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Nitric oxide reduces oxidative stress in cancers cells by forming dinitrosyliron complexes

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Abstract

The chelatable iron pool (CIP) is a small but chemically significant fraction of total cellular iron. While this dynamic population of iron is limited, it is redox active and capable of generating reactive oxygen species (ROS) that can lead to oxidative stress which is associated with various pathologies. Nitric oxide ($\bullet\text{NO}$), is a free radical signalling molecule that regulates numerous physiological and pathological conditions. We have previously shown that macrophages exposed to endogenously generated or exogenously administered nitric oxide ($\bullet\text{NO}$) results in its interaction with CIP to form dinitrosyliron complexes with thiol containing ligands (DNICs). In this study we assessed the consequences of DNIC formation in cancer cells as $\bullet\text{NO}$ is known to be associated with numerous malignancies. Incubation of cancer cells with $\bullet\text{NO}$ led to a time and dose dependent increase in formation of DNICs. The formation of DNICs results in the sequestration of the CIP which is a major source of iron for redox reactions and reactive oxygen species (ROS) generation. Therefore, we set out to test the antioxidant effect of $\bullet\text{NO}$ by measuring the ability of DNICs to protect cells against oxidative stress. We observed that cancer cells treated with $\bullet\text{NO}$ were partially protected against H_2O_2 mediated cytotoxicity. This correlated to a concomitant decrease in the formation of oxidants when $\bullet\text{NO}$ was present during H_2O_2 treatment. Similar protective effects were achieved by treating cells with iron chelators in the presence of H_2O_2 . Interestingly, $\bullet\text{NO}$ decreased the rate of cellular metabolism of H_2O_2 suggesting that a proportion of H_2O_2 is consumed *via* reactions with cellular iron. When the CIP was artificially increased by supplementation of cells with iron, a significant decrease in the cytoprotective effect of $\bullet\text{NO}$ was observed. Notably, $\bullet\text{NO}$ concentrations, at which cytoprotective and antioxidant effects were observed, correlated with concentration-dependent increases in DNIC formation. Collectively, these results demonstrate that $\bullet\text{NO}$ has antioxidant properties by its ability to sequester cellular iron. This could play a significant role in variety of diseases involving ROS mediated toxicity like cancer and neurodegenerative disorders where $\bullet\text{NO}$ has been shown to be an important etiologic factor.

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Graphical abstract

Nitric Oxide converts redox active iron into antioxidant cytoprotective DNIC.

Keywords

Nitric oxide; dinitrosyliron complexes; chelatable iron; oxidative stress; cancer

1. INTRODUCTION

Nitric oxide ($\bullet\text{NO}$) is a small free radical signalling molecule present in various biological systems (1). It is a vital effector and messenger molecule that plays role in variety of biological processes including smooth muscle tone, immune response, angiogenesis, apoptosis, and synaptic communication (1). In biological systems, nitric oxide leads to production of a complex array of reactive nitrogen oxide species (2), but it directly reacts with very small number of targets (3). These targets are either other free radicals (*e.g.*, dioxygen, superoxide) or transition metals (*e.g.*, Fe^{2+}) (3). This reactivity is due to the ability of these targets to stabilize the unpaired electron on $\bullet\text{NO}$ (4).

Iron is an essential metal ion crucial to a variety intracellular processes such as oxygen transport, enzyme catalysis, and electron transfer reactions *etc.* (5). Nitric oxide is known to react with transition metals (*e.g.*, iron) in a biological system to form various complexes (4,6). Nitric oxide is also known to react with the intracellular chelatable iron pool (CIP) which leads to the formation of dinitrosyliron complexes (DNIC) with thiol-containing ligands (7–10). The chelatable iron pool is methodologically defined because it is accessible to chemical iron chelators like desferrioxamine and accounts for minor proportion of total cellular iron (1–5%) (11,12). It is comprised by both Fe^{2+} and Fe^{3+} iron and it is associated with a diverse population of high and low molecular weight cytosolic ligands such as organic anions (phosphates and carboxylates), polypeptides, and surface component of membranes (*e.g.*, phospholipid head groups) (12). The CIP is a dynamic pool, but under normal physiological conditions its concentration is regulated within a narrow range by homeostatic mechanisms (typically involving iron regulatory proteins, IRE-IRP pathway) (11).

Importantly the CIP forms a chemically significant portion of total intracellular iron, as it is an important source of redox active iron complexes which are known to be involved in

reactions leading to the formation of reactive oxygen species *e.g.*, the Fenton reaction (12,13). While H_2O_2 reacts slowly with biological molecules, the Fenton reaction results in the formation of highly oxidizing hypervalent metal-oxo complexes and hydroxyl radical (14). These species can react at diffusion controlled rates with various macromolecule in cells leading to lipid peroxidation, DNA base modifications, DNA strand breaks, and protein oxidation (15). These damaging events are considered crucial pathogenic factors for numerous diseases, like neurodegenerative disorders (Alzheimer's and Parkinson's disease), ischemia-reperfusion injury, cancer, *etc.* (11).

The biological and pathological consequences of $\bullet\text{NO}$ can be either deleterious or cytoprotective which depend on a variety of complex micro-environmental factors (16,17). Pro- or anti-oxidant effects of $\bullet\text{NO}$ depend on its local concentration, cell phenotype, and oxygen concentration as well as the presence of other reactive species in the biological milieu (16,18–20). At high local cellular concentration of $\bullet\text{NO}$, it can react with a number of other reactive oxygen and nitrogen oxide species to cause deleterious effects on cellular components. In contrast, low physiological doses of $\bullet\text{NO}$ are shown to abate the oxidative potential of reactive oxygen species perhaps through the formation of metal-nitrosyl complexes which can lead to a decrease in the formation of Fenton related oxidants (16,21). In addition, $\bullet\text{NO}$ has been shown to protect cell against death caused by oxidizing agents such as hydrogen peroxide, alkylhydroperoxides (22,23).

In cancer biology, $\bullet\text{NO}$ plays numerous roles (24) ranging from tumor progression (25,26), DNA mutations (27), migration/invasion (28), epigenetic regulation (29–31) to even treatment (32,33). For many cancer types, however, the presence of $\bullet\text{NO}$ or the expression of nitric oxide synthase (NOS) is consider a negative prognostic indicator that correlates with worse patient outcomes. Although numerous cell signalling mechanisms have been reported to explain these associations we postulate that a simpler mechanism could be significant contributing factor; the ability of $\bullet\text{NO}$ to function as an antioxidant. Herein, we provide evidence that protection of cancer cells in the presence of oxidants results from the ability of $\bullet\text{NO}$ to sequester chelatable iron in the form of DNIC.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Diethylenetriamine nonoate (DETA/NO) and Spermine/nonoate (Sper/NO) were a generous gift from Dr Joseph A. Hrabie (National Institute of Health, NCI). Ferric ammonium citrate (FAC), Diethylenetriamine-pentaacetic acids (DTPAC), phosphate buffer saline (PBS), desferrioxamine mesylate, CelLytic™ M cell lysis reagent, ferrous sulfate, Phenylmethanesulfonyl fluoride (PMSF), potassium iodide, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium (Ferrozine™), Neocuproine, ammonium acetate, sodium hydroxide, acetic acid, hydrochloric acid, ascorbic acid, and lipopolysacchride (LPS) were purchased from Sigma-Aldrich (St Louis, MO). Chemical iron chelator, 2,2'-dipyridal (DP), was purchased from Acros Organics (NJ). Bio-rad DC™ protein assay reagents were purchased from Bio-rad (Hercules, CA). Dihyrorhodamine 123 was purchased from Alexis Biochemicals (San Diego, CA) and alamarBlue® dye was purchased from Life Technologies (Waltham, MA). Protease inhibitor cocktail set III was

purchased from Merck Millipore (Bellerica, MA). All cell culture supplies were purchased from either Invitrogen or Fischer Scientific (Pittsburgh, PA), unless otherwise specified.

2.2 Cell Culture

HCC 1806 and MDA-MB-231 breast cancer cells and HT-29 colon cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 murine macrophage cells were also obtained from ATCC. All cell lines were cultured at 37°C temperature and 5% CO₂ concentration in a tissue culture incubator. The cells were grown to 80-90% confluence in tissue culture plates in either DMEM (for HCC 1806 and HT-29) or RPMI (for MDA-MB-231 and RAW 264.7) growth medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Prior to treatments, growth media was replaced with serum-free growth medium for 16 hours. All experiments were conducted under these culture conditions.

Iron supplementation: The cells were treated with ferric ammonium citrate (150 µg/mL) for various time points. Plates were washed with PBS containing DTPAC (100 µM) in order to remove excess extracellular iron and fresh growth medium was added to the plate prior to treatments.

2.3 Total iron determination

Total iron levels were determined using a previously established method (34) with slight modifications. Briefly, cells were lysed using lysis buffer (CellLytic™ M Cell lysis reagent with 1% protease inhibitor cocktail and 1mM PMSF). The samples were centrifuged and the supernatant was collected for further analysis. Aliquots (100 µL) of cell lysates were mixed with 100µL of 10mM HCl (the solvent of the iron standard FeCl₃), and 100µL of the iron-releasing reagent (a freshly mixed solution of equal volumes of 1.4M HCl and 4.5% (w/v) KMnO₄ in H₂O) for 2 hour at 60 °C. After the mixtures were cooled to room temperature, 30 µL of the iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1M ascorbic acid dissolved in water) was added. After 30 minutes, 250 µL of the solution was transferred into a well of a 96-well plate and the absorbance was measured at 550 nm on a microplate reader.

Iron concentrations were estimated by comparing the absorbance of the sample to that of a known standard of FeCl₃ (mixture of 100 µL of FeCl₃ standards (0–300 µM) in 10 mM HCl, 100 µL lysis buffer, 100 µL releasing reagent, and 30 µL detection reagent). The intracellular iron concentrations determined were normalized to the amount of protein in the sample.

2.4 Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR Spectroscopy was performed using our established method (8,28). Briefly, cells were trypsinized and re-suspended in equal volumes of PBS. DNIC signals were measured by EPR at g = 2.03 using a Bruker X-band EMX Plus EPR spectrometer, fitted with a liquid nitrogen dewar (at 77°K) with the following settings: modulation amplitude 10G, 200G scan range, 90 sec scan time, 1 scan. DNIC concentrations were estimated by comparing the signal amplitudes to that of a known standard of GSH-DNIC. For quantitative studies a microwave power saturation profile for EPR signal amplitudes was conducted to ensure we

were operating below the saturation. Protein samples were collected from each experiment so that DNIC could be quantitatively compared between individual runs (pmol DNIC/mg protein).

For the CIP quantification, cells were treated with 1 mM desferrioxamine (DFO) for 4 h and then harvested for EPR analysis. The concentration of the CIP was calculated by comparing the signal amplitude to the amplitude of known concentrations of a DFO-Fe(III) standard prepared as previously described (8,24). EPR settings: $g = 4.03$, modulation amplitude of 10G, 200 G scan range, 30 sec scan time, 4 scans.

2.5 GSH-DNIC synthesis

The synthetic DNICs were synthesized using previously described method (35). 1 ml of 0.5 mM FeSO_4 (dissolved in degassed water to avoid rapid oxidation to the ferric state) was added to 10 mL (final volume) of 0.1 M of degassed PBS buffer, pH 7.4, containing 20 mM GSH and 2 mM GSNO (25 °C) under hypoxic conditions. After 45 minutes, the reaction was almost 70% complete, and the resulting stock solution of GSH-DNIC was quantified either by UV-Vis spectroscopy ($\epsilon_{403}=3000$) or EPR spectroscopy.

2.6 Cell Viability Assay

Cell viability was assessed using an established protocol (36). Briefly, cells (10×10^3 /well) were plated in 96-well plate 16 hour before the experiment. The cells were treated with Sper/NO for 1 h followed by H_2O_2 for 2 h. They were further incubated in FBS supplemented DMEM media. After 48 hours, media was replaced with media containing 10% alamarBlue® dye for 2–3 h. Absorbance was recorded at 570nm using 600nm as reference wavelength.

2.7 DHR fluorescence assay

The levels of cellular oxidants was determined using DHR fluorescence assay (37). Cells (7.5×10^3 /well) were plated in 96-well plate 16 h before the experiment. The cells were incubated with PBS containing DHR123 (100 μM) for 1 h at 37°C. The cells were washed once with PBS and incubated with the culture medium containing Sper/NO for 1 h. H_2O_2 were added to the medium and incubated for 1 h. The fluorescence was measured by Spectra MAX GeminiEM plate reader (excitation: 485nm, emission: 530nm).

2.8 Cellular H_2O_2 metabolism

HCC 1806 cells were trypsinized and put into a reaction chamber at 6×10^6 cells/ml of serum-free media. Cell suspensions were constantly stirred in a sealed, water-jacketed, temperature-controlled (37 °C) chamber. The reaction chamber was equipped with a hydrogen peroxide electrode connected to an Apollo 4000 free radical analyser (World Precision Instruments, Sarasota, FL, USA). Headspace in the vessel was negligible. Reactions were initiated by bolus injection of H_2O_2 with a gas-tight syringe and metabolism was measured in real time.

2.9 Statistical Analysis

One way ANOVA analysis with Fisher's LSD posthoc test was done using OriginPro8.1 software, OriginLab (Northampton, MA). The data were reported as the Mean $\pm$ Standard Error of Mean (S.E.M.).

3. RESULTS

3.1 The amounts and distributions of Cellular Iron differ between Cancer Cell types

Iron is a primary biological target for $\bullet$ NO in most cell types including cancers. For this reason we set out to assess the levels of total iron, CIP, and DNIC in 3 phenotypically different cancer cell types (HCC 1806 and MDA-MB-231 breast cancer cells and HT-29 colon cancer cells). Figure 1A demonstrates that the amounts of total cellular iron vary significantly between the 3 cancer cell lines with twice as much iron in the MDA-MB-231 cells compared to the HCC1806. Also, the concentration of iron in the CIP as well as the percentage total iron that made up the CIP were distinctly different between cell types (Fig. 1B,C). Interestingly, despite both breast cancer cell types (HCC 1806 and MDA-MB-231) having different levels of total cellular iron (8.6 vs. 19.2 nmol/mg protein) a similar percentage of iron was measured in the CIP ($\sim$ 1%). The HT-29 colon cancer cells had both the largest CIP (>500 pmol/mg protein) as well as 4 times the amount of total cellular iron in the CIP ($>4\%$).

3.2 DNIC formation is a function of the amount of iron in the CIP as well as $\bullet$ NO exposure time and concentration

We have shown previously in macrophages that DNICs are formed rapidly upon cellular exposure to $\bullet$ NO (8). However, the parameters of $\bullet$ NO exposure leading to DNIC formation in cancer cells has not been extensively studied. The three cell lines in culture were treated with the $\bullet$ NO-donor Sper/NO for 1 h and DNIC levels were assessed (Fig. 1D). Under these conditions significant amount of DNIC (500–900 pmoles/mg protein) were formed. Markedly higher levels of DNICs were observed in HT-29 cells compared to both HCC 1806 and MDA-MB-231 cells which paralleled differences in their respective CIP levels (Fig. 1B). In the case of the HCC 1806 cells, levels of DNIC exceeded measured amounts of the CIP (CIP 100 pmol vs. DNIC 450 pmol).

To better understand the relationship between $\bullet$ NO concentration and exposure time on DNIC formation we treated HCC 1806 cells with two different $\bullet$ NO-donors that have different $\bullet$ NO-release kinetics (Sper/NO and DETA/NO). Sper/NO has a relatively short half-life (39 min at 37°C) and provides a burst of $\bullet$ NO whereas DETA/NO has a significantly longer half-life (20 h at 37°C) and provides a continuous steady-state concentration of $\bullet$ NO for a prolonged period of time (38). The cells were incubated with Sper/NO (10–50 μ M) for 3 h which we have determined gives a physiological steady-state $\bullet$ NO concentration of approximately 3–300 nM (8,38,39). Under these conditions, significant amounts DNIC were detected at Sper/NO concentrations above 20 μ M (Fig. 2A). Exposure of HCC 1806 cells to DETA/NO (500–1000 μ M, [$\bullet$ NO]_{ss} = 25–2,000 nM) for 4 h also lead to a dose-dependent increase in the formation of DNICs (Fig. 2B). At 500 μ M DETA/NO the levels of DNIC closely matched the amount of iron in the CIP, however, at

higher •NO concentrations the amounts of DNIC exceeded the levels of the CIP (Fig. 1B, 2B). As the CIP is the primary source of iron for DNIC assembly this suggests that higher amounts of •NO facilitate the release of iron from other intracellular sources making it available for reaction with •NO.

To determine temporal effects of continuous •NO exposure on DNIC formation we treated HCC 1806 cells with DETA/NO (1 mM) for 12 hours and measured DNIC over time. Figure 2C demonstrates that DNICs were rapidly formed after a 1 h of •NO exposure with maximal amounts of DNICs detected by 4 hours. No increases in DNIC were observed after 4 hours but maximal amounts of DNIC were maintained throughout the duration of •NO exposure. Although the amounts of DNIC formed were 3–4 folds higher than the level of iron in the CIP a saturation level of DNIC was achieved by 4 hours even in the presence of continued •NO exposure (Fig. 1B, 2C).

3.3 Stability of Intracellular DNICs in Cancer Cells

Before we could look at downstream phenotypic effects of DNIC in cancer cells it was important to understand their stability (biological lifetime). To determine this the three cancer cell lines (HCC 1806, MDA-MB-231 and HT-29) were treated in culture with high concentrations of •NO (Sper/NO, 1 mM) to allow maximal DNIC formation. After 1 hour the •NO source was removed by washing the cells and replacing with fresh growth media. Samples were then harvested for DNIC analysis at various time points (0–4 h) after •NO removal (time = zero). Over a 4 hour period cellular DNICs decreased with a half-life of approximately 1 hour for all cell types (Fig. 2D). Taken together, these results (Fig. 2A–D) demonstrate that exposure of cancer cells to •NO results in rapid formation of DNICs and these complexes have a significant life-span in the absence of •NO.

3.4 Nitric oxide inhibits hydrogen peroxide-mediated oxidation

Although the CIP is a small fraction of total cellular iron it is redox active and pathologically significant due to its capacity to generate oxidizing reactive oxygen species (ROS) (12,13). The CIP is also the primary sources of iron for DNIC assembly. Therefore, we wanted to test whether sequestration of CIP iron *via* DNIC assembly would render the iron less redox active. Hydrogen peroxide (H₂O₂), by itself, is a relatively mild oxidant. In the presence of ferrous iron, however, Fenton chemistry converts H₂O₂ into highly oxidizing species (13–15,34,35). First HCC 1806 cells were pre-treated with increasing concentrations of •NO to form DNIC (Sper/NO, 0–1,000 μM, 1h). Cells were then exposed to a bolus of hydrogen peroxide (200 μM) for 2 h and intercellular oxidation was determined by measuring the conversion of dihydrorhodamine 123 (DHR123) to the highly fluorescent product rhodamine. Figure 3A demonstrates a significant ($p < 0.001$ – 0.05) dose dependent decrease in H₂O₂-mediated oxidation in cells pre-incubated with •NO. No significant oxidation was measured in cells incubated with •NO alone (Fig. 3A). In another set of experiments, cells were incubated with increasing concentration of hydrogen peroxide (0–400 μM) in the presence or absence of low amounts of •NO (50 μM Sper/NO) (Fig. 3B). Incubation of cells with hydrogen peroxide alone leads to a dose dependent increase in formation of cellular oxidants (Fig. 3B). Importantly, in presence of •NO results in

significant ($p < 0.05$) suppression in peroxide mediated increase in cellular oxidant levels at all concentrations of peroxide examined.

Figures 3A, B demonstrate a proof of concept that •NO could decrease the amount of oxidation induced by H₂O₂. In general these concentrations of •NO were superphysiologic/pathologic. To model a more physiologic relevant scenario we examined the ability of endogenously produced •NO to abate H₂O₂-mediated oxidation. RAW 264.7 macrophage cells were activated with lipopolysaccharide (LPS; 1 µg/mL) for 16 h which we have shown induces iNOS expression and •NO production (8). These cells were then loaded with DHR 123 followed by treatment with hydrogen peroxide (500 or 1,000 µM) for 2 h. Under these conditions there was a significant decrease in intercellular oxidation in the •NO-producing cells compared to the control cells following H₂O₂ exposure (Fig. 3C).

Next, in order to ascertain the role of CIP in •NO-mediate anti-oxidant effects, studies were conducted with cancer cells supplemented with iron. Notably, we have previously demonstrated that supplementation of HCC 1806 cancer cells with ferric ammonium citrate (FAC) can significantly increase the CIP levels in these cells (28). Thus, the effect of CIP supplementation (*via* FAC) on production of oxidants was examined. HCC 1806 cells were incubated with FAC then loaded with DHR 123 dye. The cells were then treated with the •NO-donor (Sper/NO; 25 µM) for 1 h followed by hydrogen peroxide (200 µM) for 2 h. In the absence of •NO there was a significant increase in oxidant production in the cells supplemented with. However, oxidation was considerably less in cells pre-treated with Sper/NO in both the iron supplemented and non-supplemented cells (Fig. 3D). Collectively, these results (Fig. 3A–D) demonstrate that •NO can interact with the CIP to mitigate its redox ability, potentially *via* formation of DNICs.

3.5 Nitric Oxide decreases the rate of cellular H₂O₂ metabolism

It is well-known that a significant proportion of cellular hydrogen peroxide is metabolized by the enzyme catalase and we have shown that this is fairly rapid and by first-order kinetics (19). Interestingly, studies have also demonstrated that •NO can be oxidated by compound I of catalase as well as reversibly inhibit catalase activity (40). Therefore, in addition to forming DNICs, it was important to determine if the protective effects of •NO were linked to its ability to alter H₂O₂ metabolism. We examined the effect of •NO on hydrogen peroxide metabolism using electrochemical detection where the rate of H₂O₂ disappearance in the presence of cells could be monitored in real time. Notably, the rate of cellular hydrogen peroxide consumption was twice as fast in the control cells compared cells exposed to •NO (Fig. 3E). These results suggest that •NO interacts with H₂O₂ catabolic pathways and that although •NO is protective against oxidative damage it paradoxically increases both the half-life of H₂O₂ and the cellular exposure.

3.6 Cytoprotective effects of •NO

The damage caused CIP-generated oxidants can result in DNA strand breaks, base modifications, protein oxidation, and lipid peroxidation. Studies herein have demonstrated that •NO can suppress the oxidant levels in cancer cells potentially *via* its ability to interact with the CIP. Hence, we set out to assess the ability of •NO to alter H₂O₂-mediated

cytotoxicity. To examine this, HCC 1806 cells were pre-incubated with increasing concentrations of the •NO-donor Sper/NO (10–100 µM) for 1 h followed by incubation with hydrogen peroxide (200 µM) for 2 h. Cells were then washed and supplemented with fresh growth media. After 48 hours, cellular viability was measured. The incubation of cells with hydrogen peroxide alone lead to a significant ($p < 0.001$) decrease in cell survival compared to control cells (Fig. 4A). Notably, pre-incubation with Sper/NO (25–100 µM) resulted in a significant ($p < 0.001$) decrease in peroxide-mediated cytotoxicity (Fig. 4A).

Our theory is that •NO behaves similar to chemical iron chelators by virtue of its ability to sequester iron in the form of DNICs. Thus we next sought to determine if chemical iron chelators could similarly prevent peroxide mediated cytotoxicity. HCC 1806 cells were incubated with iron chelator Dipyrldal (100–200 µM) for 1 h, followed by treatment with hydrogen peroxide (200 µM) for 2 h. The cells were washed and supplemented with fresh growth media and after 48 h cell viability was measured. As in figure 4A peroxide alone resulted in a marked decrease in cellular viability. However, similar to Sper/NO, preincubation with Dipyrldal lead to a significant decrease in peroxide mediated cytotoxicity (Fig. 4B). These results indicate that the protective effects of •NO and iron chelators may be due to their ability to interact with the same pool of iron (*i.e.*, the CIP).

3.7 Nitric Oxide Mediated Cytoprotection Against Oxidative Stress is Due to its Interaction with CIP

To further elucidate the role of the CIP in •NO-mediated cytoprotection we supplemented the cells with iron to augment the CIP (FAC 150 µg/mL for 4 h). These cells were then treated with low amounts of •NO (Sper/NO 25 or 50 µM) for 1 h followed by the addition of hydrogen peroxide (200 µM) for 2 h. The cells were washed and supplemented with fresh growth medium for 48 h and cell viability was measured. Iron supplemented cells were significantly more susceptible to H₂O₂-mediated toxicity compared HCC 1806 cells with basal CIP levels. Although both iron supplemented and non-iron supplemented cells were partially protected from H₂O₂ by •NO, greater amounts of •NO were required in the cells supplemented with iron (Fig. 4C). Collectively, these results (Fig. 4A–C) demonstrate that •NO can interact with CIP and prevents oxidative stress mediated cell death, potentially *via* formation of DNICs.

4. DISCUSSION

DNIC were first detected in cancers as far back as the 1960s but the source of their formation and their biological functions were not known. The roles of •NO in caners are complex but in many cases it is associated with deleterious patient outcomes. In this short study, we looked into some basic parameters that contribute to DNIC formation and decay as well as potential phenotypic consequences in cancer cells. It is well-known that the CIP is a main source of iron required for the formation of these complexes (3). The major evidence for this is the observation that treatment of cells with iron chelators completely ablates DNIC assembly (8). As iron chelators display antioxidant properties due to their ability to sequester iron and render it less redox active, so to might •NO via the formation of DNIC.

Thus, a potential role for •NO in cancers is to protect the tumours from oxidative host-defences mechanisms.

Although the CIP is the source of iron for DNIC assembly, we have shown that in cancer cells exposed to higher prolonged concentrations of •NO the concentrations of DNIC significantly exceed the CIP (8). This implies that •NO is capable of liberating iron from other sources such as iron storage and iron-sulphur cluster containing proteins. This indicates that a function of •NO may be to increase the availability of “free iron” (CIP) which, although not tested in this study, could potentially have pro-oxidant effects upon the removal of •NO. This would be partially dictated by the stability of DNIC upon •NO removal as well as the ability of the cells to re-establish normal iron homeostasis to bring the CIP back to basal levels. We observed that once the •NO source was removed, the DNIC signal disappeared with a half-life of approximately 60 minutes. We use the term disappear over decay because at this stage of our investigations we can only speculate on the mechanism(s) of disappearance of the EPR =2.04 DNIC signal. Potential explanations for the loss of this EPR signal could be due to multitude of reasons: conversion to non-paramagnetic •NO-iron complexes, dimeric-DNIC, decay of DNIC, and cellular export of DNIC. Turella *et al* showed that GST extended the half-life of DNIC in cell lysates from 2.8 minutes to 4.5-8 h (41). Another study demonstrated that Cys-DNIC was less stable under ambient air, but persists for hours under deoxygenated conditions (42). Watts *et al* suggested that there is an equilibrium between low and high molecular weight DNIC and the latter are exported out of the cell resulting in loss of the EPR signal (43). These results highlight the complexity •NO/iron interactions and suggest that loss of DNIC is a multifactorial process that is a function of microenvironmental conditions as well as differences in the contribution of specific thiol ligands (protein vs. non-protein).

One of the classical and probably best-studied redox reactions associated with oxidative stress the Fenton reaction. Under biological conditions, the CIP is assumed to be the major source of iron to catalyse the formation of strong oxidants by this reaction (13). Cancer cells are known to produce much higher level of H₂O₂ and H₂O₂-mediated oxidative stress is known to play an important role in carcinogenesis by inducing DNA strand breaks, mutations, and genomic instability (15). There are reports, however, demonstrating cytoprotective effects of nitric oxide against hydrogen peroxide mediated insults (22). In the current study, we tested whether •NO could abate iron-mediated H₂O₂ toxicity *via* its ability to sequester iron in the form of DNIC. It was demonstrated that cancer cells pretreated with •NO or endogenously synthesizing •NO showed both significantly less intercellular oxidation and toxicity in response to hydrogen peroxide compared to cells not exposed to •NO. These results could largely be replicated by treating cells with iron chelators instead of •NO suggesting that the mechanisms of protection are similar for both molecules (*i.e.*, the CIP is the only target shared by both •NO and iron chelators). Further evidence for a role of DNIC in the protective effects of •NO comes from the observation that artificially increasing in the CIP *via* iron supplementation led to increased level of oxidant production and increased cell death. High amounts of •NO could partially overcome this excess iron-attributable toxicity.

We concluded that in cancer cells DNIC formation is an important intracellular event and their predominant function may be to protect cells from oxidative stress. One could envision a scenario where early oxidative stress induces oncogenic transformation but tumours that later acquire the ability to synthesize •NO exhibit a selective survival advantage against host-defences by being protected from iron-mediated toxicity. This compensatory mechanism could be one explanation why •NO-associated tumours correlate to increased aggressiveness and worse patient outcome.

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ABBREVIATIONS

DNIC	Dinitrosyliron Complexes
NO	Nitric Oxide
CIP	Chelatable Iron Pool

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HIGHLIGHTS

- Nitric oxide reacting with the CIP rapidly forms DNIC in cancer cells
- The CIP is a major source of cellular oxidant generation upon H₂O₂ exposure
- DNIC reduce H₂O₂-mediated oxidation and cell death

Figure 1. Comparing amounts of total Iron, chelatable Iron, and dinitrosyliron complexes in 3 cancer cell lines

(A) Quantification of total intracellular iron. Cell lysates from three different cancer cell types (HCC 1806, HT29 and MDA-MB-231) were digested with acidic KMnO_4 solution to release iron from all intracellular stores. The lysates containing free released iron were then treated with iron detection reagent (*i.e.*, 6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1M ascorbic acid dissolved in water). Absorbance was measured at 550 nm and compared to known iron standard. **(B) Quantification of chelatable iron.** The intracellular CIP ($\text{Fe}^{2+}/\text{Fe}^{3+}$) was measured by treating the cells with desferrioxamine (DFO, 4h) which complexes with the CIP (DFO-Fe^{3+}) to give a distinct EPR signal at $g=4.3$. This was compared to standard DFO-Fe^{3+} complex as described previously (28). **(C) Percentage CIP of total cellular iron.** **(D) DNIC quantification.** HCC 1806, HT29 and MDA-MB-231 cell were treated with the $\bullet\text{NO}$ -donor Sper/NO (1 mM) for 1 h. DNICs were detected in whole cells by EPR analysis ($g=2.03$) and quantified by comparing to a standard curve generated with a chemically synthesized small molecular weight glutathione DNIC (GSH-DNIC), as shown previously (28). ($n = 3$ for all experiments)

Figure 2. Kinetics of DNIC assembly and degradation in Cancer Cells

(A–C) Quantification of DNIC after NO exposure in relation to the CIP. HCC 1806 cells were treated with (A) Sper/NO (0–50 μM) for 4 h or (B) DETA/NO (0–1 mM) for 4 h or (C) DETA/NO (1 mM) for indicated time points. The cells were harvested and DNIC were measured by EPR. DNIC are expressed in terms of total cellular protein (100 pmoles/mg protein ~ 5–10 μM DNIC samples). *, $p < 0.05$, **, $p < 0.01$. **(D) Stability of DNIC.**

HCC1806, MDA-MB-231, HT-29 cells were treated with •NO-donor Sper/NO (1 mM) for 1 h then washed and supplemented with fresh media (time = 0). The time points indicate time after removal of •NO source. ##, $p < 0.01$ with respect to DNIC levels at 0 h time point for HCC 1806 cells. ^, $p < 0.05$, ^^, $p < 0.01$ with respect to DNIC levels at 0 h time point for MDA-MB-231 cells. **, $p < 0.01$ with respect to DNIC levels at 0 h time point for HT-29 cells. 100 pmoles/mg protein ~ 5–10 μM DNIC samples. (n = 3 for all experiments)

Figure 3. Nitric oxide decreases H_2O_2 -mediated cellular oxidation

(A) Nitric Oxide reduces cellular oxidation mediated by H_2O_2 in a dose dependent manner. HCC 1806 cells were loaded with fluorescent dye DHR 123 (100 μM). The cells were then treated with $\bullet\text{NO}$ -donor Sper/NO (10–1,000 μM) for 1 h followed by hydrogen peroxide (200 μM) for 2 h. Fluorescence was measurement and compared to untreated controls **(B) Low amounts of NO diminish oxidation in the presence of increasing concentrations of H_2O_2 .** Cells were loaded with fluorescent dye, DHR 123 (100 μM) and then treated with the $\bullet\text{NO}$ -donor Sper/NO (50 μM , 1h) followed by hydrogen peroxide (100–400 μM , 2h). **(C) Endogenous $\bullet\text{NO}$ decreases intracellular oxidation.** RAW 264.7 cells were activated with LPS (1 $\mu\text{g}/\text{mL}$) for 16 h. The cells were then loaded with fluorescent dye, DHR 123 (100 μM) and treated with hydrogen peroxide (0.5–1 mM, 2h). Fluorescence was measurement and compared with respect to individual non-LPS treated control. **(D) Cellular iron supplementation augments H_2O_2 -mediated oxidation.** HCC 1806 cells were incubated with increasing concentration of ferrous ammonium citrate (62.5–125 $\mu\text{g}/\text{mL}$) which increases the CIP. They were then loaded with DHR 123 and treated with Sper/NO (0 or 25 μM) for 1 h followed by peroxide for 2 h. with respect to individual non-iron supplemented control. **(E) The effect of $\bullet\text{NO}$ on hydrogen peroxide metabolism in cancer cells.** HCC 1806 cells in suspension (constantly stirred) were treated with NO (25 μM Sper/NO) followed by bolus addition of H_2O_2 (1 μM). H_2O_2 concentration was monitored in real time, representative electrochemical trace. H_2O_2 did not disappear in the

absence of cells +/- •NO (data not shown). (n = 3 for all experiments, *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$)

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Figure 4. Cytoprotective effect of NO are due to its interaction with CIP in cancer cells
 Cellular viability measurements. **(A) Nitric oxide suppresses peroxide mediated cytotoxicity in cancer cells.** HCC 1806 cells were treated with increasing concentration of Sper/NO (10–100 μM) for 1 h followed by hydrogen peroxide (200 μM) for 2 h. Survival was measured 48 h after removal of hydrogen peroxide. ***, $p < 0.01$ as indicated. **(B) Iron chelator suppresses H_2O_2 mediated cytotoxicity.** HCC 1806 cells were treated with the iron chelator Dipyridal (100 or 200 μM) for 1 h followed by hydrogen peroxide (200 μM) for 2 h. Survival was measured 48 h after removal of hydrogen peroxide. ***, $p < 0.01$ as indicated. **(C) NO suppresses H_2O_2 mediated cell death after iron supplementation.** HCC 1806 cells were treated with FAC (150 $\mu\text{g}/\text{mL}$) for 4 h to increase the CIP. Cells were then treated with Sper/NO (0–50 μM) for 1 h followed by the addition of hydrogen peroxide (200 μM) for 2 h. Survival was measured 48 h after removal of hydrogen peroxide. ***, $p <$

0.001 with respect to no treatment (NT) control. ^{###}, $p < 0.001$ with respect to corresponding no Sper/NO (0 μ M) control. ^{^^}, $p < 0.001$ with respect to corresponding treatments with no FAC supplementation. (n = 3 for all experiments)

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