BACILLUS ANTHRACIS CO-OPTS NITRIC OXIDE AND HOST SERUM ALBUMIN FOR PATHOGENICITY IN HYPOXIC CONDITIONS

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Date: 4/30/13

Spring Semester 2013
George Mason University
Fairfax, VA

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DEDICATION

This is dedicated to my family and friends for all the support and to Dr. Popov to guide me through the maze known as the understanding of *Bacillus anthracis*.

ACKNOWLEDGEMENTS

This work was made possible due to the support from George Mason University College of Science (Dean Dr. V. Chandhoke). Thank Dr. Weidong Zhao and Aarthi Narayanan for help with MS experiments. Thanks to Myung Chung-Chul for help with RT-PCR results. Thanks to Ryan Blower whose help and team work was instrumental especially with the AR results. Thanks to Dr. Taissia Popova whose previous work laid a great foundation for further study. Last but not least thanks to my Committee Dr. Daniel Cox, Dr. Geraldine Grant, and Dr. Serguei Popov whose guidance has made me a better scientist today.

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LIST OF ABBREVIATIONS OR SYMBOLS

Nitric Oxide ..... NO
Bacterial Nitric Oxide Synthase ..... baNOS
Peroxynitrite ..... PN
Superoxide Dismutase ..... SD
Human Small Airway Epithelial Cells ..... HSEACs
Anthrolysin O ..... ALO
Succinic Acid ..... SA
Lethal Toxin ..... LT
Edema Toxin ..... ET
Protective Antigen ..... PA
Bovine Serum Albumin ..... BSA
Bacterial Supernatant ..... Sups
Nitrate/Nitrite ..... NN
L-N^{ω}-nitroarginine methyl ester ..... L-NAME
5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrinato Iron (III) ..... FeTPPS
Mn (III) tetrakis (4-benzoic acid) porphyrin ..... MnTBAP
Hydrogen Peroxide ..... H_2O_2
Reactive Oxygen Species ..... ROS
Reactive Nitrogen Species ..... RNS
Horseradish Peroxidase ..... HRP
Amplex Red ..... AR
3-morpholinisydnonimide SIN-1
Mitogen-Activated Protein Kinase Kinase ..... MAPKK
5,5'-dithiobis-2-nitrobenzoic acid ..... DTNB
Dithiothreitol ..... DTT

ABSTRACT

BACILLUS ANTHRACIS CO-OPTS NITRIC OXIDE AND HOST SERUM ALBUMIN FOR PATHOGENICITY IN HYPOXIC CONDITIONS

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George Mason University, 2013

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Bacillus anthracis is a dangerous pathogen of humans and many animal species. Its virulence has been mainly attributed to the production of Lethal and Edema toxins as well as the antiphagocytic capsule. Recent data indicate that the nitric oxide (NO) synthase (baNOS) plays an important pathogenic role at the early stage of disease by protecting bacteria from the host reactive species and S-nytrosylating the mitochondrial proteins in macrophages. Another toxin-independent mechanism relevant to late-stage anthrax was shown with non-phagocytic host cells exposed to pathogenic factors secreted by *B. anthracis* in microaerobic (hypoxic) conditions. In addition to synergistic effect of the pore-forming hemolysin (anthrolysin O) perforating the host cell and the fermentation metabolite (succinic acid) fueling the release of reactive oxygen species from mitochondria, this mechanism involves the activity of NO-derived toxic product(s). In this study we for the first time present evidence that NO produced by baNOS participates

in the generation of highly reactive oxidizing species which could be abolished by the NOS inhibitor L-NAME, free thiols, and superoxide dismutase but not catalase. The formation of toxicants is a result of the simultaneous formation of NO and superoxide leading to a labile peroxynitrite and its stable decomposition product, nitrogen dioxide. The toxicity of bacteria could be potentiated in the presence of bovine serum albumin, which serves as a trap of a volatile NO accelerating its reactions. Our data suggest that during infection in the hypoxic environment of pre-mortal host the accumulated NO is expected to have a broad toxic impact on host cell functions.

INTRODUCTION

Bacillus anthracis is the causative agent of anthrax. Although the incidence of disease among people in the developed countries is low, it remains important as a biodefense threat. Antibiotics are the only approved drugs for anthrax treatment, which is effective only at the early stages of infection. Patients with the advanced disease have about 50% chance of survival (Inglesby et al., 2002). Therefore, further understanding of *B. anthracis* toxicity is required for the acceleration of progress in the development of novel anthrax therapies and prophylaxes.

The disease can be initiated by three major routes: inhalation, ingestion of spores, as well as a direct contact of spores with damaged skin (Inglesby, 2002). During inhalational anthrax, *B. anthracis* spores are phagocytosed by resident phagocytes (alveolar macrophages or dendritic cells) and transported to the regional lymph nodes (Dixon et al., 2000; Guidi-Rontani, 2002). Inside macrophages, some internalized spores survive a bactericidal environment and ultimately initiate disease escaping from macrophages (Cote et al., 2008). The spores also demonstrate a capacity of invading the lung epithelium directly at low frequency (Russell et al., 2008). During vegetative growth, bacterium produces several virulence factors including the toxins, such as the Lethal Toxin (LT) and Edema Toxin (ET), and a poly-γ-D-glutamic acid capsule [reviewed in

(Guichard et al., 2012; Moayeri and Leppla, 2009)]. LT and ET consist of the receptor-binding protective antigen (PA) associated with the catalytic subunits, Lethal Factor and Edema Factor, respectively. The toxins' genes are expressed from plasmid XO1, while the capsule gene is located on the plasmid XO2. In macrophages, LT causes intracellular proteolytic cleavage of members of the mitogen-activated protein kinase kinase (MAPKK) family. ET is a calcium- and calmodulin-dependent adenylyl cyclase that converts cytosolic ATP to cAMP (Moayeri and Leppla, 2009). Accumulated evidence demonstrates that LT and ET influence many important cellular processes including the host's innate immune response; however, mechanisms by which *B. anthracis* kills the host are not fully understood.

Current understanding of initiation of anthrax has been the result of animal model studies. Since capsule and toxin proteins created by the bacteria have arguably significantly contributed to the mortality of the host importance of their roles need to be further comprehended. *In vivo* studies in non-human primates are arguably the closest model to human infection but pose major complications due to the cost, the lack of large scale studies and the limited amount of genetic and immunological tools for a sufficient study (Heninger et al., 2006, Levy et al., 2012a, and Weiner and Glomski, 2012). Therefore, studies have been done in guinea pigs, rabbits and murine models. Most important characteristics are reproduced in murine models of infection although they show a high sensitivity to capsule (Heninger et al., 2006, Levy et al., 2012a and Weiner and Glomski, 2012). This sensitivity requires studies to use the attenuated noncapsulated Sterne strain in murine models. Recent

data obtained in animal models of anthrax using virulent strains with deletions of LT and ET genes show that *B. anthracis* possess pathogenic factors which can surpass the effects of these toxins (Heninger et al., 2006; Chand et al., 2009; Lovchik et al., 2012; Levy et al., 2012b, 2012a). For example, Heninger et al. (2006) demonstrate that the LT and ET are not required for a full toxicity in Ames or UT500 strains upon an inhalation administration of spores. Furthermore, the production of LT and ET were not necessary for escaping the lung suggesting a toxin-independent mechanism is required for trafficking of the pathogen. Similarly, Levy et al. (2006), demonstrate that in rabbit and guinea pig models, the absence of ET and LT had minimal effect on virulence of fully virulent Vollum strain. Instead, virulence was dependent on route of infection and animal model (Levy et al, 2012a, 2012b). However, these studies provided no mechanistic interpretation of their results. Mechanism aside, these studies corroborate in the absence of vital pathogenic factors other important virulence factors are commonly masked. For instance, our previous studies confirm *B. anthracis* produces at least four individual proteases that promote deterioration of host tissues (such as fibronectin, collagen, gelatin and laminin) for bacterial dissemination. During the intratracheal challenge with supernatants of B. anthracis, containing these proteases, DBA/2 mice display hemorrhaging and even death within 2 to 3 days (Chung et al. 2006, Popov et al. 2005). While, LT and ET aren't necessary for mortality, they most likely modify the pathophysiology factoring in the eventual outcome of the host (Henringer et al. 2006, Levy et al. 2012a, 2012b), Survival of *B. anthracis* during the infectious process greatly relies on the collective cooperation of all known and unidentified virulence factors, especially during growth and dissemination.

Since *B. anthracis* is a non-motile bacteria, the dissemination beyond initial site of infection requires transportation by the host. It is commonly accepted that vegetative spores are required to escape the lymphatic system to continue pathogenesis (Haninger et al., 2006, and Weiner and Glomski, 2012). The most widely recognized model for dissemination of *B. anthracis* is the Trojan Horse model. In this model dormant resident phagocytes transport spores to draining lymph nodes where spores germinate producing capsule and exotoxins overwhelming the lymph node allowing the bacteria to invade the bloodstream producing bacteremia and possible host death (Weiner and Glomski 2012, Weiner et al. 2012). Route of infection plays a vital role as both subcutaneous and gastrointestinal form in murine systems show vegetative growth at initial spore site. Weiner et al. determined that debridement (removal of infected tissue) of tissue at the initial site of infection increased survival of mice when subjected to subcutaneous anthrax. If the Trojan Horse model were a complete model for all forms of infection debridement would have negligible effect once spores enter the lymph nodes in early stages of infection, which wasn't the situation. This implies that lethality in the different forms of anthrax manifest upstream of the lymph nodes (Weiner et al. 2012). In light of this data modification to the Trojan Horse model needed to be completed. This lead to the new model termed "Jailbreak Model" in which exotoxins and spore germination occur at initial site of infection producing a failure in cellular barriers in the endothelium to obtain entry into the lymphatic system then further progressing to the circulatory system (Weiner and Glomski 2012). Though the totality of this model hasn't

been investigated in all animal models it provides a more complete understanding of pathogenic mechanisms.

We have been interested in investigation of the pathogenic mechanisms contributing to the LT-independent virulence with a particular focus on the contribution of *B. anthracis* nitric oxide synthase (baNOS). Similar to mammalian NOSs, the bacterial homolog generates nitric oxide (NO) from L-arginine in the presence of oxygen (Sudhamsu and Crane, 2009; Crane et al., 2010). NO is a relatively unreactive free radical and easy diffusion of nitric oxide through membranes (Denicola et al., 1996b) makes possible its interactions with intracellular targets. In the host cells, NO and other reactive nitrogen species (RNS) derived from NO participate in numerous biological events such as glycolysis, growth, signal transduction, stress response and maintenance of homeostasis by S-nitrosylation of protein thiol groups and nitration of tyrosine residues (Habib and Ali, 2011). S-nitrosylation is a ubiquitous posttranslational, enzyme-independent, redox-sensitive modification that serves as a major effector of NO-mediated biochemistry regulating broad spectrum of proteins. NO can also react with superoxide ($O_2^{\bullet -}$) and form highly toxic peroxynitrite ($ONOO^-$, PN) playing an important role in different inflammatory diseases. PN is formed during sepsis, inflammation, excitotoxicity, and ischemia-reperfusion of tissues, conditions under which the cellular production of nitric oxide and superoxide increase (Pacher et al., 2007), and participates in reactions related with the pathological expression of these processes. PN induces nitration of protein tyrosine residues (3-nitrotyrosine) resulting in modulation of catalytic activity, cell signaling, and cytoskeletal organization (Pacher et al., 2007).

Available data indicate that baNOS plays an essential role in *B. anthracis* virulence through different mechanisms relevant to the early stage of disease involving interaction of bacteria with macrophages as well as the late, pre-mortal stage characterized by the interaction of pathogenic factors with non-phagocytic host cells. One of these mechanisms confers protection of *B. anthracis* from the host reactive species within macrophages by preventing DNA damage during the Fenton reaction of ferrous ion with hydrogen peroxide (Shatalin et al., 2008). We recently reported that S-nitrosylation of mitochondrial proteins by baNOS-derived NO leads to depletion of the macrophage bioenergetics resulting in cell death (Chung et al., 2012). In agreement with these observations, the *B. anthracis* mutant with a deletion of baNOS gene is strongly attenuated in mice (Shatalin et al., 2008).

Another LT-independent mechanism likely relevant to late-stage anthrax was demonstrated in experiments with non-phagocytic host cells exposed to pathogenic factors generated by *B. anthracis* Sterne and dSterne strains grown in microaerobic conditions (Popova et al., 2011). Compared to the fully aerobic cultures, the oxygen pressure in microaerobic cultures is reduced, but not completely abated, while the pressure of carbon dioxide is increased. As stated previously, the Sterne strain is fully toxigenic but attenuated due to the absence of capsule. The dSterne strain is a derivative of Sterne producing neither LT, ET nor the capsule. It was found that the intoxication depends on the expression of the pore-forming hemolysin, anthrolysin O (ALO). The killing activity of ALO is synergistically enhanced by the bacterial metabolite, succinic

acid (SA), released from bacteria into environment as a result of anaerobic fermentation. Host cells exposed to bacterial culture supernatants (Sups), demonstrate the onset of acute oxidative stress, which can be attenuated by the mimetics superoxide dismutase (SOD) and catalase, indicating the involvement of the superoxide radical or other reactive oxygen species (ROS). As a substrate of the mitochondrial complex II, SA can stimulate generation of ROS by the host cells in hypoxic conditions (Quinlan et al., 2012). The toxic role of ALO in this process likely consists in perforating the cytoplasmic membrane and creating pores for the delivery of SA and other bacterial products into the target cells (Popova et al., 2011).

However, the ALO-based mechanism contributes only to about a half of the Sups' toxicity as it follows from the effect of ALO inhibition by cholesterol (Popova et al., 2011). The partial protective effect of the iron porphyirin derivative, FeTPPS, used in previous studies to catalyze decomposition of PN (Belik et al., 2010; Valez et al., 2012) indicates that this RNS may also play a part in the toxicity of Sups. Due to the transient nature of PN, it is difficult to observe it directly within the cells. In the conditions of oxidative stress, this unstable cytotoxic compound can be formed in the fast reaction of superoxide with NO derived from the host NOS or baNOS (Pacher et al., 2007). Another source of NO may consist in denitrification of nitrate or nitrite in the conditions of anaerobic respiration demonstrated for a number of bacterial species (Kraft et al., 2011; Bueno et al., 2012).

In this follow-up study, we thought to characterize the nature of cytotoxic species formed in Sups during a microaerobic growth of *B. anthracis*. We present evidence consistent with the notion that NO produced by baNOS can participate in the generation of *B. anthracis* toxicity to non-phagocytic cells through formation of PN and its reaction products. During experimentation with culture media used for bacterial growth we found that the presence of bovine serum albumin (BSA) potentiated the toxicity of bacteria, and explored the mechanistic features of this phenomenon. The results indicate that BSA serves as a trap stabilizing a volatile NO produced by *B. anthracis* in a relatively stable form. We conclude that the role of NO in anthrax is not limited to its low-level production within macrophages. During infection the bacteria are likely to accumulate NO in circulation due to the high content of albumin in serum. In a hypoxic environment of the pre-mortal host accompanied by the presence of ROS the accumulated NO is expected to have a broad systemic impact on different cell types through cytotoxicity of its downstream products and protein chemical modifications causing deterioration of host cell functions.

MATERIALS AND METHODS

Reagents

All reagents including superoxide dismutase from *E. coli*, peroxidase from horseradish, catalase from bovine liver, and rabbit anti-nitrotyrosine antibody were from Sigma-Aldrich. BSA was of >98% purity, essentially free from fatty acids and globulin. AR and Alamar Blue dyes were from Invitrogen. Anti-rabbit IgG, HRP-linked antibody was from Cell Signaling Technology. All cell culture reagents and formulated media were purchased from Mediatech, Inc., VA.

HSAEC culture and bacterial strains

HSAECs (Cambrex, Inc., MD) were grown in Ham's F12 medium supplemented with non-essential amino acids, sodium pyruvate, and 10% fetal calf serum at 37^{o}C, 5% CO_2 in the presence of 100 µg/ml of streptomycin and 100 U/ml of penicillin. Confluent HSAECs were seeded into the 96-well culture plates (unless indicated otherwise), washed 3 times with Hepes-buffered standard saline (HBSS), and incubated with bacterial Sups at 37^{o}C, 5% CO_2. Sups were produced by inoculation of spores (final $6x10^{6}$ spores/ml) into the DMEM/F12 culture medium supplemented with non-essential amino acids,

pyruvate, glutamine, BSA, and 5 µM nitrate (complete DMEM/F12), unless indicated otherwise. The cultures were incubated at 37^{o}C, 5% CO_2 in 12-well tissue culture plates in static conditions or with shaking for 24 h. Bacterial growth was measured by optical density at 600 nm (OD_{600}) using 96-well plates and 200 µl of suspension per well. Bacteria were removed by centrifugation, and Sups were supplemented with 100 µg/ml of streptomycin and 100 U/ml of penicillin to exclude growth of any contaminating bacteria. In some experiments we used a Complete Serum-Free Medium® (CSFM), which is a proprietary serum-free and low-protein formulation based on DMEM/F12, RPMI 1640, and McCoy's 5A. It does not contain any insulin, transferrin, cholesterol, growth or attachment factors. The manufacturer indicates that the medium contains trace elements, high-molecular-weight carbohydrates, extra vitamins, a high-quality bovine serum albumin (1 g/L). Our analysis shows that it contains *ca.* 300 µM nitrate.

The Sterne strain 34F2 ($pXO1^{+}$, $pXO2^{-}$) was obtained from the Colorado Serum Co. (Boulder, CO). The generation and characterization of the plasmidless delta Sterne strain were described in (Bradburne et al., 2008). The spores of *B. anthracis* strains were prepared as described (Popov et al., 2002).

The viability of cells after exposure to Sups for 2 h at 37^{o}C, 5% CO_2 was routinely determined using the redox dye Alamar Blue. Briefly, the cells were washed with HBSS, Alamar Blue in CSFM was added (1:10 ratio) for 1 h incubation, and its fluorescence was measured at 530/584 nm. The viability was calculated relative to mock-treated cells.

In the experiments with L-NAME, different concentrations of the inhibitor were made by mixing 1 M stock solution in water with a complete DMEM/F12 buffered with 50 mM HEPES to maintain a uniform pH of 6.95-7.0 of all mixtures.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Bacteria were cultured in DMEM containing 10% fetal calf serum and 2 mM glutamine at 37^{o}C, 5% CO_2 incubator without agitation or with agitation at 200 rpm. Trizol solution with bacterial enhancement reagent (Invitrogen) was used to isolate total RNAs from *B. anthracis*. Random primed cDNA was prepared from 1 µg total bacterial RNA using Superscript II reverse transcriptase (Invitrogen). Semi-quantitative PCR of the cDNA was performed using Platinum Supermix (Invitrogen) and primers specific for baNOS [5'CTT GTC TTT CCA TAA TGT ACC' (sense) and 5'TAA ATA TGC AAC GAA CGA CG (antisense)] to yield a 540-bp amplicon.

Mass spectrometry

The Sups were dried with SpeedVac, reconstituted in 8 M urea, reduced by 10 mM DTT for 30 min, alkylated by 50 mM iodoacetamide for 30 min, and digested by trypsin at 37^{o}C overnight. Tryptic peptides were further purified by Zip-Tip (Millipore) and analyzed by LC-MS/MS using a linear ion-trap mass spectrometer (LTQ, Orbitrap). After

sample injection, the column was washed for 5 min with mobile phase A (0.4% acetic acid) and peptides eluted using a linear gradient of 0% mobile phase B (0.4% acetic acid, 80% acetonitrile) to 50% mobile phase B in 30 min at 250 nl/min, then to 100% mobile phase B for an additional 5 min. The LTQ mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by five MS/MS scans where the five most abundant molecular ions were dynamically selected for collision-induced dissociation using normalized collision energy of 35%. Tandem mass spectra were searched against SEQUEST database using tryptic cleavage constraints. High-confidence peptide identifications were obtained by applying the following filters to the search results: cross-correlation score 1.9 for 1+, 2.2 for 2+, 3.5 for 3+ ions, and a maximum probability for a random identification of 0.01.

AR assay

The enzymatic determination of hydrogen peroxide can be accomplished with high sensitivity and specificity using Amplex® Red (N-acetyl-3,7-dihydroxyphenoxazine, AR), a highly sensitive and chemically stable fluorogenic probe. Enzyme-catalyzed oxidation of AR, which is a colorless and nonfluorescent derivative of dihydroresorufin, produces highly fluorescent resorufin, which is detected by absorbance at 571 nm.

The experiments with AR measured the accumulation of resorufin in bacterial cultures grown as described above. AR and horseradish peroxidase (HRP) were added to culture

medium prior to incubation at final concentrations of 0.1 mM and 0.2 U/ml, respectively. Samples of cultures were taken at specific time points, the bacteria were pelleted by centrifugation at 10000 g for 5 min, and absorbance of Sups was read at 571 nm. The results in particular cultivation conditions demonstrated a satisfactory reproducibility between independent experiments. However, we detected some variation in the shape of the accumulation curves depending on the culture plate well size, volume and nature of medium in the wells likely caused by disturbance in the gas-liquid exchange of the volatile reaction products such as NO, NO_2 upon handling of the microaerobic cultures.

Preparation of modified BSA

Sodium nitrite (400 mg/ml) was acidified to pH 5 using 0.5 M HCl, and mixed with equal volume of BSA solution (200 mg/ml in water). The mixture was incubated for 1 h at room temperature in the dark, and the protein was precipitated with 4 volumes of ice-cold acetone. After incubating at minus 20 ^{O}C for 20 min the protein was pelleted, washed 4x with 70% acetone, air dried and resuspended in 100 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine 1% Tween 20, pH.

Statistical analyses

All measurements were made in triplicates, and all experiments were repeated at least twice with consistent results. Error bars in the figures indicate standard deviations (n=3) or 95% confidence intervals (two-tail t-test).

RESULTS

BSA increases accumulation of NO reaction products and the toxicity of Sups

The microaerobic cultures of the toxigenic (Sterne) and non-toxigenic (dSterne) strains upon their static growth on top of the HSAEC monolayers in the atmosphere of 5% CO_2 demonstrate an acute cytotoxicity. The bacterial factors responsible for the toxicity are present in the bacterial culture supernatants (Sups) grown until late stationary phase (>20 h) (Popova et al., 2011). To elucidate contribution of baNOS to the toxicity of Sups we characterized the production of NO by this enzyme. We first demonstrated using RT-PCR that both strains expressed the baNOS gene in the stationary phase of growth (Figure 1A). Next, we analyzed Sups for accumulation of NO in the form of its end oxidation products, nitrite and nitrate (NN). To avoid masking of the released NN by the nitrate concentration in the culture medium such as CSFM (which contains *ca*. 300 µM nitrate), we used DMEM/F12 medium with low nitrate content (*ca.* 0.1 µM). In the aerated cultures of both strains the concentration of NN did not exceeded a few µM, but strongly increased in the case when the medium was supplemented with BSA (Figure 1B). The inhibitor of NOS (L-N^{ω}-nitroarginine methyl ester, L-NAME) reduced the NN content to the background level. These observations demonstrated that the accumulation

of NN resulted from the NO-generating activity of baNOS in the presence of BSA and suggested that the enhancing property of BSA might contribute to the toxicity of Sups. We tested the viability of HSAECs after incubation with Sups of bacterial cultures grown in the presence of different concentrations of BSA. Figure 2 shows a profound decrease of the HSAEC viability dependent on the concentration BSA in culture medium upon incubation with dSterne Sups. Similar results were obtained with the Sterne strain (not shown). Finally, we tested if BSA was a novel globular protein that increases dSterne Sup toxicity. Gelatin is a ubiquitous mix of multiple proteins, which served as a good control. Figure 3 demonstrates that BSA is a novel globular protein able to increase Sup toxicity while gelatin remained at background levels. The presence of both BSA and gelatin did not result in substantial changes of the optical densities of cultures (OD_{600} 0.30 ±0.06 SD, *n*=5). Therefore, the effect of BSA could not be attributed just to the larger number of bacteria. Instead, the increased acidity of Sups indicated changes in the production of acidic metabolic products (Figure 2).

B. anthracis **microaerobic cultures generate NO-derived oxidants**

To explain the enhancing effect of BSA on the toxicity of Sups we suggested that BSA might trap a volatile and highly diffusible NO as intermediate product(s) of chemical reactions with the protein side groups (Pacher et al., 2007; Denicola et al., 1996b; Hakim et al., 1996; Foster et al., 2009). It is also known that the BSA hydrophobic interior can

reversibly absorb NO, thus decreasing its dissipation from solution and increasing the rate of NO autoxidation into intermediate species such as N_2O_3 or $NO_2^{\bullet}$ (Rafikova et al., 2002) (Figure 4). In the presence of a sufficient amount of superoxide, which may originate in Sups during bacterial growth from various sources including the leakage of the respiratory chain (Dröse and Brandt, 2012; González-Flecha and Demple, 1995; Messner and Imlay, 2002, 1999), the trapped NO will be quickly converted to PN and its decomposition products (Reaction **4** in Figure 4).

The ROS/RNS could also be released from the Sup-exposed host cells (Popova et al., 2011). These reactive species in turn can rapidly oxidize a number of biological compounds, such as thiols, which can act as RNS/ROS scavengers (Pacher et al., 2007; Wouters et al., 2011). In agreement with these considerations, incubation of Sups with DTT or cysteine decreased the Sups' toxicity toward HSAECs (Figure 5).

To elucidate the nature of reactive species in Sups formed during bacterial growth we used the properties of various dyes to form characteristic products upon interaction with ROS and RNS. Amplex Red (10-Acetyl-3,7-dihydroxyphenoxazine, AR) is a sensitive dye typically used to detect hydrogen peroxide (H_2O_2) due to the formation of a highly fluorescent and colored product resorufin in the presence of horseradish peroxidase (HRP) (Zhou et al., 1997). This enzyme can also catalyze rapid oxidation of the dye by PN (formed in reaction of NO with superoxide, $O_2^{\bullet-}$) in the presence or absence of carbon dioxide (Floris et al., 1993). However, distinguishing between PN and H_2O_2-dependent mechanisms can be done based on the effect of inhibitors (*e.g.* L-NAME, superoxide

dismutase, and catalase). The assay is quite specific regarding the nature of the oxidant and does not generate significant amounts of fluorescent products when exposed to 100 μM HOCl, xanthine/oxidase-generated $O_2^{\bullet-}$, an anaerobic $NO^{\cdot}$ or H_2O_2 in the absence of HRP (Palazzolo-Ballance et al., 2007).

To reliably detect transient reactive species we used a cumulative experimental setup in which AR and HRP were present in the medium during the whole period of bacterial growth. Aliquots of bacterial culture were withdrawn at certain time points, bacteria pelleted, and the Sups analyzed spectrophotometrically. Upon growth in static cultures both the toxigenic Sterne and the non-toxigenic dSterne strains were able to oxidize a colorless AR into a red-colored resorufin detected by absorbance in culture medium seeded with the indicated amount of spores. Accumulation of resorufin due to the release of oxidants took place in the spore dose-dependent manner and was followed by a gradual decline (data not shown). In order to elucidate the mechanism behind the decline we first demonstrated that addition of H_2O_2 to Sups during the declination phase resulted in the appearance of color in accordance with the calibration curve obtained for H_2O_2. This indicated that the assay was able to respond to the additional oxidant and its components were not depleted (not shown). On the other hand, it is known that peroxidases can further oxidize resorufin into a colorless product (Towne et al., 2004). Therefore, we tentatively attributed this effect to the activity of HRP and limited our observations to the initial ascending parts of the curves

Effects of catalase and SOD are consistent with the transient formation of PN

To distinguish between the release of H_2O_2 and other oxidants like PN, the test was supplemented with catalase (up to 20 µg/ml). Data in Figure 6 show that the intensity of resorufin remained unchanged in the presence of a wide range of catalase concentrations thus excluding H_2O_2 as the reactive species generated by *B. anthracis*. On the other hand, it was reported that HRP can use PN as substrate in the catalase-insensitive process (Floris et al., 1993). Experiments with the addition of 10 U/ml of *E. coli* Mn-superoxide dismutase (Mn-SOD) strongly inhibited oxidation (Figure 7a). This effect, however, cannot be interpreted as evidence of the direct interaction of superoxide radical with SOD because the dismutation product, H_2O_2, would readily react with AR thus maintaining its oxidation at the same level (Zielonka et al., 2012). It is also considered unlikely that SOD would be able to compete effectively with the extremely fast conversion of superoxide into PN in the presence of NO (Pacher et al., 2007). Therefore, we favor the mechanism in which Mn-SOD is involved in a direct catalytic inactivation of PN (Quijano et al., 2001)(Surmeli et al., 2010).

Activity of baNOS is involved in the production of oxidizing and toxic species in Sups

One might expect that the formation of PN or other NO-derived RNS should depend on the activity of baNOS as a major source of NO. Indeed, addition of L-NAME to the

culture medium strongly reduced the oxidation of AR (Figure 7B). We also tested if the reduced oxidation in the presence of baNOS correlated with the reduced toxicity of Sups to HSAECs. The culture media were supplemented with different concentrations of L-NAME and inoculated with equal amounts of dSterne spores. The static cultures were grown for 24 h. It was found that inhibition of baNOS affected the bacterial growth and increased the generation of acidic products (Figure 8). The mechanism of this effect has not been reported and the nature of the metabolic changes is currently unknown. In the viability test we wanted to compare Sups grown without the additional effect of pH on HSAECs that would mask the effect of other Sup components. For this purpose all samples were titrated with a small volume of NaOH to the acidity of Sups without L-NAME (pH 5.1). The HSAEC viability test showed that the initial assayed concentration of L-NAME increased toxicity in correlation with the toxic effect of L-NAME on control untreated HSAECs. There was also a considerable increase in the amount of bacteria. However, further concentrations of L-NAME caused a strong protective effect up to the level of control cells treated with L-NAME only (Figure 8).

One caveat of the above experiments was that during the viability test the L-NAME present in Sups might potentially inhibit the host cell NOS along with the baNOS. To clarify this possibility, HSAECs were pre-incubated with L-NAME for 1 h, the inhibitor was removed, and the cells were exposed to the Sups of cultures grown without L-NAME. In contrast to the effect of L-NAME on bacteria, the viability of HSAECs was

decreased considerably, indicating the protective effect of the host NOS against the toxic substances of Sups.

Tyrosine nitration and oxidation of dihydrorhodamine 123 (DHR) confirm generation of PN

Current methodologies for detection of PN are based on the reactions of radical species formed from its decomposition. The formation of nitrotyrosine ($TyrNO_2$) from tyrosine residues of proteins is commonly considered as evidence of the transient PN presence resulting in the release of intracellular $^{\bullet}NO_2$ (Pacher et al., 2007; Ferrer-Sueta and Radi, 2009). The results of the AR/HRP test showed that the addition of BSA to the culture medium resulted in a partial consumption of the RNS formed in the Sups (Figure 9A) indicating that BSA could be a convenient substrate to detect possible reactions of Tyr nitration. Therefore we analyzed the chemical modification of BSA in the dSups by western blotting with anti-$TyrNO_2$ antibodies. We detected a band corresponding to the nitrated BSA in the Sups (Figure 9B) but not in the original medium. As a positive control we used a partial nitration of BSA by an acidified sodium nitrite which accompanies the main reaction of protein S-nytrosylation [Stamler et al. (1992) and our data presented below].

DHR serves as a sensitive fluorogenic probe for PN-derived RNS (NO_2, hydroxyl radical $OH^{\bullet}$; or carbonate radical $CO_3^{\bullet-}$) (Zielonka et al., 2012; Wrona et al., 2005). Initial experiments with DHR added to the bacterial cultures growing in complete DMEM/F12

demonstrated a strong quenching the DHR signal; therefore, a modified protocol was used. Bacteria grown for a certain period of time were pelleted, washed with PBS, finally resuspended in PBS containing DHR and incubated at 37^{o}C, 5% CO_2 for the appearance of fluorescence. Figure 9C shows that bacteria-generated species were able to convert DHR into fluorescent product indicating the transient formation of PN, which is unlikely to accumulate in Sups due to its short lifetime.

BSA cysteine residues are not in involved in the generation of toxicity

Cys-34 is one of the BSA residues highly susceptible to various chemical modifications by ROS and RNS, because it is located in the hydrophobic cavity formed by the protein tertiary structure and is not involved in the formation of disulfide bridges with other Cys residues of the protein (Christodoulou et al., 1995; Rafikova et al., 2002). Serum albumin isolated from plasma contains only about 65% of reduced thiol (Era et al., 1995; King, 1961). The oxidized portion of Cys-34 was thought to be in a mixed disulfide form, mostly with cysteine, cysteinyl-glycine (Cys-Gly) (a degradation product of glutathione), and glutathione (GSH) to a lesser extent (King, 1961) (Yasuhara and Nokihara, 1998). It has been previously suggested that the thiol content of serum albumin is important for the transport and stabilization of NO through the formation of intermediate nitrosothiol derivatives (Foster et al., 2009).

Our results of AR test presented above showed that *B. anthracis* microaerobic cultures represent an oxidizing environment. Therefore we expected that the free thiol groups of Cys-34 would be oxidized or otherwise chemically modified during the bacterial growth and wanted to test if such modifications would contribute to the toxicity of Sups. First, we tested the viability of HSAECs after incubation of the BSA with acidified nitrite, which results mainly in the S-nitrosylation of Cys-34 (Stamler et al., 1992). There was only a marginal effect of nitrosylated BSA on cell viability detected at the concentration of 8 mg/ml at the pH 7.4 corresponding to the culture medium or pH 5.3 -5.5 corresponding to Sups (Figure 10). This result excluded S-nitrosylation from the candidate BSA modifications resulting in the generation of toxic species in the Sups and suggested an overall protective effect of free thiols due to consumption of NO in this reaction.

To test the above suggestion, we increased the free-thiol content of BSA by reducing it with 5 mM DTT for 30 min followed by an extensive dialysis against PBS. The SH content of BSA determined using a thiol-specific Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB) was increased two-fold after the DTT treatment (to about 90% of total Cys-34), but the Sup prepared using this BSA was found to be less toxic than the one with the untreated BSA (Figure 11). However, this effect cannot be attributed solely to the chemical reactivity of SH groups. The cultures supplemented with the reduced BSA had almost a 3-fold reduction in bacterial growth and a decreased acidity in

comparison with the non-reduced BSA (data not shown) indicating substantial metabolic changes in the reduced environment.

Finally, we chose to block Cys-34 residues by N-ethylmaleimide (NEM) and compare the toxicity of Sups supplemented with the modified and unmodified BSA. After the NEM treatment the free-thiol content of the BSA dropped to <5%, compared with the amount found in the unmodified BSA. The cultures with the modified and unmodified BSA showed no substantial differences between each other in the growth of bacteria, final pH, and the toxicity of Sups (Figure 11). Overall, we concluded that free SH groups of BSA were not required for the accumulation of toxicity during bacterial growth and even antagonized it similar to the effects of DTT and cysteine after incubation with Sups (Figure 5).

Mass spectroscopy (MS) analysis of BSA modification products in Sups confirms the presence of NO-derived chemical modifications

For the MS analysis we used the Sterne Sup grown in CSFM. This medium contains 1 mg/ml of BSA. The Sups were treated with trypsin, and the peptide fragments with S-nitrosylated Cys and nitrated Tyr were identified by the LC-MS/MS. Controls included the untreated BSA as well as the BSA modified with sodium nitrite in acidic conditions. Table 1 shows that the nitrite-modified BSA digest contained a single S-nitrosylated, Cys -34-containing peptide along with several Tyr-nitrated ones. The static Sterne culture demonstrated selective S-nitrosylation of Cys-34 and nitration of Tyr-30. No nitration

was found in the aerated culture, which might reflect a reduced amount of PN formed in comparison with the hypoxic conditions of static culture in agreement with the AR test results (Data not shown). However, S-nitrosylation of Cys-34 was still detectable, indicating that PN was not required for this reaction.

PN scavengers FeTPPS and MnTBAP abrogate Sup toxicity

PN is a strong oxidant [E'_o 1.4 v for PN/NO_3^- (Zakharova et al., 2007)]. However, the many of its biological effects can be due to the reactions of its decomposition product, NO_2 (Pacher et al., 2007) [E'_o 0.8 v for NO_2/NO_3^- (Standard electrode potential data)]. We suggested that reactive species scavengers or scavengers of PN will be able to abrogate Sup toxicity by converting these species to a nontoxic nitrate. 5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrinato Iron (III) (FeTPPS) and Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) are known peroxynitrite catalysts capable of eliminating both ROS and decomposition of PN to nitrate (Loukili et al., 2011). Neither compound forms complex with nitric oxide while only FeTPPS exhibits little SOD mimeric activity. We incubated dSterne Sups with a range of FeTPPS and MnTBAP concentrations for 30 min after Sup retrieval and found a strong cytoprotection of HSAECs from Sup toxicity in as little as 100μM (Figure 12 A, B). In both cases a slight decrease in cell viability was seen in controls, which is to be expected, and the data were normalized to the control for each concentration. The decrease in Sup toxicity

suggests that FeTPPS and MnTBAP serve as genuine scavengers of peroxynitrite rather than ROS.

DISCUSSION

In this study we further elucidated the recently discovered mechanism of *B. anthracis* toxicity toward the host cells (Popova et al., 2011). The soluble factors secreted by bacteria in the microaerobic conditions of reduced oxygen pressure quickly decrease the viability of the exposed cells independently of the LT and ET expression by the challenge strains. The toxic effect culminating in the cell oxidative stress and mitochondrial damage has a multifactorial nature. It requires a hypoxic environment for bacterial growth, an accumulation of acidic metabolic products (such as SA), as well as the secretion of pore-forming toxin, ALO. Although ALO and SA synergize in the host cell killing, the magnitude of their activity does not account for the full toxicity of the bacterial culture. It was therefore suggested that an additional toxic effect may come from the transient formation of PN because a catalyst of PN decomposition partially protects the exposed cells (Popova et al., 2011).

This study presents evidence in favor of this hypothesis. We show that *B. anthracis* was able to generate micromolar concentrations of NO in the process sensitive to the baNOS inhibitor, L-NAME (Figure 1). Unexpectedly, we found that accumulation of the NN (as products tracing the presence of NO) required a supplementation of the culture medium with BSA. We suggested that this effect reflects the property of BSA to absorb NO into

the hydrophobic interior of its globule and thus facilitate the autoxidation of NO in the presence of oxygen (Rafikova et al., 2002). Estimates show that the acceleration effect due to the increased concentrations of reactants can reach $>10^4$ times (Rafikova et al., 2002). Although the fine details of the oxidation mechanism are still disputed, all authors agree that NO_2 is an intermediate (Kharitonov et al., 1995; Ford et al., 1993; Lewis and Deen; Lancaster, 2006; Goldstein and Czapski, 1995) which can be formed in several reactions (Figure 4). Further interaction of NO with NO_2 can give rise to N_2O_3. However, the latter species can be present only at very low concentrations compared to NO_2 (Espey et al., 2002a), which is known to be an active oxidant of a number of biological species including sulfhydryl compounds (Goldstein and Czapski, 1995; Wink et al., 1993). Therefore the process of BSA interaction with NO in the presence of oxygen will likely involve chemical reaction of NO or NO_2 with the BSA side chain groups, such as the free thiol group of Cys-34. According to (Rafikova et al., 2002), about 5% of the absorbed NO becomes converted to a relatively stable S-nitrosoalbumin. Literature data (Ishima et al., 2009) and our experiments (Figure 10) show that these BSA derivatives are not acutely toxic and are expected to act as NO_2 sinks, removing a stoichiometric amount of oxidant from the system. Similar reactions can explain the effect of free Cys or DTT on the Sup activity (Figure 5). It is expected that accumulation of the NO-related toxicity in Sups would take place only after chemical saturation of the free BSA thiols (and possibly other reactive protein groups).

Regardless of the reactive intermediates involved, nitrosation *via* NO autoxidation may be the dominant route when relatively high rates of NO formation occur in the presence of oxygen without significant O_2^- generation (Wink et al., 1993; Espey et al., 2001; Liu et al., 1998). In contrast, the formation of PN may chiefly prevail under conditions where O_2^- is present, but not in great excess of NO (Zielonka et al., 2012; Pacher et al., 2007) (Reaction **4**). The rapid reaction between O_2^- and NO (Kissner et al., 1997; Koppenol et al., 1992; Goldstein and Czapski, 1995) has prompted numerous investigators to focus on PN and the roles it may play in oxidation and nitration of susceptible molecules (Radi et al., 2001). The protonated PN anion can decompose leading to secondary formation of other oxidants, chiefly NO_2 and hydroxyl radical ($OH^\bullet$, reaction 5) (Kissner et al., 1997; Koppenol et al., 1992; Goldstein et al., 1998). While NO_2 is capable of permeating cells (Espey et al., 2002b; Liu et al., 1998), it is likely that the highly reactive $OH^\bullet$ molecule would not have sufficient lifetime for diffusion into the cell and will recombine with NO to form a stable nitric acid (reaction 6)(Espey et al., 2002a).

An important facet to consider in the biochemistry of NO and $O_2^{\bullet-}$ is the influence of CO_2 on this system. Several groups (Lymar et al., 1996; Khairutdinov et al., 2000; Denicola et al., 1996a; Romero et al., 1999; Zhang et al., 2001; Jourd'heuil et al., 1999) have demonstrated that $ONOO^-$ can be rapidly consumed by CO_2 to form the CO_2 adduct nitrosuperoxocarbonate ($ONOOCO_2^-$) (reaction 8). This reaction pathway would circumvent the putative homolysis of ONOOH to NO_2 (Reaction **5**) and $^\bullet OH$ in favor of $ONOOCO_2^-$ decomposition into NO_2 and carbonate radical (CO_3^-, Reaction **9**). The CO_2

reaction pathway may result in an enhanced level of NO_2 formation relative to that produced by alternate routes of PN catabolism. The literature data argue against $ONOO^-$, ONOOH or $ONOOCO_2^-$ as significant cell permeable species relative to NO_2 (Lymar et al., 1996; Khairutdinov et al., 2000). The commonality of NO_2 as a putative product of the ONOOH or $ONOOCO_2^-$ decomposition pathways in combination with the ability of NO_2 to diffuse into cells and mediate oxidation (Khairutdinov et al., 2000) suggest it as one of key toxic agents in Sups.

Experiments with AR confirmed that *B. anthracis* grown in microaerobic cultures generated oxidizing species. Based on the accumulation of resorufin in the presence of catalase the properties of this species were distinct from H_2O_2. In addition, the inhibiting effect of SOD indicated that this species was not the superoxide, because the SOD-catalyzed dismutation of the latter would give rise to H_2O_2 still capable of the AR oxidation. We also found that the oxidation of AR was inhibited by L-NAME and therefore concluded that the major reactive species originated from NO (which itself does not oxidize AR). In the case of NO autoxidation being a predominant reaction, one would expect an acceleration of AR conversion to resorufin upon aeration of cultures. Remarkably, we observed an opposite effect. A background oxidation without bacteria indeed increased; however, it was not increased in the dSterne culture (not shown) and even decreased in the Sterne one. Overall, the results are completely consistent with the consumption of NO in fast reaction **4** effectively competing with reaction 1 and leading to the transient formation of PN. We did not investigate the origin of the superoxide in *B.*

anthracis cultures. Similar to other bacteria, it may consist in the respiratory chain leakage or the activity of metabolic enzymes such as fumarate reductase (Messner and Imlay, 2002).

The formation of PN provides an explanation for the increased fluorescence of DHR in response to bacterial products. DHR has been most widely used to measure intracellular oxidants. This probe does not directly react with H_2O_2 or PN, but becomes oxidized via radical mechanism involving $OH^{\bullet}$, NO_2, or $CO_3^{-\bullet}$ in the presence of carbon dioxide (reactions **5, 8, 9**)(Wrona et al., 2005).

In the case PN and NO_2 are the major reactive species participating in the oxidation of AR, one should expect that their interaction with BSA would result in the formation of nitrated Tyr residues. Indeed, such a modification was detected using a specific antibody (Figure 9B) as well as the MS analysis (Table) in static but not in aerobic conditions, consistent with the absence of bacteria-produced oxidizing species in aerated cultures tested with AR (Figure 6, Figure 7 A&B).

Finally, we carried out experiments with L-NAME added to the medium of bacterial cultures to demonstrate that inhibition of baNOS resulted in the reduced toxicity of Sups (Figure 8) correlating with the capacity of L-NAME to decrease the formation of AR-oxidizing species. We showed that effective protection of HSAECs could be accomplished in reaction with the peroxynitrite scavengers FeTPPS and MnTBAP as true

scavengers of PN (Pacher et al 2007, Popova et al. 2010). When coupled with DHR, these results suggest that the overall end product of PN is the likely RNS damaging species. While oxidation of NO_2 can result in a relatively inert nitrate, a reduction of NO_2 using antioxidants has a potential to recover a chemically reactive nitrite or biologically active NO.

Our data obtained in the *in vitro* system using culture medium supplemented with BSA raise a question whether the toxic effect of *B. anthracis* would also take place *in vivo* during the bacterimic stage of infection when bacteria can be found in serum. To address this question one can carry out experiments with the addition of fetal bovine serum (FBS) to the culture medium. Our preliminary data (not shown) indicate that the presence of FBS delayed the toxic effect of Sups relative to BSA to 48 h due to unidentified factors. We suggest that among these factors the antibacterial mechanisms in serum could delay spore germination and bacterial growth. In order to counteract *B. anthracis* toxicity different animal hosts have various means and defenses to prevent and eradicate spore infection. Host serum has been long established to contain components to have effects on spore replication, germination, and dissemination of the bacteria from site of infection into circulation (Bensman et al. 2012). Sera from different species (human, mouse, or rabbit) demonstrate different levels of antibacterial activity, and human serum was implicated in the strong inhibition of the spore germination (Rooijakkers et al. 2010, Bensman et al. 2012). On the other hand, saturation with NO of a large amount of BSA present in FBS would certainly decrease the formation of PN and RNS. No information is

available regarding the effects of serum on the expression of *B. anthracis* pathogenic factors relevant to the NO-mediated toxicity in microaerobic conditions. Together all results reiterate the multifactorial nature of anthrax toxicity and pathogenicity. Further understanding of how serum interacts with different bacterial processes such as the production of acidic metabolic products (such as SA), the secretion of pore-forming toxin like ALO, the activity of baNOS is necessary to comprehend progression of host-pathogen interaction *in vivo*.

The mechanism we describe was not previously identified for bacterial pathogens capable of producing NO either through the activity of NOS or denitrification. However, very similar cytotoxic effects supporting our observations were reported in the case of neural PC12 cells exposed to PN generated during oxidation of 3-morpholinisydnonimide (SIN-1) (Konishi et al., 2009; Shirai et al., 2012). The exposure of cells to the culture medium obtained after complete SIN-1 decomposition demonstrates almost the same level of cytotoxicity as did fresh SIN-1. Although the reactive species were not identified, their properties closely mirror our observations. The cytotoxicity is dependent on the presence of serum and could be abolished by thiols. The presence of SOD but not catalase during the SIN-1 decomposition prevents the formation of cytotoxic substances. The authors concluded that the formation of toxicants is a result of the simultaneous formation of NO and superoxide. In agreement with our considerations regarding the mechanism of PN reactivity, it was found that CO_2 plays a critical role in the cytotoxicity of the SIN-1 decomposition products (Shirai et al., 2012). Surprisingly, the authors misinterpreted the

effect of BSA when they found that addition of fresh serum (or BSA) antagonizes the cytotoxicity. As we show, BSA consumes a portion of reactive species in chemical reactions with its side groups and therefore needs to be chemically “saturated” in order to display a maximal toxic effect.

In summary, we present novel data regarding the property of baNOS to participate in the formation of cytotoxic substances through generation of NO which gives rise to the transient formation of reactive species with the properties of PN in the conditions of reduced oxygen availability. This product is known to quickly decompose with the accumulation of stable but chemically reactive NO_2. Our data are consistent with the notion of NO_2 as the major toxic species in the Sups. The role of BSA consists in the trapping of NO and NO_2 in the hydrophobic interior of the protein globule accompanied by chemical reactions of the BSA side chains. This mechanism is expected to function along with the toxic effect ALO and SA discovered in our previous studies. The experiments are in progress to elucidate a possible synergism between NO, ALO, and SA as pathogenic factors of *B. anthracis*.

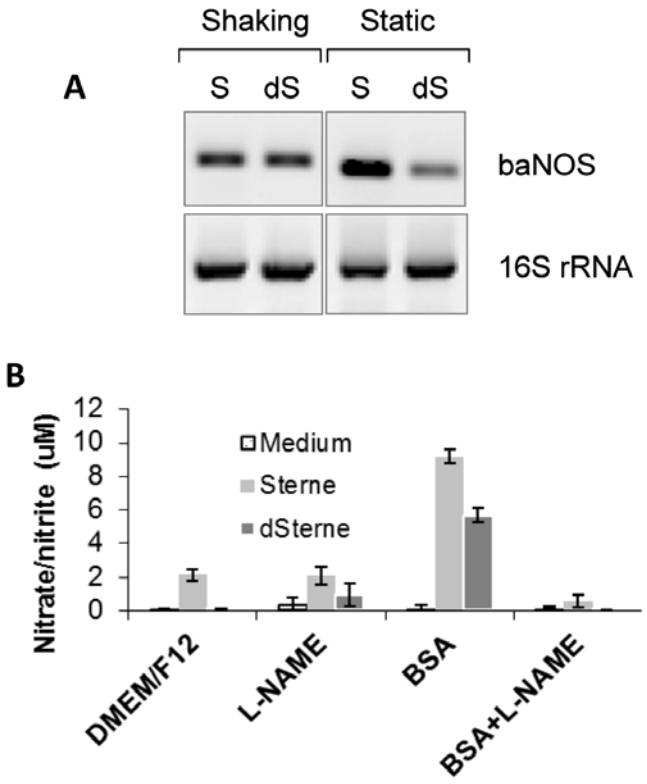


Figure 1. baNOS is expressed in aerated and static cultures, which accumulate nitrate/nitrite (NN) as trace products of NO in the presence of BSA. (**A**) RT-PCR of baNOS mRNA in different culture conditions. Aerated culture was grown in 2 ml of medium per well of a 6-well plate at 300 rpm at 37^{o}C, 5% CO_2 for 20 h. Static culture was grown in 10 ml of medium per well of a 6-well plate without shaking at 37^{o}C, 5% CO_2 for 20 h. 12.5 ng of bacterial RNA isolated with the TrizolMax kit (Invitrogen) were used per 50 µl reactions in 38 PCR cycles. No bands were detected in the negative controls without the RT step (not shown). Data obtained by M.Chung-Chul. (**B**) Cultures were growth in 5 ml of DMEM/F12 medium per well of a 12-well plate with shaking at 200 rpm at 37^{o}C, 5% CO_2 for 24 h. Cultures were supplemented with BSA (1 mg/ml) and/or NO-synthase inhibitor L-NAME (10 mM). NN concentration was measured with a colorimetric assay kit (Cayman Chemical). Error bars indicate 95% confidence intervals. Data obtained by T. Popova.

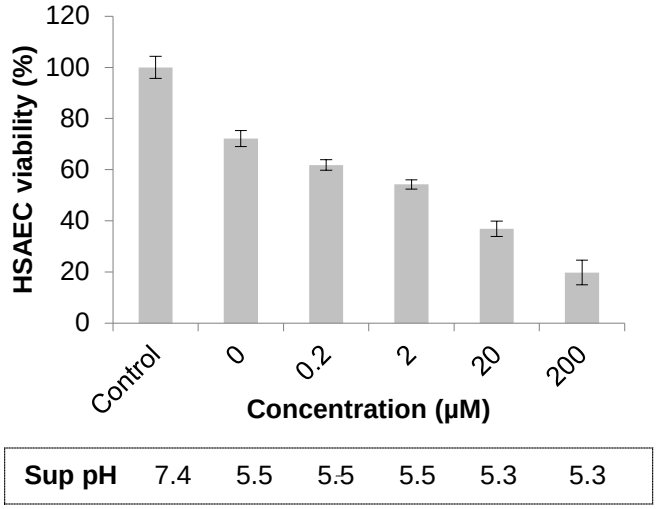


Figure 2**.** Supplementation of complete DMEM/F12 culture medium with BSA increases the toxicity of Sups of dSterne cultures grown in microaerobic conditions for 24 h. HSAECs were exposed to Sups for 2 h. Untreated cells served as control. OD_{600} of Sups was measured in 96-well plate reader (200 µl per well). Error bars indicate 95% confidence intervals.

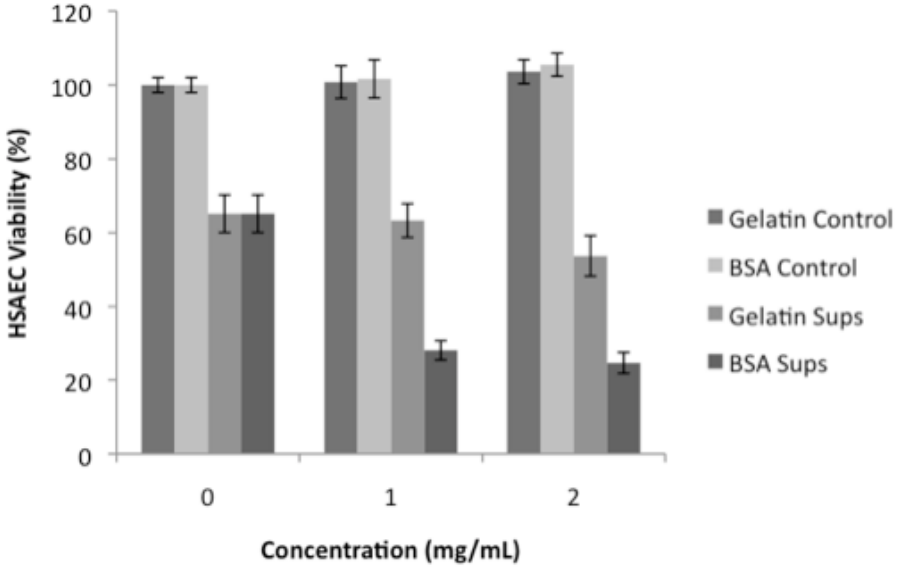


Figure 3. . BSA is a specific protein that increases dSterne Sup toxicity. dSterne Sups were made with the addition of BSA or gelatin (negative control) into complete DMEM/F12 containing 300 µM nitrate for 24 h. HSAECs were exposed to Sups for 2 h. Viability was tested using Alamar Blue. Error bars indicate 95% confidence intervals.

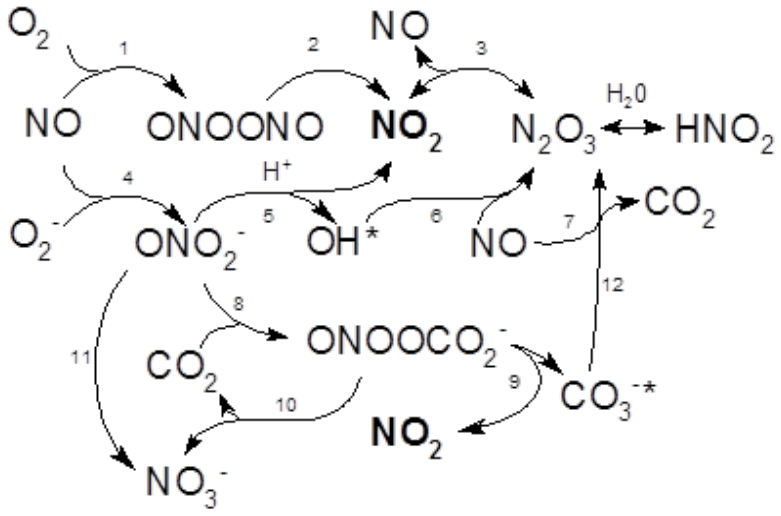


Figure 4. Schematic of biologically relevant chemical reactions of NO with oxygen and superoxide radical in the presence of carbon dioxide according to the results of (Kharitonov et al., 1995; Ford et al., 1993; Lewis and Deen; Lancaster, 2006; Goldstein and Czapski, 1995; Espey et al., 2002a; Wink et al., 1993; Goldstein et al., 1998; Goldstein, S. & Grabski, 1995). NO_2 is a major toxic species expected to accumulate in the system before its conversion to stable nitrite and nitrate. The stoichoimetric coefficients are not shown.

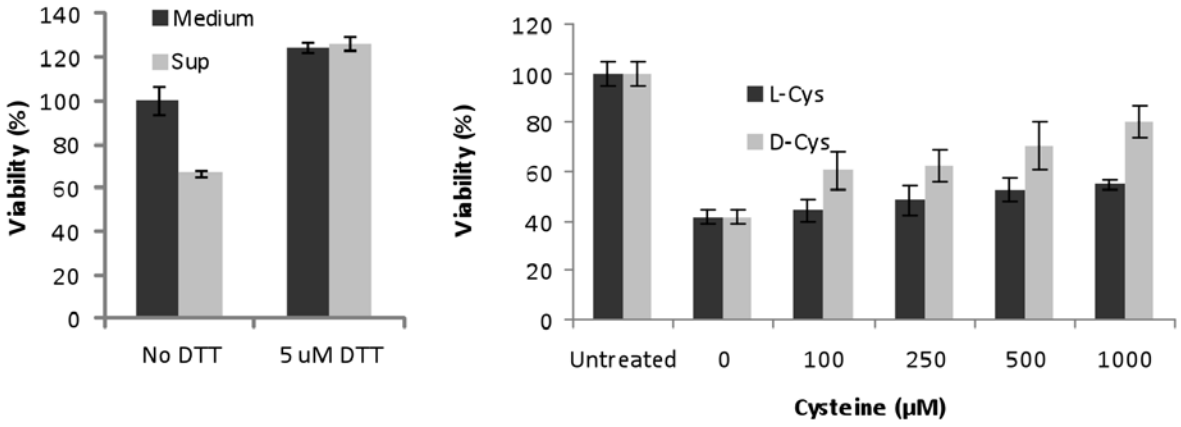


Figure 5. Antioxidant treatment of Sups protects HSAECs. The dSterne Sups grown in complete DMEM/F12 were incubated with 5 mM DTT (for 30 min) or cysteine (for 1 h). HSAECs were exposed to the treated Sups for 2 h and the viability of cells was tested using Alamar Blue. In control experiments, addition of Cys had no effect on viability of untreated cells, and no cytoprotection was detected with other L-amino acids tested (Val, Leu, Ala) (not shown). Error bars indicate 95% confidence intervals.

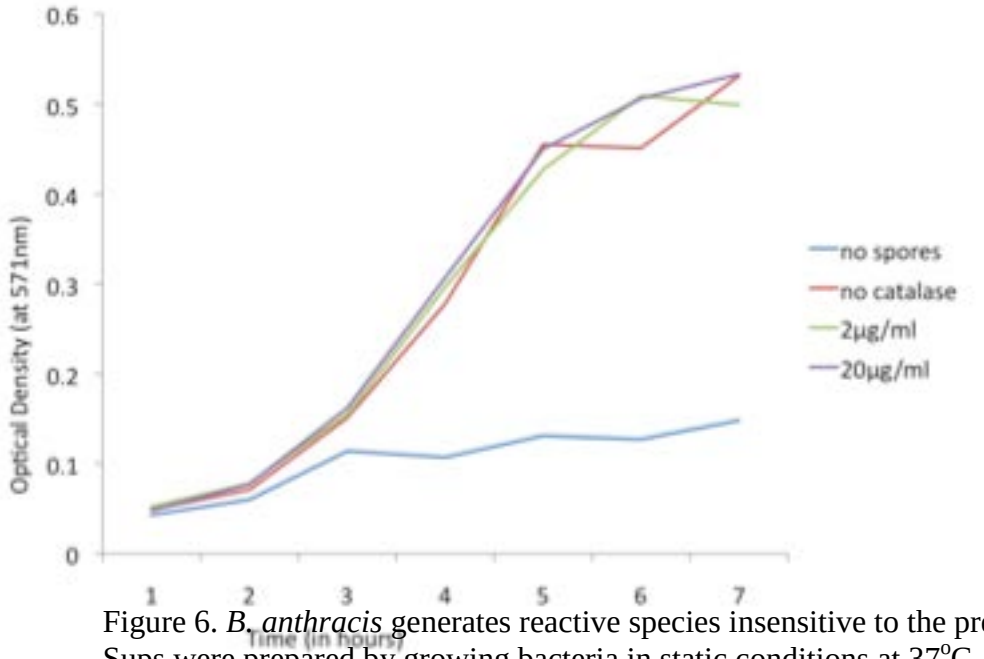


Figure 6. *B. anthracis* generates reactive species insensitive to the presence of catalase. Sups were prepared by growing bacteria in static conditions at 37^{o}C, 5% CO_2 in 24-well plate containing 1.5 ml of DMEM/F-12 medium per well or 12-well plate containing 5 ml of DMEM/F12 medium per well. Spores were seeded to a final concentration of 6x10^{6}/ml. Catalase were added final concentrations were 2µg/mL and 20µg/mL correspondingly. Before spectrophotometric measurements, bacteria were pelleted by centrifugation and Sups were diluted 1:1 with 10x PBS, pH 7.4, to exclude the effect of pH change on the absorbance of resorufin. In the presence of catalase (20 µg/ml) the response is indistinguishable from that of Sterne strain only. Data was obtained with R. Blower.

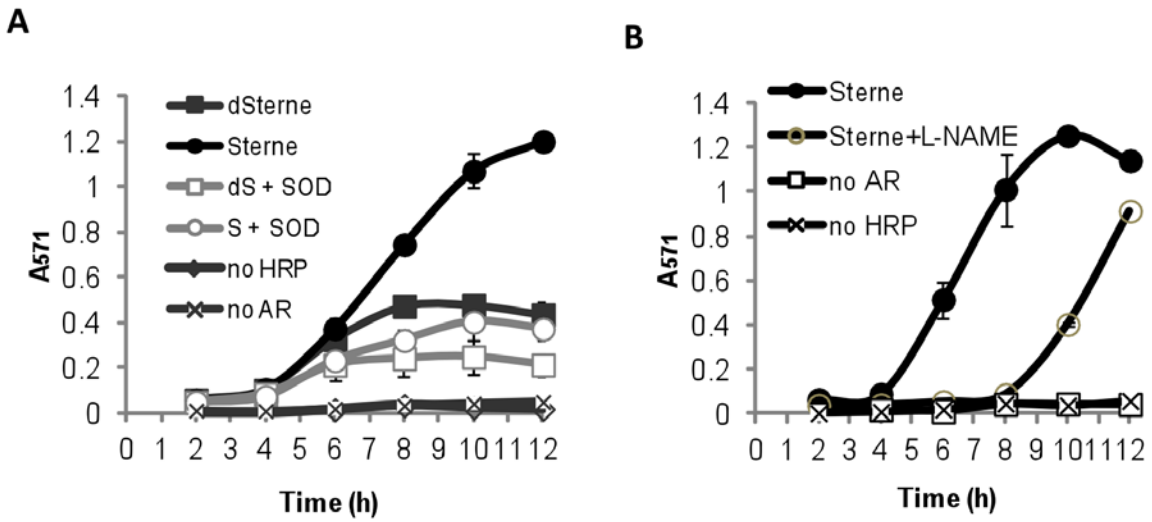


Figure 7. SOD and L-NAME inhibit oxidation of AR during bacterial growth in microaerobic conditions. (**A**) Effect of SOD. (**B**) Effect of L-NAME. Sups were prepared by growing bacteria in static conditions at 37^{o}C, 5% CO_2 in 24-well plate containing 1.5 ml of in DMEM/F-12 medium per well (**A**) or 12-well plate containing 5 ml of DMEM/F12 medium per well (**B**). Spores were seeded to a final concentration of 6x10^{6}/ml. SOD, HRP, AR, and L-NAME final concentrations were 10 U/ml, 0.2 U/ml, 0.1mM, and 10 mM, correspondingly. Before spectrophotometric measurements, bacteria were pelleted by centrifugation and Sups were diluted 1:1 with 10x PBS, pH 7.4, to exclude the effect of pH change on the absorbance of resorufin. Error bars indicate standard deviations (*n*=3). Data was obtained with R. Blower.

Sup Properties				
L-NAME (mM)	0	10	50	100
OD600	0.61	0.92	0.43	0.35
pH	5.1	5.1	4.9	4.6

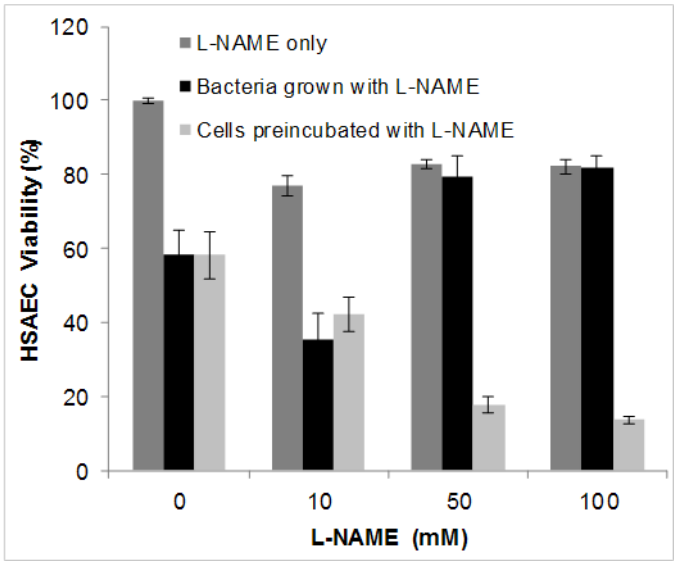


Figure 8. Inhibition of baNOS with L-NAME decreases toxicity of Sups to HSAECs. Static cultures of dSterne strain were grown in complete DMEM/F12 as described in Materials and methods with or without the indicated concentrations of L-NAME. The table shows the ODs and pHs of 24-h cultures. The Sups were added for 2 h to HSAECs pretreated or not with the indicated concentrations of L-NAME for 1 h, and the viability of the cells was assayed with Alamar Blue. Controls included the culture medium with L-NAME without bacteria. All tested samples were titrated with HCl to the pH 5.1 of Sups without L-NAME. Error bars indicate 95% confidence intervals.

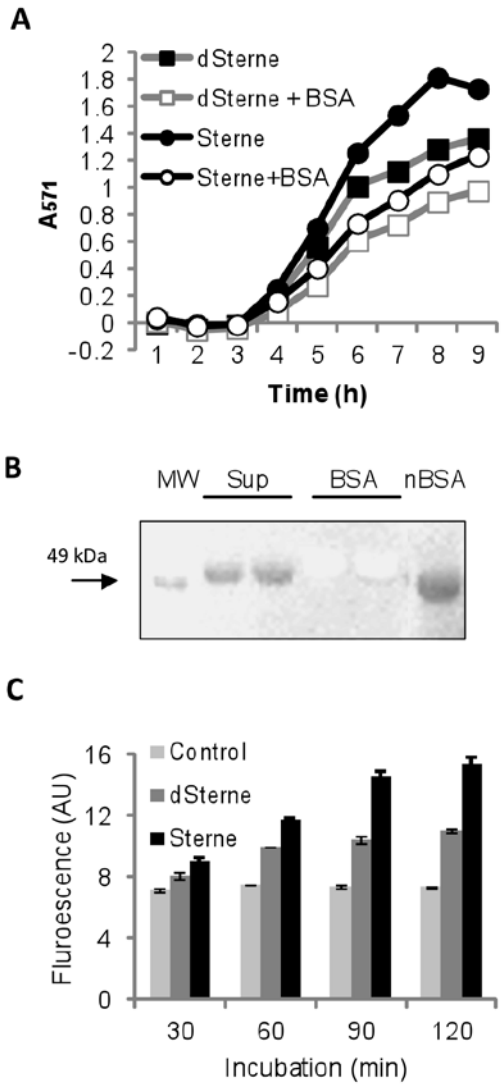


Figure 9. Bacterial growth in microaerobic conditions generates oxidizing species with the properties of PN. (A) BSA inhibits the oxidation of AR catalyzed by HRP in dSterne cultures grown as in Figure 6 in 12-well plates with the addition of 1 mg/ml of BSA in DMEM/F12 medium. Data obtained by R. Blower (B) Western blot of dSterne Sups, unmodified BSA, and nitrated BSA (nBSA) using anti-nitrotyrosine antibodies. Sups were prepared by inoculation of dSterne spores (final concentration of 6x 10^6 spores/ml) into DMEM/F12 with 1 mg/ml of BSA. After 24 h, bacteria were removed by centrifugation and gel electrophoresis was performed followed by membrane transfer. Anti-rabbit HRP-linked secondary antibody was used. Data was obtained by R. Blower (C) Fluorescence of DHR indicates the release of PN in *B. anthracis* cultures. Bacteria grown statically for 18 h in complete DMEM/F12 medium were pelleted, washed with PBS, resuspended in PBS containing DHR, and incubated at 37^{o}C, 5% CO_2 for the indicate periods of time for detection of fluorescence at 500/540 nm. Error bars indicate 95% confidence intervals.

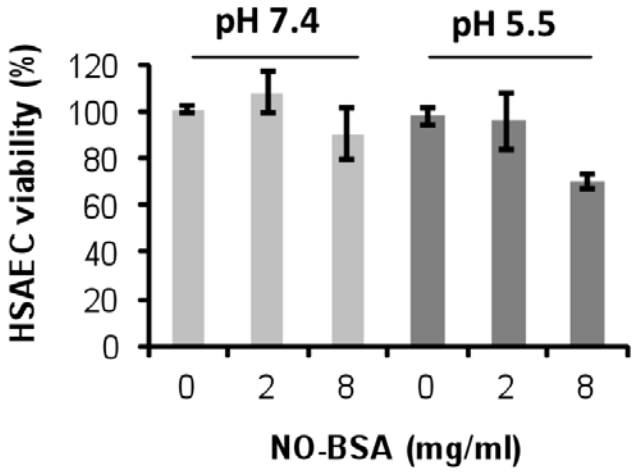


Figure 10. Nitrosylated BSA (NO-BSA) is not acutely toxic. HSAECS were incubated with NO-BSA at indicated concentrations and pH for 2 h in DMEM/F12 medium, and the viability of HSAECs was tested using Alamar Blue. Error bars indicate 95% confidence intervals. Data was obtained with R. Blower.

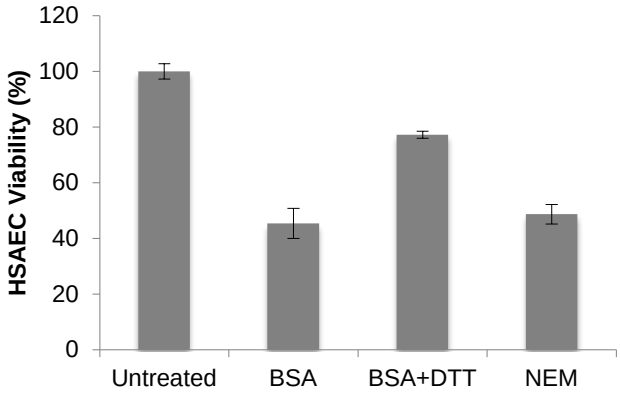


Figure 11. Blocking of free SH groups of BSA does not reduce the toxicity of dSterne Sup grown in the presence of BSA. The protein was dissolved in 10 ml of PBS, treated with 5 mM DTT for 30 min, and then dialyzed against two changes of 1 L of PBS for 24 h. The reduced BSA was then modified by NEM (125 µM) for 1h at 37^{o}C and dialyzed against two changes of 1 L of PBS overnight. The modified and mock-treated BSA was used to supplement the DMEM/F12 medium at 2 mg/ml. Bacterial cultures were grown for 24 h and Sups were tested for toxicity using HSAECs and the Alamar Blue viability assay. Error bars indicate 95% confidence intervals.

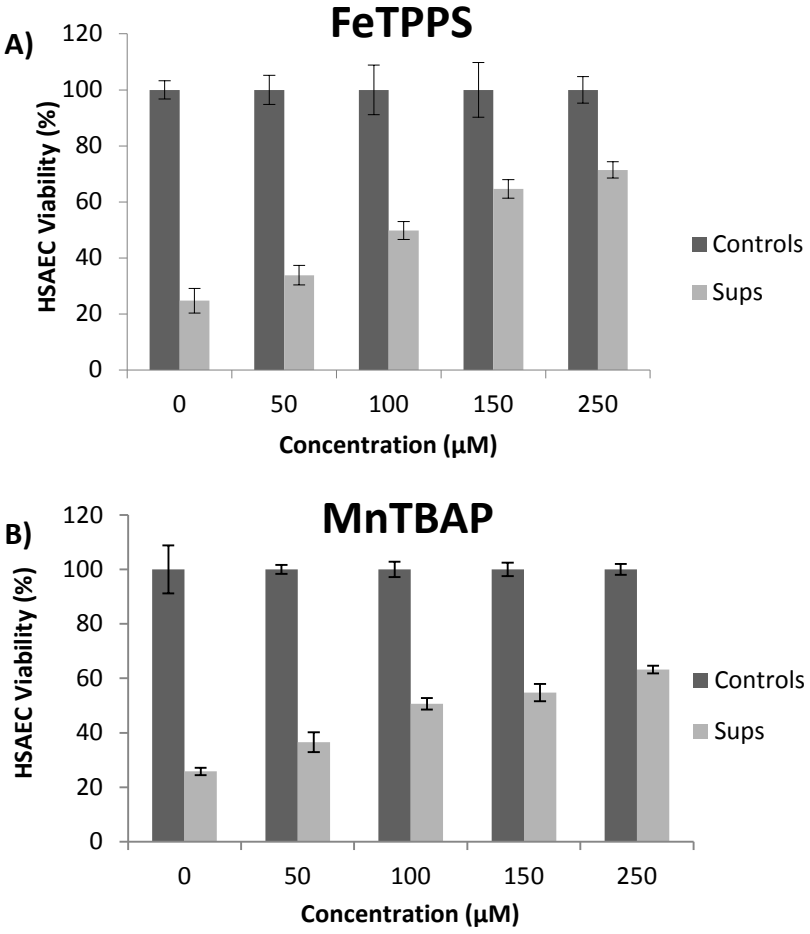


Figure 12. PN scavengers decrease dSterne Sup toxicity by decomposition of PN and ROS. (**A**) dSterne Sups incubated in complete DMEM/F-12 medium was incubated for 30 min with FeTPPS at 37^{O}C after Sup retrieval. (**B**) dSterne Sups incubated in complete DMEM/F-12 medium was incubated for 30 min with FeTPPS at 37^{O}C after Sup retrieval. In both tests the HSAECs were exposed to these Sups for 2 h and viability was tested using Alamar Blue. Error bars indicate 95% confidence intervals.

Table 1. Chemical modifications of BSA identified in Sups of *B. anthracis* Sterne cultures grown in CSFM*

Sample	Peptides with S-nitrosylation	Peptides with Tyr-nitration
Untreated BSA	None	None

Nitrite-treated BSA (positive control)	G[21]LVLIAFSQY[30]LQQC#PFDEHVK	RHPEY@AVSVLLR HPYFY@APELLYYANK HPYFYAPELLY@YANK HPYFYAPELLYY@ANK RHPY@FY@APELLYYANK LGEY@GFQNALIVR DAFLGSFLY@EYSR DAFLGSFLYEY@SR
Static culture	GLVLIAFSQYLQQC#PFDEHVK	GLVLIAFSQY@LQQC#PFDEHVK
Aerated culture	GLVLIAFSQYLQQC#PFDEHVK	None

* CSFM contains 1 mg/ml of BSA
# indicates S-nitrosylation
@ indicates Tyr nitration
Data obtained by A. Narayanan

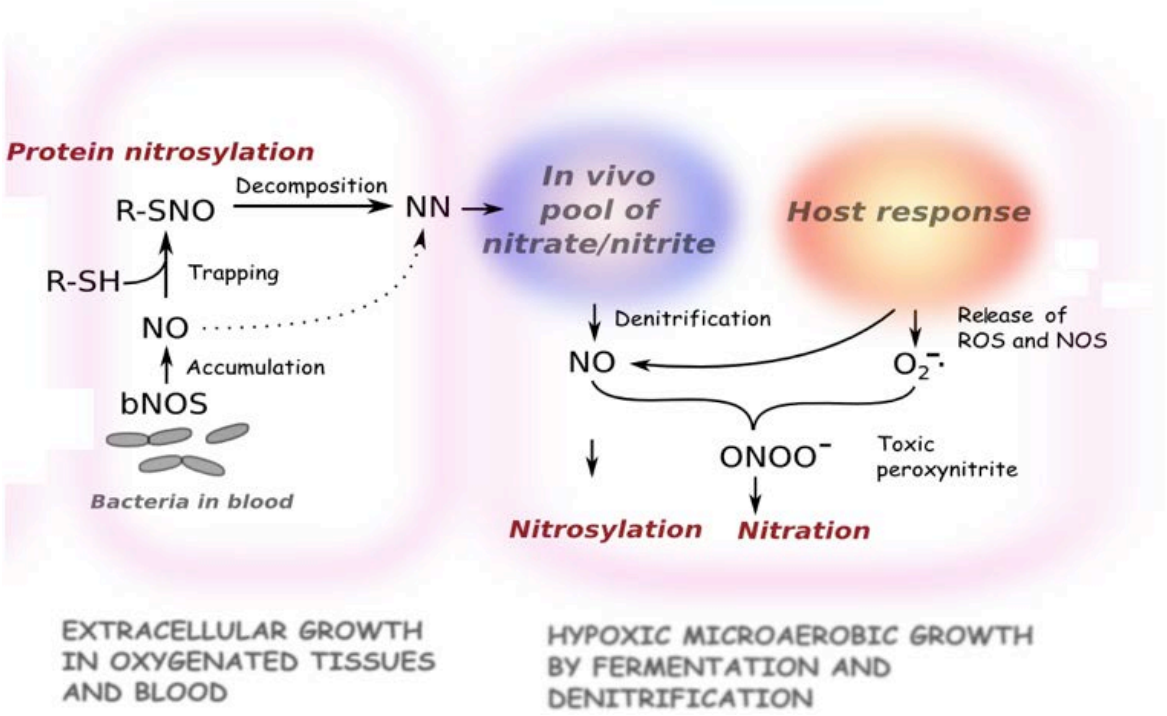
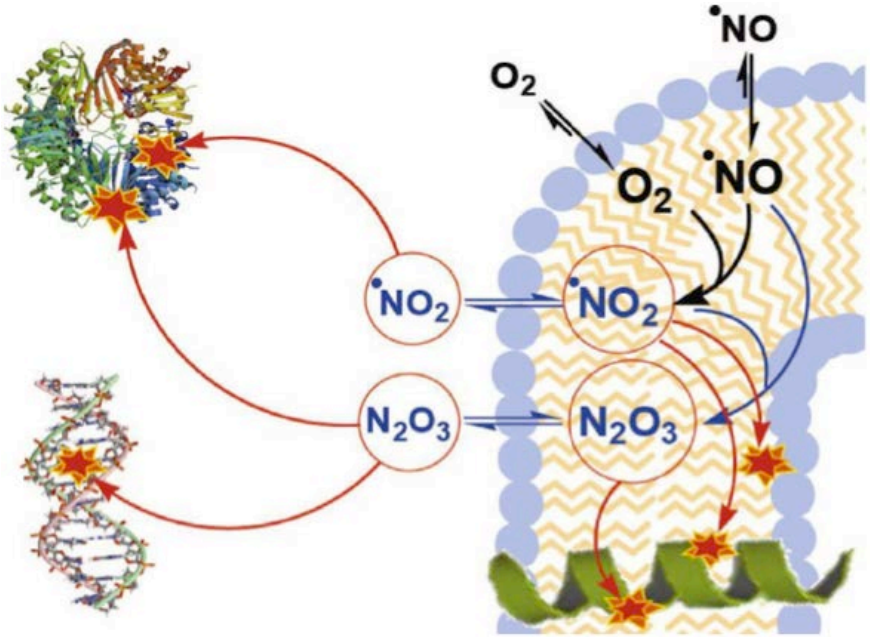


Figure 13. LT/ET-independent mechanism of *B. anthracis* toxicity.

A)



B)

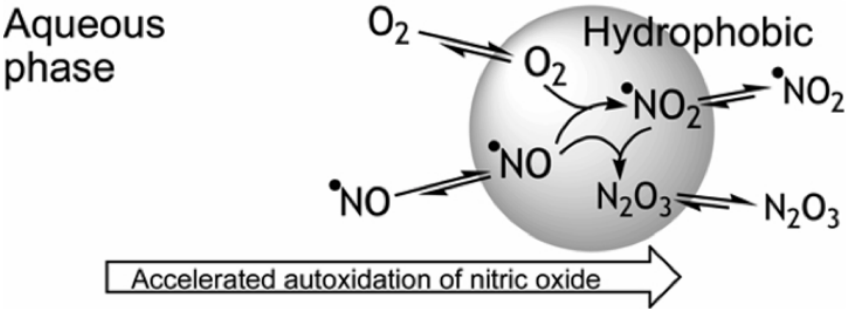


Figure 14. Interaction between NO and BSA. (A) Autoxidation of NO in BSA leading to protein nitrosylation and DNA damage. (B) The hydrophobic pocket of BSA allows for accelerated autoxidation of NO.

REFERENCES

Belik, J., Stevens, D., Pan, J., McIntyre, B. A. S., Kantores, C., Ivanovska, J., Xu, E. Z., Ibrahim, C., Panama, B. K., Backx, P. H., et al. (2010). Pulmonary vascular and cardiac effects of peroxynitrite decomposition in newborn rats. *Free radical biology & medicine* 49, 1306–14.

Bensman, M.D., Mackie, R.S., Minter, Z.A., Gutting, B.W. (2012). Effect of animal sera on *Bacillus anthracis* Sterne spore germination and vegetative cell growth. Journal of Applied Microbiology 113(2), 276-283.

Bradburne, C., Chung, M.-C., Zong, Q., Schlauch, K., Liu, D., Popova, T., Popova, A., Bailey, C., Soppet, D., and Popov, S. (2008). Transcriptional and apoptotic responses of THP-1 cells to challenge with toxigenic, and non-toxigenic Bacillus anthracis. *BMC immunology* 9, 67.

Bueno, E., Mesa, S., Bedmar, E. J., Richardson, D. J., and Delgado, M. J. (2012). Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control. *Antioxidants & redox signaling* 16, 819–52.

Chand, H. S., Drysdale, M., Lovchik, J., Koehler, T. M., Lipscomb, M. F., and Lyons, C. R. (2009). Discriminating virulence mechanisms among Bacillus anthracis strains by using a murine subcutaneous infection model. *Infection and immunity* 77, 429–35.

Christodoulou, J., Sadler, P. J., and Tucker, A. (1995). 1H NMR of albumin in human blood plasma: drug binding and redox reactions at Cys34. *FEBS letters* 376, 1–5.

Chung, M.-C., Popova, T. G., Millis, B.A., Mukherrie, D.V., Zhou, W., Liotta, L.A., Petricoin, E.F., Chandhoke, V., Bailey, C., Popov, S.G. (2006). Secreted Neutral Metalloproteases of *Bacillus anthracis* as Candidate Pathogenic Factors. *Journal of biological chemistry* 281, 31408-31418.

Chung, M.-C., Narayanan, A., Popova, T. G., Kashanchi, F., Bailey, C. L., and Popov, S. G. (2012). Bacillus anthracis-derived Nitric oxide Induces Protein S-nitrosylation

Contributing to Macrophage Death. *Biochemical and biophysical research communications*.

Cote, C. K., DiMezzo, T. L., Banks, D. J., France, B., Bradley, K. A., and Welkos, S. L. (2008). Early interactions between fully virulent Bacillus anthracis and macrophages that influence the balance between spore clearance and development of a lethal infection. *Microbes and infection / Institut Pasteur* 10, 613–9.

Crane, B. R., Sudhamsu, J., and Patel, B. A. (2010). Bacterial nitric oxide synthases. *Annual review of biochemistry* 79, 445–70.

Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996a). Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Archives of biochemistry and biophysics* 333, 49–58.

Denicola, A., Souza, J. M., Radi, R., and Lissi, E. (1996b). Nitric oxide diffusion in membranes determined by fluorescence quenching. *Archives of biochemistry and biophysics* 328, 208–12.

Dixon, T. C., Fadl, A. A., Koehler, T. M., Swanson, J. A., and Hanna, P. C. (2000). Early Bacillus anthracis-macrophage interactions: intracellular survival survival and escape. *Cellular microbiology* 2, 453–63.

Dröse, S., and Brandt, U. (2012). Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Advances in experimental medicine and biology* 748, 145–69.

Elza A. Zakharova, T. A. Y. B. F. N., and Gregory G. Wildgoose and Richard G. Comptonb The voltammetric determination of peroxynitrite at a mercury film electrode. *New J. Chem., 2007, 31, 394–400*. Available at: http://pubs.rsc.org/en/content/articlepdf/2007/NJ/B615188D.

Era, S., Kuwata, K., Imai, H., Nakamura, K., Hayashi, T., and Sogami, M. (1995). Age-related change in redox state of human serum albumin. *Biochimica et biophysica acta* 1247, 12–6.

Espey, M. G., Miranda, K. M., Thomas, D. D., and Wink, D. A. (2001). Distinction between nitrosating mechanisms within human cells and aqueous solution. *The Journal of biological chemistry* 276, 30085–91.

Espey, M. G., Thomas, D. D., Miranda, K. M., and Wink, D. A. (2002a). Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11127–32.

Espey, M. G., Xavier, S., Thomas, D. D., Miranda, K. M., and Wink, D. A. (2002b). Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* 99, 3481–6.

Ferrer-Sueta, G., and Radi, R. (2009). Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS chemical biology* 4, 161–77.

Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993). Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horseradish peroxidase and catalase. *European journal of biochemistry / FEBS* 215, 767–75.

Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS letters* 326, 1–3.

Foster, M. W., Hess, D. T., and Stamler, J. S. (2009). Protein S-nitrosylation in health and disease: a current perspective. *Trends in molecular medicine* 15, 391–404.

Goldstein, S., and Czapski, G. (1995). The reaction of NO. with O2.- and HO2.: a pulse radiolysis study. *Free radical biology & medicine* 19, 505–10.

Goldstein, S., Czapski, G., Lind, J., and Merenyi, G. (1998). Mechanism of Decomposition of Peroxynitric Ion (O(2)NOO(-)): Evidence for the Formation of O(2)(*-) and (*)NO(2) Radicals. *Inorganic chemistry* 37, 3943–3947.

Goldstein, S. & Grabski, G. (1995). Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants: the nature of the oxidizing intermediates. *J. Am. Chem. Soc.* 117, 12078–12084.

González-Flecha, B., and Demple, B. (1995). Metabolic sources of hydrogen peroxide in aerobically growing Escherichia coli. *The Journal of biological chemistry* 270, 13681–7.

Guichard, A., Nizet, V., and Bier, E. (2012). New insights into the biological effects of anthrax toxins: linking cellular to organismal responses. *Microbes and infection / Institut Pasteur* 14, 97–118.

Guidi-Rontani, C. (2002). The alveolar macrophage: the Trojan horse of Bacillus anthracis. *Trends in microbiology* 10, 405–9.

Habib, S., and Ali, A. (2011). Biochemistry of nitric oxide. *Indian journal of clinical biochemistry* 26, 13–17.

Hakim, T. S., Sugimori, K., Camporesi, E. M., and Anderson, G. (1996). Half-life of nitric oxide in aqueous solutions with and without haemoglobin. *Physiological measurement* 17, 267–77.

Heninger, S., Drysdale, M., Lovchik, J., Hutt, J., Lipscomb, M. F., Koehler, T. M., and Lyons, C. R. (2006). Toxin-deficient mutants of Bacillus anthracis are lethal in a murine model for pulmonary anthrax. *Infection and immunity* 74, 6067–74.

Inglesby, T. V, O'Toole, T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Friedlander, A. M., Gerberding, J., Hauer, J., Hughes, J., et al. (2002). Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA : the journal of the American Medical Association* 287, 2236–52.

Inglesby, T. V. (2002). Anthrax as a Biological Weapon, 2002: Updated Recommendations for Management. *JAMA: The Journal of the American Medical Association* 287, 2236–2252.

Ishima, Y., Kragh-Hansen, U., Maruyama, T., and Otagiri, M. (2009). Albumin as a nitric oxide-traffic protein: characterization, biochemistry and possible future therapeutic applications. *Drug metabolism and pharmacokinetics* 24, 308–17.

Jourd'heuil, D., Miranda, K. M., Kim, S. M., Espey, M. G., Vodovotz, Y., Laroux, S., Mai, C. T., Miles, A. M., Grisham, M. B., and Wink, D. A. (1999). The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. *Archives of biochemistry and biophysics* 365, 92–100.

Khairutdinov, R. F., Coddington, J. W., and Hurst, J. K. (2000). Permeation of phospholipid membranes by peroxynitrite. *Biochemistry* 39, 14238–49.

Kharitonov, V. G., Sundquist, A. R., and Sharma, V. S. (1995). Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *The Journal of biological chemistry* 270, 28158–64.

King, T. P. (1961). On the sulfhydryl group of human plasma albumin. *The Journal of biological chemistry* 236, PC5.

Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chemical research in toxicology* 10, 1285–92.

Konishi, K., Watanabe, N., and Arai, T. (2009). SIN-1 cytotoxicity to PC12 cells is mediated by thiol-sensitive short-lived substances generated through SIN-1 decomposition in culture medium. *Nitric oxide* □*: biology and ch* *journal of the Nitric Oxide Society* 20, 270–8.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chemical research in toxicology* 5, 834–42.

Kraft, B., Strous, M., and Tegetmeyer, H. E. (2011). Microbial nitrate respiration – Genes, enzymes and environmental distribution. *Journal of Biotechnology* 155, 104–117.

Lancaster, J. R. (2006). Nitroxidative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. *Chemical research in toxicology* 19, 1160–74.

Levy, H., Weiss, S., Altboum, Z., Schlomovitz, J., Glinert, I., Sittner, A., Shafferman, A., and Kobiler, D. (2012a). Differential Contribution of Bacillus anthracis Toxins to Pathogenicity in Two Animal Models. *Infection and immunity*.

Levy, H., Weiss, S., Altboum, Z., Schlomovitz, J., Rothschild, N., Glinert, I., Sittner, A., and Kobiler, D. (2012b). The effect of deletion of the edema factor on Bacillus anthracis pathogenicity in guinea pigs and rabbits. *Microbial pathogenesis* 52, 55–60.

Lewis, R. S., and Deen, W. M. Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chemical research in toxicology* 7, 568–74.

Liu, X., Miller, M. J., Joshi, M. S., Thomas, D. D., and Lancaster, J. R. (1998). Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proceedings of the National Academy of Sciences of the United States of America* 95, 2175–9.

Loukili, N., Rossenblatt-Velin, N., Li, J., Clerc, S., Pacher, P., Feihl, F., Waeber, B., Laudet, L. (2011). Peroxynitrite induces HMGB1 release by cardiac cells in vitro and HMGB1 upregulation in the infarcted myocardium in vivo. *Cardiovascular Research.* 89, 586-594.

Lovchik, J. A., Drysdale, M., Koehler, T. M., Hutt, J. A., and Lyons, C. R. (2012). Expression of either Lethal Toxin or Edema Toxin by Bacillus anthracis is Sufficient for Virulence in a Rabbit Model of Inhalational Anthrax. *Infection and immunity*.

Lymar, S. V, Jiang, Q., and Hurst, J. K. (1996). Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* 35, 7855–61.

Messner, K. R., and Imlay, J. A. (2002). Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. *The Journal of biological chemistry* 277, 42563–71.

Messner, K. R., and Imlay, J. A. (1999). The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of Escherichia coli. *The Journal of biological chemistry* 274, 10119–28.

Moayeri, M., and Leppla, S. H. (2009). Cellular and systemic effects of anthrax lethal toxin and edema toxin. *Molecular aspects of medicine* 30, 439–55.

Pacher, P., Beckman, J. S., and Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiological reviews* 87, 315–424.

Palazzolo-Ballance, A. M., Suquet, C., and Hurst, J. K. (2007). Pathways for intracellular generation of oxidants and tyrosine nitration by a macrophage cell line. *Biochemistry* 46, 7536–48.

Popov, S. G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Wu, A., Alibek, D., Bailey, C., and Alibek, K. (2002). Lethal toxin of Bacillus anthracis causes apoptosis of macrophages. *Biochemical and biophysical research communications* 293, 349–55.

Popov, S.G., Popova, T.G., Hopkins, S., Weinstein, R.S., MacAfee, R., Fryxell, K.J., Chandhoke, V., Bailey, C., Alibek, K. (2005). Effective antiproteases-antibiotic treatment of experimental anthrax. *BMC Infectious Disease* 8, 5-25.

Popova, T. G., Millis, B., Chung, M.-C., Bailey, C., and Popov, S. G. (2011). Anthrolysin O and fermentation products mediate the toxicity of Bacillus anthracis to lung epithelial cells under microaerobic conditions. *FEMS immunology and medical microbiology* 61, 15–27.

Quijano, C., Hernandez-Saavedra, D., Castro, L., McCord, J. M., Freeman, B. A., and Radi, R. (2001). Reaction of peroxynitrite with Mn-superoxide dismutase. Role of the metal center in decomposition kinetics and nitration. *The Journal of biological chemistry* 276, 11631–8.

Quinlan, C. L., Orr, A. L., Perevoshchikova, I. V, Treberg, J. R., Ackrell, B. A., and Brand, M. D. (2012). Mitochondrial complex II can generate reactive oxygen

species at high rates in both the forward and reverse reactions. *The Journal of biological chemistry* 287, 27255–64.

Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M., and Cayota, A. (2001). Unraveling peroxynitrite formation in biological systems. *Free radical biology & medicine* 30, 463–88.

Rafikova, O., Rafikov, R., and Nudler, E. (2002). Catalysis of S-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proceedings of the National Academy of Sciences of the United States of America* 99, 5913–8.

Romero, N., Denicola, A., Souza, J. M., and Radi, R. (1999). Diffusion of peroxynitrite in the presence of carbon dioxide. *Archives of biochemistry and biophysics* 368, 23–30.

Rooijakkers, Suzan H. M., Suzanne L. Rasmussen, Shauna M. McGillivray, Thomas B. Bartnikas, Anne B. Mason, Arthur M. Friedlander, and Victor Nizet. (2010). Human Transferrin Confers Serum Resistance Against Bacillus Anthracis. *Journal of Biological Chemistry* 36, 27609–27613.

Russell, B. H., Liu, Q., Jenkins, S. A., Tuvim, M. J., Dickey, B. F., and Xu, Y. (2008). In vivo demonstration and quantification of intracellular Bacillus anthracis in lung epithelial cells. *Infection and immunity* 76, 3975–83.

Shatalin, K., Gusarov, I., Avetissova, E., Shatalina, Y., McQuade, L. E., Lippard, S. J., and Nudler, E. (2008). Bacillus anthracis-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 105, 1009–13.

Shirai, K., Okada, T., Konishi, K., Murata, H., Akashi, S., Sugawara, F., Watanabe, N., and Arai, T. (2012). Bicarbonate plays a critical role in the generation of cytotoxicity during SIN-1 decomposition in culture medium. *Oxidative medicine and cellular longevity* 2012, 326731.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proceedings of the National Academy of Sciences of the United States of America* 89, 444–448.

Standard electrode potential (data page) - Wikipedia, the free encyclopedia Available at: http://en.wikipedia.org/wiki/Standard_electrode_potential_(data_page) [Accessed December 25, 2012].

Sturzbecher, M., Kissner, R., Nauser, T., and Koppenol, W. H. (2007). Homolysis of the peroxynitrite anion detected with permanganate. *Inorganic chemistry* 46, 10655–8.

Sudhamsu, J., and Crane, B. R. (2009). Bacterial nitric oxide synthases: what are they good for? *Trends in microbiology* 17, 212–8.

Surmeli, N. B., Litterman, N. K., Miller, A.-F., and Groves, J. T. (2010). Peroxynitrite Mediates Active Site Tyrosine Nitration in Manganese Superoxide Dismutase. Evidence of a Role for the Carbonate Radical Anion. *Journal of the American Chemical Society*.

Towne, V., Will, M., Oswald, B., and Zhao, Q. (2004). Complexities in horseradish peroxidase-catalyzed oxidation of dihydroxyphenoxazine derivatives: appropriate ranges for pH values and hydrogen peroxide concentrations in quantitative analysis. *Analytical biochemistry* 334, 290–6.

Valez, V., Cassina, A., Batinic-Haberle, I., Kalyanaraman, B., Ferrer-Sueta, G., and Radi, R. (2012). Peroxynitrite formation in nitric oxide-exposed submitochondrial particles: Detection, oxidative damage and catalytic removal by Mn-porphyrins. *Archives of biochemistry and biophysics*.

Weiner, Z.P., Boyer, A.E., Gallegos-Candela, M., Cardani, A.N., Barr, J.R., et al. (2012). Debridement Increases Survival in a Mouse Model of Subcutaneous Anthrax. PLoS ONE 7(2): e30201. doi:10.1371/journal.pone.0030201

Weiner, Z.P. and Glomski, I.J. (2012). Updating persspectives on the initation of *Bacillus anthracis* growth and dissemination through its host. Infection and Immunity 80, 1626-1633.

Wink, D. A., Darbyshire, J. F., Nims, R. W., Saavedra, J. E., and Ford, P. C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. *Chemical research in toxicology* 6, 23–7.

Wouters, M. A., Iismaa, S., Fan, S. W., and Haworth, N. L. (2011). Thiol-based redox signalling: rust never sleeps. *The international journal of biochemistry & cell biology* 43, 1079–85.

Wrona, M., Patel, K., and Wardman, P. (2005). Reactivity of 2',7'-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms toward carbonate, nitrogen dioxide, and hydroxyl radicals. *Free radical biology & medicine* 38, 262–70.

Yasuhara, T., and Nokihara, K. (1998). Quantitative determination of biological sulfhydryl groups by postcolumn derivatization and elucidation of microheterogeneity of serum albumins. *Analytical chemistry* 70, 3505–9.

Zhang, H., Joseph, J., Feix, J., Hogg, N., and Kalyanaraman, B. (2001). Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: comparison with nitration and oxidation of tyrosine by peroxynitrite in aqueous solution. *Biochemistry* 40, 7675–86.

Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R. P. (1997). A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Analytical biochemistry* 253, 162–8.

Zielonka, J., Zielonka, M., Sikora, A., Adamus, J., Joseph, J., Hardy, M., Ouari, O., Dranka, B. P., and Kalyanaraman, B. (2012). Global profiling of reactive oxygen and nitrogen species in biological systems: high-throughput real-time analyses. *The Journal of biological chemistry* 287, 2984–95.

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