Salmonella enterica serovar Typhimurium sseK3 induces apoptosis and enhances glycolysis in macrophages

Chuan Yu1,2,3†, Fuyu Du1,2†, Chunjie Zhang1,2*, Yinju Li1,2, Chengshui Liao1,2, Lei He1,2, Xiangchao Cheng1,2,3 and Xiaojie Zhang1,2

Abstract
Background: Salmonella enterica serovar Typhimurium (S. Typhimurium) is an important infectious disease pathogen that can survive and replicate in macrophages. Glycolysis is essential for immune responses against S. Typhimurium infection in macrophages, and is also associated with apoptosis. S. Typhimurium secreted effector K3 (SseK3) was recently identified as a novel translated and secreted protein. However, there is no study about the role of sseK3 in the relationship between apoptosis and glycolysis in cells infected with S. Typhimurium. It is unclear whether this protein exerts a significant role in the progress of apoptosis and glycolysis in S. Typhimurium-infected macrophages.

Results: Macrophages were infected with S. Typhimurium SL1344 wild-type (WT), ΔsseK3 mutant or sseK3-complemented strain, and the effects of sseK3 on apoptosis and glycolysis were determined. The adherence and invasion in the ΔsseK3 mutant group were similar to that in the WT and sseK3-complemented groups, indicating that SseK3 was not essential for the adherence and invasion of S. Typhimurium in macrophages. However, the percentage of apoptosis in the ΔsseK3 mutant group was much lower than that in the WT and sseK3-complemented groups. Caspase-3, caspase-8, and caspase-9 enzyme activity in the ΔsseK3 mutant group were significantly lower than in the WT group and sseK3-complemented groups, indicating that sseK3 could improve the caspase-3, caspase-8, and caspase-9 enzyme activity. We also found that there were no significant differences in pyruvic acid levels between the three groups, but the lactic acid level in the ΔsseK3 mutant group was much lower than that in the WT and sseK3-complemented groups. The ATP levels in the ΔsseK3 mutant group were remarkably higher than those in the WT and sseK3-complemented groups. These indicated that the sseK3 enhanced the level of glycolysis in macrophages infected by S. Typhimurium.

Conclusions: S. Typhimurium sseK3 is likely involved in promoting macrophage apoptosis and modulating glycolysis in macrophages. Our results could improve our understanding of the relationship between apoptosis and glycolysis in macrophages induced by S. Typhimurium sseK3.

Keywords: S. Typhimurium, sseK3, Macrophages apoptosis, Glycolysis

* Correspondence: cjzhang@sina.com
† Chuan Yu and Fuyu Du contributed equally to this work.
1 The Key Lab of Animal Disease and Public Health, Henan University of Science and Technology, 263 Kaiyuan Avenue, Luoyang 471023, Henan, China
2 Luoyang Key Laboratory of Live Carrier Biomaterial and Animal Disease Prevention and Control, Luoyang 471023, Henan, China
Full list of author information is available at the end of the article

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Background
Salmonella enterica serovar Typhimurium (S. Typhimurium) is a zoonotic pathogen that can infect humans [1]. Salmonella can be transmitted to humans via contaminated animal products, causing illness and potentially deaths [1]. S. Typhimurium can survive and replicate in macrophages, which could carry bacteria from the Peyer’s patches to adjacent lymph nodes, the spleen and the liver in mouse models [2].

Glycolysis is an essential cellular metabolic pathway [3]. Under anaerobic conditions, the pyruvate is eventually converted into lactic acid. However, under aerobic conditions, pyruvic acid enters the tricarboxylic acid cycle (TCA cycle) and is oxidized to CO₂ and H₂O [4]. Apoptosis is closely associated with glycolysis activities [5, 6]. Inhibition of the glycolysis with iodoacetate was associated with macrophage apoptosis [7], and genetic silencing of hypoxia inducible factor-1α (HIF-1α) repressed imiquimod-induced aerobic glycolysis and sensitized cells to imiquimod-induced apoptosis owing to faster ATP and Mcl-1 depletion [5]. Sirtuin 6 modulates hypoxia-induced apoptosis in osteoblasts via inhibition of glycolysis, and hypoxia-induced apoptosis of osteoblasts is dependent on glycolytic activity [8]. Several molecules that function intracellularly as enzymes are involved in glycolysis, and become externalized to the surface of apoptotic cells, causing apoptotic recognition and the triggering innate apoptotic immunity [9]. Thus, glycolysis is closely related to apoptosis.

Immune cells were able to detect the metabolic abnormalities caused by Salmonella through inflammatory signals, and glycolysis was essential for this process in S. Typhimurium-infected with macrophages [10, 11]. Salmonella pathogenicity island 2 (SPI-2)-encoded type III secretion system 2 (T3SS2), which delivers 28 effector proteins into the host cell, is one of the key virulence determinants after invasion or phagocytic uptake of Salmonella [12–14]. Previous studies have identified that SseK3 is a novel translated and secreted protein of S. Typhimurium, and is encoded by the sseK3 gene [15]. SseK3 is a glycosyltransferase, and could transfer an N-acetyl-glucosamine moiety onto the guanidino group of a target arginine, thereby regulating host cell function [16]. It belongs to the glycosyltransferase type-A family of glycosyltransferase enzymes and binds ligands in a metal-ion-dependent manner via a DXD motif [16]. SseK3 is co-regulated with the T3SS2 in host cells and is injected into infected host cells [17]. However, the mechanisms underlying SseK3 activity during S. Typhimurium infection and the role of sseK3 in macrophage glycolysis remain unclear.

In this study, we aimed to determine the role of S. Typhimurium sseK3 on macrophage apoptosis and glycolysis after S. Typhimurium infection. Our data showed that the sseK3 of S. Typhimurium could promote macrophage apoptosis and improve glycolysis levels. These results would provide a better understanding of the relationship between glycolysis and apoptosis in S. Typhimurium-infected macrophages.

Results
Adherence and invasion
The invasive and adhesive abilities of the sseK3 mutant and complemented strain were similar to those of the WT strain (Fig. S1A, Fig. S1B); there were no significant differences between the WT, ΔsseK3 mutant, and sseK3-complemented groups (P > 0.05). This suggests that sseK3 does not play a prominent role in promoting the attachment and invasion of S. Typhimurium into the macrophages. Because the same number of bacteria invaded macrophages of the different infection groups, the intracellular load was consistent, and improved the accuracy of subsequent experiments.

Apoptosis
Apoptosis assays were performed as previously described [18, 19]. The percentage of apoptotic cells of ΔsseK3 mutant group was much lower than that in the WT and sseK3-complemented groups. In the ΔsseK3 mutant group, the percentage of FITC-Annexin V positive cells was 3.78%, while in the WT group, it was 14.64% (Fig. 1), suggesting that SseK3 plays an important role in S. Typhimurium-induced macrophage apoptosis.

Caspase activity
Caspases play an essential role in apoptosis [20]. Caspase-3, 8, and 9 activity was therefore measured at different time points (Fig. 2), and was much lower in the mock group than in the infection groups (ΔsseK3 mutant, WT, and sseK3-complemented groups), indicating that S. Typhimurium infection could stimulate the activity of caspase-3, 8, and 9 in macrophages. However, it could be seen from Fig. 2 that caspase-8 and caspase-9 activity was significantly lower in the ΔsseK3 mutant group than that in the WT and sseK3-complemented groups at 2 h, 4 h, 6 h, and 8 h (P < 0.001), suggesting that SseK3 could induce the activation of caspase-8 and caspase-9. There was no significant difference in caspase-3 activity among the ΔsseK3 mutant, WT, and sseK3-complemented groups at 2 h (P > 0.05). However, significant differences in caspase-3 activity were observed among the infection groups at 4 h, 6 h, and 8 h (P < 0.001). Caspase-3 activity was significantly lower in the ΔsseK3 mutant group than in the WT and sseK3-complemented groups. These results illustrated that SseK3 likely played a pivotal role in the process of inducing macrophage apoptosis by S. Typhimurium.
Glycolysis
To determine whether SseK3 could affect glycolysis in macrophages infected with S. Typhimurium, pyruvic acid, lactic acid, and ATP levels were detected as previously described [21–23] (Fig. 3). The results showed that there were no significant differences in pyruvic acid levels between different groups at 2 h, 4 h, 6 h, and 8 h (\(P > 0.05\)). However, there were significant differences in lactic acid levels between the mock and infection groups at 2 h, 4 h, 6 h, and 8 h (\(P < 0.001\)). Macrophages glycolysis was significantly increased in the infection groups than in the mock group, and lactic acid levels were significantly lower in the ΔsseK3 mutant group than in the WT and sseK3-complemented groups at 4 h, 6 h, and 8 h (\(P < 0.001\)). These results illustrated that SseK3 encoded by sseK3 could improve glycolysis in macrophages by modulating lactic acid and the ATP levels.

Discussion
Many Salmonella secreted proteins exert different virulence functions in the process of bacterial survival and replication in host cells [24]. In Salmonella SCVs T3SS2 helps Salmonella transport virulence (effector) proteins into host cells [25]. S. Typhimurium expresses the SseK family of proteins, including SseK1, SseK2 and SseK3, which are T3SS effectors [1, 26]. SseK3 is essential for cellular Salmonella infection, and plays a pivotal role in the natural host immune process [15, 17]. Several secretory proteins of S. Typhimurium contribute to the considerable stress observed during apoptosis [24, 25, 27, 28]. There were several studies about the function of SseK3. Joshua et al. found that SseK3 targeted death domain proteins in the TNF and TRAIL signaling pathways and employed the glycosyltransferase effectors to antagonize different components of death receptor signaling [29]. Yang et al. found that SseK3 could bind an E3 ubiquitin ligase (TRIM32) and influence NF-κB activity [15]. But Günstner et al. found that SseK3 caused weak GlcNAcylation of TNFR1-associated death domain protein (TRADD) and inhibited TNF-α-induced apoptosis in Salmonella-infected macrophages [25]. These researches were contradictory. Nonetheless, there has been
Fig. 2 Caspase-3, caspase-8, and caspase-9 activity in RAW264.7 cells infected with WT S. Typhimurium, ΔsseK3 mutant, or sseK3-complemented strains. Caspase-3, −8, and −9 activity was measured by colorimetric assay and analyzed using two-way ANOVA with Bonferroni’s multiple-comparison test (*P < 0.05, **P < 0.01, ***P < 0.001). Results are shown as mean ± SD.

Fig. 3 Glycolysis in RAW264.7 cells infected with WT, ΔsseK3 mutant, or sseK3-complemented strains. The pyruvic acid, lactic acid, and ATP levels were measured by colorimetric assay and analyzed using two-way ANOVA with Bonferroni’s multiple-comparison test (*P < 0.05, **P < 0.01, ***P < 0.001). Results are shown as mean ± SD.
Apoptosis and glycolysis are closely related. Comin-Anduix et al. found that fermented wheat germ extract (FWGE) inhibited glycolysis and induced apoptosis in tumor cells [40]. Jeong et al. found that the modification of glycolysis could change the sensitivity of apoptosis via the mitochondrial pathway [41]. Recently, research has shown that inhibition of glycolysis could block cell apoptosis [42]. Methyl jasmonate could inhibit the glycolysis and induce apoptosis of tumor cells [43], whereas propofol could improve glycolysis of cells and cause the apoptosis [44]. Pyruvic acid, lactic acid and ATP are important components in the process of cellular glycolysis. Various metabolic pathways produce pyruvic acid [45]. Pyruvic acid could enter the TCA cycle and be completely oxidized in aerobic conditions, or become lactic acid under anaerobic conditions [46, 47]. HIF-1α could prevent the production of ATP and thus regulate the glycolysis of mouse granulosa cells [48]. We studied glycolysis in macrophages infected with WT, ΔsseK3 mutant, or sseK3-complemented by detecting the levels of pyruvic acid, lactic acid, and ATP. There were no significant differences in intracellular pyruvic acid levels among the groups (P > 0.05). Compared to the WT and sseK3-complemented groups, the lactic acid levels of macrophages infected by ΔsseK3 mutant strain did not significantly change at 2 h (P > 0.05). However, there were significant differences between the WT and ΔsseK3 mutant groups at 4 h (P < 0.001), 6 h (P < 0.001), and 8 h (P < 0.001), suggesting that the deletion of sseK3 may reduce S. Typhimurium-induced glycolysis in macrophages, and SseK3 likely enhances glycolysis. Furthermore, lactic acid levels were significantly higher in the WT, ΔsseK3 mutant, and sseK3-complemented strains, than in the mock group (P < 0.001), which illustrated that the anaerobic pathway was enhanced in macrophages of the infection groups. There was no significant difference in ATP levels among the groups at 2 h; however, the ATP levels were significantly higher in the ΔsseK3 mutant group than in the WT and sseK3-complemented groups at 4 h (P < 0.001), 6 h (P < 0.001), and 8 h (P < 0.001), indicating that glycolysis was lower in the ΔsseK3 mutant group, and SseK3 likely enhanced glycolysis. Moreover, the ATP levels of macrophages in the mock group were significantly higher than those in the infections groups at 4 h (P < 0.001), 6 h (P < 0.001), and 8 h (P < 0.001), which suggested that aerobic metabolism was predominant in the mock group, since less ATP is produced by glycolysis than by aerobic oxidation. Therefore, the deletion of sseK3 likely decreased glycolysis in the ΔsseK3 group compared with WT group, and SseK3 could improve glycolysis in macrophages infected by S. Typhimurium and thereby induce apoptosis. This conclusion was consistent with the results of Sumi et al'
[44] and Ding et al’ [32], showing that glycolysis and apoptosis of cells could be improved simultaneously. But it was not in agreement with the findings of Li et al’ [43] and Comin-Anduix et al’ [40], who showing that glycolysis of cells was inhibited and apoptosis of cells was enhanced, possible owing to differences in the type of cell. Glycolysis is the main source of energy in cancer cells, which use this metabolic pathway for ATP generation. Altered energy metabolism is a biochemical fingerprint of cancer cells, and is a “hallmark of cancer” [49]. Rosmarinic acid induces apoptosis in HepG2 cells, mainly via inactivation of the glycolytic pathway [50]. However, macrophages show a high level of plasticity, with the ability to undergo dynamic transition between M1 and M2 polarized phenotypes [51]. Lipopolysaccharide (LPS) of bacteria could induce RAW264.7 macrophages into the classical proinflammatory phenotype (M1) [52]. Activation of inflammatory responses could cause the apoptosis by mediating inactivation of the PI3K/Akt/GSK-3β signaling pathway [53]. Moreover, the proinflammatory stimulus, LPS, suppresses myelocytomatosis viral oncogene (Myc) expression and cell proliferation and engages a HIF1α-dependent transcriptional program responsible for heightened glycolysis [54]. We speculate that S. Typhimurium SseK3 might activate the M1 phenotype of macrophages and thereby activate HIF1-α, which enhances glycolysis and finally causes apoptosis. There could be several different signaling pathways involved in SseK3-mediated apoptosis and glycolysis of macrophages, which merit further study. We aim to further clarify the relationship between glycolysis and apoptosis in future studies.

Conclusions
Our results cumulatively showed that SseK3 of S. Typhimurium could induce apoptosis and improve glycolysis in macrophages. Our findings may help to illustrate the mechanism by which S. Typhimurium induces macrophage apoptosis and provide a better understanding of the putative relationship between SseK3-induced macrophage apoptosis and glycolysis.

Methods
Bacterial strains, cells, and culture
S. Typhimurium SL1344, S. Typhimurium SL1344 ΔsseK3 mutant (with deletion of sseK3), and sseK3-complemented bacterial strains used in this study were available in our laboratory. The ΔsseK3 mutant was constructed using counter-selectable suicide vectors. The sseK3 gene was cloned into the pBR322 plasmid for complementation studies. RAW264.7 macrophage cells were obtained from the American type culture collection (ATCC, Manassas, VA), and cultured in Dulbecco’s modified Eagle medium (DMEM)/high-glucose medium (HyClone, USA) containing 10% fetal calf serum (FCS) in an incubator at 37 °C and 5% CO2.

Adherence and invasion assay
Adhesion and invasion of RAW264.7 cells was assessed as previously described [55, 56]. A 24-well cell culture plate was inoculated with 1 × 10^6 RAW264.7 cells per well. The WT, ΔsseK3 mutant and sseK3-complemented strains were then added to RAW264.7 cells at a multiplicity of infection (MOI) of 100:1, with three replicate wells per strain. To allow complete contact between the bacteria and RAW264.7 cells, the plates were centrifuged at 1000 rpm and incubated in 5% CO2 for 2 h at 37 °C. For the adherence assay, the supernatants were aspirated, and the cells were washed three times with phosphate buffered saline (PBS). Subsequently, the cells were digested with 0.25% trypsin, plated in a gradient dilution and counted. For the invasion assay, the supernatants were aspirated, cells were washed three times with PBS, gentamicin-containing medium (100 μg/mL) was added, and the cells were incubated at 37 °C with 5% CO2. After incubation, the supernatants were aspirated, and the cells were washed three times with PBS. Subsequently, the cells were lysed using 0.1% Triton X-100, plated with a gradient dilution and counted.

Flow cytometry assay
A 6-well cell culture plate was inoculated with 1 × 10^6 RAW264.7 cells per well and incubated for 16 h. WT, ΔsseK3 mutant and sseK3-complemented strains were incubated with RAW264.7 cells at a multiplicity of infection (MOI) of 100:1, with three replicate wells per strain. To allow complete contact between bacteria and RAW264.7 cells, the plates were centrifuged at 1000 rpm. Gentamicin-containing medium (100 μg/mL) was then added and the plates incubated at 37 °C with 5% CO2. After incubation, the supernatants were aspirated, and the cells were washed three times with PBS. The percentage of cells undergoing apoptosis was detected by flow cytometry using an Annexin V-FITC/PI apoptosis detection kit (KeyGEN BioTECH, Jiangsu, China). Cells from the infected and mock groups were digested with 0.25% trypsin, washed three times with ice-cold PBS, and suspended in 500 μL binding buffer. 5 μL Annexin V-FITC and 5 μL Propidium Iodide (PI) were added, and the solution was incubated in a dark room for 15 min at room temperature and immediately analyzed by flow cytometry (Beckman Coulter, Inc., Fullerton, CA, US).

Caspase-3, caspase-8, and caspase-9 activity assay
Caspase-3, caspase-8, and caspase-9 activity was measured using a Caspase-3 Assay Kit, Caspase-8 Assay Kit, and Caspase-9 Assay Kit (Beyotime, Shanghai, China),
Glycolysis assay
The glycolysis levels were measured using pyruvic acid, lactic acid, and ATP analysis kits, which were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). WT, Δssek3 mutant, and ssek3-complemented strains were treated as above similar methods. After incubation with gentamicin-containing medium (100 μg/mL), the supernatants were aspirated, and the cells were washed three times with PBS. The protein concentration in each group was detected using the Bradford protein assay kit (Beyotime, Shanghai, China), and absorbance values for pyruvic acid analysis, lactic acid, and ATP analysis were read at 505 nm, 530 nm, and 636 nm, respectively in a microplate spectrophotometer (Infinite 200 PRO NanoQuant, Tecan, Switzerland).

Statistical analysis
Data were presented as the mean ± standard deviation (SD) of three independent experiments. Two-way analysis of variance (ANOVA) with a post-hoc test (Bonferroni’s multiple-comparison test) was used to compare and assess statistical significance among all groups. P < 0.05 was considered statistically significant.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01838-z.

Additional file 1: Figure S1. Adherence and invasion assays for WT, Δssek3 mutant, and ssek3-complemented strains in RAW264.7 cells. Bonferroni’s multiple-comparison test showed no significant differences between the groups (P > 0.05).

Abbreviations
Ssek3: S. Typhimurium secreted effector K3; WT: Wild-type; HIF-1α: Hypoxia inducible factor-1α; SPI-2: Salmonella pathogenicity island 2; T3SS2: Type III secretion system 2; TRADD: TNFR1-associated death domain protein; FWGE: Fermented wheat germ extract; Myc: Myelocytomatosis viral oncogene; ATCC: American type culture collection; DMEM: Dulbecco’s modified Eagle medium; FCS: Fetal calf serum; PBS: Phosphate buffered saline; MOI: Multiplicity of infection; PI: Propidium Iodide; SD: Standard deviation; ANOVA: Analysis of variance

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Authors’ contributions
CY and FD contributed equally to this work. They performed the experiments, analyzed the experimental results and wrote manuscript. FD, CY, and CZ conceived of and designed the experiments. FD, CY, CZ, and YL analyzed experiment performance. CL, LH, and XC analyzed and interpreted all of the data. YL, CL, LH, and XZ performed the statistical analyse. CY, CZ, YL, and XC did the supervised study. All authors read and approved the manuscript.

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Availability of data and materials
The data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing of interests.

Author details
1The Key Lab of Animal Disease and Public Health, Henan University of Science and Technology, 263 Kaiyuan Avenue, Luoyang 471023, Henan, China. 2Luoyang Key Laboratory of Live Carrier Biomaterial and Animal Disease Prevention and Control, Luoyang 471023, Henan, China. 3Luoyang Polytechnic, 6 Airport Road, Luoyang 471023, Henan, China.

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