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INHIBITION OF NAD(P)H:QUINONE OXIDOREDUCTASE$_1$ IN ETHACRYNIC ACID-RESISTANT HUMAN COLON CARCINOMA CELLS

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Abstract—Human colon carcinoma HT29 cells sensitive (WT) and resistant (HT/M and HT/S) to ethacrynic acid (EA) were used to investigate the role of NAD(P)H:quinone oxidoreductase$_1$ (NQO$_1$) in drug resistance. Significant decreases in the levels of NQO$_1$ activity were observed in resistant cells as compared with the sensitive cells. However, the decreased activities of NQO$_1$ in resistant cells were found to be due to inhibition of the enzyme by EA. Human NQO$_1$ cDNA-derived protein in monkey kidney COS1 cell extract was used to demonstrate that *in vitro* inhibition of NQO$_1$ activity by EA was rapid, reversible and concentration dependent, with an IC_{50} value of 250 μM. These results suggest that NQO$_1$ may not have a role in EA resistance of human colon carcinoma HT29 cells and that EA is an inhibitor of NQO$_1$ activity.

Key words: colon carcinoma cells; drug resistance; ethacrynic acid; NAD(P)H:quinone oxidoreductase$_1$

Drug resistance, either intrinsic or acquired, severely limits the effectiveness of chemotherapy. The development and maintenance of drug resistance are associated with numerous biochemical and genetic alterations [reviewed in Ref. 1]. Detoxifying enzymes, such as glutathione *S*-transferases, have been implicated in the resistant phenotype of the cells [2, 3]. An increase in the levels of glutathione *S*-transferase π subunit transcript is reported to be one of the factors responsible for resistance to EA† in subpopulations of HT29 human colon carcinoma cells [4]. The role of detoxifying enzymes in drug resistance has been emphasized because several genes including glutathione *S*-transferase and NQO$_1$, also known as DT diaphorase (EC 1.6.99.2), are expressed at much higher levels in tumor cells than in normal cells of the same origin [5–7]. The significance of elevated glutathione *S*-transferase activity in tumor cells with respect to drug resistance is relatively well understood [8]. However, information regarding the role of elevated NQO$_1$ activity in drug-resistant tumor cells is scant. NQO$_1$ catalyzes two-electron reductions of various quinones to the stable, relatively less reactive hydroquinones and thus protects the system against mutagenicity and toxicity of semiquinones and oxygen free radicals that might be generated by the action of one-electron reducing enzymes such as cytochrome P450 reductase [9].

We have cloned and sequenced the cDNA encoding human 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible cytosolic NQO$_1$ [10]. Subsequently, the NQO$_1$ cDNA-derived protein in monkey kidney COS1 cells was shown to be identical to that of human DT diaphorase [11]. It has been clearly established that cDNA-expressed protein in COS1 cells could replace the purified protein for studies on its kinetics and role in drug metabolism [11, 12]. This is mainly because COS1 cells deficient in NQO$_1$ activity express more than a 1000-fold increase in active NQO$_1$ enzyme upon transfection with NQO$_1$ cDNA [11]. In the present report, we demonstrated that human colon carcinoma cells resistant to EA had significantly lower levels of NQO$_1$ activity as compared with sensitive cells. In addition, we showed that the decrease in NQO$_1$ in resistant cells was due to inhibition of the enzyme activity by EA. Human NQO$_1$ cDNA-derived protein in monkey kidney COS1 cells also was used to demonstrate that EA inhibition of NQO$_1$ activity was rapid, reversible and concentration dependent.

MATERIALS AND METHODS

EA-sensitive and -resistant cell lines

EA-sensitive (WT) and -resistant (HT/M and HT/S) human colon carcinoma HT29 cells were provided by Dr. K. D. Tew, Fox Chase Cancer Center, Philadelphia. The development of resistant cells has been described previously [4]. Subpopulations of HT29 cells resistant to EA were selected with or

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† Trivial names and abbreviations: EA, ethacrynic acid; NQO$_1$, the dioxin-inducible cytosolic form of NAD(P)H:quinone oxidoreductase (EC 1.6.99.2, also known as DT diaphorase and abbreviated by others as NMO1, NMOR, QR and QAO); WT, ethacrynic acid-sensitive human colon carcinoma HT29 cells; HT/M, ethacrynic acid-resistant human colon carcinoma HT29 cells. These cells were selected in ethacrynic acid after treatment with the mutagen ethyl methanesulfonate; HT/S, ethacrynic acid-resistant human colon carcinoma HT29 cells (one-step selection in ethacrynic acid); and DCPIP, 2,6-dichlorophenolindophenol.

without prior exposure to the mutagen ethyl methanesulfonate [4] and designated as HT/M or HT/S, respectively. Selection of resistant phenotype was done in medium containing 72 μM EA. Both clones displayed 2- to 3-fold resistance to the selection agent and required its constant presence for maintenance of the resistant phenotype [4].

Cell culture

Human colon carcinoma cell lines sensitive to EA (WT) were cultured in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U penicillin/mL and 50 μg streptomycin/mL. The cells were grown at 37° under a humidified atmosphere with 5% CO_2. The EA-resistant cells (HT/M and HT/S) were grown in a similar medium containing 72 μM EA. Monkey kidney COS1 cells were also maintained under similar experimental conditions except that Dulbecco's Modified Eagle's Medium (DMEM) was used as the growth medium.

Ethacrynic acid treatment

The sensitive cells (WT) were treated with 72 μM EA for 24 hr. In a related experiment, the resistant cells (HT/M and HT/S) were treated with medium that did not contain EA for 24 hr. After the incubation period, the cells were washed, scraped, and homogenized.

NQO_1 enzyme activity

The colon carcinoma cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline without calcium and magnesium, scraped and collected by centrifugation at 800 *g* for 5 min. The cell pellets were homogenized in 0.25 M sucrose containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to a protein concentration of 1–5 mg/mL. The cell homogenates were centrifuged at 14,000 rpm in an Eppendorf centrifuge at 4° for 15 min, and the supernatant was removed for enzyme analysis.

NQO_1 activity was determined by previously described procedures [13, 14]. The final assay mixture contained 25 mM Tris–HCl, pH 7.4, 0.18 mg/mL BSA, 5 μM FAD, 0.01% Tween 20, 200 μM NADH and 50 μM 2,6-dichlorophenolindophenol (DCPIP). The reaction rate was monitored in a Beckman spectrophotometer by measuring the decrease in absorbance at 600 nm due to reduction of the substrate and oxidation of NADH at 340 nm. NQO_1 activity in the 14,000 rpm supernatants of HT29 cell homogenates was determined in the absence and presence of 10 μM dicoumarol, a highly potent inhibitor of cytosolic NQO_1 activity [13]. The 14,000 rpm supernatant fraction was incubated with dicoumarol for 2 min prior to enzyme assay.

Transfection of COS1 cells with NQO_1 cDNA

COS1 cells in culture were transiently transfected with cDNA for human NQO_1 by the DEAE-Dextran and chloroquine method, as described previously [11]. Stated briefly, COS1 cells grown as a monolayer to approximately 70% confluency in 100 mm petri dishes were allowed to grow for about 3 hr in fresh DMEM medium. The medium was removed by aspiration, and 4 mL of a solution containing 10 μg of NQO_1 cDNA, 0.1 M Tris–HCl (pH 7.6), and 2.5 mg/mL DEAE-Dextran was poured onto the cells. After an incubation period of 8 hr at 37°, the medium was removed, and the cells were washed once with fresh medium. Four milliliters of DMSO shock medium (10%, v/v) was added to the cells for 1.5 min followed by 5 mL of DMEM containing 100 μM chloroquine for 2 hr. After 2 hr, the medium was removed and the cells were washed twice with fresh medium. Fresh DMEM (10 mL) was added to the cells, and the cells were grown for 72 hr prior to harvesting for determination of protein concentration and NQO_1 activity.

In vitro *effect of ethacrynic acid on cDNA-derived NQO_1 activity*

After transfection with NQO_1 cDNA, COS1 cells were washed, scraped and collected by centrifugation as described for the colon carcinoma cells. The transfected COS1 cells were homogenized in 0.25 M sucrose containing 0.1 mM PMSF to a protein concentration of 2–5 mg/mL. The homogenates were centrifuged at 100,000 *g* for 1 hr, and the resulting supernatants (cytosols) containing human NQO_1 cDNA-derived protein were used in all of the following experiments to determine the kinetics of ethacrynic acid inhibition of NQO_1 activity.

Time and concentration-dependent inhibition of NQO_1 activity. To determine the optimum time required to inhibit NQO_1 activity by EA, the inhibitor at a final concentration of 500 μM was mixed with COS1 cell cytosol (2 μg protein) and incubated for time intervals ranging from 0 min to 4 hr. At the end of the incubation period, the reaction was started by the addition of all the required cofactors and substrate, and the reduction of DCPIP was followed spectrophotometrically. Similarly, increasing concentrations of EA ranging from 3.125 μM to 4 mM were mixed with the cytosol and incubated in the assay buffer for 2 min, and the disappearance rate of DCPIP was followed.

Reversibility of inhibition. To determine if the inhibition of NQO_1 activity by EA is reversible, 500 μL of COS1 cell cytosol (12.5 μg protein) was incubated with 5 mM EA at room temperature for 15 min. At the end of the incubation period, aliquots were withdrawn for determination of NQO_1 activity. The remaining cytosol was dialyzed against 2 L of 50 mM Tris buffer (pH 7.4) for 14 hr in the cold. At the end of the dialysis period, NQO_1 activity was determined in various samples. Appropriate control experiments without the inhibitor were also conducted concurrently.

RESULTS

The structure of ethacrynic acid (EA) is shown in Fig. 1. The EA-resistant cells (HT/M and HT/S) showed lower levels of total and dicoumarol-inhibitable NQO_1 activities than did the sensitive (WT) cells (Fig. 2). The decrease in NQO_1 levels in the resistant cells amounted to 30–50% of the activity in the sensitive cells. Interestingly, the treatment of sensitive (WT) cells with 72 μM EA (the concentration required to maintain the resistant phenotype of HT/M and HT/S cells) resulted in an

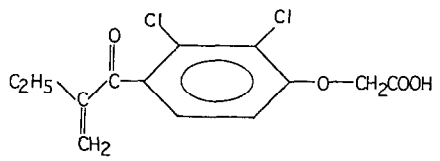

Fig. 1. Chemical structure of ethacrynic acid.

approximately 50% decrease in NQO_1 activity (Fig. 3). Increasing the EA concentration in the medium to more than 72 μM for treatment of sensitive (WT) cells did not result in a further decrease in NQO_1 activity (data not shown). In a related experiment, the use of normal medium instead of medium containing EA to culture the resistant colon carcinoma cells (HT/M and HT/S) increased the NQO_1 level to that of the sensitive (WT) cells (Fig. 3).

We used the COS1 cell system [11] to express NQO_1 cDNA in order to study the EA inhibition of NQO_1 activity. The protein and activity expressed from NQO_1 cDNA into COS1 cells have been characterized earlier [11]. COS1 cells transfected with human NQO_1 cDNA produced large amounts of cDNA-derived NQO_1 activity (Table 1). EA treatment of COS1 cell cytosolic extract (100,000 g supernatant) containing cDNA-derived NQO_1 protein caused significant inhibition of NQO_1 activity (Figs. 4 and 5). The inhibition of NQO_1 activity by EA did not increase with increasing incubation time from 0 to 240 min (Fig. 4), and was concentration

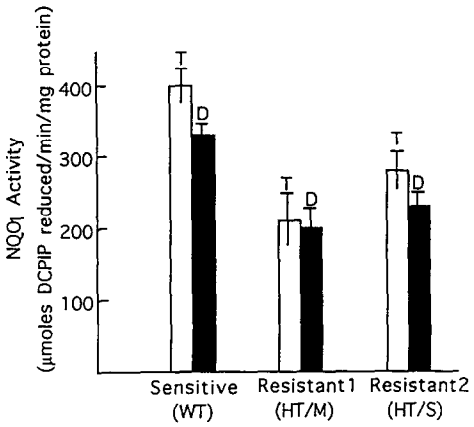

Fig. 2. NQO_1 activity in ethacrynic acid-sensitive and -resistant human colon carcinoma cell lines. EA-sensitive (WT) and -resistant (HT/M and HT/S) human colon carcinoma cells were grown in tissue culture flasks. The resistant cells were grown in the presence of 72 μM EA to maintain the resistant phenotype. After 80% confluency, cells were harvested and analyzed for total (T) and dicoumarol-inhibitable (D) NQO_1 activities. DCPIP = 2,6-dichlorophenolindophenol. Data are the means ± SEM of five independent experiments.

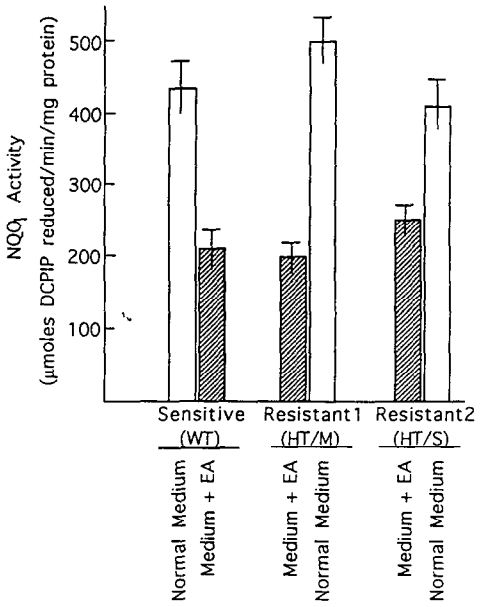

Fig. 3. Effect of ethacrynic acid on NQO_1 activity in sensitive and resistant human colon carcinoma cells. Sensitive (WT) human colon carcinoma HT29 cells were treated with 72 μM EA (medium + EA) or solvent to dissolve EA (ethanol:water, 1:1, v/v) (normal medium) for 24 hr prior to measuring the activity of the NQO_1 enzyme. Resistant cells were grown either in medium containing 72 μM EA or in normal medium for 24 hr before measuring the NQO_1 activity. Data represent the means ± SEM of five different experiments.

Table 1. Expression of human NAD(P)H:quinone oxidoreductase$_1$ cDNA in monkey kidney COS1 cells

COS1 cells	NQO_1 activity (μmol DCPIP reduced/min/mg protein)
Untransfected	0.036 ± 0.002
Transfected	34.66 ± 1.18

NQO_1 activity was assayed by procedures described in Materials and Methods. Values are means ± SEM of three separate transfections.

dependent (Fig. 5). Approximately 85% of the NQO_1 activity was inhibited by a 4 mM concentration of EA. Dialysis of the cytosol incubated with EA restored NQO_1 enzyme activity to 80% of the control activity (Fig. 6).

DISCUSSION

Drug resistance is of great concern in modern medicine. Numerous biochemical alterations, including altered levels of glutathione *S*-transferases, have been shown to be associated with drug resistance [1–3, 8]. Recently, a 2- to 3-fold increase in resistance of human colon carcinoma cells to EA was shown

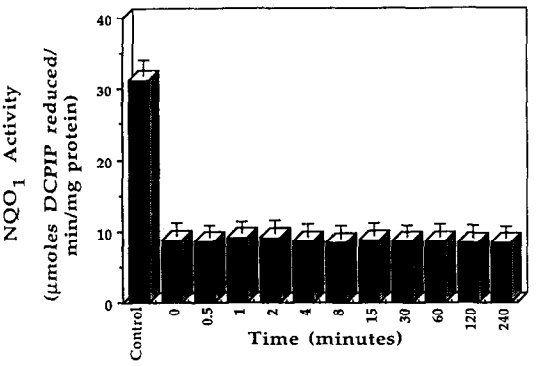

Fig. 4. Time-course of inhibition of NQO_1 activity by ethacrynic acid. Cytosol (100,000 *g* supernatant) of COS1 cells transfected with cDNA for NQO_1 was mixed and incubated with EA at a final concentration of 500 μM. NQO_1 activity was determined at different time intervals by procedures described in Materials and Methods. Results are means ± SEM of three different transfections.

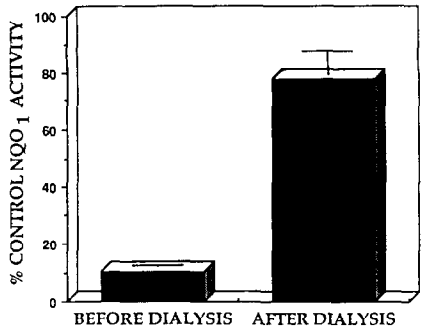

Fig. 6. Effect of dialysis on the inhibition of NQO_1 activity by ethacrynic acid. Five hundred microliters of cytosol (100,000 *g* supernatant) of COS1 cells transfected with cDNA for NQO_1 was incubated with 10 mM EA for 15 min. The cytosol was dialyzed against 25 mM Tris buffer (pH 7.4) for 14 hr at 4°. NQO_1 activity was determined before and after dialysis, and the results are expressed as percent of control activities (mean ± SEM) of three incubations. Control activities were 32.45 ± 2.65 μmol of DCPIP reduced/min/mg protein (mean ± SEM).

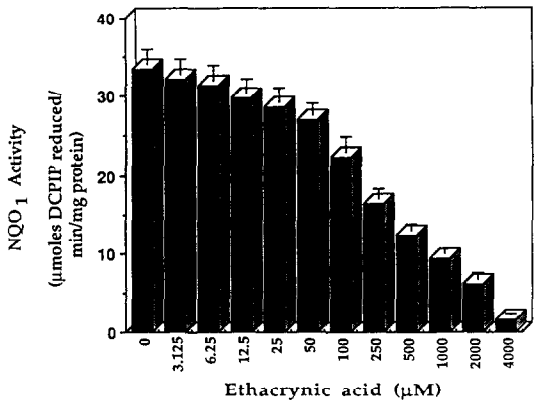

Fig. 5. Concentration-dependent inhibition of NQO_1 activity by ethacrynic acid. Cytosol (100,000 *g* supernatant) of COS1 cells transfected with cDNA for human NQO_1 was incubated at room temperature for 2 min with ethacrynic acid at final concentrations as indicated. NQO_1 activity was determined spectrophotometrically at the end of the incubation periods, as described in Materials and Methods. Results are means ± SEM of three different transfections.

to be related to an increase in glutathione *S*-transferase π transcript [4]. Because of several reasons, NQO_1, like glutathione *S*-transferase is expected to play an important role in drug resistance. Both NQO_1 and glutathione *S*-transferase genes are expressed at much higher levels in various tumor tissues than in normal tissues of the same origin [5–7]. Also, both of these genes are regulated by similar mechanisms that involve the antioxidant response element (ARE) and products of the proto-oncogenes Jun and Fos and the xenobiotic response element (XRE) and the Ah receptor [reviewed in Refs. 15 and 16]. Both NQO_1 and glutathione *S*-transferases have been shown to detoxify as well as activate drugs and xenobiotics [9]. The differences in the sensitivity of Chinese hamster V79 and Walker 256 rat carcinoma cells to the monofunctional alkylating agent CB1954 have been attributed to differences in NQO_1 activities [17]. A selected subpopulation of the same Walker cells expresses resistance to a broad range of nitrogen mustards and thus has been linked to an elevated expression of the Yc (α family) subunit of glutathione *S*-transferase [18, 19]. Similarly, treatment of the Walker cells with EA sensitizes them to bifunctional nitrogen mustards through an inhibition of glutathione *S*-transferase and a concomitant depletion of glutathione (GSH) [20]. We used EA-sensitive (WT) and -resistant (HT/M and HT/S) human colon carcinoma cells to investigate the role of NQO_1 in EA resistance.

Significantly decreased levels of NQO_1 activities were observed in EA-resistant colon carcinoma cells (HT/M and HT/S) as compared with sensitive (WT) cells. These results are different from those reported for glutathione *S*-transferase, whose activity is increased in EA-resistant colon carcinoma cells [4]. Several observations indicate that the decreased levels of NQO_1 activity observed in EA-resistant colon carcinoma cells are due to inhibition of enzyme activity by EA and are not associated with drug resistance. These include inhibition of NQO_1 activity in WT cells by EA (Fig. 3), loss of inhibition of NQO_1 activity in resistant cells when grown in medium without EA (Fig. 3), and inhibition of cDNA-derived NQO_1 activity by EA (Figs. 4 and 5).

The inhibition of NQO_1 activity by EA was not due to its interference in the activity assay because two different assay procedures (reduction of DCPIP at 600 nm and oxidation of NADH at 340 nm) revealed similar results (data on oxidation of NADH are not shown). It is noteworthy that only 40–50%

of the total NQO_1 activity was inhibited in EA-resistant cells (Fig. 2) and WT colon carcinoma cells treated with EA (Fig. 3). The remaining NQO_1 activity seen in cytosolic extracts of these cells may be because of the reversal of inhibition of the enzyme due to dilution of EA during homogenization. In addition, a part of the remaining activity may also be due to activities of enzymes other than NQO_1. *In vitro* studies with cytosolic extracts from COS1 cells transfected with NQO_1 cDNA clearly demonstrated that inhibition of the NQO_1 activity by EA was rapid, reversible and concentration dependent with an IC_{50} value of 250 μM. The NQO_1 enzyme is known to detoxify quinones and protect the cells against their cytotoxicity [13]. Therefore, the discovery of EA as an inhibitor of NQO_1 activity could have an impact on its use as a diuretic agent.

In conclusion, NQO_1 does not seem to play a role in EA resistance of human colon carcinoma cells, although it is an inhibitor of NQO_1.

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