**S-nitrosylation of Peroxiredoxin 1 Contributes to Viability of Lung Epithelial Cells during
Bacillus anthracis Infection**

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Running title: S-nitrosylation effects on host viability during anthrax infection

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; bNOS, bacterial nitric oxide
synthase; Prx, peroxiredoxin; HSAECs, human small airway epithelial cells; MOI, multiplicity
of infection; BST, Biotin-Switch technique; L-NAME, L-Nω-nitroarginine methyl ester; DAF-
2DA, 4,5-diaminofluorescein diacetate; SNOC, S-nitrosocysteine.

Keywords: *Bacillus anthracis*; Nitric oxide synthase; Peroxiredoxin 1

Abstract

Background

Using *Bacillus anthracis* as a model gram-positive bacterium, we investigated the effects of host protein S-nitrosylation during bacterial infection. *B. anthracis* possess a bacterial nitric oxide synthase (bNOS) that is important for its virulence and survival. However, the role of S-nitrosylation of host cell proteins during *B. anthracis* infection has not been determined.

Methods

Nitrosoproteomic analysis of human small airway epithelial cells (HSAECs) infected with toxigenic *B. anthracis* Sterne was performed, identifying peroxiredoxin 1 (Prx1) as one predominant target. Peroxidase activity of Prx during infection was measured using 2-Cys-Peroxiredoxin activity assay. Chaperone activity of S-nitrosylated Prx1 was measured by insulin aggregation assay, and analysis of formation of multimeric species using Native PAGE. Griess assay and DAF-2DA fluorescence assay were used to measure NO production. Cell viability was measured using the Alamar Blue assay and the ATPlite assay (Perkin Elmer).

Results

S-nitrosylation of Prx1 in Sterne-infected HSAECs leads to a decrease in its peroxidase activity while enhancing its chaperone function. Treatment with bNOS inhibitor, or infection with bNOS deletion strain, reduces S-nitrosylation of Prx1 and decreases host cell survival. Consistent with this, siRNA knockdown of Prx1 lowers bNOS-dependent protection of HSAEC viability.

Conclusions

46 Anthrax infection results in S-nitrosylation of multiple host proteins, including Prx1. The nitrosylation-
47 dependent decrease in peroxidase activity of Prx1 and increase in its chaperone activity is one factor
48 contributing to enhancing infected cell viability.

49
50
51 General significance

52 These results provide a new venue of mechanistic investigation for inhalational anthrax that could lead to
53 novel and potentially effective countermeasures.

Author Summary

Mechanisms of host cell survival play a central role during infection with microbial pathogens, including the etiologic agent of anthrax, *B. anthracis*. The critical role of S-nitrosylation (nitric oxide attachment to cysteine residue) in regulating cell viability has been demonstrated by some studies. S-nitrosylation of proteins is mediated by the nitric oxide Synthase (NOS) gene, and although *B. anthracis* is one of the few bacterial species that produces NO (bNO), very little is known about the contribution of its bNO activity to host cell survival. Here, we present the first demonstration of bNOS-dependent S-nitrosylation of specific host cell proteins during infection with *B. anthracis* and its significant contribution to host cell viability. Of the identified hits, we focused on Peroxiredoxin 1 (Prx1), which is a key regulator of oxidative/nitrosative stress. S-nitrosylation of Prx1 following infection decreased its peroxidase activity but enhanced its chaperone function. Treatment with bNOS inhibitor, or infection with a bNOS deletion strain, decreased S-nitrosylation of Prx1 and also reduced host cell survival. Furthermore, lowering the levels of Prx1 lowered the level of bNOS-dependent protection of host cells. We propose that the nitrosylation-dependent enhancement of Prx1 chaperone activity during *B. anthracis* infection is one major contributing factor for enhancing host cell survival.

1. Introduction

The important role of protein S-nitrosylation during signal transduction and regulation of protein function has garnered much recent interest. In vitro modification of the functions of many proteins through S-nitrosylation has been demonstrated and has been well-characterized in a number of cases [1]. Furthermore, many studies have identified important roles for S-nitrosylation for carrying out normal cellular function and recently numerous pathophysiological settings have been ascribed to aberrant S-nitrosylation of proteins [2–6]. Unlike phosphorylation, S-nitrosylation does not directly rely on the recognition of target structure by an enzyme for specificity, but rather on the chemical reactivity between the nitrosylating agent and the target protein [7]. S-nitrosylation can be selective and specific, but the exact mechanisms are not yet fully understood.

The importance of nitric oxide (NO) during infection has also been demonstrated, where NO derived from either host or bacteria can induce post-translational modification (PTM) of proteins by S-nitrosylation of thiol groups of cysteine residues [2,8,9]. *B. anthracis*, the causative agent of anthrax disease, is one of the few species of bacteria that produces NO (bNO), in addition to producing toxins that enable bacilli to evade immune defenses and cause host cell death [10–12]. In this context, effects on cell viability of the host during infection with *B. anthracis* are important to study, and little is known with regard to how nitrosylation effects influence host cell viability during anthrax infection. It has been recently reported that bNO contributes to death of macrophages by S-nitrosylation of bioenergetic-related proteins within mitochondria [13]. However, the landscape of host nitrosylation effects is complex and clearly much remains to be discovered about the different cellular effects that are mediated through this PTM process, including the cell viability effects tied to anthrax infection. *B. anthracis* produces bNO by the gene that encodes for the bacterial nitric oxide synthase (bNOS) [2,14]. The bNOS gene is

119 located on the chromosome [2,14], in contrast to the genes that encode for the major virulence
120 factors toxin and capsule, which are carried on two plasmids (pXO1 and pXO2, respectively)
121 [15]. bNO is a key bacterial defense mechanism, protecting bacterial DNA against the oxidative
122 burst in infected macrophages [14–16]. Unlike eukaryotic NOS, bNOS lacks the essential
123 reductase domain and instead uses eukaryotic cellular reductases as a dedicated redox partner to
124 generate nitric oxide *in vivo* [17–19]. Functional bNOS is known to protect *B. anthracis* against
125 oxidative stress and is essential to its virulence in macrophages [14–16]. The production of bNO
126 has been demonstrated to occur very early after *B. anthracis* infection, while the host NO
127 formation occurs at later time points [8,11,14]. However, the mechanism of the virulent effect of
128 bNO with respect to host cell protein modification has not been previously investigated.

129 The S-nitrosylation of different peroxiredoxins (Prxs), which function to degrade peroxide or
130 act as a chaperone, have been shown to have both positive and negative influences on different
131 diseases, such as cardiac and neurological disorders [20–22]. For instance, S-nitrosylation of
132 Prx2 during Parkinson's disease causes the inhibition of its peroxidase activity and thus
133 contributes to the neurodegeneration process, while during cardiac disease S-nitrosylation of
134 Prx2 is proposed as a protective strategy against nitrosative stress [21,22]. Highly homologous to
135 Prx2 is the Prx1 protein, which has many different roles in the cells: chaperone activity, signal
136 mediation, peroxidase activity, and H₂O₂ scavenging activity [23,24]. Multiple structural and
137 conformational changes occur to regulate the different roles that Prx1 executes throughout the
138 different stages of metabolism or in different environments [23,25,26]. Although the regulations
139 are delicate and complex, over-oxidation and S-nitrosylation are two important post-translational
140 modifications that help regulate Prx1 activity [26–28].

The functional switch of Prx1 from peroxidase to chaperone is correlated with the oligomerization status of the enzyme [26–28]. The change in oligomerization status modifies Prx function and allows for an efficient switch mechanism for controlling signaling via H₂O₂. Under lower oxidation conditions, Prx1 is in a monomeric or dimeric form, resulting in high peroxidase activity [26–29]. In over-oxidation conditions, Prx1 is converted to high-molecular-weight complexes and acts as a chaperone, with significantly lower peroxidase activity [24,27]. In addition, S-nitrosylation of Prx1, like other post-translational modifications, causes an increase in oligomerization [26,29].

In this study, we investigated S-nitrosylation of host proteins during early *B. anthracis* infection and how this modification could play a role during anthrax pathogenesis, in particular with respect to cell viability effects discussed above. Through nitrosoproteomic analysis, Prx1 was found to be a predominant target for S-nitrosylation during toxigenic *B. anthracis* Sterne infection. Therefore, we hypothesized that bNO contributes to the determination of the function of host Prx1 as either a chaperone or peroxidase through S-nitrosylation, and leads to an effect on the cell viability of the infected host as a consequence. Our results demonstrate that nitrosylation of Prx1 enhances its chaperone activity in the early stage of *B. anthracis* infection, and suggest a model in which this enhanced chaperone activity contributes to enhanced cell viability within the infected lung. Our results demonstrate the need for further investigation into the S-nitrosylation of host proteins and suggest that such studies will lead to a deeper understanding of inhalational anthrax and the potential development of improved therapeutic treatments.

2. Materials and methods

2.1. Bacterial strains and HSAECs

B. anthracis non-encapsulated Sterne strain 34F2 (devoid of pXO2) was obtained from the Colorado Serum Company. The non-toxigenic Δ Sterne strain (devoid of both pXO1 and pXO2) was obtained from the collection of the National Center for Biodefense and Infectious Diseases (George Mason University, VA, USA). The Δ bNOS strain has been constructed and characterized by two of the authors for this study [11]. HSAECs were obtained from Cambrex Inc. (Walkersville, MD) and maintained according to the vendor's protocol.

2.2. Reagents

Purified Prx1 protein used in this study was purchased from Abcam (ab74172). Antibodies used in this study include: Rabbit α -Prx1 antibody (Abcam, ab41906), Rabbit α -Prx-SO3 antibody (Abcam, ab16830), Rabbit α -biotin antibody (Abcam, ab53494), α -B-actin (Abcam, ab8227), α -B-tubulin (Abcam, ab6046), α -elomase 1 (Abcam, ab85086), and α -GAPDH (Abcam, ab9485). Hs-PRDX1_1 Flexitube siRNA (cat#- 1027415, sequence: AAGGCTGATGAAGGCATCTCG), and control Luciferase GL2 siRNA (Cat#: 301704, Sequence: AACGTACGCGGAATACTTCGA) were purchased from Qiagen.

2.3. Bacterial infection of HSAECs

HSAECs were incubated with *B. anthracis* spores using a multiplicity of infection (MOI) of 210 cfu per HSAECs. HSAECs were maintained in Ham's F12 medium (ATCC) containing 10% fetal bovine serum, 2 mM L-glutamine, 2 mM non-essential amino acid, 60 μ M β -mercaptoethanol, and 2 mM sodium pyruvate (HSAECs medium) at 37°C in an atmosphere of 95% air and 5% CO₂, according to vendor's protocol. HSAECs were cultured and grown in medium with 100 U/ml penicillin-streptomycin (ATCC),

but at least 2 h prior to challenging the cells with *B. anthracis* spores, the medium was changed to medium without penicillin-streptomycin.

2.4. Biotin-switch technique (BST)

For the BST assay, cells were either left uninfected or incubated with the spores for 6 hours. The live cells were subsequently washed 3 times with PBS and then lysed using lysis buffer provided by the commercial BST kit (Cayman Chemical). The cell lysate was processed using the BST kit according to the manufacturer's instructions, as detailed by Jaffrey *et al.* [29], with a few modifications. After the BST reaction, the acetone-precipitated protein pellets were resuspended in neutralization buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% TritonX-100). Twenty percent of the sample was saved as "input," while 80% was further purified with either streptavidin-agarose or streptavidin-Sepharose beads. For purification, the sample was incubated with the beads for 1 h at room temperature with gentle agitation. The beads were then washed five times with neutralization buffer containing 600 mM NaCl. The bound proteins were then eluted in an elution buffer (20 mM HEPES, pH 7.7, 100 mM NaCl, 1mM EDTA, and 100 mM 2-mercaptoethanol) by boiling for 10 min. The samples were centrifuged and the supernatant was incubated in 2 volumes of cold acetone at -20°C for precipitation of the proteins prior to Western blot analysis and silver staining. In order to quantify percent protein recovery following the BST procedure, BCA assay was performed to compare protein quantification of the starting cell lysate, the material obtained post biotin switch, and the material post streptavidin pulldown (Table S1).

2.5. SDS-PAGE and Western blot analysis

209 Either total cell lysates, or streptavidin-agarose- or streptavidin-Sepharose-purified proteins
210 (biotin-switched) from normal (control) and spore-infected HSAECs, were boiled in 2X SDS
211 sample buffer containing either 60 mM dithiothreitol or 30 mM 2-mercaptoethanol for 5 min.
212 The samples were loaded and run on 4-12% Bis-Tris Gel (Invitrogen) and transferred onto
213 nitrocellulose membrane using the iBlot Transfer System (Invitrogen). Following primary and
214 HRP-conjugated secondary antibody incubations, protein bands were detected using the
215 SuperSignal West Femto Maximum Sensitivity Substrate System (Pierce) and the Molecular
216 Imager ChemiDoc XRS System (Bio-Rad) to visualize the bands.

218 2.6. Mass spectrometry

219 Cells were challenged with Sterne spores (MOI 10) for 6 hours and live cells were
220 subsequently harvested for analysis. Streptavidin-agarose purified S-nitrosylated proteins (biotin-
221 switched) were analyzed by reverse-phase liquid chromatography nanospray tandem MS using a
222 linear ion-trap mass spectrometer (LTQ, Thermo Electron), as described by Chung *et al.* [30].
223 Tandem mass spectra were searched against the human database (the National Center for
224 Biotechnology Information) with SEQUEST. For a peptide to be considered legitimately
225 identified, it had to achieve cross-correlation scores of 1.5 for $[M+H]^+$, 2.0 for $[M+2H]^{2+}$, and
226 2.5 for $[M+3H]^{3+}$, and a maximum probability of randomized identification of 0.001.

228 2.7. Native PAGE Oligomerization Study

229 Cell lysates from uninfected HSAECs, uninfected SNOC-treated HSAECs, and cells
230 infected with either *B. anthracis* Sterne strain (MOI 2) or *B. anthracis* Δ BNOS spores (MOI 2)
231 and collected at 3 hours post infection, were run using Native PAGE (Invitrogen, BN1004BOX),

following the manufacturer's instructions. Briefly, samples were prepared using the Native PAGE sample buffer (Invitrogen, BN2008) and after addition of G250 loading buffer (Invitrogen, BN2004) to each sample they were run on a Native PAGE gel and transferred to PVDF membrane. Western blot analysis was performed using a rabbit anti-Prx1 primary antibody (Abcam, ab41906) and HRP-conjugated goat anti rabbit secondary antibody, and visualized using the ECL kit (Thermo Scientific, Cat# 34095). Recombinant Prx1 protein, and recombinant Prx1 treated with SNOC for 3 hours were also run as controls.

2.8. Peroxiredoxin activity

Cell lysates from uninfected cells and from cells infected with toxigenic Sterne spores (MOI 10) were prepared at 3 hour post infection. Peroxidase activity was measured by the reduction of NADPH by 2-Cys-Peroxiredoxins, using *E. coli* thioredoxin and mammalian thioredoxin reductase to provide the reducing power. The 2-Cys-Peroxiredoxin activity assay kit (Redoxica) was used per manufacturer's instruction. For assay monitoring, OD₃₄₀ values were measured using a Helios γ spectrophotometer (Thermo Electron) at 30 sec intervals for 3 min.

2.9. Determination of NO production

For fluorescence microscopy, cells were cultured in an eight-chamber slide overnight. Cells were pretreated with 2 mM L-N ω -nitroarginine methyl ester (L-NAME) (Sigma-Aldrich) for 30 min. Sterne spores (MOI 10) were then added for either 2 hours or 6 hours. Following each incubation period, the spores were washed out using Hanks balanced salt solution (HBSS, Invitrogen). 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem) at a concentration of 15 μ M in HBSS was added to the chambers and incubated for 15 min in 5% CO₂ incubator at 37°C. The cells were then washed three times with HBSS, and the amount of nitric oxide for each

condition was visually analyzed using a Nikon Eclipse 90i fluorescence microscope. The levels of NO in culture supernatants of infected cells were measured using Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemicals), according to manufacturer's instructions.

2.10. *S-nitrosocysteine (SNOC) preparation*

Stock solutions of SNOC were prepared fresh for each experiment by allowing 10 mM L-cysteine to react with 20 mM sodium nitrite in the presence of 20 mM HCl and 1 mM EDTA. Uncharged nitrous acid was removed by adding 0.1% ammonium sulfamate. As SNOC solution is more stable at acidic pH, dilutions into the reaction buffers (pH 7.4) were made immediately before the assay. The final concentration of SNOC was determined from absorbance at 334 nm, using the extinction coefficient of $900 \text{ M}^{-1} \cdot \text{cm}^{-1}$. For control, the SNOC buffer was made without sodium nitrite.

2.11. *Chaperone activity*

Insulin was used as a substrate for measuring the chaperone activity of Prx1, using 5 mM DTT-induced chemical aggregation of insulin (52 μM) as described [27,31]. Changes in turbidity due to substrate aggregation were monitored by measuring absorbance at 650 nm using a GENESYS 20 UV spectrophotometer (Thermo Fisher Scientific). A molar ratio of 0.25:1 (Prx1:insulin) was used. Prior to measuring the activity, the samples containing 200 μM SNOC were pretreated for 30 min. The samples in the absence of Prx1 were pretreated with 60 mM HEPES (pH 7). For determining chaperone activity of cell lysates, 25 μl of cell lysates (0.2 mg/ml proteins) were added to the reaction mixture instead of Prx1. Chaperone activity was calculated based on turbidity changes at 650 nm after a 2 h incubation.

2.12. Cell viability

To test cell viability of S-nitrosylated HSAECs in the presence or absence of H_2O_2 , cells were pretreated with either 200 μM fresh SNOC or 200 μM 2-month-old SNOC for 30 min. Medium with or without 1 mM H_2O_2 was replaced with fresh media and the cells were further cultured for 24 hours. Cell viability was measured using the Alamar Blue assay (Fig 3C). Following addition of 10% Alamar Blue (Invitrogen) to the culture media, the cells were incubated for 2 hours and the amount of Fluorescence was measured using a Fluoroskan II fluorescent plate reader at the excitation wavelength of 530 nm and emission wavelength of 584 nm.

For measuring S-nitrosylation effects on viability of Sterne infected HSAECs (Fig 5B and 5D), cells were pretreated for 30 min with either media alone or media containing either 5 mM L-NAME, 200 μM fresh SNOC, 200 μM Old SNOC, or SNOC buffer, followed by addition of either media alone or media containing Sterne spores or ΔbNOS spores (MOI 2) for 6hours. The cells were incubated for 1 h in 20 μg gentamicin, 100 units penicillin, and 100 μg streptomycin per ml before performing the ATPlite assay (Perkin Elmer) according to manufacturer's instructions. The ATPlite assay is not sensitive to nitrous oxide and is therefore an appropriate cell viability assay for our studies.

To measure cell viability for the siRNA experiments (Fig 5D), after a 24-h transfection with Prx1 or control siRNA, HSAECs were pretreated with either media only or media containing 200 μM fresh SNOC for 30 min, followed by the addition of either media only or media containing Sterne spores or ΔbNOS spores (MOI 2). Cell viability was measured using the ATPlite assay 6 hours post infection, as described above.

2.13. *siRNA Knock down of Prx1*

HSAECs were seeded (5×10^4 cells/well) in a 24-well plate in Ham's F-12 medium containing serum and antibiotics. Twenty-four hours after seeding, cells were transfected with FlexiTube siRNA (Qiagen) according to the manufacturer's instructions, using 0.3 μ g of Prx1 siRNA and 2.4 μ l of RNAiFect or HiPerFect transfection reagents (Qiagen). As experimental control, cells were also transfected with the same amount of non-related luciferase GL2 siRNA (Qiagen). Following a 24-h incubation period, the effect of transfection was verified by Western blot analysis using anti-human Prx1 polyclonal antibody.

2.14. *Statistics*

All experiments presented in this study were validated with a minimum of 3 biological replicates (independent runs). All data are expressed as arithmetic means $\pm$ standard deviations. Comparisons between groups were carried out using the Student's t-test and Kruskal-Wallis rank sum tests, in order to determine statistical significance. Significance was set at $P < 0.05$.

3. Results

3.1. *S-nitrosylation of proteins in HSAECs during toxigenic B. anthracis infection*

We first determined the dependency of the death of HSAECs on infection duration and the multiplicity of bacterial infection (Fig. S1). At lower MOIs (2 and 5), significant cell death was observed starting at 5 hours post infection; however, at higher MOI (10), significant cell death was observed by 1 hour post infection. At 5 and 6 hours post infection, an increase in MOI caused statistically significant increases in cell death although at MOI 10 there was no difference

in cell death levels between the two time points. We next determined the correlation between HSAECs death and protein S-nitrosylation during *B. anthracis* infection, by comparative analysis of total cell extracts from uninfected cells with those from cells that were infected with Sterne spores for 2 hours. Using a commercial BST assay kit, S-nitrosylated proteins were converted to S-biotinylated proteins, and were analyzed by both silver staining and Western analysis against biotin. Equivalent protein band intensities on the silver-stained gel showed that the total protein levels in the two samples were similar (Fig. S2A and C). Probing for biotin showed that the S-nitrosylation of proteins increased during Sterne infection compared to uninfected control (Fig. S2B and D), suggesting that the S-nitrosylation due to toxigenic Sterne infection is specific.

In order to profile the S-nitrosylated proteins, mass spectrometry analysis was performed. Total cell lysates from live uninfected, Δ Sterne-challenged, or Sterne spore-challenged HSAECs were collected after 6 hours of infection and S-nitrosylated proteins were converted to biotinylated forms using the BST and purified by streptavidin beads. The Δ Sterne strain served as an appropriate control since we have shown that unlike the Sterne strain the Δ Sterne derivative does not express bNOS mRNA at either early (4 h) or late (20 h) post infection time points [13]. Total purified S-biotinylated proteins were trypsinized and identified by LTQ tandem mass spectrometry (MS), and the MS analysis was repeated using an independent sample set (biological replicate) to verify the reproducibility of the data. A complete listing of the identified S-nitrosylated host proteins are provided in Supplementary Table S2. The MS analysis identified 141 S-nitrosylated proteins in HSAECs infected with the Sterne strain. From these, we selected a list of proteins with significant increase in S-nitrosylation after Sterne challenge based on the stringent criterion of having identified multiple peptides as compared to none or only a

few peptides for the corresponding uninfected or Δ Sterne-challenged controls (Table 1). These selected proteins, implicated by both MS runs, were categorized based on their known functionalities (Table 1). The major functional classes of these proteins were chaperones, cytoskeletal proteins, metabolic enzymes, protein synthesis proteins, and signaling proteins.

3.2. *Prx-1 is S-nitrosylated by B. anthracis infection*

To further validate the MS identification of the selected proteins with an increase in S-nitrosylation during toxigenic Sterne infection, specific proteins from each of the functional groups were chosen for further analysis. Beta-actin and β -tubulin from the cytoskeleton category, enolase I and glycerol-3-aldehyde dehydrogenase (GAPDH) from the metabolic enzyme category, and Prx1 from the signaling category were selected. Western blots against Prx1, β -actin, β -tubulin, enolase I, and GAPDH were performed after the cell lysates were S-biotinylated using BST and subsequently purified by streptavidin-agarose beads (Fig. 1A). Fully consistent with the MS findings, the Western data showed that these proteins were S-nitrosylated to much higher levels during Sterne infection compared to the uninfected or Δ Sterne-challenged conditions. From the identified MS hits, Prx1 was chosen for further functional study, considering its important function as a key regulator of oxidative/nitrosative stress and also its chaperone activity during the normal cellular life cycle. Furthermore, Prx1 appeared to be significantly S-nitrosylated after Sterne challenge (Table 1). Two fully independent MS runs both demonstrated a dramatic difference between the identified number of peptide hits for Prx1 following Sterne challenge compared to no infection or infection with Δ Sterne controls; therefore, even though the MS analysis was qualitative these dramatic differences nevertheless suggested the importance of S-nitrosylation of Prx1 during toxigenic Sterne infection.

Furthermore, this is confirmed by Prx1 Western analysis following BST of lysates from cells infected with WT Sterne compared to cells infected with Δ Sterne or with a bNOS deletion mutant (Δ bNOS) derived from the Sterne strain, which lacks the ability to S-nitrosylate proteins. We have demonstrated previously that the Δ bNOS strain has invasion and germination characteristics in macrophages that are similar to the Sterne strain from which it has been derived [11]. Furthermore, it has been shown previously that the Δ bNOS strain has similar growth rate to the Sterne strain in bacterial cultures [32]. Therefore, the Δ bNOS strain serves as an appropriate control for our studies. As shown in Fig. 1B, Prx-1 was S-nitrosylated by Sterne WT, but not by bNOS mutant (Δ N) or Δ Sterne mutant (Δ S). Taken together, the results suggest that Prx-1 is a major S-nitrosylated protein during *B. anthracis* infection.

3.3. NOS inhibition and bNOS deletion decrease S-nitrosylation of Prx1

To determine NO levels during infection, nitrate/nitrite levels in culture supernatants were determined using Griess assay. Sterne infection increased nitrate/nitrite levels in culture supernatants, with higher levels observed with an increase in infection time (Fig 2A). In contrast, infection with the bNOS mutant (Δ N) or Δ Sterne mutant (Δ S) did not cause any increase in comparison to uninfected control (Fig. 2A). These results demonstrate that under our infection conditions the increase in NO levels during *B. anthracis* infection is due to the function of the bacterial bNOS gene. We also observed an increase in the intracellular NO levels in HSAECs at 6 h post infection as indicated by DAF-2DA staining, a cell-permeable fluorescent dye that is used to measure intracellular NO levels. However, treatment with L-NAME, a pan-inhibitor of nitric oxide synthase (NOS), blocked accumulation of intracellular NO during infection (Fig. 2B). To ensure that the S-nitrosylation of Prx1 can be inhibited by L-NAME, total lysates from

live cells that were pre-treated for 30 min with 2 mM L-NAME and subsequently challenged with Sterne spores for 6 hours were used for analysis. The cell lysates were S-biotinylated using BST and following purification by streptavidin-agarose beads were analyzed by Western blotting against Prx1. The results showed that L-NAME does inhibit S-nitrosylation of Prx1, whereas in the absence of L-NAME Prx1 continues to be S-nitrosylated during Sterne infection (Fig. 2C). The expression of total Prx1 shows little change during several post infection times analyzed, indicating that the S-nitrosylation changes observed during toxigenic Sterne infection are not related to changes in total Prx1 expression levels (Fig. 2D). The slight oscillation observed at the 4 and 6 h post infection time points might correspond to the oxidation–reduction cycles of peroxiredoxin proteins that constitute a universal marker for circadian rhythms in organisms [33,34].

3.4. S-nitrosylation of Prx1 causes a decrease in its peroxidase activity

To study the effects of S-nitrosylation on Prx activity of HSAECs during anthrax infection, the total activity of 2-Cys Prxs was assayed by monitoring NADPH reduction in a thioredoxin reductase-coupled reaction. The 2-Cys Prx activity was decreased after 3 hours of toxigenic Sterne treatment when compared to uninfected HSAECs (Fig. 3A). In line with this observation, the ability of recombinant Prx1 to catalyze H_2O_2 was inhibited following treatment with SNOC, which ensures that the protein's cysteine residues are S-nitrosylated (Fig. 3B). SNOC treatment decreased Prx1 peroxidase activity by about 10-fold. In the absence of Prx1, SNOC itself did not change the H_2O_2 levels in the reaction mixture, showing that the observed effects were due to the Prx1 activity (Fig. 3B). These results confirm that S-nitrosylation does indeed inactivate the peroxidase activity of Prx1.

We next determined whether the S-nitrosylation of Prx1 has an effect on cell viability during Sterne infection. First, we analyzed the effect of S-nitrosylation on cell death induced by oxidative stress. HSAECs were pretreated with either 200 μ M fresh SNOC or 200 μ M old (inactive) SNOC for 30 min prior to H₂O₂ treatment. As shown in Fig. 3C, in the absence of S-nitrosylation (absence of SNOC treatment) HSAECs were resistant to H₂O₂ even at the high H₂O₂ concentration of 1 mM (ca. 73% viability). However, HSAECs pre-treated with fresh SNOC showed a further decrease in viability (ca. 23%) compared to HSAECs treated with old (inactive) SNOC. These results suggest that protein S-nitrosylation by SNOC sensitizes HSAECs to H₂O₂-mediated cell death.

3.5. Prx1 S-nitrosylation increases its chaperone activity

The chaperone activity of Prx relies on the ability of conserved cysteines to form disulfide bonds to ultimately form a homodecamer or dodecamer. Therefore, as part of the effort to determine whether the S-nitrosylation of Prx1 also leads to an increase in its chaperone function, we performed Native PAGE analysis under both reducing and non-reducing conditions to compare the untreated and SNOC-treated (nitrosylated) forms of purified Prx-1 and also cell extracts from uninfected cells, SNOC-treated uninfected cells, Sterne-infected cells, and Δ bNOS-infected cells (Fig. 4A). Our Native PAGE results demonstrate that there is a clear increase in the oxidized dimer form of Prx1 following SNOC treatment of the recombinant protein samples as well as uninfected cells. In addition, a similar increase in the Prx-1 oxidized dimer form is observed in the Sterne infected cells compared to the uninfected or Δ bNOS-infected control conditions. These findings are fully consistent with published reports that SNOC treatment increases the oxidized dimer form of Prx1 and that Prx1 dimer formation in cells increases over

time by treatment with CysNO or by treatment with increasing concentrations of CysNO [26]. In addition, consistent with our findings for Prx1 during anthrax infection, it has been shown that the oxidized dimer form of Prx1 has reduced peroxidase activity and that S-nitrosylation of Prx1 inhibits its peroxidase activity and increases dimerization of Prx1 [26]. Furthermore, and most significantly, the SNOC-treated and Sterne-infected cells clearly show increased levels of high molecular weight species of Prx1 corresponding to the decamer and dodecamer forms that are not present in the control or Δ bNOS-infected cells and disappear under reducing conditions (Fig. 4A), indicating a shift from the lower molecular weight enzymatic active form of Prx1 to the higher molecular weight forms of the protein that have been shown to signify increased chaperone activity [25,28] . Thus, these results demonstrate that bNOS-induced S-nitrosylation of Prx1 leads to formation of higher molecular weight species that have been linked to increased chaperone function.

We also tested for increased chaperone activity of S-nitrosylated Prx1 by a direct assay, using insulin as a folding substrate [27,28]. Changes in turbidity, which are due to substrate aggregation as a result of inability to fold properly, were monitored by measuring absorbance at 650 nm every 2.5 minutes for 2 hours. The results showed that S-nitrosylation of Prx1 by SNOC treatment increased the chaperone activity of Prx1, as indicated by the lower turbidity of the sample (Fig. 4B). Furthermore, Sterne infected cell lysates displayed higher chaperone activity compared to uninfected or bNOS-infected cell lysates (Fig. 4C). This observed increase in Prx1 chaperone activity during infection is presumably due to the S-nitrosylation of Prx1 resulting in its multimerization or stabilization of the multimers. Overall, these results demonstrate that S-nitrosylation of Prx1 causes an increase in its chaperone function during infection. It has been reported that in addition to S-nitrosylation over-oxidation of Prx1 can also lead to inhibition of

its peroxidase activity [3,7,14,26]. Therefore, we determined whether the S-sulfonylation of Prx1 also occurs during infection with *B. anthracis*. HSAECs were challenged with Sterne spores for 6 hrs and were subsequently left either untreated or treated with 1 mM H₂O₂ for 30 min. Western blot analysis of the cell extracts for SO₃-Prx and Prx1 demonstrated that S-sulfonylation of Prx1 does not take place during the post infection time under study and furthermore that Sterne infection prevents H₂O₂-mediated over-oxidation of Prx1, as shown by the lack of a SO₃-Prx signal under these conditions (Fig. S3). Therefore, S-sulfonylation of Prx1 does not occur during infection with *B. anthracis*.

3.6. *Prx1* contributes to NO-dependent protection of cell viability

To determine if protein S-nitrosylation has an effect on cell viability during *B. anthracis* Sterne infection, HSAECs were infected with the bNOS mutant or WT Sterne spores (MOI 2). We observe similar levels of viability for cells infected with either WT or Δ bNOS mutant strain at 2 hrs and 4 hrs post infection (Fig. 5A). However, decreased cell viability is observed starting at the 6 hr time point, with the cells infected with Δ bNOS showing a more dramatic decrease ($P < 0.01$) in viability (Fig. 5A). Consistent with this observation, pretreatment of HSAECs either with old SNOC or SNOC buffer only, or with 5 mM L-NAME inhibitor for 30 min prior to infection with *B. anthracis* spores, decreased cell viability. Conversely, pretreatment with fresh SNOC significantly increased cell viability in both Sterne and Δ bNOS infected cells, up to 75% in Sterne-infected cells and, as expected, to a lesser degree (64%) in cells infected with the bNOS mutant strain (Fig. 5B). These results suggest that protein S-nitrosylation overall, including S-nitrosylation mediated by bNOS, inhibits host cell death arising from *B. anthracis* infection.

To explore whether the S-nitrosylation of Prx1 plays a role in the observed NO-dependent protection of cell viability, siRNA knockdown of Prx1 was performed to lower the level of S-nitrosylated species. Efficient knockdown was achieved by using 0.3 µg of Prx1 siRNA (Fig. 5C). Cell viability assays indicated that while Prx1 knockdown had no effect on the viability of uninfected cells it significantly lowered the viability of Sterne-infected cells (Fig. 5D). Furthermore, pretreatment of cells with SNOC significantly increased cell viability of infected cells or cells treated with control siRNA compared to the non-SNOC treated cells, whereas it did not show the same significant increase in the cells treated with Prx1 siRNA (Fig. 5D). Thus, there is a significant jump in viability when comparing Sterne infection (64%) or Control siRNA + Sterne infection (68%) with those same conditions when SNOC is added (80-82%), whereas there is no significant increase in viability when Prx1 siRNA + Sterne infection (39%) is compared with the same condition when SNOC is added (51%). These results strongly suggest that S-nitrosylated Prx1 contributes to NO-dependent protection of cell viability.

4. Discussion

The important role of protein S-nitrosylation in signal transduction and regulation of protein function has been established in a variety of cellular contexts [2–4]. Furthermore, the importance of S-nitrosylation in determining the viability of macrophages infected with *B. anthracis* has been demonstrated by our previous work [13]. In this study, we profiled the host proteins that undergo S-nitrosylation during infection with *B. anthracis* and functionally characterized one of the top hits (Prx1 protein) in order to gain a mechanistic understanding of the viability effects of S-nitrosylation. Our results show that infection with *B. anthracis* contributes to the determination of Prx1 function via the process of S-nitrosylation, and results in improved host cell viability. It

has been demonstrated that during the very early phase of infection (within the first several hours post infection), the nitrosylation effects are due to the function of bNOS [11,13]. Therefore, considering that our analyses have been performed within this time frame, the S-nitrosylation effects on Prx1 are attributable to the bNOS function. In agreement with this suggestion, only Sterne strain infection, and not infection with the Δ bNOS strain or Δ Sterne, showed an increased protein S-nitrosylation in HSAECs, and infection with Sterne resulted in higher cell viability compared to Δ bNOS infection. Future studies are required to examine further the exact role of bNOS during this early S-nitrosylation phase following infection. As suggested previously, the ability of bNOS to produce nitric oxide faster and more efficiently than mammalian NOS gives *B. anthracis* an advantage over its host [11,13].

Some proteins common to other S-nitrosylation studies are present in the selected list of proteins with significantly increased S-nitrosylation during Sterne infection, which we derived from our nitrosoproteomic data: β -actin, α/β -tubulin, enolase 1, GAPDH, and pyruvate kinase [35,36]. These proteins have been shown to be S-nitrosylated in different diseases, such as heart and neurological disorders [20,21,29]. Enolase and pyruvate kinase, metabolic enzymes required for glycolysis, have been found to be S-nitrosylated in endotoxin-treated macrophages [35]. However, the effect of S-nitrosylation on the function of these two proteins has not been determined. With regard to GAPDH, Hara *et al.* [36] demonstrated that S-nitrosylation of GAPDH augments its binding to Siah 1 (an E3 ubiquitin ligase), through which the nuclear translocation of GAPDH is mediated. This suggests that the NO-S-nitrosylation-GAPDH-Siah1 cascade may represent an important molecular mechanism of cytotoxicity [36]. S-Nitrosylation of GAPDH has also been shown to inhibit its glycolytic activity [3], and in the context of anthrax infection bacterial GAPDH expression on the cell surface has been shown to be a plasminogen

binding protein [37]. In this regard, the S-nitrosylation of GAPDH in HSAECs and its role in *B. anthracis* infection and cell cytotoxicity merits further investigation. While our study focused on the contribution of S-nitrosylated Prx1 to the viability of lung epithelial cells during anthrax infection, clearly our findings do not exclude potential contributions from other factors as well.

As discussed previously, Prx1 has many different roles in the cell: chaperone activity, signal mediation, peroxidase activity, and hydrogen peroxide scavenging function [23,24]. The multiple structural and conformational changes that occur to regulate the different roles of Prx1 throughout the different stages of metabolism, or in different cellular environments, are delicate and complex. Over-oxidation and S-nitrosylation are two important post-translational modifications that help regulate Prx1 activity [26,27]. The oligomeric state controls the switch from peroxidase to chaperone function in human Prx1 and Prx2 [38]. When Prx1 is S-nitrosylated or over-oxidized, it acts more as a chaperone and is in a high-molecular-weight configuration of homodecamer [28,39]. However, when Prx1 is acting more as a peroxidase, it is in a low-molecular-weight configuration of oligomers or monomer [40]. Our studies show that during *Sterne* infection the inhibition of the peroxidase function of Prx1 is induced by S-nitrosylation and not over-oxidation. Our results also show that the S-nitrosylation of Prx1 during anthrax infection leads to formation of higher molecular weight (decamer) species, which have been shown to carry increased chaperone function and lower peroxidase activity [28]. Consistent with this, our assay results show lower peroxidase activity and higher chaperone function for S-nitrosylated Prx1. Gel filtration analysis and mass spectrometry studies have revealed that Prx2 as well as Prx1 mutant C83S exist as dimers, whereas wild type Prx1 can form a homodecamer [28]. In the case of Prx1, an additional cysteine (Cys83) is present that plays an important role at the dimer-dimer interface where van der Waals interactions and hydrophobic

force have been shown to be important for the formation of the homodecamer [27,28,40]. Although the details of Prx1 structural changes were not a focus of our studies, our results suggest that the multimerization states of Prx1 are favored by the nitrosative stress during *B. anthracis* infection, as indicated by the S-nitrosylation-dependent inhibition of peroxidase activity, enhancement of the chaperone activity of Prx1, and generation of multimeric forms of Prx1.

In its over-oxidation state, Prx1 forms multiple different complexes with signaling partners, including kinases c-Abl and JNK, p38 and the phosphatase PTEN [41]. In the absence of over-oxidation, Prx1 causes the activation of c-Abl and JNK and inactivates the phosphatase activity of PTEN [22,42–45]. This suggests that the increased Prx1 chaperone activity during *B. anthracis* infection, which is attributable to its S-nitrosylation, may be coupled to the function of Prx1 within one or more of these complexes in order to assist in increased viability of infected cells. In addition, it has been shown that Prx1 can act as a RNA binding chaperone that functions to promote cell survival under stressed conditions [46], so this is also potentially one mechanism by which the increased chaperone activity of Prx1 during anthrax infection may be improving viability. Our results show that the siRNA knockdown of Prx1 does not have any impact on cell viability in the absence of infection. In contrast, *B. anthracis* infection of cells in which the levels of S-nitrosylated Prx1 species has been lowered by Prx1 knockdown results in decreased cell viability. Consistent with this observation, SNOC treatment improves cell viability during *B. anthracis* infection and the level of improvement is reduced under the conditions of Prx1 knockdown. Furthermore, cell viability is significantly reduced during infection with the Δ bNOS strain, which lacks the ability to S-nitrosylate the host proteins. Together, these results support the conclusion that when Prx1 is at normal levels the improved cell viability observed during *B.*

576 *anthracis* infection can be correlated with the S-nitrosylation of Prx1. It should also be pointed
577 out that in addition to Prx1 the S-nitrosylation of other proteins may, and likely do, play a role in
578 prevention of full scale cell death during early *B. anthracis* infection, and future studies are
579 required to address this issue. Delayed cell death induced by a toxigenic strain of *B. anthracis*
580 has been shown to be caused by up-regulation of anti-apoptotic genes cFLAR and NF- κ B [47].
581 This apoptotic delay during anthrax infection may allow for improved bacterial survival and
582 dissemination, resulting in systemic disease. Also, S-nitrosylation of bacterial toxins has been
583 demonstrated to play a protective role against cell death [48] and therefore the possible
584 contributions of nitrosylated bacterial proteins in modulating cell viability following anthrax
585 infection should be considered in future studies as well. The results presented here show that the
586 S-nitrosylation of Prx1 is another mechanism for the suppression of cell death during early
587 anthrax infection, thereby contributing to the regulation of the progression of the disease.

588 From this study, a plausible model can be proposed to explain the effects of S-
589 nitrosylation of Prx1 in HSAECs (Fig. 6). During normal cellular metabolism, Prx1 encounters
590 H₂O₂ and forms an intramolecular disulfide bond between Cys52 and Cys173 to form a
591 homodimer [26,28]. With the help of Trx enzyme and NADPH, the intramolecular disulfide
592 bonds are broken and monomers of Prx1 are regenerated that are able to degrade another
593 molecule of H₂O₂. During infection, NO is produced by *B. anthracis* either outside or inside of
594 the host cells. Our model proposes that Prx1 nitrosylation occurs as a result of the nitric oxide
595 production by the bNOS gene of *B. anthracis*. NO binds to one or more of the cysteines in Prx1,
596 causing it to form a homodecamer. As a result, its peroxidase activity is decreased and its
597 chaperone activity is increased. Given that other NO target proteins such as GADPH have been
598 shown to have a negative influence on cell viability [36], and based on the findings for the

peroxidase-to-chaperone functional switch [26–28,38], such as enhancement of yeast resistance to heat shock due to the chaperone function of yeast cPrx1 and 2 [28], we propose that the increase in cell viability observed during *B. anthracis* infection is correlated to the increase in chaperone activity of Prx1 (Fig. 6). These results provide a novel mechanism of host response to *B. anthracis* infection, through bacterial-derived NO and the resulting protein S-nitrosylation in the lung epithelial cells. In summary, these findings suggest the potential of analyzing host S-nitrosylation changes for development of novel or improved therapeutic measures to treat inhalation anthrax.

5. References

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Table 1. Categorized summary of selected HSAECs proteins with significantly higher level of S-nitrosylation during Sterne challenge.

Function	Protein	Acc. No.	Number of Peptide Hits		
			Sterne	ΔSterne	Control
Chaperones	peroxiredoxin 1	4505591	15, 9	0, 0	1, 1
	peroxiredoxin 2, isoform a	32189392	3, 2	0, 0	1, 1
	heat shock protein 90 kDa	153792590	4, 3	0, 1	0, 0
Cytoskeletons	beta-actin	4501885	27, 15	3, 2	3, 2
	tubulin, beta	29788785	3, 3	0, 1	1, 1
	tubulin, alpha	57013276	4, 3	0, 2	0, 2
	actinin, alpha	12025678	3, 1	1, 0	0, 0
	annexin A2	209862831	6, 4	3, 2	2, 2
	profilin 1	4826898	4, 3	0, 2	0, 1
	filamin 1	116063573	2, 1	0, 0	0, 0
	hypothetical protein LOC112714 (tubulin, a3e)	46409270	3, 3	1, 0	0, 0
Metabolic Enzymes	enolase 1	4503571	3, 3	1, 0	1, 1
	glyceraldehyde-3-phosphate dehydrogenase	7669492	3, 4	1, 0	1, 1
	pyruvate kinase 3	33286418	7, 5	1, 1	1, 1
	lactate dehydrogenase A	5031857	2, 1	1, 0	1, 0
Protein synthesis	ribosomal protein L31	4506633	2, 2	0, 0	0, 0
	ribosomal protein L8	15431306	2, 2	0, 0	0, 0

Total cell lysates from live uninfected, ΔSterne-challenged, or Sterne spore-challenged HSAECs were collected at 6 hours post infection and S-nitrosylated proteins were converted to biotinylated forms using

the BST and purified by streptavidin beads. Total purified S-biotinylated proteins were trypsinized and identified by LTQ tandem MS.

FIGURE LEGENDS

Fig. 1. Validation of protein S-nitrosylation in *B. anthracis*-infected HSAECs identified by MS analysis. (A) Western blot analysis of S-nitrosylated proteins in cell extracts from uninfected control cells (C), Sterne spore (S)-, and Δ Sterne spore-challenged (Δ S) HSAECs. 2 μ g of purified biotin-switched proteins were purified by streptavidin-agarose beads and detected using specific antibodies for Prx1 or several housekeeping proteins (β -actin-tubulin, enolase 1, and GAPDH) that were identified by the MS analysis. (B) Western blot analysis of Prx1 (top), β -actin (middle) and S-nitrosylated proteins (bottom) in cell extracts from uninfected control cells (C), Sterne spore (S)-challenged, Δ Sterne spore (Δ S)-challenged, and bNOS mutant (Δ N)-infected HSAECs. For a negative control, a reducing agent containing ascorbate was omitted during BST.

Fig. 2. L-NAME inhibition of the S-nitrosylation of Prx1 induced by *B. anthracis* infection. (A) NO levels in culture supernatants of bacteria were determined by Griess assay after 2, 4 and 6 h infection. (B) HSAECs in the presence or absence of 2 μ M L-NAME were analyzed for the amount of nitric oxide production (green color) during Sterne-spore challenge (MOI 10), using DAF-2DA. (C) HSAECs, with or without treatment with 2 μ M L-NAME, were challenged with Sterne spores (MOI 10) for 6 hours. Total cell lysates were S-biotinylated by BST and following purification by streptavidin-agarose beads Western blot analysis against Prx1 was performed. (D)

Total Prx1 protein levels following Sterne infection of HSAECs (MOI 10) was analyzed by Western analysis of cell lysates obtained at 0, 2, 4, and 6 hours post infection. Cell lysates from uninfected HSAECs obtained at the same post-infection time points were included as control.

Fig. 3. Changes in 2-Cys peroxiredoxin peroxidase activity during anthrax infection of HASECs and modulation of peroxidase of Prx1 following S-nitrosylation. (A) Peroxidase activity of Prx1 was analyzed by measuring the level of reduction of NADPH in the presence of thioredoxin and thioredoxin reductase. Total cell extracts were prepared from uninfected HSAECs and HSAECs challenged with Sterne spores for 3 hours (MOI 10), and the degradation of NADPH was monitored by measuring absorbance at 340 nm over a period of 360 seconds. Assay buffer without Trx was used as a blank control for the absorbance measurements. Prx activities of uninfected (UI) control without TrxR, UI control with TrxR, and Sterne-infected HSAECs were compared (left panel). Sterne infection abrogated peroxidase activity of Prx1 at 3 h post infection (right panel). The assay was performed in triplicates. (B) Chemical reduction of H_2O_2 by recombinant wild type (WT) Prx-1 in the presence or absence of 200 μM SNOC was measured. The samples were tested in triplicates. Error bars in graph represent standard deviations. (C) HSAECs were pretreated for 30 min with either media only (Control) or media containing 200 μM fresh SNOC or 200 μM “old” SNOC (SNOC control), followed by addition of medium containing hydrogen peroxide (1 mM) for 24 hours. The Alamar Blue assay was used to measure cell viability. Assays were performed in triplicates. Error bars in graphs represent standard deviations.

Fig. 4. Sterne infected HSAECs increase chaperone activity of Prx1. (A) Prx1 was incubated with SNOC (250 μ M) for 3 h, and HSAECs were infected with bacteria (MOI 10) for 3 h. Total cell lysates, non-treated Prx1, and SNOC-treated Prx1 were subjected to Western blot analysis using α -Prx1 antibody to analyze the formation of Prx1 multimeric forms under non-reducing (top) and reducing (bottom) Native PAGE conditions. The “UI” lanes correspond to uninfected cell samples, the “S” lane is for the Sterne-infected sample, and the Δ N lane is for the Δ bNOS-infected sample. (B) The chaperone activity of Prx1 was measured using insulin as substrate. Substrate aggregation was monitored by measuring the light scattering at A_{650nm} . Prx1 and 0.3 mg/mL Insulin were used. (C) Relative chaperone activity of cell lysates prepared from uninfected HSAECs, HSAECs infected with Δ bNOS mutant, and Sterne-infected HSAECs were measured. Chaperone activity was measured at 2 h post incubation and calculated by net decrease of A_{650nm} ($n = 3$).

Fig. 5. Effect of Prx1 S-nitrosylation on cell viability in Sterne-infected HSAECs. (A) HSAECs were infected with Δ bNOS mutant or Sterne spores (MOI 2), and cell viability was measured using the ATPlite assay. (B) HSAECs were pretreated for 30 min with either media only or media containing either 200 μ M fresh SNOC, 200 μ M Old SNOC, SNOC buffer, or 5 mM L-NAME, followed by 6 hour incubation with either media alone or media containing Sterne spores (MOI 2). Cell viability was measured using the ATPlite assay. (C) HSAECs were treated with 0.3 μ g of Prx1-targeted siRNA and Prx1 protein levels were determined by Western blot analysis for verification of effective knockdown; GL2 luciferase siRNA was used as a negative control. (D) After 24hour transfection with Prx1 or control siRNA, HSAECs were pretreated with either media only or media containing 200 μ M fresh SNOC for 30 min, followed

829 by the addition of either media only or media containing Sterne spores or Δ bNOS spore (MOI 2)
830 for 6 hours. Cell viability was measured using the ATPlite assay 6 hours post infection. Data
831 represent mean % cell viability $\pm$ SEM.

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833 **Fig. 6. Proposed Model of Prx1 function in HSAECs during *B. anthracis* infection.** Nitric
834 oxide (NO) produced by *B. anthracis* inhibits the oxidation of host Prx1 and causes its S-
835 nitrosylation. The S-nitrosylation of Prx1 increases the chaperone activity of Prx1, leading to
836 increased cell viability through as yet to be determined mechanisms.

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