR  | 788.4           |    | 2        |
| 157–169  | QLEILELITQETR  | 1574.8          |    | 12       |
| 170–197  | ELHNPHHTTWDPFPEPASFLNLFKVF | 3339.7 | 3337.2 | 18     |
| 200–221  | ESGSLSPHEHPPVHNCASAGIR | 2175.1 | 2174.3 | 15     |
| 200–221  | ESGSLSPHEHPPVHNCASAGIR+1.2-NQ | 2331.1 | 2330.1 | 4-6     |
| 222–237  | SGTFCADTCLLMDK | 1730.8          |    | 13       |
| 256–268  | FMRGLQTADQLR   | 1564.8          |    | 11       |
| 258–268  | MGLTQADQLR     | 1245.7          |    | 8        |
| 269–279  | FSYLAVIEAGK    | 1197.7          |    | 7        |
| 280–292  | FIMGDSVQDQWKK | 1540.7          |    | 10       |
| 293–314  | ELSIHDELEPPHEHPPPRPPK | 2508.3 | 2507.1 | 16     |
Arylation of PTP1B by 1,2-NQ

1,2-NQ exhibits a concentration dependence such that, at 5 μM, Cys-121 arylation is much faster than that of Cys-215, whereas at 50 μM the rates approach each other.

In other studies, 1,2-NQ has been shown to cause the contraction of tracheal smooth muscle in the same concentration range, through activation of the EGFR system (19) leading ultimately to stimulation of the vanillin receptor to cause smooth muscle contraction. In vivo experiments in which 1,2-NQ was intratracheally administered to ovalbumin-sensitized mice resulted in enhanced airway sensitivity and goblet cell hyperplasia, two actions that may indicate airway hyper-responsiveness that is associated with lung disease (12). Because these tissue and whole animal responses are occurring at comparable concentrations, it appears likely that the in vivo effects are mediated by initial inactivation of PTP1B.

The present study reports, for the first time, that chemical knockdown of PTP1B occurs by Michael addition of 1,2-NQ to the His-25, Cys-121, and Cys-215 residues. This reaction is irreversible so that recovery of PTP1B function in dephosphorylation of phosphorylated EGFR requires PTP1B resynthesis. Thus, the rate of recovery will depend on the turnover rate of the protein. If the rate is slow, chronic exposure to low concentrations of compounds like 1,2-NQ will have a cumulative effect on PTP activity in the cell. The EGFR has been demonstrated to play a key role in bronchial epithelial repair, remodeling, and control of airway inflammation (35), and effects on this system by environmental agents could be relevant to their known effects on airway diseases. Samet et al. (36) reported previously that exposure of airway epithelial cells to the ambient particulate matter cause a significant transactivation of EGFR. They subsequently showed that contaminating metals are capable of phosphorylating EGFR through a ligand-independent mechanism. The work reported here indicates that electrophiles such as 1,2-NQ, which are present in ambient particulate matter, can cause long term effects on the EGFR system and may contribute to the adverse health effects associated with airborne pollutants.

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