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**Siniperca chuatsi** rhabdovirus (SCRV) induces autophagy via PI3K/Akt-mTOR pathway in CPB cells

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**ABSTRACT**

Autophagy is an important mechanism for organisms to eliminate viruses and other intracellular pathogens. *Siniperca chuatsi* rhabdovirus (SCRV) is an agent that has caused devastating losses in Chinese perch (*Siniperca chuatsi*) industry. But the role of autophagy in *Siniperca chuatsi* rhabdovirus (SCRV) infection is not clearly understood. In this study, we identified that SCRV infection triggered autophagy in CPB cells, which was demonstrated by the appearance of the membrane vesicles, GFP-LC3 punctuate pattern, conversion of LC3-I to LC3-II, and the co-localization of autophagosomes and lysosomes. The changes of autophagy flux in SCRV infection indicated that autophagy was inhibited at the early stage of SCRV infection, but was promoted at the late stage. UV-inactivated SCRV can induce autophagy, suggesting that SCRV replication is not essential for the induction of autophagy. Furthermore, we found inducing autophagy with Rapa inhibited SCRV proliferation, but inhibiting autophagy with 3-MA or CQ increased SCRV production in CPB cells. Then we assessed the effects of PI3K/Akt-mTOR signaling pathway on SCRV induced autophagy. We found that SCRV infection activated PI3K/AKT signaling pathway at 4 hpi, but inhibited it at 8 hpi. SCRV-N mRNA and protein level were decreased by inhibiting PI3K with LY294002, but increased by activating PI3K with 740Y–P. Those results indicated that SCRV infection induced autophagy via the PI3K/Akt-mTOR signal pathway, which will provide new insights into SCRV pathogenesis and antiviral treatment strategies.

**1. Introduction**

*Siniperca chuatsi* rhabdovirus (SCRV), as one of the piscine rhabdoviruses, has caused great losses to the Chinese perch aquaculture industry [1,2]. The viral genome is a negative single-stranded RNA with the length about 11 kb, which encodes five structural proteins, including RNA-dependent RNA polymerase protein (L), Glycoprotein (G), nucleoprotein (N), Phosphoprotein (P) and Matrix protein (M) [3]. SCRV infection can lead to visceral and skin bleeding, high morbidity and mortality [2].

Autophagy is a ubiquitous mechanism in eukaryotes that aims at eliminating useless or harmful substrates through catabolism to maintain cell homeostasis [4,5]. Under the normal physiology condition, autophagy maintains a low level to deliver damaged proteins and organelles to lysosomes. However, during stress response (hypoxia, starvation, endoplasmic reticulum stress or invading pathogens), autophagy is dramatically up-regulated [6]. This process starts with the formation of a cup-shaped membrane structure, termed the phagophore, and ends up with cargo degradation and release, termed the autophagosome-lysosome. In recent years, many studies have shown that autophagy plays a significant role in antiviral immunity [7,8]. Virus can not only induce autophagy of host cells, but also evade the host immune clearance by membrane vesicles. In return, host cells capture the intracellular viruses by autophagosome and degrade them by autolysosome [9]. It has been found that Autophagy was triggered by viral hemorrhagic septicemia virus (VHSV) and spring viræma of carp virus (SVCV) [10]. SVCV induced autophagy to facilitate viral RNA replication and virions release in epithelioma papulosum cyprini (EPC) cells [11]. Snakehead fish vesiculovirus (SHVV) induced apparent autophagy in SSN-1 cells and autophagy inhibited virus replication [12]. Autophagy can be repressed by PI3K/Akt/mTOR pathway in tumor cells [13]. Recent studies showed that PI3K/Akt/mTOR pathway is also...
involved in the virus-induced autophagy [7]. Many viruses can activate the PI3K/AKT pathway to promote viral infection, such as Hepatitis C Virus (HCV) [14], Marek’s Disease Virus (MDV) [15], Newcastle disease virus (NDV) [16], Dengue Virus(DENV) [17], Herpes simplex virus (HSV) [18], Zaire Ebola virus (EBOV) [19], and Coxsackievirus B3(CVB3) [20]. However, the role of PI3K/Akt/mTOR signal pathway in the modulation of SCRV-induced-autophagy remains uncharacterized clearly.

In this study, we investigated the relationship between autophagy and SCRV replication in Chinese perch brain (CPB) cell line. Furthermore, the PI3K/Akt/mTOR signal pathway involved in the SCRV-induced autophagy was identified. These results will provide new insights into SCRV pathogenesis and antiviral treatment strategies.

2. Materials and methods

2.1. Cell line and virus strains

The Chinese perch brain cells (CPB), originated from mandarin fish (S. chuiasi) brain, was established in our laboratory and were propagated and maintained at 28 °C in Leibovitz’s L-15 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA) [21]. SCRV was isolated in our laboratory and propagated in CPB cells at 28 °C [2]. Virus stocks were stored at −80 °C.

2.2. Pharmaceuticals and antibodies

Rapamycin (Rapa), 3-methyladenine (3-MA), Chloroquine (CQ), LY294004 (LY), and 740Y-P were purchased from Sigma Aldrich (USA) and solubilized in DMSO except CQ solubilized in phosphate-buffered saline (PBS). p-Akt (Ser473) rabbit mAb, p-PI3K p85 (Tyr458)/p55 (Tyr199) antibody, and mTOR (7C10) rabbit mAb were purchased from CST (USA). p-mTOR (ser2448) antibody, LC3A/B antibody, α-tubulin antibody, and β-actin antibody were purchased from Abcam (USA). p-Akt (Ser473) rabbit mAb, p-PI3K p85 (Tyr458)/p55 (Tyr199) antibody, and mTOR (7C10) rabbit mAb were purchased from CST (USA). p-mTOR (ser2448) antibody, LC3A/B antibody, β-actin antibody, and β-actin antibody were purchased from Abcam (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibodies were purchased from KPL (USA). The rabbit polyclonal antibody of SCRV nucleoprotein (SCRV-N) was stored in our laboratory.

2.3. Viral infection and sample collection

CPB cells were infected with SCRV (MOI = 1). Following 1 h of adsorption at 28 °C, the inoculum was removed and the cells were washed twice with Hank’s Balanced Salt Solution (HBSS) before adding L-15 medium with 2% (v/v) FBS. The SCRV-infected and mock-infected cells were sampled at indicated times.

2.4. Plasmid transfection and confocal fluorescence microscopy observation

The recombinant plasmid GFP-LC3 was constructed and stored in our laboratory [22]. The log phase CPB cells were transfected by recombinant plasmid GFP-LC3 at the concentration of 2 μg per 100 μL FuGENE®6 Transfection Reagent (Promega) diluted with Opti-MEM (GIBCO). The 100 μL mixture was added dropwise onto CPB cells cultured in the 35-mm glass-bottomed culture dishes (NEST), then mixed gently, and incubated for 15min. The cells were disposed with Rapa or virus at 48 h post-transfection, then fixed with 4% paraformaldehyde for 15min, and permeabilized in 0.3% Triton X-100 for 15min. Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI) for 5min. Images were acquired under a laser confocal fluorescence microscope (Olympus fluoView 1200).

2.5. SCRV inactivation by UV

To obtain inactivated SCRV, the viral suspensions (5 mL) were exposed to ultraviolet (UV) at clean bench for 2 h and shaken every 15min. Then virus infectious activity was tested using three blind passages in CPB cells. Briefly, the UV-treated viruses (1 mL) were initially inoculated onto three 25 cm² cell culture flasks at 28 °C and cell monolayer was observed daily to verify the occurrence of CPE for 10 days. Then the viral supernatant freeze-thawed three times was inoculated onto the new flasks every 7 days, and repeated twice. The absence of CPEs during this period confirmed that the virus was inactivated.

2.6. Transmission electron microscopy observation

The CPB cells with the confluence of 80–90% were infected with SCRV at a MOI of 1 and incubated for 8 h. Rapa-treated Cells were used as a positive control and untreated cells as a negative control. These cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.4) for 24 h at 4 °C and then post-fixed in 0.1 M phosphate buffer containing 1% osmium tetroxide for 1 h. Ultrathin sections were stained with uranyl acetate-lead citrate and examined by a Philips CM10 electron microscopy.

2.7. MTS assay

The cell viability was assessed using MTS assay according to CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) protocol. Briefly, cells were seeded (5 × 10⁴ cells/well) in 96-well plates and allowed to attach overnight. Subsequently, cells were washed once with PBS and then fed fresh medium supplemented with Rapa (0, 0.05, 0.1, 0.2, 0.5 μM), or 3-MA (0, 10, 50, 100, 500 μM, and 1, 5 mM), or CQ (0, 1, 5, 10 μM), or LY294002 (0, 5, 10, 20, 50 μM), or 740Y-P (0, 10, 20, 30, 40, 50 μM). At 24, 48, 72 and 96 h post-treatment, 20 μL MTS solution was added into each well, and incubated for 3 h at 28 °C. Then cell viability was determined by recording the OD₄₉₀ nm in an ELISA microplate reader (Infinite M200 Pro, Tecan, Switzerland). Medium without pharmaceuticals was used as control.

2.8. Pharmaceuticals treatment experiment

CPB cells were seeded into 6-well plates. When the cell confluence of 80–90% was reached, cells were washed and treated with different autophagy regulators at the optimal working concentration and pre-treatment time. Then cells were infected with SCRV at a MOI of 1. Cells or supernatants were harvested at 12 h post-infection (hpi) for qRT-PCR, Western blot, and virus titration detection.

2.9. qRT-PCR assay

To assess gene mRNA level, the total RNA of cell samples with virus or pharmaceutical treated were extracted and the cDNA were synthesized as mentioned above. qPCR was performed using SYBR Premix Ex Taq kit (Takara). The 18S rRNA was used as the internal control. The primers were listed in Table 1. The relative expression ratio was calculated using the 2⁻ΔΔCT method.
formed in a 20 μl volume, including 10 μl 2 × SYBR® Premix Ex Taq™, 0.4 μl each forward and reverse primer(10 μM), 0.4 μl ROX reference dye II, and 6.8 μl DEPC water and 2 μl cDNA. All reactions were performed in triplicate and the cycling parameters were designed according to the instructions.

2.10. Western blot analysis

Cells were collected and lysed in RIPA buffer with 1 mM PMSF. Proteins were separated by 12% or 7% SDS-PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, USA). Blots were incubated with the indicated primary antibody (dilution concentration according to the recommendation), and subsequently incubated with peroxidase-conjugated goat-anti-rabbit IgG (1:5000 dilution). Immunoreactive proteins were visualized by chemiluminescence using Thermo Scientific Pierce Western Blot ECL Plus (Thermo, USA). Densitometry of bands representing protein expression was done using Sigma Scan Pro5 software. For each immunoblot, the band intensity of each lane was normalized relative to the loading control β-Tubulin or β-Actin.

2.11. Determination of virus titration in supernatants

Supernatants were harvested at 12 h post-infection (hpi), and viral titers were measured by tissue culture infectious dose (TCID50). Samples were diluted through a 0.22 μm filter and serially diluted 10-fold in L-15. CPB cells were inoculated with the diluted samples for 7 d. CPE was observed and recorded every day. The TCID50 was calculated using the Karber method.

2.12. Statistical analysis

Results are expressed as the means ± standard deviation (SD) from at least 3 experiments. All tests were conducted using SPSS software (version 21.0). Values were considered statistically significant at P < 0.05 and extremely significant at P < 0.01.

3. Results

3.1. SCRV infection induced autophagy in CPB cells

To determine whether autophagy is triggered in SCRV-infected cells, we directly detected visualized formation of autophagy-related structures by transmission electron microscope. As Fig. 1A showed that a lot of membrane vesicles were apparently observed in the cytoplasm of SCRV-infected and Rapa-treated cells, but rarely observed in mock cells.

To observe the formation of autophagosomes in infected CPB cells, we cloned Siniperca chuatsi LC3 gene and constructed plasmid, which was transfected into CPB cells. As shown in Fig. 1B, the accumulation of GFP-LC3 fluorescent dots was observed in the transiently transfected CPB cells infected with SCRV or treated with Rapa. In contrast, there was almost no fluorescent dot formation in the mock cells. Furthermore, the fusion of autophagosomes with lysosomes was verified by labeling lysosomes with an acidified compartments marker LysoTracker. As shown in Fig. 1C, the co-localization of GFP-LC3-tagged autophagosomes and LysoTracker-stained lysosomes can be detected in SCRV-infected CPB cells, while mock cells exhibited almost no overlap. These results indicated that cells underwent a complete autophagic process following SCRV infection.

The levels of LC3-II, a form conjugated with phosphatidylethanolamine and present on membranes and autophagosomes, correlates with autophagy formation [23]. SCRV infection triggered the autophagy by detecting LC3 protein. As shown in Fig. 1D, compared to the mock group, LC3-II/LC3-I in the infection group was gradually decreased at 2 hpi and 4 hpi, but then LC3-II/LC3-I was progressively increased at 8 hpi. It indicated that SCRV infection inhibited autophagy at the early stage of life cycle (0–4 hpi), and promoted autophagy at the late stage of life cycle (8–12 hpi). Above results indicated that SCRV infection triggered autophagy in CPB cells.

To further clarify whether SCRV replication is required for the induction of autophagy, we inactivated SCRV by ultraviolet (UV) radiation and examined its capability of inducing autophagy. The results of three blind passages verified that UV-treated SCRV was inactivated completely (data not shown). As shown in Fig. 1E, the relative amounts of LC3-II were increased in CPB cells inoculated with UV-inactivated SCRV compared to the levels in mock-infected cells at 2 hpi, 4 hpi and 8 hpi, suggesting that SCRV replication is not essential for the induction of autophagy in CPB cells.

3.2. Autophagy inhibited SCRV replication in CPB cells

SCRV infection induced autophagy in CPB cells. Thus, we want to know whether SCRV replication is regulated by autophagy. The cytotoxicity test results showed that the CPB cells were inoculated with 500 nM Rapa, 500 μM 3-MA and 1 μM CQ, respectively, the cell viability was more than 70% (Fig. 2A). Considering that 3-MA effect of different treated concentration or time is uncertainty in different cells, and Rapa induced autophagic effect is not ideal in some cell lines [24], we explored the effective working conditions according to LC3 protein level. The results showed that Rapa and 3-MA had a strong effect at a higher working concentration of 0.5 μM and 0.5 mM, respectively (Fig. 2B). The treated time results showed that the inhibition effect of 3-MA with 3h pre-treatment was better and induction effect of Rapa with 12h pre-treatment is more obvious (Fig. 2C). Thus the working concentration and pre-treatment time of Rapa and 3-MA are 0.5 μM for 12h and 0.5 mM for 3h, respectively. CQ working concentration and pre-treatment time was 1 μM for 4h referring to our previous published paper [22].

As shown in Fig. 3A, compared to the SCRV-infected cells at 12 hpi, gene transcription of SCRV-G, SCRV-M, and SCRV-N was significantly inhibited in the Rapa-treated cells. On the contrary, gene transcription of SCRV-G, SCRV-M, and SCRV-N was significantly increased upon 3-MA or CQ treatment. SCRV-N protein level in the Rapa-treated group was significantly lower than that in the 3-MA or CQ treated group, which is consistent with the results of gene transcription level (Fig. 3B). Furthermore, viral titer results exhibited that titer of SCRV supernatant was increased after blocking autophagy by 3-MA or CQ (Fig. 3C). However, activating autophagy with Rapa reduced the SCRV titer (Fig. 3C). Together, these results indicated that autophagy inhibited SCRV replication in CPB cells.

3.3. SCRV infection induced autophagy by depressing PI3K/Akt/mTOR signaling pathway in CPB cells

Subsequently, we investigated whether PI3K/Akt/mTOR signaling pathway was involved in SCRV-induced autophagy. As shown in Fig. 4A, compared to the control groups, SCRV infection induced the up-regulation of p-PI3K, p-AKT, p-mTOR and down-regulation of LC3-II at 4 hpi, but the opposite result was observed at 8 hpi. Then, the PI3K inhibitor LY294002 (20 μM) and the activator 740Y–P (10 μM) were used to assess the effect of PI3K/Akt/mTOR on autophagy in CPB cells (Fig. 4B). In the 740Y–P treatment group, the expression level of p-PI3K, p-AKT, and p-mTOR was up-regulated but LC3-II was down-regulated, but in the LY294002 treatment group, the opposite result was observed. These results proved LY294002 and 740Y–P were effective in CPB cells and could be used for later experiments. Next, SCRV replication was determined in CPB cells treated with LY294002 and 740Y–P. As shown in Fig. 4C, compared to the control group, qRT-PCR and western blotting results showed that the SCRV/N mRNA and protein expression were significantly decreased in the 740Y–P group, but
A

Mock

Rapa

SCRV

B

GFP-LC3

DAPI

Merge

Mock

SCRV

Rapa

C

GFP-LC3

LysoTracker

Overlay

Mock

SCRV

(D)

LC3-I

LC3-II

β-Tubulin

2h 4h 8h

Mock SCRV Mock SCRV Mock SCRV

(D) LC3-I/LC3-I

2h 4h 8h

Mock SCRV

(E)

Mock Inactivated SCRV

LC3-I

LC3-II

β-Tubulin

2h 4h 8h

Mock SCRV

(E) LC3-II/β-Tubulin

2h 4h 8h

Mock SCRV

(caption on next page)
they were significantly increased in the LY294002 group, indicating that inhibiting PI3K can promote SCRV replication, but activating PI3K can inhibit SCRV replication. Above results indicated that SCRV infection induced autophagy via PI3K/Akt/mTOR signaling pathway.

4. Discussion

Autophagy is an evolutionarily conserved membrane-trafficking process and maintains the cellular metabolic homeostasis. Besides its role in healthy catabolic processes, many viruses induce autophagy for their own benefit during virus infection, such as spring viraemia of carp virus (SVCV), Newcastle disease virus (NDV), etc [11,28,29]. But some viruses block autophagy for their multiplication, such as canine distemper virus (CDV), human parainfluenza virus type 3 (HPIV3), etc [30,31]. Generally, it is well demonstrated that influenza A virus (IAV) infection triggers autophagosome formation for providing a replicative niche for IAV, but inhibits the fusion of autophagosomes with lysosomes avoiding the host's defenses [7]. In this study, we found that LC3-II/LC3-I in the SCRV infection group was firstly gradually decreased at 2 hpi and 4 hpi, but then progressively increased at 8 hpi. Our previous studies have showed that SCRV completed entering and transcription at the early stage of life cycle (0–4 hpi), and completed assembly and

Fig. 2. Optimization of concentration and treatment time of autophagy regulators. (A) The effects of autophagy regulators on CPB cell viability. CPB cells were cultured for 24 h, 48 h, 72 h, 96 h in the presence of 0, 25, 50, 100, 200, 500 nM rapamycin, 0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 mM 3-MA, or 0, 1, 5, 10 μM CQ. The cell viability was determined using the MTS assay. Three parallel samples were pooled as biological replicates. *p < 0.05; **p < 0.01. (B) Confirmation of the autophagy regulators working concentration. CPB cells were treated with 0.1, 0.2, 0.5 μM rapamycin, or 0.1, 0.2, 0.5 mM 3-MA. LC3 protein level was detected by western blotting. (C) Confirmation of the autophagy regulators treatment time. CPB cells were treated with 0.5 mM 3-MA for 2 h, 3 h, 4 h, or 0.5 μM rapamycin for 4 h, 12 h. LC3 protein level was detected by western blotting. CPB cells at 0 h post-treatment were used as mock.
cells [32]. These results indicated that SCRV infection inhibited autophagy at early stage of life cycle, but promoted autophagy at late stage of life cycle.

For host defense system, autophagy serves as an important function in innate immunity by eliminating intracellular pathogens. For example, herpes simplex virus-1 (HSV-1) activating host autophagy lead to its degradation in host cells [33]. Autophagy inhibits the replication of vesicular stomatitis virus (VSV) both in vitro and in vivo [34]. In this study, we found that SCRV multiplication (mRNA, protein and viral titer) was increased when inhibiting autophagy with 3-MA or CQ, but it was decreased when promoting autophagy with Rapa. Those indicated that induction of autophagy inhibited SCRV proliferation and inhibition of autophagy promoted SCRV proliferation, inferring autophagy in CPB cells played an antiviral role in SCRV infection. These results were similar to the researches on VHSV in ZF4 cells [10], and SHVV in SSN-1 cells [12].

Autophagy is negatively regulated by the PI3K-AKT-mTOR pathway [35]. Some studies have shown that activating PI3K/AKT pathway can promote viral infection and replication, such as porcine reproductive and respiratory syndrome virus (PRRSV) and Middle East respiratory syndrome coronavirus (MERS-CoV) [36]. It is reported that PI3K/Akt/mTOR signaling pathway participates in the process of autophagy induced by CVB3 infection, and PI3K inhibition alleviated autophagy and decreased CVB3 mRNA replication and VP1 expression [20]. In contrast, some viruses replications were inhibited by activating the PI3K-AKT-mTOR pathway, such as hepatitis E virus (HEV) [37]. In this paper, we found that PI3K/Akt/mTOR signaling pathway was activated at 4 hpi, but depressed at 8 hpi, accompanied with LC3-I/II-down-regulation at 4 hpi or up-regulation at 8 hpi. It indicated that SCRV infection promoted PI3K/Akt/mTOR signaling pathway at the early stage of life cycle, and inhibited this pathway at the late stage of life cycle, which was exactly contrary to the change trend of SCRV-induced autophagy. And SCRV-N mRNA and protein levels at 8 hpi were increased by activating PI3K with 740Y-P, but decreased by inhibiting PI3K with LY294002, which further indicated that SCRV infection induced autophagy via PI3K-AKT-mTOR pathway and activating the PI3K-AKT-mTOR pathway promoted SCRV replication.

In conclusion, the relationship between SCRV and autophagy was elicited based on the CPB cell model. Those results revealed that SCRV infection induced autophagy. Promotion of autophagy decreased SCRV multiplication, and inhibition of autophagy increased the SCRV proliferation. Furthermore it was showed that PI3K-Akt-mTOR signaling pathway participated in virus-induced autophagy. All of above results will provide new insights into SCRV pathogenesis and antiviral treatment strategies.

CRediT authorship contribution statement

Xiaozhe Fu: Methodology, Resources, Validation, Formal analysis, Data curation, Visualization, Writing - original draft. Yue Ming: Validation, Formal analysis, Data curation, Visualization, Writing - original draft. Chen Li: Validation, Formal analysis, Data curation, Visualization, Writing - original draft. Yinjie Niu: Methodology, Formal analysis, Writing - review & editing. Qiang Lin: Methodology, Resources. Lihui Liu: Resources. Hongru Liang: Methodology. Zhibin Huang: Methodology. Ningqiu Li: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Fig. 4. SCRV induced autophagy by activating PI3K/Akt-mTOR pathway. (A) Effect of SCRV infection on the PI3K/Akt-mTOR pathway. CPB cells were infected with SCRV and sampled at 4 hpi or 8 hpi. The expression level of p-PI3K, p-AKT, mTOR, p-mTOR, LC3 protein was determined by Western blotting. (B) The effects of PI3K inhibitor LY294002 and PI3K activator 740Y-P on PI3K/Akt-mTOR pathway. The cell viability treated with LY294002 and 740Y-P was determined using the MTS assay. Then CPB cells treated with LY294002 (20 μM) and 740Y-P (10 μM) were sampled at 8 hpi. The expression level of p-PI3K, p-AKT, mTOR, p-mTOR, LC3 protein was determined by Western blotting. (C) Effect on SCRV replication in CPB cells treated with LY294002 or 740Y-P. CPB cells treated with LY294002 or 740Y-P were infected with SCRV and sampled at 8 hpi. The expression level of SCRV-N mRNA and protein was determined by qRT-PCR and Western blotting. *p < 0.05; **p < 0.01.
