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A Novel Plasma Membrane Quinone Reductase and NAD(P)H:Quinone Oxidoreductase 1 are Upregulated by Serum Withdrawal in Human Promyelocytic HL-60 Cells

Nathalie Forthoffer,[1] Consuelo Gómez-Díaz,[1,2] Rosario I. Bello,[1] María I. Burón,[1] Sergio F. Martín,[1,3] Juan C. Rodríguez-Aguilera,[1,2] Plácido Navas,[1,2] and José M. Villalba[1,4]

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We have studied changes in plasma membrane NAD(P)H:quinone oxidoreductases of HL-60 cells under serum withdrawal conditions, as a model to analyze cell responses to oxidative stress. Highly enriched plasma membrane fractions were obtained from cell homogenates. A major part of NADH-quinone oxidoreductase in the plasma membrane was insensitive to micromolar concentrations of dicumarol, a specific inhibitor of the NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), and only a minor portion was characterized as DT-diaphorase. An enzyme with properties of a cytochrome b_5 reductase accounted for most dicumarol-resistant quinone reductase activity in HL-60 plasma membranes. The enzyme used mainly NADH as donor, it reduced coenzyme Q_0 through a one-electron mechanism with generation of superoxide, and its inhibition profile by *p*-hydroxymercuribenzoate was similar to that of authentic cytochrome b_5 reductase. Both NQO1 and a novel dicumarol-insensitive quinone reductase that was not accounted by a cytochrome b_5 reductase were significantly increased in plasma membranes after serum deprivation, showing a peak at 32 h of treatment. The reductase was specific for NADH, did not generate superoxide during quinone reduction, and was significantly resistant to *p*-hydroxymercuribenzoate. The function of this novel quinone reductase remains to be elucidated whereas dicumarol inhibition of NQO1 strongly potentiated growth arrest and decreased viability of HL-60 cells in the absence of serum. Our results demonstrate that upregulation of two-electron quinone reductases at the plasma membrane is a mechanism evoked by cells for defense against oxidative stress caused by serum withdrawal.

KEY WORDS: Cell death; NQO1 (DT-diaphorase); oxidative stress; plasma membrane; quinone reductase; serum-withdrawal.

INTRODUCTION

Quinone reductases are flavoenzymes that catalyze the transfer of two electrons from cellular donors (mainly NADH or NADPH) to a variety of quinone acceptors. According to mechanisms by which quinones become reduced, these enzymes can be classified into two categories. In reactions catalyzed by the so-called *one-electron quinone reductases*, such as the microsomal enzymes cytochrome b_5 and cytochrome P450 reductases, two electrons are sequentially donated with the concomitant generation of semiquinones (Nakamura and Hayashi, 1994). These compounds react readily with molecular oxygen to generate superoxide and other highly reactive oxygen species (ROS) that can cause oxidative stress and damage to cellular structures (Joseph and Jaiswal, 1994). On the other hand, cells also contain *two-electron quinone reductases* which are mainly cytosolic enzymes such as NAD(P)H:(quinone acceptor) oxidoreductase 1 (NQO1,

[1] Departamento de Biología Celular, Fisiología e Inmunología, Facultad de Ciencias, Universidad de Córdoba, Campus Rabanales, Edificio C-6, 3ª planta, 14014 Córdoba, Spain.
[2] Present address: Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, Sevilla, Spain.
[3] Present address: Department of Biomedical Engineering, School of Medicine, Baltimore, Maryland.
[4] To whom correspondence should be addressed; e-mail: bc1vimoj@uco.es.

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DT-diaphorase) (Cadenas, 1995; Jaiswal, 2000a). Reactions catalyzed by these enzymes are characterized by their obligatory two-electron reaction mechanism, resulting in a direct hydride transfer to a variety of quinone substrates to give their corresponding hydroquinones (Faig *et al.*, 2000; Li *et al.*, 1995). Some hydroquinones are unstable and can undergo further redox cycling to generate ROS whereas, many hydroquinones are relatively stable, and thus two-electron quinone reductases are generally regarded as antioxidant enzymes (Cadenas, 1995). Accordingly, recent reports have indicated that NQO1 activity maintains the reduced states of ubiquinones (Beyer *et al.*, 1996; Landi *et al.*, 1997; Navarro *et al.*, 1998) and α-tocopherolquinone (Siegel *et al.*, 1997) thereby promoting their antioxidant function in membranes.

Because the plasma membrane is the initial site of attack of extracellular oxidants, those redox systems able to regenerate antioxidants at this location are of extreme importance, because they could protect the intracellular components from oxidant damage. In this way, a plasma membrane ascorbate free-radical reductase (Villalba *et al.*, 1993) has been recently recognized as an efficient mechanism to preserve ascorbate in a location where it can recycle α-tocopherol and thus prevent lipid peroxidation (May *et al.*, 1998, 2001).

Previous results obtained in our laboratory have documented changes of several quinone reductases in plasma membranes under conditions of chronic oxidative stress. Liver plasma membranes from vitamin E and selenium-deficient rats contained elevated levels of cytochrome b_5 reductase and, during establishment of deficiency, similar induction patterns were observed for both this enzyme and ubiquinone present in this membrane. More remarkably, a dramatic increase in NQO1 activity associated to the plasma membrane occurs in liver cells from vitamin E and selenium-deficient animals (Navarro *et al.*, 1998, 1999). Also, significant increases in soluble and membrane-bound DT-diaphorase were found in liver of rats fed with a diet deficient in selenium (Olsson *et al.*, 1993). The increase of quinone reductases at the plasma membrane may lead to higher levels of ubiquinols in the presence of NAD(P)H, and thus account for enhanced antioxidant protection (Navarro *et al.*, 1998).

Several lines of evidence support important roles for quinone reductases as regulators of signaling cascades which control cell growth and death (Asher *et al.*, 2001; Bello *et al.*, in press; Collin *et al.*, 2001; Cross *et al.*, 1999; Gaikwad *et al.*, 2001; Siemankowski *et al.*, 2000). Furthermore, the control of quinone/hydroquinone ratio by plasma membrane quinone reductases could play important regulatory roles in some models of oxidative stress-mediated apoptopsis, such as cell death induced by serum withdrawal (Martín *et al.*, 2001; Villalba *et al.*, 2000, 2001). However, very little is known about the response of these enzymes to serum deprivation. The aim of this work was to study quinone reductase activities in plasma membranes from HL-60 cells under control (10% serum) and serum removal conditions, as a model for oxidative stress-related apoptosis (Slater *et al.*, 1996). Our results have shown that the bulk of quinone reductase present in plasma membranes from control cells is resistant to the NQO1 inhibitor dicumarol, and it can be attributed to a cytochrome b_5 reductase. Serum-withdrawal induces a significant increase in the amount of two-electron quinone reductases at the plasma membrane, such as NQO1 and a novel dicumarol-resistant NADH-specific quinone reductase, which can be distinguished from the cytochrome b_5 reductase. NQO1 activity is required to sustain growth and viability of HL-60 cells under serum deprivation conditions.

MATERIALS AND METHODS

Culture Conditions

HL-60, a human promyelocytic cell line, was cultured in RPMI-1640 medium (BioWhittaker, U.S.A.) supplemented with 10% fetal calf serum (FCS, Linus, Europe), 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Sigma, Spain), and 2 mM of L-Glutamine (Sigma, Spain) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For serum-withdrawal experiments, cells were concentrated from stock cultures by centrifugation at $1000 \times g$ for 5 min, washed twice in serum-free RPMI-1640 medium and then cultured in the same medium without FCS. Stock solutions of the NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) inhibitor dicumarol (2 mM) were prepared in 6 mM NaOH and added to cells to a final concentration of 5 μM. The same amount of vehicle was added to controls. Cell viability was determined by the Trypan blue exclusion method.

Preparation of Cytosolic Fractions

All procedures were carried out at 4°C. About 10^7 cultured cells were concentrated by centrifugation at $1000 \times g$ for 5 min and washed with cold 130 mM Tris-HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, and 1 mM PMSF. Cells were centrifuged again and resuspended in 1 mL of hypotonic lysis buffer (10 mM Tris-HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, 1 mM

PMSF, and 20 μg/μL each of chymostatin, leupeptin, antipain, and pepstatin A (CLAP)). Homogenization of cells was carried out for 5 min with the aid of a glass-glass potter and then for 30 s. with a mechanical cell homogenizer. After disruption of cells, the concentration of the lysis buffer was raised to 100 mM Tris by adding enough volume of 250 mM Tris buffer, pH 7.6 containing 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF, and CLAP. Unbroken cells and debris were separated by centrifugation at $800 \times g$ for 5 min and the supernatant was saved. Cytosolic fractions were separated from membranous material by ultracentrifugation at $100{,}000 \times g$ for 30 min.

Preparation of Plasma Membrane Fractions

All steps were carried out at 4°C. Briefly, about 5×10^8 cultured cells were disrupted as described above in 4 mL of hypotonic buffer. After homogenization, 4 mL of hypertonic buffer were added to restore osmotic conditions. Nuclei and unbroken cells were then discarded by a 10 min centrifugation at $1000 \times g$ and the supernatants so obtained were centrifuged at $40{,}000 \times g$ for 1 h to obtain a fraction of crude membranes. Plasma membranes were then isolated from crude membranes by the aqueous two-phase partition method in dextran and polyethylene glycol (Albertsson *et al.*, 1982). Briefly, the pellet of crude membranes was resuspended in cold water and mixed with 6.6% (w/w) dextran T-500 (Pharmacia, Sweden), 6.6% (w/w) poly(ethylene glycol) 3350 (PolySciences, U.S.A.), 0.25 M sucrose and 5 mM potassium phosphate (pH 7.2). After 5 min centrifugation at $750 \times g$, plasma membrane-enriched upper phase was washed with 1 mM bicarbonate and collected by centrifugation at $40{,}000 \times g$. Plasma membranes were resuspended in 50 mM Tris-HCl, pH 7.6, containing 10% glycerol, 1 mM PMSF and 1 mM DTT, and stored at −80°C until needed. Purity of plasma membrane fractions was checked by immunoblotting techniques using a battery of marker antibodies specific for different endomembranes and the plasma membrane (see below).

Polyacrylamide Gel Electrophoresis and Western Blotting

Whole HL-60 cells or isolated plasma membranes were denatured in SDS/DTT loading buffer (60 mM Tris-HCl, pH 6.8, 0.1 mg/mL bromophenol blue, 10% (w/v) sucrose, 2 mM EDTA, 20 mM DTT, and 1.5% SDS). Heating of the samples in loading buffer was limited to 42°C for 10 min to avoid the aggregation of membrane proteins at higher temperatures. After denaturation, proteins (30 μg) were separated by SDS-PAGE on 10 or 12.5% acrylamide gels and electrotransferred to sheets of nitrocellulose (Millipore, U.S.A.). Quantitative transfer to nitrocellulose membranes was checked by Poceau-S staining of total protein in lanes. Protein determinations were carried out by the dye-binding method described by Stoscheck (1990) using bovine γ-globulin as standard.

Immunostaining and Quantification of Antibody Binding

Prior to staining, nitrocellulose membranes were blocked in 25 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 0.03% Tween 20, and 2% skimmed milk for 30 min and then incubated with the corresponding primary antibody diluted in blocking buffer. The following marker primary antibodies were used: rabbit antihuman Calnexin (StressGen Biotech., Victoria, Canada) as marker for endoplasmic reticulum; mouse antihuman KDEL receptor (StressGen Biotech., Victoria, Canada) as marker for Golgi apparatus membranes; mouse antihuman cytochrome *c* oxidase (RDI Research Diagnostics, Flanders, NJ) as marker for mitochondria, and rabbit antipig Na^+/K^+ ATPase α-subunit (Villalba *et al.*, 1994) as marker for the plasma membrane. Commercial marker antibodies were employed according to general reccomendations of the manufacturers. Rabbit antipig Na^+/K^+ ATPase α-subunit was used at a 1:1000 dilution. Detection of cytochrome b_5 reductase in membranes was accomplished by staining with a specific polyclonal antiserum against pig liver cytochrome b_5 reductase at a 1:1000 dilution (Navarro *et al.*, 1995).

After incubation with the corresponding primary antibody (2–4 h at room temperature), nitrocellulose membranes were washed three times with blocking buffer and then incubated 45 min at room temperature with respective secondary antibodies (horseradish peroxidase-conjugated antimouse IgG, or alkaline phosphate-conjugated antirabbit IgG). Horseradish peroxidase-conjugated antibody was revealed by enhanced chemiluminescence, whereas alkaline phosphatase-conjugated antibody was revealed by the colorimetric revelation of alkaline phosphatase. Photographic films or blots of stained nitrocellulose were scanned to obtain digital images for quantification of intensity reaction using Quantiscan software (Biosoft, USA). Quantification was performed both in specifically stained bands and in Poceau-S-stained lanes. Reaction intensity of antibody-stained bands was normalized to that of the corresponding lane stained with Ponceau-S to correct for protein loading.

Enzyme Activities

All assays were performed at 37°C with continuous gentle stirring in a total volume of 1 mL using an UV-Visible spectrophotometer (DU-650, Beckman, U.S.A.).

NAD(P)H-coenzyme Q reductase activity was assayed by measuring NAD(P)H (0.2 mM) oxidation at 340 nm in a reaction mixture which contained 50 mM Tris-HCl buffer pH 7.6, 400 μM coenzyme Q_0 (CoQ_0), 5 μM rotenone, 0.02% Triton X-100, and protein sample (30 μg plasma membrane protein or 0.5 μg of purified cytochrome b_5 reductase). The activity was measured both in the presence and absence of dicoumarol at 10 μM, an inhibitor of NQO1 activity (Lind *et al.*, 1990), and/or the thiol reagent *p*-hydroxy mercuribenzoate (*p*HMB), a strong inhibitor of the cytochrome b_5 reductase (Barham *et al.*, 1996) at 1, 10, or 100 μM. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used in calculations of specific activities.

NQO1 activity was measured at 550 nm by calculating the dicoumarol (10 μM)-sensitive coupled reduction of menadione-cytochrome *c*, in the presence of NADPH. Assays were carried out in 50 mM Tris-HCl buffer pH 7.6, containing 0.02% Triton X-100, 5 μM rotenone, 10 μM menadione, 0.2 mM DFO, 76 μM cytochrome *c*, 30 μg plasma membrane proteins and 0.2 mM NADPH. An extinction coefficient of 29.5 mM^{-1} cm^{-1} was used in calculations of specific activities.

Reduction of acetylated cytochrome c. The CoQ-dependent reduction of acetylated cytochrome *c* was assayed as an estimation of superoxide generation by CoQ-reductase. Assays were carried out in 50 mM phosphate buffer pH7.4, containing 0.2 mM DFO, 0.2 mM NADH, 10 μM dicumarol, 5 μM rotenone, 0.02% Triton X-100, 0.125 mg of acetylated cytochrome *c*, 30 μg plasma membrane protein, and 20 μM CoQ_0, and the reaction was monitored at 550 nm. Absorbance changes were monitored either in the presence or in the absence of 50–200 U/mL superoxide dismutase (SOD). An extinction coefficient of 29.5 mM^{-1} cm^{-1} was used to calculate specific activities.

Purification of Cytochrome b_5 Reductase

Pig liver plasma membranes were treated with 0.5 M KCl in 25 mM Tris-HCl, pH 7.6 containing 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT, and 10% glycerol to remove peripheral proteins, and then integral proteins were extracted by solubilization with 2.5% 3-[(cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS). The protein extract was separated from the membrane residue by centrifugation at 105,000*g* for 1 h at 4°C. The cytochrome b_5 reductase was then purified by size exclusion chromatography on Sephacryl S-300 HR, ion-exchange chromatography on DEAE-Sepharose 6B CL, and affinity chromatography on 5′ADP agarose followed by elution with NADH (Arroyo *et al.*, 2000; Villalba *et al.*, 1995). Purification was monitored by SDS-PAGE in 10% acrylamide.

RESULTS

Plasma Membrane Fractions

Fractions isolated from HL-60 cells by two-phase partition were highly enriched in plasma membranes, and virtually devoid from significant contamination with endomembranes. As shown in Fig. 1, antigens used as markers for endomembranes were easily detected with their corresponding specific antibodies in whole cells, but no significant staining was observed in plasma membrane fractions. On the contrary, a strong staining with the plasma membrane marker (anti-Na^+/K^+ ATPase α-subunit) was observed in HL-60 cells plasma membrane. Quantification of stained bands (Table I) revealed a very high degree of enrichment (more than 300-fold) of the plasma membrane marker in these fractions. These data indicate that plasma membranes obtained from HL-60 cells by two-phase partition are an adequate material to study

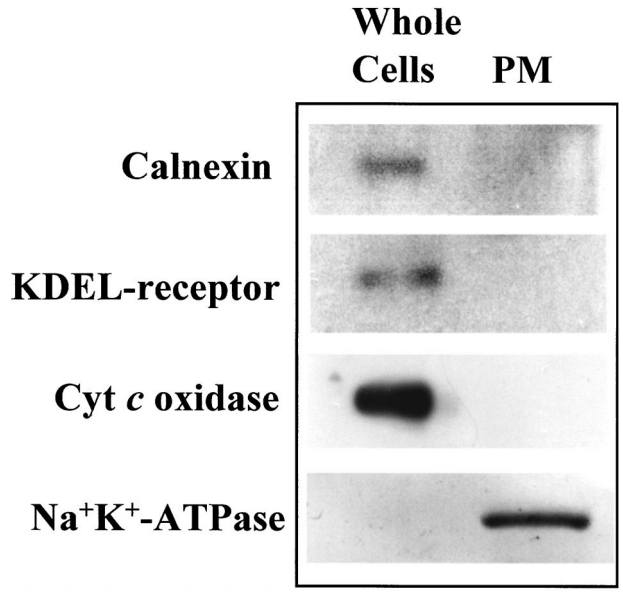

Fig. 1. Western blot immunostaining of whole HL-60 cells and plasma membrane fractions with marker antibodies. For details, see footnote of Table I.

Table I. Assessment of Purity of HL-60 Plasma Membranes by Immunological Markers[a]

Marker antigen	Subcellular localization	Whole cells (A.U.)	PM (A.U.)
Calnexin	Endoplasmic reticulum	100,270	N.D.
KDEL-receptor	Golgi apparatus	55,062	N.D.
Cytochrome *c* oxidase	Mitochondria	943,540	542
Na^+,K^+-ATPase (α subunit)	Plasma membrane	339	121,247

[a] HL-60 cells and isolated plasma membrane fractions were denatured in SDS-PAGE loading buffer. Equal amounts of protein (30 μg) were loaded onto 10 or 12% polyacrylamide gels, electrophoresed, and then electrotransferred to nicrocellulose for immunolabeling with corresponding marker antibody. Antibody binding was visualized either by enhanced chemiluminescence (calnexin, KDEL-receptor, and cytochrome *c* oxidase), or by colorimetric development of alkaline phosphatase (Na^+,K^+-ATPase). Photographic films or stained nitrocellulose were scanned to obtain digital images for quantification of intensity reaction of bands (expressed in arbitrary units, A.U.). N.D. not deteced.

NAD(P)H:quinone reductase activities because contamination with mitochondrial or microsomal reductases is minimized.

Induction of NADH:Quinone Oxidoreductases Upon Serum Withdrawal

HL-60 cells plasma membrane was assayed for NADH:quinone oxidoreductase activity using the benzoquinone coenzyme Q_0 (CoQ_0) as electron acceptor. Quinone reductase was assayed both in the absence and the presence of dicumarol to estimate the contribution of NQO1 to the whole activity. Before serum withdrawal, basal dicumarol-resistant CoQ_0-reductase activity was 83.2 ± 1.7 nmoles min^{-1} mg^{-1}, whereas dicumarol-sensitive activity was much lower, 4.8 ± 0.1 nmoles min^{-1} mg^{-1}. After serum withdrawal, the activities of both dicumarol-resistant and -sensitive quinone reductases were significantly increased, showing a peak after 32 h without serum (Fig. 2). Dicumarol-sensitive quinone reductase exhibited a maximal increase of 5–6-fold, while the increase of dicumarol-resistant reductase was about 2–3-fold. Both activities declined at 48 h, but remained twofold higher than initial values before serum removal.

Characterization of Dicumarol-Resistant Quinone Reductase in HL-60 Cells Plasma Membrane

When plasma membranes were assayed for NQO1 (DT-diaphorase) activity according to the specific assay

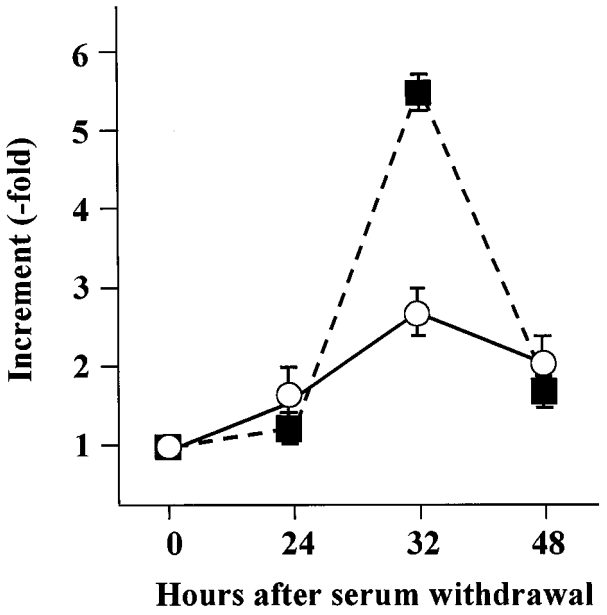

Fig. 2. Increase of quinone reductases upon serum withdrawal. HL-60 cells were cultured in serum-free medium for the periods indicated, and then collected and disrupted for plasma membrane preparation. Both dicumarol-sensitive (DT-diaphorase) (■) and -resistant (○) quinone reductases were assayed in plasma membrane fractions using NADH as donor and CoQ_0 as electron acceptor. Values were refered to initial activities at zero time. Three independent experiments (three determinations each) were carried out. Results are mean ± SD.

described by Lind *et al.* (1990), results were very similar to those obtained for dicumarol-sensitive CoQ_0-reductase, both in control membranes, and membranes obtained from serum-deprived cells (not shown). Thus, it is clear that the quinone reductase sensitive to micromolar concentrations of dicumarol can be characterized as NQO1. Since most of NQO1 activity resides in the cytosol, we tested whether or not the bulk of cytosolic NQO1 activity was also induced by serum removal. As found for plasma membrane-bound NQO1, cytosolic NQO1 significantly increased (about fivefold) after 32 h without serum and then decreased at 48 h (8.21 ± 2.51 nmol min^{-1} mg^{-1} in control cells, 42.76 ± 8.28 nmol min^{-1} mg^{-1} at 32 h without serum, and 31.25 ± 0.4 nmol min^{-1} mg^{-1} at 48 h). Thus, it is clear that the increase in plasma membrane-associated NQO1 is related to a general increase of NQO1 expression.

The identity of the enzyme(s) responsible for dicumarol-resistant quinone reductase is not so evident. As an initial characterization of this activity in HL-60 cells plasma membrane, we tested several properties of the enzyme including its susbtrate specificity, mechanism of reaction, and sensitivity to inhibitor. For these experiments, plasma membrane fractions were obtained from

cells that had been grown either in the presence of 10% serum on in a serum-free medium for 32 h. All assays were carried out in the presence of 10 μM dicumarol to exclude contribution of NQO1 to the measured activity.

Substrate Specificity

We assayed dicumarol-resistant CoQ_0 reductase using NADH or NADPH as electron donors. The enzyme in plasma membrane from both control and serum-depleted cells used NADH much better than NADPH (Fig. 3(A)). However, a difference between both activities in the two samples used should be noted. Serum-withdrawal induced a significant increase in the activity measured with NADH, but NADPH-dependent activity was almost unaffected. As a consequence, the ratio of both activities (NADH- *versus* NADPH-dependent) was about six in control membranes, and this value was increased to about 8.5 in plasma membrane from serum-depleted cells. This result indicates that serum-withdrawal induced the expression or activated enzyme(s) showing a high degree of specificity towards NADH.

Reaction Mechanism

As shown before, a dicumarol-resistant enzyme activity was increased in HL-60 cells plasma membranes after serum withdrawal, which was specific for NADH as electron donor. This feature fits with reported properties of NADH-cytochrome b_5 reductase (Barham *et al.*, 1996), an enzyme that can reduce a variety of quinones by a one-electron mechanism to generate the corresponding semiquinones and hydroquinones in the absence of cytochrome b_5 (Nakamura and Hayashi, 1994; Villalba *et al.*, 2000). To test whether or not dicumarol-resistant NADH-specific quinone reductase of plasma membrane from HL-60 cells was due to a cytochrome b_5 reductase, we measured superoxide generation (owing to autoxidation of semiquinones generated by the one-electron reduction of quinones) as the quinone-dependent, SOD dismutase-inhibitable reduction of cytochrome *c* (Fig. 3(B)). Superoxide was generated during quinone reduction by quinone reductase in control plasma membranes with NADH, but much less amount of superoxide was generated with NADPH. This indicates that the enzyme present in control plasma membrane is a one-electron quinone reductase, similar to the cytochrome b_5 reductase. However, the twofold increase in quinone reductase activity that took place after 32 h without serum (Fig. 3(A)) did not result in a similar increase of superoxide generation (Fig. 3(B)), suggesting that the enzyme upregulated at the plasma

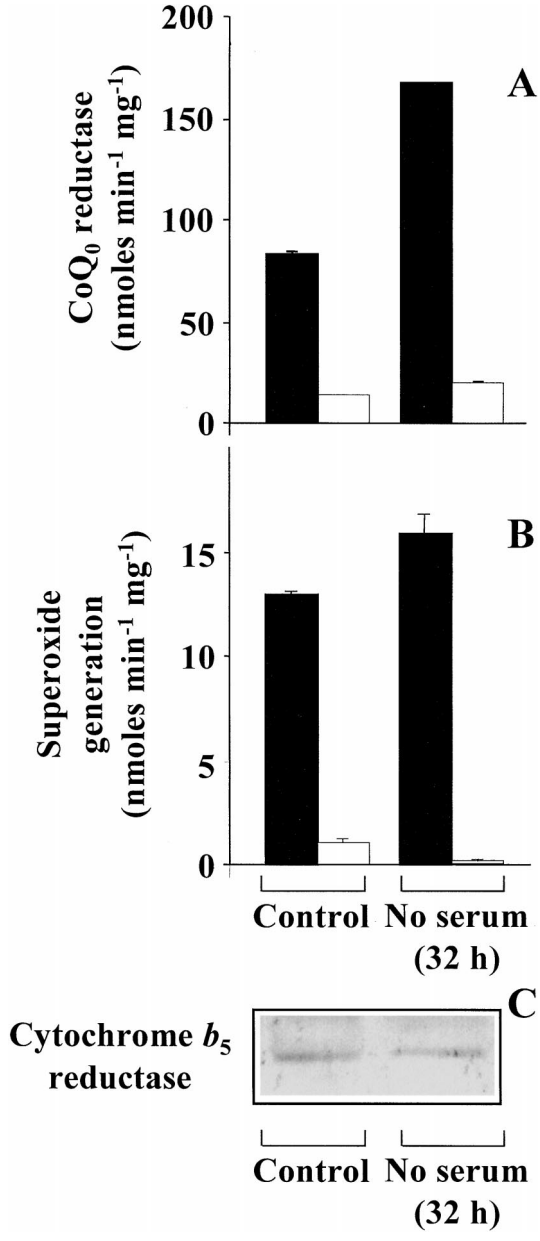

Fig. 3. Donor specificity and reaction mechanisms of dicumarol-resistant quinone reductases in HL-60 cells plasma membranes. Assays were carried out with plasma membranes isolated from control and serum-deprived (32 h) cells in the presence of 10 μM dicumarol to inhibit NQO1 activity. Activities were recorded with NADH (■) or NADPH (□) as electron donors. (A) Coenzyme Q_0 reductase. (B) Superoxide generation. The reaction was initiated by addition of CoQ_0 to the reaction medium. Superoxide was measured from the activity that was sensitive to SOD. Two independent experiments (three determinations each) were carried out. Results are mean ± SD. (C) Detection of cytochrome b_5 reductase polypeptide in western blots of electrophoresed membrane proteins from control and serum-depleted (32 h) HL-60 cells. Quantification of stained bands with reference to total protein loading (see Material and Methods) gave values of 2791 and 2021 arbitrary units respectively.

membrane by serum withdrawal is a two-electron quinone reductase. Moreover, no generation of superoxide was measured with NAPDH in plasma membrane obtained from serum-depleted cells, which agrees with the observed increase of NQO1, another two-electron quinone reductase.

Inhibition by p-Hydroxy Mercuribenzoate

The results described above are consistent with the idea that a cytochrome b_5 reductase accounts for the bulk of dicumarol-resistant quinone reductase activity which is constitutively present at the plasma membrane in HL-60 cells, but dicumarol-resistant quinone reductase that was upregulated by serum withdrawal is not due to a cytochrome b_5 reductase. To confirm this fact we test its sensitivity to the thiol reagent *p*HMB, a strong inhibitor of this enzyme. Inhibition assays were also carried out with the cytochrome b_5 reductase purified to homogeneity from pig liver plasma membrane, to allow us for making a comparison with HL-60 plasma membrane in terms of *p*HMB sensitivity. As shown in Fig. 4, inhibition patterns of quinone reductase activity of purified cytochrome b_5 reductase and plasma membrane isolated from control HL-60 cells were very similar. While *p*HMB at concentrations of 1–10 μM strongly inhibited quinone reductase of both control plasma membrane and cytochrome b_5 reductase, these concentrations had little or no effect on dicumarol-resistant NADH-specific quinone reductase of plasma membrane isolated from serum-depleted cells. These results clearly distinguish the upregulated enzyme from the cytochrome b_5 reductase. Accordingly, immunostaining of western blots with specific antiserum against cytochrome b_5 reductase showed that cytochrome b_5 reductase polypeptide did not increase in plasma membranes isolated from serum-depleted (32 h) cells (Fig. 3(C)).

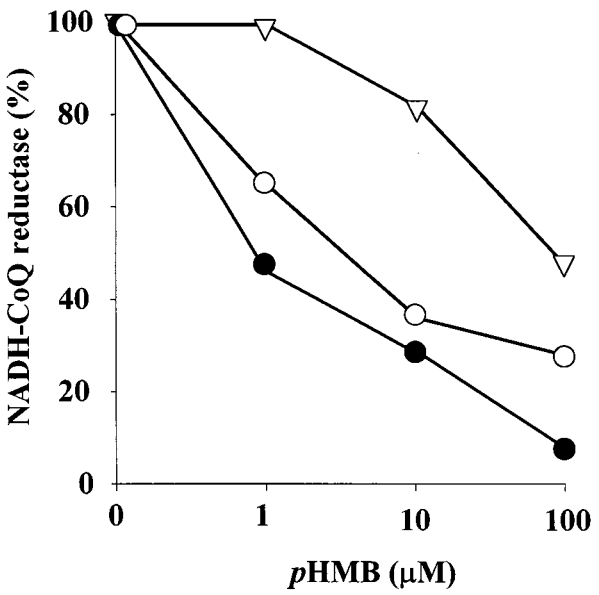

Fig. 4. Sensitivity to *p*HMB of dicumarol-resistant NADH:quinone oxidoreductases. Assays for NADH-CoQ_0 reductase were carried out in the presence of 10 μM dicumarol with control plasma membranes (○), plasma membranes from serum-deprived (32 h) HL-60 cells (▽), and purified NADH-cytochrome b_5 reductase (●). Values of activity were referred to controls without *p*HMB and expressed in percentage. Two independent experiments (three determinations each) were carried out. Results are mean ± SD.

Requirement for NQO1 Activity Under Serum-Deprivation Conditions

Recent reports have documented that quinone reductases play important roles in the regulation of both cell growth and death (Asher *et al.*, 2001; Cross *et al.*, 1999; Siemankowski *et al.*, 2000), most likely through their ability to control the intracellular redox evironment by modulating the NAD(P)H:$NAD(P)^+$ ratio (Gaikwad *et al.*, 2001), and increased quinone reductase could either potentiate (Asher *et al.*, 2001; Siemankowski *et al.*, 2000) or inhibit (Cross *et al.*, 1999) cell death. We have taken advantage of the extreme sensitivity of NQO1 to dicumarol in the micromolar range to analyse the putative role of the activity increase in either the signaling cascade leading to death of HL-60 cells or the protection of cells under serum deprivation conditions. For these experiments we chose a concentration of 5 μM dicumarol, which fully inhibits NQO1 activity without affecting significantly the activity of other cellular quinone reductases. As shown in Fig. 5(A), serum withdrawal led to a significant decrease in the growth rate of HL-60 cells, and addition of dicumarol to serum-free cultures strongly potentiated growth arrest. In addition, a decrease in the viablility of HL-60 cells was observed after 72 h culture in serum-free medium containing 5 μM dicumarol (Fig. 5(B)). The NQO1 inhibitor had no effect on both the growth rate and viability of HL-60 cells when cultured in medium containing 10% serum (Fig. 5(A) and (B)). Thus, our results demostrate that NQO1 activity is required to sustain viability and promote the growth of serum-deprived HL-60 cells.

DISCUSSION

Compelling evidences have accumulated during the last years to support important roles for quinone reductases

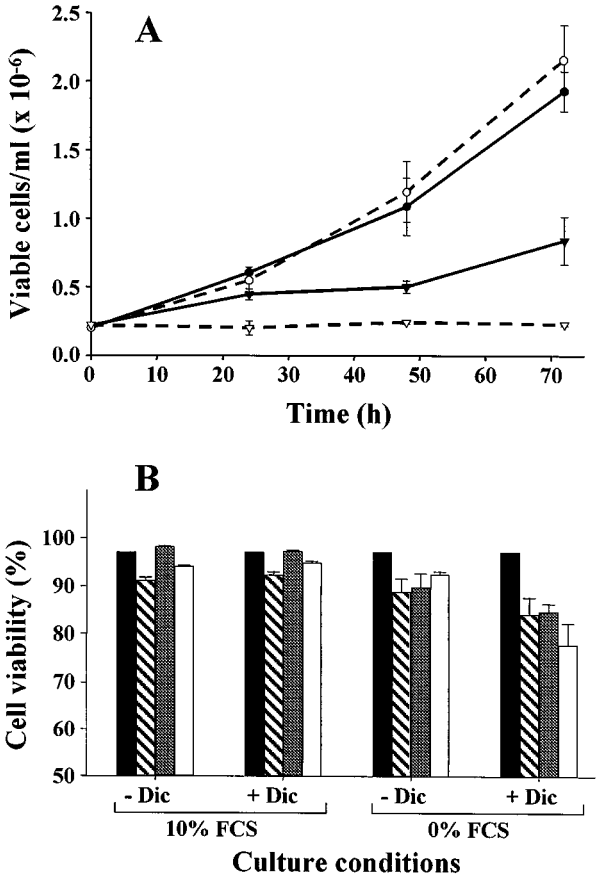

Fig. 5. Potentiation of serum withdrawal-induced growth arrest by inhibition of NQO1 activity in HL-60 cells. Cells were cultured in RPMI medium containing 10% serum or in serum-free medium, either in the absence or in the presence of 5 μM dicumarol. At the indicated times, aliquots were withdrawn and used for counting of viable cells by the Tripan blue exclusion method. (A) Number of viable cells (●), 10% serum; (○) 10% serum plus 5 μM dicumarol; (▼) serum-free medium; (▽) serum-free medium plus 5 μM dicumarol. (B) Cell viability of cultures at 0 (■), 24 (▨), 48 (▩), and 72 h (□). Two independent experiments (three determinations each) were carried out. Results are mean ± SD.

in protection against oxidative stress (Beyer *et al.*, 1996; Cadenas, 1995; Navarro *et al.*, 1998; Villalba *et al.*, 2000, 2001). Also, recent reports have pointed novel functions of quinone reductases in the control of intracellular redox balance permissive for signaling, thus affecting the regulation of cell death and growth (Asher *et al.*, 2001; Bello *et al.*, in press; Collin *et al.*, 2001; Cross *et al.*, 1999; Gaikwad *et al.*, 2001; Siemankowski *et al.*, 2000). Among the different quinone reductases, those located at the plasma membrane are of great importance for cells because this membrane is the initial site of attack of extracellular oxidants (May *et al.*, 2001). Moreover, plasma membrane quinone reductases are in a favorable position to control in situ quinone/hydroquinone ratios (Navarro *et al.*, 1998), which can affect signal transduction processes (Martín *et al.*, 2001).

In this work, we have analyzed plasma membrane quinone reductases of HL-60 cells under control and serum deprivation conditions, as an attempt to characterize how these enzymes paticipate in the cellular response to oxidative stress. Our results have shown that a cytochrome b_5 reductase accounts for the bulk of quinone reductase activity in plasma membrane from control cells. This enzyme can function as a one-electron quinone reductase (Nakamura and Hayashi, 1994). Its role in the reduction of quinones, such as ubiquinone, and in the recycling of α-tocopherol has been documented for both liver plasma membrane (Navarro *et al.*, 1995; Villalba *et al.*, 1995) and erythrocyte ghosts (Constantinescu *et al.*, 1993, 1994). In addition, HL-60 cells plasma membrane contained low levels of NQO1 activity. Although the bulk of NQO1 resides in the cytosol, a minor portion is usually associated with membranes (Lind *et al.*, 1990). Furthermore, its assay in vitro requires detergent for optimal activity, which is consistent with an interaction of NQO1 with membrane components at the membrane-cytosol interphase (Beyer *et al.*, 1996).

Upon serum withdrawal, plasma membrane-associated quinone reductases increased significantly to reach a maximum at about 32 h after serum deprivation and decreased at about 48 h. Two quinone reductases were upregulated by serum withdrawal, and were characterized as NQO1, and a novel dicumarol-resistant NADH-specific reductase. The latter also reduced quinones through a two-electron mechanism and was distinguished from the cytochrome b_5 reductase. The increase of plasma membrane-bound NQO1 was likely due to a general activation of NQO1 expression, since similar increases were observed for the bulk of cytosolic NQO1 activity. Properties of the novel quinone reductase, such as preference for NADH as electron donor and resistance to inhibition by dicumarol, resembled those reported for a NAD(P)H:quinone oxidoreductase recently identified in rat liver microsomes, which was considered immunologically related to cytosolic NQO1 (Jaiswal, 2000b). Dicumarol-resistant NADH-specific quinone reductase upregulated by serum deprivation remains to be fully characterized from a biochemical and molecular point of view. However, coincidence of expression patterns with those of NQO1 also argues in favor of some relationship between both enzymes. Our results constitute the first demonstration that this novel enzyme is upregulated at the plasma membrane by serum withdrawal, and that it does not generate superoxide during quinone reduction. An increase of two-electron quinone reductases may

be beneficial to cells, particularly under prooxidant conditions, because they can avoid spurious generation of ROS by redox cycling of semiquinones (Jaiswal, 2000a).

Interestingly, activities of other antioxidant enzymes such as catalase, glutathione S-transferase and glutathione peroxidase increase in F-MEL cells after serum deprivation to reach the maximum at 24–72 h, and then decrease to initial or lower levels (Kim *et al.*, 2000a). Also, glutathione levels increase at 24 h, and then decrease significantly. As hypothesized for environmental oxidative stress conditions (Jaiswal, 1994), the coincidence of expression patterns of plasma membrane quinone reductases and other cellular antioxidant enzymes is in agreement with the idea that serum withdrawal triggers the coordinated induction of several defensive gene products to provide the required protection of cells.

The increase of cytosolic and plasma membrane-associated NQO1 activity in serum-deprived cells was not unexpected. NQO1 is transcriptionally induced by oxidants and antioxidants able to undergo redox cycling to generate ROS (Favreau and Pickett, 1991; Jaiswal, 2000a; Li and Jaiswal, 1992, 1994; Rushmore *et al.*, 1991). Furthermore, the amount of membrane-bound NQO1 also increases under oxidative stress conditions. A dramatic increase in NQO1 activity associated to the plasma membrane has been observed in liver cells from vitamin E and selenium-deficient rats (Navarro *et al.*, 1998, 1999), and a significant increase in soluble and membrane-bound NQO1 has been also found in liver of rats fed with a diet deficient in selenium (Olsson *et al.*, 1993). Prooxidant conditions prevailing under serum deprivation (Slater *et al.*, 1996) could be related to the observed increase in NQO1. A plausible candidate for mediating this increase is peroxide, which increases in F-MEL cells at 24–48 h after serum withdrawal (Kim *et al.*, 2000a,b) and stimulates NQO1 expression (Bello *et al.*, in press; Collin *et al.*, 2001; Favreau and Pickett, 1991; Jaiswal, 2000a; Li and Jaiswal, 1994; Rushmore *et al.*, 1991).

A lowering of quinone reductase activity was observed at 48 h after serum deprivation in HL-60 cells, although levels remained about twofold higher than initial ones. Serum growth factors are required to maintain elevated NQO1 levels in high-density BALB/c 3T3 cells (Schlager *et al.*, 1993), and primary cultures of normal human osteoblastic cells (Collin *et al.*, 2001). After the initial increase of plasma membrane quinone reductases, most likely due to oxidative stress conditions related to serum deprivation, a late decrease of these enzymes could be due to the prolonged lack of growth factors. Lowering of quinone reductases also correlates with a late decrease of other antioxidant enzymes previously observed in F-MEL cells under serum deprivation, which has been related to expression of c-Jun (Kim *et al.*, 2000a,b). Interestingly, c-Jun is also coordinately induced with NQO1 in response to xenobiotics and redox cycling antioxidants (Radjendirane and Jaiswal, 1999), and thus it could be also related to the late decrease in quinone reductase activity. Accordingly, Jun/Fos and Jun/Fra1 heterodimers are repressors that can bind to the antioxidant responsive element (ARE) present in NQO1 gene promoter with a high affinity (Venugopal and Jaiswal, 1996). Since Jun proteins by themselves are ineffective in repression of ARE-mediated gene expression, and since repression is apparently due to c-Fos and Fra1 (Venugopal and Jaiswal, 1996; Wilkinson *et al.*, 1998), it remains for further investigation to elucidate whether or not a c-Jun increase could play a role in the late decrease of quinone reductase after serum deprivation, as has been demonstrated for other antioxidant enzymes under the same conditions (Kim *et al.*, 2000a,b).

According to results presented previously by several authors, increased quinone reductase could either potentiate (Asher *et al.*, 2001; Siemankowski *et al.*, 2000) or inhibit (Cross *et al.*, 1999) cell death. To test the particular role played by NQO1 in the growth of HL-60 cells under serum deprivation, we treated cells with dicumarol at 5 μM, a concentration which fully inhibits NQO1 without affecting other cellular quinone reductases (Lind *et al.*, 1990; Preusch *et al.*, 1991; Takahashi *et al.*, 1992; Wu *et al.*, 1997). Dicumarol treatment strongly potentiated growth arrest and produced a decrease in the viability of HL-60 cells in the absence of serum, which is consistent with a protective role for NQO1. Accordingly, dicumarol also potentiates tumor necrosis factor α (TNFα)-induced apoptosis in HeLa cells (Cross *et al.*, 1999), and NQO1 activity is required to maintain the viability of HeLa cells grown to high-density (Bello *et al.*, in press). Further, ubiquinone, a substrate for plasma membrane quinone reductases (Sun *et al.*, 1992; Villalba *et al.*, 1995), stimulates cell growth in the absence of serum in several cell lines such as HeLa, BALB/3T3, HL-60, and K-562 (Barroso *et al.*, 1997; Crane *et al.*, 1993; Sun *et al.*, 1995; Villalba *et al.*, 1996). No effect of dicumarol at 5 μM was observed in the presence of 10% serum. Serum antioxidants and albumin can contribute to this protective effect. Albumin is also a scavenger of ROS such as peroxide (Brown *et al.*, 1989), and it is very likely that a substantial part of protection is the result of a complexation of dicumarol by serum albumin, which prevents its uptake by cells (Bello *et al.*, in press; Petitpas *et al.*, 2001; Wosilait *et al.*, 1981).

How are signaling processes which control cell growth and death regulated by quinone reductases? Potentiation of TNFα-induced cell death by dicumarol has been related to the unbalancing of intracellular redox state

permissive for antiapoptotic signaling by NFκB (Cross *et al.*, 1999). On the other hand, plasma membrane-associated quinone reductases could play additional regulatory roles. For instance, inhibition of a plasma membrane redox system by a number of quinone analogues and inhibitors of quinone reductases is known to induce cell death (Macho *et al.*, 1998, 1999; Wolvetang *et al.*, 1996). A plasma membrane neutral magnesium-dependent sphingomyelinase (nSMase) is activated upon serum withdrawal, and ceramide, the sphingomyelin hydrolysis product, causes cell cycle arrest or apoptosis (Hannun, 1996; Hannun and Luberto, 2000; Jayadev *et al.*, 1995). Interestingly, ubiquinone addition to HL-60 cells prevents ceramide accumulation and decreases cell death induced by serum deprivation (Barroso *et al.*, 1997). Furthermore, the nSMase is strongly inhibited by the hydroquinone ubiquinol in isolated plasma membranes (Martín *et al.*, 2001). It is thus conceivable that inhibition of NQO1 with dicumarol may lead to increased generation of ceramide at the plasma membrane, thus potentiating growth arrest and cell death.

According to Jaiswal (2000b), the microsomal NAD(P)H:quinone oxidoreductase could provide additional protection to membranes containing one-electron quinone reductases, particularly in the case of loss of cytosolic enzymes. Our results suggest that this enzyme may be not sufficient to overcome a lack of NQO1 activity under serum deprivation conditions. The elucidation of the particular role played by dicumarol-resistant quinone reductase upregulated in the absence of serum remains for further investigations, once specific inhibitors of this novel enzyme have been identified.

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