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Cellular Antioxidant Properties of Human Natural Killer Enhancing Factor B

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The protein, NKEF (natural killer enhancing factor), has been identified as a member of an antioxidant family of proteins capable of protecting against protein oxidation in cell-free assay systems. The mechanism of action for this family of proteins appears to involve scavenging or suppressing formation of protein thiyl radicals. In the present study we investigated the antioxidant protective properties of the NKEF-B protein overexpressed in an endothelial cell line (ECV304). *Nkef*-B-transfected cells displayed significantly lower levels of reactive oxygen species (ROS) compared with control or vector-transfected cells. Tert-Butylhydroperoxide-induced ROS was 15% lower in *nkef*-B-transfected cells and cytotoxicity was slightly, though not significantly, lower. NKEF-B had no effect on ROS induced by menadione or xanthine plus xanthine oxidase. NKEF-B overexpression resulted in slightly (≈ 10%) lower levels of cellular glutathione (GSH) and had no effect on rate or extent of GSH depletion following either diethylmaleate (DEM) or buthionine sulfoximine (BSO) treatment. Lipid peroxidation, assessed as thiobarbituric acid-reactive substances, was 40% lower in n*kef*-B-transfected cells compared with vector-only-transfected cells. DEM-induced lipid peroxidation was suppressed by NKEF-B at DEM concentrations of 20 μM to 1 mM. At 10 mM DEM, lipid peroxidation was unaffected by NKEF-B. NKEF-B expression also protected cells against menadione-induced inhibition of [^{3}H]-thymidine uptake. The NKEF-B protein appears most effective in suppressing basal low-level oxidative injury such as that produced during normal metabolism. These results indicate that overexpression of the NKEF-B protein promotes resistance to oxidative stress in this endothelial cell line.

Keywords: Antioxidant, reactive oxygen species, Glutathione, lipid peroxidation

INTRODUCTION

Cellular anti-oxidant defense systems are increasingly recognized as important regulators of cell function and viability. The constant burden imposed by high metabolic activity involving electron transport in the presence of oxygen as well as exposure to xenobiotic toxins mandates the expression of multiple types of constitutive and inducible antioxidant strategies (for review see 1).

NKEF proteins are members of a recently discovered family of cellular anti-oxidants which were originally characterized as sulfur radical scavengers based on their ability to protect enzymes from oxidative inactivation.[2-6] Since

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this protective activity lacks both sequence homology and enzymatic activity associated with known antioxidant enzymes such as catalase and superoxide dismutase, a novel mechanism of action must be assumed.

Previous cell-free studies have revealed an antioxidant protective effect of recombinant NKEF on the enzyme glutamine synthetase.[2] This effect was similar to those described for other members of this family of proteins characterized in rat and yeast. However, the actions of the human NKEF proteins in an intracellular system have not been extensively characterized. In the present report we describe biochemical effects of the NKEF-B protein stably overexpressed in a transfected human endothelial cell line, ECV304. The results lend further support to the hypothesis of a primary anti-oxidant function for this protein and the ability to protect against cell death.

MATERIALS AND METHODS

An expression vector for *nkef*-B was generated as a pCE2 plasmid construct containing a CMV enhancer, EF-1α promoter, and a hygromycin B selection marker. The *nkef*-B gene was obtained from a cDNA clone by PCR amplification of a 620 bp Asp 718I-Bam HI fragment.

Stably transformed cell lines were derived using the endothelial cell line ECV304 (American Type Culture Collection, Rockville, MD). Plasmids pCE2 (vector control) or pCE2:*nkef*-B plasmids were linearized with Spe I and transfected by electroporation using a Cell-Porator™ (Life Technologies, Gaithersburg, MD) as described by Cachianes *et al.*[7] Transfected clones were selected by culture with 200 μg/ml Hygromycin B. Northern blot analysis of transfected cells revealed a 30-fold increase in NKEF-B mRNA and Western blot analysis revealed an 11-fold increase in NKEF-B protein.[8]

Cells were cultured in RPMI 1640 supplemented 10% fetal bovine serum, 1% penicillin-streptomycin and 50 μg/ml hygromycin B. Cells were incubated at 37°C in humidified chambers gassed with 10% CO_2 – 90% air. Cells were passaged once per week in multiwell plates, coated with poly-L-lysine were seeded at a density of 8×10^4 cells per well (12-well plate).

Reactive oxygen species (ROS) were measured using 2,7-dichlorofluorescein-diacetate (DCF-DA).[9-11] Cells in multiwell plates were preloaded with 20 μg/ml in HEPES-buffered Krebs Ringer (KR) for 20 min at room temperature. After washing, cells were kept in the dark at room temperature in KR containing various toxins. After one hour fluorescence (Ex = 485 Em = 530) was measured using a Cytofluor 2300 plate reader (PerSeptive Biosystems, Framingham, MA). Cell viability was then determined by adding 50 μg/ml (final) propidium iodide and after 20 min measuring fluorescence at Ex = 530, Em = 590. Twenty minutes following addition of 160 μM digitonin, fluorescence at Ex = 530, Em = 590 is remeasured to assess total cell number. This value is used as a denominator to determine percent dead cells and to normalize DCF fluorescence with respect to number of cells.

Levels of reduced glutathione were evaluated using the fluorescent probe, monochlorobimane (MCB). Cells were exposed to 40 μM MCB in KR for 15 min at room temp. Fluorescence was measured at Ex = 395, Em = 460 and values were normalized to propidium iodide fluorescence in the presence of digitonin as above.

Lipid peroxidation was measured using the thiobarbituric acid-reactive substances (TBARS) method by a modification of the method described previously.[12] Cells in 12-well plates were treated 4hr with 10 mM diethylmaleate (DEM) in KR. After washing cells twice, 250 μl 0.1% Triton X-100 was added with agitation for 10 min. Then cell extracts were combined with 100 μl thiobarbituric acid (TBA) in glass tubes and incubated for 1 hr at 95°C. After cooling, samples were extracted with 0.5 ml n-butanol and 250 μl of the separated butanol phase was placed in a 48-well culture plate for measurement of fluorescence (Ex = 530, Em = 590 at sensitivity = 4). Standard curves were

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obtained using 1,1,3,3-tetraethoxypropane as aldehyde source and data were calculated as nmoles TBARS per 10^5 cells.

[^{3}H]-Thymidine incorporation was measured in cells cultured at a density of 10^4 cells/well in 96-well plates. Cells were incubated with various concentrations of menadione for 20 h at 37°C. Cells were then labelled with 0.5 μCi/well of [^{3}H]-thymidine (6.7 Ci/mmol) for 4 hr and then harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA). Membrane filters were counted in a β-counter using Bio Safe II scintillation fluid.

For most experiments data were analyzed using the Student's t-test with unpaired data. Data from lipid peroxidation experiments were analyzed by 2-way ANOVA using the SAS analysis program.

RESULTS

Tert-Butylhydroperoxide (tBOOH) produces a time- and concentration-dependent increase in DCF oxidation in the ECV304 endothelial cell line. 500 μM tBOOH produced a 6.8-fold increase in DCF oxidation in control cells (Fig. 1A). In *nkef*-B-transfected cells, DCF oxidation was 63% lower than in control cells under basal conditions and 46% lower at 500 μM tBOOH. The decreased ROS evident in *nkef*-B-transfected cells was associated with decreased cytotoxicity (Fig. 1B). The percentage of dead cells assessed by propidium iodide (indicating necrotic death) was 23 ± 5% in control cells and 13 ± 2% in *nkef*-B-transfected cells for cells treated 1 hr with 500 μM tBOOH.

In a separate series of experiments comparing vector-transfected versus *nkef-B*-transfected cells, the effects of various toxins on DCF oxidation and viability were examined. NKEF-B overexpression decreased ROS levels by 15% ($p < 0.005$) after 1 hr in KR (Table I). T-BOOH-induced ROS was also partially suppressed by NKEF-B which produced a 15% reduction after 1 hr exposure to 50 μM T-BOOH. The degree of protection against cytotoxicity afforded by NKEF-B was approximately the same (4–6%) in the presence or absence of tBOOH. NKEF-B had no protective effect against ROS induced by menadione or xanthine oxidase plus xanthine. Neither methyl mercury nor diethylmaleate induced appreciable increases in ROS in this cell line.

Levels of reduced glutathione (GSH) were examined using the fluorogenic compound, monochlorobimane. GSH levels were slightly (5–10%) lower in *nkef-B*-transfected cells compared with controls although this difference was not quite statistically significant. Exposure of

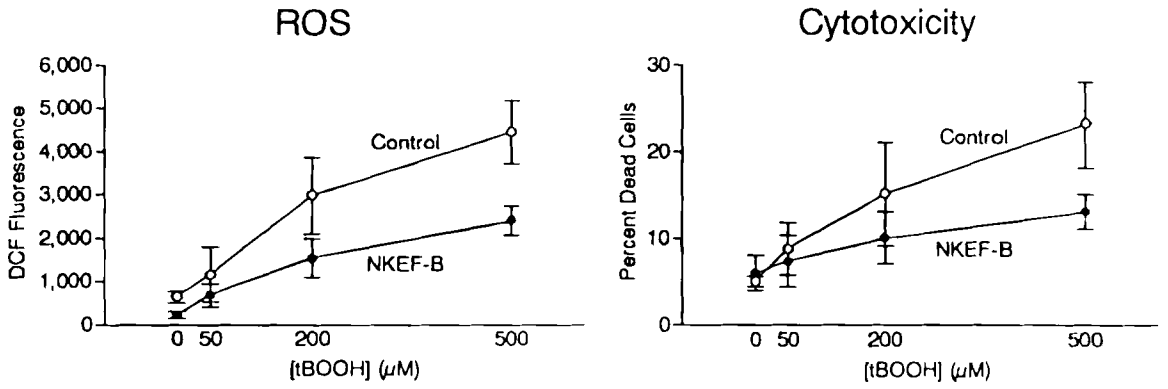

FIGURE 1 Effect of *nkef*-B overexpression on tBOOH-induced ROS (A) and viability (B). ECV 304 cells were treated with the indicated amounts of tBOOH in KR buffer for 1 hr following DCF-DA loading. DCF oxidation and viability were measured as described under Methods. Control cells were untransfected ECV cells. $p < 0.05$ comparing ROS values for control and NKEF-B cells at O and 500 μM tBOOH. $p < 0.05$ comparing viability values at 200 and 500 μM tBOOH ($n = 3$).

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TABLE I

	DCF Oxidation		CELL Viability	
Toxin	Control	NKEF-B	Control	NKEF-B
Control	1294 ± 42.8[a] (43)	1099 ± 37.5[b] (42)	88 ± 0.6 (43)	92 ± 0.6[c] (42)
tBOOH [100 μM]	3928 ± 426 (24)	3320 ± 425[b] (24)	79 ± 1.8 (24)	85 ± 1.6[c] (24)
Menadione [50 μM]	2589 ± 214 (23)	2538 ± 311 (22)	87 ± 0.8 (23)	90 ± 0.8 (23)
MeHg [50 μM]	1109 ± 77 (11)	1081 ± 93 (12)	91 ± 0.6 (11)	93 ± 0.6 (12)
X/XO [100 μM][5 mU]	2171 ± 95 (12)	2062 ± 104 (12)	89 ± 0.4 (12)	93 ± 0.5 (12)
DEM [20 mM]	694 ± 76[d] (8)	652 ± 68[d] (8)	82 ± 3.5 (8)	87 ± 2.8 (8)

[a]DCF (ROS) and PI (viability) fluorescence values obtained after 1 hr exposure to indicated toxins in KR at room temperature were normalized to PI fluorescence in the presence of digitonin and are expressed as means ± SEM (N).
[b]$p < 0.005$ compared with control using Student's t-test.
[c]$p < 0.05$ compared with control.
[d]DEM exposures were included in only a subset of these experiments accounting for mean ROS values being substantially lower than controls

cells to diethylmaleate (DEM) produced similar rates of decline in GSH in the two cell types (Fig. 2) over a period of several hours. The rate of GSH decline over 48 hours following buthionine sulfoximine (BSO) treatment was also unaffected by NKEF-B (Fig. 2B). Steady-state levels of GSH attained following these treatments were also similar.

Overexpression of NKEF-B resulted in significantly lower (40%) levels of TBARS in cultured ECV 304 cells maintained in KR for 4 h at room temperature (Fig. 4). These values presumably represent basal, unstressed levels of lipid peroxidation in these cells. NKEF-B expression resulted in reduction of TBARS valves by 32%, 48%, and 19% for 20 μM, 100 μM, and 1 mM diethylmaleate, respectively. Levels of GSH declined in a concentration-dependent manner which was similar in both cell types. Four hours exposure to 10 mM diethylmaleate in HEPES-buffered Kreb's Ringer increased TBARS to approximately the same level (≈ 35 p moles/10^5 cells) for both *nkef-B*- and vector-transfected cells.

Exposure of cells to low concentrations (0–10 μM) of menadione for 20 h produced a concentration-dependent suppression of [^{3}H]-thymidine uptake, indicating purturbation of general cell function, energetics and viability (Fig. 3). NKEF-B over-expression resulted in significant protection from menadione-induced cytotoxicity at concentrations below 10 μM. Sensitivity to menadione was similar for wild type and vector-transfected cells. Maximal protection by NKEF-B was observed at 6 μM menadione.

DISCUSSION

The recently discovered NKEF proteins have been inferred to possess anti-oxidant properties

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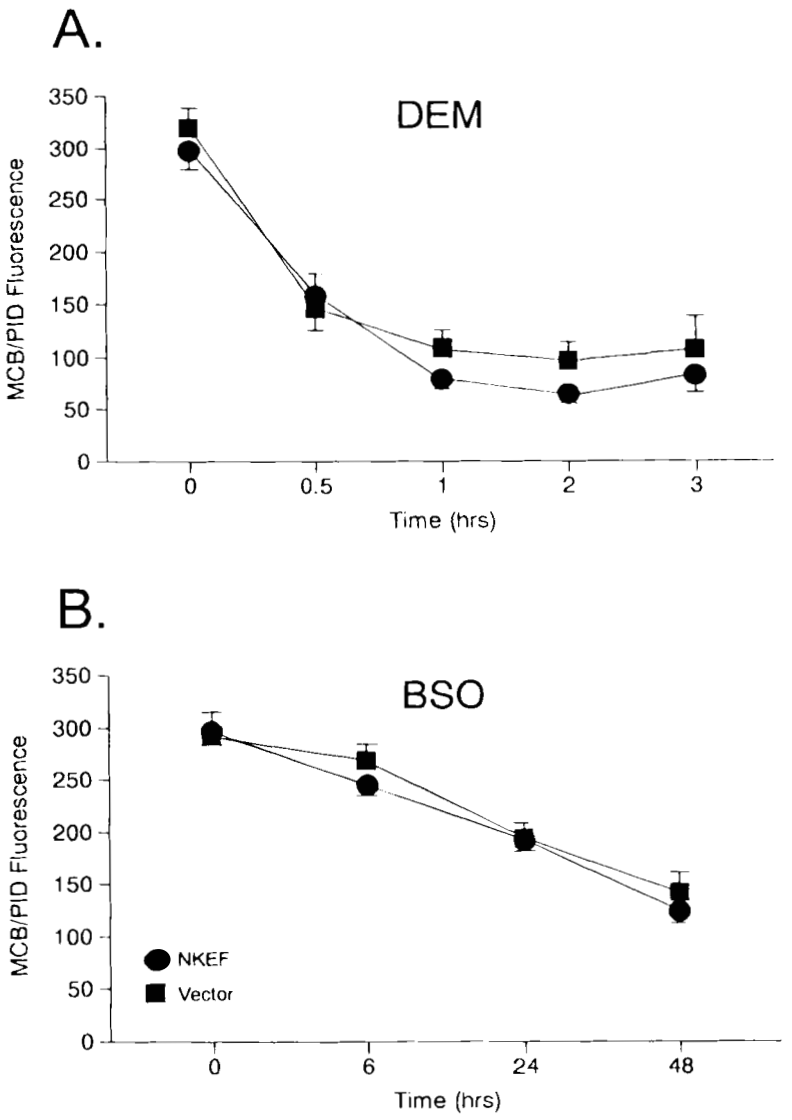

FIGURE 2 Intracellular levels of reduced glutathione (GSH) during treatment with 5 mM DEM (A) or 200 μM BSO (B). Values are expressed as MCB Fluorescence normalized to PI fluorescence in the presence of digitonin and represent means of 20–30 determinations ± SEM obtained in 4–7 experiments. $p > 0.05$ for all determinations comparing NKEF-B-transfected to vector-transfected controls.

on the basis of (1) sequence homology to the conserved thiol specific anti-oxidant family of proteins,[3] (2) inducible expression upon exposure to pro-oxidants,[13,14] and (3) ability to protect against glutamine synthetase inactivation in a cell-free assay system.[2]

The present studies characterize anti-oxidant effects of NKEF-B in a cellular model system, i.e. a human endothelial cell line, ECV304. Despite maintaining slightly lower basal levels of GSH, *Nkef-B*-transfected cells displayed lower rates of ROS-mediated DCF oxidation and lower levels of lipid peroxidation. DCF can be oxidized by H_2O_2 and hydroxyl radical but not directly by superoxide anion, $\cdot O_2^-$. However, intracellularly generated $\cdot O_2^-$ is converted to H_2O_2 and can thus

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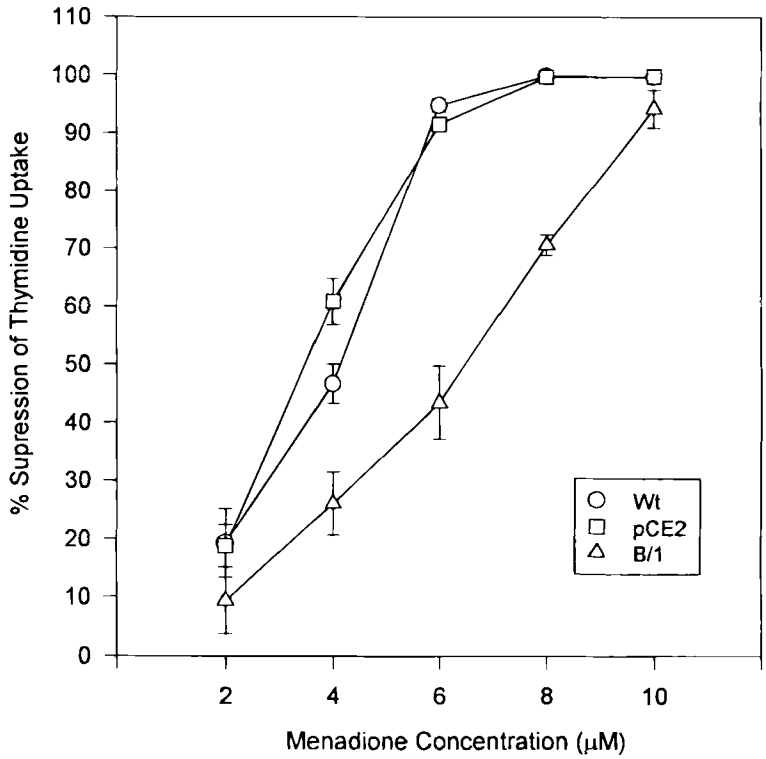

FIGURE 3 Effect of menadione on cellular [^{3}H]-thymidine uptake. Thymidine uptake studies were performed as described under Materials and Methods and were interpreted to represent a general indicator of cell viability and function. Values are means of triplicate determinations ± SEM. Four repeats of this experiment produced similar results. Wt = wild type; pCE2 = vector-transfected, B/1 = NKEF-B-transfected cells.

indirectly oxidize DCF. The lower rate of DCF oxidation in NKEF-B-transfected cells suggests that NKEF suppresses intracellular accumulation of these ROS under basal, unstressed conditions. Several pro-oxidant agents and other toxins are able to oxidize DCF either directly or by elevating intracellular ROS.[10,15] T-BOOH-induced DCF oxidation was significantly diminished in NKEF-B-transfected cells when compared with control cells. This observation is consistent with a previous report indicating that the yeast homolog of NKEF known as thiol-specific anti-oxidant protects yeast from growth-inhibitory effects of t-butylhydroperoxide. The inability of NKEF-B to diminish ROS accumulation produced by menadione or xanthine, oxidase plus xanthine however, suggests that anti-oxidant activities of this protein are limited and are restricted to peroxide-generating agents in this acute exposure paradigm.

Since the NKEF-B family of proteins is believed to act primarily by suppressing protein thiyl radical generation, a link between protein thiyl radicals and peroxide-dependent DCF oxidation must be inferred. One possible mechanism for such linkage would be the activation or increased stabilization of appropriate anti-oxidant defense proteins such as catalase or glutathione peroxidase. However, NKEF-B-transfected cells showed no change in activities of catalase, glutathione reductase, glutathione peroxidase or glutathione-S-transferase.[8] The ability of NKEF and thiol-specific antioxidant proteins to protect glutamine synthetase against oxidative inactivation has been established and serves as an exemplary model for

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understanding intracellular stabilization of enzyme activity. Oxidation of glutamine synthetase involves binding of Fe^{2+} to a divalent cation site on the enzyme followed by H_2O_2 dependent generation of ROS.[16] Oxidation of adjacent basic amino acid residues then renders the enzyme susceptible to proteolytic degradation. The NKEF-B protein and its homologs are able to interfere with a critical step in this sequence. Recent reports indicate that the thiol-specific antioxidant protein can be reduced by thioredoxin when thioredoxin reductase and NADPH are present.[17-19] Evidence suggests these components may represent the biological electron transfer pathway allowing the thiol-specific antioxidant to eliminate cellular peroxides. Accordingly this enzyme has been renamed thioredoxin peroxidase.

Assessment of lipid peroxidation using the TBARS assay revealed significantly lower levels of membrane oxidation in NKEF-B overexpressing cells compared with controls. Following 4 hours of exposure to HEPES-buffered Krebs Ringer at room temperature, TBARS levels were 40% lower in NKEF-B-transfected cells. Exposure to 10 mM DEM over this period resulted in TBARS levels which were similar in both cell types. As with ROS, basal levels of oxidative stress were lower in NKEF-B-transfected cells while pro-oxidant-induced injury was unaffected or only slightly affected by NKEF-B.

Thymidine-uptake studies, indicative of general cellular injury, allowed for analysis of prolonged low-dose exposure to oxidant stress. Whereas NKEF-B provided no apparent protection from 1 hr exposure to 50 μM menadione in the ROS assay, significant protection was observed following 20 hr exposure to 4–8 μM menadione in the [^{3}H]-thymidine uptake assay. This apparent paradox is probably due to an inability of NKEF-B to suppress oxidative injury in an acute, high-dose paradigm such as the ROS assay where the functional activity of NKEF-B may be overwhelmed. A similar high-dose/low-dose discrepancy was observed with the glutathione-depleting agent DEM in the lipoperoxide assay (Fig. 4) and N-ethylmaleimide in the thymidine uptake assay (data not shown). However, high concentrations of tBOOH do not seem to counteract the protective anti-oxidant action of NKEF-B (Fig. 1 and Table I).

At higher concentrations, DEM will react readily with protein sulfhydryls. Since the NKEF family of proteins has an absolute requirement for two critical cysteine residues (cys 47 and cys 170

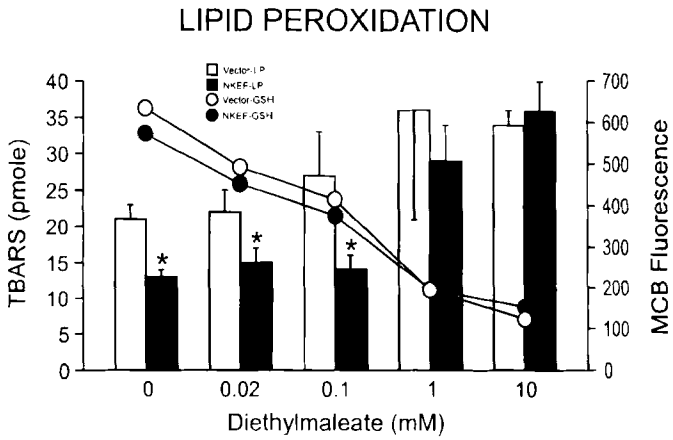

FIGURE 4 Effect of DEM on lipid peroxidation (vertical bars) and intracellular glutathione levels (linear graphs). Lipid peroxidation values represent means of 13–43 determinations ± SEM obtained from 10 experiments. GSH values represent means of 8 determinations obtained from 2 experiments (SEM ≤ 10%).

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TABLE II CELLULAR LIPID PEROXIDATION

	CELLS	
	Control	NKEF-B
Control	21.3 ± 2.8[a]	12.7 ± 1.8[b]
DEM	34.0 ± 2.4	36.3 ± 4.5

[a]Values represent pmoles TBARS per 10^5 cells per well and are expressed as means of 20-32 determinations ± SEM obtained from 5 different experiments.
[b]$p < 0.01$ compared with vector-transfected controls using student's T-test.

for NKEF-B), it is likely that NKEF-B is inactivated at higher concentrations of the sulfhydryl-reactive agents.

Despite having little or no effect on intracellular levels of reduced GSH, NKEF-B was able to confer protection against both ROS accumulation and lipid peroxidation and to help maintain cell viability. With the exception of t-butylhydroperoxide, acutely-administered toxins produced effects which were not influenced by NKEF-B. These results suggest that the NKEF-B protein most effectively suppresses chronic, low-level oxidative damage generated as a consequence of basal metabolism.

Such activity would be highly advantageous if expressed in long-lived cells such as neurons which may gradually accumulate oxidized or aggregated proteins. Normal aging and neurologic disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis are characterized by gradual accumulation of insoluble proteinaceous and lipoidal materials such as β-amyloid, lipofuscin and cytoskeletal aggregates.[20,21] Increased lipid peroxidation and other signs of oxidative injury have been reported in Alzheimer's and other neurologic disorders.[22,23] These biochemical disturbances likely play a significant role in the pathogenesis of these disorders. The ability to pharmacologically manipulate the expression of an anti-oxidant gene such as *nkef-B* may have a significant therapeutic effect in ameliorating or delaying progression of these degenerative disorders.

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