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Nitric oxide protects against mitochondrial permeabilization induced by glutathione depletion: Role of S-nitrosylation? ☆

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Abstract

Nitric oxide (NO) is known to mediate a multitude of biological effects including inhibition of respiration at cytochrome *c* oxidase (COX), formation of peroxynitrite ($ONOO^-$) by reaction with mitochondrial superoxide ($O_2^{\cdot -}$), and S-nitrosylation of proteins. In this study, we investigated pathways of NO metabolism in lymphoblastic leukemic CEM cells in response to glutathione (GSH) depletion. We found that NO blocked mitochondrial protein thiol oxidation, membrane permeabilization, and cell death. The effects of NO were: (1) independent of respiratory chain inhibition since protection was also observed in CEM cells lacking mitochondrial DNA (ρ^0) which do not possess a functional respiratory chain and (2) independent of $ONOO^-$ formation since nitrotyrosine (a marker for $ONOO^-$ formation) was not detected in extracts from cells treated with NO after GSH depletion. However, NO increased the level of mitochondrial protein S-nitrosylation (SNO) determined by the Biotin Switch assay and by the release of NO from mitochondrial fractions treated with mercuric chloride (which cleaves SNO bonds to release NO). In conclusion, these results indicate that NO blocks cell death after GSH depletion by preserving the redox status of mitochondrial protein thiols probably by a mechanism that involves S-nitrosylation of mitochondrial protein thiols.

Keywords: Nitric oxide; Glutathione; S-nitrosylation; Mitochondrial membrane permeabilization; Peroxynitrite; Superoxide; Nitrotyrosine; Reactive oxygen species

Nitric oxide (NO), a free radical gas with a diverse range of cellular actions (reviewed in [1,2]), is extremely hydrophobic and typically nonreactive which allows it to diffuse relatively large distances in the hydrophobic interior of cell membranes [3]. Because mitochondria are extensively membranous, they are key targets for NO [4]. In particular, NO is well known to bind to the binuclear metallic center of cytochrome *c* oxidase (COX), the terminal electron acceptor of the electron transport chain (ETC), and reversibly inhibit mitochondrial respiration [5–7].

NO also reacts with reactive oxygen species (ROS) including the superoxide anion ($O_2^{\cdot -}$) which is generated during respiration by electron leakage at respiratory complexes I and III [8–10]. The product of this reaction is the potent oxidant peroxynitrite ($ONOO^-$) [11,12]. Since the second-order rate constant ($k = \sim 1 \times 10^{10}\ M\ s^{-1}$) for this

☆ *Abbreviations:* ANT, adenine nucleotide translocator; BgK, bongkrekic acid; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3 oxide; COX, cytochrome *c* oxidase; DEM, diethylmalate; DEA-nonoate, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; DEM, diethylmaleate; DETA-nonoate, (*Z*)-1-[2-(2-Aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; spermine-nonoate, (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}- diazen-1-ium-1,2-diolate; 5,5′-dithiobis(2-nitrobenzoic acid), DTNB; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; ETC, electron transport chain; FBS, fetal bovine serum; MnSOD, manganese superoxide dismutase; MPT, mitochondrial permeability transition; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NO, nitric oxide; PVT, protein vicinal thiols; RNS, reactive nitrogen species; SD, standard deviation of the mean.

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reaction exceeds the rate constant for the dismutation of $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2) catalyzed by manganese superoxide dismutase (MnSOD) ($k = \sim 2 \times 10^9$ M s^{-1}) [13], it suggests that in respiring mitochondria, $ONOO^-$, rather than H_2O_2, may be generated if NO is present [14,15].

Another key feature of NO biology is post translational modification of protein thiols by S-nitrosylation which requires oxygen or $O_2^{\bullet -}$ and the intermediate formation of a nitrosonium ion (NO^+) [16,17]. There are also a variety of putative oxygen independent mechanisms leading to S-nitrosylation including transnitrosylation (direct transfer of NO^+ from compounds such as *S*-nitrosoglutathione (GSNO) to thiolate) [18,19] or direct reaction of NO with a thiolate [20].

However, although it is clear that NO targets mitochondria and can undergo a variety of different reactions, it is not clear which reactions predominate during conditions of severe redox stress as occurs during glutathione (GSH) depletion. Since GSH loss is a common feature in many diseases, identification of pathways of mitochondrial NO metabolism after GSH depletion may provide clues as to the potential use of NO based compounds for therapeutic purposes [21].

Materials and methods

Materials and cell culture. All chemicals were of reagent grade and were obtained from Sigma. Diethylmaleate (DEM) was used as a concentration of 5 mM. Tetra-methyl rhodamine methyl ester (TMRM) and dichlorofluorescein diacetate (DCFDA) were obtained from Molecular Probes (Eugene Oregon). DEA-nonoate (DEANO) and spermine-nonoate (sperNO) were used at concentrations of 100 μM and the NO scavenger carboxyPTIO (cPTIO) was used at 1 mM (Alexis Biochemicals, Lausen, Switzerland). Lymphoblastic leukemic CEM parental (ρ^+) cells were cultured in RPMI, 10% FBS, and supplements as described previously [22]. Cells were passaged daily to maintain them in log-phase growth and kept at a nominal concentration of $2.5–5 \times 10^5$/ml. CEM ρ^0 cells were derived from CEM cells by culturing in the presence of 50 ng/ml ethidium bromide for 6 weeks. ρ^0 cells were then cultured in RPMI with 1 mM pyruvate and 50 μg/ml uridine added, and regularly monitored for oxygen consumption.

Biochemical methods. Mitochondrial fraction isolation was performed according to [23] with modification. Protein concentration of each fraction was determined by the D*c* protein assay (Bio-Rad, Hercules, CA). Oxygen consumption was determined polarographically using a Clarke-type oxygen electrode (Hansatech Instruments, Norfolk, England); DTNB reactive protein thiols in control mitochondrial fractions were estimated after subtracting DTNB non-protein reactive thiols (including GSH). DTNB reactive mitochondrial protein thiols were determined by the method of Ellman with modification [24]. GSH and GSSG levels were measured using a commercial kit (Cayman Chemicals, Ann Arbor, MI, USA); the redox potential (E_h) was calculated by the Nernst equation using cell volume as 7 μL/million cells [25] and E_0 at pH 7.0 was taken as −240 mV [26].

Determination of nitrotyrosine. CEM cells were cultured in RPMI (control), RPMI + DEM; or RPMI + DEM + sperNO (1–1000 μM) for 120 min and cell extracts were prepared with protease inhibitors as described previously [27]. Hydrogen peroxide-free $ONOO^-$ was synthesized as described previously and quantified in 1 N NaOH at 302 nm ($\varepsilon = 1670$ M^{-1} cm^{-1}) [27]. Rabbit polyclonal anti-nitrotyrosine antibodies were from either Upstate Biotechnology (Lake Placid, NY, USA; #12-348) or Biomol (Plymouth Meeting, PA, USA; #SA-297). Peroxidase-conjugated secondary antibodies for Western blotting were purchased from Promega. For positive controls, cell lysates were treated with $ONOO^-$ for 5 min with protease inhibitors and 1 mM $ONOO^-$ was added to BSA to generate nitrated BSA as previously described [27]. Western blotting for nitrotyrosine containing proteins was conducted as described in [27] using polyclonal anti-nitrotyrosine antibodies with an enhanced chemiluminescence detection kit (GE Healthcare Bio-sciences, Amersham Place, Buckinghamshire, England) followed by analysis using a Kodak Image Analyser (IS440CF, NEN Life Science, Boston, MA, USA). Protein concentration was determined using Bio-Rad protein assay.

Electron microscopy. CEM cells in the logarithmic proliferation phase were cultured in either RPMI (control), RPMI + DEM; or RPMI + DEM and sperNO (100 μM) for 120 min and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 1 h. The cells were washed with 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer. Finally, the cells were dehydrated with graded series of ethanol, and embedded in LX112. Thin sections were prepared and stained with uranyl acetate. Specimens were examined on a JEOL 1000X electron microscope operating at 80 keV.

Flow cytometric analyses. Flow cytometry determinations for cellular ROS formation and $\Delta\psi_m$ were performed as described previously [22]. Briefly, for ROS determination, cells were loaded with 10 μM DCFDA for 15 min, washed with phosphate-buffered saline containing 10 mM glucose, and analyzed immediately by flow cytometry using the FL-2 setting (log mode). In each analysis, 10,000 events were recorded. For determination of $\Delta\psi_m$, cells were loaded with 250 nM TMRM (TMRM is a cationic dye which accumulates within mitochondria in accordance with the $\Delta\psi_m$ Nernst potential) for 15 min and red fluorescence was determined by flow cytometry using the FL-3 setting [22]. In each analysis, 10,000 events were recorded.

Biotin Switch for the determination of S-nitrosylated proteins. CEM mitochondrial fractions were isolated by differential centrifugation and protein S-nitrosylation was determined using the NitroGlo Nitrosylation System (PerkinElmer, Boston, MA, USA) according to the manufacturer's instructions with modification. In brief, 300 μl of blocker solution was added to each SDS cell extract and reacted for 1 h at 50 °C in the dark to block free thiols. The proteins were then precipitated, washed with cooled acetone, and solubilized in solubilization buffer. Nitrosylated proteins were reduced to free thiols using 50 mM ascorbic acid and biotinylated using pyridyldithiol-biotin for 1 h at room temperature. The samples were precipitated and electrophoresed on a 10% SDS gel under nonreducing conditions, at a constant 18 mA/h. Proteins were then transferred onto a PVDF membrane at 30 V for overnight in a cold room. The PVDF membrane was blocked with 1% BSA + PBST and incubated in primary anti-biotin antibody (1:500) overnight. This was followed by incubation in anti-biotin-HRP-conjugated secondary antibody (1:500) for 1 h and the blots were developed using ECL kit reagent.

Determination of NO released from mitochondrial fractions using mercuric chloride ($HgCl_2$). Mitochondrial fractions were treated with $HgCl_2$ (which cleaves the SNO bond of S-nitrosylated proteins) and releases gaseous NO. Briefly, mitochondrial fractions were transferred to plastic vials containing 1.0 ml PBS at 25 °C. NO production was then measured using an Apollo 4000 Free Radical Analyser (World Precision Instruments, Sarasota, FL, USA) equipped with an ISO-NOP3020 nitric oxide selective electrode after the addition of 1 mM $HgCl_2$.

Statistical analysis. Data are expressed as means ± standard deviation of the mean (SD) of three or more separate experiments performed in duplicate. For significance testing, ANOVA was used ($P < 0.05$).

Results

sperNO blocks cell death and preserves mitochondrial protein redox status after GSH depletion

Co-incubation of cells with sperNO completely preserved cell viability after treatment with DEM (to deplete GSH) (Fig. 1A). This effect was reversed by co-incubation of cells with cPTIO (a NO scavenger) (Fig. 1A). Whereas,

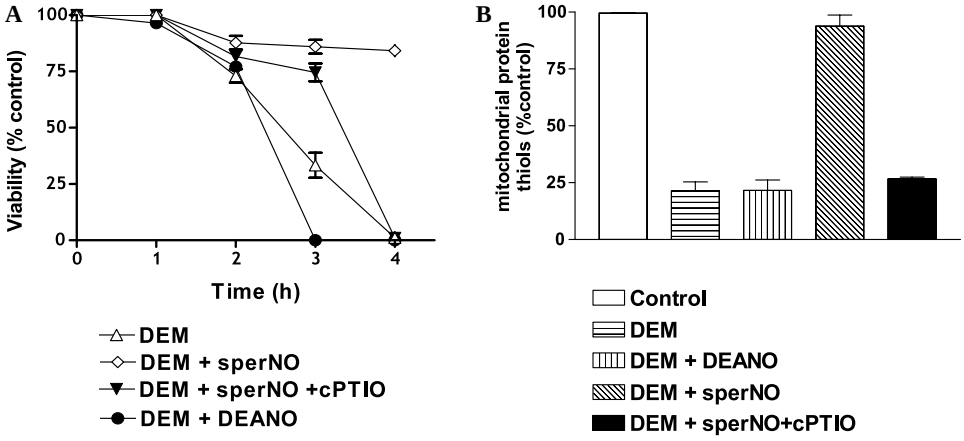

Fig. 1. (A) NO prevents loss of cell viability after GSH depletion. CEM cells were treated with (a) DEM, (b) DEM + sperNO, (c) DEM + DEANO or (d) DEM + sperNO + cPTIO. Cell viability was determined by trypan blue analysis. Data are expressed as means ± SD of three or more separate experiments performed in duplicate. (B) NO preserves mitochondrial protein thiol redox status. Cells were treated as described above with RPMI control, DEM, DEM + 100 μM sperNO, DEM + 100 μM sperNO + cPTIO or DEM + 100 μM DEANO and mitochondrial DTNB reactive thiols were determined as described under Materials and methods. Data are expressed as percentage of control (means ± SD of three independent experiments).

DEANO did not prevent loss of cell viability after treatment with DEM (Fig. 1A).

Mitochondrial protein thiol redox status of cells treated with DEM was significantly reduced compared to controls (21 ± 4.0% of control) (Fig. 1B). Mitochondrial protein thiol redox status of cells treated with DEM + sperNO was not significantly different to controls (94 ± 4.9% of control) (Fig. 1B). Mitochondria protein thiol redox status of cells treated with DEM + sperNO + cPTIO or DEM + DEANO was significantly reduced compared to controls (26 ± 0.79% of control) and 21 ± 4.56% of control, respectively (Fig. 1B). These results show that sperNO, but not DEANO, prevents (1) loss of cell viability after GSH depletion and (2) mitochondrial protein thiol oxidation after GSH depletion. GSH and GSSG levels and GSH redox (E_h) potential were determined on aliquots of CEM cells treated with DEM ± sperNO.

DEM treatment for 120 min significantly reduced total GSH levels (>95%) in the cells from 60.9 ± 2.4 nmol/mg protein to 0.2 ± 0.04 nmol/mg protein ($P < 0.05$). Co-treatment of cells with sperNO did not prevent GSH depletion after DEM treatment since GSH levels were 0.3 ± 0.03 nmol/mg protein ($P < 0.05$) (Table 1). DEM treatment for 120 min significantly reduced mitochondrial GSH (>95%) in CEM cells from 5.8 ± 0.17 to 0.12 ± 0.01 nmol/mg protein ($P < 0.05$). Co-treatment of cells with sperNO did not prevent mitochondrial GSH depletion after DEM treatment since GSH levels were 0.15 ± 0.02 nmol/mg protein ($P < 0.05$) (Table 1). The GSH Nernst redox potential (E_h) increased ~126 mV in cells treated with DEM compared to control cells and ~123 mV in cells co-treated with DEM + sperNO compared to control cells. $E_h = -265 \pm 8.9$ mV in control cells compared with $E_h = -139 \pm 5.5$ mV in DEM treated cells and $E_h = -142 \pm 6.7$ mV in CEM cells treated with DEM + sperNO (Table 1).

sperNO preserves mitochondrial membrane potential ($\Delta\psi_m$) and mitochondrial ultrastructure

Mitochondrial ultrastructure (electron microscopic (EM) analysis) and $\Delta\psi_m$ was performed on cells treated with DEM ± sperNO. CEM cells in the logarithmic proliferation phase were cultured in either RPMI (control), DEM; or DEM + sperNO for 120 min and processed as described under Materials and methods. Fig. 2A, caption "1" shows mitochondria in control cells; inset arrowheads point to mitochondrial cristae. Caption "2" shows mitochondria of cells treated with DEM; inset arrowheads point to (a) fragmented electron dense mito-

Table 1
Aliquots of CEM cells (approximately 4×10^6/ml) treated with either RPMI (control), DEM or DEM + sperNO for 120 min, and GSH and GSSG concentrations were determined as described under Materials and methods

	Nernst GSH redox potential E_h (mV)	Total GSH/GSSG (nmol/mg protein)	Mitochondrial GSH (nmol/mg protein)
Control	−265 ± 8.9	60.9 ± 2.4/1.1 ± 0.1	5.8 ± 0.17
DEM	−139 ± 5.5*	0.2 ± 0.04/0.8 ± 0.03*	0.12 ± 0.01*
DEM + sperNO	−142 ± 6.7*	0.3 ± 0.03/0.8 ± 0.08*	0.15 ± 0.02*

The GSH redox potential (E_h) in mV was calculated using the Nernst equation as described under Materials and methods. Data are expressed as means ± SEM ($n = 3$). (*$P < 0.05$) GSH/GSSG and GSH Nernst Redox values (E_h) in CEM cells.

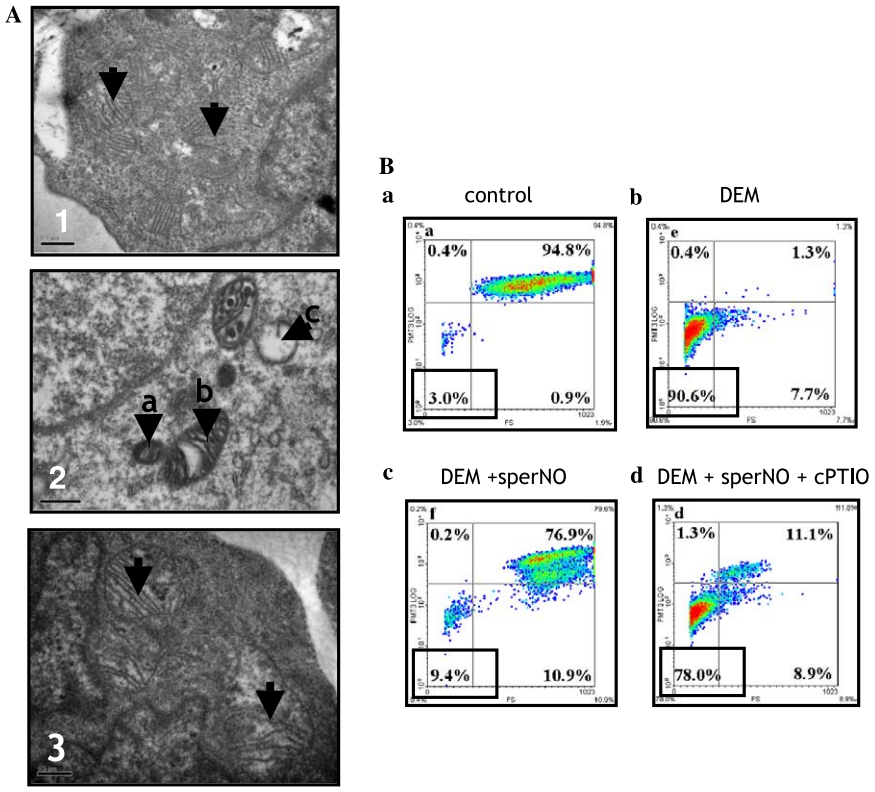

Fig. 2. (A) NO preserves mitochondrial ultrastructure after GSH depletion. Caption 1 shows mitochondria of untreated control cells; inset arrowheads point to mitochondrial cristae. Caption 2 shows mitochondria of cells treated with DEM; inset arrowheads point to (a) fragmented electron dense mitochondrion, (b) mitochondrion showing increased electron density of mitochondrial cristae, and (c) vacuolated mitochondrion. Caption 3 shows mitochondria of cells treated with DEM + sperNO; inset arrowheads point to mitochondrial cristae. (B) NO prevents loss of $\Delta\psi_m$ after GSH depletion. A representative example of $\Delta\psi_m$ determined by monitoring TMRM fluorescence of cells treated with DEM ± sperNO as described under Materials and methods. A representative example of TMRM fluorescence of cells treated with (a) RPMI (control); (b) DEM; (c) DEM + sperNO and (d) DEM + sperNO + cPTIO for 120 min. A TMRM two-dimensional color density plot showing percentage number of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top right quadrant) versus percentage number of cells with reduced $\Delta\psi_m$ (TMRM fluorescence in bottom left quadrant). Results show that DEM treatment for 120 min caused loss of TMRM fluorescence in ~91% of cell population compared to ~3% in control cells. Treatment with DEM + sperNO caused loss of TMRM fluorescence in ~9% of cell population while treatment with DEM + sperNO + cPTIO caused loss of TMRM fluorescence in ~78% of cells. Data are representative of three or more independent experiments.

chondrion, (b) mitochondrion showing increased electron density of mitochondrial cristae, and (c) vacuolated mitochondrion. Caption "3" shows mitochondria of cells treated with DEM + sperNO; inset arrowheads point to mitochondrial cristae. EM results indicate that sperNO preserves mitochondrial cristae (the sites of electron transport and oxidative phosphorylation) after GSH depletion.

Fig. 2B shows a representative example of TMRM fluorescence of cells treated with: (a) RPMI (control); (b) DEM; (c) DEM + sperNO and (d) DEM + sperNO + cPTIO for 120 min. TMRM two-dimensional color density plot showing percentage number of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top right quadrant) versus percentage number of cells with reduced $\Delta\psi_m$ (TMRM fluorescence in bottom left quadrant). Results show that DEM treatment for 120 min caused loss of TMRM fluorescence in ~91% of cell population compared to ~3% in control cells. Treatment with DEM + sperNO caused loss of TMRM fluorescence in ~9% of cell population while treatment with DEM + sperNO + cPTIO caused loss of TMRM fluorescence in ~78% of cells.

Nitrotyrosine not detected after treatment with DEM and sperNO

Since GSH depletion is known to increase mitochondrial (ROS) production, we considered that under these conditions NO could react with mitochondrial $O_2^{\cdot-}$ generating $ONOO^-$. We determined nitrotyrosine (a marker for $ONOO^-$) levels by immunoblotting [27]. Fig. 3A shows that nitrotyrosine levels were not increased in extracts of cells treated with sperNO (over a range of concentrations from 1 to 1000 μM) + DEM, suggesting that $ONOO^-$ was not formed in significant quantities in cells treated with sperNO after GSH depletion. These results indicated that NO was not scavenging mitochondrial ROS ($O_2^{\cdot-}$) and generating the reactive nitrogen species $ONOO^-$.

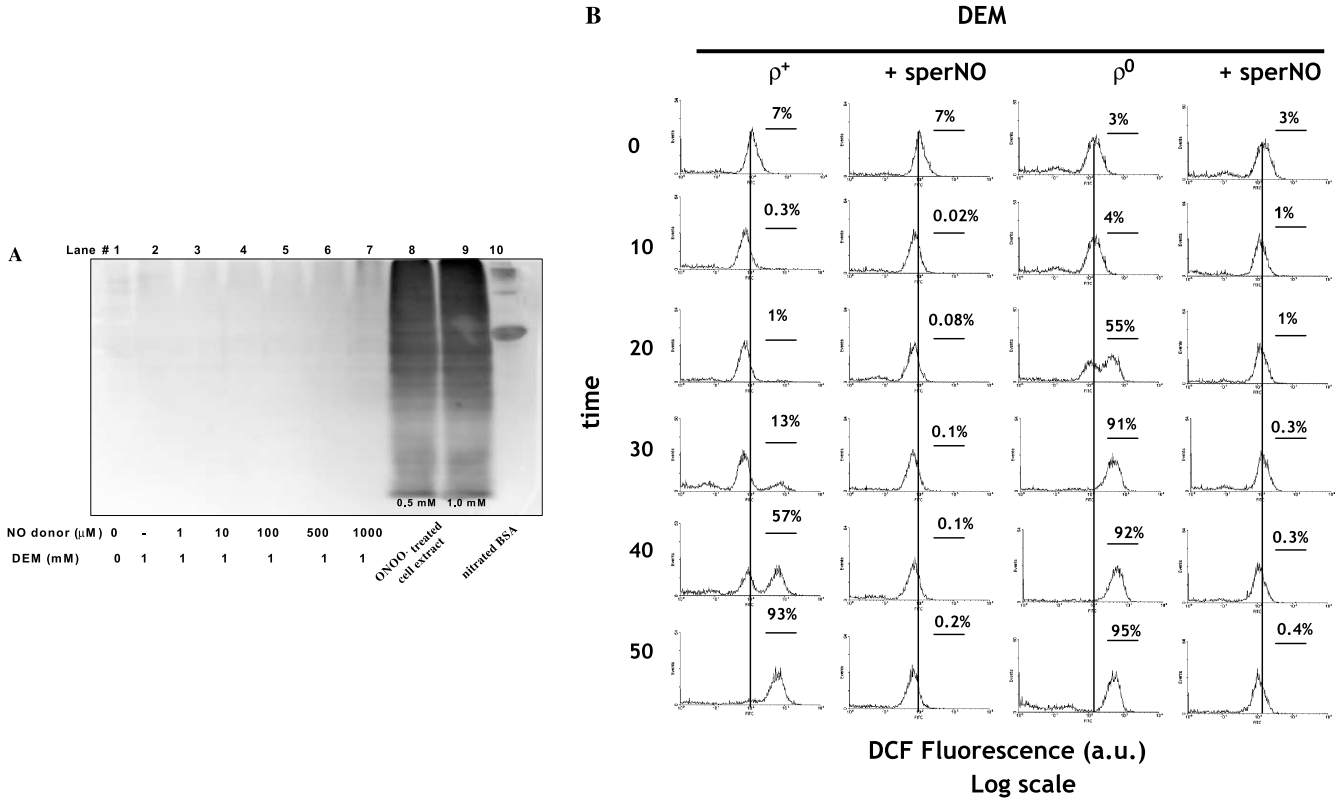

Fig. 3. (A) Nitrotyrosine not detected after treatment with DEM and sperNO. CEM cells were incubated with sperNO ± DEM at a range of concentrations: lanes: (1) control (RPMI), (2) DEM (alone), (3) 1 μM sperNO, (4) 10 μM sperNO, (5) 100 μM sperNO, (6) 500 μM sperNO, and (7) 1000 μM sperNO. Lanes 8–10: CEM cell lysates exposed to (8) 500 μM $ONOO^-$, (9) 1000 μM $ONOO^-$, and (10) BSA treated with 1 mM $ONOO^-$. The formation of 3-nitrotyrosine was estimated by immunoblotting as described under Materials and methods. Data are representative of two separate experiments. (B) NO blocks oxidation in CEM parental ρ^+ cells and cells lacking mitochondrial DNA (ρ^0) after GSH depletion. Representative DCF fluorescence histograms of CEM cells treated with DEM ± sperNO and determined by FACS analysis (FL-2 settings) as described under Materials and methods. After treatment, DCF fluorescence was measured on aliquots of ρ^+ and ρ^0 cells at 10 min intervals over 50 min. Results show that GSH depletion caused time-dependent increase in DCF fluorescence in both cell lines and this fluorescence increase was blocked by sperNO.

sperNO blocks DCF oxidation in parental CEM (ρ^+) cells and cells lacking mitochondrial DNA (ρ^0)

We next considered that NO might protect cell viability after GSH depletion by its effects on cell respiration (i.e., by inhibition of COX) since we have previously shown that specific mitochondrial inhibitors of respiration are cytoprotective after GSH depletion [10,22]. To investigate this, we created a CEM cell line lacking mitochondrial DNA (ρ^0) which does not possess a functional electron transport chain (ETC). Cells (ρ^+ and ρ^0) were treated with DEM ± sperNO and DCF fluorescence was measured by flow cytometry. Results show that GSH depletion caused progressive increase in DCF fluorescence in both ρ^+ and ρ^0 cells (indicating increased oxidation) which was completely blocked by sperNO (Fig. 3B). sperNO also preserved cell viability (determined by trypan blue exclusion) after DEM treatment in both ρ^+ and ρ^0 cell lines (data not shown). These results indicated that the cytoprotective effects of sperNO did not depend on its inhibitory action on mitochondrial respiration.

sperNO increases the level of mitochondrial protein S-nitrosylation after DEM treatment

We next considered that NO might prevent mitochondrial thiol oxidation and thereby preserve cell viability induced by GSH depletion by modifying the structure of mitochondrial protein thiols by S-nitrosylation [1,18,19]. Fig. 4A shows results of the BIOTIN SWITCH assay for S-nitrosylated proteins in mitochondrial fractions from cells treated with: lane 1, RPMI media (control); lane 2, DEM; lane 3, DEM + sperNO and lane 4, DEM + DEANO. Results show that sperNO, but not DEANO, increased the level of S-nitrosylated proteins determined by immunoblot. As a second measure of S-nitrosylation, mitochondrial fractions were treated with $HgCl_2$ (to cleave SNO bonds and release gaseous NO) and monitored for NO release using an NO selective electrode (see Materials and methods). Fig. 4B shows a representative trace of NO released from mitochondrial fractions of cells treated with: DEM alone, DEM + sperNO, DEM + sperNO + cPTIO, and DEM + DEANO. Mitochondria from cells treated with DEM + sperNO showed a robust release

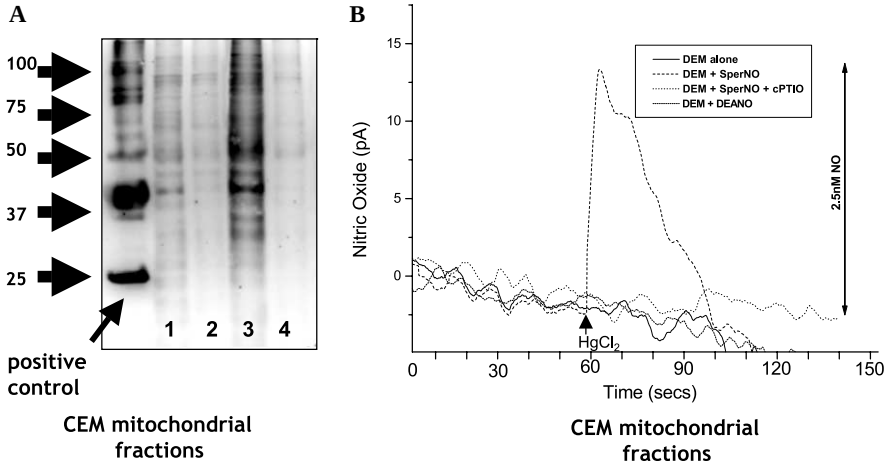

Fig. 4. (A) SperNO increases the level of mitochondrial protein S-nitrosylation after DEM treatment. Representative results of the BIOTIN-SWITCH assay for SNOs performed on the mitochondrial fractions of cells treated with: lane 1, RPMI media (control); lane 2, DEM; lane 3, DEM + 100 μM sperNO and lane 4, DEM + 100 μM DEA-nonoate. Data are representative of two experiments. (B) $HgCl_2$ causes release of NO from mitochondrial fractions of CEM cells treated with sperNO. Representative traces of NO released from mitochondrial fractions of cells incubated with DEM, DEM + sperNO, DEM + sperNO + cPTIO or DEM + DEANO and treated with $HgCl_2$ (to cleave the SNO bond). NO release was monitored using a NO sensitive electrode (see Materials and methods). NO released from the mitochondrial fractions of DEM + sperNO treated cells = 2.5 ± 0.3 nmol/ mg protein; whereas NO released from mitochondrial fractions of DEM, DEM + sperNO + cPTIO or DEM + DEANO treated cells was undetectable. Data show a representative trace of NO released from mitochondrial fractions (from three independent experiments).

of NO (2.5 ± 0.3 nmol/mg protein) upon treatment with $HgCl_2$ compared to mitochondrial fractions from cells treated with either DEM, DEM + sperNO + cPTIO or DEM + DEANO which did not release NO. These results show that NO is released from CEM cells protected from GSH depletion by sperNO indicating increased S-nitrosylation.

Discussion

In this study, we investigated pathways of NO metabolism in cells with increased oxidative stress caused by GSH depletion. Our results show that NO prevented mitochondrial protein thiol oxidation, mitochondrial permeabilization, and cell death by preserving the mitochondrial protein thiol redox-status.

For NO donors we used the diazeniumdiolates (a chemical species containing the [N(O)NO]2 functional group) since they decompose spontaneously in aqueous media to release NO and possess a range of half-lives (from minutes to hours) which allowed us to determine the relative relationship between the kinetics of NO release and level of cytoprotection after GSH depletion. Also, these compounds release NO in a redox-insensitive manner which was considered necessary since we were investigating NO metabolism in GSH-redox modulated cells [28,29]. We found that sperNO, which has a relatively long half-life ($t_{1/2} \sim 30$ min), completely protected cells after GSH depletion; whereas, DEANO with a short half-life ($t_{1/2} \sim 2$ min) was not protective. This indicated that sustained release of NO was required for cytoprotection after GSH. Rosenburg et al. also found that long half-life NO donors, but not short half-life donors, were cytoprotective in oligodendrocytes depleted of the amino acid cystine (which also results in GSH depletion) [30].

We first considered that NO might be cytoprotective by preserving the GSH levels of cells treated with DEM, but found no significant difference in GSH levels or GSH Nernst redox potential (E_h) after DEM treatment either in the presence or absence of NO donor which indicated that the effect of NO was independent of the GSH-redox status (Table 1). Since NO did not prevent GSH redox-change, we considered that ROS formed as a consequence of GSH depletion could react with NO and form the oxidant $ONOO^-$ [13]. Indeed, Miles et al. [31] showed that sperNO + $O_2^{\bullet -}$ (generated by xanthine + xanthine oxidase) oxidized the fluorescent dye dihydrorhodamine which suggested that $ONOO^-$ was responsible. Since $ONOO^-$ is known to react with protein tyrosine groups and form nitrotyrosine, we determined nitrotyrosine levels on cell extracts from cells treated with sperNO ± DEM by immunoblotting, but did not observe a significant increase in nitrotyrosinated proteins (Fig. 3A). Since relative levels of NO and $O_2^{\bullet -}$ are suggested to be critical for $ONOO^-$ production, our result suggests that after GSH depletion, $O_2^{\bullet -}$ flux in CEM cells is insufficient to generate this potent nitrosative oxidant [31]. We next considered that NO might be protective by inhibition of respiration at COX, since pharmacological inhibitors of mitochondrial electron transport have been shown to be cytoprotective after GSH depletion [10,22]. Therefore, we created a CEM (ρ^0) cell line lacking mitochondrial DNA which are respiration deficient and performed experiments to determine whether NO was protective in ρ^0 cells. We found that DEM increased oxidation (determined by measuring DCF fluorescence) in respiration deficient ρ^0 cells as well as parental ρ^+ cells which was blocked equally effectively in both cell

lines by sperNO. These results indicated that GSH depletion resulted in cell oxidation independently of mitochondrial ROS formation since ρ^0 cells are not expected to generate mitochondrial ROS because they lack a functional ETC [32,33]. Recent work by Jakubowski and Bartosz [34] has indicated that DCF is a general fluorescent probe for oxidative stress rather than a specific probe for mitochondrial ROS, suggesting that its oxidation results from GSH depletion and protein thiol oxidation as well as from increased mitochondrial ROS production. Taken together, our results indicated that the cytoprotective effect of NO involved targeted protection of mitochondrial protein thiols rather than from effects on either cell respiration or formation or other reactive species.

NO is known to target proteins and modify their function through post-translational modification including S-nitrosylation [1,16–19]. Therefore, we considered that NO might be preventing mitochondrial protein thiol oxidation by this modification and thereby regulating mitochondrial integrity. To determine whether mitochondrial proteins were S-nitrosylated after treatment with sperNO, we used the Biotin-Switch assay [35]. As a second measure of S-nitrosylation, we determined NO release from the mitochondrial fractions of cells treated with NO + DEM using $HgCl_2$ to cleave the SNO bond [36,37]. NO was released from the mitochondrial fractions of cells treated with DEM + sperNO, but not from cells treated with DEM + DEANO which indicated that increased mitochondrial protein S-nitrosylation correlated with protection from mitochondrial permeabilization and cell death after GSH depletion (Fig. 4B). Together, our results implicate a role for S-nitrosylation in regulating mitochondrial integrity and blocking cell death after GSH depletion.

Since GSH loss and premature cell death are common features of diseases such as Parkinson's disease and Alzheimer's disease, this work shows a potential beneficial role of NO based releasing compounds for therapeutic use in these and similar diseases.

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