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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