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Dual effects of N-acetyl-l-cysteine dependent on NQO1 activity: Suppressive or promotive of 9,10-phenanthrenequinone-induced toxicity

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ABSTRACT

A typical antioxidant, N-acetyl-L-cysteine (NAC) generally protects cells from oxidative damage induced by reactive oxygen species (ROS). 9,10-Phenanthrenequinone (9,10-PQ), a major quinone in diesel exhaust particles, produces ROS in redox cycling following two-electron reduction by NAD(P)H:quinone oxidoreductase 1 (NQO1), which has been considered as a cause of its cyto- and genotoxicity. In this study, we show that NAC unexpectedly augments the toxicity of 9,10-PQ in cells with low NQO1 activity. In four human skin cell lines, the expression and the activity of NQO1 were lower than in human adenocarcinoma cell lines, A549 and MCF7. In the skin cells, the cytotoxicity of 9,10-PQ was significantly enhanced by addition of NAC. The formation of DNA double strand breaks accompanying phosphorylation of histone H2AX, was also remarkably augmented. On the other hand, the cyto- and genotoxicity were suppressed by addition of NAC in the adenocarcinoma cells. Two contrasting experiments: overexpression of NQO1 in CHO-K1 cells which originally expressed low NQO1 levels, and knock-down of NQO1 in the adenocarcinoma cell line A549 by transfection of RNAi, also showed that NAC suppressed 9,10-PQ-induced toxicity in cell lines expressing high NQO1 activity and enhanced it in cell lines with low NQO1 activity. The results suggested that dual effects of NAC on the cyto- and genotoxicity of 9,10-PQ were dependent on tissue-specific NQO1 activity.

Introduction

Quinones are a ubiquitous class of organic compounds of substantial toxicological interest, capable of causing inflammation and carcinogenesis (Bolton et al., 2000). 9,10-Phenanthrenequinone (9,10-PQ) has been found in the particulate fractions of air pollution including diesel exhaust particles (Cho et al.,2004; Jakober et al., 2007), and reported to have several toxic actions; reactions with certain proteins (Rodriguez et al., 2004, 2005), down-regulation of anti-oxidative enzymes (Sugimoto et al., 2005), induction of DNA deletions (Rodriguez et al., 2008), and modulation of allergic inflammation (Hiyoshi et al., 2005).

The toxicity of quinones has been attributed to two major mechanisms. The first is a direct effect on nucleophiles such as thiols in vital cellular components, leading to covalent modification. 9,10-PQ reacts with dithiols like dithiothreitol (DTT) and 2,3-dimercapto-1-propanol, and modifies protein thiols (Kumagai et al., 2002). The second is the production of reactive oxygen species (ROS) in a redox cycling process after enzymatic one- or two-electron reduction using NAD(P)H (Bolton et al., 2000; Brunmark and Cadenas, 1989; O'Brien, 1991). One-electron reduction by NADPH-cytochrome P450 reductase, and two-electron reduction by NAD(P)H:quinone oxidoreductase 1 (NQO1) and aldoketo reductase (AKR), were reported to convert 9,10-PQ to the 9,10-phenanthrene semiquinone radical (9,10-PQ$^{\bullet -}$) and 9,10-dihydroxyphenanthrene (9,10-PQH_2), respectively (Matsunaga et al., 2009; Taguchi et al., 2007, 2008). In general, two-electron reduction that changes quinones to hydroquinones is regarded as an important detoxification process because the resultant hydroquinones are removed via the formation of conjugates with glutathione (GSH), UDP-glucuronic acid, and other moieties. Inactivation of the two-electron reduction enzymes NQO1 and 2, elevated the incidence and multiplicity of chemically induced skin tumors and myeloma (Iskander and Jaiswal, 2005; Shen et al., 2010). On the other hand, certain quinones become remarkably cytotoxic when reduced. The reduction produces unstable and highly reactive semiquinone intermediates, which generates ROS such as superoxide anion radicals and hydroxyl radicals, leading to high cytotoxicity. For example, the quinone-containing anticancer agent β-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) is reduced to its corresponding hydroquinone form by NQO1, then converted back to its parental structure using two oxygen molecules, leading to the generation of cytotoxic ROS (Bey et al., 2007; Pink et al., 2000). Taguchi et al.

Abbreviations: BSFGE, biased sinusoidal field gel electrophoresis; DCFH-DA, 6-carboxy-2, 7′-diclorodihydrofluorescein diacetate, di(acetoxymethyl ester); DCPIP, 2,6-dichlorophenol-indophenol; DSBs, double strand breaks; DTT, dithiothreitol; GSH, glutathione; γ-H2AX, phosphorylated histone H2AX; NAC, N-acetyl-L-cysteine; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; FCM, flow cytometer; NQO1, NAD(P)H:quinone oxidoreductase 1; PH, phenanthrene; 9,10-PQ, 9,10-phenanthrenequinone; 9,10-PQH_2, 9,10-dihydroxyphenanthrene; ROS, reactive oxygen species.

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(2007, 2008) reported that 9,10-PQ caused protein oxidation through production of ROS via redox cycling after two-electron reduction of 9,10-PQ to 9,10-PQH_2 which was eventually eliminated from the body as a monoglucuronide. Bücker et al. (1979) found that 9,10-PQH_2 was somewhat more mutagenic than 9,10-PQ itself in a rec assay. This might also be due to the generation of ROS via production of a semiquinone radical (9,10-$PQ^{\bullet -}$) that reacts with molecular oxygen, as reported by Taguchi et al. (2007, 2008).

If the generation of ROS is key to the cytotoxicity of 9,10-PQ, the addition of thiol antioxidants should quench the ROS and suppress the toxicity. As 9,10-PQ was reported to react with dithiols but not monothiol compounds like GSH and N-acetyl-L-cysteine (NAC) (Kumagai et al., 2002), we used NAC as a ROS scavenger as the starting point of this study, and examined its effect on 9,10-PQ-induced cytotoxicity. Unexpectedly, the cytotoxicity of 9,10-PQ was not suppressed by NAC, but rather remarkably enhanced in human skin cell lines. This is important to the risk evaluation of quinones and antioxidants because 9,10-PQ in the presence of NAC induced serious DNA damage, including double strand breaks (DSBs). Furthermore, the enhanced genotoxicity of 9,10-PQ was dependent on the activity of NQO1. Herein, we report the dual nature of NAC's effect on the cyto- and genotoxicity of 9,10-PQ and its correlation with tissue-dependent NQO1 activity.

Materials and methods

Cells and cell culture conditions. The immortalized human keratinocytes, HaCaT, and normal human skin fibroblasts, ASF4-1, were kindly provided by Dr. N. Fusening (German Cancer Research Center, Germany) and Dr. K. Kaji (University of Shizuoka, Japan), respectively. The cell lines of human squamous cell carcinoma, HSC-1, human breast adenocarcinoma, MCF-7, human lung adenocarcinoma epithelium, A549, and Chinese hamster ovary, CHO-K1, were purchased from the Japanese Collection of Research Bioresources (Japan). These cells were cultured in Dulbecco's modified Eagle medium (DMEM) or Ham's F-12 (CHO-K1) containing 10% fetal bovine serum (FBS) (20% for HSC-1) and 100 units/ml of penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2. The cell line G-361 derived from malignant human skin melanoma, was purchased from the Health Science Research Resources Bank (Japan) and cultured in MEM supplemented with 10% FBS. CHO-hNQO1 cells were established by stable transfection of CHO cells with a vector containing full-length cDNA encoding human NQO1 under transcriptional control of the human cytomegalovirus immediate-early (CMV) promoter (De Haan et al., 2002). CHO-hNQO1 cells were cultured in Ham's F-12/ DMEM with 200 μg/ml of zeocin. All experiments were performed with exponentially growing cells.

Treatment with 9,10-PQ and/or NAC and determination of viability. Cells grown on 35 mm culture dishes were treated with NAC (~25 mM) for 0.5 h, followed by 9,10-PQ for 2 h. The cells were washed with culture medium and further cultured for 24 h. Cell viability was evaluated by the fluorescein diacetate (FDA) assay. FDA is hydrolyzed by cytoplasmic esterases into fluorescent fluorescein in living cells. Cells treated with 9,10-PQ and/or NAC were harvested and suspended in PBS containing FDA (0.1 mg/ml). They were incubated for 15 min at 37 °C. The viability of cells was determined by measuring the fluorescence intensity of FDA inside the cells using a flow cytometer (FCM) (FACS Canto II, BD, Franklin Lakes, NJ).

Immunofluorescence staining for detection of phosphorylated histone H2AX (γ-H2AX). The cells treated with 9,10-PQ and/or NAC in Lab-Tek chamber slides (Nalge Nunc, IL) were immediately fixed in 2% paraformaldehyde for 30 min at room temperature and then in 100% methanol for 20 min at −20 °C. Fixed cells were immersed in buffer containing 100 mM Tris–HCl, 50 mM EDTA, and 0.5% Triton X-100 for 20 min at room temperature for better permeabilization, and blocked with 1% bovine serum albumin for 30 min at 37 °C. Cells were incubated with a primary antibody against γ-H2AX (Millipore, Bedford, MA, 1:200) for 2 h, then with a secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson Immuno Research Laboratories, PA). To confirm the distribution of foci, the nucleus was stained with PI (20 μg/ml). Images were acquired on a fluorescence microscope (BX51, Olympus Co., Japan).

Western blot analysis. The cells treated with 9,10-PQ and/or NAC were lysed in lysis buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The samples were separated by 12.5% SDS-PAGE, and blotted onto polyvinylidine fluoride (PVDF) membranes. After blocking with 1% non-fat milk, the membranes were incubated overnight at 4 °C with a primary antibody against γ-H2AX (1:1000), NQO1 or actin (Santa Cruz Biotechnol. Inc., CA, 1:1000), and then with a secondary antibody conjugated with HRP (Jackson Immuno Research Laboratories, PA) for 1 h. Protein expression was visualized with an enhanced chemiluminescence detection kit (GE Healthcare Ltd. UK).

Detection of DSBs. DSBs were detected with a biased sinusoidal field gel electrophoresis (BSFGE) system (Atto, Japan) as described previously (Toyooka et al., 2004). In brief, the cells treated with 9,10-PQ and/or NAC were solidified in 1% low-melting agarose. The agarose plugs were treated with proteinase K (0.5 mg/ml) and ribonuclease A (1 mg/ml), and electrophoresed in a 0.8% agarose gel. The gel was visualized by staining with ethidium bromide.

Flow cytometric detection of intracellular ROS. The intracellular generation of ROS was investigated using the 6-carboxy-2,7′-diclorodi hydrofluorescein diacetate, di(acetoxymethyl ester) (DCFH-DA) (Molecular Probes, Eugene, OR). Cells preincubated in the presence of 10 μM of DCFH-DA for 0.5 h were treated with 9,10-PQ (~50 μM) for 2 h. The fluorescence intensity of DCFH-DA inside the cells was determined using a FCM.

Measurement of NQO1 activity. NQO1 activity was determined spectrophotometrically by monitoring reduction of the standard electron acceptor, 2,6-dichlorophenol-indophenol (DCPIP), at 600 nm. In brief, cells were harvested by trypsinization and lysed in buffer containing 100 mM Tris–HCl (pH 7.5), 0.25 mM sucrose, 20% glycerol, and 1.95% chaps. The reaction was started by the addition of reaction buffer (25 mM Tris–HCl (pH 7.4), 0.02% bovine serum albumin, 0.01% Tween 20, 5 μM flavin adenine dinucleotide, 200 μM NADH, and 50 μM DCPIP) to cell lysate (5 μg of protein), and the decrease in absorbance at 600 nm was measured for 5 min at room temperature in the presence or absence of 20 μM dicoumarol. The dicoumarol-inhibitable part of DCPIP's reduction was used to calculate NQO1 activity expressed as μg DCPIP/μg protein/min.

Knock-down of NQO1. A549 cells (30% confluent on a 60 mm dish) were transfected with stealth RNAi (Invitrogen, Carlsbad, CA) against NQO1 or negative universal control stealth RNAi (Invitrogen) (50 pmol/ml) using Lipofectamine™ RNAiMAX2000 (Invitrogen), according to the manufacturer's instructions. After 48 h, the transfected cells were split and cultured for further 48 h for experiments. The efficiency with which NOQ1 was knocked down was confirmed by Western blot analysis and the NQO1 activity assay as described above.

Statistics. All experiments were repeated two or three times. Data are presented as the mean ± S.D. ($n = 3–5$). Data were analyzed by a one-way ANOVA followed by Dunnett's *t* test for comparisons