Rapamycin and ZSTK474 can have differential effects at different post-infection time-points regarding CVB3 replication and CVB3-induced autophagy

HUAN CHANG[1], LANG TIAN[1], JIA CHEN[1], ANLIU TANG[2], CHUNYUN LI[1], ZHUOYING LI[1] and ZUOCHENG YANG[1]

Departments of [1]Pediatrics and [2]Gastroenterology, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, P.R. China

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Abstract. Coxsackievirus B3 (CVB3) infection has been shown to stimulate autophagy. We have demonstrated that the inhibition of phosphoinositide 3-kinase (PI3K)/protein kinase B/mammalian target of rapamycin complex (mTORC) signaling pathway could affect the autophagic reaction induced by CVB3 infection in our previous study. However, the processes associating autophagy and CVB3 replication remain to be determined. In the present study, CVB3-induced autophagy and its impact on viral replication were investigated. Rapamycin (inhibitor of mTOR) and ZSTK474 (inhibitor of PI3K) were used to change the autophagic reaction caused by CVB3 in Hela cells at different post-infection (p.i.) time points (6, 9, 12 and 24 h p.i.), meanwhile, we detected the CVB3 mRNA replication and CVB3 capsid protein VP1 expression following the change of autophagy. Here, it was showed that ZSTK474 and Rapamycin promoted CVB3-induced autophagy, as well as decreasing CVB3 mRNA replication and CVB3 capsid protein VP1 expression at 6 and 9 h p.i. ZSTK474 also alleviated CVB3-induced autophagy, and decreased CVB3 mRNA replication and VP1 expression at 12 and 24 h p.i. However, Rapamycin continued to promote CVB3-induced autophagy and increase CVB3 mRNA replication at 12 and 24 h p.i, as well as increase VP1 expression at 12 h, but not at 24 h, p.i. In the present study, we found Rapamycin and ZSTK474 have differential effects at different p.i. time-points regarding CVB3 replication and CVB3-induced autophagy. This indicates that the association between CVB3-induced autophagy and viral replication depends on the infection time. During the early course of infection, autophagy may help host cells clear the virus, thereby providing protection, whereas when the infection time increases, autophagy may be exploited for viral replication.

Correspondence to: Professor Zuocheng Yang, Department of Pediatrics, The Third Xiangya Hospital, Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, P.R. China
E-mail: yang_zcr@126.com

Abbreviations: CPE, cytopathic effect; CVB3, Coxsackievirus B3; PI3K, Phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin

Key words: autophagy, Coxsackievirus B3, virus replication

Introduction

Autophagy is a membrane trafficking process that leads to the degradation of long-lived cytoplasmic proteins and excess or damaged organelles by lysosomal hydrolyases. The hallmark of autophagy is the emergence of double membrane vesicles called autophagosomes, which begins with the elongation of crescent-shaped double-membrane structures known as the phagophores, during which a portion of the cytoplasm, including organelles, is sequestrated. Matured autophagosomes eventually fuse with lysosomes, known as autolysosome, for protein and pathogen degradation and contents recycling (1). Macroautophagy (hereafter referred to as autophagy) is a homeostatic process in cells, whereby long-lived proteins and damaged organelles are degraded within lysosomes (2). Autophagy is an important physiological response that alters during exercise and over aging (3-6). It also serves as an important function in innate host defense by eliminating intracellular pathogens (7,8). Autophagy plays a key role in mediating ssRNA virus detection and interferon-alpha secretion by plasmacytoid dendritic cells (pDCs) (9,10), and contributes to the regulation and function of innate and adaptive immune responses (11). Autophagy genes have been shown to play a protective role *in vivo* against many of these pathogens. Disturbances in autophagy are widely implicated in many human diseases, including cancer, neurodegeneration and infectious disease, as well as cardiovascular and pulmonary disease (7,12-14). Autophagy also contributes to the regulation of innate and adaptive immune responses (7,8,15-20), and is implicated in clearance of pathogens and antigen presentation, thereby playing a protective role during many viral and bacterial infections (21-23). The mechanisms by which intracellular bacteria and viruses are targeted to autophagosomes for degradation have been extensively investigated (24,25), with

bacteria, virus and parasite autophagy termed Xenophagy (15). However, evolution has led to several viruses developing mechanisms to evade their inhibition by autophagy, with some virus able to subvert autophagy for their own replication (26). Coxsackieviruses B3 (CVB3) belongs to the Enterovirus genus, within the Picornaviridae family. CVB3 has a positive-stranded RNA genome packaged in a nonenveloped icosahedral viral capsid and infects cells via the coxsackievirus and adenovirus receptor (CAR) or decay-accelerating factor (DAF). CVB3 entry induces a direct cytopathic effect (CPE) and even cell death in host cells (27,28). CVB3 can induce autophagic reaction *in vivo* and *in vitro* (29-33), although the processes linking CVB3-induced autophagy and its impact on CVB3 replication still have to be determined.

Materials and methods

Western blot analysis. Western blotting was performed as described previously (34). Polyclonal antibody against LC3-I/II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and antibody against β-actin (Proteintech Group, Inc., Chicago, IL, USA) were used at a dilution of 1:1,000. Monoclonal anti-enterovirus antibody (Dako Co.; Agilent Technologies, Inc. Inc., Santa Clara, CA, USA) was used at a dilution of 1:100. Goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Cwbio) and goat anti-rabbit IgG horseradish peroxidase-Cwbio were used at a dilution of 1:2,000. Rapamycin was used at a concentration of 10 nM and ZSTK474 (both Selleck Chemicals, Houston, TX, USA) was used at a concentration of 50 nM.

Virus infection. CVB3 was propagated in HeLa cells in DMEM medium supplemented with 2% FBS, and the virus titer was routinely determined by plaque assay. HeLa cells were maintained in DMEM medium containing 10% FBS and infected with CVB3 at 10 tissue culture infective dose (TCID50) values, then cells were harvested at different post-infection (p.i.) time-points for subsequent experiments. In drug intervention groups, HeLa cells were pretreated with the Rapamycin or ZSTK474 for 2 h before viral infection, and DMEM contained 10% FBS acted as a sham.

Cell transfection. HeLa cells that stably expressed GFP-LC3 were produced by transfecting HeLa cells with pEGFP-LC3 followed by Geneticin (G418) selection. 4 mg of expression plasmid combined with 10 μl Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added to HeLa cells according to the manufacturer's instructions. At 5 h post-transfection, the cells were refreshed by the DMEM containing 10% FBS. 24 h later, G418 was added at concentrations of 1,000 and 500 μg/ml for maintenance of transfection.

Semi-quantitative PCR. Extraction of total RNA reverse transcription was performed following the method of the RNA Extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). cDNA was subjected to Semi-quantitative PCR with the following primers: CVB3 [referring to Li *et al* (34)] forward, 5'-TGGTGGGCTATG GAGTATGG-3' and reverse, 5'-CACTGGATGGG GTGTTGT CT-3' and β-actin forward, 5'-CTAAGGCCAACCGTGAAA AGATGAC -3' and reverse, 5'-TGGGTACATGGTGGTGCC ACCAGAC-3'. The amplification profile was 2 min and 30 sec at 95˚C, 40 sec at 94˚C, 40 sec at 59˚C and 72˚C at 40 sec for 35 cycles, 5 min at 72˚C.

Statistical analysis. All data are presented as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance followed Duncan's post hoc analysis. A $P<0.05$ was considered to be indicate a statistically significant difference.

Results

Rapamycin and ZSTK474 promote CVB3-induced CPE. The mTOR signal pathway modulates viral replication (29). In order to elucidate the role of the mTOR pathway in CVB3-induced CPE, HeLa cells were pretreated with Rapamycin (10 nM) or ZSTK474 (50 nM) or 0.1% DMSO for 2 h before viral infection, with DMEM containing 10% FBS acting as a sham. 2 h later, CBV3 infected cells at 10 TCID50, were observed by microscope for CPE at 6 h p.i., 9 h p.i., 12 h p.i., and 24 h p.i. At 12 h p.i, the infected cells changed in morphology, as indicated by cell rounding, cell shrinkage, cell floating and cell detachment, which was more obvious in cells pretreated with Rapamycin or ZSTK474 (Fig. 1).

Role of Rapamycin and ZSTK474 in CVB3-induced autophagy. Our previous work has demonstrated that pretreating the virus-infected HeLa cells with Rapamycin and ZSTK474 impacts on CVB3-induced autophagy. In order to investigate any temporal aspects to this, we dealt with the HeLa cells with Rapamycin (10 nM) or ZSTK474 (50 nM) or 0.1% DMSO following the method described in paragraph 3.1. 2 h later, we infected the cells with CVB3 at 10 TCID50. At 6, 9, 12 and 24 h p.i, cells were harvested for western blotting. GFP-LC3 dots formation was also observed with fluorescent microscopy. At 6 h p.i., we observed an obvious LC3II/LC3I ratio increase and GFP-LC3 dots formation in CVB3-infected groups. Rapamycin increased CVB3-induced autophagy over all time-points, as indicated by increased LC3II/LC3I ratio and the green fluorescent dots formation vs. the DMSO group ($P<0.05$). At 6 and 9 h p.i., ZSTK474 also increased CVB3-induced LC3II/LC3I ratio and increased the GFP-LC3 dots formation. However, at 12 and 24 h p.i., ZSTK474 invert this effect on virus infected cells (Fig. 2).

Role of Rapamycin and ZSTK474 in virus replication. To further observe the association of autophagy and virus replication at different infected time, HeLa cells were pretreated with Rapamycin or ZSTK474 or DMSO for 2 h just as described in paragraph 3.1, then the cells were infected with CVB3 at 10 TCID50, and harvested at 6, 9, 12 and 24 h p.i, for CVB3 mRNA and viral capsid protein VP-1 measurement by semi-quantitative PCR and Western blot analysis (Fig. 3). At 6 and 9 h p.i, Rapamycin and ZSTK474 increased CVB3-induced autophagy as well as decreasing VP-1 protein expression and CVB3 mRNA synthesis ($P<0.05$). At 12 and 24 h p.i, ZSTK474 mitigated CVB3-induced autophagy as well as decreasing

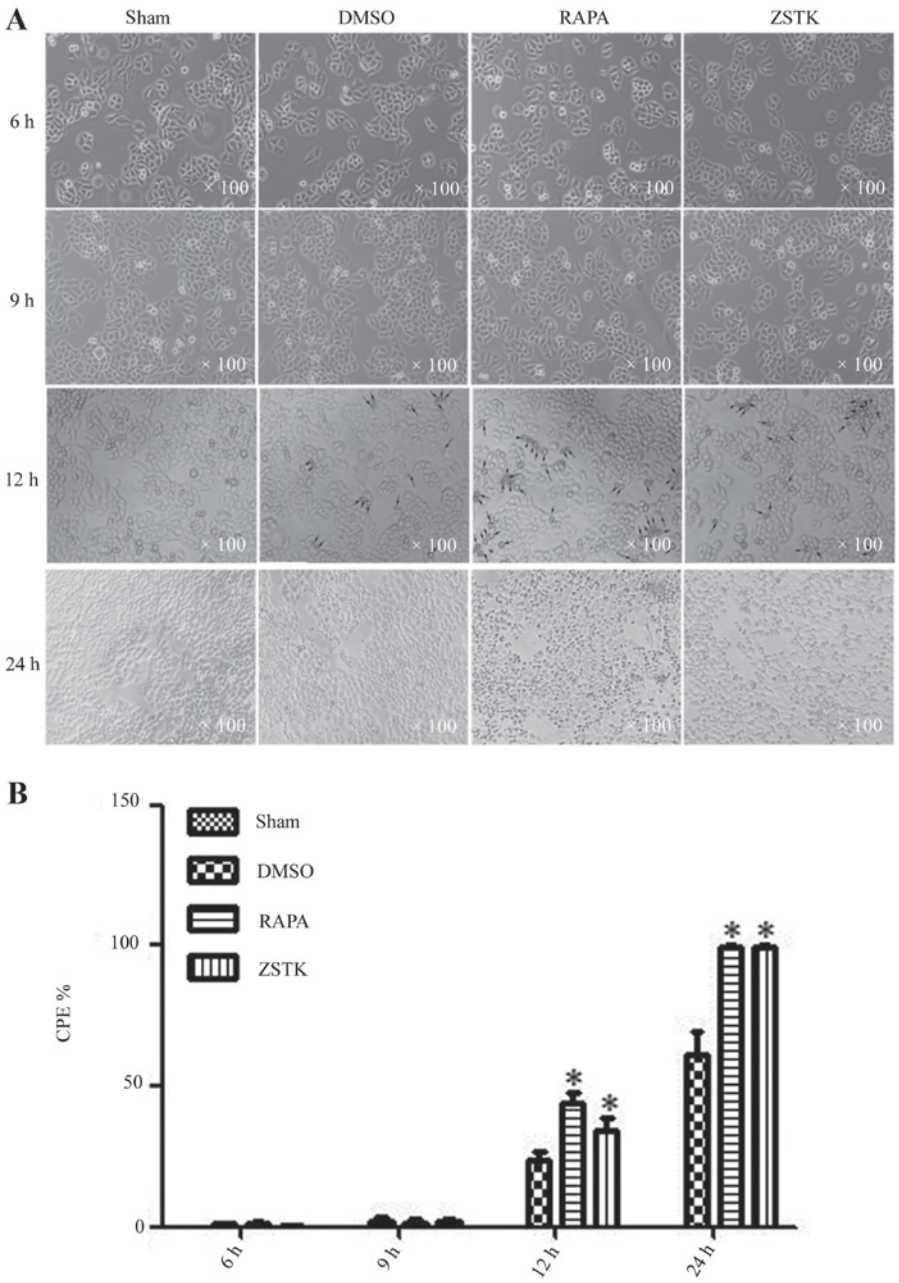

Figure 1. Rapamycin and ZSTK474 promote CVB3-induced CPE. Rapamycin and ZSTK474 have impacts on GFP-LC3 dots formation after CVB3 infection. HeLa cells were divided into sham, Rapamycin (10 nM), ZSTK474 (50 nM), and DMSO (0.1%) groups, then infected with CVB3 at 10 TCID50. (A) Collected at 6, 9, 12, and 24 h p.i. CPE was determined by cellular change under microscopy. (B) There was no obvious CPE at 6 and 9 h p.i., while it could be seen at 12 and 24 h p.i. Compared with the control/DMSO group, CPE at 12 and 24 h p.i was more evident in the Rapamycin and ZSTK474 group. *P<0.05 compared with the control group. CVB3, coxsackievirus B3; CPE, cytopathic effect; TCID50, tissue culture infective dose.

VP-1 protein expression and CVB3 mRNA synthesis (P<0.05). At 12 and 24 h p.i., Rapamycin increased CVB3-induced autophagy and promoted CVB3 mRNA synthesis (P<0.05), whilst at 12 h (P<0.05), but not 24 h (P>0.05), p.i., Rapamycin increased VP-1 protein expression.

Discussion

Since autophagy serves as an important function in innate host defense by eliminating intracellular pathogens, Predictably, evolution has led to several viruses developing mechanisms by which to evade the inhibitory effects of the autophagy. Investigating the course of autophagy is therefore of some importance. The present study demonstrates differential temporal effects of important intracellular pathways in CVB3-induced autophagy and virus replication. Firstly, autophagy was determined by the microscopic observation of the cytopathetic effect, the calculation of the LC3II/LC3I ratio and the counting of GFP-LC3 dots. CVB3 replication was analysed by measuring VP-1 protein expression and CVB3 mRNA synthesis. CVB3 clearly provoked autophagy, as indicated by an increased LC3II/LC3I ratio and GFP-LC3 dots count after infection. Rapamycin promoted CVB3-induced autophagy during all p.i. time-points, whilst ZSTK474 also increased CVB3-induced autophagy at 6 and 9 h p.i, but not at 12 and 24 h p.i. CVB3 virus infection

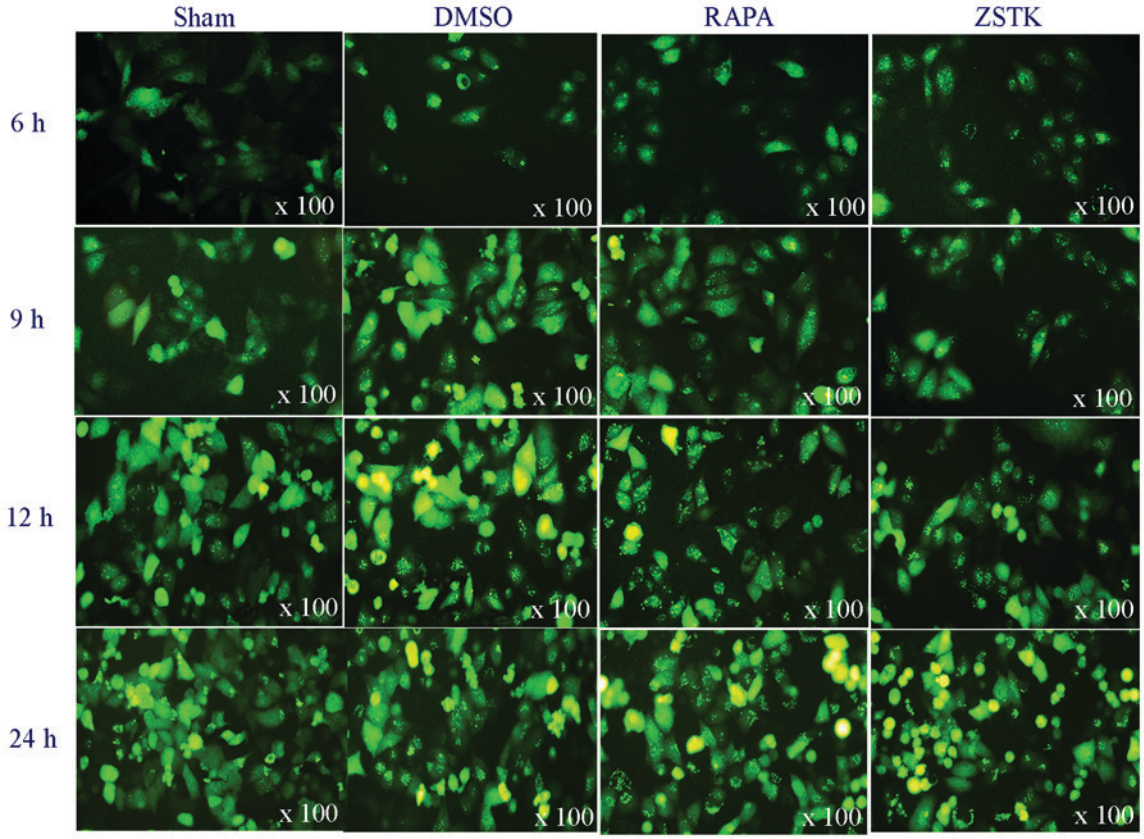

Figure 2. Rapamycin and ZSTK474 have impacts on GFP-LC3 dots formation after CVB3 infection. HeLa cells were divided into sham, Rapamycin (10 nM), ZSTK474 (50 nM), and DMSO (0.1%) groups, then infected with CVB3 at 10 TCID50. Collected at 6, 9, 12, and 24 h p.i, GFP-LC3 dots formation was observed under fluorescent microscopy. At 6 h p.i, GFP-LC3 dots formed in virus infected cells. When compared with control/DMSO group, the dots were more obvious in the Rapamycin group, and at 6 and 9 h p.i, the GFP-LC3 dot numbers increased in ZSTK474 group, vs. the control group. At 12 and 24 h p.i, the GFP-LC3 dot numbers decreased in the ZSTK474 group, vs. the control group. CVB3, coxsackievirus B3; CPE, cytopathic effect; TCID50, tissue culture infective dose.

can lead to the cleaving of the RasGAP protein, in turn driving activation of the Ras/Raf/mitogen/extracellular signal-regulated kinase (MEK)/ERK pathway, in association with increasing the levels of cytoplasmic calcium, resulting in mitochondrial damage, a decrease in ATP concentration and the activation of the AMP-activated protein kinase (AMPK)/MEK/ERK pathway (35). As our previous work indicated that the phosphoinositide 3-kinase (PI3K)/AKT/mTOR signal pathway is involved in CVB3-induced autophagy, it seems likely that CVB3-driven autophagy involves the interaction of multiple signaling pathways.

Previous research indicates that both the mTOR inhibitor, Rapamycin, and PI3K inhibitor, LY294002, can promote CVB3-induced CPE (29). The MEK/ERK pathway and the PI3K/Akt pathway are known to interact in some circumstances, with the inhibition of one leading to the activation of the other (36). Rapamycin can provoke autophagy by the modulation of rapamycin complex (mTORC)1, which also plays a role in the regulation of mTORC2. Being upstream of mTORC1, the inhibition of mTORC2 could inhibit mTORC1, thereby further aggravating the autophagic reaction induced by CVB3 infection. What's more, it may be speculated that the inhibition of mTORC1 with Rapamycin may drive feedback on the MEK/ERK signaling pathway, thereby further promoting autophagy following CVB3 infection. However, at 24 h p.i., we did not find Rapamycin to affect VP-1 protein expression, possibly as a consequence of energy and amino acid loss following virus infection, with mTOR being further inhibited by Rapamycin, resulting in the decreased phosphorylation of 4EBP1 and the inhibition of cap-dependent translation. ZSTK474 aggravated autophagy by inhibiting the PI3K signaling pathway initially. However, as infection time increased, the continuous consumption of energy and cellular content may lead to changes in the signaling pathways involved in CVB3-induced autophagy. ZSTK474 may block PI3K completely so that CVB3 can not trigger autophagy through the PI3K/AKT/mTOR signal pathway, under energy deprivation or amino acid loss, although PI3KC3 can trigger autophagy directly. Consequently, future research should determine as to whether the PI3KC3 pathway takes part in the later CVB3-induced autophagy, as well as to whether ZSTK474 can also have effects on PI3KC3.

In the present study, we showed that ZSTK474 and Rapamycin could promote CVB3-induced autophagy, decrease CVB3 mRNA replication and VP1 expression at 6 and 9 h p.i. When combined with our previous work showing that p62 protein expression decreases at the beginning of CVB3 infection, this may be taken to indicate that CVB3-induced autophagy can help host cells to initially clear the virus, and therefore to be initially protective. As infection time increases, we found changes in the processes linked to autophagy, with CVB3 mRNA replication and VP1 expression at 12 and 24 h.p.i altered. When combined with our previous work

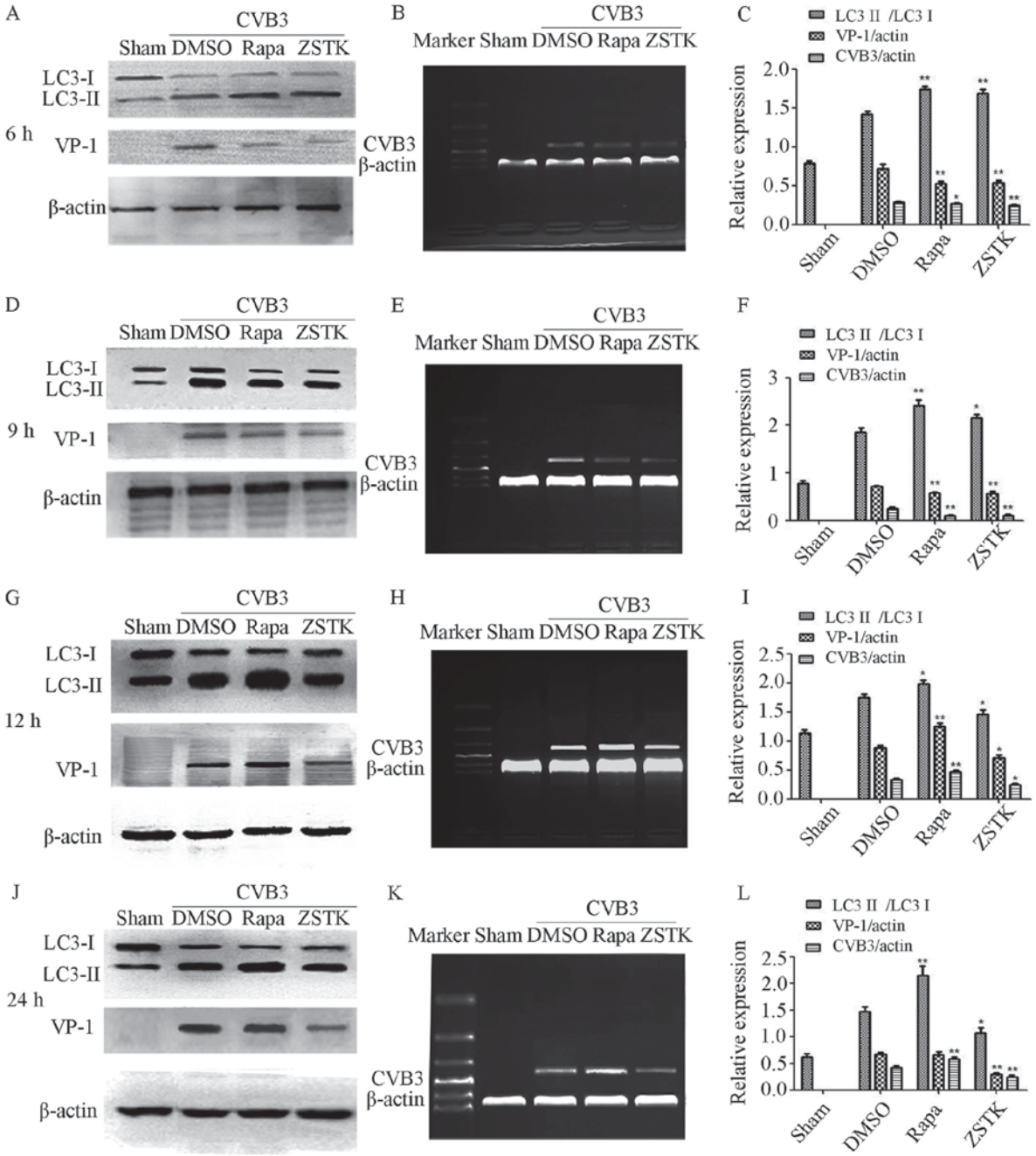

Figure 3. Role of autophagy in CVB3 replication. HeLa cells were divided into sham, Rapamycin (10 nM), ZSTK474 (50 nM), and DMSO (0.1%) groups, then infected with CVB3 at 10 TCID50 and harvested at 6, 9, 12 and 24 h p.i for CVB3 mRNA and viral capsid protein VP-1 analysis. (A) The protein change at 6 h p.i in different group, shows the LC3II/LC3I ratio increased in all virus infected group, Rapamycin and ZSTK474 increased the LC3II/LC3I ratio further, accompanied by decreased VP-1 protein expression. (B) Rapamycin and ZSTK474 decreased CVB3 mRNA synthesis at 6 h p.i. (C) The statistical outcome at 6 h p.i. in the different groups. (D) The protein change at 9 h p.i in different groups, LC3II/LC3I ratio increased in all virus infected group. Rapamycin and ZSTK474 increased the LC3II/LC3I ratio further, accompanied by decreased VP-1 protein expression. (E) Rapamycin and ZSTK474 could decrease CVB3 mRNA synthesis at 9 h p.i. (F) The statistical outcome at 9 h p.i. in the different groups. (G) The protein change at 12 h p.i in the different groups, shows the LC3II/LC3I ratio increased in all virus infected group. Rapamycin increase the LC3II/LC3I ratio and promoted VP-1 protein expression, in contrast to ZSTK474, which decreased the LC3II/LC3I ratio and VP-1 protein expression. (H) Rapamycin promoted CVB3 mRNA synthesis but ZSTK474 weaken it at 12 h p.i. (I) The statistical outcome at 12 h p.i. in the different groups. (J) The protein change at 24 h p.i. Rapamycin increased the LC3II/LC3I ratio, in contrast to ZSTK474, which decreased the LC3II/LC3I ratio and VP-1 protein expression. (K) When compared with control/DMSO group, Rapamycin promoted CVB3 mRNA synthesis, with ZSTK474 weakening it at 24 h p.i. (L) The statistical outcome at 24 h p.i. in the different groups. *P<0.05 compared with the control group. **P<0.05 compared with the control group. CVB3, coxsackievirus B3; TCID50, tissue culture infective dose.

showing that the p62 protein expression increased gradually following CVB3 infection, we speculate that following CBV3 virus infection, the accumulated virus and damaged organelles exceed the ability of lysosome to cope, coupled to the direct damage of the virus to lysosome, all leading to a change of autophagic flux, resulting in an incomplete autophagy process. Subsequently, autophagy can be detrimental, which might be exploited for viral replication. Our findings may supply a clue that autophagy has been implicated as a mechanism for the CVB3 induced myocarditis during the virus infection.

The role of autophagy during this process may be of great importance to the resultant of the disease. At the beginning, the role of autophagy may be protective helping host cells clear the virus, alternations in autophagy following CVB3 infection may play a role in the viral persistence and pathogenesis in the host cells.

In conclusion, we demonstrate that two important intracellular pathways can have differential effects at different time-points in CVB3-induced autophagy, contributing to autophagy initially helping host cells clear the virus, but being detrimental at later time-points, which may be exploited for viral replication.

Our research indicates Rapamycin and ZSTK474 can have differential effects at different p.i. time-points regarding CVB3 replication and CVB3-induced autophagy. Early during the course of infection, autophagy may help host cells clear the virus, thereby affording protection, whereas when infection time increases, autophagy may be exploited for viral replication.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

HC and ZY designed the study, analyzed the data and wrote the manuscript. HC, LT, JC, AT, CL, ZL and ZY were involved in data interpretation, discussion and preparation of the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Mizushima N: Autophagy: Process and function. Genes Dev 21: 2861-2873, 2007.
2. Dice JF: Chaperone-mediated autophagy. Autophagy 3: 295-299, 2007.
3. Pauly M, Assense A, Rondon A, Thomas A, Dubouchaud H, Freyssenet D, Benoit H, Castells J and Flore P: High intensity aerobic exercise training improves chronic intermittent hypoxia-induced insulin resistance without basal autophagy modulation. Sci Rep 7: 43663, 2017.
4. Eisenstein M: Molecular biology: Remove, reuse, recycle. Nature 514: S2-S4, 2014.
5. Garber K: Autophagy. Explaining exercise. Science 335: 281, 2012.
6. García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, Ruiz-Bonilla V, Gutarra S, Ballestar E, Serrano AL, *et al*: Autophagy maintains stemness by preventing senescence. Nature 529: 37-42, 2016.
7. Levine B, Mizushima N and Virgin HW: Autophagy in immunity and inflammation. Nature 469: 323-335, 2011.
8. Randow F and Münz C: Autophagy in the regulation of pathogen replication and adaptive immunity. Trends Immunol 33: 475-487, 2012.
9. Lee HK, Lund JM, Ramanathan B, Mizushima N and Iwasaki A: Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 315: 1398-1401, 2007.
10. Su C, Zhan G and Zheng C: Evasion of host antiviral innate immunity by HSV-1, an update. Virol J 13: 38, 2016.
11. Kudchodkar SB and Levine B: Viruses and autophagy. Rev Med Virol 19: 359-378, 2009.
12. Liao YH, Xia N, Zhou SF, Tang TT, Yan XX, Lv BJ, Nie SF, Wang J, Iwakura Y, Xiao H, *et al*: Interleukin-17A contributes to myocardial ischemia/reperfusion injury by regulating cardiomyocyte apoptosis and neutrophil infiltration. J Am Coll Cardiol 59: 420-429, 2012.
13. O'Connell D and Liang C: Autophagy interaction with herpes simplex virus type-1 infection. Autophagy 12: 451-459, 2016.
14. Doria A, Gatto M and Punzi L: Autophagy in human health and disease. N Engl J Med 368: 1845, 2013.
15. Deretic V and Levine B: Autophagy, immunity, and microbial adaptations. Cell Host Microbe 5: 527-549, 2009.
16. Virgin HW and Levine B: Autophagy genes in immunity. Nat Immunol 10: 461-470, 2009.
17. Fader CM, Aguilera MO and Colombo MI: Autophagy response: Manipulating the mTOR-controlled machinery by amino acids and pathogens. Amino Acids 47: 2101-2112, 2015.
18. Zhou XJ and Zhang H: Autophagy in immunity: Implications in etiology of autoimmune/autoinflammatory diseases. Autophagy 8: 1286-1299, 2012.
19. Scharl M and Rogler G: Inflammatory bowel disease: Dysfunction of autophagy? Dig Dis 30 (Suppl 3): S12-S19, 2012.
20. Puri P and Chandra A: Autophagy modulation as a potential therapeutic target for liver diseases. J Clin Exp Hepatol 4: 51-59, 2014.
21. Zhong L, Hu J, Shu W, Gao B and Xiong S: Epigallocatechin-3-gallate opposes HBV-induced incomplete autophagy by enhancing lysosomal acidification, which is unfavorable for HBV replication. Cell Death Dis 6: e1770, 2015.
22. De Leo A, Colavita F, Ciccosanti F, Fimia GM, Lieberman PM and Mattia E: Inhibition of autophagy in EBV-positive Burkitt's lymphoma cells enhances EBV lytic genes expression and replication. Cell Death Dis 6: e1876, 2015.
23. Li J, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H and Yuan Z: Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol 85: 6319-6333, 2011.
24. Watson RO, Manzanillo PS and Cox JS: Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell 150: 803-815, 2012.
25. Orvedahl A, Sumpter R Jr, Xiao G, Ng A, Zou Z, Tang Y, Narimatsu M, Gilpin C, Sun Q, Roth M, *et al*: Image-based genome-wide siRNA screen identifies selective autophagy factors. Nature 480: 113-117, 2011.
26. Liu J, Wang H, Gu J, Deng T, Yuan Z, Hu B, Xu Y, Yan Y, Zan J, Liao M, *et al*: BECN1-dependent CASP2 incomplete autophagy induction by binding to rabies virus phosphoprotein. Autophagy 13: 739-753, 2017.
27. Garmaroudi FS, Marchant D, Hendry R, Luo H, Yang D, Ye X, Shi J and McManus BM: Coxsackievirus B3 replication and pathogenesis. Future Microbiol 10: 629-653, 2015.
28. Sin J, Mangale V, Thienphrapa W, Gottlieb RA and Feuer R: Recent progress in understanding coxsackievirus replication, dissemination, and pathogenesis. Virology 484: 288-304, 2015.

29. Chen Z, Yang L, Liu Y, Tang A, Li X, Zhang J and Yang Z: LY294002 and Rapamycin promote coxsackievirus-induced cytopathic effect and apoptosis via inhibition of PI3K/AKT/mTOR signaling pathway. Mol Cell Biochem 385: 169-177, 2014.
30. Zhang HM, Ye X, Su Y, Yuan J, Liu Z, Stein DA and Yang D: Coxsackievirus B3 infection activates the unfolded protein response and induces apoptosis through downregulation of p58IPK and activation of CHOP and SREBP1. J Virol 84: 8446-8459, 2010.
31. Zhai X, Bai B, Yu B, Wang T, Wang H, Wang Y, Li H, Tong L, Wang Y, Zhang F, *et al*: Coxsackievirus B3 induces autophagic response in cardiac myocytes in vivo. Biochemistry (Mosc) 80: 1001-1009, 2015.
32. Xin L, Xiao Z, Ma X, He F, Yao H and Liu Z: Coxsackievirus B3 induces crosstalk between autophagy and apoptosis to benefit its release after replicating in autophagosomes through a mechanism involving caspase cleavage of autophagy-related proteins. Infect Genet Evol 26: 95-102, 2014.
33. Robinson SM, Tsueng G, Sin J, Mangale V, Rahawi S, McIntyre LL, Williams W, Kha N, Cruz C, Hancock BM, *et al*: Coxsackievirus B exits the host cell in shed microvesicles displaying autophagosomal markers. PLoS Pathog 10: e1004045, 2014.
34. Li X, Li Z, Zhou W, Xing X, Huang L, Tian L, Chen J, Chen C, Ma X and Yang Z: Overexpression of 4EBP1, p70S6K, Akt1 or Akt2 differentially promotes Coxsackievirus B3-induced apoptosis in HeLa cells. Cell Death Dis 4: e803-e809, 2013.
35. Xin L, Ma X, Xiao Z, Yao H and Liu Z: Coxsackievirus B3 induces autophagy in HeLa cells via the AMPK/MEK/ERK and Ras/Raf/MEK/ERK signaling pathways. Infect Genet Evol 36: 46-54, 2015.
36. Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, Januario T, Savage H, Punnoose E, Truong T, *et al*: In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin Cancer Res 15: 4649-4664, 2009.