CaMKII promotes ROS-dependent apoptosis induced by Suilysin in PK-15 cells

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Research Article

Keywords: CaMKII, Suilysin, Apoptosis, ROS
Abstract

Background Activation of the multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) is a common intermediate of diverse stimuli-induced cell death. Suilysin(Sly) has toxicity on a variety of cells, however, the underlying mechanism of its effect remains unclear, and the mechanism of CaMKII in Sly-induced cell death has not been reported.

Methods CaMKII expression in porcine kidney-15 (PK-15) was detected by RT-qPCR analysis and Western blotting. Morphological analysis, and CCK-8 assay were done to verify that CaMKII promotes cytotoxicity induced by Sly. AO/EB staining, and flow cytometry were used to probe into the role of CaMKII and reactive oxygen species (ROS) in Sly-induced apoptosis. The effect of CaMKII on Sly-induced toxicity in mice was evaluated by pathological tissue slices analysis.

Results CaMKII was phosphorylated by Sly in PK-15, and inhibition or knockdown of CaMKII resulted in increased resistance to Sly. In PK-15 pretreated with a CaMKII inhibitor (KN93), Sly bound to the cell membrane was reduced, and the Sly-induced ROS, apoptosis were alleviated. Moreover, pretreatment with N-acetyl-L cysteine (NAC), a ROS scavenger, also blocked Sly-induced apoptosis. In summary, our study demonstrated that CaMKII activation and ROS production were involved in Sly-induced apoptosis. In addition, we identified that KN93 attenuated the damage of Sly to the viscera.

Conclusion CaMKII participates in Sly-induced ROS-dependent apoptosis and the toxic effects of Sly in mice.

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a ubiquitous Ser/Thr protein kinase in mammalian cells with four different isoforms (CaMKII\textalpha, CaMKII\textbeta, CaMKII\textdelta, and CaMKII\textgamma), which are encoded by four different genes (CAMK2A, CAMK2B, CAMK2D, CAMK2G). Each of these isoforms consists of four distinct domains: a catalytic domain containing CaMKII kinase activity, a regulatory domain containing a self-inhibitory region and a binding site for the Ca\textsuperscript{2+}/CaM complex, a variable domain and an association domain (Nicole and Pacary 2020). The regulation of CaMKII is involved in a variety of functions, including carbohydrate, fat and amino acid metabolism (Chen et al. 2022), neurotransmitter synthesis and release (Zalcman et al. 2018), calcium homeostasis (Toussaint et al. 2015), cytoskeletal function (Lin and Redmond 2008; Easley et al. 2008; McVicker et al. 2015), and plays an essential role in pathophysiological processes such as oxidative stress (Franklin et al. 2006).

It has been reported that the activation of CaMKII is a critical mediator of apoptosis induced by diverse death stimuli and plays different roles under different conditions. Several studies had shown that CaMKII promotes cell survival, when CaMKII activity was inhibited, c-FLIP expression was reduced and cells became sensitive to Fas-mediated apoptosis (Fladmark et al. 2002). CaMKII had been shown in other studies to promote cell death, the rapid pacing (RP) promoted CaMKII activation and ROS production, which led to the activation of the apoptotic cascade and cardiac cell death (Sepúlveda et al. 2013).
CaMKII activation at the beginning of reperfusion had a deleterious effect on the I/R injury, participating in a cascade of events that led to apoptosis and necrosis (Vila-Petroff et al. 2007), and the inhibition of CaMKII prevented isoproterenol-mediated and Ang II-mediated cardiomyopathy (He et al. 2011).

Suilysin (Sly) is a toxin protein secreted by Streptococcus suis (S.suis), which is a serious zoonotic pathogen, and Sly is considered an important virulence factor of the pathogenesis (Gottschalk and Segura 2000). It belongs to the cholesterol-dependent cytolysin (CDC) family, and contains an N-terminal portion (domains 1 and 3), a connection domain (domain 2), and a C-terminal domain (domain 4). Domain 4 is the region that binds to the target cell membrane (Xu et al. 2010; Vötsch et al. 2019). Sly can bind directly to the cholesterol-rich cell membrane to punch holes, and pore formation triggers numerous signaling cascades, such as changes in osmotic pressure, ionic composition, and intracellular calcium concentration (Aroian and van der Goot 2007), ultimately leading to cell lysis, necrosis, or apoptosis (Tenenbaum et al. 2016). In addition, in vitro studies elucidated the toxic effects of Sly in different epithelial cells (HEp-2, LLC-PK, A549, MDCK, HeLa, PK) (Seitz et al. 2013; Lalonde et al. 2000). In vivo, Sly causes aggregation of platelets and neutrophils, releases cytokines such as IL-6, IL-8, IL-10, and TNF-α, and triggers inflammation (Tenenbaum et al. 2016; Zhang et al. 2016).

To date, the role of CaMKII in Sly-induced apoptosis has not been investigated. KN93 is a CaMKII-binding specific antagonist that competitively inhibits the activity of CaMKII (Vila-Petroff et al. 2007). The present study was conducted to observe the protective effect of inhibiting CaMKII activity on Sly-induced injury in vitro and in vivo, the results showed that CaMKII inhibition could reduce the binding of Sly to cell membrane and alleviate Sly-induced ROS production to decrease apoptosis. Together, these findings demonstrate that CaMKII promotes ROS-dependent apoptosis induced by Sly.

**Materials And Methods**

**Chemicals and Reagents**

PK-15 cells were purchased from the Cell Culture Centre, Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing, China). KN93, Enhanced Cell Counting Kit-8 (CCK-8), Reactive Oxygen Species (ROS) Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). anti-p-CaMKII antibody was purchased from Cell Signaling Technologies (Beverly, MA, USA). anti-CaMKII antibody and anti-GAPDH antibody were obtained from Proteintech (Wuhan, China). The anti-rabbit and anti-mouse secondary antibodies were purchased from Boster (Wuhan, China). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (San Diego, CA, USA).

**Animals**

All animal experiments were performed according to the Care and Use of Laboratory Animals of the Animal Ethics Committee of Sichuan Agricultural University (Ya'an, China) (Approval No.SYXK 2019 – 187). Eight weeks of C57BL/6 male mice(18-22g) were obtained from Chengdu Dossy Experimental
Animal Co., Ltd (Chengdu, China). All mice were housed in an appropriate temperature animal facility and had unlimited access to water and food.

Expression and purification of recombinant Sly

Recombinant Suilysin (rSly) was expressed in *E. coli* BL21 (DE3) (Biomed, Beijing, China). *E. coli* BL21 (DE3) (pCold l-sly) was grown in LB medium until achieving the OD<sub>600nm</sub> of 0.6–0.8, and isopropyl thio-D-galactopyranoside (IPTG) (Solarbio, Beijing, China) was used to induce the expression of rSly at 18°C, 180 r/min for 24 h. After the bacteria were collected by centrifugation at 4°C and lysed by sonication, the supernatant was obtained by centrifugation. Ni<sup>2+</sup> column (Bio-Rad, Hercules, CA, USA) was used to purify His-tagged rSly, and rSly was eluted with 240 mM imidazole. Purified proteins were controlled by separation in SDS-polyacrylamide gels and immunoblot analysis using anti-Sly antibody (self-made by the research group).

Recombinant Suilysin D4 was expressed and preserved by our laboratory (Sichuan Agricultural University, Chengdu, China).

Cell culture and stimulation

The PK-15 cell line was incubated in DMEM (Gibco, Beijing, China) containing 10% fetal bovine serum (Gibco, Beijing, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Solarbio, Beijing, China) at 37°C with 5% CO<sub>2</sub>. PK-15 cells were pretreated with different doses of KN93 (1, 5, 10, 15, and 20 µM) for 24 h to select the minimum safe concentration, and then different concentrations of rSly (0.3 µg/mL, 0.6 µg/mL) were incubated for 24 h. Finally, the cells were collected for subsequent experiments. In the control group, PK-15 cells were only incubated with 0.1% DMSO (Solarbio, Beijing, China).

Quantitative real-time PCR assay

The expression levels of genes (*CAMK2A*, *CAMK2B*, *CAMK2D*, *CAMK2G*) were evaluated by reverse transcription qPCR. The primer sequences are listed in Table 1. Total RNA in PK-15 was extracted with the Axygen Total RNA Miniprep Kit (Jiangsu, China) according to the manufacturer's instructions. The RNA concentration was determined. cDNA was synthesized with a reverse transcription kit (Yeasen, Shanghai, China) and amplified with a PCR instrument. qPCR was conducted using an SYBR Green PCR kit (Yeasen, Shanghai, China) on a Roche Real-Time PCR System (Basel, Switzerland). The reaction conditions were as follows: preheating at 94°C for 10 min, denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s for 40 cycles. GAPDH was used as a control for normalization of qPCR results.
Table 1
The qPCR primers

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Western blot analysis

Cells were lysed in RIPA buffer (Solarbio, Beijing, China) containing protease (Solarbio, Beijing, China) and phosphatase inhibitors (Beyotime, Nanjing, China). Equal amounts of protein extract were loaded and separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) were blocked for 2 h at room temperature with 5% non-fat milk (Sangon Biotech, Shanghai, China) in TBST, and then incubated with primary antibodies anti-CaMKII (1:5000, 45–60 kDa), anti-phospho-CaMKII Thr286 (1:1000, 50, 60 kDa) and anti-GAPDH (1:5000, 37kDa) overnight at 4°C, followed by HRP-labeled secondary antibodies. Labeled proteins were visualized with an enhanced chemiluminescence system. For quantification optical densities of proteins were determined by Gel-Pro analyzer software (Media Cybernetics, Rockville, MD, USA).

Cell cytotoxicity and viability analysis.

About 1 × 10^4 cells/well were seeded in 96-well flat bottom plates and incubated at 37°C, 5% CO₂ for 24 h to obtain 80%-90% confluence. rSly was added to all the test sample cells except the control group and incubated for 24 h. The CCK-8 stock solution was diluted 1:10 in unsupplemented DMEM to make the CCK-8 working solution. 100 µL of the CCK-8 working solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 450 nm was measured using a multifunctional chemical immunometer (Thermo Fisher Scientific, MA, USA).

Generation of PK-15 KO cell lines by CRISPR/Cas9 technology
The sgRNA sequence targeting the porcine \textit{CAMK2A}, \textit{CAMK2B}, \textit{CAMK2D}, \textit{CAMK2G} were cloned into the linearized lentiCRISPRv2, respectively, and then lentivirus was assembled to infect PK-15 cells. 10 µg/mL puromycin (Solarbio, Beijing, China) was used to select positive KO cells. Approximately 7 days after screening, the cells colonies were analyzed by extracting genomic DNA, and the nucleotide sequences were revealed by Sanger sequencing.

Immunofluorescence staining

PK-15 cells were cultured on coverslips in 12 well plates for 24 h to obtain 50%-60% confluence, KN93 was treated or untreated for 24 h, rSly was diluted to 1 µg/mL with cold PBS, and rSly was allowed to bind to the membrane for 2 h at 4°C. Cells were fixed in 4% paraformaldehyde for 10 min. After washing with PBS, blocked with 2% BSA in PBS for 1 h. Then cells were incubated with Sly antibody overnight at 4°C. Then cells were incubated with secondary antibodies. After washing, the cells were incubated with DAPI to stain the nucleus. Images were acquired using a confocal microscope (Olympus, Tokyo, Japan).

ROS assay

Approximately 2 × 10^5 PK-15 cells/well were seeded in 6-well plates for 24 h to obtain 50%-60% confluence. The wells were treated or untreated with 10 µM KN93 at 37°C for 24 h, then incubated with rSly for 24 h. Intracellular ROS was measured by the DCFH-DA fluorescent dye. Cells were incubated with 10 µM DCFH-DA diluted in serum-free culture media for 30 min in the dark. And cells were collected after washing with PBS, then the fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

AO/EB staining

Acridine orange (AO) and ethidium bromide (EB) are fluorescent intercalating DNA dyes that allow for differentiation of live, apoptotic, and necrotic cells. AO can penetrate cells with intact membrane, and EB can only penetrate cells with damaged membrane, causing cells to emit green or red fluorescence respectively (Braun et al. 2016). PK-15 cells were plated at a density of 2 × 10^5 cells per well in 6-well plates and then incubated. After exposure to NAC or KN93 for 24 h, then cells were treated with 0.6 µg/mL rSly for 24 h. Floating cells were collected with 0.6 µg/mL rSly for 24 h. Floating cells were collected with 100 µL PBS, AO and EB were mixed at a ratio of 1:1 to make a working solution. 2 µL working solution was used to dye cells, then the dyeing cells were dropped on a slide and covered with a coverslip. The images were photographed by fluorescence microscope, and counting 20–200 cells to analyze.

Apoptosis analysis

To measure apoptosis of cells, Annexin V and PI double staining was performed by using an Annexin V/PI apoptosis detection kit according to the supplier's manual. Cells were detached with 0.05% trypsin and counted 1 × 10^5 cells. Cells were then centrifuged in the binding buffer supplied by the kit, and immunostained with Annexin V-FITF (5 µL) and PI (5 µL) for 15 min at room temperature in dark. Cells
were measured by flow cytometry (BD Biosciences, San Diego, CA, USA), and the percentage of apoptosis was analyzed by Flowjo software (Flowjo LLC, Ashland, OR, USA).

KN93 anti-suilsin in vivo experiment

To assess the effect of KN93 on Suilysin-induced injury, C57BL/6 mice were used for in vivo experiments. Mice were stratified into three groups, namely, control (PBS), dimethyl sulfoxide (DMSO), KN93. There were ten mice in each group. Each group was inoculated with PBS, DMSO, KN93 respectively. Daily injection of KN93 (10 µmol/kg, i.p.) or an equivalent volume of DMSO or PBS for 7 d (Zhang et al. 2016). Mice were monitored body weight at the same time daily. 24 h later, the minimum lethal dose of rSly (2.5 µg/g) was injected into mice i.p.

Statistical analysis

Statistical analysis was performed by the Graphpad Prism 8 software (GraphPad, San Diego, CA, USA). The means ± S.D. were determined for each treatment group. Two-tailed Student's t-test was used to determine significant differences between the treatment and control groups (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns, no significant).

Results

Sly induced CaMKII phosphorylation in PK-15 cells

To determine the role of CaMKII family in rSly-induced PK-15 cytotoxicity, PK-15 cells were exposed to 0.3 µg/mL, 0.6 µg/mL rSly for 12 h and 24 h, the levels of \textit{CAMK2A}, \textit{CAMK2B}, \textit{CAMK2D} and \textit{CAMK2G} mRNAs were measured by real-time RT-PCR, and p-CaMKII/CaMKII level was measured by Western blotting. Among the genes encoding the four isoforms of CaMKII, the mRNA expression of \textit{CAMK2A}, \textit{CAMK2B} were downregulated in PK-15 cells with rSly exposure, and no significant changes in the levels of \textit{CAMK2D} and \textit{CAMK2G} (Fig. 1a). Based on the above findings, it showed that there was a link between rSly and the CaMKII family. At the protein level, this conclusion was also confirmed by downregulation of total CaMKII level and upregulation of phospho-CaMKII level (Fig. 1b). Furthermore, rSly-elevated phospho-CaMKII level was consistent with decreased cell viability (Fig. 1c), suggesting that rSly-induced PK-15 cell death might be associated with its induction of CaMKII phosphorylation.

Inhibition or knockdown of CaMKII significantly mitigated Sly-induced cell death

To investigate whether rSly induction of CaMKII phosphorylation is correlated with cell death in PK-15, PK-15 cells were exposed to 0.6 µg/mL rSly for 24 h after pretreatment with the CaMKII inhibitor KN93 (10 µM) for 24 h. We found that rSly induced phosphorylation of CaMKII was significantly attenuated by KN93 in PK-15 cells (Fig. 2a, b). Morphological analysis (Fig. 2c) showed that KN93 itself did not change cell shape, rSly alone caused cell rupture, however, KN93 partially prevented rSly-induced cell death.
Results from the CCK-8 assay (Fig. 2d) further demonstrated that KN93 significantly suppressed rSly-induced loss of viability in PK-15 cells.

Utilizing the CRISPR/Cas9 genome-editing system, we created knockdown mutants for the four genes \((CAMK2A, CAMK2B, CAMK2D, CAMK2G)\) in PK-15 cells. The successful disruption of each gene was confirmed by sequencing (Fig. 1S). CCK-8 assay of rSly-induced cell death in the four knockdown cells indicated that only knock down the genes \((CAMK2A, CAMK2B)\) conferred significant resistance to rSly-induced cell death (Fig. 2e). Collectively, these results showed that CaMKII was involved in rSly-induced cell death, and mainly CaMK2\(\alpha\) isoform and CaMK2\(\beta\) isoform played the key roles.

Inhibition of CaMKII reduced sly binding to the cell membrane

Since rSly is a cholesterol-dependent pore-forming cytolysin, its binding to the membrane is the first and most critical step for its toxic effect. To investigate how inhibition of CaMKII improved cell survival, we then detected the binding of rSly to the cell membrane by immunofluorescent staining. The results indicated that cells pretreated with CaMKII inhibitor showed significantly less membrane binding after rSly exposure compared with unpretreated cells (Fig. 3a, b). Next, we incubated PK-15 cells with truncated expression of GFP-rSly D4, and the results were consistent with the binding of full-length rSly to the cell membrane. The unpretreated PK-15 cell membrane showed bright GFP, while cells pretreated with KN93 inhibitor had almost no GFP bound to the membrane surface (Fig. 3c,d). In summary, these findings suggested that CaMKII mediated the binding of rSly to cell membrane.

KN93 reduced sly-induced apoptosis and ROS generation in PK-15 cells

ROS production is often thought to be associated with the occurrence of apoptosis. To further elucidate the protective effect of KN93, we examined total ROS by the DCFH-DA probe. As shown in Fig. 4a, KN93 significantly protected the production of ROS in rSly-treated PK-15 cells, suggesting that activation of CaMKII may promote ROS generation. Next, we determined whether inhibition CaMKII reduced apoptosis in PK-15 cells by attenuating ROS, PK-15 cells were pretreated with the ROS scavenger, NAC. The AO/EB staining (Fig. 4b, c) and Annexin V-FITC/PI assay (Fig. 4d) showed that inhibition of CaMKII and pretreatment NAC was able to reverse rSly-induced apoptosis. Taken together, these results suggested that CaMKII inhibition reduced rSly binding to the cell membrane and further reduced intracellular ROS production, which had a protective effect on rSly-induced oxidative damage (Fig. 4e).

CaMKII inhibitor attenuates the sly-induced damage in vivo

Given the above findings that KN93 could mitigate the toxic effects of rSly on porcine kidney cells, we investigated the effects of the KN93 inhibitor during exposure to rSly in vivo. To this end, an in vivo inhibition model of CaMKII was established in mice pretreated with 10 \(\mu\)mol/kg KN93 for 7 days, and the control group was injected with DMSO at the same dose of KN93 daily, then a lethal dose of rSly was injected intraperitoneally on day 8 (Fig. 5a). The results showed that there was no significant difference in body weights of mice between the DMSO and KN93 groups (Fig. 5b), indicating that KN93 and DMSO
had no obvious toxic effects on mice. Comparisons of tissue slices showed that the group of DMSO mice after rSly administration mainly represented hemorrhage and congestion in the heart, liver, spleen, lung, and kidney. In the PALS region of the spleen occurred T cells lost, the number of cells obviously decreased. In addition, cardiomyocytes, hepatocytes, and kidney cells degenerated seriously. Similarly, these symptoms could be observed in mice pretreated with KN93, while the KN93 inhibitor attenuated rSly-induced pathological damage (Fig. 5c). Taken together, these results indicated KN93 had a certain protective effect against rSly-induced toxicity in C57BL/6 mice.

Discussion

*S. suis* is a serious zoonotic pathogen that can cause multiple diseases and fatal infections in both humans and animals, such as arthritis, septicemia, meningitis, and pneumonia. It has a detrimental impact on public health and pork industries worldwide. Sly is the virulence factor of *S. suis* and plays a vital role in the pathogenic process. Acacetin could protect cells from *S. suis*-mediated infection by inhibiting Sly activity and downregulating inflammatory response (Xie et al. 2022). Silibinin, a potent antagonist of Sly-mediated hemolysis, was found to alleviate *S. suis*-induced J774 cells injury and protect mice from *S. suis* infection (Shen et al. 2019). By comparing a Sly-deficient mutant with a *S. suis* wt strain, the loss of adherence and invasion, and the reduction of cytotoxicity and apoptosis had been found (Meng et al. 2016). ST1 strain with higher Sly-production than ST104 strain could contribute to the higher bacterial density and enhanced inflammation in brain and increased mortality (Takeuchi et al. 2014). The overexpression of Sly in ST1 strain (P1/7-Sly) obviously increased inflammasome activation, causing streptococcal toxic shock-like syndrome (STSLS). However, the strain (P1/7-mSly) overexpressing mutant Sly could not cause a cytokine storm and STSLS (Xu et al. 2021). Therefore, selecting a target Sly can provide a new idea and a feasible strategy for preventing the *S. suis* infections.

The first step for Sly to play its cytotoxic role is to bind to the cell membrane. In this study, we found that inhibition of CaMKII reduced the binding of Sly to PK-15 cells and also reduced the binding of Sly D4, which is responsible for binding to cell membrane. It was speculated that CaMKII might regulate the membrane components of PK-15 cells, particularly changing the cholesterol content of membrane. Interestingly, cholesterol is an essential component of mammalian cell membranes, and its acquisition is primarily uptake of low density lipoprotein (LDL) from the extracellular environment through receptor-mediated mechanisms, then LDL synthesized cholesterol that is finally transferred by vesicular trafficking along the cytoskeletal tracks to plasma membrane (Ikonen 2008). Previous research had reported that inhibition of CaMKII with KN93 abolished LDL endocytosis and mobilization of low-density lipoprotein receptors (LDLRs) to the cell surface (Chen et al. 2022). While CaMKII binds to F-actin tightly and participates in actin reorganization (Wang et al. 2019). The model of CaMKII binding to stable F-actin is important for providing a molecular basis for the actin cytoskeleton and maintaining polymerized F-actin (Lin and Redmond 2008). Thus, the reasons why inhibiting CaMKII reduced the binding of Sly to the cell membrane, whether it affected the cytoskeleton or changed the membrane composition, which is worthy to further investigation.
ROS is one of the common proapoptotic molecules in apoptosis. ROS can cause oxidative damage to cells. The interaction between ROS and CaMKII has been reported previously. Calcitonin gene-related peptide (CGRP) attenuated Ang II-induced ROS generation and apoptosis in VSMCs by inhibiting the CaMKII/CREB signaling pathway (Luo et al. 2020). The CaMKII-Drp1 pathway played a major role in ROS-dependent apoptosis induced by blue light (Yang et al. 2021). KN93 had a protective effect on the pancreatic acinar cell necroptosis by inhibiting ROS production and decreasing the expression of RIP3 and p-MLKL (Zhu et al. 2021). The RIP3-CaMKII pathway mediated I/R- and Dox-induced ROS production, leading to cardiac oxidative injury. Collectively, CaMKII works as a sensor of ROS increments.

In vitro, we had demonstrated that the KN93 inhibitor had a protective effect against Sly toxicity, but did not improve the survival of mice in vivo. In experiments, we found that all dead mice exhibited severe small intestinal congestion and edema, and the intestinal lumen was filled with yellowish fluid, which may be the main cause of death in mice. Due to the presence of large amounts of fat in the abdominal cavity and the lethal dose of purified Sly treated by intraperitoneal injection, Sly molecules could accumulate in the abdominal cavity in large quantities and rapidly, thus causing severe damage to the intestinal tract. In production clinics, the transmission route of S.suis is mainly through the respiratory tract and wounds, and secreted Sly is limited, which could explain why KN93 did not obviously reduce the lethality of Sly in mice. Overall, CaMKII is still expected to be a potential target against S.suis.

Conclusions

Here we demonstrated that KN93, an inhibitor of CaMKII, could mitigate rSly-induced cell death, reduce the binding of rSly to the plasma membrane, and decrease the levels of oxidative stress products. Our findings suggested that both ROS production and CaMKII activity play a key role in the pathogenesis of rSly.

Declarations

Acknowledgments The authors would like to acknowledge Hua Li (Institute of Animal Nutrition, Sichuan Agricultural University) for providing guidance and support for FCM analysis.

Funding This work was supported by the Sichuan Science and Technology Program (grant numbers 2021ZDZX0010).

Author Contributions Shixin Dai and Qin Zhao conceived and designed the experiments; Hang Xiao and Zhongsen Lin contributed to material preparation and data analysis. Shixin Dai wrote original draft preparation. Senyan Du, Yiping Wen, Rui Wu, Qigui Yan, Xiaobo Huang, Yiping Wang, Yifei Lang, Shan Zhao, and Xiaoping Ma commented on previous versions of the manuscript. Sanjie Cao performed the funding acquisition, conceived and supervised the study. All authors have read and approved the final manuscript.
Data availability
Some of the data supporting the findings of this study are not publicly available due to the possibility of compromising the personal privacy of research participants, but are available from the corresponding author on reasonable request.

Code availability
Not applicable.

Ethical statement
Informed consent was obtained from all individual participants. All animal experiments were performed according to the Care and Use of Laboratory Animals of the Animal Ethics Committee of Sichuan Agricultural University (Ya'an, China) (Approval No.SYXK 2019-187).

Consent for publication
Not applicable.

Competing Interests
The authors have no relevant financial or non-financial interests to disclose.

References


Figure 1

rSly induced CaMKII phosphorylation in PK-15 cells. a RT-qPCR detected the transcription level of \textit{CAMK2A}, \textit{CAMK2B}, \textit{CAMK2D}, and \textit{CAMK2G} that PK-15 cells were treated with 0-0.6 $\mu$g/mL rSly 12 h or 24 h. b Western blotting verified rSly induced phosphorylation of CaMKII in PK-15 cells. PK-15 cells were treated with 0-0.6 $\mu$g/mL rSly for 24 h, and total cell lysates were subjected to Western blotting using indicated antibodies. GAPDH was used as a loading control. c Cell viability of PK-15 cells treated with...
different concentrations of rSly for 12 h or 24 h was evaluated using the CCK-8 assay. Significant differences between groups are indicated by * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001, ns, not significant. p values were determined with two-tailed Student’s t-tests

**Figure 2**
CaMKII activity is required for rSly-induced cell death. a, KN93 obviously blocked rSly-induced CaMKII phosphorylation in PK-15 cells. c The morphology of PK-15 cells was visualized under a Nikon inverted microscope equipped with digital camera (magnification 400 ×). KN93 partially rescued cells from rSly-induced cell death. d Cell viability for PK-15 cells was measured by the CCK-8 assay. e Cell viability assays for WT and KO cell pool of four genes after rSly exposure at 0-4.8 µg/mL for 24 h. ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant. p values were determined with two-tailed Student's t-tests.

Figure 3

Immunofluorescence staining of the ability of rSly bound to the PK-15 cell membrane. a, b Full-length rSly protein bound to the cell membrane. Scale bar, 100 µm. c, d Truncated rSly D4 protein bound to the cell
membrane. Scale bar, 100 μm. Relative fluorescence intensity calculated using ImageJ software. ** p < 0.01, *** p < 0.001. p values were determined with two-tailed Student’s t-tests.

**Figure 4**

Effect of CaMKII on ROS-dependent apoptosis induced by rSly. **a** Effect of CaMKII on rSly-induced ROS generation. KN93 pretreated or unpretreated cells were incubated with rSly (0.3 and 0.6μg/mL) for 24 h. **b**
c AO/EB staining detected cell apoptosis. NAC (10 mM) and KN93 (10 μM), respectively pretreated or unpretreated, then using 0.6 μg/mL rSly treated for 24 h. The apoptosis ratio was calculated by ImageJ software. Scale bar, 200 μm. d The percentage of apoptotic cells relative to the total cell population was quantified, and the induction of apoptosis was analyzed by double staining with Annexin V-FITC/PI.

e Proposed schematic diagram of CaMKII and ROS in rSly-exposed cells. The rSly molecular is green. * p < 0.05, *** p < 0.01, ns, not significant. p values were determined with two-tailed Students’ t-tests.

Figure 5

KN93 alleviated rSly-induced toxicity in mice. a Schematic of establishing an in vivo inhibition model of CaMKII. b Body weights of C57BL/6 mice during KN93 or DMSO treatment. c Representative images of heart, liver, spleen, lung, and kidney tissues with H&E staining in different groups (magnification 400 ×, scar bar=50 μM)

Supplementary Files
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- SupplementaryMaterials.docx