Salmonella enterica serovar Typhimurium sseK3 induce cell apoptosis and enhance glycolysis in macrophages

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Abstract

Background: *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is an important infectious disease pathogen. Previous studies have identified that *S. Typhimurium* secreted effector K3 (SseK3) is a novel translated and secreted protein, but it is unclear whether this protein exerts a significant role in the progress of apoptosis and glycolysis in macrophages. **Results:** The *S. Typhimurium* SL1344 wild-type (WT) group, Δ sseK3 mutant group and sseK3-complemented group were used to infect macrophages and the effects of sseK3 on apoptosis and glycolysis of macrophages were investigated. The adherence and invasion of Δ sseK3 mutant group for macrophages were similar to WT group and sseK3-complemented group, indicating that SseK3 did not play an important role in the adherence and invasion of *S. Typhimurium* for macrophages. However, the apoptosis percentage of the Δ sseK3 mutant group was much lower than WT group and sseK3-complemented group using flow cytometry. The caspase-3, caspase-8 and caspase-9 enzyme activity of the Δ sseK3 mutant group were decreased significantly compared with WT group and sseK3-complemented group, which suggested that sseK3 could improve the activities of caspase-3, caspase-8 and caspase-9 enzyme. We also found that the pyruvic acid level did not significantly change among Δ sseK3 mutant group, WT group and sseK3-complemented group, but the lactic acid level of Δ sseK3 mutant group was much lower than WT group and sseK3-complemented group. The ATP level of Δ sseK3 mutant group was remarkably higher than WT group and sseK3-complemented group. These indicated that the sseK3 enhanced the level of glycolysis of macrophages infected by *S. Typhimurium*. **Conclusions:** Our data showed that the sseK3 of *S. Typhimurium* can promote macrophages apoptosis and influence glycolysis levels of macrophages. These results may give a better clue about the relationship between apoptosis and glycolysis in macrophages induced by *S. Typhimurium* sseK3. **Keywords:** *S. Typhimurium*, sseK3, macrophages apoptosis, glycolysis

Background

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is one of the most significant zoonotic pathogens that pose a threat to humans [1]. After being contaminated by *Salmonella*, animal products can be transmitted to humans through the food chain, threatening human health, and potentially leading to deaths [1, 2]. Besides, *S. Typhimurium* can survive and replicate in macrophages. The macrophages or dendritic cells were reported to be able to carry bacteria from the Peyer's patches to adjacent lymph nodes, spleen and liver in mouse models [3].

One of the most essential metabolic pathways in cells is glycolysis [4]. Under anaerobic condition, the pyruvate is eventually converted into lactic acid. However, under aerobic conditions, pyruvic acid enters the tricarboxylic acid cycle (TCA cycle) and eventually is oxidized to CO₂ and H₂O [5]. Liu found that Resveratrol reduced the production of lactic acid to inhibit glycolysis and induce cell apoptosis [6]. Kok found that Sirtuin6 reduced the production of HK2 during glycolysis, thereby regulating apoptosis induced by hypoxia [7]. Hypoxia inducible factor-1 α (HIF-1 α) could inhibit the production of Adenosine triphosphate (ATP) and thus inhibit the glycolysis of mouse granulosa cells [8]. Moreover, studies have

shown that *S. Typhimurium* can induce cell apoptosis [9, 10]. There is increasing evidence in recent years that the apoptosis is closely related to glycolysis activities [11-13]. Therefore, the glycolysis is closely related to cell apoptosis.

The immune cells were able to detect the metabolic abnormalities caused by *Salmonella* with the help of inflammatory signals, and glycolysis was essential in *S. Typhimurium* infection with macrophages [14, 15]. Following invasion or phagocytic uptake into the host cell, one of the key virulence determinants is the *Salmonella* pathogenicity island 2 (SPI-2)-encoded type III secretion system 2 (T3SS2), which delivers 28 effector proteins into the host cell [16-18]. Previous studies have identified that *Salmonella* secreted effector K3 (SseK3) is a novel translated and secreted protein of *S. Typhimurium*, which is encoded by *sseK3* gene [19]. SseK3 was a glycosyltransferase, which could transfer an N-acetyl-glucosamine moiety onto the guanidino group of a target arginine, regulating host cell function. The protein belongs to the glycosyltransferase type-A family of glycosyltransferase enzymes and binds the ligand in a metal ion-dependent manner via a DXD motif [20]. SseK3 was co-regulated with the T3SS2 inside host cells and was injected into infected host cells. [21]. However, the mechanism of activity of SseK3 during *S. Typhimurium* infection was completely unclear, especially there is no research about *sseK3* affecting macrophages apoptosis and glycolysis.

In this study, we determined that *sseK3* from *S. Typhimurium* induced macrophages death and glycolysis. Our data showed that the *sseK3* of *S. Typhimurium* could promote macrophage apoptosis and improve glycolysis levels. These results would supply a critical step to provide a better understanding of the relationship between glycolysis and apoptosis in *S. Typhimurium*-infected macrophages.

Results

1. Analysis of adherence and invasion

Adherence and invasion to RAW264.7 cells of the host could ensure the same level of infecting cells at the post experiments. Compared to WT, the $\Delta sseK3$ mutant exhibited similar adhesion to and invasion of RAW264.7 cells. However, the *sseK3*-complemented strain regained the adherence and invasion ability of WT (Fig.1). This result suggests that *sseK3* does not play an important role in the adherence and invasion of RAW264.7 cells by *S. Typhimurium*. Moreover, the WT, the $\Delta sseK3$ mutant and *sseK3*-complemented strains have the similar level of infecting RAW264.7 cells.

2. Analysis of cell apoptosis

The apoptosis assay was performed as previously described [22, 23]. Apoptosis assay showed that the apoptosis percent of $\Delta sseK3$ mutant group was much lower than that of the WT and *sseK3*-complemented groups. In the $\Delta sseK3$ mutant group, the FITC-Annexin V positive cells were 3.78%, while in the WT group, it was 14.64% (Fig.2), suggesting that SseK3 encoded by *sseK3* plays an important role in the process of inducing macrophages apoptosis by *S. Typhimurium*.

3. Analysis of caspase activity

In the process of apoptosis, the caspase played an essential role [24]. Caspase-3, 8, 9 activities were measured at different time points respectively (Fig.3). The caspase-3, caspase-8 and caspase-9 activities of mock group was much lower than infection groups ($\Delta sseK3$ mutant, WT and *sseK3*-complemented groups), which indicated that the infection groups could stimulate the activities of caspase-3,8,9 in macrophages. However, it could be seen from Fig.3 that the caspase-8 and caspase-9 activities of $\Delta sseK3$ mutant group significantly lower than WT group and *sseK3*-complemented group 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. As for caspase-3, there were no significant difference among $\Delta sseK3$ mutant group ($P>0.05$), WT group and *sseK3*-complemented group at 2 h. But significant difference among infection groups was observed at 4 h, 6 h and 8 h ($***P<0.001$), the caspase-3 activity of $\Delta sseK3$ mutant group significantly lower than WT group and *sseK3*-complemented group. These results illustrated that SseK3 served a pivotal role in the process of inducing macrophage apoptosis by *S. Typhimurium*.

4. Analysis of glycolysis

The analysis of glycolysis was performed as previously described [25-27]. To determine the SseK3 whether can affect the glycolysis of macrophages infected by *S. Typhimurium*, the level of pyruvic acid, lactic acid and ATP were detected respectively (Fig.4). The results showed that the level of pyruvic acid was no significant difference at 2 h, 4 h, 6 h and 8 h ($P>0.05$) among each group. However, as for lactic acid, there was significant difference between mock group and infection group at 2 h, 4 h, 6 h and 8 h ($***P<0.001$). The glycolysis of macrophages of infection group were remarkably increased compared with mock group, and the lactic acid level of $\Delta sseK3$ mutant group was significantly lower than WT and *sseK3*-complemented groups at 4 h, 6 h and 8 h ($***P<0.001$). The investigations of glycolysis in macrophages suggested SseK3 encoded by *sseK3* could boost the lactic acid of macrophages. We also found that the level of ATP in infection groups were significantly lower than in mock group at 4 h, 6 h and 8 h ($***P<0.001$), and the level of ATP in the $\Delta sseK3$ mutant group was much higher than in 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 the glycolysis of macrophages through lactic acid and the ATP level changing.

Discussion

Many secreted proteins of *Salmonella* exert different virulence functions in the process of survival and replication in cells [28]. T3SS2 helps *Salmonella* transport virulence (effector) proteins in SCVs into host cells [29]. *S. Typhimurium* possesses the SseK family of proteins, including SseK1, SseK2 and SseK3, which are T3SS effectors [1, 30]. SseK3 is an important protein during *Salmonella* infection of cells and plays a pivotal role in the natural host immune process [19, 21]. Multiple secretory proteins of *S. Typhimurium* exerted enormous stress during the period of apoptosis of cells and related to cell apoptosis [28, 29, 31, 32]. Nonetheless, the role of SseK3 in the process of cells apoptosis and glycolysis

is unclear. This research is the first to show that SseK3 encoded by *sseK3* of *S. Typhimurium* could improve cell apoptosis and boost glycolysis in macrophage.

Santo found that early sipB-dependent and delayed sipB-independent mechanisms could cause the apoptosis induced by *S. Typhimurium* [33]. Kasinskas investigated that the lacking ribose chemoreceptors of *S. Typhimurium* localized in tumor quiescence and induced apoptosis [34]. Besides, there had shown that the cAMP receptor protein of *S. Typhimurium* could induce the apoptosis of macrophages [35]. Therefore, in order to determine the relationship between *S. Typhimurium sseK3* and macrophages apoptosis, we used the WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains to infect the macrophages. In our study, we found that the adherence percentage and invasion percentage of $\Delta sseK3$ mutant group were similar to WT group and *sseK3*-complemented group during the infecting period ($P>0.05$), which ensured that the WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains had the same level going through into the macrophages. Subsequently, the apoptosis in different infection groups were detected by the assay of flow cytometry. The results showed that the apoptosis percentage of $\Delta sseK3$ mutant group (3.78%) was much lower than WT group (14.64%) and *sseK3*-complemented group (11.81%). These indicated that deletion of the *sseK3* gene significantly affected the cell apoptosis.

The caspases activities were further to be detected. The activation of caspases exerts essential role on the cells apoptosis induced by the loss of mitochondria membrane [36]. There had shown that the SopB of *Salmonella* could protect host cell from apoptosis induced by caspase-3 [37]. The activation of caspase-3 and caspase-9 showed that the apoptosis was activated in the macrophages infected by *Escherichia coli* bacteria [38]. Besides, the caspase-8 influenced the synthesis of pro-IL-1 β and was essential for the apoptosis of cells induced by *Salmonella* [39]. As these previously researches, we investigated the caspase-3, caspase-8 and caspase-9 activity during the apoptosis of macrophages infected by *S. Typhimurium*. We found that caspase-3, caspase-8 and caspase-9 activity were remarkably increased in infection groups compared with mock group at 2 h, 4 h, 6 h and 8 h ($***P<0.001$), indicating that the apoptosis were activated in the macrophages apoptosis. Furthermore, the activity of caspase-3, caspase-8 and caspase-9 in $\Delta sseK3$ mutant group were much lower than in WT group and *sseK3*-complemented group, which suggested that SseK3 may be a pivotal component for *S. Typhimurium* activating apoptosis in macrophages.

There were increasing evidences that the apoptosis and glycolysis had closely relationship. Previously, Comin-Anduix found that fermented wheat germ extract (FWGE) inhibited the glycolysis and induced the apoptosis of tumor cells [40]. Jeong found that the modification of glycolysis could change the sensitivity of apoptosis through mitochondria pathway [41]. Recently, There has shown that the glycolysis could be inhibited and the cell death was further blocked [42]. Li found that methyl jasmonate could inhibit the glycolysis and induce apoptosis of tumor cells [43]. Sumi found that propofol could improve glycolysis of cells and lead to the apoptosis of cells [44]. The pyruvic acid, lactic acid and ATP were important components in the process of cell glycolysis. There have various metabolic pathway to produce pyruvic acid [45]. Pyruvic acid could enter the TCA cycle to be completely oxidized in aerobic condition, whereas it could become lactic acid under anaerobic oxygen condition [46, 47]. HIF-1 α could

prohibit the production of ATP and thus regulate the glycolysis of mouse granulosa cells [8]. Thus, WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains were used to explore the ability of macrophage glycolysis through detecting the levels of pyruvic acid, lactic acid and ATP. We found that the levels of intracellular pyruvic acid did not significantly change among all groups from 2 h to 8 h ($P>0.05$), but the lactic acid and ATP were different, respectively, among these groups. In the beginning stage, compared with WT and *sseK3*-complemented groups, the level of lactic acid of macrophages infected by $\Delta sseK3$ mutant strain did not significantly change at 2 h ($P>0.05$), while there was significant difference between WT and $\Delta sseK3$ mutant group at 4 h ($***P<0.001$), 6 h ($***P<0.001$) and 8 h ($***P<0.001$), suggesting the deletion of *sseK3* may reduce the glycolysis of macrophages induced by *S. Typhimurium* and SseK3 encoded by *sseK3* may enhance the glycolysis. Furthermore, the levels of lactic acid of WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains were significantly larger than mock group from 2 h to 8 h ($***P<0.001$), which illustrated that the anaerobic pathway of macrophages was enhanced among infection groups. Besides, as for ATP, there was no significant difference among all groups at 2 h, while the ATP levels of macrophages of $\Delta sseK3$ mutant group were remarkably larger than that of WT and *sseK3*-complemented groups at 4 h ($***P<0.001$), 6 h ($**P<0.01$) and 8 h ($***P<0.001$), indicating that glycolysis level of WT and *sseK3*-complemented groups were lower than $\Delta sseK3$ mutant group and SseK3 encoded by *sseK3* may enhance the glycolysis. Moreover, the ATP levels of macrophages of mock group were significantly higher than infections groups at 4 h ($***P<0.001$), 6 h ($***P<0.01$) and 8 h ($***P<0.001$), respectively, which suggested that the mock group may mainly undergoing the aerobic metabolism, for the prominent reason may be that the ATP produced by glycolysis is much lower than ATP produced by aerobic oxidation pathway. Therefore, the deletion of *sseK3* decreased the glycolysis of $\Delta sseK3$ group compared with WT group and SseK3 could improve the level glycolysis of macrophages infected by *S. Typhimurium*. In a word, the SseK3 of *S. Typhimurium* could induce apoptosis and enhance the glycolysis of macrophages. The conclusion was consistent with Sumi's [44] and Ding's [35] results, but it was contradict with Li's [43] and Comin-Anduix's [40] results. We speculate that there may be other different pathways involved in the apoptosis and glycolysis of cells, which need more experiments to explain this problem. In our future work, we will further explore the relationship between glycolysis and apoptosis.

Conclusions

In summary, from what has been discussed above, we could conclude that the SseK3 of *S. Typhimurium* could induce cell apoptosis and boost glycolysis in macrophages. This finding may help to illustrate the mechanism that *S. Typhimurium* induces macrophages apoptosis and provide a better understanding of the possible relationship between macrophage apoptosis and glycolysis induced by SseK3.

Methods

Bacterial Strains, cells and culture

S.Typhimurium SL1344, the deletion of *sseK3* *S.Typhimurium* SL1344 ($\Delta sseK3$ mutant) and its complemented (*sseK3*-complemented) were used in this study. Macrophages RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and cells were 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 [48, 49]. A 24-well cell culture plate was inoculated with 1×10^5 RAW264.7 cells per well and incubated for 16 h. WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains were coincubated with RAW264.7 cells at a multiplicity of infection (MOI) of 100:1, with three replicate wells per strain. To allow the bacteria to fully contact the RAW264.7 cells, the plates were centrifuged and incubated with 5% CO₂ for 2 h at 37 °C. For the adherence assay, the supernatants were aspirated, and the cells were washed three times with PBS. Subsequently, the cells were digested with 0.25% trypsin and plated in a gradient dilution and counted. For the invasion assay, the supernatants were aspirated, the cells were washed three times with PBS, and gentamicin-containing medium (100 µg/mL) was added and 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 and 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, $\Delta sseK3$ mutant and *sseK3*-complemented strains were coincubated with RAW264.7 cells at a multiplicity of infection (MOI) of 100:1, with three replicate wells per strain. To allow the bacteria to fully contact the RAW264.7 cells, the plates were centrifuged with 1000 rpm/min. After 4 h, the percent of cells undergoing apoptosis was detected by flow cytometry using Annexin V-FITC/PI apoptosis detection kit (KeyGEN BioTECH Jiangsu China). The cells of infected and mock groups were digested with 0.25% trypsin and washed three times with ice-cold phosphate buffered saline (PBS) and suspended in Binding Buffer with 500 µL, followed by adding 5 µL Annexin V-FITC and 5 µL Propidium Iodide (PI). Then the solution was placed in the dark room for 15 min at room temperature followed by immediately analysis using flow cytometry (Beckman Coulter, Inc., Fullerton, CA, US).

Caspase-3, caspase-8 and caspase-9 activity assay

The activity of caspase-3, caspase-8, and caspase-9 was measured by Caspase-3 Assay Kit, Caspase-8 Assay Kit, Caspase-9 Assay Kit (Beyotime, Shanghai, China), respectively. The cells of infected and mock groups were digested by trypsinization without EDTA and washed three times with ice-cold lysis buffer 3 times, followed by adding 100µL lysis buffer on ice. After incubated for 15 minutes, the concentration of

protein was detected using the Bradford protein assay kit (Beyotime, Shanghai, China). Subsequently, after the cell lysates were incubated with Ac-DEVD-pNA for 4 h at 37°C, the samples were read at 405 nm.

Glycolysis level assay

The glycolysis levels were measured using pyruvic acid analysis kit, lactic acid analysis kit and ATP analysis kit respectively, which were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). WT, Δ *sseK3* mutant and *sseK3*-complemented groups were processed based on manufacturer's instruction at 2 h, 4 h, 6 h and 8 h, respectively. The concentration of protein each group was detected using the Bradford protein assay kit (Beyotime, Shanghai, China). Finally, the absorbance values of pyruvic acid analysis kit, lactic acid analysis kit and ATP analysis kit were read at 505 nm, 530 nm and 636 nm in a microplate spectrophotometer, respectively.

Statistical analysis

The data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments, as based on triplicates assays. Two-way analysis of variance (ANOVA) with a post-hoc test (Bonferroni's multiple-comparison test) was used to compare and assess significance of the differences among all groups. The value of $*P<0.05$, $**P<0.01$ or $***P<0.001$ was considered significant.

Declarations

Ethics approval and consent to participate

Not applicable.

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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.

Consent for publication

Not applicable.

Authors' contributions

FD and CY 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.

Competing interests

The authors declare that they have no competing of interests.

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Figures

Figure 1

Adherence and invasion assays for WT, $\Delta sseK3$ mutant and *sseK3*-complemented strains to RAW264.7 cells. There were no significant differences using Bonferroni's multiple-comparison test among all groups ($P > 0.05$).

Figure 2

The apoptosis percent of RAW264.1 cells infected by WT, $\Delta sseK3$ mutant and its complemented strains. Apoptosis was assessed by using Annexin V-FITC/PI double staining.

Figure 3

The activity of caspase-3, caspase-8 and caspase-9 in RAW264.7 cells of all groups. The activity of caspase-3, -8, and -9 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 means $\pm$ SD.

Figure 4

The glycolysis of RAW264.7 infected by WT, $\Delta sseK3$ mutant and sseK3-complemented strains. The content of pyruvic acid, lactic acid and ATP 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 means $\pm$ SD.