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***Bacillus Anthracis* Endospores Regulate Ornithine Decarboxylase and Inducible Nitric Oxide Synthase Through ERK1/2 and p38 Mitogen-Activated Protein Kinases**

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Abstract

Interactions between *Bacillus anthracis* (*B. anthracis*) and host cells are of particular interest given the implications of anthrax as a biological weapon. Inhaled *B. anthracis* endospores encounter alveolar macrophages as the first line of defense in the innate immune response. Yet, the consequences of this interaction remain unclear. We have demonstrated that *B. anthracis* uses arginase, inherent in the endospores, to reduce the ability of macrophages to produce nitric oxide (*NO) from inducible nitric oxide synthase (NOS2) by competing for L-arginine, producing L-ornithine at the expense of *NO. In the current study, we used genetically engineered *B. anthracis* endospores to evaluate the contribution of germination and the lethal toxin (LT) in mediating signaling pathways responsible for the induction of NOS2 and ornithine decarboxylase (ODC), which is the rate-limiting enzyme in the conversion of L-ornithine into polyamines. We found that induction of NOS2 and ODC expression in macrophages exposed to *B. anthracis* occurs through the activation of p38 and ERK1/2 MAP kinases, respectively. Optimal induction of NOS2 was observed following exposure to germination-competent endospores, whereas ODC induction occurred irrespective of the endospores' germination capabilities and was more prominent in macrophages exposed to endospores lacking LT. Our findings suggest that activation of kinase signaling cascades that determine macrophage defense responses against *B. anthracis* infection occurs through distinct mechanisms.

Introduction

Bacillus anthracis (*B. anthracis*) is a spore-forming Gram-positive bacterium that is the etiologic agent of anthrax [18]. Upon entering the body through a number of possible routes, endospores are phagocytosed by host macrophages. Therein, endospores evade host defenses, germinate into the vegetative form of bacteria eventually causing characteristic anthrax toxicity [2]. Germination is prerequisite to replication, toxin formation, and the subsequent dissemination of the bacterium. Production of virulence factors, including exotoxins, is associated with disseminated infection, which can lead to septicemia, toxemia, and death.

Recently, we reported that macrophages, upon phagocytizing *B. anthracis* endospores, up-regulate the inducible isoform of nitric oxide synthase (NOS2), which oxidizes L-arginine to nitric oxide (*NO) and this free radical is an important factor in controlling infection by the host [16]. The bacterium also expresses an active arginase [19], which, we have demonstrated, competes with NOS2 for L-arginine, thereby evading host defenses [16]. In so doing, these opposing enzymatic pathways dictate the fate of *B. anthracis*. With arginase activity found in both the exosporium and the bacterium, protection from macrophage-mediated killing of *B. anthracis* by decreasing the levels of *NO has been observed [23]. L-arginine is a key player in the activation of T cells, and its depletion by an arginase in *H. pylori* has been shown to result in T-cell dysfunction [26]. However, other than what is described above, little is known about the fate of L-arginine when encountered by *B. anthracis*.

The germinated bacterium expresses lethal toxin (LT), which is the central effector of *B. anthracis*-mediated toxicity and has a potential negative effect on the ornithine decarboxylase (ODC) pathway [4]. Lethal toxin (LT) is composed of protective antigen (PA) and lethal factor (LF), which contains a protease that cleaves peptide hormones and mitogen-activated protein (MAP) kinase pathway proteins [6,12]. LF cleavage of MAP kinase kinase (MKK) proteins

prevents activation of MAP kinases, such as ERK1/2 and p38 isoforms. The MAP kinases are essential for induction of the oxidative burst and pro-inflammatory cytokine expression. Therefore, disruption of MAP kinases by LT neutralizes macrophage activation, favoring bacterial escape from lymph nodes during the initial phase of infection [2]. While exogenous addition of LT disrupts MAP kinase signaling in macrophages, it is unknown whether disruption of MAP kinase signaling holds true in the presence of *B. anthracis* endospores.

In the current study, we used *B. anthracis* endospores that were genetically engineered to be germination deficient or lack the expression of LT and edema toxin (ET) to determine the mechanisms for induction of NOS2 and ODC in the context of *B. anthracis* infection. We show that *B. anthracis* induction of NOS2 and ODC in RAW 264.7 macrophages is mediated through p38 MAP kinase and ERK proteins, respectively, and that maximal induction occurs in response to endospores that are capable of germinating but lack the toxins involved in virulence.

Materials and Methods

Reagents

U0126 and SB203580 were purchased from Calbiochem (La Jolla, CA). *cis*-3,4-Di-(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (PROXYL 1) was prepared as described [21]. The antibody against phosphorylated p38 MAP kinase was purchased from Cell Signaling Technology (Danvers, MA). α -Difluoromethylornithine (DFMO), 3-[2(2-maleimidoethoxy)ethylcarbamoyl]-pyrrolidinyloxyl (PROXYL 2), cytochalasin D and antibodies for phosphorylated ERK1/2, ODC, and α -tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were purchased from Life Technologies (Grand Island, NY). The NOS2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Catalog no. TIB-71, Manassas, VA) and used up to passage 10 for all experiments. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μ g/ml), and 1 mM sodium pyruvate (complete medium) at 37°C in a humidified 5% CO₂ [9]. For bacterial inoculation, medium was replaced with complete medium with or without *B. anthracis* endospores at a MOI of 1:1. After 3, 6, or 12 h, the cells were analyzed for nitrite or ODC activities, polyamine production, and protein expression. Cell viability was monitored by Trypan blue dye exclusion after 12 h incubation with media with antibiotics and the various endospores.

B. anthracis Endospores

Endospores used include; Sterne 34F2, Sterne 34F2 Δ T (SdT), and the Δ *gerH* (the congenic *gerH*-null) strain, constructed from Sterne 34F2 [24]. The Sterne 34F2 lacks plasmid, pX02, which encodes a peptide-based capsule, but retains a second virulence plasmid called pX01, which encodes LT and ET. The congenic *gerHA*-null strain was constructed from the Sterne 34F2 strain using a deletion construct in *gerHA* containing an Erm resistance cassette [25]. This strain retains pX01 but is unable to germinate, which is needed for LT and ET production. The germination-competent SdT strain is derived from Sterne 34F2 and was cured of both pX01 and pX02 plasmids [14]; provided by Drs. Leppla and Pomerantsev (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). Endospores were prepared from the *B. anthracis* strains, as previously described [1]. Viable endospore titer was determined by dilution plating before and after heat killing (65°C for 30 min) of vegetative cells.

Phagocytosis of Endospores

Macrophages were incubated with $\Delta gerH$, SdT, and Sterne 34F2 endospores at a MOI of 1:1. For detecting endospore phagocytosis by macrophages, EPR spectroscopy was used as previously described [21]. PROXYL 1-labeled $\Delta gerH$ or Sterne 34F2 endospores and PROXYL 2-labeled SdT endospore (5×10^6 /ml) were prepared as reported [21] and added to macrophages for 12 h at 37°C. Phagocytosis was confirmed by pre-incubating cells with cytochalasin D (10 μ g/ml), which eliminated the PROXYL 1- or PROXYL 2 EPR spectra.

Nitrite Measurement

Estimates of *NO concentration during the course of *B. anthracis* infection of macrophages were determined by measuring nitrite levels in the media at various times after treatment by the Griess reaction assay as previously described [16].

Measurement of ODC Activity

ODC activity was determined by measuring the amount of $^{14}\text{CO}_2$ liberated from [^{14}C]ornithine as previously described [22] with modifications. Macrophages were washed with cold PBS and scraped into 20 mM Tris-HCl buffer (pH 7.4) containing 0.05 mM EDTA, 0.05 mM pyridoxal 5' phosphate, and 5 mM dithiothreitol. After three freeze/thaw cycles, cells were sonicated, centrifuged, and lysates were added to tubes with a septum cap containing 2.3 nmol of [^{14}C] ornithine (57.1 mCi/mmol) at 37°C for 15 min. Controls contained an equivalent volume of deionized water instead of lysates. The reaction was stopped with 10% trichloroacetic acid and liberated [^{14}C]CO $_2$ was trapped on a piece of filter paper previously impregnated with 2 N NaOH in the center well above the reaction mixture. Radioactivity was measured by scintillation counting. Protein content of the cell lysates was determined using the BCA Protein Assay (Pierce). ODC activity was expressed as pmoles of CO $_2$ produced h $^{-1}$ mg protein $^{-1}$.

Polyamine Measurements

Polyamine levels in *B. anthracis*-infected macrophage were analyzed by HPLC following a dansyl derivatization as previously reported [10] with modifications. Macrophages were washed with PBS followed by the addition of 5% perchloric acid and three freeze/thaw cycles. Samples were centrifuged and supernatant plus internal standard (10 nmole of 1,7-diaminoheptane) were mixed with saturated sodium bicarbonate and of dansyl chloride (20 mg/ml in acetone). The tubes were capped, vortexed, and incubated at 60°C for 60 min. *l*-alanine (100 mg/ml) was added to react with excess dansyl chloride and incubated for 30 min. The acetone was evaporated and toluene was added followed by vortexing and centrifugation. The organic phase was evaporated under N $_2$ and methanol was added to dissolve the polyamines. Polyamines were determined by a liquid chromatographic system (Waters 2695 Separation Module and Waters 2475 Multi λ Fluorescence Detector with excitation and emission at 314 and 515 nm, respectively). Polyamines were separated by Symmetry C18 (Waters, 5 μ m, 4.6 mm $\times$ 100 mm) using gradient elution with the following solvent systems: Solvent A—acetonitrile/water/glacial acetic acid/triethylamine (80/20/0.02/0.001), Solvent B—acetonitrile/water/glacial acetic acid/triethylamine (95/5/0.02/0.05) and Solvent C—acetonitrile/water (70/30).

Immunoblot Analysis

Protein lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted as described [16]. Proteins were detected using enhanced chemiluminescence reagent (PerkinElmer Life-Sciences).

Statistics

Statistical significance between treatment groups ($P < 0.05$) was done by analysis of variance (ANOVA).

Results and Discussion

Previously, we showed that expression of NOS2 and subsequent production of NO was induced in RAW 264.7 macrophages or freshly isolated murine macrophages exposed to *B. anthracis* [16,23]. Herein, we sought to further evaluate the consequences of NO production following *B. anthracis* infection using the Sterne 34F2, SdT, and ΔgerH mutant strains that are deficient in germination and/or production of LT or ET. First, we determined whether each of the endospores was phagocytosed by macrophages. Complete media containing penicillin and streptomycin were used and washed before experiments were conducted. As such, we could follow the cell response to up-take of endospores, as only the vegetative bacilli are sensitive to antibiotics. Cell viability, using Trypan blue exclusion, was $>92\%$ in control and endospore-treated cells. PROXYL 1-labeled (for ΔgerH and Sterne 34F2) or PROXYL 2-labeled (for SdT) endospores were incubated with macrophages. As earlier reported [21], each endospore was phagocytosed by RAW 264.7 cells and this was inhibited by cytochalasin D suggesting an actin-driven process (data not shown).

We then incubated macrophages with each of the endospore strains at a MOI of 1:1 for up to 12 h, using media containing antibiotics. At various time points, nitrite levels in media, which reflected NO production, were estimated. Exposure to all three strains of endospores resulted in NO generation, with a robust increase in NO between 6 and 12 h (Fig. 1a). Macrophages infected with SdT endospores, which lack LT, showed the highest NO levels. This result may be due to the inability of this strain to inhibit the MAP kinase signaling pathways.

Arginase catalyzes the metabolism of L-arginine to L-ornithine. Subsequent decarboxylation by ornithine decarboxylase (ODC) affords putrescine, which upon addition of aminopropyl groups, form spermidine and spermine, respectively. These polyamines appear to be essential for cell proliferation and differentiation [11,13]. Ornithine decarboxylase activity and polyamine production were determined in macrophages infected by endospores. As expected ODC activity was elevated at the 3 h time point following exposure to SdT endospores (Fig. 1b), suggesting that ODC activity is negatively regulated by LT. ODC activity was dramatically reduced by either α -difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, or L-arginine-depleted medium. These findings confirm the importance of L-arginine as the preferred substrate (data not shown).

Based on data with *H. pylori* infected RAW 264.7 cells [5], we expected that an increase in ODC activity would lead to elevated production of spermidine and spermine. Putrescine levels increased over time with the highest level achieved with SdT (LT/ET negative) endospores (Fig. 1c). Likewise, Sterne 34F2 (LT/ET positive) endospores enhanced putrescine levels, but it was delayed compared to SdT endospores (Fig. 1c). Macrophages exposed to ΔgerH (germination negative) endospores had the smallest elevation in putrescine levels (Fig. 1c). In contrast, concentrations of spermine (Fig. 1d) and spermidine (data not shown) were unchanged over time. This suggests that while ODC activity in RAW 264.7 cells exposed to endospores were enhanced, transferase enzymes required for addition of aminopropyl to form spermidine and spermine were not active or were inhibited.

The role of NO and ODC activation on ERK1/2 and p38 α MAP kinases, important mediators of cellular responses to infection, were examined. Macrophages exposed to either SdT or Sterne 34F2 endospores showed an increase in p38 α after 3 h exposure that further increased after 12 h, as compared to ΔgerH endospores (Fig. 2a). This response was transient and declined to

basal levels after 18–24 h. In contrast, p38 α activation was observed after macrophage exposure to $\Delta gerH$ endospores for 18–24 h, suggesting a delayed response (data not shown). These findings demonstrate the importance of germination for activating this kinase at early time points. The activation of ERK1/2 was also observed following exposure to SdT and Sterne 34F2 endospores as compared to $\Delta gerH$ endospores (Fig. 2a). As with p38 α , peak activation occurred after 12 h exposure followed by a decline to basal levels after 18–24 h exposure (data not shown). It is unclear why macrophages exposed to Sterne 34F2 endospores, containing LT, would cause what appears to be a more robust activation of ERK1/2 and p38 α MAP kinases as compared to SdT endospores (Fig. 2a). Nonetheless, the observation that activation of p38 α and ERK MAP kinases was more robust in macrophages infected with Sterne 34F2 or SdT endospores as compared to $\Delta gerH$ suggested that the ability to germinate is an essential trigger for activating signaling pathways involved in host response.

The requirement for the p38 and ERK1/2 MAP kinase pathways in mediating $\bullet$ NO production from NOS2 or ODC activation in response to endospores was examined using pharmacological inhibitors. Pretreatment of macrophages with SB203580, a p38 MAP kinase inhibitor, caused a significant reduction in $\bullet$ NO production, following exposure to SdT and Sterne 34F2 endospores, as compared to controls (Fig. 2b). In contrast, no effect of the inhibitor was noted when macrophages were exposed to $\Delta gerH$ endospores (Fig. 2b). Inhibition of ERK1/2 activation with the MEK1/2 inhibitor, U0126, resulted in enhanced production of $\bullet$ NO following exposure to all three endospores, as compared to controls (Fig. 2b). Whereas SB203580 had no significant effect on endospore-induced ODC activity, U0126 caused a dramatic reduction (Fig. 2c). The antagonism of ODC by ERK pathway inhibition may increase levels of L-arginine to be metabolized by NOS2. These findings could help explain the observed increase in $\bullet$ NO, following treatment with U0126 (Fig. 2b). These data support a mechanism by which p38 and ERK1/2 MAP kinases mediate $\bullet$ NO and ODC activation, respectively, in RAW 264.7 macrophages exposed to endospores.

NOS2 and ODC protein levels were examined in the presence of SB203580 and U0126. RAW 264.7 cells exposed to either SdT or Sterne 34F2 endospores showed the largest induction of NOS2 expression as compared to macrophages exposed to $\Delta gerH$ endospores (Fig. 2d). Pretreatment with the p38 MAP kinase inhibitor effectively attenuated the induction of NOS2 (Fig. 2d). In contrast, inhibition of ERK1/2 activation caused an increase in NOS2 expression that was most apparent in macrophages exposed to SdT or Sterne 34F2 endospores (Fig. 2d). These observations are consistent with the elevated levels of $\bullet$ NO observed following treatment with U0126 (Fig. 2b) and support a mechanism where p38 MAP kinase activation by germinant competent endospores regulates NOS2 induction. These findings also suggest that ERK1/2 is a negative regulator of endospore-induced NOS2.

Exposure of macrophages to each of the endospores increased ODC expression, although the highest induction of ODC was observed following exposure to SdT endospores (Fig. 2d). The lowest induction of ODC occurred in cells exposed to Sterne 34F2 endospores (Fig. 2d), which correlated with ODC activities (Fig. 1b, 2c). The induction of ODC expression was completely blocked in cells treated with U0126, but not affected in macrophages pretreated with SB203580 (Fig. 2d). These data support a role for ERK1/2, but not p38 MAP kinase, in regulating ODC induction in macrophages exposed to *B. anthracis* endospores.

In conclusion, infection of macrophages by *B. anthracis* endospores leads to an activation of kinase signaling cascades and cytokine production, an important element in host immune response [3,8]. Our data indicate that distinct MAP kinase signaling pathways are responsible for regulating the expression of NOS2 and ODC in host macrophages exposed to endospores. The functional significance of ERK-mediated ODC expression and activity in host-cell response to infection with endospores is unclear. While it was expected that polyamine

synthesis would be elevated, only the first polyamine in the pathway, putrescine, was shown to increase following exposure to endospores. The significance of the limited polyamine biosynthetic pathway and termination after putrescine synthesis may be related to reported inhibition of LF by spermine [7] or polyamine induction of macrophage apoptosis [17,20]. Thus, by limiting polyamine formation, the endospores prolong host-cell survival and the bacterium has more time to germinate and replicate within the macrophage, thus maximizing its chances of LF induction and establishing an infection. In support of this theory, Popov et al. observed that *B. anthracis*-infected mice treated with apoptosis inhibitors showed much higher bacterial numbers in their spleens compared to untreated controls [15]. Finally, our findings provide new insights into how competition for L-arginine by macrophages and endospores control cellular levels of this amino acid. Through competing metabolic pathways, bacterial arginase limits macrophage NOS2 and ODC activities and macrophages coordinate MAP kinase signaling events that control proteins involved in an initial host defense response to *B. anthracis* endospores.

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Fig. 1.

Induction of nitrite production and ODC activity in RAW 264.7 macrophages exposed to *B. anthracis* endospores. Macrophages were incubated with SdT, $\Delta gerH$, or Sterne 34F2 endospores at a MOI of 1:1 for 0–12 h. Media and cells were harvested at 0, 3, 6, and 12 h for analysis of nitrite production (**a**) or ODC activity (**b**). Open bars $\Delta gerH$; light gray bars SdT; dark gray bars Sterne 34F2 endospores. Putrescine (**c**) or spermine (**d**) levels from macrophages exposed to SdT, $\Delta gerH$, or Sterne 34F2 endospores for 0, 6, 12, 18, and 24 h. Circles $\Delta gerH$; closed squares SdT; open squares Sterne 34F2 endospores. Time 0 samples are macrophage cells in the absence of endospore exposure. Data represent the mean and standard deviation from 3–5 independent experiments. *denotes statistical significance as compared to the other endospore treatments at the same time point ($P < 0.05$)

Fig. 2.

Role of MAP kinases in mediating nitrite production and ODC activity in RAW 264.7 macrophages exposed to *B. anthracis* endospores. **a** Immunoblots for active p38α MAP kinase (p-p38, *top panel*), p44ERK1 and p42ERK2 (ppERK1/2, *middle panel*) from untreated (–) or macrophages exposed to SdT, Δ*gerH*, or Sterne 34F2 endospores for 0, 3, 6, or 12 h. α-tubulin was used for a protein loading control (*bottom panel*). The asterisk (*) indicates the position of active ERK2. Effects of MAP kinase inhibition on nitrite production (**b**) and ODC activation (**c**) induced by *B. anthracis* endospores. Macrophages were pre-treated for 10 min with or without SB230580 (20 μM) or U0126 (20 μM) and then exposed to Δ*gerH*, SdT, and Sterne 34F2 endospores for 12 h. Data represent the mean and standard error from 3–5 independent experiments. *Dark gray bars* endospores only; *light gray bars* endospores plus SB230580; *white bars* endospores plus U0126. *denotes statistical significance compared to untreated endospores ($P < 0.05$). **d** Immunoblots of NOS2 (*top panel*) or ODC (*middle panel*) expression in macrophages that were untreated (–) or exposed to SdT, Δ*gerH* or Sterne 34F2 endospores for 12 h in the absence or presence of U0126 (U) or SB230580 (SB) at the doses indicated in (**b**) and (**c**). α-tubulin expression is shown as a protein loading control. Data are representative of 3–4 independent experiments