HHS Public Access

Author manuscript

Nitric Oxide. Author manuscript; available in PMC 2021 November 01.

Published in final edited form as:

Nitric Oxide. 2020 November 01; 104-105: 36–43. doi:10.1016/j.niox.2020.08.005.

MYOGLOBIN PROMOTES NITRITE-DEPENDENT MITOCHONDRIAL S-NITROSATION TO MEDIATE CYTOPROTECTION AFTER HYPOXIA/REOXYGENATION

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Abstract

It is well established that myoglobin supports mitochondrial respiration through the storage and transport of oxygen as well as through the scavenging of nitric oxide. However, during ischemia/reperfusion (I/R), myoglobin and mitochondria both propagate myocardial injury through the production of oxidants. Nitrite, an endogenous signaling molecule and dietary constituent, mediates potent cardioprotection after I/R and this effect relies on its interaction with both myoglobin and mitochondria. While independent mechanistic studies have demonstrated that nitrite-mediated cardioprotection requires the presence of myoglobin and the post-translational S-nitrosation of critical cysteine residues on mitochondrial complex I, it is unclear whether myoglobin directly catalyzes the S-nitrosation of complex I or whether mitochondrial-dependent nitrite reductase activity contributes to S-nitrosation. Herein, using purified myoglobin and isolated mitochondria, we characterize and directly compare the nitrite reductase activities of mitochondria and myoglobin and assess their contribution to mitochondrial S-nitrosation. We demonstrate that myoglobin is a significantly more efficient nitrite reductase than isolated mitochondria. Further, deoxygenated myoglobin catalyzes the nitrite-dependent S-nitrosation of mitochondrial proteins. This reaction is enhanced in the presence of oxidized (Fe^{3+}) myoglobin and not significantly affected by inhibitors of mitochondrial respiration. Using a Chinese Hamster Ovary cell model stably transfected with human myoglobin, we show that both myoglobin and mitochondrial complex I expression are required for nitrite-dependent attenuation of cell death

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DISCLOSURES

None

after anoxia/reoxygenation. These data expand the understanding of myoglobin's role both as a nitrite reductase to a mediator of S-nitrosation and as a regulator of mitochondrial function, and have implications for nitrite-mediated cardioprotection after I/R.

INTRODUCTION

Myoglobin and mitochondria are closely associated in the heart and together form a functional metabolome to sustain cardiac bioenergetics [1; 2; 3; 4]. Physiologically, mitochondria consume oxygen, which is coupled to the generation of ATP to supply 99% of the energy required for normal cardiac function. The monomeric heme protein, myoglobin, sustains oxidative phosphorylation through the storage and transport of oxygen to the mitochondrion within the myocyte [5; 6]. However, during prolonged periods of ischemia and subsequent reperfusion (I/R), this functional relationship is disrupted and mitochondria and myoglobin both contribute to the pathogenesis of I/R injury [7]. Specifically, mitochondrial ATP generation is diminished during ischemia, leading to depleted free energy supply for cellular homeostasis. Upon reperfusion, the accumulation of reducing substrates and oxygen stimulates excessive mitochondrial reactive oxygen species (ROS) generation, leading to oxidation of mitochondrial protein complexes, the release of cytochrome c, and apoptosis [8; 9]. Myoglobin potentiates this reperfusion-induced oxidative damage through its auto-oxidation and catalysis of superoxide generation [10; 11].

Nitrite (NO_2^-), the one electron oxidation product of nitric oxide (NO), is an endogenous signaling molecule that confers potent cardioprotection in numerous *ex vivo* and *in vivo* cell, isolated heart and animal models of myocardial I/R [12; 13; 14; 15; 16]. While the precise mechanisms underlying nitrite's cytoprotective effects after I/R are still being elucidated, two seminal observations implicate myoglobin and mitochondria as independent sub-cellular components that are central to nitrite-mediated protection: 1) nitrite covalently modifies a critical cysteine residue (cysteine 39 of the ND3 subunit) on mitochondrial electron transport chain complex I by S-nitrosation during I/R). This post-translational modification results in the inhibition of electron entry and transport in the mitochondrion, effectively attenuating reperfusion ROS generation and preventing protein oxidation and apoptosis [17; 18]. 2) Myoglobin expression is required for nitrite-mediated cardioprotection as demonstrated by the inability of nitrite to decrease infarct size or protect cardiac function in myoglobin knockout mice subjected to myocardial infarction [19; 20]. This necessity for myoglobin in nitrite-mediated cytoprotection is attributed to its efficient hypoxic nitrite reductase activity, whereby deoxygenated myoglobin (deoxyMb) reduces nitrite to bioavailable NO via the following reaction:

$$\text{Nitrite} + \text{deoxyMb}\left(Fe^{2+}\right) + H^+ \rightarrow NO + \text{metMb}\left(Fe^{3+}\right) + OH^-\left(k = 12.4 M^{-1}s^{-1}; \text{pH } 7,\ 37^{\circ}C\right)$$

[21; 22]

Despite the recognition that myoglobin-dependent nitrite reduction and mitochondrial S-nitrosation are both required for nitrite-induced cytoprotection, it is unknown whether myoglobin mediates mitochondrial S-nitrosation.

In the context of S-nitrosation, it is important to note that NO does not directly modify reduced protein thiols to form S-nitrosothiols but can be converted to nitrosating species in aerobic conditions [23; 24]. Relevant to hypoxic S-nitrosation, nitrite is known to catalyze reductive nitrosylation, involving the reduction of metmyoglobin (Fe^{3+}) by NO [25]. Further, a reductive anhydrase reaction has also been proposed as a potential mechanism in which metheme (Fe^{3+}) proteins react with nitrite. Both these reactions require metheme and result in the formation of the potent nitrosating species dinitrogen trioxide (N_2O_3) [26; 27]. In this regard, heme proteins such as hemoglobin and mitochondrial cytochrome c have been shown to promote S-nitrosation [28; 29; 30]. However, the ability of myoglobin to catalyze these reactions in a physiological milieu, with physiological levels of nitrite, and its impact on mitochondrial protein modification has previously not been assessed. Additionally, several studies demonstrate that components of the mitochondrial electron transport chain can directly reduce nitrite to NO [22; 28; 31], but the efficiency of this activity has never been compared to that of myoglobin.

Herein, we directly compare the nitrite reductase activity of mitochondria and myoglobin and show that myoglobin is a significantly more efficient nitrite reductase. We demonstrate that myoglobin promotes mitochondrial S-nitrosation in purified proteins and in a cell system and demonstrate that this is essential to nitrite-mediated protection from hypoxia/reoxygenation. We discuss the implications of myoglobin-dependent mitochondrial S-nitrosation for the regulation of metabolism in physiology and hypoxic/ischemic disease.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were purchased from Sigma unless otherwise specified. For purified myoglobin experiments, horse heart myoglobin was purchased from Sigma and its concentration measured by visible absorption spectroscopy as previously described [22].

Mitochondrial Isolation

Mitochondria were isolated by differential centrifugation, as previously described [32; 33] from the hearts of male Sprague-Dawley rats. All rats (male aged 9–12 weeks) were housed and fed ad libitum in compliance with the guidelines of the Animal Care and Use Committee of the University of Pittsburgh (where all animal work took place). The experimental protocol was in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of Pittsburgh IACUC (Protocol #15127354). Rats were euthanized by the removal of the heart under anesthesia (ketamine/xylazine) in accordance with the approved protocol.

Measurement of NO generation

Mitochondria, myoglobin or cells were suspended in PBS or respiratory buffer for mitochondria (120mM KCl; 25mM sucrose; 10mM HEPES; 1mM EGTA; 1mM KH_2PO_4; 5mM $MgCl_2$, pH7.4) and purged with helium to achieve anoxia prior to the addition of nitrite. Succinate (50mM) and ADP (100mM) were added to isolated mitochondria as substrates and Rotenone (15μM) was added to inhibit complex I. NO formation in the

headspace of the reaction vessel was measured throughout by a NO chemiluminescence analyzer (Sievers, GE Analytical Instruments) as previously described [22; 34].

Cell Culture & Forced Myoglobin Expression

The human myoglobin gene from an E.Coli expression vector (kindly provided by Dr Steven Boxer, Stanford University) was sub-cloned into a pCMV-Tag 3 mammalian expression vector. Chinese hamster ovary (CHO) cells were stably transfected with the myc-tagged human myoglobin construct under the control of the CMV promoter. Cells were maintained under selection pressure (500μg/mL geneticin) in Ham's F-12 growth medium containing 10% FBS, 2.5μM hemin in 100mM NaOH and 1% Penicillin/Streptomycin. Mb overexpression was confirmed using antibody targeted to the MYC tag (9B11, Cell Signaling Technology). For siRNA experiments, the cells were transfected with NDUFAF1 siRNA (50nM; Dharmacon, USA) using Mirus TransIT-X2 Transfection reagent (Mirus Bio). Control cells were transfected in the same manner with 50nM scrambled siRNA (Dharmacon, USA). Complex I activity assay and Western blot was performed 72h after transfection.

Hypoxia/Reoxygenation (H/R)

All cells were cultured at 21% O_2, 5% CO_2 and cells in the control group were maintained in these conditions throughout. For H/R, cells were subjected to 16 h of hypoxia (1% O_2, 5% CO_2) in Esumi buffer as described in [35] (137mM NaCl, 12mM KCl, 0.5mM $MgCl_2$, 0.9Mm $CaCl_2$, 20mM HEPES, 20mM 2-Deoxy-D-Gluc, pH 6.4). Cells were then reoxygenated for 1 h at 20% O_2, 5% CO in the same buffer. In nitrite treated groups, nitrite was present throughout H/R.

Absorbance Spectrophotometry to assess myoglobin concentration

The absorbance spectrum of CHO-R and CHO-Mb cell lysate (500μg) was measured using a Diode Array spectrophotometer (Beckman Coulter). Difference spectra between the two cell lines was calculated and the resultant absorbance peak at 410nm was indicative of the presence of myoglobin and used to calculate the concentration of Mb expressed in CHO-Mb cells (Extinction coefficient$_{415nm}$ =125 $mM^{-1}cm^{-1}$). For isolated myoglobin, the concentration and percentage of each heme species (oxyMb, deoxyMb, and metMb) was analyzed by deconvoluting the spectrum into components from basis spectra using a least squares method similar to that described in [22].

S-nitrosation Detection

S-nitrosation was detected by tri-iodide based reductive chemiluminescence in the presence and absence of acidified sulfanilamide as previously described [18]. Briefly, after treatment of cells or mitochondria with nitrite (in the presence and absence of myoglobin), SNO was stabilized with 100 μM Diethylenetriaminopentaacetic acid (DTPA), 4 mM ferricyanide, 10 mM N-Ethylmaleimide, and 1% NP-40. The sample was then divided into three parts: one left untreated, one treated with 10% acidified sulfanilamide (v/v) to eliminate nitrite, and one treated with 5mM mercuric chloride and 10% acidified sulfanilamide to eliminate SNO

and nitrite. The fractions were then injected into a triiodide-containing vessel that was connected to an NO chemiluminescence detector (Sievers).

Mitochondrial Isolation from Cells

Complex I was immunoprecipitated from isolated mitochondria using agarose beads and anti-NDUFB3 (sc-135317, Santa Cruz Biotechnologies) and SNO was measured in the isolated protein as previously described [18].

Lactate Dehydrogenase Release (LDH) Assay

LDH activity was assessed by measuring the rate of NADH oxidation at 340nm. LDH release in the media was quantified as a percentage of total LDH activity in the media plus cell lysates.

Complex I Activity

Once treated, cells were subjected to two cycles of rapid freeze-thaw (in light protected conditions to preserve SNO) and then complex I activity was measured by spectrophotometrically monitoring the rotenone sensitive oxidation of NADH at 340nm in the presence of Coenzyme Q_2 as previously described in [18].

Hydrogen Peroxide Production

Hydrogen peroxide production was measured by monitoring the rotenone-sensitive oxidation of Amplex red in the presence of horseradish peroxidase at 585nm. Measurements were made in intact cells immediately after treatment, thus this assessment was that of H_2O_2 leaving the cell.

Statistical analysis

All data are reported as mean ± SEM. Data were analyzed using an unpaired, two-tailed student's t-test for single comparisons or ANOVA for multiple comparisons. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Myoglobin is a more efficient nitrite reductase than isolated mitochondria

In the first series of experiments we compared the efficiency of isolated mitochondria and purified myoglobin as catalysts of hypoxic nitrite reduction. Isolated rat heart mitochondria (0–20mg/ml) were first incubated with nitrite (1mM) in anoxia and NO generation measured in the headspace of the reaction. Consistent with previous reports [28; 31; 36], isolated mitochondria demonstrated a significant rate of nitrite-mediated NO generation that was dependent on the concentration of mitochondrial protein present (Figure 1A). We next determined the rate of myoglobin-catalyzed nitrite reduction to NO. Consistent with prior studies [22], deoxygenated ferrous myoglobin (0–100μM), generated significant concentrations of NO when incubated with nitrite (1mM). NO generation was dependent on the presence of ferrous (Fe^{2+}) myoglobin, as oxidation (Fe^{3+}) of 25% of the total heme present significantly decreased the rate of NO production. Oxidation of 100% of the

myoglobin completely inhibited nitrite reduction to NO (Figure 1B). Given that mitochondria and myoglobin both reduced nitrite to NO in a concentration dependent manner, we next sought to directly compare the efficiency of these NO generating systems. Comparison of 2mg/ml of mitochondria (containing approximately 25μM cytochrome c oxidase) [37; 38] with myoglobin (25μM) showed that myoglobin generated significantly more NO gas than mitochondria over the concentration range of nitrite (0–1mM) tested (Figure 1C).

To determine whether the NO generation observed was associated with the S-nitrosation of mitochondrial cysteine residues, isolated mitochondria (5mg/ml) were incubated with nitrite (0–100μM) in the absence or presence of purified myoglobin (25μM). After 10 minutes of anoxia, the reaction mixture was collected and mitochondrial S-nitrosation measured in normoxia. In the absence of myoglobin, nitrite concentration-dependently S-nitrosated isolated mitochondria (Figure 1D). When myoglobin was added into the reaction mixture, mitochondrial S-nitrosation was significantly increased at every nitrite concentration examined (Figure 1E) and this effect was dependent on the concentration of myoglobin (Figure 1E). To determine whether the SNO formation was solely due to NO formation, 25% of the myoglobin present was oxidized to metmyoglobin. Though decreased NO production was observed with 25% metmyoglobin (Figure 1B), greater concentrations of mitochondrial S-nitrosation were measured in the presence of metmyoglobin than with ferrous myoglobin alone (Figure 1E), consistent with potential reductive nitrosylation or anhydrase mechanisms. Collectively, these data demonstrate that myoglobin is a more efficient nitrite reductase than mitochondria and that myoglobin promotes nitrite-dependent mitochondrial S-nitrosation that is enhanced in the presence of metheme.

Myoglobin promotes nitrite-dependent NO and SNO formation in intact cells

We next utilized an isogenic cell model with differential myoglobin expression to determine whether the myoglobin-dependent S-nitrosation observed with the purified enzyme was present in a more physiological system. Chinese Hamster Ovary (CHO) cells, which lack endogenous myoglobin expression, were stably transfected with either a vector control (CHO-R) or a myc-tagged human myoglobin construct (CHO-Mb). The absence of myoglobin in the CHO-R cells and the presence of myoglobin in the CHO-Mb cells was confirmed by Western blot (Figure 2A) and absorbance spectrophotometry was used to confirm the presence of myoglobin-derived heme in the CHO-Mb cells. Cells were lysed and treated with potassium ferricyanide (100μM) to oxidize all ferrous heme. The difference in the spectra between CHO-R and CHO-Mb cells demonstrated that the CHO-Mb cells showed a metmyoglobin spectrum with an absorbance peak at 415 nm (Figure 2B). Calculation of myoglobin concentration from these spectra showed that the CHO-Mb cells contained 5.5±1.2 μM myoglobin.

To determine whether myoglobin expression contributes to cellular hypoxic NO production, CHO-R or CHO-Mb cells were incubated with or without nitrite (1mM) in anoxia and NO gas generation was continuously measured (Figure 2C). While CHO-R cells had a measurable rate of NO generation (3.1±.6pmol/min/mg), CHO-Mb cells generated significantly more NO (7.2±0.7pmol/min/mg) in the presence of nitrite (1mM). Notably, NO

generation by the CHO-R cells was inhibited by approximately 80% in the presence of the mitochondrial inhibitors myxathiazol and cyanide, suggesting that the majority of nitrite reduction by wildtype CHO-R cells was mitochondrial (Figure 2D). In contrast, mitochondrial inhibitors had no significant effect on NO generation by CHO-Mb cells, while potassium ferricyanide (100μM) treatment, resulting in heme oxidation, significantly abrogated NO generation (Figure 2D). Measurement of total cellular and mitochondrial RSNO in the cells after anoxic nitrite (25 μM) treatment demonstrated that CHO-Mb cells contained a significantly greater concentration of cellular (4.98±0.51pmol/mg) and mitochondrial (3.23±0.37 pmol/mg) RSNO than CHO-R cells (2.13±0.72pmol/mg and 0.58±0.23 pmol/mg respectively) (Figure 2E). These data demonstrate that even low concentrations of myoglobin significantly enhance hypoxic nitrite-dependent cellular NO generation as well as S-nitrosation. Further, these data suggest that the mitochondrion is a significant target for myoglobin-mediated S-nitrosation in intact cells.

Myoglobin promotes S-nitrosation in intact cells to mediate cytoprotection after hypoxia/reoxygenation

It is well established that nitrite mediates cytoprotection after I/R. Mechanistically, this protective effect is due to the S-nitrosation of a critical cysteine residue on mitochondrial complex I, resulting in the inhibition of mitochondrial complex I enzymatic activity and attenuated mitochondrial ROS generation at reperfusion [17; 18]. Thus, we next sought to determine whether the enhanced mitochondrial S-nitrosation observed in the presence of myoglobin augmented mitochondrial complex I S-nitrosation and cytoprotection. We established an *in vitro* cellular model of hypoxia/reoxygenation (H/R) to simulate *in vivo* I/R. CHO-R and CHO-Mb cells were subjected to prolonged hypoxia (1% O_2, 16 hours), after which they were reoxygenated (1hour) and cell death, measured by lactate dehydrogenase release, was assessed. Exposure to H/R alone induced similar levels of cell death in CHO-R and CHO-Mb cells (63.2±5.7% versus 69.8±4.9% cell death). However, treatment of the cells with sodium nitrite (10–100μM) during the hypoxic period concentration-dependently decreased cell death only in the CHO-Mb cells, while no statistically significant effect on viability was observed in CHO-R cells at concentrations lower than 100μM (Figure 3A).

Measurement of complex I activity at reoxygenation demonstrated that both cell lines showed decreased complex I activity after H/R due to oxidative stress. However, nitrite (25 μM) further inhibited complex I activity in CHO-Mb cells but had no significant effect on CHO-R cells (Figure 3B). Consistent with the effect on complex I, CHO-Mb cells treated with nitrite generated significantly lower rates of mitochondrial H_2O_2 at reoxygenation (Figure 3C). To determine whether the inhibition of complex I activity and cytoprotection was associated with S-nitrosation in this model, we next measured the concentration of Complex I SNO in CHO-R and CHO-Mb cells before and after H/R. Consistent with a role for myoglobin-dependent nitrite mediated S-nitrosation, CHO-Mb cells contained significantly greater levels of complex I SNO than CHO-R cells at reoxygenation. Notably, the level of mitochondrial S-nitrosation in CHO-Mb cells was significantly higher (5.2 ±0.4

pmol/mg) at the time of reoxygenation and returned to baseline levels 120 min after reoxygenation, consistent with the reversibility of S-nitrosation (Figure 3D).

To directly determine whether complex I S-nitrosation was responsible for the myoglobin-dependent nitrite mediated cytoprotection, we next used a siRNA approach to decrease the expression of the complex I assembly factor NDUFAF1 in CHO-R and CHO-Mb cells. Using this approach, we observed an approximately 50% decrease in the expression of the protein (Figure 4A) and a decrease in complex I activity of similar magnitude (Figure 4B), with no significant change in citrate synthase activity (data not shown). Further, knockdown of complex I did not significantly change the rate of nitrite reduction to NO in the CHO-Mb cells (Figure 4C). However, while nitrite (20μM) protected cells transfected with scrambled siRNA after H/R, this protection was lost in cells CHO-Mb cells lacking NDUFAF1 (Figure 4D).

DISCUSSION

The primary findings of this study are that 1) myoglobin is a more efficient nitrite reductase than the mitochondrion and that 2) myoglobin promotes the nitrite-dependent hypoxic S-nitrosation of mitochondrial proteins. The physiological relevance of this biochemistry is demonstrated in the context of ischemia/reperfusion by showing that myoglobin expression significantly enhances nitrite-mediated S-nitrosation and protection after hypoxia/reoxygenation of isolated mitochondria and intact cells (Figure 5).

It is well established that nitrite mediates cardioprotection during I/R, and that the reduction of nitrite is required for this protection. While mitochondria and myoglobin have independently been described as nitrite reductase enzymes [22; 28; 31; 36; 39], this study is the first to directly compare the nitrite reduction catalyzed by these two tightly linked functional components of oxygen metabolism, and assess their independent contribution to nitrite-mediated cytoprotection after I/R. While the data presented here confirm that both myoglobin and mitochondria reduce nitrite, the rate of nitrite-dependent NO generation by myoglobin is significantly greater than that of mitochondria. Further, prior studies have attributed mitochondrial nitrite reduction to both the Q cycle and to cytochrome c oxidase [28; 31]. Notably, though myoglobin and mitochondria are both highly abundant in cardiac tissue, the expression of myoglobin (150–300 nmoles/g (wet)) is approximately five to ten fold greater than the expression of cytochrome c oxidase (30nmoles/g (wet)). This is in agreement with prior studies demonstrating that oxidation or genetic deletion of myoglobin significantly decreases nitrite-dependent NO production in the heart [19; 34; 39]. Importantly, nitrite-dependent mitochondrial S-nitrosation occurs in tissues such as the liver and brain which do not contain myoglobin [13; 14]. This is due to the predominance of different nitrite reductases in these tissues[34]. The relative role of other nitrite reductase proteins in modulating mitochondrial S-nitrosation and their efficacy in comparison to cytochrome c oxidase is yet to be defined.

We show here that in addition to the generation of bioavailable NO, myoglobin mediates nitrite-dependent S-nitrosation in hypoxic conditions. In the present study, mitochondrial RSNO was measured in each case after reoxygenation, therefore it is possible that oxidation

of NO derived from nitrite reduction resulted in nitrosative chemistry. However, this is unlikely since S-nitrosation was enhanced in the presence of partially oxidized myoglobin (25% metmyoglobin) compared to 100% ferrous myoglobin. This suggests that myoglobin potentially demonstrates reductive anhydrase chemistry (Reaction 2) or reductive nitrosylation (Reaction 3), in which metheme catalyzes the generation of the potent nitrosating agent N_2O_3 [29; 33].

$$Mb(Fe^{3+}) - NO_2^- + NO \leftrightarrow Mb(Fe^{2+}) + N_2O_3 \quad \text{(Reaction 2)}$$

$$Mb(Fe^{3+}) - NO + NO_2^- \leftrightarrow Mb(Fe^{2+}) + N_2O_3 \quad \text{(Reaction 3)}$$

Importantly, the kinetics and energetics of the anhydrase chemistry are highly controversial [40]. Thus, further investigation is required to determine the specific chemical mechanism and kinetics of myoglobin-mediated S-nitrosation as well as the extent to which this pathway predominates with endogenous sources of nitrite.

We have previously demonstrated that NO generated by myoglobin-catalyzed nitrite reduction inhibits mitochondrial respiration through the reversible binding of NO to the heme a3 of cytochrome c oxidase [19; 22]. Here, we extend this paradigm to demonstrate that myoglobin also promotes nitrite-dependent S-nitrosation of the mitochondrion and that this modification contributes to nitrite-mediated cytoprotection. While here we focused on complex I, as its role in cytoprotection after I/R is well established, regulation of the mitochondrion by myoglobin-mediated post-translational modification may be relevant in physiology as well as other pathological conditions. For example, several Krebs Cycle enzymes are known targets for S-nitrosation. Aconitase, α-ketoglutarate, and succinate dehydrogenase activity have been reported to be inhibited by S-nitrosation [32; 41]. Myoglobin and mitochondria have fluctuating protein levels depending on tissue type, disease state, and oxygen tension, suggesting a highly environmental context for myoglobin-mediated mitochondrial regulation. Mitochondrial biogenesis occurs ubiquitously during exercise, while alterations in myoglobin protein level are more controversial and dynamic, apparently based on tissue. Further, myoglobin expression has recently been detected in solid tumors of epithelial origin where decreased mitochondrial function is a hallmark of disease [42; 43]. The inhibition of mitochondrial function via myoglobin-mediated post-translational modifications may be an important mechanism of mitochondrial regulation in these physiological states and this warrants further investigation in the future.

In summary, the studies presented here demonstrate that myoglobin is a more efficient nitrite reductase than mitochondria and that myoglobin expressed in a cellular milieu propagates nitrite-dependent S-nitrosation of mitochondria. These data expand the role of myoglobin as a regulator of mitochondrial function and lay the foundation to further investigate myoglobin mediated mitochondrial S-nitrosation in physiology and disease.

AKNOWLEDGEMENTS

We would like to thank Dr. Netanya Y. Spencer for creating the CHO-Mb cells.

FUNDING SOURCES

This work was funded by NIH/NHLBI RO1HL133003-01A1 to SS.

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Highlights

- Myoglobin is a more efficient nitrite reductase than mitochondrial cytochromes
- Myoglobin catalyzes nitrite-dependent S-nitrosation of mitochondria
- Partial oxidation of myoglobin enhances nitrite-dependent S-nitrosation

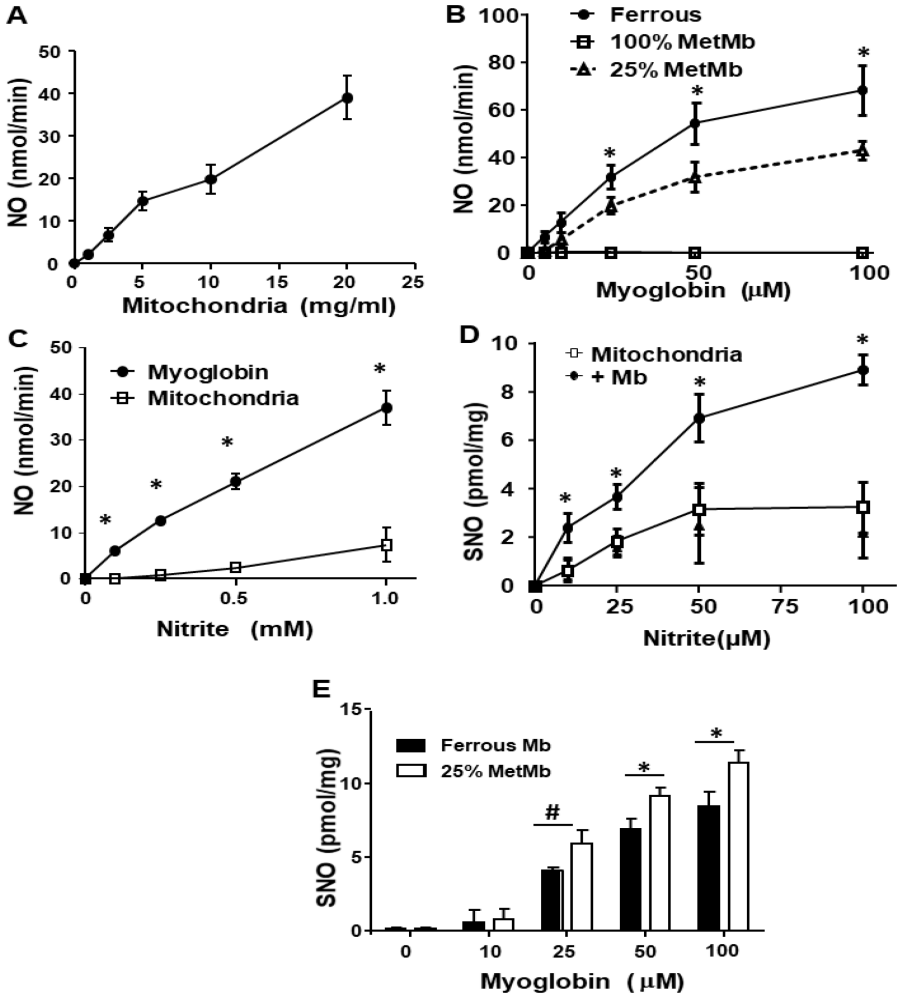

Figure 1: Myoglobin is a more efficient nitrite reductase than mitochondria that promotes SNO formation.

(A) The rate of NO generated in the headspace of the reaction of anoxic mitochondria (0–20mg/ml) with nitrite (1mM). **(B)** NO generated in the headspace of the reaction of ferrous (closed dot), 25% oxidized (triangles) or 100% oxidized (squares) myoglobin (0–100μM) with nitrite (1mM). **(C)** The rate of NO generation by isolated mitochondria (2mg/ml) or ferrous myoglobin (25 μM) in the presence of nitrite (0–1mM) in anoxia. **(D)** Total mitochondrial SNO generated after 10 minutes of incubation of nitrite (0–100 μM) with mitochondria alone (2mg/ml; open squares) or mitochondria with ferrous myoglobin (25 μM; closed circles) in anoxia. **(E)** The concentration of mitochondrial SNO generated after incubation of 100% ferrous (closed bars) or 25% metmyoglobin (open bars) with nitrite (25 μM). *p<0.01 and #p<0.05 versus mitochondria alone. *p<0.01 and #p<0.05 versus ferrous myoglobin for panels C and F. Data are means ± SEM. n=5 for all panels.

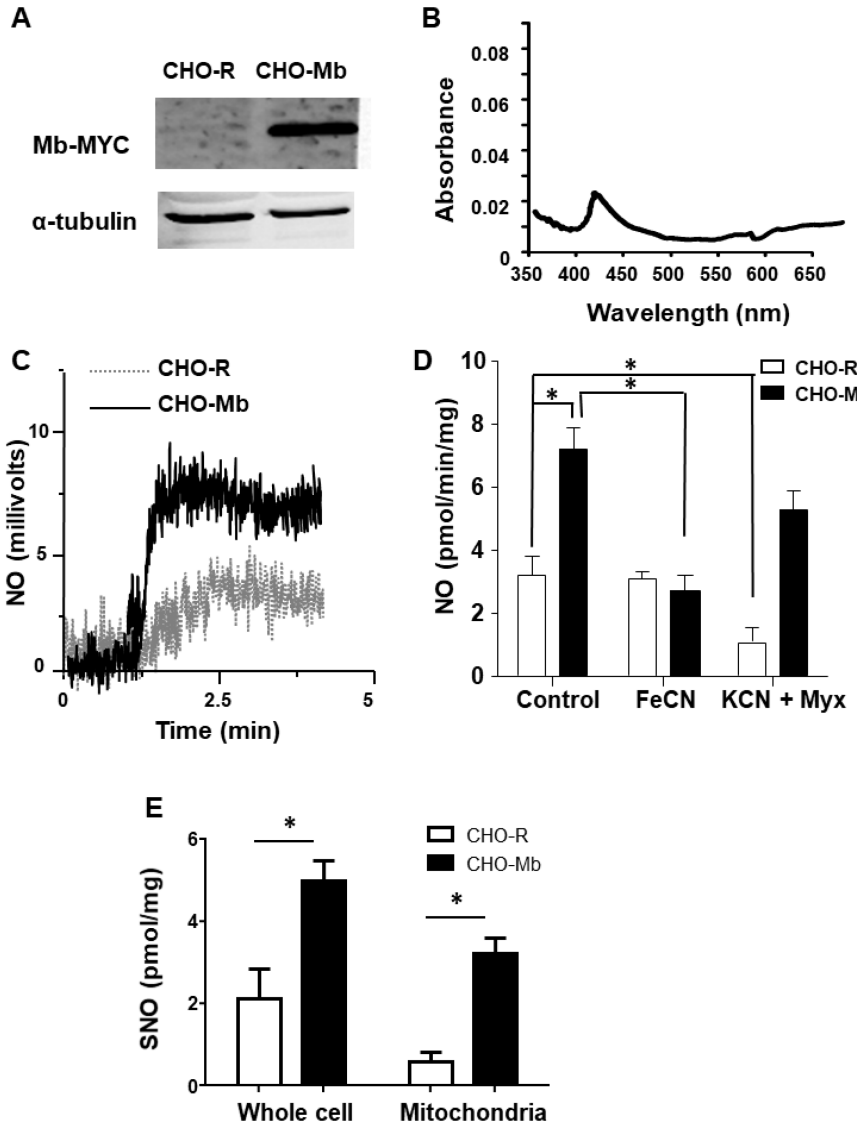

Figure 2: Myoglobin enhances cellular NO generation and S-nitrosation.

(A) Representative Western blot demonstrating myoglobin expression in CHO-Mb cells and lack of expression in CHO-R cells. Western blot shows MYC tag on myoglobin and α-tubulin control in each cell type. **(B)** The difference spectra between CHO-R and CHO-Mb cells. **(C)** Representative chemiluminescence trace showing NO generation by CHO-R (gray) and CHO-Mb (black) cells. **(D)** Quantitation of NO generation rate from traces like those shown in (C) of untreated (control) CHO-R (open bars) and CHO-Mb (filled bars) cells and those treated with potassium ferricyanide (100μM) or potassium cyanide (25μM) and Myxathiazol (20μM). **(E)** S-nitrosothiol concentration measured in the whole CHO-R or CHO-Mb cell or mitochondria isolated from those cells after anoxic treatment with nitrite (25μM) for 10 min. *$p<0.01$. Data are means ± SEM. n=5 for panels D and E.

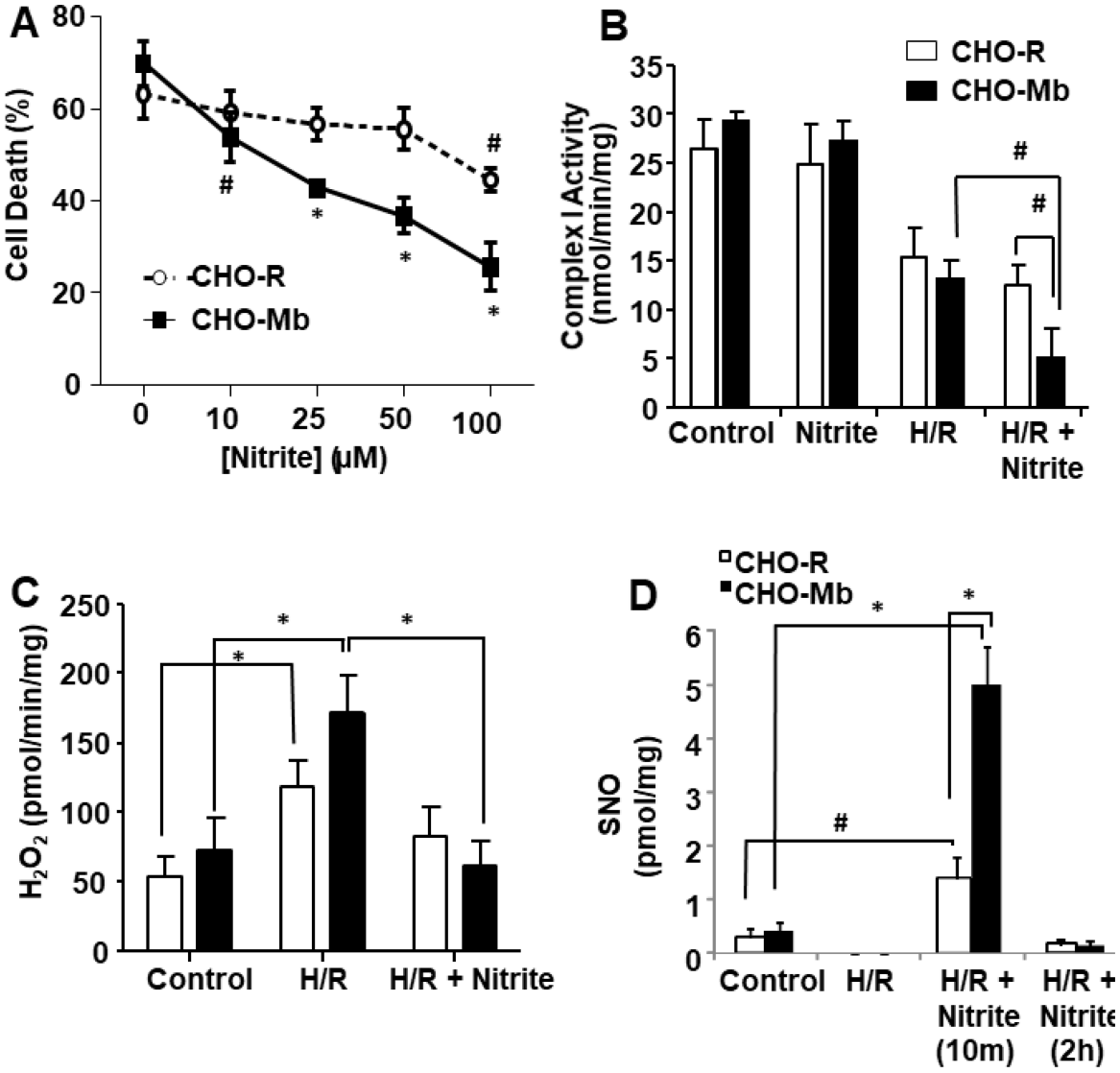

Figure 3: Myoglobin expression enhances nitrite-dependent attenuation of cell death after hypoxia/reoxygenation.

(A) Cell death (percent of lactate dehydrogenase release) in CHO-R (open circles) and CHO-Mb (filled squares) cells after hypoxia/reoxygenation with nitrite treatment (0–100μM). **(B)** Mitochondrial complex I activity in CHO-R (open bars) and CHO-Mb (filled bars) cells in normoxia (control), after nitrite (25μM) treatment in normoxia (Nitrite), after hypoxia/reoxygenation (H/R), or after hypoxia/reoxygenation in the presence of nitrite (25μM; H/R + Nitrite). **(C)** Hydrogen peroxide production by CHO-R (open bars) and CHO-Mb (filled bars) cells in normoxia (Control), after hypoxia/reoxygenation (H/R), or after hypoxia/reoxygenation in the presence of nitrite (25μM; H/R + Nitrite). **(D)** S-nitrosothiol levels of mitochondria isolated from CHO-R and CHO-Mb cells in normoxia (control) after hypoxia/reoxygenation (H/R), or hypoxia/reoxygenation in the presence of nitrite either 10 minutes (10m) or 2 hours (2h) after reoxygenation. *p<0.01; #p<0.05. Data are means ± SEM. n=5 for all panels.

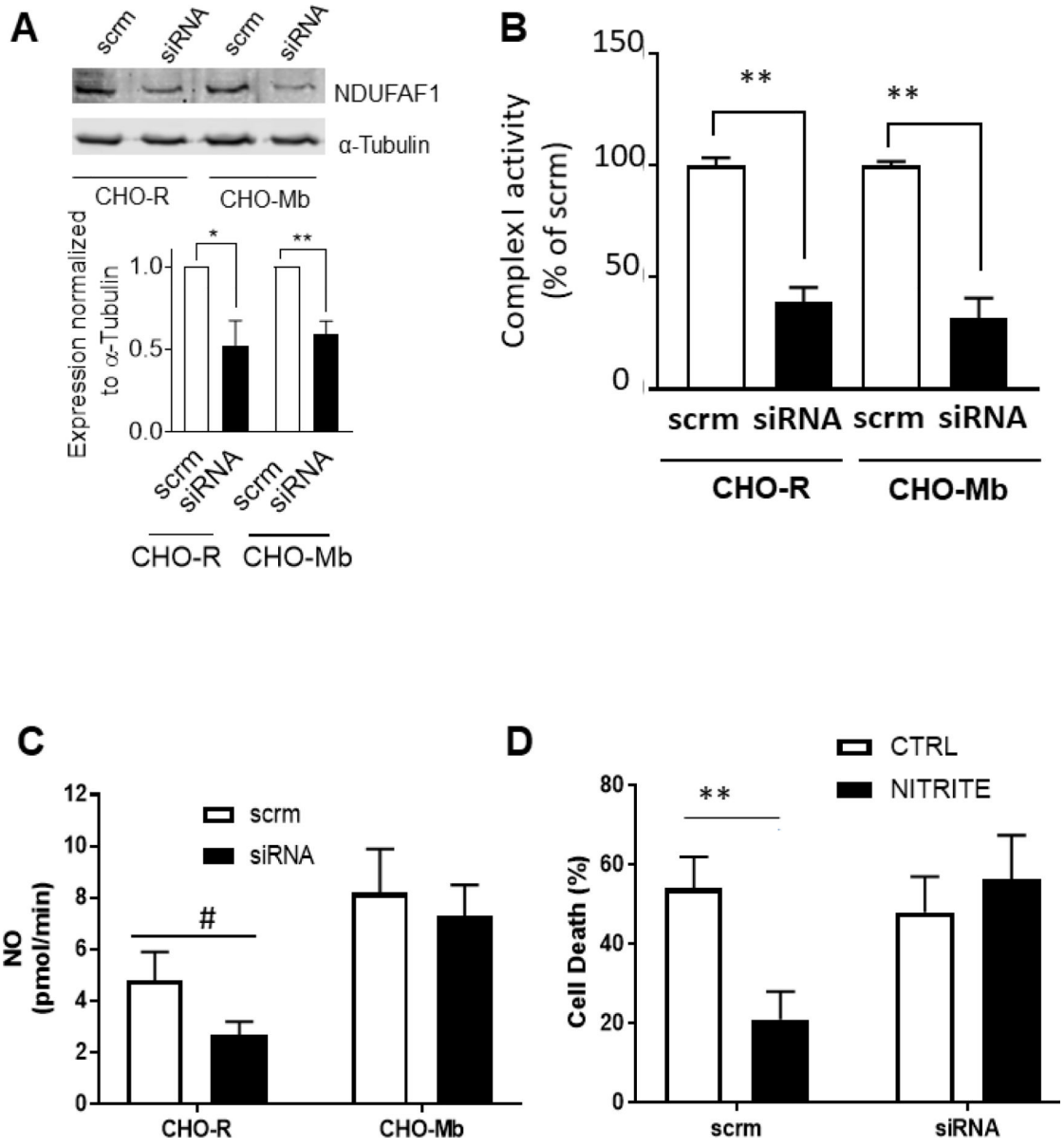

Figure 4: Complex I is required for nitrite and myoglobin dependent protection from hypoxia/ reoxygenation.

(A) Representative Western blot and quantification of complex I assembly factor NDUFAF1 protein expression following 72h of incubation with scrambled (scrm) or siRNA targeted to NDUFAF1. **(B)** Complex 1 activity (rate of rotenone-sensitive NADH consumption) following NDUFAF1 knockdown, normalized as a percentage of cells treated with the scramble control (scrm). **(C)** NO production by CHO-R and CHO-Mb cells treated with scrambled (open bar) or siRNA to NDUFAF1 in the presence of nitrite (1mM) in anoxia. **(D)** Quantification of cell death (LDH release) as a percent of normoxic cells in CHO-Mb with NDUFAF1 (scrm) or after siRNA to NDUFAF1 (siRNA) after H/R in the presence or absence (ctrl) of 20uM nitrite. *p<0.01; #p<0.05. Data are means ± SEM. n>3 for all panels.

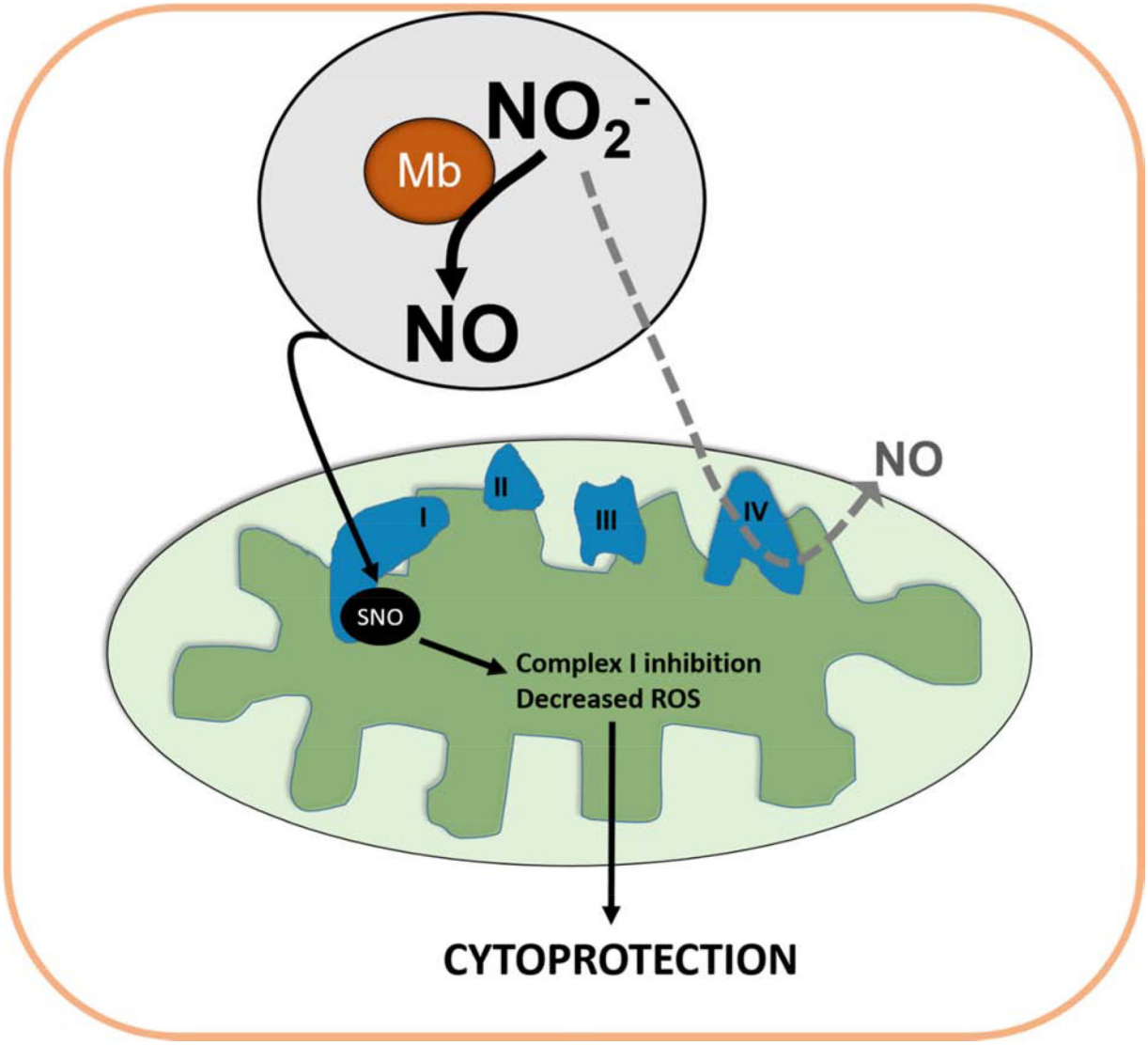

Figure 5: The reaction between nitrite and myoglobin S-nitrosates mitochondrial complex I to mediate cytoprotection.

During hypoxia, nitrite in the cell is reduced to NO to some extent by mitochondrial complex IV (gray dotted arrow), but with greater efficiency by myoglobin (black arrow). This reaction between nitrite and myoglobin results in the S-nitrosation (SNO) of mitochondrial complex I, which inhibits complex I activity and results in decreased mitochondrial ROS production, leading to cytoprotection of cardiomyocytes.