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The poly-γ-D-glutamic acid capsule surrogate of the *Bacillus anthracis* capsule induces nitric oxide production via the platelet activating factor receptor signaling pathway

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ABSTRACT

The poly-γ-D-glutamic acid (PGA) capsule, a major virulence factor of *Bacillus anthracis*, confers protection of the bacillus from phagocytosis and allows its unimpeded growth in the host. PGA capsules released from *B. anthracis* are associated with lethal toxin in the blood of experimentally infected animals and enhance the cytotoxic effect of lethal toxin on macrophages. In addition, PGA capsule itself activates macrophages and dendritic cells to produce proinflammatory cytokine such as IL-1β, indicating multiple roles of PGA capsule in anthrax pathogenesis. Here we report that PGA capsule of *Bacillus licheniformis*, a surrogate of *B. anthracis* capsule, induces production of nitric oxide (NO) in RAW264.7 cells and bone marrow—derived macrophages. NO production was induced by PGA in a dose-dependent manner and was markedly reduced by inhibitors of inducible NO synthase (iNOS), suggesting iNOS-dependent production of NO. Induction of NO production by PGA was not observed in macrophages from TLR2-deficient mice and was also substantially inhibited in RAW264.7 cells by pretreatment of TLR2 blocking antibody. Subsequently, the downstream signaling events such as ERK, JNK and p38 of MAPK pathways as well as NF-κB activation were required for PGA-induced NO production. In addition, the induced NO production was significantly suppressed by treatment with antagonists of platelet activating factor receptor (PAFR) or PAFR siRNA, and mediated through PAFR/Jak2/STAT-1 signaling pathway. These findings suggest that PGA capsule induces NO production in macrophages by triggering both TLR2 and PAFR signaling pathways which lead to activation of NF-kB and STAT-1, respectively.

1. Introduction

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore-forming rod-shaped bacterium (Inglesby et al., 2002). Virulent strains of *B. anthracis* carry two plasmids, pXO1 and pXO2, which encode genes for the three major virulence factors: lethal toxin, edema toxin and the antiphagocytic capsule (Little and Ivins, 1999). These virulence factors play essential roles in pathogenesis and lethality of anthrax infection by suppressing host immunity, enabling the pathogen to proliferate to high number as well as directly damaging host vital systems such as the cardiovascular system and the liver (Liu et al., 2014).

B. anthracis capsule, synthesized by genes encoded in the pXO2 plasmid, is composed of poly-γ-D-glutamic acid (PGA) and protects the bacteria from phagocytosis by the immune cells (Leppla et al., 2002). During anthrax infection, capsule is released by *B. anthracis* into the blood circulation (Makino et al., 2002). High concentrations of PGA have been detected in the sera, and this has coincided with the emergence of bacteremia in a murine model of inhalation anthrax (Kozel et al., 2004; Sutherland et al., 2008). Capsule released in blood is associated with LT in experimentally infected animals at the terminal stages of anthrax infection (Ezzell et al., 2009). The PGA capsule has been shown to activate caspase-1 and induces the release of IL-1β from THP-1 cells that were differentiated into macrophages and from human monocyte-derived

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dendritic cells (DCs) (Cho et al., 2010). PGA also enhances the cytotoxic effect of LT on macrophages and increases mortality of mice challenged with LT (Jang et al., 2011). These reports suggest that the PGA capsule plays multiple roles in the pathogenesis of anthrax infection, including not only classical anti-phagocytic activity at the early stage of infection but also augmentation of LT-mediated cytotoxicity and induction of inflammatory cytokines that may contribute to systemic inflammation and sepsis at the terminal stage of infection.

Nitric oxide (NO) is a ubiquitous biological molecule produced by numerous cell types and has been implicated in various physiological and pathological processes exerting both detrimental and beneficial effects at the cellular and vascular levels (Nathan, 1992; Bogdan, 2001). Ample evidence supports that NO plays a critical role in the pathogenesis of septic shock (Chandra et al., 2006). High plasma levels of nitrite/nitrate, the stable end product of nitric oxide, were observed in septic patients (Ochoa et al., 1991). A massive release of NO in vascular smooth muscle causes profound vasodilation and hypotension in sepsis (Moncada and Higgs, 1993). Mice deficient in inducible nitric oxide synthase (iNOS) have been shown to be resistant to LPS-induced mortality (Wei et al., 1995) and vascular hypocontractility (Gunnett et al., 1998). Moreover, inhibitors of NO production have shown beneficial effects to patients with severe sepsis as well as to animals in experimental sepsis (Wu et al., 1996; Liaudet et al., 1997; Petros et al., 1994). Exposure of immune cells to various inflammatory cytokines, bacteria, or bacterial components such as LPS, lipoteichoic acid (LTA), and peptidoglycan generates NO production from L-arginine via inducible nitric oxide synthase (iNOS) expression (Bogdan, 2001).

Although ample evidence demonstrated potential roles of PGA especially anthrax infection, the involvement of the PGA capsule itself in the production of NO has not been studied yet. In this study, we investigated the effect of the PGA capsule on NO production in murine macrophage RAW264.7 cells and the intracellular signaling pathway involved. We also identified the cellular receptors that are involved in PGA-induced NO production in macrophages. Our experimental results demonstrate the mechanisms by which PGA stimulates NO production.

2. Materials and methods

2.1. Reagents and chemicals

N-ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), *N*-ω-nitro-D-arginine methyl ester hydrochloride (D-NAME), ultrapure LPS from *Escherichia coli*, and Parthenolide were obtained from Sigma–Aldrich (St. Louis, MO). 2-Iminopiperidine hydrochloride (2-IPD) was purchased from Santa Cruz Biotechnology (Dallas, TX). Janus kinase 2 protein (JAK2) inhibitor (AG490), p38 MAP kinase inhibitor (SB202190), and ERK inhibitor (PD98059) were purchased from A.G. Scientific, Inc. (San Diego, CA). JNK Inhibitor V (JNKV) was purchased from Calbiochem (Darmstadt, Germany). Platelet activating factor receptor (PAFR) inhibitors (CV6209 and CV3988) were purchased from Enzo Life Sciences (Farmingdale, NY). Purified mouse anti-iNOS/NOS TYPE II was purchased from BD Transduction Laboratories (San Diego, CA). Rabbit polyclonal antibodies specific for IκB-α, β-actin, p38, ERK1/2, SAPK/JNK, STAT-1 (Ser727, Tyr701), and phosphorylated forms of p38, ERK1/2, SAPK/JNK and STAT-1 (Ser727, Tyr701) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibody specific for PAFR (H-300) was obtained from Santa Cruz Biotechnology (Dallas, TX). Horseradish peroxidase–conjugated anti–rabbit IgG and anti–mouse IgG were purchased from Sigma–Aldrich. All the reagents for reverse transcription (RT)-PCR and PAFR small interfering RNA (siRNA) were purchased from Promega (Madison, WI) and Dharmacon (Lafayette, CO), respectively. CpG oligodeoxynucleotides (ODN) 2395, purified monoclonal IgG antibody to mouse TLR2, and mouse IgG2a isotype control were obtained from Invivogen (San Diego, CA). Murine macrophage RAW264.7 cells and *Staphylococcus aureus* lipoteichoic acid (LTA) were kindly provided by Dr. Han of Seoul National University (Seoul, Republic of Korea).

Bacillus licheniformis ATCC 9945a was grown in E medium and PGA purification from the culture supernatant was performed as described (Jang et al., 2011). Usually under these culture condition, *B. licheniformis* 9945a has been reported to produce PGA with between 80% and 90% D-glutamate repeat units (Birrer et al., 1994). As the molecular mass of purified PGA was ~500 kDa, PGA was fragmented with acid hydrolysis, and PGA with molecular mass between 50 and 100 kDa was used in this experiment (Jang et al., 2011). Before use, endotoxin level in the purified PGA was measured using a *Limulus* amebocyte lysate assay kit (Lonza, Walkersville, MN, USA). According to this assay, 100 μg/ml PGA contained <0.1 EU/ml. For the purity of PGA from *B. licheniformis*, additional analysis was performed by ^{1}H nuclear magnetic resonance spectroscopy (NMR). Purified PGA with ^{1}H NMR showed standard peak patterns for γ-D-PGA (data not shown).

2.2. RAW264.7 cell culture

The mouse macrophage cell line RAW264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% (v/v) penicillin (100 U/ml)-streptomycin (100 μg/ml) (Invitrogen) at 37 °C in a 5% humidified incubator. Cells were plated in culture plates and incubated with PGA and/or various reagents as described for each experiment. At the end of stimulation, the accumulated nitrite in culture supernatants was quantified.

2.3. Preparation of mouse bone marrow–derived macrophages (BMDMs)

Six-week-old female C57BL/6 wild-type and TLR2-deficient mice were purchased from The Jackson Laboratory (Sacramento, CA). Mouse bone marrow cells were prepared as described (Park et al., 2013). In brief, bone marrow cells were isolated from tibiae and femora and placed in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% (v/v) penicillin (100 U/ml)-streptomycin (100 μg/ml) (Invitrogen). Subsequently, red blood cells were removed using red blood cell lysing buffer (Sigma–Aldrich). To differentiate the bone marrow cells into macrophages, the cells were cultured in complete DMEM in the presence of 30% (v/v) supernatant from a culture of mouse fibroblast cell line L929 and 50 μM 2-mercaptoethanol (Invitrogen) for 6–8 days. The animal study protocol (KCDC-007-13-1A) was approved by the Institutional Animal Care and Use Committee of the Korea National Institute of Health. Animals were housed in specific pathogen-free facilities until bone marrow cells were isolated. BMDMs were harvested, resuspended, and plated in culture plates and stimulated with PGA as indicated for each experiment. At the end of stimulation, culture supernatant was harvested and analyzed for NO production.

2.4. Determination of NO production

Nitrite accumulation was measured as an indicator of NO production in the culture medium using the Griess Reagent System (Promega) following the manufacturer's protocol. Briefly, the cell culture supernatants were obtained, and an equal volume of 1% sulfanilamide was added to the supernatants. After 5–10 min of incubation, an equal volume of 0.1% *N*-1-napthylethylenediamine