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Bacillus anthracis-derived nitric oxide induces protein S-nitrosylation contributing to macrophage death

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ABSTRACT

Bacillus anthracis, a causative agent of anthrax, is able to germinate and survive within macrophages. A recent study suggested that *B. anthracis*-derived nitric oxide (bNO) is a key aspect of bacterial defense that protects bacterial DNA from oxidative burst in the macrophages. However, the virulent effect of bNO in host cells has not been investigated. Here, we report that bNO contributes macrophage killing by S-nitrosylation of bioenergetic-relating proteins within mitochondria. Toxigenic Sterne induces expression of the *bnos* gene and produces bNO during early stage of infection. Nitroso-proteomic analysis coupled with a biotin-switch technique demonstrated that toxigenic infection induces protein S-nitrosylation in *B. anthracis*-susceptible RAW264.7. For each target enzyme tested (complex I, complex III and complex IV), infection by *B. anthracis* Sterne caused enzyme inhibition. *N*ω-nitro-L-arginine methyl ester, a NO synthase inhibitor, reduced S-nitrosylation and partially restored cell viability evaluated by intracellular ATP levels in macrophages. Our data suggest that bNO leads to energy depletion driven by impaired mitochondrial bioenergetic machinery that ultimately contributes to macrophage death. This novel mechanism of anthrax pathogenesis may offer specific approach to the development of therapeutics.

1. Introduction

Bacillus anthracis is an aerobic, gram-positive, soil-born, spore-forming bacterium that causes three distinct forms of anthrax: cutaneous, inhalational and gastrointestinal. In inhalational anthrax, *B. anthracis* spores are phagocytosed by alveolar macrophages and transported to the regional lymph nodes [1,2]. In macrophages, lethal toxin (LT), a major toxin, causes intracellular proteolytic cleavage of members of the mitogen-activated protein kinase kinase (MAPKK) family [3,4]. Edema toxin (ET), another toxin, is a calcium- and calmodulin-dependent adenylyl cyclase that converts cytosolic ATP to cAMP, interfering with cellular signaling and membrane permeability regulation [5]. The accumulated evidence suggests that the activities of LT and ET allow bacteria to evade the host's innate immune response (reviewed by [6,7]), nevertheless the mechanism by which *B. anthracis* evades immune attack are not yet fully understood.

Bacteria-derived nitric oxide (bNO) has recently gained attention as an executor of toxin-independent evasion of the host innate immune response because of the potential of bacteria against host oxidative stress defense system [8]. In mammals, NO is involved in many biologic processes that range from protection against pathogens to blood pressure regulation [9]. NO is synthesized from L-arginine by nitric oxide synthase (NOS) isozymes, namely neuronal (nNOS), inducible (iNOS), and endothelial NOS (eNOS) [10]. Recent genome sequencing effort has also identified bacterial protein sequences with substantial similarity to those of mammalian NOSs. Bacterial NOSs (bNOSs) found in strains of *Bacillus* [10,11], *Dienococcus* [12], *Staphylococcus* [13] and *Streptomyces* [14] are structurally similar to mammalian NOSs and *bona fide* bNO production has been demonstrated in several bacterial species. In the case of non-pathogenic *B. subtillis*, the bacterium produces bNO to gain rapid protection against oxidative damage by preventing the reaction of hydrogen peroxide with DNA (Fenton reaction) and activating a catalase [15]. This observation was extended to elucidate pathogenic mechanism of *B. anthracis* [15]. The NOS-deficient strain of *B. anthracis* demonstrated reduced virulence in an A/J mouse model of systemic infection correlating with a reduced spore survival in macrophages, suggesting that bNO acts as a defense against the im-

Abbreviations: bNO, *B. anthracis*-derived nitric oxide; bNOS, bacterial nitric oxide synthase; BST, biotin-switch technique; LT, lethal toxin; ET, edema toxin; ETC, electron transfer complexes; ROS, reactive oxygen species; L-NAME, *N*ω-nitro-L-arginine methyl ester; MOI, multiplicity of infection.

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mune oxidative burst and plays an essential role in pathogen virulence [15]. However, the mechanism of the virulent effect of bNO with respect to host cell protein modification has not been previously investigated.

In this study, we sought to examine the role of bNO as a pathogenic factor in host cells. NO exerts its effect by chemical modification of target proteins through S-nitrosylation of cysteine residues and nitration of tyrosine residues. S-nitrosylation of proteins by NOS activation can be induced by different treatments such as endotoxins and cytokines, or in pathophysiologic conditions such as hypoxia and combustion smoke [16]. This modification contributes to a large part of the influence of NO on cellular signal transduction and the etiology and symptomatology of an increasing number of human diseases including infectious diseases [17]. Since production of bNO has been reported in experiments with recombinant *B. anthracis* NOS (bNOS) [18] as well as in spore-infected macrophages [15], we expected to detect protein S-nitrosylation in *B. anthracis*-infected host cells. Indeed, we identified S-nitrosylated host proteins including electron transfer complexes (ETCs) in mitochondria by nitrosoproteomic analysis. Inhibition of S-nitrosylation of these proteins restored cell viability, which could be a novel mechanism of macrophage killing by the bNO-mediated mitochondrial impairment, in addition to the previously proposed mechanism involving LT [19].

2. Materials and methods

2.1. Bacterial strains and RAW264.7 cells

B. anthracis non-encapsulated Sterne strain $34F_2$ (devoid of pXO2) was obtained from the Colorado Serum Company. The non-toxigenic delta-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). Murine macrophage cell lines RAW264.7 from American Type Culture Collection (ATCC) were maintained in DMEM medium (ATCC) containing 10% fetal calf serum, 2 mM L-glutamine at 37 °C in an atmosphere of 95% air and 5% CO_2.

2.2. Biotin-switch technique (BST)

RAW264.7 cells were infected with spores (MOI 10) for 6 h and were lysed by HEN buffer (250 mM HEPES, pH 7.9, 1 mM EDTA, 0.1 mM neocuproine) containing 0.4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS). Control nitrosylation with S-nitrosyl glutathione (GSNO) was performed by preincubation of untreated control proteins with 100 μM GSNO for 2 h. BST was performed by the procedure detailed by Jaffrey et al. [20].

2.3. Two-dimensional (2D) gel electrophoresis

Immediately after the BST, the purified proteins were suspended in a buffer (2D protein solubilizer 1, Invitrogen) without reducing agent (i.e. dithiothreitol). An aliquot of 100 μg of protein isolated by the BST was loaded onto an 11-cm isoelectric focusing strip, pH 4–7. Focusing was conducted on isoelectric focusing cells at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 30 min. Strips were then equilibrated in 1× NuPAGE LDS sample buffer. Fifteen minutes later, strips were equilibrated for an additional 15 min and then loaded onto the second dimension using 4–12% NuPAGE (Invitrogen). Gels were then stained using a silver staining kit (Pierce).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Bacteria were cultured in DMEM containing 10% fetal calf serum and 2 mM glutamine in a 5% CO_2 incubator without agitation, or in Luria broth (LB) containing 0.5% glycerol to inhibit sporulation at 28 °C or 37 °C in a 5% CO_2 incubator or in a regular culture shaker (under air condition) with agitation (200 rpm). TRIzol solution with bacterial enhancement reagent (Invitrogen) was used to isolate total RNAs from *B. anthracis* cells. Random primed cDNA was prepared from 1 μg total bacterial RNA using Superscript II reverse transcriptase (Invitrogen). Semiquantitative PCR of the cDNA was performed using Platinum Supermix (Invitrogen) and primers specific for bNOS [5′-CTT GTC TTT CCA TAA TGT ACC-3′ (sense) and 5′-TAA ATA TGC AAC GAA CGA CG-3′ (antisense)] to yield a 540-bp amplicon.

2.5. Mass spectrometry

Streptavidin-agarose purified S-biotinylated proteins were separated by SDS–PAGE, and visualized by silver staining (Pierce). Protein bands were excised from the gel and digested with trypsin (Promega) according to published procedure [21].

2.6. Enzyme assays

Confluent RAW264.7 cells were infected with Sterne or delta-Sterne spores (MOI 10) for 2 and 6 h. Cell lysates were prepared in each assay buffer by repeating freeze–thaw or sonication for 10–20 s with ~50% power. Protein concentration was determined colorimetrically using the Bradford protein assay reagent (Bio-Rad) and the bovine serum albumin as standard. Spectrophotometric assays were performed to measure the complex I and III activities as described [22]. Complex IV activity was assayed by a cytochrome c oxidase assay kit (Sigma) according to the technical recommendation.

2.7. Measurement of NO and intracellular ATP levels

Total levels of NO in culture medium were determined by nitrate/nitrite fluorometric assay kit (Cayman Chemical) using the flow-through of a 30 kDa molecular weight cut-off filter. Intracellular ATP levels were measured by assaying luciferase activity in the presence of cell lysates according to the manufacturer's instructions (ATPLite-M; Packard). RAW264.7 cells cultured in a 96-well plate were treated with Sterne spores (MOI 10) for 2, 4 and 6 h in the presence of *N*ω-nitro-L-arginine methyl ester (L-NAME; 0, 1, 5, and 10 mM). After adding lysis buffer and luciferase substrate, luminescence was measured by Fluoroskan Ascent FL luminometer (Labsystems).

2.8. Statistical analysis

All data are expressed as arithmetic means ± standard deviations. Comparisons between groups were carried out using the unpaired Student *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Toxigenic Sterne only induces expression of the bnos gene during infection

We first examine whether toxigenic Sterne upregulates the expression of *bnos* gene in the infection conditions. The bNOS mRNAs of Sterne strain analyzed by RT-PCR were detectable beginning at 4 h post infection and high at 20 h, whereas delta-Sterne

did not express bNOS at both time points (Fig. 1A). Experiments with LB in a shaking incubator (air condition) revealed lower bNOS expression, which was increased in the presence of CO_2 at 28 °C, where toxin genes are not expressed [26], as well as at 37 °C (Fig. 1B). We measured the concentrations of nitrate/nitrite in culture supernatants of spore-infection macrophages as tracers of NO generation. The nitrate/nitrite content of culture supernatants was increased at 4 h post Sterne infection (Fig. 1C). However, iNOS or nNOS was not detectable within 6 h post infection (Fig. 1D), suggesting that bNOS mainly contributes to NO production in an early infection.

3.2. Sterne infection induces protein S-nitrosylation in RAW264.7 cells

In order to examine the role of NO produced by *B. anthracis* infection, we examined protein S-nitrosylation, a major modification by NO. Murine RAW264.7 macrophages were infected with spores of toxigenic Sterne and non-toxigenic deltaSterne strains and cell extracts were prepared. The cell extracts were submitted to BST, and the resulting S-biotinylated proteins were separated by 2D-PAGE followed by Western blot analysis using anti-biotin antibody. The results showed that toxigenic Sterne infection (Fig. 2D) induced more protein S-nitrosylation than uninfected control and non-toxigenic deltaSterne infection (Fig. 2B and F, respectively), suggesting a higher S-nitrosylation in toxigenic infection.

3.3. bNO targets bioenergetic machinery in macrophages

We next sought to identify S-nitrosylated proteins produced by toxigenic Sterne infection using a BST [20]. The S-nitrosylated proteins identified by MS analysis were chaperones and stress response proteins, proteins involved in oxidative phosphorylation, proteins regulating cellular energy supply, cytoskeletal proteins, and antioxidants (Supplemental Table S1). A recent study suggests that NO targets mitochondrial proteins to induce S-nitrosylation and results in modulation of mitochondrial respiratory functions by eliciting changes in O_2 consumption, energy-conservation processes and free radical production [23]. Therefore, we chose the enzymes mainly responsible for mitochondrial respiration as the most likely S-nitrosylation targets for further verification. As shown in Fig. 3A, the proteins immunodetected in the Streptavidin bead eluates from Sterne-infected cells, but not from delta-Sterne-infected ones included UQCRc2 and Ndufs3. Mitochondrial ATP5A1 was detected in the case of both infections in streptavidin-purified fractions. Beta-actin was not detected in Western blot analysis, presumably due to protein degradation during cell death at 6 h post infection (Fig. 3A, *actin input*), although β-actin was reported as a target for S-nitrosylation [29–31].

We also examined whether *B. anthracis* infection directly alters the activities of the identified candidate proteins. Sterne infection significantly decreased complex I activity in a time-dependent manner. Similarly, delta-Sterne infection decreased complex I activity in a lesser extent than Sterne infection (Fig. 3B). Complex III and IV activities were also decreased in Sterne-infected cell extracts with statistical significance ($P < 0.05$ and $P < 0.01$, respectively) at 6 h post infection (Fig. 3D). These results suggest that activities of core protein in the mitochondrial respiration chain complexes are impaired during infection.

3.4. Cell viability is partially restored by NOS inhibition

In order to examine the effect of bNO on mitochondria and the eventual cytotoxicity to microphages, we measured cell viability in the presence of L-NAME (a pan-NOS inhibitor) at 2, 4 and 6 h post infection. Sterne infection decreased intracellular ATP levels, the indicator of cell viability, by an average of 95% compared to uninfected control at 6 h post infection (Fig. 4A). Nevertheless, L-NAME treatment partially restored macrophage viability in a concentration-dependent manner (Fig. 4B). Of note, cell viability at 4 h post infection was recovered up to 60% of uninfected control by 10 mM L-NAME treatment from 20% (no inhibitor treatment), indicating the restoration of cell viability by bNOS inhibition. Together with decreases of complex I, III, and IV activity, these results suggest that bNO is involved in energetic impairment, resulting in cytotoxicity.

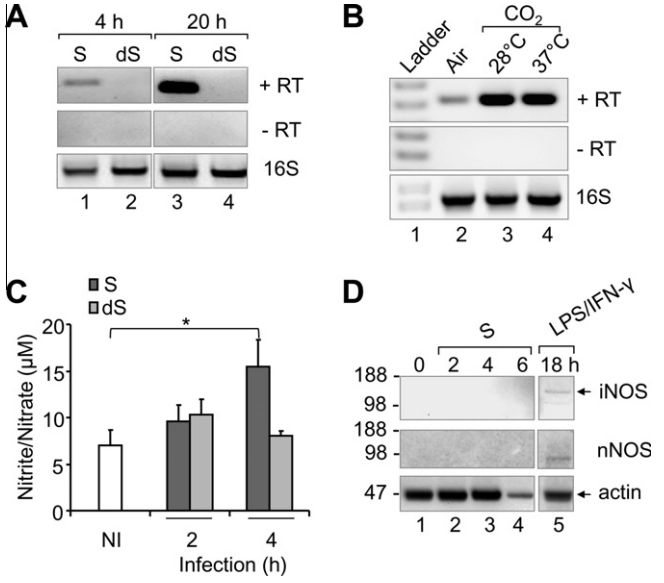

Fig. 1. NOS expression in bacteria and macrophages. (A) Semiquantitative RT-PCR of bacterial RNAs isolated from Sterne (S) and deltaSterne (dS) vegetative cells cultured in DMEM containing 10% fetal calf serum and 2 mM glutamine in 5% CO_2 at 37 °C. RT-PCR was performed in the presence (+RT) or absence (−RT) of reverse transcriptase. (B) Semiquantitative RT-PCR of bacterial RNAs isolated from Sterne vegetative cells cultured in LB containing 0.5% glycerol in 5% CO_2 incubator at 37 or 28 °C, and in rotary culture shaker at 37 °C under air condition (Air), with agitation (200 rpm). (C) Total NO production assayed by nitrate/nitrite in the culture supernatants of spore-infected macrophages. $^*P < 0.05$ compared to control treatment. D, Western blots of infected cell lysates against iNOS, nNOS and actin antibody.

4. Discussion

Infection of toxigenic *B. anthracis* Sterne strains increased NO accumulation in macrophages [15]. This prompted us to examine the expression of bNOS responsible for the production of bacterial origin of NO. Transcription of bNOS was present at 4 h (early stage of infection) and high at 20 h in macrophage medium, in the presence of CO_2. Air condition decreased significantly the expression in LB, a bacterial culture medium [24]. It has also been reported that the highest levels of transcription of toxin genes occurred at 37 °C, but not at 28 °C, under CO_2/bicarbonate equilibrium condition [25]. The maximum levels of transcription of toxin genes occurred at the end of exponential phase, which is similar as NOS gene expression. This suggests that some of the regulatory networks in regulation of the three toxin genes could be shared with NOS gene. Indeed, NO was abundantly synthesized by vegetative bacteria inside cells. This environment is probably relatively low O_2 and high CO_2 levels compared to air conditions, which could be relevant to NOS expression inside macrophages. On the other hand, exposure of RAW264.7 macrophages to a congenic germination-defficient *B. anthracis* Δ*gerH* endospores upregulated iNOS expression 12 h

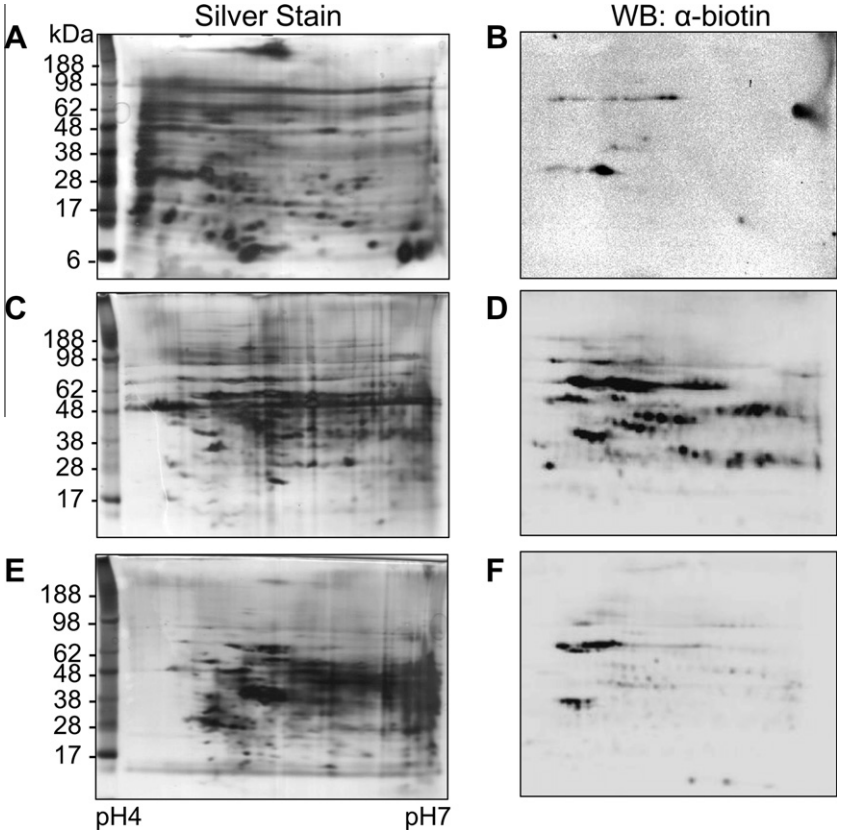

Fig. 2. 2D-PAGE analysis of S-nitrosylated proteins. Comparison of S-nitrosylated proteins in uninfected (A, B), Sterne- (C, D) and deltaSterne-infected (E, F) extracts by 2D PAGE. The proteins were silver stained (A, C and E), and were visualized by Western blot with anti-biotin antibodies (B, D and F), respectively.

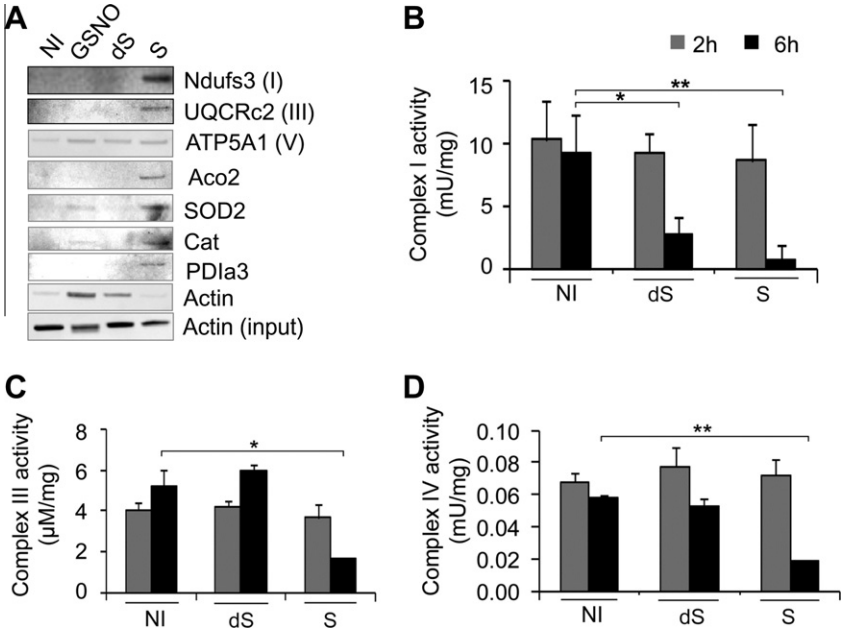

Fig. 3. Validation of S-nitrosylation and enzyme assays of ETC proteins. (A) Western blot analysis of S-nitrosylated proteins purified from extracts of uninfected (NI), GSNO-treated, deltaSterne (dS)- or Sterne (S)-infected RAW264.7 cells. (B–D) Enzyme activities of complex I (B), complex III (C), and complex IV (D) in extracts prepared from spore-infected RAW264.7 cells. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to control treatments at 6 h.

after exposure [26]. Interestingly, sonicated spores (without exosporium) set ahead significant NO generation up to 6 h post infection, suggesting the importance of exosporium in iNOS induction. In addition, injection of vegetative *B. anthracis* cells into nonhuman primate induced a marked iNOS expression and increased protein tyrosine nitration in lung tissues of dead animals [27]. These studies on regulation of iNOS expression by *B. anthracis* were performed at late stage of infection *in vitro* and *in vivo*. However, the

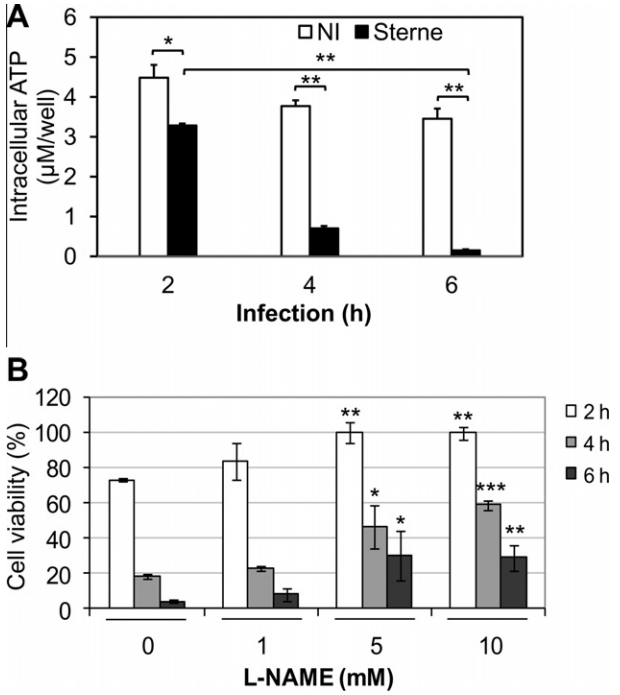

Fig. 4. Partial recovery of intracellular ATP depletion and macrophage viability by Sterne spores. (A) Intracellular ATP levels of RAW264.7 cells infected with Sterne spores (MOI 10). $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to uninfected control at each time point. (B) Cell viability of RAW264.7 cells infected with Sterne spores (MOI 10) in the presence of L-NAME (0, 1, 5, and 10 mM). The values (cell viability, %) represent intracellular ATP levels relative to each uninfected control cells in the presence of L-NAME. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to Sterne treatment without L-NAME at each time point.

studies ruled out the involvement of bNOS in pathogenicity during early stages of infection. Sterne spores deficient in bNOS lost their virulence in A/J mouse model of systemic infection and exhibited severely compromised survival when germinating within macrophages [15]. Thus, we conclude that major NO producer at an early stage of infection is only ingested vegetative bacteria, while at a late stage of infection is host NOS or both.

Using immunodetection in conjugation with proteomic analyses, we identified several ETC proteins, which become significantly S-nitrosylated after Sterne infection. The S-nitrosylated proteins include ETC proteins such as NADH dehydrogenase (ubiquinone) Fe–S protein 3 (a component of complex I), ubiquinole cytochrome c reductase core protein 2 (a component of complex III) and mitochondrial H^+-transporting F1-ATP synthase subunits α1 and β, and F0-ATP synthase (components of complex V). Above enzymes including cytochrome c oxidase (a component of complex IV), which were identified by mass spectrometry, were inactivated in Sterne-infected macrophages. This result suggests that the ETC is a major target for S-nitrosylation during NO-mediated mitochondrial damage. S-nitrosylation takes place in mitochondria as an important regulatory mechanism, because mitochondria contain sizeable thiol pools, are abundant in transition metals, and are internal alkaline pH, all of which are known to modulate SNO biochemistry [28]. In addition, since NO is lipophilic and forms the putative S-nitrosylating intermediate N_2O_3 within the membrane, mitochondrial respiratory chain could be an ideal target for S-nitrosylation. Complex I is the first enzyme of the mitochondrial respiratory chain and is S-nitrosylated under pathophysiological state [29]. In neurodegenerative diseases and hypoxia, complex I is S-nitrosylated, resulting in enzyme inactivation. Experimental evidence suggests that inhibition of complex I activity through S-nitrosylation is not due to NO itself, but to species formed in tissue-specific mitochondrial metabolism. It has been reported that $ONOO^-$ is the main species involved in complex I damage [30]. When a more severe and persistent cytochrome-c oxidase inhibition by NO occurs, the mitochondrion produces an excess of O_2^- that directly reacts with NO to yield $ONOO^-$ [31], which in turn causes irreversible inhibition of respiration. These circumstances lead to a pathological condition, termed "nitroxia" in which cellular bioenergetics in severely impaired, such as low ATP levels [32]. In spore-infected macrophages, NO produced by the vegetative cells may be converted to $ONOO^-$ in the presence of O_2, which is involved in protein S-nitrosylation and/or nitration. Thus, $ONOO^-$-mediated complex I S-nitrosylation and inactivation is a plausible hypothesis.

Of particular interest, we observed that Sterne infection resulted in increased S-nitrosylation of the mitochondrial F1-ATPase. It has recently been reported that *B. anthracis* lethal toxin upregulates 2.1-fold ATP5B in protein levels [33], while spore infection downregulates ATP5B expression resulting in reduction (~50%) of ATP activity [34]. Depletion of intracellular ATP levels by Sterne infection suggests that lethal toxin treatment differs from spore infection in terms of regulation of intracellular ATP levels by ATP5B, and that ATP depletion by spore infection requires another blocking system, such as S-nitrosylation of energetic machinery. This was partly supported by restoration of intracellular ATP levels with treatment of L-NAME, an inhibitor of pan-NOS. Although we did not target ATPase activity specifically, overall blockage of S-nitrosylation of ETC proteins by L-NAME restored intracellular ATP levels in a concentration-dependent manner. Thus, ATP depletion during *B. anthracis* infection could be a novel mechanism for cell killing, which is alternative, toxin-independent.

In conclusion, our nitroso-proteomic analysis provides a novel mechanism that bNO abrogates ETC machinery at an early stage of macrophage infection by protein inactivation through S-nitrosylation. S-nitrosylation of ETC proteins depleted intracellular ATP, resulting in macrophage death. Because bNO renders bacteria resistant to immune oxidative attack and host cell damage, inhibitors of bNOS can be used for antimicrobial as well as anti-inflammatory therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.042.

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