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Modulation of the Toxicity and Macromolecular Binding of Benzene Metabolites by NAD(P)H:Quinone Oxidoreductase in Transfected HL-60 Cells

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Benzene is oxidized in the liver to produce a series of hydroxylated metabolites, including hydroquinone and 1,2,4-benzenetriol. These metabolites are activated to toxic and genotoxic species in the bone marrow via oxidation by myeloperoxidase (MPO). NAD(P)H:quinone oxidoreductase (NQO1) is an enzyme capable of reducing the oxidized quinone metabolites and thereby potentially reducing their toxicities. We introduced the NQO1 gene into the HL-60 cell line to create a high MPO-, high NQO1-expressing cell line, and tested its response in assays of benzene metabolite toxicity. NQO1 expression reduced a class of hydroquinone- and benzenetriol-induced DNA adducts by 79−86%. The cytotoxicity and apoptosis caused by hydroquinone were modestly reduced, while protein binding was unchanged and the rate of glutathione depletion increased. NQO1's activity in reducing a class of benzene metabolite-induced DNA adducts may be related to its known activities in maintaining membrane-bound endogenous antioxidants in reduced form. Alternatively, NQO1 activity may prevent the formation of adducts which result from polymerized products of the quinones. In either case, this protection by NQO1 may be an important mechanism in the observation that a lack of NQO1 activity affords an increased risk of benzene poisoning in exposed individuals [Rothman, N., et al. (1997) *Cancer Res.* **57**, 2839−2842].

Introduction

Benzene is a recognized myelotoxicant and leukemogen (*1*, *2*). It is metabolized in the liver into a series of hydroxy metabolites, including phenol, hydroquinone, catechol, and benzenetriol (*3*). These metabolites are relatively stable in the blood and enter tissues throughout the body. The bone marrow is thought to be the primary organ of toxicity for several reasons, including its propensity for peroxidative metabolism of benzene metabolites (*4*, *5*), the presence of metabolic activating enzymes in both progenitor and supportive stromal cells (*6*, *7*), and high rates of cell proliferation.

Hydroquinone, which is thought to be benzene's primary toxic metabolite, is activated to toxic and genotoxic

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species via its oxidation to 1,4-benzoquinone. Metabolism of hydroquinone is dependent on peroxidase activity (*5*, *7*, *8*), and the major peroxidase of bone marrow is myeloperoxidase (MPO).[1] 1,4-Benzoquinone is a reactive electrophile which may adduct to cellular proteins or DNA, yet is also a good substrate for NAD(P)H:quinone oxidoreductase (NQO1).

NQO1 catalyzes an obligate two-electron reduction of a wide variety of quinones using NADH or NADPH as the reducing cofactor (*9*). NQO1 is ubiquitously expressed (*10*), and can be induced by compounds and conditions under which the antioxidant response element or xenobiotic response element is activated (*11*). NQO1 is generally known for its detoxifying properties, such as with the chemicals menadione (*12*, *13*) and benzo[*a*]pyrene quinone (*14*), but can also function as an activation enzyme, such as with streptonigrin (*15*) and the indoloquinone E09 (*16*). For benzene-induced toxicity, we are concerned with the NQO1-mediated conversion of benzoquinones to hydroxyquinones, which may reduce the level of quinone and semiquinone intermediates and thus protect against toxicity. Semiquinones may also autoxidize back to benzoquinones, forming reactive oxygen species in a redox cycle pathway. However, the NQO1-mediated two-electron reduction of benzoquinones forms more hydroxyquinones which may then be oxidized again by MPO, thus also potentially facilitating redox cycling. The kinetics of these two pathways, along with the relative toxicities and targets of various metabolites, would ultimately determine the protective effect of NQO1 in the bone marrow.

A recently described variant of NQO1 consisting of a base pair mutation results in a loss of activity (*17*). This variant was found to be polymorphic, with 34−52% of the population being a heterozygote, and 4−20% homozygous for the mutant allele (*18*−*20*). In a study of benzene-exposed Chinese workers, individuals homozygous for the mutant allele were found to be more likely to be poisoned (exhibited by low white blood cell counts) than individuals heterozygous or homozygous for the wild-type allele (*21*). We therefore sought to explore the role of NQO1 in benzene toxicity in an in vitro study. Other studies in which the role of NQO1 in benzene toxicity has been considered have utilized comparisons between different cell types or inducers or inhibitors of NQO1 expression (*22*−*25*). These studies generally suggest that NQO1 protects cells from benzene toxicity, but have not isolated NQO1 effects from those of other factors such as glutathione and peroxidase. In this paper, we explore the role of NQO1 by altering its expression in HL-60 cells by recombinant DNA means, thus avoiding the side effects of other methods of altering NQO1 expression or activity. The effects of NQO1 expression in benzene metabolite toxicity, apoptosis, glutathione depletion, [^{14}C]hydroquinone binding to proteins, and DNA adduct formation are examined.

Materials and Methods

Chemicals and Enzymes. Hydroquinone, benzoquinone, and benzenetriol (all 99%) were from Aldrich (Milwaukee, WI); streptonigrin, sodium arsenite, vincristine sulfate, deferoxamine mesylate, and dicoumarol were from Sigma (St. Louis, MO). **Caution:** *Sodium arsenite is a human carcinogen and a poison. Benzoquinone, streptonigrin, vincristine, and dicoumarol are all acutely toxic and should be handled appropriately*. Hydroquinone, benzoquinone, benzenetriol, and sodium arsenite were freshly dissolved in phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, NY), sterile filtered, and used immediately. Stock solutions of streptonigrin were prepared in dimethyl sulfoxide (DMSO), and vincristine sulfate in ethanol. These were diluted in PBS prior to addition to cultures. Vehicle (DMSO or ethanol) controls showed no difference from PBS controls in the TD_{50} assay.

Cell Culture. The HL-60 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in "complete media" [RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin supplement (Gibco BRL)]. Transfected cell lines were maintained in the presence of 500 μg/mL Geneticin sulfate (Gibco BRL). Cells were passaged 1:4 twice weekly and used for experiments between passages 10 and 30. The day prior to experiments, cell lines were adjusted to a concentration of 1×10^6 cells/mL. HT-29 cells were obtained from ATCC, and grown in McCoy's medium with 10% FBS.

Creation of the 33C Cell Line. The NQO1 gene was obtained as a reverse transcriptase-polymerase chain reaction (RT-PCR) product from HL-60 RNA. An NQO1 cDNA was created by using an NQO1 downstream primer, GAT GAT CAT TTT CTA GCT TTG ATC TGG T (NQ-B1), in the presence of Maloney-Murine Leukemia Virus Reverse Transcriptase (Mu-MLV RT) (Gibco BRL). A fraction of this RT product was amplified using an upstream primer, AGC CAT GGT CGG CAG AAG AGC A (NQ-A1), and NQ-B1 (standard PCR conditions, 94 °C for 50 s, 58 °C for 1 min, and 72 °C for 1.5 min, 35 cycles, *Taq* polymerase from Perkin-Elmer, Palo Alto, CA). The PCR product was inserted into pUC118, and a single subclone was sequenced. The clone matched exactly the wild-type sequence for NQO1 (Genbank J03934). Upon obtaining the correct sequence, we excised the insert from the plasmid, blunt-ended with Klenow DNA polymerase, and *Not*I linkers were added with T4 DNA ligase. The insert was digested with excess *Not*I and cloned into the *Not*I site in pRSV1 (Stratagene, La Jolla, CA). The resulting plasmid, pRSV1-NQO1, was linearized with *Dra*III, and ethanol precipitated. The plasmid (5 μg) was transfected into HL-60 cells (1×10^7 cells) in 250 μL of RPMI. The cells were pulsed at 250 mV and 960 μF in 4 mm cuvettes (Gene Pulser, Bio-Rad, Hercules, CA) and, on the following day, were plated in 96-well plates at a density of 5×10^5 cells per plate in the presence of 800 μg/mL Geneticin. Approximately 150 μL of medium was aspirated with a micropipet tip attatched to a pasteur pipet and replaced weekly.

Geneticin-resistant clones (appoximately 15 per each 96-well plate) were screened for expression of NQO1 by semiquantitative RT-PCR. One hundred thousand cells were washed in PBS and added directly to Mu-MLV RT buffer [50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, and 1 mM DTT] and 50 ng of random hexamer oligonucleotides (Promega) and heated to 70 °C for 5 min. Tubes were cooled on ice, and MuMLV RT was added with 10 μmol each of dATP, dGTP, dCTP, and dTTP and 5 units of RNAsin (Promega) in a total volume of 20 μL. The reaction was allowed to proceed for 1 h, and 3 μL was used for subsequent PCR. PCR primers were added for a duplex reaction: β-actin primers BA2 (TAC ATG GCT GGG GTG TTG AA) and BA3 (AAG AGA GGC ATC CTC ACC CT) (*26*) and pRSVI primers LAC-START (GAC CAT TCA CCA CAT TGG TGT GC) and LACSWPREX (GCG GCC GCA AGC TTG GTT GGA) (PCR, 94 °C for 50 s, 58 °C for 1 min, and 72 °C for 5 s, 25−29 cycles). The products of both of these PCRs were cDNA (rather than genomic DNA) in origin since they both extend over an intron (intron/exon data for pRSVI are available from Stratagene). The actin product (218 bp) was compared in intensity to the pRSVI product (119 bp); only clones exhibiting a strong pRSVI band

[1] Abbreviations: MPO, myeloperoxidase; NQO1, NAD(P)H:quinone oxidoreductase; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; Mu-MLV RT, Maloney-Murine Leukemia Virus Reverse Transcriptase; GSH, glutathione; GSSG, glutathione disulfide; FSC, forward scatter; SSC, side scatter.

in the linear PCR amplification range were taken for further analysis. These positive clones were maintained in the presence of 500 μg/mL Geneticin to maintain expression of the transgene. Geneticin was removed from the media prior to experiments.

Positive clones were grown and assayed for NQO1 enzyme activity using 1,2-dichlorophenolindolephenol (DCPIP) as a substrate exactly as described previously (*17*). Protein was quantified using the Bradford method (Bio-Rad protein assay), and NQO1 activity is expressed as nanomoles of DCPIP reduced per minute per milligram of protein. The best-expressing clones were further analyzed by Northern blots.

Northern blots were performed essentially as described previously (*27*). RNA (20 μg) was electrophoresed in 30% formaldehyde gels, and transferred to Nylon membranes (Genescreen Plus, New England Nuclear). Blots were probed with the original NQO1 insert which was labeled with [^{32}P]ATP by random priming (*27*). Blots were hybridized using QuikHyb (Stratagene), and washed to a stringency of 0.2× SSC and 0.1% SDS at 60 °C. The membranes were then autoradiographed on X-ray film (Kodak, Rochester, NY), and the autoradiographs were scanned into TIFF format with a Hewlett-Packard 4C scanner. RNA was quantified using a densitometry program (NIH Image 1.60).

Western blot analysis was performed with a monoclonal antibody to human NQO1 kindly provided by D. Siegel and D. Ross (University of Colorado, Boulder, CO). Cell proteins (25 μg, prepared as described above for NQO1 activity) were subjected to SDS−PAGE (10% polyacrylamide), and electroblotted onto PVDF membranes (Immobilon, DuPont). The membranes were preblocked in 5% nonfat dry milk in PBS with 0.5% Tween-20 (PBS-T) for 1−2 h. The NQO1 monoclonal antibody was diluted 1:1000 in PBS-T, and membranes were rocked for 1 h. Membranes were washed four times in PBS-T, followed by a 30 min incubation in goat anti-mouse horseradish peroxidase-conjugated IgG (Sigma) (diluted 1:5000 in PBS-T with 5% nonfat dry milk). Membranes were again washed four times with PBS-T, and detection was performed using an Amersham enhanced chemiluminescence kit (ECL). Controls include purified NQO1 protein (also from D. Siegel and D. Ross) and protein isolated from HT-29, an NQO1-expressing colon cancer cell line (*17*).

The C15 cell line, another HL-60 derivitave, has 46% of the level of MPO activity of the parent cell line. C15 cells contain an antisense construct to MPO (*28*).

MPO and Glutathione Determination. MPO was assayed by monitoring the conversion of guaiacol to tetraguaiacol. Cells (0.08 mL of 5×10^5 cells) were incubated with 0.02 mL of 0.02% acetyltrimethylammonium bromide for 5 min at room temperature. A sample of 0.9 mL of assay buffer [10 mM sodium phosphate (pH 7.0), 0.003% H_2O_2, and 14 mM guaiacol] was added, and the optical density at 470 nm was monitored for 30 s with a Perkin-Elmer Lambda 3B spectrophotometer. Four moles of H_2O_2 is required to produce 1 mol of tetraguaiacol product which has an extinction coefficient of 26.6 mM^{-1} cm^{-1}. MPO units of activity were calculated as follows:

$$\text{units/mL} = (\text{OD} \times V_t \times 4)/(E \times t \times V_s)$$

where V_t is the total volume (milliliters), V_s is the sample volume (milliliters), OD is the density change, and t is the time of measurement (minutes).

For determination of glutathione (GSH) and glutathione disulfide (GSSG) levels, cells were treated in triplicate at a density of 3×10^6 cells/mL in Hank's Balanced Salt Solution supplemented with 12.5 mM HEPES (pH 7.3). One milliliter of cells was removed and lysed with 200 μL of 100% perchloric acid (Aldrich). GSH and GSSG were derivatized by the method of Reed et al., and analyzed as iodoacetate derivitaves with high-pressure liquid chromatography (HPLC) (*29*). The protein concentration was measured by the Lowry method, and the GSH content was expressed in terms of nanomoles per milligram of protein.

For time series experiments, GSH was analyzed by a fluorometric method that is more suitable for large sample collections. This assay is dependent on the specific reaction of cellular GSH to *o*-phthaldehyde at pH 8.0 and was performed as described previously (*30*). For this assay, the protein content was assayed by the Bradford method (Bio-Rad protein assay), and as described above, the GSH content was expressed as nanomoles per milligram of protein. Cells were treated at a concentration of 3×10^6 cells/mL in Hank's Balanced Salt Solution with 50 μM hydroquinone**.** Assays were performed in triplicate on two separate occasions.

Toxic Dose (TD_{50}) Levels. The TD_{50} level is defined as the concentration (in micromolar) at which 50% of cells remain viable 24 h after exposure to the chemical compared to control cells. Cells were washed in PBS, and placed in TI-25 flasks at a concentration of 1×10^6 cells/mL in PBS. Chemicals were added and the cells placed in a 37 °C incubator for 2 h. The cells were washed in PBS and returned to complete media. The apoptosis experiments described below demonstrated that the majority of apoptosis caused by the chemicals occurred within the first 24 h after exposure, and the subsequent apoptotic fragmentation of cells rendered them unrecordable. It should be noted that apoptotic cells are viable by the standard of trypan blue exclusion, even when they begin to fragment. At no point was necrosis (i.e., loss of cell membrane integrity) significantly induced by any chemical in these experiments, and therefore, viability immediately following treatment was not a valid measure of toxicity. At 24 h, when apoptotic cells were removed by fragmentation, the number of live cells remaining was recorded. The values of four dose−response experiments (eight total sampling points for each dose, and five doses per chemical) were plotted, and the best fit least-squares regression line was plotted through the points (SYSTAT statistical program). Only those dose points ranging from the dose immediately preceding toxicity (i.e., the maximal dose of "no effect") to the dose at which <10% of the cells remained were used for the analysis. "Statistical significance" is the lack of crossover by 95% or 99% confidence intervals.

Apoptosis. Cells were treated in the same fashion as in TD_{50} assays. Cells were treated with chemical for 2 h, and then returned to complete media. In preliminary experiments, the extent of apoptosis was determined to peak between 8 and 12 h following exposure, and apoptotic cells degraded by 24 h. In subsequent experiments, analyses were performed 12 h following treatments. Apoptosis was studied using the terminal deoxynucleotidyl-transferase (TdT) assay for DNA breaks. Cell staining was performed exactly as described previously (*31*). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickenson, San Jose, CA) using Lysis II software. Four attributes of the cells were measured: forward scatter (FSC, indicating cell size), side scatter (SSC, indicating granularity), green fluorescence (fluorescein or biotin−dUTP incorporation in this assay), and red fluorescence (propidium iodide or DNA content). Cells were gated on FSC and SSC to include only uniform size cells, thus excluding partially degraded or shrunken cells. Thus, the assay includes only normally sized cells in active stages of growth or apoptosis. It thus differs from many flow cytometric assays of apoptosis, which measure particles that may well be debris from necrotic cells. Green fluorescence was displayed on log scale and red on linear scale.

Protein Binding. Protein binding by ^{14}C-labeled hydroquinone was performed essentially as described previously (*32*, *33*) except that lysates were applied to glass fiber filters and washed rather than repeatedly centrifuged. In preliminary experiments, the simpler glass fiber filter method proved to be as good at, or better than, washing out noncovalently associated radioactivity as the established centrifuge method. Cells were washed and resuspended in RPMI at a concentration of 1×10^6 cells/mL in triplicate 2 mL cultures in 24-well plates. ^{14}C-labeled hydroquinone (Wizard Laboratories, Davis, CA; 22.2 mCi/mmol) was mixed with an excess of unlabeled hydroquinone, and cells were treated in a total concentration of 10 μM for 3 h. The

cultures were then transferred to 15 mL polypropylene tubes, and centrifuged. Cells were washed once with 10 mL of PBS, then resuspended in 2 mL of PBS. Trichloroacetic acid (200 μL) was added while vortexing to lyse cells and precipitate macromolecules. The solution was applied to glass fiber filters (GF-A, Whatman) under vacuum. The filters were washed twice in succession with 2 mL each of 5% TCA in water, 1:1 methanol/water, and finally 1:1 ethanol/ether. The filters were then placed in a scintillation counter vial, and air-dried. One milliliter of 1 M NaOH was added, and the vials were kept at 37 °C overnight. The solution was neutralized with 1 N HCl, and an aliquot removed for protein determination by the Bradford method (Bio-Rad protein assay). Scintillation fluid (10 mL) was added to the vials, and the vials were counted in a scintillation counter (Beckman). Protein binding is expressed as picomoles of hydroquinone equivalents bound per milligram of protein. The assay was repeated once.

DNA Adducts. For the determination of DNA adducts, cells were treated essentially as previously described (*34*). Briefly, cells were centrifuged and resuspended in fresh complete media in 30 mL cultures in TI-75 flasks at a density of 1×10^6 cells/mL. After a 6 h equilibration period in the incubator, hydroquinone and benzenetriol were added. After a 24 h incubation, cell counts and viabilities were determined, and DNA was extracted from the cells. Cells were lysed in 0.1 M Tris, 0.1 M NaCl, 50 mM EDTA (pH 8.0), and 1% SDS on ice and then extracted twice with equal volumes of chloroform/isoamyl alcohol (24:1). The aqueous supernatant was incubated with RNAse A and RNAse T1 (250 μg/mL, Sigma) at 37 °C for 60 min followed by digestion with proteinase K (10 μg/mL, Boehringer) at 37 °C for 60 min. The digest was again extracted with chloroform/isoamyl alcohol as described above, after which sodium acetate (0.4 M final) was added to the supernatant. DNA was ethanol precipitated, washed, and dissolved in 0.1 M SSC. The quantity of DNA was determined fluorometrically. DNA (4 μg) was enzymatically digested to completion with micrococcal nuclease (Worthington Biochemicals, Lakewood, NJ) and spleen phosphodiesterase (Pharmacia, Piscataway, NJ) (*35*). Samples were treated with nuclease P_1 (Pharmacia) and converted to ^{32}P-labeled 3′5′dpNps by incubation with 150 μCi of [^{32}P]ATP (6000 Ci/mmol, New England Nuclear) and 2.5 μL of T_4 polynucleotide kinase (Pharmacia). The total volume of ^{32}P-labeled nucleotides was applied to a 10 cm × 10 cm PEI-cellulose plate and chromatographed as described previously (*35*). Adducts 1−3 were resolved by using the conditions previously reported (*34*). Adducts with relatively less mobility, i.e., they do not run out of the origin in the method of Lévay and Bodell, were also characterized (adducts 4−6) (*36*). In this method, a lower concentration (1.0 M rather than 1.8 M) of lithium chloride was used in the D1 chromotography step. Levels of adducts were determined by scraping adduct spots and scintillation counting. Areas adjacent to and with dimensions equal to those of the adduct spots were used as controls, and adduct levels were calculated using the formula described previously (*35*).

Results

Characterization of the NQO1 Model. Nine out of 119 Geneticin-resistant clones were positive for transfected NQO1 activity as determined by semiquantitative RT-PCR. These nine clones were assayed for NQO1 enzyme activity and RNA expression (Figure 1 and Table 1). By Spearman rank correlation, the level of expression and NQO1 enzyme activity were correlated by a factor of 0.906. Clone 33C had both the highest level of expression and activity, with a 34-fold higher NQO1 activity than the control. All subsequent studies were conducted with the 33C cell line.

Western analysis of NQO1 protein using a monoclonal antibody revealed a single 30 kDa band in 33C cells (Figure 2). HT-29 is a high NQO1-expressing cell line

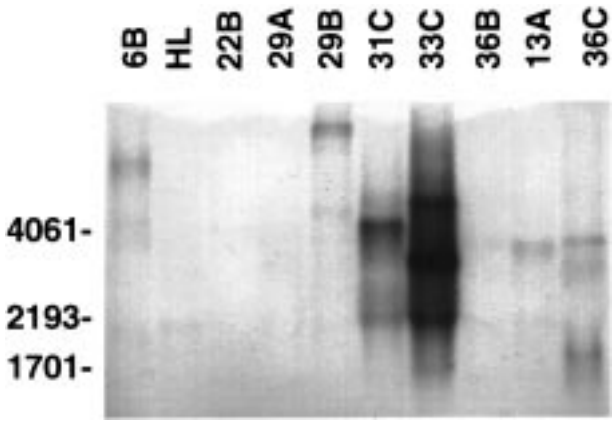

Figure 1. Northern blot of RT-PCR-positive NQO1 cell lines. HL-60 cells were transfected with a plasmid containing the *NQO1* gene as described in Materials and Methods. Geneticin-resistant clones were screened for *NQO1* expression by semiquantitative RT-PCR, and those showing the strongest levels of expression were analyzed by Northern blots, using the *NQO1* cDNA clone as a probe. Lane markers denote specific clones; HL represents HL-60 cells as a control. Numbers denote RNA marker bands (New England Biolabs).

Table 1. RNA Content and Enzyme Activity in RT-PCR-Positive HL-60 NQO1-Transfected Cell Lines

cell line	RNA content[a]	NQO1 activity[b]	fold elevated[c]
HL-60 control	200	6.2	1.0
6B	2374	30.7	4.9
13A	1884	40.1	6.5
22B	160	14.2	2.3
29A	220	4.5	0.73
29B	496	11.7	1.9
31C	10681	38.4	6.2
33C	25765	210.0	33.9
36B	565	26.0	4.2
36C	6706	49.6	8.0

[a] RNA content is an arbitrary number from densitometric analysis. [b] In nanomoles of DCPIP reduced per minute per milligram of protein. [c] NQ01 activity relative to HL-60 cells.

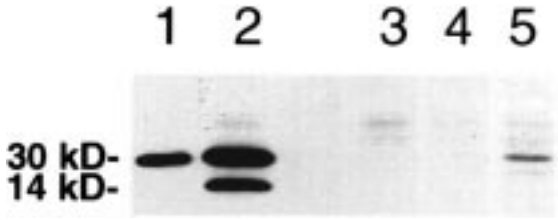

Figure 2. Western analysis of NQO1 expression in various cell lines. Cell proteins (25 μg) from HT-29 cells (lane 2), HL-60 cells (lane 3), C15 cells (lane 4), and 33C cells (lane 5) were separated by SDS−PAGE, blotted, and probed with a monoclonal antibody to NQO1. Lane 1 contains purified NQO1 protein. The 14 kDa band in lane 2 may represent a breakdown product of NQO1.

(*17*), and demonstrated a high reactivity to the NQO1 antibody. An additional lower-molecular mass protein reactive to the antibody was also evident, and may represent a breakdown product of NQO1. The HL-60 and C15 cell lines did not demonstrate detectable NQO1 protein. It should be noted that HL-60 is heterozygous for the NQO1 polymorphism at nucleotide 609 (*18*) (data not shown), but this fact has no relevance here since the NQO1 gene is not detectably expressed in HL-60 cells.

Enzyme and Glutathione Levels in the Three Cell Lines. HL-60 and 33C both have high MPO levels (Table 2), while the C15 cell line contains only 46% of the MPO level found in HL-60 cells (*28*). NQO1 is virtually undetectable in HL-60 and C15 cell lines, while ap-

Table 2. Glutathione, MPO, and NQO1 Activity in HL-60 and 33C Cell Lines

cell line	glutathione[a]	MPO[b]	NQO1[c]
HL-60	28.5 ± 2.2	24.6 ± 3.2	6.2 ± 1.1
33C	28.0 ± 1.6	26.2 ± 2.6	210.0 ± 12.4

[a] In nanomoles per milligram of protein. [b] In units per milliliter. [c] In nanomoles of DCPIP reduced per minute per milligram of protein.

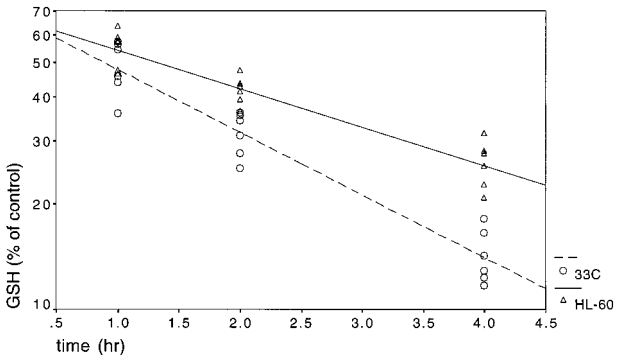

Figure 3. Glutathione depletion in HL-60 and 33C cells in response to 50 μM hydroquinone. Cells were treated with hydroquinone and assayed for intracellular glutathione content as described in Materials and Methods.

preciable NQO1 activity is present in the 33C cell line. 33C had 34% of the NQO1 activity of HT29, which metabolized 618 nmol of DCPIP min^{-1} (mg of protein)$^{-1}$.

Decreases in glutathione concentrations following exposure of cells to hydroquinone were assayed by an HPLC method (*29*), and also by reduction of *o*-phthaldehyde at pH 8.0 (*30*). Glutathione levels were equivalent in the two cell lines, as determined by HPLC (Table 2). The HPLC method was also able to detect GSSG, but no GSSG was detected after exposure to hydroquinone (data not shown). The extent of GSH depletion was measured by the simpler fluorometric assay for time series data. The level of GSH in HL-60 cells fell below 50% of control levels between 1 and 2 h after the addition of hydroquinone, and to 26% after 4 h (Figure 3). GSH was depleted by hydroquinone at a significantly greater rate in 33C cells than in HL-60 cells (Figure 3, least-squares linear regression, $p < 0.001$). The extent of glutathione depletion by 10 h postexposure was equivalent in the two cell lines with approximately 12% of the GSH of control levels remaining (data not shown).

TD_{50} Levels. For TD_{50} levels, cells were exposed to chemical in PBS for 2 h, returned to complete media for 24 h, and counted. The viability of cultures 24 h postexposure never fell below 85% (data not shown), demonstrating that necrosis was not induced. In agreement with this, a recent study in which HL-60 cells were used also showed that the predominant cell death pathway resulting from benzene metabolite exposure resulted from apoptosis and not from necrosis (*37*). 33C cells were protected from hydroquinone toxicity, although the protection was only modest and not statistically significant (Table 3). The oxidized form of hydroquinone, 1,4-benzoquinone, was also tested to determine the effect of NQO1. Benzoquinone was 1 order of magnitude more toxic to the cell lines than hydroquinone, and the 33C cell line was protected slightly against benzoquinone toxicity compared to HL-60 cells (Table 3; TD_{50} of 3.5 vs 3.0 μM, a statistically significant difference that may lack biological significance). Benzenetriol is another benzene metabolite which can be oxidized to a quinone. Benzenetriol toxicity, however, did not differ among the two cell lines.

Table 3. Fifty Percent Survival Values (TD_{50}) for HL-60 and 33C Cell Lines Exposed to Different Chemicals

	TD_{50} (μM)	
treatment	HL-60	33C
hydroquinone	45 ± 3.2[a]	51 ± 4.3
benzoquinone	3.0 ± 0.21	3.5[b] ± 0.18
benzenetriol	16 ± 1.3	16 ± 1.6
streptonigrin	0.14 ± 0.018	0.075[c] ± 0.011
vincristine	2.1 ± 0.12	2.0 ± 0.08
arsenite	75 ± 4.1	79 ± 3.6

[a] Mean ± 95% confidence limits. [b] Significant difference from the HL-60 value when $p < 0.05$. [c] Significant difference from the HL-60 value when $p < 0.01$.

Streptonigrin was chosen to be a positive control for the 33C cell line; this chemical is activated to toxic species by NQO1 (*15*). Predictably, it was twice as toxic to 33C as HL-60 cells. Negative controls included vincristine and arsenite. Vincristine is a substrate for MPO (*28*), but not a known substrate of NQO1. Arsenite is a thiol-depleting agent which was used as a control for the toxic effects of nonenzymatic thiol depletion. Both vincristine and sodium arsenite were equitoxic to HL-60 and 33C cells.

Apoptosis. Apoptotic cells are identified as the cells with high green fluorescence, indicating incorporation of biotin−dUTP by the enzyme TdT to the ends of DNA cleaved by apoptotic endonucleases. An increased level of apoptosis was clearly demonstrated in response to hydroquinone treatment by the TdT assay (Figure 4A,B). Apoptosis by hydroquinone is evident in all phases of the cell cycle; i.e., green fluorescence occurs in cell populations of G_1, S, and G_2/M phases of the cell cycle (Figure 4B). The kinetics of apoptotic formation in HL-60 cells was assayed by measuring apoptosis at various time points after cessation of hydroquinone treatment. A peak of apoptosis occurred around 8−12 h, and by 24 h, apoptotic cells disintegrated and were lost to analysis (Figure 5). Apoptosis induced by 50 μM hydroquinone at the 12 h time point peak was used for comparison between the three cell lines.

Hydroquinone (50 μM) induced significantly less apoptosis in 33C cells than in HL-60 cells (Figure 6). The level of apoptosis in response to arsenite was lower in 33C cells, but not significantly. Arsenite induced apoptosis in all phases of the cell cycle in a manner similar to that of hydroquinone (data not shown). Interestingly, the apoptosis data presented here closely mirror the TD_{50} results listed in Table 4, suggesting that apoptosis is the main form of cell death induced by these chemicals in HL-60 cells.

Protein Binding. Both of the cell lines had equivalent level of protein binding in the presence of radiolabeled hydroquinone. HL-60 cells bound 790 ± 24 pmol of hydroquinone equivalents per milligram of protein, while 33C cells correspondingly bound 864 ± 65 pmol per milligram of protein. As an assay control, C15 cells were also treated with radiolabeled hydoquinone and included in the assay. C15 cells demonstrated lower counts of 625 ± 63 pmol per milligram of protein, in correlation with the reduced level of expression of MPO.

DNA Adducts. One set of high-mobility polyphenolic adducts were resolved as described by Levay and Bodell (*34*). Single adducts were produced in response to hy-

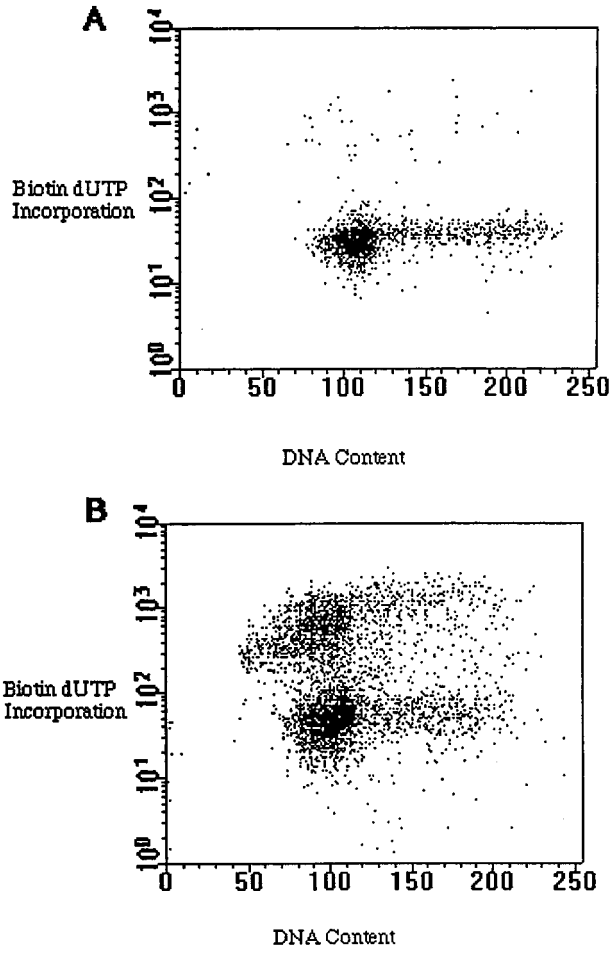

Figure 4. TdT assay of apoptosis. Response of HL-60 cells to 50 μM hydroquinone. (A) Untreated HL-60 cells demonstrate very few cells staining for biotin−dUTP. (B) Treated HL-60 cells demonstrate a large increase in the number of cells staining for biotin−dUTP, thus exhibiting apoptotic DNA breaks.

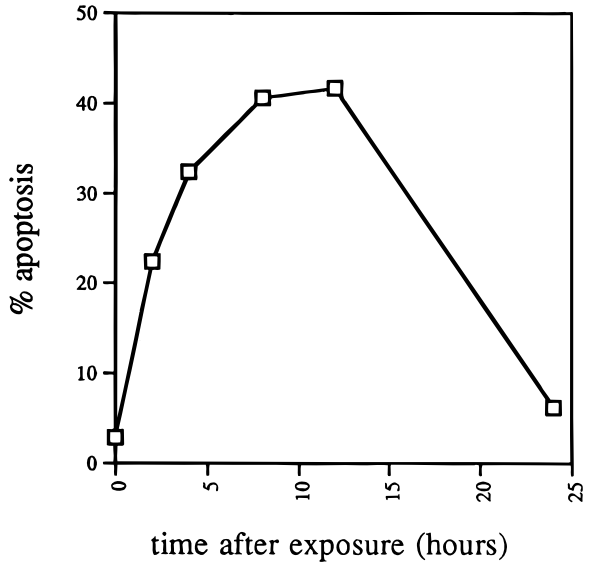

Figure 5. Time course of apoptosis of HL-60 cells following hydroquinone exposure. HL-60 cells were treated with 50 μM hydroquinone for 2 h, returned to complete media, and immediately prepared for DNA break analysis by the TdT assay. Cells were removed at various intervals over the next 24 h and analyzed.

droquinone and benzenetriol individually, and three adducts were found in response to the combination

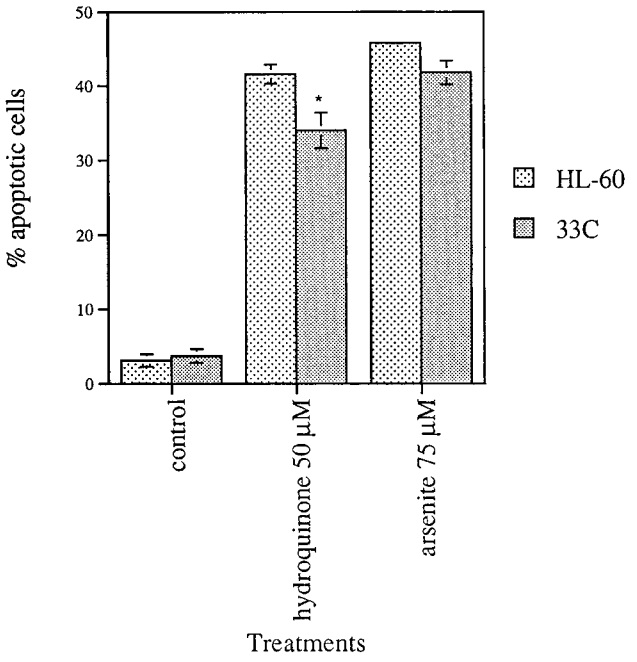

Figure 6. Induction of apoptosis by hydroquinone and arsenite as analyzed with the TdT assay. HL-60 and 33C cells were treated with the indicated chemicals in PBS, returned to complete media, and assayed for apoptosis 12 h later. The asterisk indicates that $p < 0.05$ for the difference between HL-60 and 33C cells.

Table 4. Low-Mobility DNA Adducts of Hydroquinone- and Benzenetriol-Treated Cell Lines[a]

cell line	treatment	adduct 4	adduct 5	adduct 6	total adducts ± SEM
HL-60	50 μM HQ	295	141	−[b]	436 ± 6.5
33C		59	−	−	59 ± 25
C15		126	22	−	148 ± 28
HL-60	100 μM BT	137	23	−	159 ± 40
33C		33	1	−	34 ± 6.0
C15		82	13	−	94 ± 19
HL-60	50 μM HQ and 100 μM BT	230	110	98	437 ± 4.2
33C		65	17	1	82 ± 1.4
C15		139	81	59	235 ± 69

[a] Number of adducts per 10^9 nucleotides. [b] Adducts were undetectable.

(adducts 1−3, data not shown). Potentiation of these adducts was observed in combined treatments with catechol and benzenetriol; however, the adduct levels were not affected by NQO1 expression. Interestingly, however, the low-MPO C15 cell line displayed no detectable adducts in response to hydroquinone alone but adduct levels similar to those of HL-60 cells in combined treatments. Hydroquinone alone produced statistically similar levels of adducts in HL-60 cells (14 adducts per 10^9 nucleotides) and 33C cells (10 adducts per 10^9 nucleotides), and no detectable adducts in C15 cells. Combined hydroquinone/benzenetriol treatments produced 344, 317, and 330 adducts per 10^9 nucleotides in HL-60, 33C, and C15 cells, respectively. Benzenetriol adducts were not quantified in these experiments.

Using different chromatography conditions, a second set of higher-molecular mass, low-mobility adducts were observed (Table 4). Interestingly, these adducts were detected in the same relative positions independent of whether the cells were exposed to hydroquinone or benzenetriol or combined treatments, indicating that they

were not specific to the chemical that was added. The C15 cell line had 66% less of these adducts than HL-60 following hydroquinone treatment, 41% less for benzenetriol, and 36% for the combination (Table 4). In contrast to adducts 1−3, the 33C cell line had 86% less adducts for hydroquinone, 79% less for benzenetriol, and 81% less adducts for the combination (Table 4). This result was consistent in two separate experiments, which are combined for the data presented in Table 4. In general, levels of the lower-mobility adducts were relatively high for single treatments and lower than expected for the combined treatments (subadditive); however, there is some suggestion of synergy in combined treatments in the formation of adducts 5 and 6. No DNA adducts of any type could be detected in vehicle (PBS)-treated control cells.

Discussion

To test the specific effects of NQO1 in vitro and to gain clues to the mechanism of in vivo NQO1-mediated protection against benzene toxicity, we constructed an NQO1-expressing HL-60 subline and tested its response to chemicals versus its non-NQO1-expressing parent. A high level of NQO1 expression showed a modest protective effect against cell death in two assays (Table 3 and Figure 6), but promoted a loss of glutathione in cells in response to hydroquinone and caused no change in protein binding from [^{14}C]hydroquinone (Figures 3 and Results). The relative lack of protection against cell death and sulfhydryl binding would not support these as mechanisms for NQO1-mediated protection against benzene poisoning. Most significantly, NQO1 expression greatly inhibited the formation of a class of DNA adducts (Table 4). This effect was not correlated in extent with any other parameter and therefore may represent an effect of NQO1 on an endogenous cellular component rather than on quinones derived from the benzene metabolites. Alternatively, NQO1 may be suppressing the formation of biphenolic or polymeric products of quinones which may form this class of less mobile adducts. These results will be considered in more detail below.

NQO1's seemingly paradoxical effects in modulating toxicological parameters in response to hydroquinone are comparable with the wider literature which shows, on balance, a protective role of NQO1 in benzene toxicology. In a cell free assay, NQO1 inhibited by up to 70% of the binding of metabolites of [^{14}C]phenol to guinea pig microsomal proteins (*38*). In this system, hydroquinone was shown to be the major metabolite of phenol, with catechol produced in smaller amounts. This protection by NQO1 has not been as clear-cut in studies with living tissue. In one study, a 1.4-fold difference in toxicity between stromal cell cultures derived from DBA/2 and C57BL/6 mice was attributed to a 2-fold difference in NQO1 activity (*23*). In the study presented here, we show that a 34-fold difference in NQO1 activity resulted in only a 1.2-fold difference in cytotoxicity, arguing that other factors besides NQO1 may contribute to the difference in cytotoxicity in the mouse stromal cell cultures. In other studies, the addition of NQO1-inducing *tert*-butyl hydroquinone (*23*) and 1,2-dithiole-3-thione (*24*) were protective against hydroquinone-induced cytotoxicity. These *Ah*-inducing chemoprotectants induce a wide variety of cellular antioxidant enzymes and glutathione, however, and a specific effect from NQO1 would be difficult to glean from these experiments. The inhibition of NQO1 by dicoumarol potentiated toxicity of hydroquinone in stromal cell cultures (*24*), but the high concentration of dicoumarol that was used (40 μM for 24 h) makes interpretation difficult. Indeed, in another study, inhibiting NQO1 with dicoumarol in an NQO1-expressing cell line, ML-1, had little to no effect in potentiating hydroquinone-induced cytotoxicity (*25*). In the work for the study presented here, it was found that incubation with 25 μM dicoumarol for 2 h severely suppressed HL-60 cell proliferation and the replicative index in cells growing for a 24 h period in dicoumarol-free media after exposure (data not shown), illustrating potential difficulties with this approach.

Several parameters besides NQO1 may alter a cell's response to hydroquinone. A more critical role for glutathione rather than NQO1 is suggested in two studies concerning the toxicity of hydroquinone to cells in culture (*24*, *25*). In another study, bone marrow macrophages were found to be 100-fold more sensitive to hydroquinone toxicity than fibroblasts (*22*), and 6-fold more sensitive to protein binding by radiolabeled hydroquinone (*39*). Differences between NQO1, MPO, glutathione *S*-transferases, and UDP-glucuronosyltransferase activities were found between the two cell types (*7*). The large variation in enzyme levels, let alone those of other constituents such as glutathione, precludes a direct analysis of NQO1 to the response of these cells to hydroquinone. We have overcome some of the problems of the former studies by altering NQO1 recombinantly in one cell line without the use of chemical inducers or inhibitors, and without detectable differences in other enzyme or redox parameters. The weak protection by NQO1 seen in this study would suggest that NQO1 protection against cell toxicity (i.e., cell death in short-term assays) may not be responsible for the NQO1-mediated protection from benzene in vivo. Differences in DNA adduct levels may point to a relevant mechanism for explaining the in vivo protection.

DNA adducts were assayed in three cell lines: HL-60 cells, 33C cells, and an MPO-deficient HL-60 subline, C15 (*28*). DNA adducts were developed with two separate chromatography conditions on identical samples. C15 cells had a consistently reduced level of hydroquinone-induced adduct formation corresponding to its lower MPO activity. 33C cell adduct levels, however, were not reduced in one class of DNA adducts, but sharply reduced in the other. We consider these two sets of adducts in turn.

The single hydroquinone-induced DNA adduct assessed as described by Bodell and co-workers (*34*) was evident in HL-60 and 33C cells but undetectable in C15 cells. This result mirrors that shown in a 1993 study by Levay and Bodell, who showed that this adduct level was correlated with the level of peroxidase enzyme activity in various cell lines (*8*). In contrast, NQO1 expression (33C cells vs HL-60 cells) did not alter the extent of formation of this adduct. The three cell lines did not demonstrate a difference in adduct levels with respect to the combination of hydroquinone and benzenetriol. This result may be related to other data showing that H_2O_2 did not enhance adduct formation by benzenetriol (*40*), and benzenetriol synergistically increased adduct formation with hydroquinone (*34*). Benzenetriol in this instance is not subject to peroxidase (it is autoxidized), but is a source of H_2O_2 for hydroquinone oxidation by redox mechanisms.

Different chromatography conditions allow the resolution of a set of lower-mobility DNA adducts (Table 4). These adduct spots exhibit identical mobilities in all treatments, suggesting that they are not chemical specific. Interestingly, the same adduct spots were detected in human DNA, and their levels were increased in lung tissue from cancer patients above controls (*36*). However, with no data on the identity of the adduct spots, we cannot determine whether the adducts are truly chemical-independent. We speculate that the adducts may be formed by the reaction of the quinones or their metabolites with the cell membrane, given recent results indicating that NQO1 may have an endogenous role in reducing membrane-bound coenzyme Q (*41*) and vitamin E quinone (*42*). An endogenous product of lipid peroxidation, malondialdehyde, is known to cause DNA adducts (*43*). NQO1 may be serving an antioxidant function in inhibiting adducts associated with lipid damage. Alternatively, the lower-mobility adducts may be the product of further metabolism of the quinones into biphenolic or other polymeric products. NQO1 may inhibit such metabolism, resulting in a decreased level of higher-mobility adducts, but no change in the level of the low-mobility monoadducts. Detailed chemical analysis of the effect of NQO1 in the formation of polymeric products from simple quinones should help to clarify these issues.

The goal of this study was to create and utilize a practical and efficacious model for NQO1-mediated effects of benzene metabolite toxicity, and to help explain the significant protective effect of NQO1 in vivo (*21*). In creating a model, we were quite successful; the 33C cell line was shown to be quite similar to its HL-60 cell line parent except for a 34-fold higher NQO1 activity. 33C cells were shown to be more susceptible to toxicity from streptonigrin, a chemical known to be activated by NQO1. 33C cells also demonstrated a protection from toxicity to benzoquinone, a toxic quinone reduced to the less toxic hydroquinone by NQO1. In contrast, 33C cells were equitoxic to HL-60 cells with respect to arsenite and vincristine, chemicals not metabolized by NQO1.

In attempting to explain the in vivo protective effect of NQO1, we discovered a class of DNA adducts severely suppressed by the expression of NQO1. This class of adducts appeared to be caused by reactive byproducts of hydroquinone and benzenetriol metabolism in cells, or polymeric products of the benzene metabolites. However, the relative lack of NQO1 activity in reducing toxicity by hydroquinone in short-term tests, and the paradoxical increase in the level of glutathione depletion in NQO1-containing cells, demonstrates an apparent lack of correlation of such tests to the in vivo role of NQO1 in benzene toxicity. It is likely that benzene poisoning in vivo is less a result of the immediate toxicity of benzene metabolites to dividing cells, resulting in apoptosis and necrosis, but more importantly of damage to DNA in slower-growing stem cells which would result in the longer-term inability to adequately resupply the body with blood cells. Such toxicities would be analogous in effect to the short-term toxicity of 5-fluorouracil in the first case, and the long-term toxicity of cross-linking chemotherapeutics in the second. NQO1's suppression of a subclass of DNA adducts may be evidence of its role in protection that would lead to long-term hemostasis in the bone marrow, rather than simply short-term prevention of toxicity.

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