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Inhibition of ROS-induced apoptosis in endothelial cells by nitron spin traps *via* induction of phase II enzymes and suppression of mitochondria-dependent pro-apoptotic signaling

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Abstract

Oxidative stress is the main etiological factor behind the pathogenesis of various diseases including inflammation, cancer, cardiovascular and neurodegenerative disorders. Due to the spin trapping abilities and various pharmacological properties of nitrones, their application as therapeutic agent has been gaining attention. Though the antioxidant properties of the nitrones are well known, the mechanisms by which they modulate the cellular defense machinery against oxidative stress is not well investigated and requires further elucidation. Here, we have investigated the mechanisms of cytoprotection of the nitron spin traps against oxidative stress in bovine aortic endothelial cells (BAEC). Cytoprotective properties of both the cyclic nitron 5,5-dimethyl-pyrroline *N*-oxide (DMPO) and linear nitron alpha-phenyl *N*-tert-butyl nitron (PBN) against H₂O₂-induced cytotoxicity were investigated. Preincubation of BAEC with PBN or DMPO resulted in the inhibition of H₂O₂-mediated cytotoxicity and apoptosis. Nitron-treatment resulted in the induction and restoration of phase II antioxidant enzymes *via* nuclear translocation of NF-E2-related factor 2 (Nrf-2) in oxidatively-challenged cells. Furthermore, the nitrones were found to inhibit the mitochondrial depolarization and subsequent activation of caspase-3 induced by H₂O₂. Significant down-regulation of the pro-apoptotic proteins p53 and Bax, and up-regulation of the anti-apoptotic proteins Bcl-2 and p-Bad were observed when the cells were preincubated with the nitrones prior to H₂O₂-treatment. It was also observed that Nrf-2 silencing completely abolished the protective effects of nitrones. Hence, these findings suggest that nitrones confer protection to the endothelial cells against oxidative stress by modulating phase II antioxidant enzymes and subsequently inhibiting mitochondria-dependent apoptotic cascade.

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Keywords

nitrones; spin traps; oxidative stress; endothelial dysfunction; Nrf-2

1. Introduction

Oxidative stress has been a hallmark of pathogenesis behind a number of disorders including chronic obstructive pulmonary disease (COPD) [1–3], cardiovascular disorders [4, 5], ischemia/reperfusion injury [6], neurodegenerative disorders [7, 8] and cancer [9]. In biological systems oxidative stress is induced by reactive oxygen species (ROS), a group of highly reactive molecules that include hydroxyl radical ($\text{HO}^\bullet$), peroxy radical ($\text{ROO}^\bullet$), superoxide anion radical ($\text{O}_2^{\bullet-}$) or non-radical compounds such as hydrogen peroxide (H_2O_2) or singlet oxygen ($^1\text{O}_2$). It has been reported that approximately 1–3% of the oxygen consumed by the body is converted into ROS [10]. Increased production of ROS in the vasculature leads to endothelial dysfunction [11, 12], which acts as the main etiological factor behind several cardiovascular disorders (CVD) such as atherosclerosis, hypercholesterolemia, hypertension, diabetes, and heart failure [13, 14]. To attenuate the ROS-mediated pathogenesis against oxidative stress, use of natural antioxidants such as vitamin E, vitamin C, epigallocatechin gallate (EGCG), and theaflavins [15–17], as well as synthetic antioxidants such as SOD-mimetics (e.g. M40403 and M40419), salens (e.g. EUK134), and GPX mimetics (ebselen, BXT-51072) [17, 18], have been suggested. However dietary supplementation with natural antioxidants such as vitamins E, C, and carotenoids showed no positive outcomes in CVD clinical trials [19] and in some cases contributed to increased mortality [20–22]. Hence the use of targeted synthetic antioxidants has gained attention due to their rich chemistry and opportunities for modulating their physico-chemical properties as well as their target specificity to enzyme active sites, organelles, or cells for optimal pharmacological activity.

Nitrones are compounds with a general common moiety of $\text{R}_1\text{R}_2\text{C}=\text{N}^+\text{O}^-(\text{R}_3)$ (where $\text{R}_1 = \text{H}$; $\text{R}_1, \text{R}_2, \text{R}_3 =$ alkyl group), and are used extensively as the spin-traps for their ability to form adducts with transient free radicals [23]. To date, there are two major types of spin traps (Fig. 1) that are commonly used for spin trapping, the linear nitrones (e.g., α -phenyl *N*-tertiary-butyl nitron (PBN) (Fig. 1A) and cyclic nitrones (e.g., 5,5-dimethyl-pyrroline *N*-oxide (DMPO) (Fig. 1B). Due to their unique chemical properties and biological activity [24], nitrones have been used as therapeutic agents against several ROS-related disorders such as brain injury [25], ischemia/reperfusion-induced cardiomyopathy [26], visual impairment [27], neuronal damage [28], and cancer [29, 30]. Moreover, nitrones also showed potential as therapeutic agent against stroke [31, 32] and can lead to the improvement of cerebral blood flow in animal models [33]. Previous reports suggest that both the PBN and DMPO are capable of conferring protection to endothelial cells against oxidative stress-induced injury, but the mechanisms of this protection remains unclear [26, 34–36]. Decrease in nitric oxide (NO) bioavailability has been known to be a hallmark in ROS-induced endothelial dysfunction [37]. NO has several beneficial vascular effects such as vasodilatation, regulation of vascular smooth muscle proliferation, and expression of cellular adhesion molecules [38, 39]. Furthermore, it has been reported that the $\text{O}_2^{\bullet-}$ adduct of the nitron spin traps may undergo unimolecular decomposition to yield NO [40, 41]. Hence the application of nitrones may lead to the improvement of the vascular health by counterbalancing the loss of NO bioavailability under oxidative stress. Previous experimental evidences also indicate that application of nitrones confers protection against ROS-induced mitochondrial dysfunction by salvaging the mitochondrial bio-energetics and enzymatic activities of the mitochondrial ETC complexes [26].

The phase II antioxidant enzymes play a key role in protecting cells and tissues from oxidative and electrophilic stresses. Induction of these antioxidant enzymes is regulated primarily by the antioxidant or electrophile response elements (ARE/EpRE), which are found in the 5'-flanking region of many phase II and antioxidant genes [42–44] such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx) and glutathione reductase (GR). The coordinated action of these phase II enzymes ensures effective detoxification of oxidative and electrophilic species. For example HO-1 is known to possess antioxidant, anti-apoptotic, anti-thrombotic, and anti-inflammatory properties [45], while GPx and GR function coordinately to counterbalance oxidative stress. GPx catalyzes the direct decomposition of H_2O_2 via the oxidation of reduced glutathione (GSH) to GSSG, which is then converted to GSH by glutathione reductase (GR) [46]. The enzyme NQO1 catalyzes the two-electron reduction of electrophilic quinone compounds to form the less reactive hydroquinones and serves as an antioxidant enzyme [47].

The transcription factor NF-E2-related factor 2 (Nrf-2) plays a critical role in trans-activating phase II enzyme expression and thus has become an important therapeutic target for natural and synthetic antioxidants against oxidative stress [48–51]. Upon activation, Nrf-2 translocates to the nucleus and binds to the antioxidant response element (ARE) to modulate the expression of the important phase II enzymes [52–54]. Aside from its role in regulating carcinogen detoxification and cellular antioxidative defense machineries, Nrf-2 also has anti-inflammatory functions [55, 56] and can therefore act as a potential therapeutic target for various antioxidant drugs.

In this study, we identified the molecular mechanisms of cytoprotection by nitrones during oxidative stress. The treatment of endothelial cells with nitron resulted in inhibition of H_2O_2 -induced cytotoxicity accompanied by significant lowering of ROS generation. In addition, nitrones suppressed the H_2O_2 -induced mitochondrial-dependent activation of caspase-3 and nuclear accumulation of the transcription factor Nrf-2 with subsequent activation of the phase II antioxidant enzymes. Together, these studies uncover novel aspects of the cytoprotective mechanisms of nitrones and provide evidence of their potential use to inhibit oxidative-stress induced apoptosis.

2. Materials and methods

2.1. Materials

BAEC were purchased from Cell Systems, Kirkland, WA. Tissue culture materials such DMEM, fetal bovine serum (FBS), penicillin-streptomycin, trypsin and MEM-non essential amino acids were purchased from Gibco, Invitrogen (Grand Island, NY). DMPO was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD) and PBN was purchased from Sigma (St. Louis, MO). Primary antibodies (against p53, Bax, Bcl-2, p-Bad and Nrf-2) and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear and cytoplasmic extraction kit was purchased from Thermo Scientific (Rockford, IL), while protein estimation kit was purchased from Bio-Rad (Hercules, CA). Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (Franklin Lakes, NJ.). 2,7-dichlorofluorescein diacetate (DCF-DA), Rhodamine 123, 4',6-diamidino-2-phenylindole (DAPI), NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2,6-dichloroindophenol, EDTA, GSH, glutathione reductase, and o-phthalaldehyde were purchased from Sigma (St. Louis, MO.). The Nrf-2-siRNA and scramble siRNA-Control were purchased from Qiagen (Valencia, CA 91355).

2.2. Cell culture and treatment

BAEC were cultured in DMEM medium with 4.5 g/L D-glucose and 4 mM L-glutamine, and supplemented with 10% fetal bovine serum, 2.5 mg/L endothelial cell growth supplement, and 1% MEM-non essential amino acids at 37 °C in a humidified atmosphere of 5% CO₂ and 20% O₂. The medium was changed every 2–3 days and cells were subcultured once they reached 90–95% confluence. For cytotoxicity studies, BAEC were pretreated with nitron alone (0–100 µM) or in the presence of 100 µM H₂O₂ for 24 h, while for cytoprotection studies, cells were preincubated with nitron (0–50 µM) for 24 h and then subsequently treated with 100 µM H₂O₂ for 24 h.

2.3. Cell viability assay (MTT Assay)

Cell viability was assessed by MTT assay following the manufacturers protocol as previously described and the formation of the formazan was measured using a Varian microplate reader absorbance 570 nm (50 MPR, Foster City, CA) [57]. BAEC were grown to 80% confluency and incubated with nitron (0–100 µM) for 24 h to assess their cytotoxicity. For cytoprotection studies BAEC were preincubated with nitron (0–100 µM) for 24 h, then treated with 100 µM H₂O₂ and incubated for additional 24 h. Data were calculated as the percentage of inhibition by the following formula (eqn 1.):

$$\% \text{ inhibition} = [100 - A_t / A_s - 100] \quad (1)$$

where A_t and A_s indicated the absorbance of the test substances and solvent control, respectively.

2.4. Detection of Apoptosis

For flow cytometric analysis of apoptosis treated cells were stained with annexin FITC-conjugated annexin V (1 µg/ml) and propidium iodide (PI) (0.5 µg/ml) in a Ca²⁺-enriched binding buffer for 30 min at room temperature as previously described [58]. Percentage of apoptosis, was assessed using a Becton Dickinson FACS Calibur (San Jose, CA, USA) and data were analyzed using Cell Quest version 3.3 software. For confocal microscopy studies, BAEC were cultured over glass coverslips reaching 60–70% confluency, prior treatment, and subsequently stained with Annexin V-FITC (1 µg/ml) and DAPI (1 µM) at 37 °C for 1h. Images were captured using an Olympus FluoView-1000 confocal microscope (Olympus America Inc., Center Valley, PA) at 40 X magnification (emission filters 570 nm for DHE and 495 nm for DAPI. Fluorescence intensities of 100 cells from different fields were calculated using the software FV10-ASW 2.1 (Olympus America Inc., Center Valley, PA). Results were presented as mean ± SEM from three independent experiments (*n* = 3).

2.5. Detection of Reactive oxygen Species (ROS)

For flow cytometric analysis of ROS, treated BAEC were washed with phosphate buffered saline (PBS), and incubated with 25 µM of DCFH-DA at 37 °C for 30 min. ROS generation was assessed by flow cytometry at excitation and emission wavelengths of 488 nm and 535 nm, respectively. For the assessment of ROS by confocal microscopy studies, BAEC cells were washed after treatments with PBS three times and subsequently incubated with 25 µM of the fluorogenic probe 2',7'- dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min at 37 °C, followed by nuclei staining with DAPI (1 µM) for 1h. Images were then captured by Olympus FluoView-1000 confocal microscope (Olympus America Inc., Center Valley, PA) and fluorescence intensities of 100 cells from different fields were calculated using the method described above. Results were presented as mean ± SEM from three independent experiments (*n* = 3).

2.6. Determination of mitochondrial membrane potential ψ (MMP)

Changes in MMP in BAEC treated for varying time period (0–24 h) were determined using flow cytometry by staining cells with the fluorescent probe Rhodamine 123 (5 $\mu\text{g/mL}$). After treatment BAE cells were incubated with rhodamine 123 (5 $\mu\text{g/mL}$) for 60 min in the dark at 37 °C, harvested and suspended in PBS. The MMP was then determined using FACS by measuring the fluorescence intensity (FL-1, 530 nm) [59]. All experiments were done in triplicates and the percent of mean fluorescence intensity (% MFI) was plotted for each sample.

2.7. Determination of caspase-3 activity

Caspase-3 activity was determined by using the DEVD-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) assay as described previously [60]. After treatment, cells were incubated in a cyto-buffer (10% glycerol, 50 mM Pipes, pH 7.0, 1 mM EDTA, 1 mM DTT) containing 20 μM of the tetrapeptide substrate DEVD-AFC (Enzyme Systems Products, Livermore, CA). Release of free AFC was determined using a Cytofluor 4000 fluorometer (Perseptive Company, Framingham, MA, USA; Filters: excitation; 400 nm, emission; 508 nm).

2.8. Preparation of cell extracts, western blot analysis, and silencing

Nuclear translocation of the transcription factor Nrf-2 in nitro-treated BAEC was detected by western blotting and confocal microscopy. For confocal analysis, BAEC were fixed after treatments with 2% *para*-formaldehyde followed by incubation with permeabilization solution (0.1% Na-citrate, 0.1% Triton) for 1 h at room temperature. Cells were first blocked using 5% BSA solution o/n at 4 °C and incubated with goat polyclonal anti-Nrf-2 antibody (1:200 dilutions, Santa Cruz, sc-722) for 3 h at room temperature followed by the incubation with anti-goat FITC-conjugated IgG antibody (1:150 dilutions) and DAPI (1 μM) for 2 h at room temperature. After incubation, cells were washed with PBS and images were taken by Olympus FluoView-1000 confocal microscope (Olympus America Inc., Center Valley, PA) at 60X magnification.

Subcellular localization of Nrf-2 was analyzed by western blotting BAEC nuclear and cytoplasmic extracts. Briefly, cells were washed once with cold PBS, scraped off the dish and collected by centrifugation at $500 \times g$ for 5 min, and fractions were collected by adding extraction reagents as indicated by manufacturers at 200:11:100 (v/v) (Thermo Scientific, Rockford, IL). Equal amounts of protein were loaded and mouse monoclonal anti-glyceraldehydes-3-phosphate dehydrogenase (1:1000 dilutions, Santa Cruz, sc-35448) and mouse monoclonal anti-lamin B (1:1000 dilutions, Santa Cruz, sc-365962) antibodies were used as the loading controls for the cytosolic and nuclear fractions.

Western blot analysis was also performed to determine the expression levels of p53, Bax, Bcl-2 and p-BAD proteins in untreated, H_2O_2 -treated, and nitro-pre incubated H_2O_2 -treated BAEC. After treatment cells were harvested, washed with cold PBS (pH 7.4) and lysed with BD lysis buffer (1 mM PMSF, 10 $\mu\text{g/mL}$ aprotinin and 10 $\mu\text{g/mL}$ leupeptin, pH 7.4) for 30 min and centrifuged at $12000 \times g$ for 30 min at 4 °C. The protein concentration of the clear supernatant was measured using Bio-Rad protein assay kit (Hercules, CA), taking bovine serum albumin as standard. In separate experiments, the membrane was incubated with mouse monoclonal anti-p53 antibody (1:1000 dilution, Santa Cruz, sc-126), mouse monoclonal anti-Bax antibody (1:1000 dilution, Santa Cruz, sc-70407), mouse monoclonal anti-Bcl-2 antibody (1:1000 dilution, Santa Cruz, sc-7382), mouse monoclonal anti-p-Bad (ser 136) antibody (1:500 dilution, Santa Cruz, sc-7999) and mouse monoclonal anti- β -actin antibody (1:1000 dilution, Santa Cruz, sc-47778) overnight at 4 °C. After washing with tris-buffered saline containing 0.1% Tween-20, the membranes were then incubated with HRP conjugated anti-mouse secondary antibody (1:2000 dilution, Santa Cruz

Biotechnology). The protein bands were visualized using chemiluminescence kit from Thermo Scientific, and the bands were quantified densitometrically by Bio-Rad Chemi Doc (1708070) system using the software Quantity One (Bio-Rad).

For silencing experiments, BAEC cells were transfected at 60% confluency with 50 nM Nrf-2-siRNA (sequence for targeting Nrf-2 was 5-TTGACAATTATCATTCTATT-3', Qiagen, Cat-No: 1027423) or siRNA-control (Qiagen, Cat-No: 1027292) using DharmaFect (Dharmacon, Inc, IL, T-2002-02) following manufacturer protocols. Forty-eight hours after transfection, the cells were used to assess silencing by immunoblotting using goat polyclonal anti-Nrf-2 antibody (1:500 dilutions, Santa Cruz, sc-722) and mouse monoclonal anti- β -actin antibody (1:1000 dilution, Santa Cruz, sc-47778) as loading control and for experiments showed under results.

2.9. Assessment of phase II antioxidant enzymes

After treatment BAEC were collected, resuspended in 300 μ l of ice cold assay buffers, sonicated, followed by centrifugation at $14\text{--}18000 \times g$ for 10 min at 4 $^{\circ}\text{C}$ and the supernatant were collected and stored at -80°C . Cellular HO-1 activity was measured according to previous procedure [61]. Briefly, cells were resuspended in 100 mM potassium phosphate buffer, pH 7.4, 2 mM with MgCl_2 , followed by three cycles of freeze-thawing and sonicated on ice before centrifugation at $18000 \times g$ for 10 min at 4 $^{\circ}\text{C}$. HO-1 assay was initiated by addition of 100 μ l ($\sim 200\text{--}250$ μg protein) of cell extracts to 200 μ l of reaction mix containing 0.5 mM NADPH, 2 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 0.2 mM hemin, and 1 mg/ml rat liver cytosol (as a source of biliverdin reductase) in 100 mM potassium phosphate buffer, pH 7.4, containing 2 mM MgCl_2 . The reaction was conducted for 1 h at 37 $^{\circ}\text{C}$ in the dark and terminated by addition of 300 μ l chloroform. The extracted bilirubin was measured by the difference in absorbance between 464 nm and 530 nm (extinction coefficient = $40 \text{ mM}^{-1} \text{ cm}^{-1}$) and HO activity was expressed as pmol of bilirubin formed per mg of cellular protein.

Cellular NQO1 activity was determined using dichloroindophenol as the two-electron acceptor, as described before [46]. Ten μ l of cell extract was added to a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 0.08% Triton X-100, 0.25 mM NADPH, 80 μM 2,6-dichloroindophenol (DCIP) in absence or presence of 25 μM dicoumarol, a potent inhibitor of NQO1. The two-electron reduction of DCIP was monitored at 600 nm at 25 $^{\circ}\text{C}$ for 3 min. Specific activity of NQO1 was calculated using the extinction coefficient of $21.0 \text{ mM}^{-1} \text{ cm}^{-1}$, and expressed as nanomole of DCIP reduced per minute per milligram of cellular protein.

Cellular GPx activity was measured as previously described [46]. Ten μ l of cell extract were added to a reaction mixture containing 170 μ l of 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and 2 mM sodium azide, 30 μ l of 10 mM GSH, 30 μ l of glutathione reductase (2.4 U/ml), and 30 μ l of 1.5 mM NADPH (prepared in 0.1% NaHCO_3). The mixture was incubated at 37 $^{\circ}\text{C}$ for 3 min. and after the addition of 30 μ l of 2 mM H_2O_2 , the rate of NADPH consumption was monitored at 340 nm, 37 $^{\circ}\text{C}$ for 5 min. GPx activity was calculated using the extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$, and expressed as nanomole of NADPH consumed per minute per milligram of cellular protein.

Cellular GR activity was measured following the protocol described before [46]. Briefly, to a reaction mixture containing 230 μ l of 50 mM potassium phosphate buffer (pH 7.0) and 1 mM EDTA, 10 μ l of cell extract and 30 μ l of 20 mM GSSG were added. The mixture was pre-warmed at 37 $^{\circ}\text{C}$ for 3 min. and the reaction was started by the addition of 30 μ l of 1.5 mM NADPH (prepared in 0.1% NaHCO_3). The subsequent consumption of NADPH was monitored at 340 nm, 37 $^{\circ}\text{C}$ for 5 min. GR activity was calculated using the extinction

coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nanomole of NADPH consumed per minute per milligram of cellular protein.

2.10. Measurement of intracellular GSH

Total GSH was determined by fluorometric methods as previously described [46]. Briefly, 10 μl of cell extracts were incubated with 12.5 μl of 25% *meta*-phosphoric acid, and 37 μl of 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 8.0, at 4 °C for 10 min. The samples were centrifuged at $13000 \times g$ for 5 min at 4 °C. The resulting supernatant (10 μl) was incubated with 0.1 ml of o-phthalaldehyde solution (0.1% in methanol) and 1.89 ml of the above phosphate buffer for 15 min at room temperature. Fluorescence intensity was then measured using a Perkin-Elmer LS50B fluorimeter (2H2 INC, Ontario, Canada) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cellular GSH content was calculated using a concurrently run GSH (Sigma) standard curve, and expressed as nanomole of GSH per milligram of cellular protein.

2.11. Statistical analysis

Data are presented as the mean of at least three independent experiments along with standard error of the mean (SEM). Statistical analyses of data were done by one-way analysis of variance (ANOVA), with Student-Newman-Keul test by using Sigma plot 12.0. The p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Inhibition of H_2O_2 –induced cytotoxicity and apoptosis of BAEC by nitrones

Treatment of BAEC with nitron for 24 h did not exhibit significant cytotoxicity up to 100 μM (Fig. 2A). Incubation of BAEC with 100 μM H_2O_2 for 24 h showed loss of viability by ~ 62% (Fig. 2B). To evaluate the cytoprotective properties of nitrones, BAEC were preincubated for 24 h with different concentrations of the spin traps prior to the addition of 100 μM H_2O_2 for additional 24 h of incubation. A dose-dependent increase in cell viability was also observed when cells were pretreated with nitron (0–50 μM). Addition of 25 μM and 50 μM PBN increased viability of H_2O_2 -BAEC treated cells to 88% and 98%, respectively, while at DMPO concentrations of 25 μM and 50 μM , viability of H_2O_2 -treated cells was also increased to 57% and 80%, respectively (Fig. 2B).

The effect of nitrones in H_2O_2 -induced apoptosis in BAEC was then investigated. Flow cytometry data show pretreatment of cells with 50 μM PBN resulted in a significant decrease of apoptotic population to 5%, whereas same concentration of DMPO reduced apoptosis to 16% (Fig. 2C). In addition, the anti-apoptotic effects of nitrones were also evaluated by fluorescence microscopy (Fig. 2D). Treatment of BAEC with 100 μM H_2O_2 for 24 h resulted in 37% of cells undergoing apoptosis as illustrated by Annexin V/PI staining (Fig. 2D) compared to 3% observed in cells treated with control diluents (PBS control). Treatment of BAEC with 100 μM of H_2O_2 for 24 h resulted in a 3.5-fold significant increase in apoptotic cells as shown by the increase in Annexin V-FITC+ compared to control cells. Pretreatment with 50 μM PBN reduced the percentage of apoptotic cells to levels comparable to controls, whereas 50 μM DMPO induced a significant reduction by 1.6-fold. These results suggest that the onset of apoptosis in oxidatively challenged BAEC is significantly inhibited by the nitrones.

3.2. Decrease of ROS generation in H_2O_2 -treated BAEC by nitrones

Significant amount of ROS generation was observed in H_2O_2 -treated BAEC as indicated by both flow cytometry and confocal microscopy studies. BAEC treated with 100 μM H_2O_2 resulted in a time dependent-accumulation of ROS as observed by FACS (Fig. 3A).

Pretreatment with either 25 μM or 50 μM PBN prior to the addition of 100 μM H_2O_2 resulted in a significant decrease of ROS generation to levels compared to control as shown by the percentage of mean fluorescent intensity (MFI) of DCF-fluorescence (Fig. 3A). Similarly preincubation with 25 μM or 50 μM DMPO before the addition of H_2O_2 resulted in a decrease of fluorescence up to 1.6- and 1.2-folds respectively (Fig. 3A). Consistently, confocal microscopy studies also revealed a significant increase in ROS generation in BAEC treated with 100 μM H_2O_2 for 24 h as observed in Figs. 3B and 3C, but pretreatment with nitrones showed a drastic reduction of DCF-fluorescence in H_2O_2 -treated cells (Figs. 3B and C). Preincubation of H_2O_2 -treated BAEC with 25 μM or 50 μM PBN resulted in the decrease of DCF-fluorescence by 3.4- and 7-folds respectively, whereas pretreatment with similar concentrations of DMPO resulted in 1.4- and 3.2-folds decrease in DCF-fluorescence (Figs. 3B and C).

3.3. Inhibition of H_2O_2 – induced mitochondrial depolarization in BAEC by nitrones

To further understand the mechanisms involved in nitron cytoprotection, we determined their effect of nitrones on mitochondrial depolarization. Treatment of BAEC with 100 μM H_2O_2 for 16 h resulted in the depletion of mitochondrial membrane potential as determined by flow cytometry (Fig. 4A). A gradual decrease in MMP by almost 58% was observed in BAEC cells treated with 100 μM H_2O_2 for 16 h with rhodamine 123 fluorescence (Fig. 4A). Pretreatment of BAEC with 50 μM nitron prior H_2O_2 -treatment restored mitochondrial potential to levels found in control cells (Fig. 4A). These results suggest that nitrones inhibit H_2O_2 -induced mitochondrial depolarization.

3.4 Inhibition of H_2O_2 – induced activation of caspase-3 by in BAEC by nitron spin traps

Treatment of BAEC with 100 μM H_2O_2 showed a gradual increase of caspase-3 activity reaching a maximum level of ~ 5-fold after 24 h of H_2O_2 -treatment (Fig 4B). To determine whether nitron affects H_2O_2 -induced caspase-3 activation, BAEC were pretreated with 25 μM and 50 μM of nitron prior to the addition of H_2O_2 . Preincubation with PBN resulted in inhibition of the H_2O_2 -induced caspase-3 activity by 2- and 2.5-folds at 25 μM and 50 μM (Fig. 4C), respectively, whereas at similar doses of DMPO, ~ 3.4- and 2.5-folds decrease were observed, respectively (Fig. 4C).

3.5. Modulation of pro-and anti-apoptotic proteins by nitrones in H_2O_2 -treated BAEC

Treatment of BAEC with 100 μM H_2O_2 for 24 h resulted in a distinct increase in the levels of the pro-apoptotic proteins p53 and Bax to 1.7- and 1.5-folds respectively, while the expression of the pro-survival Bcl-2 decreased to 0.78-fold (Fig. 4D). The expression level of phospho (p)-Bad was also found to be reduced to 0.6-fold in H_2O_2 -treated cells, compared to controls. Addition of 50 μM nitron prior H_2O_2 -treatment significantly increased p-Bad and Bcl-2 levels and were accompanied by the down regulation of Bax and p53 (Figs. 4D and 4E). Since cell survival in the early phase of apoptosis depends mostly on the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family, the ratio of Bax/Bcl-2 acts as an important determinant of cell fate. Bax/Bcl-2 ratio increased in H_2O_2 -treated BAEC, but when cells were preincubated with the nitrones prior to H_2O_2 -treatment, the ratio was decreased significantly thereby shifting the balance to cell survival (Fig. 4E). These results clearly indicate that nitron-pretreatment results in the attenuation of H_2O_2 -induced apoptosis signaling in BAEC.

3.6. Nitrones regulate nuclear translocation of Nrf-2

To elucidate the antioxidant mechanism of nitron spin traps, we investigated the effect of nitrones on Nrf-2 subcellular localization. Treatment of BAEC with 50 μM PBN showed increased nuclear localization of Nrf-2 compared with PBS-controls cells (Fig 5A).

Similarly, a fraction of Nrf-2 was also found in the nucleus in cells treated with 50 μ M DMPO (Fig. 5A). Furthermore, subcellular fractionation studies showed increased nuclear localization of Nrf-2 accompanied by a decrease in the cytoplasmic fraction in PBN-treated cells (Figs. 5B and 5C). Similarly, DMPO also induced the nuclear translocation of Nrf-2 as observed by immunoblots of subcellular lysates of cells treated with 50 μ M DMPO (Figs. 5B and 5C). In addition, increase nuclear Nrf-2 was observed in BAEC cells pretreated with 50 μ M of the nitron prior to the addition of 100 μ M H₂O₂ (Fig. 5D). These results clearly indicated that nitron-treatment resulted in the nuclear translocation of Nrf-2 in the endothelial cells.

3.7. Induction of cellular phase II enzymes activity by nitrones alone in BAEC

Since activation of Nrf-2 is associated with the induction of phase II antioxidant enzymes, we determined the effect of nitrones on the activity of various phase II enzymes. Treatment of BAEC with 25 μ M or 50 μ M PBN induced HO-1 activity by 1.7- and 2.4-folds respectively (Fig. 6A), while at same concentrations of DMPO, the HO-1 activity was increased by about 1.1- and 1.8-folds, respectively (Fig. 6A). Similarly in the presence of 25 μ M and 50 μ M PBN, the activity of NQO1 increased to 1.8- and 2.2-folds, respectively, while in the presence of 25 μ M and 50 μ M DMPO, NQO1-activity was increased to 1.2- and 1.7-folds, respectively (Fig. 6B).

Similar effects were observed in GR and GPx enzyme activities (Figs. 6C and D). GR activity was also found to be highly up-regulated in PBN-treated BAEC. In the presence of 25 μ M or 50 μ M PBN, the GR activity levels were remarkably increased up to 2.5- and 3.1-folds respectively, compared to the untreated cells. At similar PBN concentrations, GPx activity levels were increased to 1.3- and 1.4-folds respectively. DMPO also showed increase in both GPx and GR activity levels (Figs. 6C and D). For example, in presence of 50 μ M DMPO, GPx and GR activity levels were increased up to 1.5- and 1.8-folds respectively. These data suggest that treatment of BAEC with the nitrones result in significant activation of the antioxidant enzymes, and that the ability of PBN to induce enzyme activity is more robust compared to DMPO.

Intracellular GSH levels were found to be significantly increased when BAEC were treated with nitron. In presence of 50 μ M PBN, GSH level was found to be increased up to 1.8-fold, while at similar concentration of DMPO, the intracellular GSH was increased to 1.4-fold, compared to control (Fig. 6E).

3.8. Induction of cellular phase II enzymes by nitrones in H₂O₂-treated BAEC

Next, we determine whether nitron affects the phase II enzyme activities during oxidative stress. Treatment of BAEC with 100 μ M H₂O₂ for 24 h resulted in significant reduction of HO-1, NQO1, GR and GPx activities by 30%, 35%, 40% and 24%, respectively, compared to control (Fig. 7). Pretreatment of BAEC with 50 μ M nitron showed that HO-1, NQO1, GR and GPx activities were increased to the levels statistically similar to control (Fig. 7). The intracellular GSH level was found to be decreased by 29% in H₂O₂-challenged BAEC, but a significant recovery of thiol levels was observed when the cells were preincubated with nitrones. Pretreatment of BAEC with 50 μ M nitron resulted in the recovery of intracellular GSH by 25% and 20%, respectively (Fig. 7E).

3.9. Nitron-induced cytoprotection is mediated by Nrf-2

To delineate the role of Nrf-2 in nitron-induced cytoprotection against oxidative stress, Nrf-2 expression was silenced in BAEC cells. BAEC cells transfected with siRNA-Nrf-2 showed a significant reduction of Nrf-2 expression compared with cells transfected with siRNA-Control (Fig. 8A). Next, the cytoprotective properties of nitron were evaluated in

siRNA-Nrf-2 or siRNA-Control BAEC cells treated with H₂O₂. Cells transfected with siRNA-Nrf-2 or siRNA-Control were treated with 50 μ M PBN or DMPO for 24 h before addition of 100 μ M H₂O₂ for 24 h. Silencing of Nrf-2 reduced cell viability by 20% compared to siRNA-Control cells (Fig. 8B). A minor but not significant loss of DMPO cytoprotection was observed in the siRNA-Control cells treated with H₂O₂, but this effect was not observed with PBN (Fig. 8B). Silencing of Nrf-2 reduced viability for 20% compared to siRNA-Control cells even in the absence of cytotoxic agents (Fig. 8B, * $p < 0.05$). Nitron-induced cytoprotection against H₂O₂ was completely abrogated in siRNA-Nrf-2 cells reaching levels found in H₂O₂-siRNA-Control treated cells (Fig. 8B, # $p > 0.1$). Collectively, these results indicate that Nrf-2 plays the key role in nitron-induced cytoprotection against oxidative stress.

4. Discussion

Structurally, spin traps are simple molecules but they impart rich chemistries and biological properties which made them relevant pharmacological agents [24]. For instance, nitrones can exhibit redox properties by virtue of their oxidation state [62], react with a variety of free radicals [63–65], decompose to NO after addition to O₂^{•-} [40, 41], can form biologically relevant species such as hydroxamic acid, aldehydes, and nitrous acid [62] and more effectively traps O₂^{•-} at mildly acidic pH than in neutral pH which makes spin trapping relevant in ischemic conditions or cancer cells [66]. Although the mechanism of nitron-protection is still obscure, earlier studies suggest that the spin trapping properties play an important role in nitron-mediated cardioprotection [67, 68] but recent experimental evidences pointed out the involvement of other mechanisms of protection other than spin trapping [26]. For example, PBN is also known to suppress the expression levels of heat shock proteins, c-Fos, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), when used against several ROS-induced disorders [32]. How nitrones modulate the antioxidant defense mechanisms and apoptosis signaling against oxidative stress are yet to be fully understood. Hence in the present study, we had investigated the nitron-mediated induction of cellular antioxidants and phase II enzymes *via* Nrf-2 pathway in BAEC treated with H₂O₂. Inhibitory effects of nitrones on the activation of mitochondrial-dependent apoptotic cascade were also explored simultaneously in H₂O₂-treated BAEC.

Nitric oxide plays an essential role in the regulation of normal vascular homeostasis and vessel integrity and most of the NO donating drugs are limited by their stability, controlled delivery and targeting properties [69]. Hence the controlled-NO releasing properties of nitrones could impart unique pharmacological properties [40], which are not associated with other widely employed anti-oxidant drugs.

We observed that pretreatment with nitron significantly inhibited H₂O₂-mediated cytotoxicity and apoptosis in BAEC (Fig. 2). Hydrogen peroxide induces damage to the whole cell mainly because of its ability to cross membranes and so is usually referred to as a global oxidant. Treatment of BAEC with 100 μ M H₂O₂ resulted in a time-dependent increase in ROS, which was found to be ameliorated by nitrones (Fig. 3). Generation of ROS in oxidatively challenged cells lead to mitochondrial dysfunction and simultaneous release of cytochrome c, which further activates caspase-3, the central executioner of apoptosis [70, 71]. However, upon preincubation of BAEC with nitron prior to H₂O₂-treatment, the mitochondrial dependent activation of caspase-3 was found to be inhibited to a significant extent (Figs. 4A–4C).

It is reported that under oxidative stress the pro-apoptotic protein Bad translocates to the mitochondria and heterodimerizes with Bcl-2 and Bcl-XL and facilitates Bax-dependent mitochondrial depolarization, finally resulting in the activation of caspase-3 [70]. Bad

remains in an inactive form due to the phosphorylation at ser-136 residue by upstream kinases like Akt, and this inhibits its association with Bcl-2 [72]. Phosphorylated (p)-Bad dissociates from Bcl-2 and is sequestered in the cytosol [73] to promote cell survival. The pro-apoptotic protein p53 is activated under oxidative stress [74] and also known to play a significant role in activation and mitochondrial translocation Bax [75]. H₂O₂-treated BAEC, showed an up-regulation of the pro-apoptotic proteins like p53 and Bax along with the concomitant down regulation of the anti-apoptotic p-Bad and Bcl-2. Preincubation with nitronone, however, reverses the effect and inhibits the up-regulation of pro-apoptotic proteins (Figs. 4D–4E).

To elucidate the mechanisms of cytoprotective property of nitrones, we investigated other antioxidative mechanisms other than direct spin trapping. Since it is well established that the transcription factor Nrf-2 can modulate the expressions and activities of several phase II antioxidant enzymes after being translocated to the nucleus [52–54]. Western blot and confocal studies were performed with BAEC treated with different concentrations of the nitronone alone. Interestingly, we observed that higher accumulation of Nrf-2 in nucleus upon treatment with higher nitronone concentration (Fig. 5). As anticipated, activities of the phase II enzymes such as HO-1, NQO1, GR, and GPx, along with the intracellular GSH levels were also increased due to nitronone-treatment (Fig. 6). Similarly depletion of the antioxidant enzyme activities in oxidatively challenged BAEC were also found to be restored due to nitronone pretreatment (Fig. 7). The fact that Nrf-2 translocation and induction of phase II enzyme activities were observed with nitronone treatment alone, and only Nrf-2 translocation with significant decrease in antioxidant activities were observed with H₂O₂ treatment alone, indicate that the nitrones confer protection through Nrf-2 activation and perhaps through salvaging of enzymes activity from direct H₂O₂-mediated enzyme inactivation. To confirm the role of Nrf-2 in nitronone-mediated cytoprotection, BAEC were transfected with siRNA-Nrf-2 and MTT assay was performed. Silencing of Nrf-2 resulted in the complete attenuation of nitronone-induced protection of BAEC against H₂O₂ (Fig. 8).

Although both PBN and DMPO had conferred significant protection against H₂O₂-toxicity, better protection was observed in PBN-treated cells. DMPO was found to be protective at a concentration of 50 μ M. On the other hand, at 25 μ M PBN showed enhanced protective properties than DMPO. Our earlier work showed that DMPO is a better spin trap for O₂^{•-} and HO₂[•] radicals than PBN-type nitrones [76] in spite that PBN confers better protection than DMPO in this study. Also as demonstrated by others where PBN was shown to confer better cardioprotection than DMPO in isolated rat hearts against doxorubicin-induced toxicity [77]. Previously, we have shown that the PBN-based nitrones confer robust protection against H₂O₂-mediated toxicity [34]. The lipophilicity of PBN was found to be much higher than that of DMPO with log k'_{w} values of 1.64 and 0.31, and that the rate of internalization inside the cells is also higher for PBN compared to DMPO [34]. The increased lipophilicity of PBN may lead to its better compartmentalization inside the cells and this may account for the enhanced antioxidant property of PBN than DMPO. DMPO has been widely employed as spin trapping agents with more superior properties than that of PBN-based nitrones in terms of reactivity to O₂^{•-} and HO₂[•] [76]. Therefore, attempts had been made to conjugate cyclic nitrones to several target specific groups in order to facilitate their delivery to specific subcellular compartments [34, 78–80] since the poor target specificity of some of the natural antioxidants such as vitamin E, vitamin C, or lipoic acid are limiting their applications against ROS-induced CVDs [19, 81, 82].

In conclusion, the present study clearly demonstrates that pretreatment of oxidatively challenged cells with nitronone results in the attenuation of H₂O₂-induced cytotoxicity and apoptosis. The underlining mechanisms of protective effects of nitrones are contributed to a combination of ROS-quenching, up-regulation of phase II enzymes *via* Nrf-2 activation and

suppression of mitochondria-mediated apoptotic cascade (Fig. 9). This study also demonstrates for the first time that a number of endogenous antioxidants and phase II enzymes are induced by nitrones to suppress ROS-mediated endothelial damage. Future investigations will be necessary to determine the upstream molecular mechanisms as well as the *in vivo* relevance of our findings. Since oxidative stress-induced endothelial cell dysfunction plays a key role in cardiovascular diseases, our findings suggested a novel application for nitrone-therapeutics in the treatment of cardiovascular diseases or cardiometabolic disorders such as hypertension or hyperglycemia which are associated with endothelial dysfunction and depletion of cellular antioxidant pool.

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Fig. 1. Chemical structures of nitron spin traps. (A) Linear nitron, α -phenyl *N*-*tert*-butyl nitron (PBN); and (B) cyclic nitron, 5,5-dimethyl-pyrroline *N*-oxide (DMPO)

Fig. 2.

Effect of nitrones on H₂O₂-induced cytotoxicity and apoptosis in BAEC. (A) Cytotoxicity of nitrone (0–100 μM) alone determined by MTT assay. Data are represented as the mean ± SEM, where $n = 6$. (B) Cell viability of BAEC treated with H₂O₂ (100 μM) alone and those preincubated with nitrone (0–50 μM) as determined by MTT assay. Data are represented as the mean ± SEM, * $p < 0.05$ vs. control (untreated cells), and # $p < 0.05$ vs. H₂O₂-treated cells, where $n = 6$. (C) Induction of apoptosis in H₂O₂-treated BAEC and subsequent inhibition by nitrone-pretreatment. Apoptotic cells were analyzed flow cytometrically, and a dot plot representation of Annexin-V-FITC-fluorescence (x -axis) vs. PI-fluorescence (y -axis) has been displayed. The figure represents the best of three independent experiments. (D) Immunofluorescence study of Annexin V-FITC positive apoptotic cells treated with H₂O₂ (100 μM) alone for 24 h and those preincubated with nitrone (0–50 μM). The right panel corresponds to the merged image of FITC-fluorescence and DAPI-fluorescence, used to stain the nuclei. Bar represents 10 μm.

Fig. 3.

Effect of nitrones on H₂O₂-induced ROS generation in BAEC. (A) BAEC were treated with 100 μM H₂O₂ alone at various time points (0–24 h) in the absence or presence of nitron (0–50 μM). After treatment, cells were incubated with 25 μM of DCFH-DA at 37 °C for 30 min in the absence of light and ROS generation was then assessed using a FACS Calibur flow cytometer (BD) with excitation at 488 and emission at 535 nm. (Inset), Mean fluorescence intensity (MFI) from the histogram analysis of all three sets such as H₂O₂-alone (various time intervals), PBN + H₂O₂ and DMPO + H₂O₂ were presented as mean ± SEM, **p* < 0.05 vs. control (untreated cells), and #*p* < 0.05 vs. H₂O₂-treated cells, where *n* = 3. (B) Confocal images of BAEC treated with H₂O₂ (100 μM) alone and those preincubated with nitron (0–50 μM). The right panel corresponds to the merged image of DCF-fluorescence and DAPI-fluorescence, bar represents 10 μm. (C) A plot of relative DCF- fluorescence obtained from the average of 100 cells. Data are represented as the mean ± SEM, **p* < 0.05 vs. control (H₂O₂-untreated cells), and #*p* < 0.05 vs. H₂O₂-treated cells, where *n* = 3.

Fig. 4.

Effect of nitrones on H₂O₂-induced mitochondrial dysfunction, caspase-3 activation and modulation of pro- and anti-apoptotic proteins in BAEC. (A) Determination of mitochondrial membrane potential (MMP). BAEC were treated with 100 μM H₂O₂ in the absence or presence of nitrone (0–50 μM) for 24 h. After treatment cells were stained with rhodamine 123 and incubated for 60 min, and MMP was measured using a FACS Calibur flow cytometer (BD) with excitation at 488 and emission at 535 nm. Results are expressed as a representative histogram analysis. (B) Time-dependent activation of caspase-3 by H₂O₂. To determine time-dependent activation of caspase-3 by H₂O₂, BAEC were treated with 100 μM H₂O₂ at various time points (0–24 h) and caspase-3 activity was determined by using the 7-amino-4-trifluoromethyl coumarin assay (AFC) linked to the tetrapeptide DEVD as described in “materials and methods” section. Results are represented as mean ± SEM, **p* < 0.05 vs. control (untreated cells). (C) Inhibition of H₂O₂-induced caspase-3 activation by nitrones. To determine nitrone-induced inhibition of caspase-3 activity, BAEC were preincubated with nitrone (0–50 μM) and then treated with 100 μM H₂O₂ for 24 h. After treatment cells were incubated with DEVD-AFC. Finally the release of free AFC was monitored using Cytofluor 4000 fluorimeter (excitation, 400 nm; emission, 508 nm). Results are represented as mean ± SEM, **p* < 0.05 vs. control (untreated cells), and #*p* < 0.05 vs. H₂O₂-treated cells, where *n* = 3. (D) Modulation of pro- and anti-apoptotic proteins by the nitrones in H₂O₂-treated BAEC. Expression levels of p53, Bax, Bcl-2 and p-Bad in BAEC

treated with H₂O₂ in the absence and presence of nitron were monitored by western blotting. (E), (F) Densitometric analysis of the expressions of p53, p-Bad and Bax/ Bcl-2 ratio. Results are represented as mean $\pm$ SEM, * p < 0.05 vs. control (untreated cells), and # p < 0.05 vs. H₂O₂-treated cells, where n = 3.

Fig. 5.

Effect of nitrones on nuclear translocation of Nrf-2. (A) BAEC were treated with nitron (0–50 μM) for 24 h. After treatment, cells were immunostained with goat polyclonal anti-Nrf-2 antibody (1:200 dilutions) followed by anti-goat FITC-conjugated IgG antibody (1:150 dilutions) and DAPI (1 μM) to stain the nucleus. After incubation, cells were washed with PBS and images were taken by Olympus FluoView-1000 confocal microscope at 60 X magnification. The right panel corresponds to the merged image of FITC-fluorescence and DAPI-fluorescence, bar represents 10 μm. The figure represents the best of three independent experiments ($n = 3$) (B) Western blot analysis of the cytoplasmic and nuclear extracts of nitron-treated BAEC, using anti-Nrf-2 primary antibody (1:500 dilution). Anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000 dilution) and anti-lamin B (1:1000 dilution) antibodies were used as loading controls for the cytoplasmic and nuclear fractions. The figure represents three independent experiments ($n = 3$) (C) Densitometric ratio of cytosolic vs. nuclear Nrf-2 in nitron-treated BAEC, calculated from the respective band intensities. Data are represented as the mean $\pm$ SEM, * $p < 0.05$ vs. control (PBN-untreated cells) and # $p < 0.05$ vs. control (DMPO-untreated cells), where $n = 3$. (D) Immunoblot analysis of the nuclear extracts of untreated, H₂O₂-treated, and nitron-preincubated H₂O₂-treated BAEC for Nrf-2, using anti-Nrf-2 primary antibody (1:500 dilution) and anti-lamin B (1:1000 dilution) as the loading control. The figure represents three independent experiments ($n = 3$).

Fig. 6.

Effect of nitrones on the activities of phase II enzymes and intracellular GSH in BAEC. BAEC were incubated with different concentrations of nitron (0–50 μ M) for 24 h, and phase II enzyme activities were spectrophotometrically assessed. Following the protocol described in “materials and methods” section, activities from the cell extracts were determined for (A) HO-1, (B) NQO1, (C) GR, (D) GPx and (E) GSH, respectively. Results are represented as mean $\pm$ SEM, * p < 0.05 vs. control (nitron-untreated cells), where n = 3.

Fig. 7.

Effect of nitrones on the activities of phase II enzymes and intracellular GSH in H_2O_2 -treated BAEC. BAEC were preincubated with different concentrations of nitron (0–50 μ M) and then challenged with 100 μ M H_2O_2 for 24 h. After treatment cell extracts were prepared and assayed for (A) HO-1, (B) NQO1, (C) GR (D) GPx and (E) GSH, respectively. Results are represented as mean $\pm$ SEM, * p < 0.05 vs. control (untreated cells), and # p < 0.05 vs. H_2O_2 -treated cells, where n = 3.

Fig. 8.

Nrf-2 is necessary for nitrosonitron cytoprotection. (A) BAEC were transfected with a specific siRNA-Nrf-2 or a scramble siRNA-Control and efficiency of silencing was evaluated by immunoblotting 48 h post-transfection. The results are a representative of three experiments ($n = 3$). (B) Cytoprotective properties of nitrosonitron was evaluated by MTT assays in BAEC transfected with siRNA-Nrf-2 or siRNA-Control treated 48 h post-transfection with 50 μ M nitrosonitron for 24 h followed by treatment with 100 μ M H_2O_2 or diluent control for 24 h. Results are represented as mean $\pm$ SEM ($n = 3$), * $p < 0.05$ siRNA-Control NT vs. siRNA-Nrf-2 NT and # $p > 0.1$ siRNA-Control H_2O_2 -treated vs. siRNA-Nrf-2 H_2O_2 -treated, PBN + H_2O_2 or DMPO + H_2O_2 -treated cells.

Fig. 9. Possible mechanism of nitrone-mediated cytoprotection against oxidative stress. In the schematic diagram activation pathways are indicated by ($\rightarrow$), and the inhibitory pathways are indicated by ($\dashv$).