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Superoxide dismutase of *Streptococcus suis* serotype 2 plays a role in anti-autophagic response by scavenging reactive oxygen species in infected macrophages

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ABSTRACT

Streptococcus suis serotype 2 (SS2) causes septic shock and meningitis. However, its pathogenesis is still not well-understood. We have recently shown that superoxide dismutase sodA of SS2 is a virulence factor probably by increasing resistance to oxidative stresses. Reactive oxygen species (ROS) are products of the respiratory burst of phagocytic cells and have been shown to activate autophagy. We wanted to know if and how SS2 explores its sodA to interfere with cell autophagic responses. A *sodA* deletion mutant (Δsod) was compared with its parent and complemented strain in autophagic response in the murine macrophage cell line RAW264.7. We found that the Δsod mutant induced significant autophagic responses in infected cells, shown as increased LC3 lipidation (LC3-II) and EGFP-LC3 punctae, than those infected by its parent or complemented strain at 1 or 2 h post-infection. Co-localization of the autophagosomal EGFP-LC3 vesicles with lysosomes was seen in cells infected with Δsod mutant and its parent strain, indicating that SS2 infection induced complete autophagic responses. Reduced autophagic responses of cells infected with the wild-type strain might be related to decreased ROS by the scavenging effect of its sodA, as shown by increased superoxide anion or ROS level in cells infected with the Δsod mutant and in the cell free xanthine oxidase–hypoxanthine ROS-generating system, as compared with its parent or complemented strain. Taken together, SS2 makes use of its sodA for survival not only by scavenging ROS but also by alleviating the host autophagic responses due to ROS stimulation.
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1. Introduction

Streptococcus suis serotype 2 (SS2) is the causative agent of several forms of illness of pigs, such as arthritis, endocarditis, meningitis and septicemia. It is also recognized as a zoonotic agent causing human meningitis (Han et al., 2001; Tang et al., 2011) and severe toxic shock syndrome. Virulence factors of SS2 so far defined include the antiphagocytic capsular polysaccharide, suilysin, cell wall-associated and extracellular proteins, fibronectin-binding proteins, serum opacity factor, *etc.* (Feng et al., 2014). However, its pathogenesis is still poorly understood.

Once in the blood stream, SS2 faces phagocytosis by neutrophils and macrophages, the first line of host defense (Wu et al., 2014). Phagocytosis-associated respiratory

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burst would generate antimicrobial reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical OH^{3-} (Ambrozova et al., 2010). The superoxide anion O_2^- is known to be the major ROS that regulates autophagy, another cellular process related to microbial infection (Scherz-Shouval and Elazar, 2011). During bacterial infection, autophagy potentially captures bacteria that have escaped from phagosomes into the cytoplasm, thereby delivering the bacteria into autophagolysosomes where they are destroyed (Campoy and Colombo, 2009). Most studies of bacterial autophagy used intracellular bacteria. Only intracellular bacteria or their products are processed by autophagy (Campoy and Colombo, 2009; Tang et al., 2014). Some pathogens, once inside the autophagosomes, could modify this compartment to establish an environment necessary for its survival (Mostowy, 2013). On the other hand, some bacterial species encode antioxidant enzymes such as superoxide dismutase and/or catalase to cope with ROS from the host for their survival (Iiyama et al., 2007).

We have recently demonstrated that the superoxide dismutase (sodA) of SS2 is functional in anti-oxidative stresses by phagocytes (Tang et al., 2012b). However, it remains unknown if autophagy is part of SS2 pathogenesis, and if sodA is explored as an anti-autophagic mechanism by reducing the superoxide anion. Here, we reveal that infection with the Δsod mutant triggered more pronounced autophagic response than its parent or complemented strain during the first two hours of infection, suggesting that sodA of SS2 was functional as an anti-autophagic factor. Since there was more intracellular O_2^- in macrophages infected with Δsod mutant than the wild-type strain and purified sodA of SS2 could scavenge ROS produced *in vitro* by the hypoxanthine–xanthine system, we conclude that scavenging superoxide anions by sodA contributed to the anti-autophagic response of *S. suis* type 2.

2. Materials and methods

2.1. Bacterial strains and plasmids

S. suis type 2 strain ZJ081101 was a clinical strain from the lung of a diseased pig. Unless otherwise indicated, SS2 strains were grown in Brain Heart Infusion (BHI, Oxoid, UK) at 37 °C and *E. coli* strains, in Luria-Bertani (LB) broth or LB agar at 37 °C. The *sodA* deletion mutant (SS2-Δsod) and complemented strain (SS2-cΔsod) carrying the expression plasmid pSET2s::sodA were constructed in our laboratory (Tang et al., 2012). Antibiotics (all from Sigma) were added, where necessary, to the culture media at the following concentrations: chloramphenicol (Cm) at 4 μg/ml for SS2 Δsod mutant and complemented strain. *E. coli* strains DH5α and BL21 were used for general manipulation of plasmids and prokaryotic expression of SS2 sodA. The recombinant eukaryotic expression vectors pcDNA-egfp and pcDNA-egfp-LC3B were constructed earlier in our laboratory (Zhu et al., 2012).

2.2. Expression and purification of recombinant SS2-sodA

The ORF of *sodA* was amplified from the genome DNA of SS2 strain ZJ081101 by PCR with the primer pairs: sod-EcoRI: CCGGAATTCATGACAAT TATTTTACCAGACCTTCCA and sod-SalI: CGCGTCGACTTTAGCTGCTTTAT AAAGTTCGTTAACC. The fragment was cloned into pET-30a (Invitrogen) using the *Eco*RI and *Sal*I sites. The recombinant plasmid pET-sodA was transformed into *E. coli* BL21 (DE3). Expression of recombinant protein (rSS2-sodA) was induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG, 1 mM/L) to the log phase culture at 37 °C for 4 h. The his-tagged rSS2-sodA was purified using HisTrap columns (GE Healthcare) according to the manufacturer instruction and concentrated by membrane ultrafiltration (Millipore) and stored at −80 °C till use.

2.3. Cells culture and bacterial infection

Infection of RAW264.7 macrophages cells or cells stably expressing EGFP-LC3B (Zhu et al., 2012) with SS2 strains followed the procedures according to an earlier paper (Cybulski et al., 2009). Briefly, the cells were grown in the 1640 medium containing 10% fetal calf serum (FCS, Gibco) in tissue culture plates at 37 °C and 5% CO_2. The bacterial strains (wild-type, Δsod mutant or complemented strain) were grown to logarithmic phase in BHI for 6 h at 37 °C, harvested by centrifugation, washed once and resuspended in sterile 10 mM PBS, pH 7.2. The confluent cell monolayers were infected at MOI (multiplicity of infection) of about 100:1 for 1 and/or 2 h (or 3 h in the initial experiments) at 37 °C and 5% CO_2, and washed twice with PBS before further analysis as described below. For confocal microscopic detection of autophagosome formation, the macrophage cells stably expressing EGFP-LC3B were cultured in a petri dish containing a coverslip (10 mm in diameter) and confluent monolayers were infected with wild-type, Δsod mutant or complemented strain (about 100:1 MOI). The cells mock-infected and rapamycin (0.5 μM, Millipore) pretreated for 8 h were used as negative and positive controls.

For analysis of co-localization between GFP-LC3 punctae and lysosomes, infection of EGFP-LC3B-expressing macrophages with the wild-type strain or its Δsod mutant was the same as above. Positive and negative controls were included by treating the uninfected cells with Rapamycin and 3-methyladenine (3-MA) (both from Sigma). 3-MA is able to block the fusion of autophagosomes and lysosomes (Petiot et al., 2000). Lysotracker (Invitrogen) was used to label the lysosomes for confocal microscopy.

2.4. SDS-PAGE and immunoblotting

The infected cells after washing were lysed for 10 min in ice-cold lysis buffer (50 mM Tris–HCl pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40) with complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell debris was pelleted by centrifugation and clear supernatants transferred to new tubes. Protein concentration was measured by BCA protein assay kit (MultiSciences Hangzhou, China) and the protein samples were either used directly for SDS-PAGE or stored at −80 °C till use. Protein samples were mixed with 5 × SDS-PAGE loading buffer and boiled for 5 min. Equal amounts of

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