0026-895X/04/6505-1238–1247$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:1238–1247, 2004

Vol. 65, No. 5
3151/1146850
Printed in U.S.A.

NAD(P)H:Quinone Oxidoreductase 1: Role as a Superoxide Scavenger

David Siegel, Daniel L. Gustafson, Donna L. Dehn, Jin Yi Han, Preecha Boonchoong, Lawrence J. Berliner, and David Ross

Department of Pharmaceutical Sciences and Cancer Center, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado (D.S., D.L.G., D.L.D., D.R.); and Department of Chemistry and Biochemistry, University of Denver, Denver, Colorado (J.Y.H., P.B., L.J.B.)

Received December 11, 2003; accepted February 5, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Experiments using purified recombinant human NAD(P)H:quinone oxidoreductase 1 (NQO1) revealed that the auto-oxidation of fully reduced protein resulted in a 1:1 stoichiometry of oxygen consumption to NADH oxidation with the production of hydrogen peroxide. The rate of auto-oxidation of fully reduced NQO1 was markedly accelerated in the presence of superoxide ($O_2^{\cdot-}$), whereas the addition of superoxide dismutase greatly inhibited the rate of auto-oxidation. The ability of reduced NQO1 to react with $O_2^{\cdot-}$ suggested a role for NQO1 in scavenging $O_2^{\cdot-}$, and this hypothesis was tested using established methods for $O_2^{\cdot-}$ production and detection. The addition of NQO1 in combination with NAD(P)H resulted in inhibition of dihydroethidium oxidation, pyrogallol auto-oxidation, and elimination of a potassium superoxide-generated ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide:$O_2^{\cdot-}$ adduct signal (electron spin resonance). Kinetic parameters for the reduction of $O_2^{\cdot-}$ by NQO1 were estimated using xanthine/xanthine oxidase as the source of $O_2^{\cdot-}$ and after NQO1-dependent NADH oxidation at 340 nm. The ability of NQO1 to scavenge $O_2^{\cdot-}$ was also examined using cell sonicates prepared from isogenic cell lines containing no NQO1 activity ($NQO1^-$) or very high levels of NQO1 activity ($NQO1^+$). We demonstrated that addition of NAD(P)H and cell sonicate from $NQO1^+$ but not $NQO1^-$ cells resulted in an increased level of $O_2^{\cdot-}$ scavenging, and this increased level of $O_2^{\cdot-}$ scavenging could be inhibited by 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione (ES936), a mechanism-based inhibitor of NQO1. NQO1 can generate hydroquinones that are redox active, and the $O_2^{\cdot-}$ scavenging activity of NQO1 may allow protection against $O_2^{\cdot-}$ at the site of hydroquinone generation. In addition, the $O_2^{\cdot-}$ scavenging activity of NQO1 may provide an additional level of protection against $O_2^{\cdot-}$-induced toxicity.

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) is an obligate two-electron reductase that catalyzes reduction of a broad range of substrates including quinones, quinone-imines, and nitro-compounds (Ross et al., 2000). NQO1 is a FAD-containing protein that exists as a homodimer and is biochemically characterized by its unique ability to use either NADH or NADPH as reducing cofactors and by its inhibition by the anticoagulant dicumarol (Ernster, 1967; Edwards et al., 1980). The enzyme is generally considered as a detoxification enzyme because of its ability to reduce reactive quinones and quinone-imines to less reactive and less toxic hydroquinones. The two-electron reduction of quinones also bypasses semiquinone production and thus prevents the generation of reactive oxygen species such as O$_2^{\cdot-}$ and by disproportionation hydrogen peroxide (Thor et al., 1982; Lind et al., 1990). The ability of NQO1 to deactivate many reactive species, including quinones, quinone-imines, and azo compounds, demonstrates its importance as a chemoprotective enzyme. The other major protective effect of NQO1 is to function as a cancer preventive enzyme, which has been recognized for more than 30 years (Huggins and Fukunishi, 1964). In humans, high levels of NQO1 have been found in most tissues except for liver (Siegel and Ross, 2000; Strassburg et al., 2002). Immunohistochemical studies have revealed that NQO1 is expressed primarily in epithelium, endothelium, and adipocytes (Siegel and Ross, 2000). In addition, NQO1 is also expressed at high levels in many epithelial-derived human tumors (Siegel et al., 1998; Siegel and Ross, 2000; Wilson et al., 2001).

The function(s) of NQO1 may not be restricted to metabo-

This work was supported by National Institute of Health grants R01-CA51210 and R01-NS44613.

ABBREVIATIONS: NQO1, NAD(P)H:quinone oxidoreductase 1; DHE, dihydroethidium; DCPIP, 2,6-dichlorophenol-indophenol; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; XO, xanthine oxidase; SOD, superoxide dismutase; HRP, horseradish peroxidase; EMPO, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide; ES936, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione; CHO, Chinese hamster ovary; ESR, electron spin resonance.

lism of xenobiotic quinones. NQO1 may play an antioxidant role via the reduction of endogenous quinones such as vitamin E quinone (Siegel et al., 1997) and coenzyme Q_{10} (Beyer et al., 1996). Reduction of these compounds by NQO1 generates stable hydroquinones with excellent antioxidant properties. Other findings suggest that NQO1 may have an even broader antioxidant role. An important observation is that x-radiation and UV radiation, which are known to generate free radicals, are some of the most potent inducers of NQO1 expression in human cells (Boothman et al., 1993; Agrawal et al., 2001). The absence of NQO1 in human liver and moderately high expression throughout most other tissues suggest that the function for NQO1 may have evolved from primarily quinone detoxification to a more broadened antioxidant role. It has been proposed that NQO1 stabilizes p53 (Asher et al., 2001), and in recent studies, we have shown that NQO1 physically associates with the tumor suppressor protein p53 in human tumor cells and primary cultures (Anwar et al., 2003). p53 is a powerful transcription factor that up-regulates a number of proteins involved in protection against oxidant stress including proteins with homology to quinone oxidoreductases/NQO1 (Polyak et al., 1997). All of these findings suggest a more direct role for NQO1 in protection against oxidative stress than considered previously. In this work, we demonstrate that NQO1 has the ability to directly scavenge $O_2^{\cdot-}$.

Materials and Methods

Chemicals and Reagents. Diethylenetriaminepentaacetic acid, deferoxamine mesylate, NADH, NADPH, potassium superoxide, dihydroethidium (DHE), pyrogallol, xanthine, 2,6-dichlorophenol-indophenol (DCPIP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), xanthine oxidase (XO), cytochrome *c* (horse heart), Cu/Zn superoxide dismutase (SOD; bovine erythrocyte), and catalase were obtained from the Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP) was obtained from Calbiochem (San Diego, CA). 2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) was obtained from Alexis Corporation (Läufelfingen, Switzerland). 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl] indole-4,7-dione (ES936) was synthesized as described previously (Beall et al., 1998). Recombinant human NQO1 was purified from *Escherichia coli* by Cibacron blue affinity chromatography as described previously (Beall et al., 1994). Purity was greater than 98% as determined by colloidal Brilliant Blue G staining (Sigma-Aldrich). Purified NQO1 had an activity of between 350 and 500 μmol of DCPIP/min/mg of protein and was stored in 25 mM Tris-HCL, pH 7.4, containing 250 mM sucrose. NQO1 activity was measured using dicumarol-inhibition of DCPIP reduction as described previously (Benson et al., 1980). In brief, NQO1 activity assays were performed on purified NQO1 and cell cytosols using the following conditions: 200 μM NADH and 40 μM DCPIP in 25 mM Tris-HCL containing 0.7 mg/ml bovine serum albumin (1-ml reaction volume). After the addition of purified protein or cytosol, the decrease in absorbance at 600 nm was monitored over 1 min at 27°C. Reactions were performed in the presence and absence of 10 μM dicumarol. Protein concentrations were determined using the method of Lowry et al. (1951).

Cell Lines. Construction of the human NQO1 transfected (stable) MDA468-NQ16 cell line from the parental MDA468 cell line has been described previously (Dehn et al., 2003). The NQO1 activity of the parental cell line was measured at less than 5 nmol of DCPIP/min/mg of protein, whereas the NQO1 activity of the MDA468/NQ16 cell line was greater than 2500 nmol of DCPIP/min/mg of protein. Both cell lines were grown in minimal essential medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% (v/v) CO_2. Construction of the human NQO1 transfected (stable) CHO-DTD812 cell line from the parental Chinese hamster ovary (CHO) cell line has been described previously (Gustafson et al., 1996). No NQO1 activity or immunoreactive NQO1 protein could be detected in the parental CHO cell line, whereas the NQO1 activity of the CHO/812 cell line was greater than 3500 nmol DCPIP/min/mg of protein. Both CHO cell lines were grown in Ham's F-12 medium supplemented with 7% (v/v) fetal bovine serum, 20 mm HEPES, pH 7.4, 1 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% (v/v) CO_2. For cytosol preparation, all cell lines were grown to three-quarters confluence in 100-mm plates, medium was removed, and the cells were washed with phosphate-buffered saline, pH 7.4. After rinsing, cells were scraped into phosphate-buffered saline and pelleted by centrifugation. The cell pellet was resuspended in 2.5 ml of Tris-HCL, pH 7.4, containing 250 mM sucrose, sonicated on ice for 5 s, and followed by centrifugation at 30,000*g* (15 min) to remove insoluble matter. Protein concentrations were determined on supernatants using the method of Lowry et al. (1951). For toxicity studies CHO cells were treated with freshly prepared pyrogallol dissolved in 20 mM HEPES, pH 8.0, for 2 h, after which the HEPES solution was replaced with fresh growth media, and the cells were allowed to divide for 4 days. The MTT-based cytotoxicity assay was performed as described previously (Dehn et al., 2003).

Auto-Oxidation of NQO1. The auto-oxidation of NQO1 was monitored in a stirred cell at 35°C using a Clark electrode (YSI Inc., Yellow Springs, OH). Reactions (4 ml) contained 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM deferoxamine, and 200 μM NADH. Before the addition of NQO1 (400 μg), a 1-ml aliquot of reaction was removed for spectrophotometric analysis at 340 nm to determine the initial NADH concentration. Oxygen consumption was monitored over 15 min after which another 1-ml aliquot was immediately removed for NADH analysis. Reactions were run in the presence and absence of SOD (5 U/ml). Oxygen consumption was linear over the length of the experiment except for an initial lag period of approximately 1 min. To determine products (superoxide and hydrogen peroxide) generated during auto-oxidation of NQO1, we used luminol-based chemiluminescence in the presence and absence of HRP (12.5 U/ml). Reactions (0.2 ml) containing 50 mM potassium phosphate buffer, pH 7.4, 200 μM NADH, 80 μg of NQO1, and 0.5 mM luminol were mixed for 1 min at 27°C after which samples were immediately placed into a luminometer. Experiments were repeated with the addition of catalase (177 U/ml) or SOD (10 U/ml).

Spectral Analysis of the Reaction of Superoxide with NQO1. Spectrophotometric analysis was used to monitor the NQO1 catalytic cycle (NADH oxidation and FAD reduction) in NQO1 in the presence and absence of potassium superoxide. Reaction conditions (0.5 ml) were: 40 nmol of recombinant human NQO1, 50 nmol of NADH, and 5 μmol of potassium superoxide. Reactions were performed in 50 mM potassium phosphate buffer, pH 7.4, at 27°C. Scans (250–500 nm) were taken every 40 s. The potassium superoxide stock solution was prepared by adding 1 g of potassium superoxide to 1 ml of anhydrous ME_2SO, vigorously mixing for 2 min, followed by centrifugation to remove excess potassium superoxide yielding a stock concentration of approximately 3.6 mM (Reiter et al., 2000).

Oxidation of Dihydroethidium. Oxidation of DHE by $O_2^{\cdot-}$ was monitored by spectroscopy using a fluorescent compatible microplate reader (Molecular Devices, Sunnyvale, CA). Reactions (1 ml) included 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid, 0.75 mM xanthine, 0.23 U of XO, and 10 μM dihydroethidium. Reactions were started by the addition of XO and were monitored at excitation and emission wavelengths of 480 and 586 nm, respectively, from 0 to 15 min at 27°C.

ESR Spectroscopy. X-band ESR spectra were recorded at room temperature on a Varian E4 spectrometer (Varian, Inc., Palo Alto, CA) equipped with a TE_{102} cavity. Typical experimental conditions were: microwave frequency, 9.27 GHz; microwave power, 20 mW;

modulation frequency, 100 KHz; modulation amplitude, 0.1 mT; and the applied magnetic field was 300 mT at a scan rate of 8 mT/2 min. Reactions (0.1 ml) were in 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM diethylenetriaminepentaacetic acid, and 25 mM EMPO, and reactions were started by the addition of 1 μl of potassium superoxide dissolved in ME_2SO (for stock concentration, see above).

Pyrogallol Auto-Oxidation. Inhibition of pyrogallol auto-oxidation was performed as described previously (Marklund and Marklund, 1974). In brief, 1-ml reactions were monitored for increased absorbance at 420 nm from 0 to 5 min at 27°C. Rates were determined from 3 to 5 min. Complete reactions contained 50 mM Tris-HCL, pH 8.0, 2 mM EDTA, 0.2 mM NADH or NADPH, 12 μg of NQO1, 2.5 μM ES936, and 0.2 mM pyrogallol. In studies with human cell lines, freshly prepared cytosol replaced purified recombinant NQO1.

Kinetic Parameters. Kinetic parameters for the reduction of $O_2^{\cdot-}$ by NQO1 were obtained using xanthine/XO as the source of $O_2^{\cdot-}$ and monitoring NQO1-dependent NADH oxidation at 340 nm. Reactions (1 ml) were performed at 27°C in 50 mM potassium phosphate buffer, pH 7.8, 0.5 mM xanthine, and 0.2 mM NADH. In these experiments, we varied the XO activity from 0 to 0.7 units and quantified the rate of $O_2^{\cdot-}$ production using the reduction of 0.05 mM cytochrome *c* (monitored at 550–540 nm). We then substituted NQO1 (20–80 μg) in place of cytochrome *c* and monitored a linear decrease in absorbance at 340 nm (NADH oxidation) for 2 min. The background rate of NADH oxidation was determined at each XO concentration in the absence of NQO1, and this was subtracted from the rate determined in the presence of NQO1. Kinetic parameters (K_m and V_{max}) were calculated from the rates of NADH oxidation using Sigma Plot and its associated enzyme kinetics module (SPSS Inc., Chicago, IL).

Results

NQO1 Auto-Oxidation. The auto-oxidation of reduced NQO1 was examined in the presence of NADH and absence of an electron acceptor. In these studies, we monitored molecular oxygen consumption and NADH oxidation simultaneously over 15 min, and results from these studies clearly demonstrated a 1:1 stoichiometry of oxygen consumption to NADH oxidation (Table 1). Interestingly, the addition of SOD significantly slowed the rate of both oxygen consumption and NADH oxidation (Table 1). To identify whether $O_2^{\cdot-}$ and/or hydrogen peroxide were formed during the auto-oxidation of NQO1, we used luminol-based chemiluminescence in the presence and absence of HRP (Fig. 1). In the absence of HRP, no chemiluminescence was detected during the auto-oxidation of NQO1, indicating that $O_2^{\cdot-}$ was not being generated in sufficient quantities to oxidize luminol. The inclusion of HRP, however, resulted in a robust signal that was greater than 95% inhibited by catalase. These data indicate that hydrogen peroxide was the major product formed during the auto-oxidation of NQO1. The inclusion of SOD along with HRP also resulted in a greater than 80% inhibition of the signal, and this can be explained by the ability of SOD to slow the overall rate of NQO1 auto-oxidation (Table 1). In control experiments, no inhibition of NQO1 activity was observed when the purified NQO1 was incubated with hydrogen peroxide (100 mM) for 1 h in the presence or absence of NADH (data not shown), indicating that the inhibition in chemiluminescence was not because of direct inactivation of NQO1 by hydrogen peroxide. The auto-oxidation of NQO1 was also examined by UV-visible spectroscopy. In these experiments, we monitored the spectrum of NADH and FAD following reduction of NQO1 in the presence and absence of potassium superoxide (Fig. 2). These data clearly show that the rate of NADH oxidation is greatly accelerated in the presence of $O_2^{\cdot-}$ (Fig. 2B); conversely, the addition of SOD significantly inhibited the rate of NADH oxidation (Table 1). No oxidation of NADH by potassium superoxide was detected in the absence of NQO1 (Fig. 2, inset), indicating that these reactions are mediated through NQO1. These data clearly demonstrated an enhanced rate of flavin oxidation in NQO1 in the presence of $O_2^{\cdot-}$ and suggested that NQO1 may be capable of efficiently catalyzing the reduction of $O_2^{\cdot-}$ to hydrogen peroxide using reduced pyridine dinucleotide cofactors.

Superoxide-Dependent Oxidation of Dihydroethidium. The ability of NQO1 to scavenge superoxide was examined using the oxidation of DHE (Fig. 3). In these experiments, the xanthine/XO system was used to generate $O_2^{\cdot-}$, which then oxidized DHE to generate a fluorescent product (Zhao et al., 2003). The addition of NADH and NQO1 significantly inhibited the oxidation of DHE by $O_2^{\cdot-}$. No inhibition was observed when NADH or NQO1 were added separately or when ES936, a mechanism-based inhibitor of NQO1 (Winski et al., 2001; Dehn et al., 2003) was added in combination with NQO1 and NADH. The addition of SOD completely inhibited xanthine/XO-dependent increase in DHE fluorescence, confirming $O_2^{\cdot-}$ as the primary oxidant of DHE (Fig. 3). Catalase had no effect on xanthine/xanthine oxidase-induced DHE fluorescence, indicating that hydrogen peroxide is not involved in these reactions (Fig. 3). Control experiments demonstrated that NQO1 and NADH could not directly reduce the oxidized product of DHE back to DHE (data not shown). Oxygen consumption studies (Clark electrode) also demonstrated that the combination of NQO1 and NADH had no effect of the rate of xanthine/XO-catalyzed molecular oxygen consumption, indicating that NQO1 and NADH do not

TABLE 1

Stoichiometric analysis of NADH oxidation and O_2 consumption resulting from the autooxidation of NQO1

Experimental conditions are described under *Materials and Methods*. Results are expressed ± S.D.

Treatments	NADH Oxidized	O_2 Consumed
	nmol	
NADH, NQO1 (n = 3)	64.6 ± 4.8	61.1 ± 2.57
NADH, NQO1, SOD (n = 4)	14.7 ± 2.8	8.7 ± 3.5

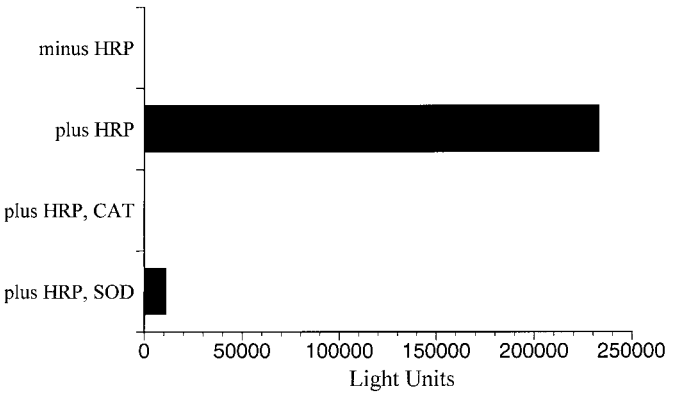
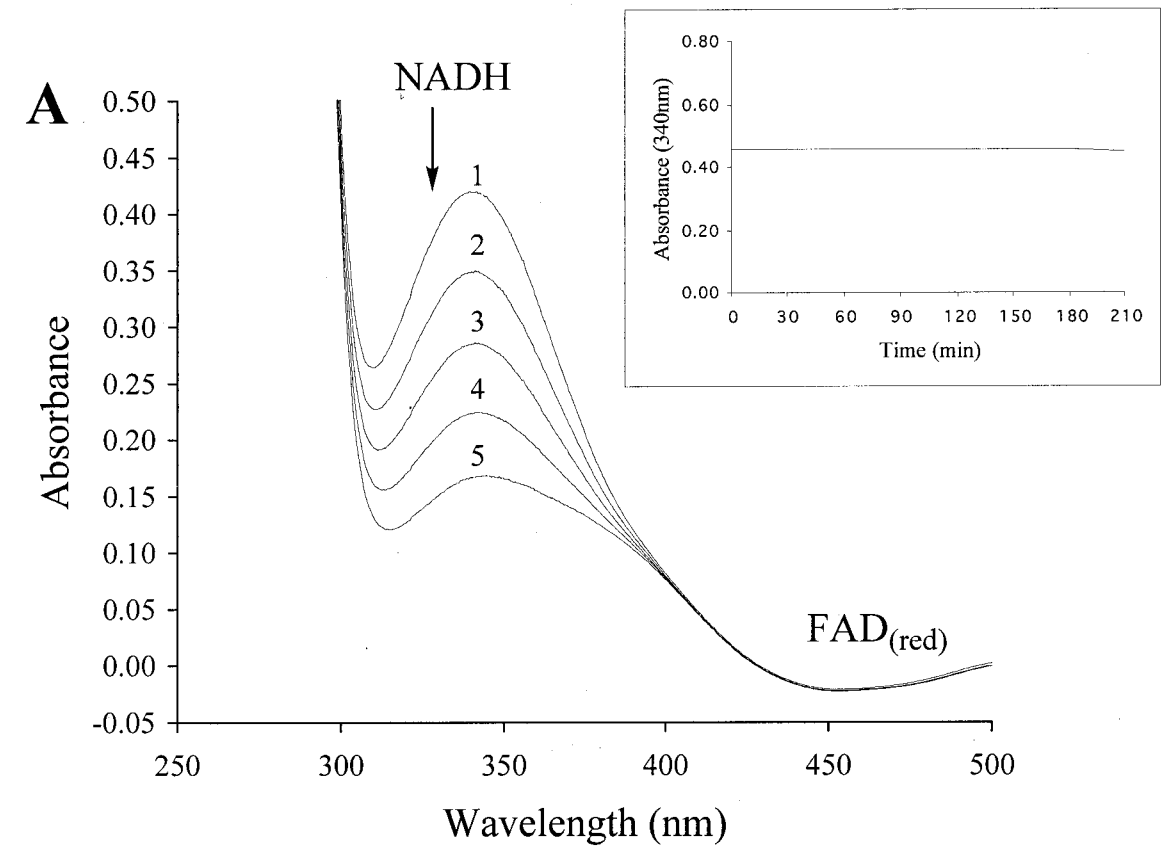


Fig. 1. Hydrogen peroxide formation during the auto-oxidation of NQO1. Luminol-based chemiluminescence was used in the presence and absence of HRP to determine the oxidation products ($O_2^{\cdot-}$ and hydrogen peroxide) formed during the auto-oxidation of NQO1. Experimental conditions are described under *Materials and Methods*. Data are representative of duplicate experiments.



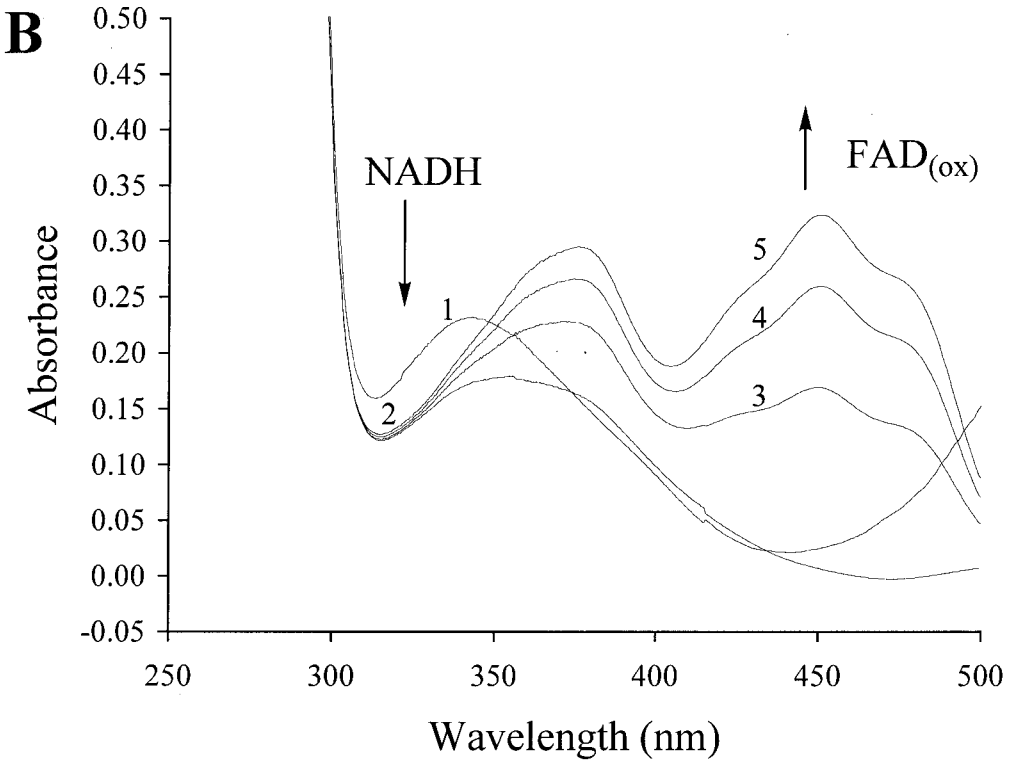


Fig. 2. Spectral analysis of the reaction of $O_2^{\cdot-}$ with NQO1. Spectrophotometric analysis was used to monitor NQO1-dependent NADH oxidation in the presence and absence of potassium superoxide. Experimental conditions are described under *Materials and Methods*. Spectra (250–500 nm) were acquired every 40 s. A, NQO1-dependent NADH oxidation in the absence of potassium superoxide. B, NQO1-dependent NADH oxidation in the presence of potassium superoxide. Inset, spectrophotometric analysis (340 nm) of the direct oxidation of NADH by potassium superoxide. No oxidation of NADH by potassium superoxide was observed in the absence of NQO1.

effect the rate of $O_2^{\cdot-}$ production by xanthine/XO (data not shown).

ESR Spectroscopy Studies. ESR spectroscopy in combination with the spin probe EMPO and potassium superoxide were used to examine the $O_2^{\cdot-}$ scavenging ability of NQO1 (Fig. 4). In these experiments, the addition of either NQO1 or NADH separately had no effect on signal intensity (Fig. 4, B and C); however, NQO1 in combination with NADH resulted in near complete elimination of the EMPO:$O_2^{\cdot-}$ adduct signal (Fig. 4D), similar to what was observed for SOD (Fig. 4F). As seen previously, the addition of ES936 reversed the inhibitory effects of NADH/NQO1 (Fig. 4E).

Auto-Oxidation of Pyrogallol. Inhibition of the auto-oxidation of pyrogallol was used as an additional measure of the ability of NQO1 to scavenge $O_2^{\cdot-}$ (Fig. 5A). In agreement with previous data, the combination of NQO1 and NADH completely prevented the auto-oxidation of pyrogallol, whereas no inhibition of pyrogallol auto-oxidation was observed with the addition of either NADH or NQO1 separately, Incubation with ES936 blocked the effect of NADH/NQO1 on pyrogallol auto-oxidation. The addition of SOD completely inhibited pyrogallol auto-oxidation, whereas the addition of catalase had no effect demonstrating that $O_2^{\cdot-}$ is the primary oxidant in this reaction. A linear relationship was observed between inhibition of pyrogallol oxidation and the NQO1 protein concentration (Fig. 5B). Oxygen consumption studies (Clark electrode) demonstrated that the combination of NQO1 and NADH had no effect of the rate of molecular oxygen consumption during the auto-oxidation of pyrogallol (data not shown).

Kinetic Analysis. Kinetic analysis of the reaction of $O_2^{\cdot-}$ with NQO1 was performed using xanthine/XO as the source of $O_2^{\cdot-}$. Kinetic parameters were determined using the NQO1-dependent oxidation of NADH. In these experiments, we added increasing quantities of XO and monitored the effect on the rate of NQO1-dependent NADH oxidation. The rate of $O_2^{\cdot-}$ production by xanthine/XO was quantified in parallel experiments using the reduction of cytochrome *c* in the absence of NQO1. Kinetic parameters (K_m and V_{max}) for the reaction of $O_2^{\cdot-}$ with NQO1 (Table 2) were calculated from reaction rate versus substrate plots (Fig. 6) fit to the classic Michaelis-Menten equation: $V = V_{max} \times [S]/K_m + [S]$. From these data, we calculated a rate constant for the reaction of $O_2^{\cdot-}$ with NQO1 to be 3.60×10^4 M/s and a K_m of 2.87 μM at pH 7.8 (Table 2).

Role of NQO1 in the $O_2^{\cdot-}$ Scavenging Activity of Human and Animal Cell Lines. To measure whether the expression of NQO1 influences $O_2^{\cdot-}$ scavenging in cells, we used isogenic cell lines that differ in the amount of NQO1 protein and activity. The parent CHO cell line (CHO) has no detectable NQO1 activity or protein in cytosolic preparations, whereas the stable clone CHO/812 overexpressing human

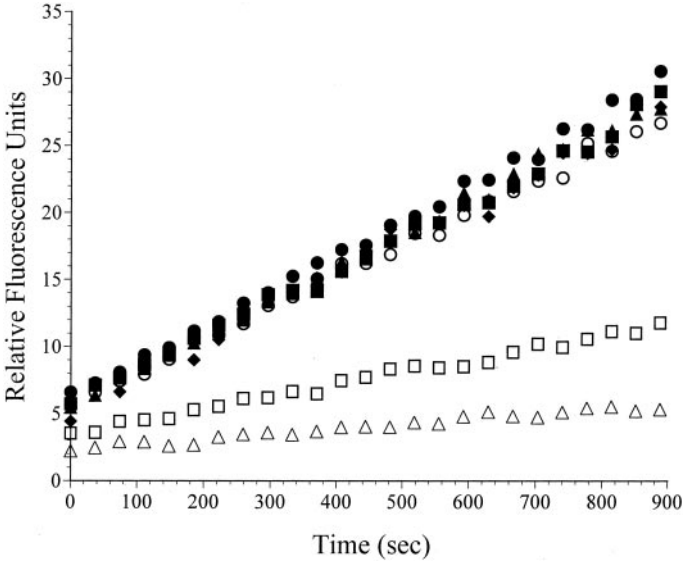


Fig. 3. The inhibition of superoxide-dependent oxidation of dihydroethidium by NQO1. Oxidation of DHE by $O_2^{\cdot-}$ generated from xanthine/XO was monitored by fluorescent spectroscopy as described under *Materials and Methods*. ■, xanthine/XO/dihydroethidium; ●, plus NADH (0.2 mM); ▲, plus NQO1 (25 μg/ml); □, plus NADH (0.2 mM) and NQO1 (25 μg/ml); ○, plus NADH (0.2 mM), NQO1 (25 μg/ml), and ES936 (4 μM); △, plus superoxide dismutase (5 U/ml); ◆, plus catalase (100 U/ml). Data are representative of five separate experiments.

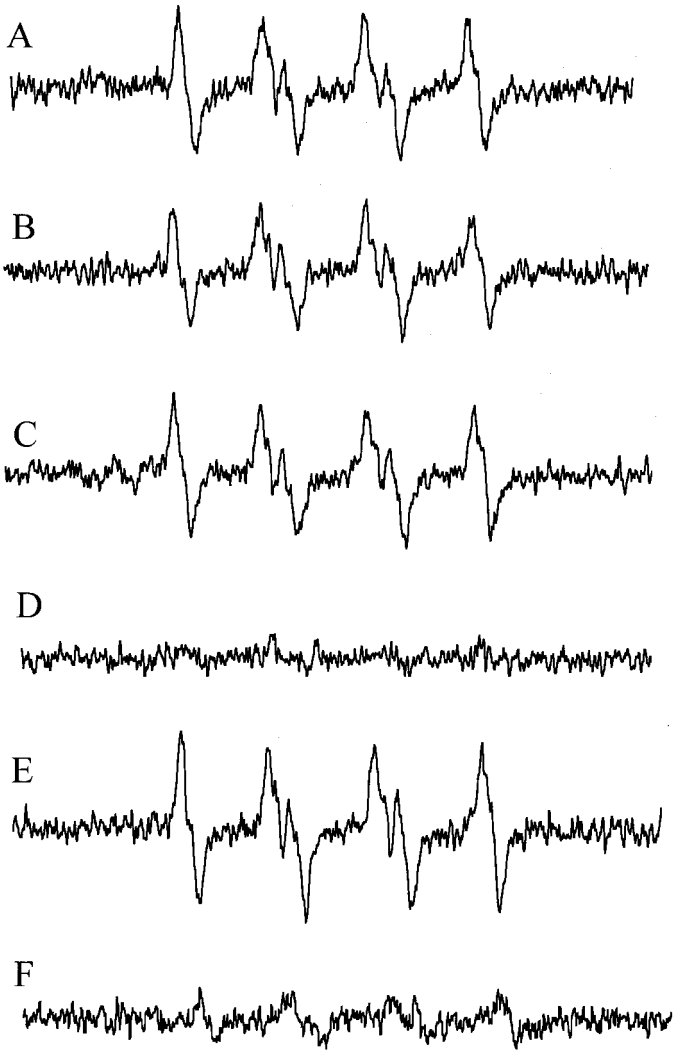


Fig. 4. The effect of NQO1 on EMPO:$O_2^{\cdot-}$ adduct formation. ESR and the spin probe EMPO were used in combination with potassium superoxide to examine the ability of NQO1 to scavenge $O_2^{\cdot-}$. Experimental conditions are described under *Materials and Methods*. In addition to potassium superoxide and EMPO, the reactions contained the following: A, no additions; B, NADH (0.5 mM); C, NQO1 (25 μg/ml); D, NADH (0.5 mM), NQO1 (25 μg/ml); E, NADH (0.5 mM), NQO1 (25 mg/ml), and ES936 (5 μM); and F, SOD (10 U/ml). Spectra were obtained 5 min after the addition of potassium superoxide.

NQO1 has an activity of greater than 3500 nmol of DCPIP/min/mg of protein. The human breast cancer cell line MDA468 (parental) has a NQO1 activity <5 nmol DCPIP/min/mg of protein, whereas the stable, NQO1 transfected cell line MDA468/NQ16 has a an activity greater than 2500 nmol DCPIP/min/mg of protein. Inhibition of pyrogallol auto-oxidation was used as a measure of $O_2^{\cdot-}$ scavenging activity (Fig. 7). The addition of NAD(P)H along with cytosol from the CHO/812 or MDA468/NQ16 cell lines resulted in a significant increase in $O_2^{\cdot-}$ scavenging activity compared with identical samples assayed in the absence of NAD(P)H, and this increase in $O_2^{\cdot-}$ scavenging activity could be inhibited by ES936. In contrast, no increase in $O_2^{\cdot-}$ scavenging activity was observed in the presence of NAD(P)H in the NQO1-null CHO and MDA468 cell lines. In the absence of reduced pyridine dinucleotide cofactor, similar $O_2^{\cdot-}$ scavenging activities were observed between the CHO and CHO/812 lines and MDA and MDA/812 lines, presumably because of equal expression of Cu/Zn SOD. Immunoblot analysis confirmed that overexpression of NQO1 in MDA468/NQ16 cells did not change the protein levels of Cu/Zn SOD compared with the parental MDA468 cell line (data not shown).

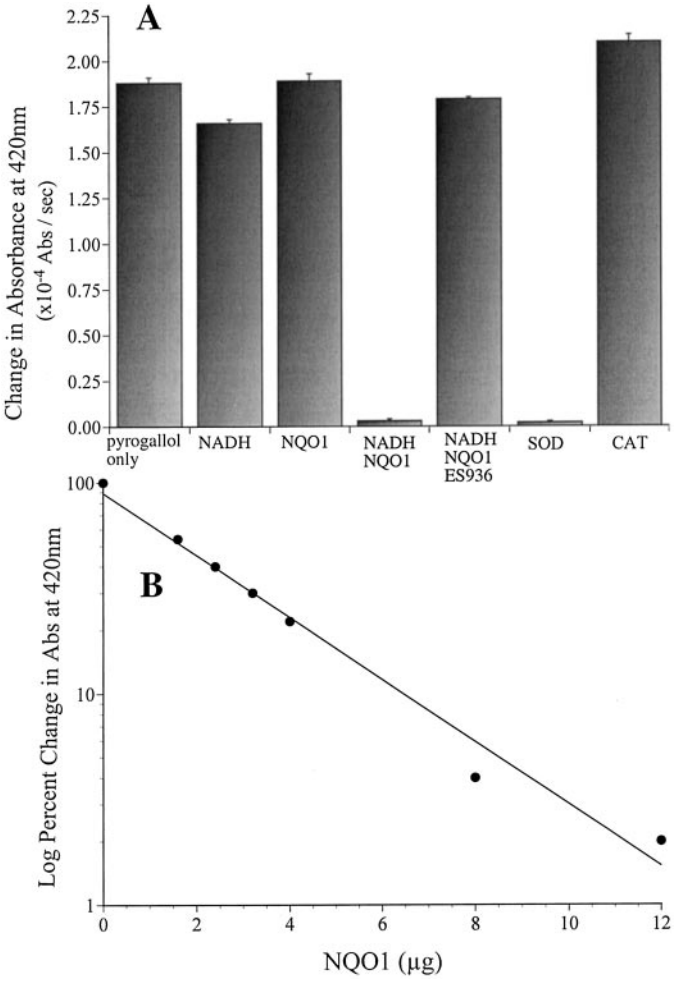


Fig. 5. The inhibition of pyrogallol auto-oxidation by NQO1. The auto-oxidation of pyrogallol was monitored spectrophotometrically at 420 nm as described under *Materials and Methods*. A, additions included NADH (0.2 mM), NQO1 (12 μg/ml), ES936 (2.5 μM), SOD (5 U/ml), and catalase (100 U/ml). Results are the mean of three separate determinations ± S.D. (error bars). B, inhibition of pyrogallol auto-oxidation as a function of NQO1 concentration.

TABLE 2

Kinetic analysis of the reaction of O<bardot;2> with NQO1

O<bardot;2> was generated using xanthine/XO as described under *Materials and Methods*. Results are the mean ± S.D. of three separate determinations.

Parameter	Value
K_m (μM)	2.87 ± 0.90
V_{max} (pmol/min)	4021 ± 361
K_{cat} (min^{-1})	3.10 ± 0.28
K_{cat}/K_m (M/s)	3.60×10^4

Effect of NQO1 Overexpression on Pyrogallol Cytotoxicity. We examined whether overexpression of NQO1 could increase protection against $O_2^{\cdot-}$-induced cytotoxicity. For these assays, we used the parental CHO cell line (no NQO1 activity) and the CHO/812 cell line overexpressing human NQO1 and exposed these cells to increasing concentrations of pyrogallol in HEPES medium (pH 8.0) for 2 h. After treatment, pyrogallol was removed, and the cells were screened for toxicity using the MTT assay. At all pyrogallol

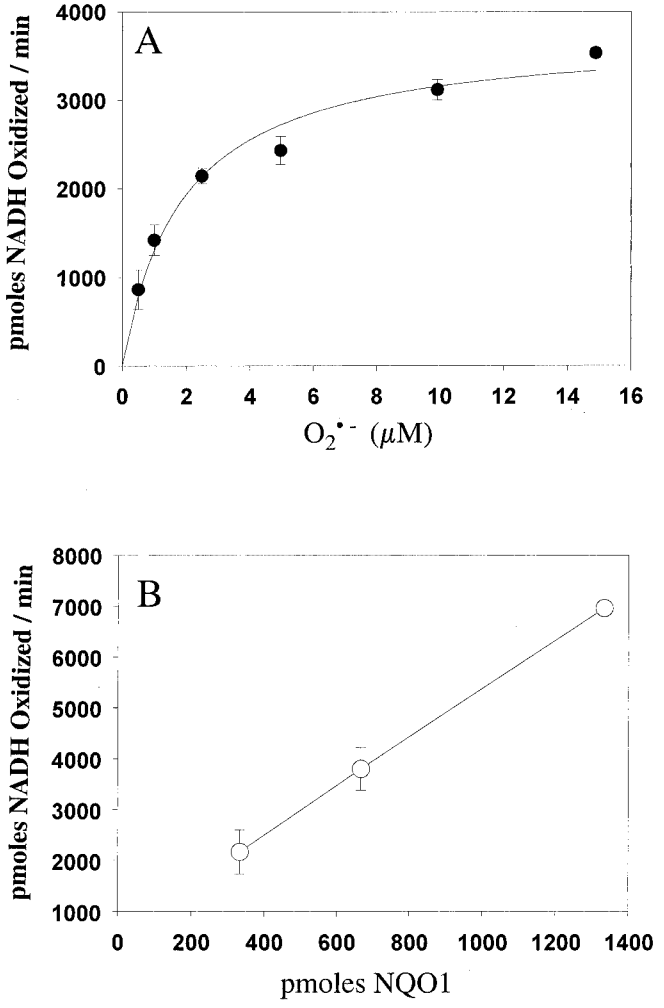


Fig. 6. A kinetic analysis of the reaction of $O_2^{\cdot-}$ with NQO1. A kinetic examination of the reaction of $O_2^{\cdot-}$ with NQO1 was performed at pH 7.8 using xanthine/XO as the source of $O_2^{\cdot-}$ and monitoring NQO1-dependent NADH oxidation at 340 nm. $O_2^{\cdot-}$ production by xanthine/XO was quantified using the reduction of cytochrome *c* in parallel experiments in the absence of NQO1. A, velocity versus substrate concentration. B, velocity versus enzyme concentration. Results are the mean of three separate determinations ± S.D. (error bars).

concentrations tested, the CHO-DTD812 cells had greater cell survival than the parental CHO cell line (Fig. 8).

Discussion

Studies on the auto-oxidation of NQO1 demonstrated that the stoichiometry of NADH oxidation to molecular oxygen consumption was approximately 1:1 and that the major product formed during the auto-oxidation of NQO1 was hydrogen peroxide. In addition, it was observed that the rate of NQO1 auto-oxidation could be accelerated in the presence of $O_2^{\cdot-}$ and that the rate of NQO1 auto-oxidation was inhibited by the inclusion of SOD. These results, although novel for NQO1, have been described previously for the auto-oxidation of free flavins and other flavoproteins (Massey et al., 1973; Muller, 1987) and are described in Scheme 1. Under this scheme, the reaction of molecular oxygen with flavinhydroquinone generates a hydroperoxyflavin (I) that decomposes rapidly to flavinsemiquinone radical and $O_2^{\cdot-}$ (II). The flavinsemiquinone can either react with molecular oxygen to form flavinquinone and $O_2^{\cdot-}$ (III) or react with $O_2^{\cdot-}$ to generate flavinquinone and hydrogen peroxide (IV). $O_2^{\cdot-}$ can also oxidize the flavinhydroquinone to flavinsemiquinone and hydrogen peroxide (V). Experiments by Massey et al. demonstrated that the addition of SOD could significantly decrease the rate of flavinhydroquinone auto-oxidation and concluded that $O_2^{\cdot-}$ was the primary oxidant responsible for the oxidation of flavinhydroquinone (Massey et al., 1973). Our data also demonstrated that the addition of SOD inhibited NQO1 auto-oxidation by greater than 75% (Table 1). The similarities between the auto-oxidation of free flavin and NQO1 are not surprising given that the FAD cofactor in NQO1 is not covalently bound to the protein. Data from X-ray crystallographic studies with NQO1 have shown that FAD is located within a highly plastic active site and held in place through hydrogen bonding and van der Waals interactions (Faig et al., 2000). An important feature of NQO1-bound FAD is that the protein environment surrounding FAD does not stabilize the flavinsemiquinone (Tedeschi et al., 1995a, 1995b). Whether $O_2^{\cdot-}$ interacts with other flavoproteins with comparably similar flavin redox chemistry to NQO1 is unknown. The ability of NQO1 bound FAD to carry out the reduction of $O_2^{\cdot-}$ in reactions similar to those described for the auto-oxidation of free flavin (IV and V) in combination with the ability of NQO1 to rapidly regenerate the flavinhydroquinone from flavinquinone via the oxidation of NAD(P)H suggested to us that NQO1 may have an embedded $O_2^{\cdot-}$ scavenging activity (Scheme 2).

In assays designed to test the $O_2^{\cdot-}$ scavenging activity of NQO1, we confirmed that NQO1 in combination with NAD(P)H could effectively inhibit a wide range of $O_2^{\cdot-}$-dependent reactions. In addition, we demonstrated that the $O_2^{\cdot-}$ scavenging activity of NQO1 could be inhibited by ES936, a mechanism-based inhibitor of NQO1. These results are important because they demonstrate that the $O_2^{\cdot-}$ scavenging activity of NQO1 is dependent on the catalytic turnover of the enzyme and not because of an unknown contaminant. Kinetic analysis of the reaction of $O_2^{\cdot-}$ with NQO1 demonstrated that the $O_2^{\cdot-}$-dependent oxidation of NADH by NQO1 is a saturable process with increasing $O_2^{\cdot-}$ concentration and that $O_2^{\cdot-}$ reduction is the rate-limiting step in this reaction. The fact that this reaction is saturable and follows classic Michaelis-Menten kinetics differentiates this mechanism of $O_2^{\cdot-}$ enzymatic detoxification from that of SOD, which is not saturable up to the experimental limits of $O_2^{\cdot-}$ concentration (Bull and Fee, 1985). The relationship between NQO1 and SOD in their kinetics of inactivation with $O_2^{\cdot-}$ has some similarities between the relationship between catalase (high-capacity, high K_m) and glutathione peroxidase (low-capacity, low K_m) for breakdown of hydrogen peroxide (Makino et al., 1994; Sasaki et al., 1998).

The primary metabolic function of NQO1 is considered to be the reduction of quinones to hydroquinones. An embedded $O_2^{\cdot-}$ scavenging activity of NQO1 is attractive because it would serve a dual purpose. Any $O_2^{\cdot-}$ generated by auto-oxidation of hydroquinones would be scavenged at its site of generation. Second, because $O_2^{\cdot-}$ is the major driving force for hydroquinone oxidation (Ollinger et al., 1990), $O_2^{\cdot-}$ removal would prevent the acceleration of HQ autoxidation helping to preserve the molecule in the hydroquinone form and facilitating excretion.

Aside from a potential role in minimizing the deleterious consequences of the generation of redox active hydroquinones, a superoxide scavenging role for NQO1 raises the issue of the relevance of this effect in cells where there are more efficient systems for superoxide removal such as SOD. The kinetic parameters obtained for the reaction of $O_2^{\cdot-}$ with NQO1 indicate that the K_{cat}/K_m ratio is 3.6×10^4 M/s, and the K_m is 2.87 μM. The rate of chemical dismutation of $O_2^{\cdot-}$ at pH 7.8 was approximated from data in a previous study (McClune and Fee, 1976). Although performed under differ-

(I) $FADH_2 + O_2 \rightarrow FADH_2O_2$

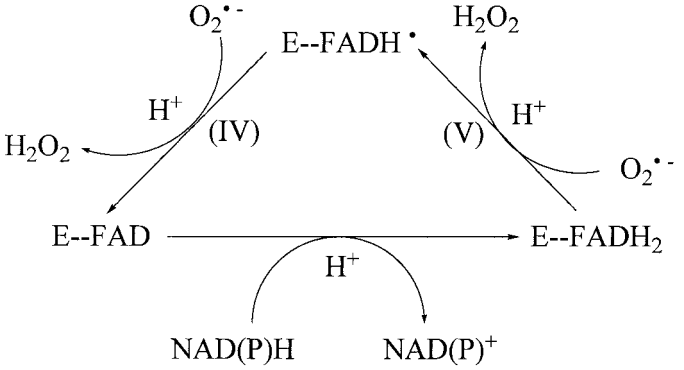
(II) $FADH_2O_2 \rightarrow FADH^{\cdot} + O_2^{\cdot-} + H^+$

(III) $FADH^{\cdot} + O_2 \rightarrow FAD + O_2^{\cdot-} + H^+$

(IV) $FADH^{\cdot} + O_2^{\cdot-} + H^+ \rightarrow FAD + H_2O_2$

(V) $FADH_2 + O_2^{\cdot-} + H^+ \rightarrow FADH^{\cdot} + H_2O_2$

Scheme 1. Reactions describing the auto-oxidation of free flavins.



Scheme 2. Reaction mechanisms describing the superoxide scavenging activity of NQO1. Numbers in parentheses indicate reactions from Scheme 1.

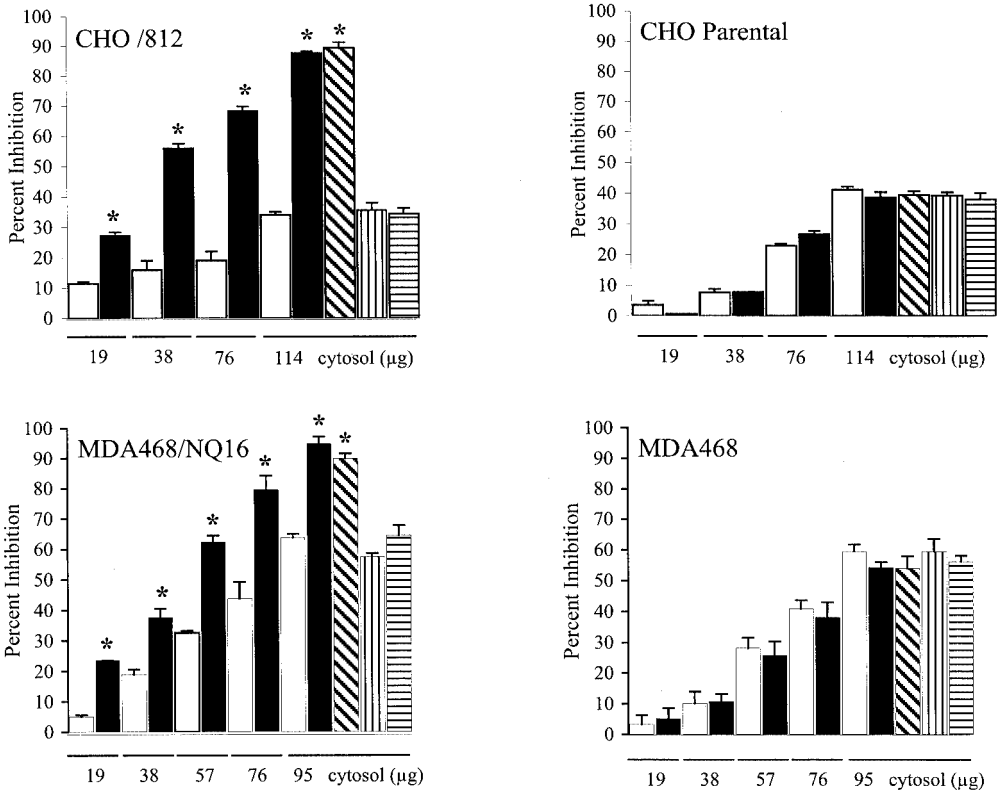


Fig. 7. The effect of NQO1 overexpression on O_2^- scavenging in CHO and MDA468 cells. CHO and MDA468 isogenic cell lines were assayed for their ability to scavenge O_2^- using the inhibition of pyrogallol auto-oxidation as described under *Materials and Methods*. Experiments were run with increasing quantities of cytosol in the presence and absence of reduced pyridine nucleotide cofactors (NADH and NADPH) and ES936. Results are the mean of three separate determinations. Open bars, minus NADH; solid bars, plus NADH; hatched bars, plus NADPH; vertical stripes, plus NADH and ES936; horizontal stripes, plus NADPH and ES936. *, significantly different from matched samples run in the absence of NAD(P)H (open bars). *, $p < 0.02$ (paired Student's t test).

ent conditions, the rate of chemical dismutation was in the order of 2.5×10^4 M/s. The kinetic constants obtained in our studies for the rate of the reaction of O_2^- with NQO1 may be artificially low because xanthine, used as a component of the O_2^- generating system, was shown to inhibit NQO1 activity by 20% (data not shown; also see Roberts et al., 1989). A more accurate measurement of the reaction kinetics for the interaction of O_2^- with NQO1 by stopped-flow methodologies using authentic O_2^- is warranted. Nevertheless, the kinetic data suggest that the rate of reaction of O_2^- with NQO1 is less than an order of magnitude higher than the rate of chemical dismutation of O_2^- and is in the order of 4 orders of magnitude less than the rate of enzymatic dismutation catalyzed by SOD (1.69×10^9 M/s). It should be borne in mind, however, that the significance of NQO1 as a cellular scavenger of superoxide will not depend solely upon the reaction rate but also on the concentration of NQO1 in cells relative to other O_2^- scavenging systems such as SOD. NQO1 is highly inducible in cells after exposure to oxidative stress, and its extensive and rapid induction would be consistent with a role for NQO1 as part of a stress or adaptive response against oxygen radicals such as superoxide. We approached the cellular relevance of the reaction of NQO1 and O_2^- using isogenic cell lines differing only in NQO1 levels. The parental CHO cell line has no NQO1 activity or protein, whereas MDA468 cells have only trace levels of NQO1 because of a homozygous genetic polymorphism (NQO1*2/*2) and were stably transfected with human NQO1 to generate CHO/812 and MDA-468NQ16 cell lines that contain very high levels of NQO1 activity. Our data demonstrated that expression of high levels of NQO1 in cell cytosols resulted in increased scavenging of O_2^-, and the specificity of this reaction for NQO1 was confirmed using ES936, a mechanism-based inhibitor of NQO1. In addition, experiments in CHO cells demonstrated that overexpression of NQO1 resulted in protection against pyrogallol-induced toxicity. These data suggest that high levels of NQO1 protein may compensate for the slower rate of reaction of O_2^- with NQO1 compared with SOD. High levels of NQO1 have been detected by immunohistochemistry in nor-

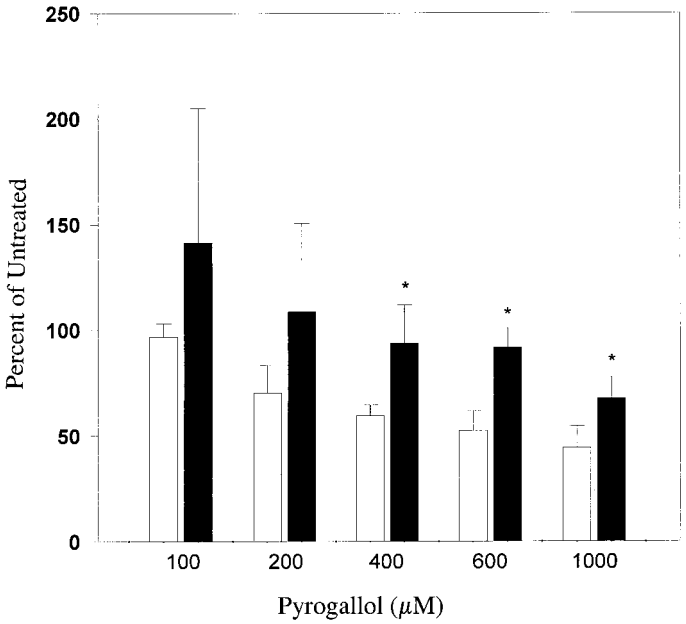


Fig. 8. The effect of NQO1 overexpression on the cytotoxicity of pyrogallol in CHO cells. The cytotoxicity of pyrogallol was assayed in wild-type CHO (CHO and NQO1 null) and CHO cells overexpressing human NQO1 (CHO/812). Open bars, CHO cells; solid bars, CHO/812 cells. *, $p < 0.05$ (paired Student's t test).

mal human tissues naturally exposed to sustained levels of molecular oxygen such as cornea and lens epithelium, vascular and alveolar endothelium, and bronchial epithelium (Siegel et al., 1998; Siegel and Ross, 2000). Based on these data, we suggest that NQO1 may function as an additional enzyme system to SOD for scavenging of $O_2^{\cdot-}$ in cellular systems, and this effect may be particularly marked in cells containing high levels of NQO1.

Consistent with a role for NQO1 in scavenging $O_2^{\cdot-}$, it was shown very recently that treatment of a pancreatic cancer cell line with the NQO1 inhibitor dicumarol increased intracellular $O_2^{\cdot-}$ production and inhibited cell growth and the in vitro malignant phenotype via a superoxide-dependent mechanism (Cullen et al., 2003).

It is conceivable that the absence of NQO1 may result in diminished protection against $O_2^{\cdot-}$ over the lifetime of an individual, leading to increased cell damage and incidence of disease. This is especially relevant to NQO1 because of a common single nucleotide polymorphism (NQO1*2). Expression of the NQO1*2 allele results in a NQO1 protein that has minimal catalytic activity and is rapidly degraded by the ubiquitin/proteasomal system (Traver et al., 1997; Siegel et al., 2001). Individuals with one variant allele have diminished NQO1 activity and protein levels, whereas individuals with two variants alleles have only trace or nondetectable levels of NQO1 protein and activity (Siegel et al., 1999). Increased rates of many cancers including lung, colon, skin, bladder, and leukemias have been found to correlate with the NQO1*2 allele frequency (Schulz et al., 1997; Clairmont et al., 1999; Larson et al., 1999; Lafuente et al., 2000; Lewis et al., 2001). The incidence of other diseases such as Parkinson's disease has also been found to correlate with NQO1*2 allele frequency (Harada et al., 2001).

In summary, we have demonstrated that NQO1 posses NAD(P)H-dependent $O_2^{\cdot-}$ scavenging activity. The reaction of $O_2^{\cdot-}$ and NQO1 is mediated through reduced forms of FAD that can rapidly be regenerated via the oxidation NAD(P)H. The ability of NQO1 to scavenge $O_2^{\cdot-}$ may be one reason for the rapid induction of this enzyme following diverse forms of oxidative stress.

References

Agrawal A, Choudhary D, Upreti M, Rath PC, and Kale RK (2001) Radiation induced oxidative stress: I. Studies in Ehrlich solid tumor in mice. *Mol Cell Biochem* **223:**71–80.

Anwar A, Dehn D, Siegel D, Kepa JK, Tang LJ, Pietenpol JA, and Ross D (2003) Interaction of human NAD(P)H:quinone oxidoreductase 1 (NQO1) with the tumor suppressor protein p53 in cells and cell-free systems. *J Biol Chem* **278:**10368–10373.

Asher G, Lotem J, Cohen B, Sachs L, and Shaul Y (2001) Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1. *Proc Natl Acad Sci USA* **98:**1188–1193.

Beall HD, Mulcahy RT, Siegel D, Traver RD, Gibson NW, and Ross D (1994) Metabolism of bioreductive antitumor compounds by purified rat and human DT-diaphorases. *Cancer Res* **54:**3196–201.

Beall HD, Winski S, Swann E, Hudnott AR, Cotterill AS, O'Sullivan N, Green SJ, Bien R, Siegel D, Ross D, et al. (1998) Indolequinone antitumor agents: correlation between quinone structure, rate of metabolism by recombinant human NAD(P)H: quinone oxidoreductase and in vitro cytotoxicity. *J Med Chem* **41:**4755–4766.

Benson AM, Hunkeler MJ, and Talalay P (1980) Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc Natl Acad Sci USA* **77:**5216–5220.

Beyer RE, Segura-Aguilar J, Di Bernardo S, Cavazzoni M, Fato R, Fiorentini D, Galli MC, Setti M, Landi L, and Lenaz G (1996) The role of DT-diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems. *Proc Natl Acad Sci USA* **93:**2528–2532.

Boothman DA, Meyers M, Fukunaga N, and Lee SW (1993) Isolation of x-ray-inducible transcripts from radioresistant human melanoma cells. *Proc Natl Acad Sci USA* **90:**7200–7204.

Bull C and Fee JA (1985) Steady-state kinetics of superoxide dismutase: properties of the iron containing protein from *Escherichia coli*. *J Am Chem Soc* **107:**3295–3304.

Clairmont A, Sies H, Ramachandran S, Lear JT, Smith AG, Bowers B, Jones PW, Fryer AA, and Strange RC (1999) Association of NAD(P)H:quinone oxidoreductase (NQO1) null with numbers of basal cell carcinomas: use of a multivariate model to rank the relative importance of this polymorphism and those at other relevant loci. *Carcinogenesis* **20:**1235–1240.

Cullen JJ, Hinkhouse MM, Grady M, Gaut AW, Liu J, Zhang YP, Weydert CJ, Domann FE, and Oberley LW (2003) Dicumarol inhibition of NADPH:quinone oxidoreductase induces growth inhibition of pancreatic cancer via a superoxide-mediated mechanism. *Cancer Res* **63:**5513–5520.

Dehn DL, Siegel D, Swann E, Moody CJ, and Ross D (2003) Biochemical, cytotoxic and genotoxic effects of ES936, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, in cellular systems. *Mol Pharmacol* **64:**714–720.

Edwards YH, Potter J, and Hopkinson DA (1980) Human FAD-dependent NAD(P)H diaphorase. *Biochem J* **187:**429–436.

Ernster L (1967) DT-diaphorase. *Methods Enzymol* **10:**309–317.

Faig M, Bianchet MA, Talalay P, Chen S, Winski S, Ross D, and Amzel LM (2000) Structures of recombinant human and mouse NAD(P)H:quinone oxidoreductases: species comparison and structural changes with substrate binding and release. *Proc Natl Acad Sci USA* **97:**3177–3182.

Gustafson DL, Beall HD, Bolton EM, Ross D, and Waldren CA (1996) Expression of human NAD(P)H: quinone oxidoreductase (DT-diaphorase) in Chinese hamster ovary cells: effect on the toxicity of antitumor quinones. *Mol Pharmacol* **50:**728–735.

Harada S, Fujii C, Hayashi A, and Ohkoshi N (2001) An association between idiopathic Parkinson's disease and polymorphisms of phase II detoxification enzymes: glutathione *S*-transferase M1 and quinone oxidoreductase 1 and 2. *Biochem Biophys Res Commun*. **288:**887–892.

Huggins C and Fukunishi R (1964) Induced protection of adrenal cortex against 7,12-dimethylbenz(alpha)anthracene: influence of ethionine: induction of menadione reductase: incorporation of thymidine H3. *J Exp Med* **119:**923–942.

Lafuente MJ, Casterad X, Trias M, Ascaso C, Molina R, Ballesta A, Zheng S, Wiencke JK, and Lafuente A (2000) NAD(P)H:quinone oxidoreductase-dependent risk for colorectal cancer and its association with the presence of K-ras mutations in tumors. *Carcinogenesis* **21:**1813–1819.

Larson RA, Wang Y, Banerjee M, Wiemels J, Hartford C, Le Beau MM, and Smith MT (1999) Prevalence of the inactivating 609C→T polymorphism in the NAD(P)H: quinone oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. *Blood* **94:**803–807.

Lewis SJ, Cherry NM, Niven RM, Barber PV, and Povey AC (2001) Polymorphisms in the NAD(P)H: quinone oxidoreductase gene and small cell lung cancer risk in a UK population. *Lung Cancer* **34:**177–183.

Lind C, Cadenas E, Hochstein P, and Ernster L (1990) DT-diaphorase: purification, properties and function. *Methods Enzymol* **186:**287–301.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Makino N, Mochizuki Y, Bannai S, and Sugita Y (1994) Kinetic studies on the removal of extracellular hydrogen peroxide by cultured fibroblasts. *J Biol Chem* **269:**1020–1025.

Marklund S and Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* **47:**469–474.

Massey V, Palmer G, and Ballou D (1973) *Oxidases and Related Redox Systems*, vol I, pp 25–49, Baltimore University Park Press, Baltimore, MD.

McClune GJ and Fee JA (1976) Stopped flow spectrophotometric observation of superoxide dismutation in aqueous solution. *FEBS Lett* **67:**294–298.

Muller F (1987) Flavin radicals: chemistry and biochemistry. *Free Radic Biol Med* **3:**215–230.

Ollinger K, Buffinton GD, Ernster L, and Cadenas E (1990) Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chem Biol Interact* **73:**53–76.

Polyak K, Xia Y, Zweier JL, Kinzler KW, and Vogelstein B (1997) A model for p53-induced apoptosis. *Nature (Lond)* **389:**300–305.

Reiter CD, Teng RJ, and Beckman JS (2000) Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* **275:**32460–32466.

Roberts JJ, Marchbank T, Kotsaki-Kovatsi VP, Boland MP, Friedlos F and Knox RJ (1989) Caffeine, aminoimidazolecarboxamide and dicoumarol, inhibitors of NAD(P)H dehydrogenase (quinone) (DT diaphorase), prevent both the cytotoxicity and DNA interstrand crosslinking produced by 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) in Walker cells. *Biochem Pharmacol* **38:**4137–4143.

Ross D, Kepa JK, Winski SL, Beall HD, Anwar A, and Siegel D (2000) NAD(P)H: quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem Biol Interact* **129:**77–97.

Sasaki K, Bannai S, and Makino N (1998) Kinetics of hydrogen peroxide elimination by human umbilical vein endothelial cells in culture. *Biochim Biophys Acta* **1380:**275–288.

Schulz WA, Krummeck A, Rosinger I, Eickelmann P, Neuhaus C, Ebert T, Schmitz-Drager BJ, and Sies H (1997) Increased frequency of a null-allele for NAD(P)H: quinone oxidoreductase in patients with urological malignancies. *Pharmacogenetics* **7:**235–239.

Siegel D, Anwar A, Winski SL, Kepa JK, Zolman KL, and Ross D (2001) Rapid polyubiquitination and proteasomal degradation of a mutant form of NAD(P)H: quinone oxidoreductase 1. *Mol Pharmacol* **59:**263–268.

Siegel D, Bolton EM, Burr JA, Liebler DC, and Ross D (1997) The reduction of alpha-tocopherolquinone by human NAD(P)H: quinone oxidoreductase: the role of α-tocopherolhydroquinone as a cellular antioxidant. *Mol Pharmacol* **52:**300–305.

Siegel D, Franklin WA, and Ross D (1998) Immunohistochemical detection of NAD-

(P)H:quinone oxidoreductase in human lung and lung tumors. *Clin Cancer Res* **4:**2065–2070.
Siegel D, McGuinness SM, Winski SL, and Ross D (1999) Genotype-phenotype relationships in studies of a polymorphism in NAD(P)H:quinone oxidoreductase. *Pharmacogenetics* **9:**113–121.
Siegel D and Ross D (2000) Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. *Free Radic Biol Med* **29:**246–253.
Strassburg A, Strassburg CP, Manns MP, and Tukey RH (2002) Differential gene expression of NAD(P)H:quinone oxidoreductase and NRH:quinone oxidoreductase in human hepatocellular and biliary tissue. *Mol Pharmacol* **61:**320–325.
Tedeschi G, Chen S, and Massey V (1995a) DT-diaphorase: redox potential, steady-state and rapid reaction studies. *J Biol Chem* **270:**1198–1204.
Tedeschi G, Deng PS, Chen HH, Forrest GL, Massey V, and Chen S (1995b) A site-directed mutagenesis study at Lys-113 of NAD(P)H:quinone-acceptor oxidoreductase: an involvement of Lys-113 in the binding of the flavin adenine dinucleotide prosthetic group. *Arch Biochem Biophys* **321:**76–82.
Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, and Orrenius S (1982) The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes: a study of the implications of oxidative stress in intact cells. *J Biol Chem* **257:** 12419–12425.
Traver RD, Siegel D, Beall HD, Phillips RM, Gibson NW, Franklin WA, and Ross D (1997) Characterization of a polymorphism in NAD(P)H: quinone oxidoreductase (DT-diaphorase). *Br J Cancer* **75:**69–75.
Wilson MW, Schelonka LP, Siegel D, Meininger A, and Ross D (2001) Immunohistochemical localization of NAD(P)H:quinone oxidoreductase in conjunctival melanomas and primary acquired melanosis. *Curr Eye Res* **22:**348–352.
Winski SL, Faig M, Bianchet MA, Siegel D, Swann E, Fung K, Duncan MW, Moody CJ, Amzel LM, and Ross D (2001) Characterization of a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1 by biochemical, X-ray crystallographic and mass spectrometric approaches. *Biochemistry* **40:**15135–15142.
Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, and Kalyanaraman B (2003) Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* **34:**1359–1368.

Address correspondence to: Dr. David Ross, Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, 4200 East 9th Ave., Denver, CO 80262. E-mail: david.ross@uchsc.edu