

Research Article

Cite this article: Qi M *et al.* (2022).

Streptococcus agalactiae-induced autophagy of bovine mammary epithelial cell via PI3K/AKT/mTOR pathway. *Journal of Dairy Research* **89**, 178–184. <https://doi.org/10.1017/S0022029922000243>

Received: 22 November 2021

Revised: 6 January 2022

Accepted: 24 January 2022

First published online: 7 April 2022

Keywords:

Autophagy; bovine mammary epithelial cell; intracellular survival; mastitis; *Streptococcus agalactiae*

Authors for correspondence:

Yongxia Liu, Email: liyongxia@sdau.edu.cn

Jianzhu Liu, Email: liujz@sdau.edu.cn

Streptococcus agalactiae-induced autophagy of bovine mammary epithelial cell via PI3K/AKT/mTOR pathway

Mengzhu Qi¹, Hao Geng¹, Na Geng¹, Yukun Cui¹, Changxi Qi¹, Guodong Cheng¹, Kaimin Song¹, Liping Hu³, Yongxia Liu^{1,2}, Jianzhu Liu¹ and Bo Han⁴

¹College of Veterinary Medicine, Shandong Agricultural University, Tai'an, Shandong 271018, China; ²Research Center for Animal Disease Control Engineering, Shandong Agricultural University, Tai'an, Shandong 271018, China; ³Shandong Provincial Center for Animal Disease Control and Prevention, Jinan, Shandong 251000, China and ⁴College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

Abstract

Streptococcus agalactiae (*S. agalactiae*) infection is a significant cause of mastitis, resulting in loss of cellular homeostasis and tissue damage. Autophagy plays an essential function in cell survival, defense, and the preservation of cellular homeostasis, and is often part of the response to pathogenic challenge. However, the effect of autophagy induced by *S. agalactiae* in bovine mammary epithelial cells (bMECs) is mainly unknown. So in this study, an intracellular *S. agalactiae* infection model was established. Through evaluating the autophagy-related indicators, we observed that after *S. agalactiae* infection, a significant quantity of LC3-I was converted to LC3-II, p62 was degraded, and levels of Beclin1 and Bcl2 increased significantly in bMECs, indicating that *S. agalactiae* induced autophagy. The increase in levels of LAMP2 and LysoTracker Deep Red fluorescent spots indicated that lysosomes had participated in the degradation of autophagic contents. After autophagy was activated by rapamycin (Rapa), the amount of p-Akt and p-mTOR decreased significantly, whilst the amount of intracellular *S. agalactiae* increased significantly. Whereas the autophagy was inhibited by 3-methyladenine (3MA), the number of intracellular pathogens decreased. In conclusion, the results demonstrated that *S. agalactiae* could induce autophagy through PI3K/Akt/mTOR pathway and utilize autophagy to survive in bMECs.

Mastitis represents a widespread disease of the mammary gland parenchyma of cows (Seegers *et al.*, 2003; Geng *et al.*, 2020b). It has a high incidence rate and prevalence in dairy cattle and causes substantial monetary losses in dairy farming globally. Mastitis is primarily driven by mechanical damage, environmental influences and especially by pathogenic microbes such as *Streptococcus agalactiae* (*S. agalactiae*) (Tong *et al.*, 2020). Bovine mammary epithelial cells (bMECs) have been proven to cope with bacterial invasion and are a significant part of the defense against mastitis and *S. agalactiae* (Niu *et al.*, 2020). *S. agalactiae* (also called group B streptococcus) is a Gram-positive coccus (De Gaetano *et al.*, 2018) which can adhere to the surface of cells and produce virulence factors that impact on the normal functioning of bMECs, in severe cases causing cell death (Pang *et al.*, 2017).

In recent years, autophagy has been considered as an essential function for organic cells to remove intracellular foreign matter (Geng *et al.*, 2020a). When the cells are stimulated, autophagy-related 13 (Atg13) would be hypophosphorylated. Subsequently, Atg proteins extract lipids from various sources, such as Golgi bodies and endoplasmic reticulum. Then, double-layered, crescent-shaped membranes are formed around the invading pathogens, the whole structure being known as a phagophore. The nascent phagophore is matured through the interaction of phosphatidylinositol 3-phosphate (PI3P), Beclin-1 and other proteins, forming an autophagosome. Mature autophagosomes are anchored to lysosomes and undergo membrane fusion to produce autolysosomal hybrid structures. Invading pathogens are degraded in acidic lysosomal compartments (Mukhopadhyay *et al.*, 2014) together with obsolete proteins and organelles (Maejima *et al.*, 2013; Mukhopadhyay *et al.*, 2014). It has been shown that *S. agalactiae* can adhere to the surface of bMECs (Tong *et al.*, 2020). The research of De Gaetano *et al.* (2018) suggested that *S. agalactiae* exploited vitronectin on the cell surface to trigger an internalization response and enter cells. It has been reported that *S. agalactiae* can generate enrichment of the proteins related to autophagy in tilapia leukocytes to affect autophagy (Zhao *et al.*, 2019). When autophagy occurs, the complex of Beclin1 and B-cell lymphoma 2 (Bcl2) will be dissociated, resulting in increased levels of Beclin1 and Bcl2 (Mukhopadhyay *et al.*, 2014).

Despite this background knowledge, autophagy of bMECs induced by *S. agalactiae* has not been covered. This study aimed to systematically describe the distinguishing features of

autophagy induced by *S. agalactiae* in bMECs and the correlation between intracellular survival of *S. agalactiae* and autophagy by testing changes in autophagy flux, assessing lysosome functions, and exploring the influence of *S. agalactiae* on the related pathways.

Materials and methods

Bacterial strains and culture conditions

S. agalactiae (ATCC27956) was employed in this experiment. First of all, the bacterial strain was inoculated in brain heart infusion broth (BHI) agar at 37 °C. After 48 h, a single bacterial colony of *S. agalactiae* was stochastically chosen and cultured in BHI and 24 h later, cells were harvested. The concentration of *S. agalactiae* was calculated by colony-counting methods.

Cell culture conditions

The bovine mammary epithelial cells (bMECs) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO₂ incubator at 37 °C. Cells that cultured to 80% fusions were used in the following experiments.

Intracellular infection model

bMECs were inoculated in tissue culture plates, and when the cells covered 80% of the culture plate, *S. agalactiae* was added at a multiplicity of infection of 50 : 1 (Niu *et al.*, 2020). After 2 h of incubation, the cells were supplied with a culture medium which contained 100 µg/ml lysozyme and 250 µg/ml ampicillin sodium. Thirty minutes later, the cells were rinsed at least three times using phosphate-buffered saline (PBS) to remove extracellular adherent bacteria. The extracellular medium was collected and cultured on a bacterial plate medium to ensure that extracellular bacteria were killed. The bMECs were again rinsed with PBS to dislodge extracellular adhered bacteria and cultured in 2% FBS-DMEM.

Cell viability assay

Cell viability was determined by the cell counting kit (CCK-8) method according to the manufacturer's instructions. bMECs were seeded into 96-well plates (1 × 10⁵ cells/well) with 100 µL per well of FBS-DMEM. Twelve hours later, cells were infected by *S. agalactiae* (multiplicity of infection = 50 : 1) for 1, 2, 4, and 6 h. After incubation, the extracellular culture media were removed and cells were gently rinsed three times with PBS. Then, 10 µl of CCK-8 operating fluid was mixed into each well, and plates were incubated for 2 h at 37 °C. Cells deprived of lysozyme and ampicillin sodium were used as a control for antibiotic addition. Non-treated cells incubated simultaneously were used as control groups. The absorbance was read at 450 nm by the Thermo Scientific Microplate Reader (Sunrise, Salzburg, Austria).

Immunoblot assay

The protein levels of sequestome 1 (p62), Beclin1, Bcl2, the serine/threonine-protein kinase p-Akt/Akt, and mammalian target of rapamycin (p-mTOR/mTOR) were analyzed quantitatively by immunoblot. The bMECs were collected gently and split on ice

in radio immunoprecipitation assay lysis buffer for 20 min. Lysates were then centrifuged at 12 000 × g for 15 min. The supernatant was collected and the protein consistency was measured by the BCA protein assay kit on the basis of the instructions. Through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins of various molecular weights were uncoupled. After being transferred to 0.45 µm polyvinylidene fluoride (PVDF) membranes using an electric transfer, the proteins were sealed with 5% skimmed milk for 1.5 h at RT. Next, the imprints was cultured with the primary antibody overnight at 4 °C, and fostered with secondary antibody at room temperature for 2 h. The images were detected by hypersensitive enhanced chemiluminescence (ECL) reagents. Immunoblots were scanned, and gray image value was analyzed using the ImageJ software.

Fluorescence microscopy

bMECs were cultivated on sterile glass sheets placed in 24-well plates. Then *S. agalactiae* was added and incubated for 2 h. Harvested cells were fixed with 4% tissue fixative for 15 min, then 0.2% Triton X-100 was added for 20 min to permeate the cells. After washing with PBS, the cells were sealed at room temperature in 5% bovine serum albumin (BSA) blocking buffer for 30 min. For immunostaining, cells were incubated overnight at 4 °C with the primary antibodies, and then incubated with secondary antibody for 1 h at room temperature. DAPI We used 4',6-diamidino-2-phenylindole (DAPI) to dye cellular and bacterial DNA. Finally, images were presented on the Leica TCS SPE confocal microscope with a 100 × oil immersion objectives.

Lysotracker deep red staining

bMECs grown on sterile glass sheets were interacted with 100 nM LysoTracker Deep Red at room temperature for 30 min after being exposed to the environment of *S. agalactiae*. Fluorescence images were examined under the spectral confocal microscope.

Intracellular survival assay

To study the influence of autophagy on the survival of *S. agalactiae* in bMECs, cells were treated with autophagy regulators such as 200 µM 3-methyladenine (3MA) and 100 nM rapamycin (Rapa) (Geng *et al.*, 2020a) while adding bacteria in the subsequent experiments. After 4, 8, 12 h, cells were rinsed with PBS and incubated with 0.1% Triton X-100 for 10 min. Intracellular *S. agalactiae* was released and collected. The number of *S. agalactiae* surviving in cells was calculated using colony-counting methods.

Statistical analysis

The values were analyzed using the GraphPad Prism 7 software. All data are displayed as the mean ± SEM. The significance of a difference between different groups was confirmed by one-way ANOVA with SPSS software analysis. Differences in the data were considered statistically significant at $P < 0.05$.

Results

Cell infection model successfully constructed

Methods used in our previous study (Niu *et al.*, 2020) were marginally modified to build an intracellular infection model, as shown in

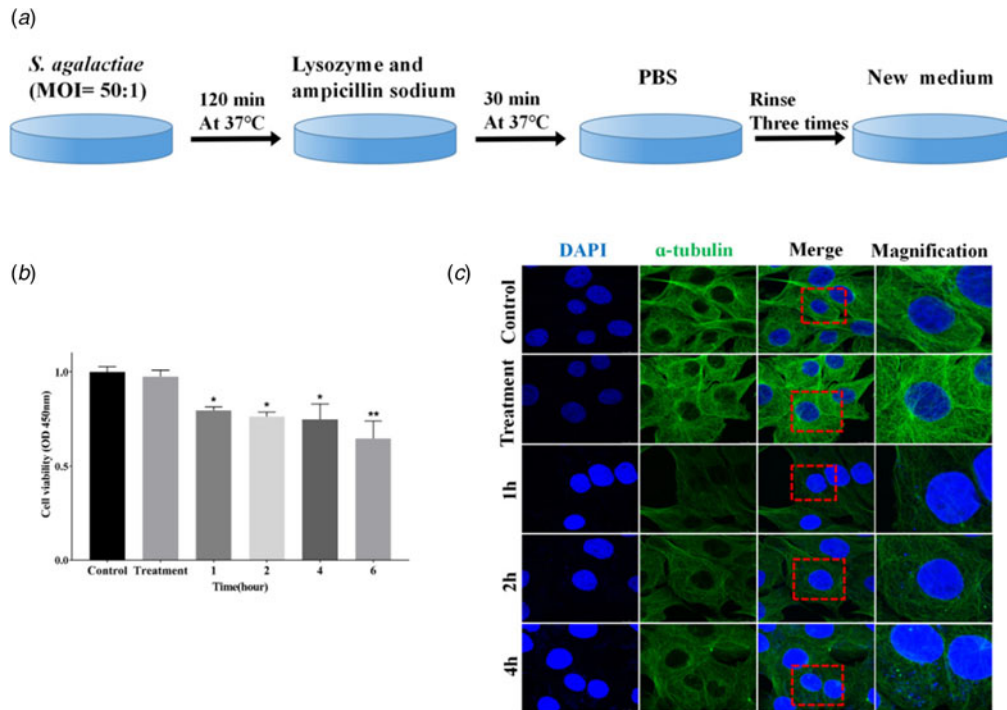


Fig. 1. Cell infection model was successfully constructed: (a) Schematic of the establishment of the intracellular infection model. (b) Effect of *S. agalactiae* invasion time on cell activity which was measured by the CCK-8 assay. All values are expressed as the means \pm SD, ($n = 3$; *, $P < 0.05$; **, $P < 0.01$). C. Typical immunofluorescence images showing colocalization of α -tubulin with DAPI. High magnification images of the outlined area are shown on the right. Scale bars: 10 μ m.

Fig. 1. Cells and *S. agalactiae* were incubated at 37 °C for 120 min. Lysozyme and ampicillin sodium were then added at 37 °C for 30 min to kill all bacteria outside the cell. After rinsing with PBS, the culture medium was changed. As shown in Fig. 1b, the viability of bMECs after incubating with intracellular *S. agalactiae* was examined by CCK-8 assay. CCK-8 assay indicated that cell activity was significantly decreased by *S. agalactiae* ($P < 0.05$) after 1 h compared with blank control without bacteria and further decreased ($P < 0.01$) after 6 h. In addition, *S. agalactiae* was located intracellularly to an increasing extent in the bMECs of the 1, 2, and 4 h groups but not in the control group (Fig. 1c).

S. agalactiae activates autophagy in bMECs

The receptor for microtubule-associated protein 1A/1B-light chain 3 (LC3-II) is located at the phagophore membrane. It plays an important role in the autophagy process and is a standard marker for autophagosomes. LC3-II is generated by the conjugation of cytosolic LC3-I to phosphatidylethanolamine on the surface of nascent autophagosomes (Runwal et al., 2019). LC3-II is associated with the relative specificity of autophagosomes and autophagic lysosomes, as it is able to promote the selective uptake and degradation of stimulating compounds such as protein aggregates (Mukhopadhyay et al., 2014). As shown in Fig. 2a, green fluorescent spots of LC3-II accumulated around the intracellular bacteria in the groups with *S. agalactiae* added. Moreover, the number of green fluorescent aggregation points increased with treatment duration, whilst the control group showed only a diffuse distribution throughout. Meanwhile, as Fig. 2b showed, the level of p62 reduced at 1, 2 and then significantly ($P < 0.05$) at 4 h. Beclin1, a protein that can combine with Bcl2, plays a crucial part in autophagy (Kang et al., 2011). As presented in Fig. 2c and d, levels of Beclin1 and Bcl2

were increasing continuously in bMECs infected by *S. agalactiae*, and this change lasted 4 h. The ratio of Beclin1/ APDH significantly increased at 2 ($P < 0.05$) and 4 h ($P < 0.01$). The ratio of Bcl2/ α -tubulin was significantly increased ($P < 0.05$) at 4 h. These results indicated that *S. agalactiae* induces autophagy in bMECs.

Changes of autophagosome and lysosome

Fluorescent protein confocal microscope and susceptible lysosome probes were used to check the autophagosome and lysosome variation in bMECs (Fig. 3). Importantly, after bMECs were infected by *S. agalactiae*, intracellular LC3-II and lysosome-associated membrane protein (LAMP) reflected green and red fluorescent spots. The intracellular red and green fluorescent speckles increased visibly within four hour after infection, and the number of overlapping parts (displayed in yellow) increased (Fig. 3a). LysoTracker Deep Red was used to evaluate the diversification of lysosome pH in bMECs. LysoTracker Deep Red displayed as a specific red fluorescence in lysosomes, and the degree of fluorescence enhancement showed a decrease in lysosome pH. As shown in Fig. 3b, the intracellular red fluorescence was enhanced within 1–4 h of infection, which means that infection with *S. agalactiae* increased the acid value in lysosomes.

Akt/mTOR signaling pathway

Several signaling pathways play a crucial function in regulating autophagy, including the PI3K/Akt/mTOR pathway (Yang et al., 2018). Autophagy is inhibited by 3MA but activated by Rapa. As represented in Fig. 4a, b, compared with that in the control group, the ratio of p-Akt/Akt and p-mTOR/mTOR significantly decreased ($P < 0.05$) in groups of Rapa + *S. agalactiae* (especially)

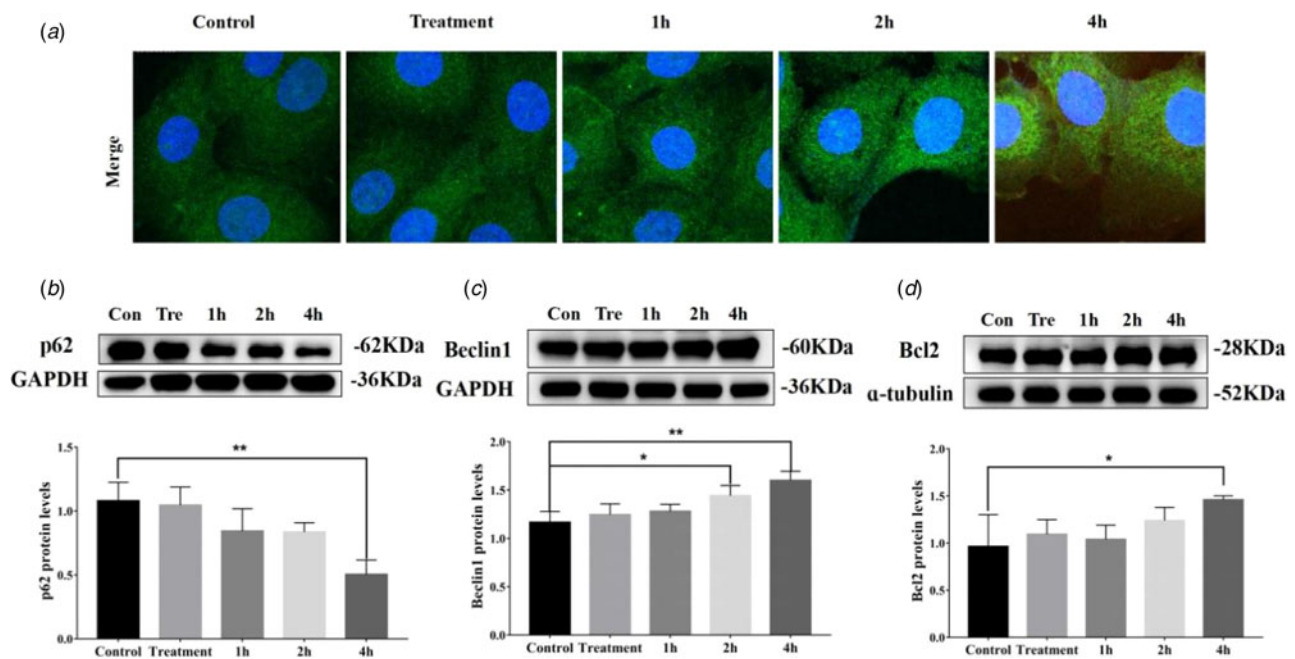


Fig. 2. Changes of autophagy flux induced by *S. agalactiae* in bMECs: (a) Observation of LC3 puncta quantitation under confocal microscopy by calculating 20 cells per state. Scale bars: 10 μ m. (b) Expression levels of p62 protein within 4 h. (c) Expression levels of Beclin1 protein within 4 h. (d) Expression levels of Bcl2 protein within 4 h. The WB results of p62, Beclin1, and Bcl2 were processed by ImageJ. The upper image is a typical western blot result, the lower image is the histogram of quantitative analysis. All values are expressed as the means \pm sd, ($n = 3$; *, $P < 0.05$; **, $P < 0.01$).

and *S. agalactiae* alone. Meanwhile, there was no significant change in 3MA treated cells, even if the value showed a numerical increase. The results suggested that *S. agalactiae*-infection inhibited the activation of p-Akt and p-mTOR, thereby inducing autophagy. Moreover, in the current study, levels of pAkt-LC3-II were detected by fluorescence microscopy (Fig. 4c). Serum can enable the activated p-Akt to enter the nucleus (Fukuda et al., 1997; Mawal-Dewan et al., 2002). LC3-II staining showed fluorescence with green color, and p-Akt showed red fluorescence. Therefore, the experimental results are consistent with the prediction. A large amount of p-Akt accumulated in the nuclei of control and treatment groups. Especially, for Rapa, Rapa + *S. agalactiae*, and *S. agalactiae* groups, the levels of p-Akt in the nucleus decreased significantly, which may be related to the inhibition of Akt activation during autophagy. These results indicate that *S. agalactiae* induces autophagy via PI3K/Akt/mTOR pathway.

S. agalactiae intracellular survival

In order to understand the function of autophagy on the survival of *S. agalactiae* in the cell, two autophagy regulators (3MA and Rapa) were added in this experiment, and the results are given in Fig. 5. Compared with the *S. agalactiae* group, the number of intracellular *S. agalactiae* in the *S. agalactiae* + Rapa group showed an extremely significant increase at 4, 8 and 12 h ($P < 0.01$). In addition, in the *S. agalactiae* + 3MA group, the number of intracellular *S. agalactiae* was significantly decreased at 8 ($P < 0.05$) and 12 h ($P < 0.01$).

Discussion

Mastitis caused by *S. agalactiae* is common in dairy cattle worldwide, and the infection rate in some areas is showing a rapid upwards trend (Jørgensen et al., 2015; Almeida et al., 2016).

Previous studies have demonstrated that autophagy is involved in the clearance of intracellular pathogens such as *S. agalactiae*. Six principal steps are involved in the process of mammalian autophagy: start over, nucleation, extension, closure, maturation and degradation or extrusion (Kang et al., 2011). Our team has confirmed that *S. aureus*, which like *S. agalactiae* is a mastitis pathogen, can induce autophagy of bovine mammary gland epithelial cells. We have also explored the role of the MAPK pathway in cell autophagy (Run et al., 2021). Autophagy is regulated by many signaling pathways. In addition to MAPK signaling, the PI3K/Akt/mTOR pathway is also a classic autophagy pathway (Feng and Qiu, 2018). However, the relationship between autophagy induced by *S. agalactiae* and PI3K/Akt/mTOR pathway is unknown.

The method of monitoring autophagy began with the initial discovery of the autophagosome or autophagolysosome (Chikte et al., 2014). LC3-II is an iconic protein of autophagy and has been extensively used to assess the degree of autophagy (Jiang and Mizushima, 2015). Under normal conditions, when autophagy is activated, LC3 protein as type I (LC3-I) in the cytoplasm is enlisted into autophagosomes and soon afterward transformed into LC3-II (Lu et al., 2018). Fluorescent protein LC3 is often used to show the levels of intracellular autophagosomes (Eskelinen, 2006). In the current research, *S. agalactiae* infection resulted in an increasing level of LC3 within 4 h, indicating that an increase in the number of autophagic vacuoles was induced. Protein p62, localized at autophagosomes, is also an autophagy marker protein (Geng et al., 2020b). Studies have reported that p62 is constantly degraded by the autophagy-lysosomal pathway as autophagic activity increases (Lu et al., 2018). In this study, the level of p62 decreased in cells treated with *S. agalactiae* compared with that in cells kept in a normal state, this being significant after 4 h. Beclin1 was discovered as a binding partner of Bcl2. Especially, the Bcl2-Beclin1 complex plays an essential part in both autophagosome formation and fusion with lysosomes (Maejima et al., 2013; Mukhopadhyay et al.,

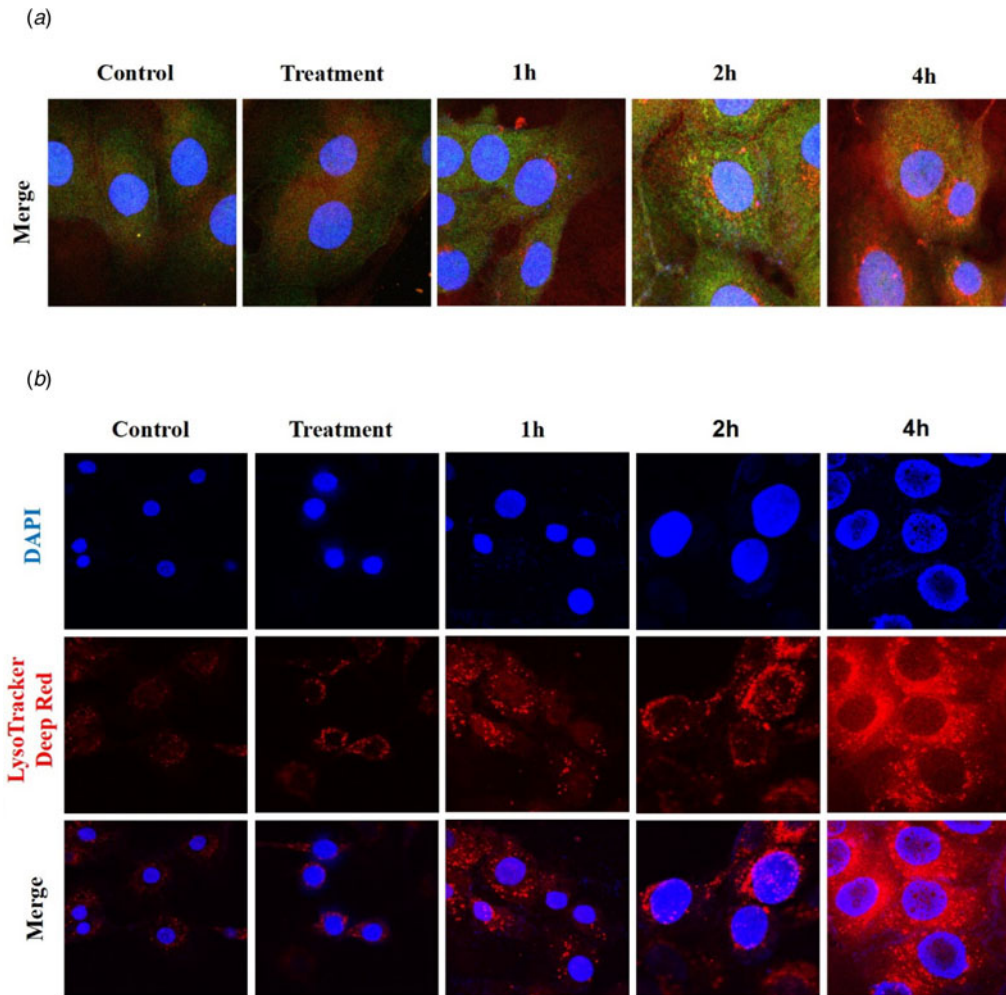


Fig. 3. Changes of autophagosome and lysosome after *S. agalactiae* infection: (a) Representative confocal images of LAMP2 and LC3 after multiple infections for 1, 2 and 4 h of *S. agalactiae*. (b) Cells were dyed with LysoTracker Deep Red for 30 min after *S. agalactiae* infection to explore the lysosomal pH. Scale bars: 10 μ m.

2014). Bcl2 is an anti-autophagy protein that restrains autophagy by directly interacting with the BH3 domain of Beclin1 (He *et al.*, 2012). Based on previous studies, when autophagy is induced, Bcl2 must be displaced from Beclin1, such that the Bcl2-Beclin1 complex is disrupted (Mukhopadhyay *et al.*, 2014). Moreover, autophagy is activated while the Beclin1 signaling pathway in cells is upregulated (Lu *et al.*, 2018). As expected, compared with that in the control group, levels of Beclin1 and Bcl2 significantly increased in our work, which suggests that Beclin1 and Bcl2 are involved in *S. agalactiae*-induced autophagy. In summary, the changes in LC3, p62, Beclin1, and Bcl2 strongly suggest that *S. agalactiae* induces autophagy in bMECs.

Lysosome-associated membrane protein-2 (LAMP-2) is a fundamental protein ingredient of the lysosome membrane. LAMP-2 promotes the maturation of autophagolysosomes by accelerating vesicular fusion on microtubules, which facilitates the degradation of the contents in the autolysosome (Tanaka *et al.*, 2000; Eskelinen, 2006). Fusion of lysosomes with autophagosomes produces autolysosomes (Babuta *et al.*, 2019). The expression level of LAMP-2 increases when the autolysosome pathway is activated (Geng *et al.*, 2020b). In this study, the intracellular red and green fluorescence spots enhanced during 1 to 4 h post-infection. The number of overlapping parts (displayed in yellow) reached

the maximum after 4 h. The results indicate that the number of autolysosomes increased with the increased degree of autophagy. Therefore, it is reasonable to speculate that the autolysosomal pathway is activated. Lysosomes are membranous cell organs involved in the degradation of intracellular proteins and external pathogens. Under a normal physiological environment, lysosomes show weak acidity (Chikte *et al.*, 2014). Lysosomes contain a large number of hydrolytic enzymes, which are characterized by optimum activity in an acidic environment with pH = 5 (Pierzyńska-Mach *et al.*, 2014). The LysoTracker Deep Red dye easily passes through cytomembrane as well as membranes of cytoplasmic vesicles and gets captured inside the acidic vesicles, such that intracellular lysosomes reflect red fluorescence. The accumulation of fluorescence probes in their interior is directly related to their acidity (Pierzyńska-Mach *et al.*, 2014). Previous work has shown that the pH of lysosomes decreases and the fluorescence intensity increases when lysosomal activity is increased. The fluorescence intensity could act as an index of variations in lysosomal pH (Martins *et al.*, 2018; Zhitomirsky *et al.*, 2018). In our current research, the intracellular red fluorescence increased continuously within 4 h post-infection, which proved that the pH in lysosomes was reduced. In short, autophagosomes fused with lysosomes, and the hydrolases in lysosomes dissolved *S. agalactiae*.

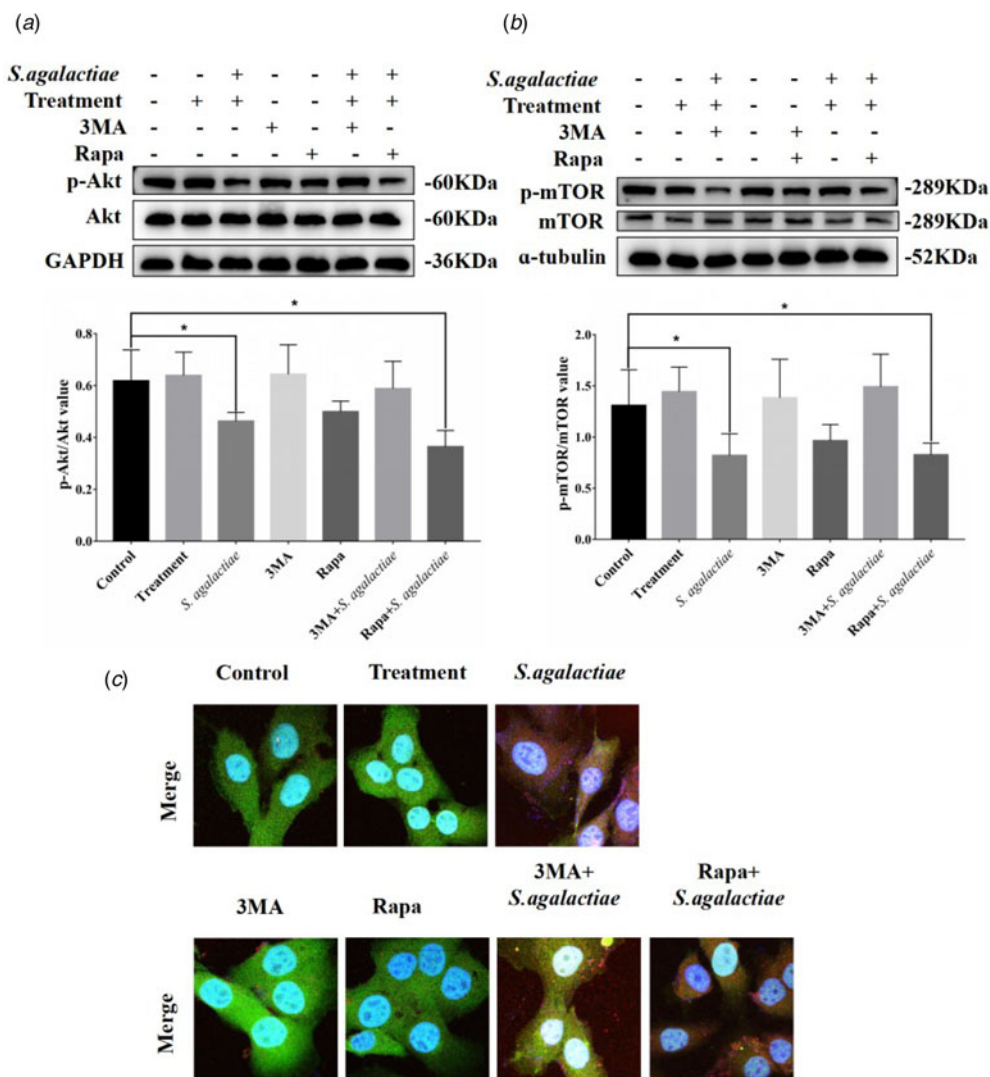


Fig. 4. Detection of Akt and mTOR after *S. agalactiae* challenge: (a) The variation of p-Akt/Akt protein expression after addition of 3MA, Rapa, and *S. agalactiae*. (b) The variation of p-mTOR/mTOR protein expression after the addition of 3MA, Rapa, and *S. agalactiae*. The immunoblot results of p-Akt/Akt and p-mTOR/mTOR were processed by ImageJ. All values are expressed as the means \pm sd, ($n = 3$; *, $P < 0.05$). (c) Representative confocal images of pAkt and LC3 after adding 3MA or Rapa. Scale bars: 10 μ m.

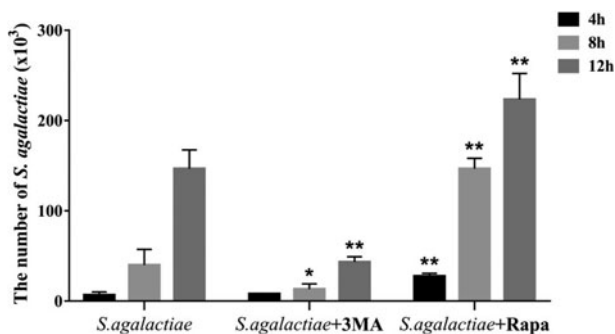


Fig. 5. The number of *S. agalactiae* surviving in BMECs: BMECs were exposed to *S. agalactiae* for 2 h. After adding 3MA or Rapa, the intracellular *S. agalactiae* quantity was calculated at 4, 8 and 12 h. All values are expressed as the means \pm sd, ($n = 3$; *, $P < 0.05$; **, $P < 0.01$).

The PI3K/Akt/mTOR pathway is a major intracellular signaling pathway involved in regulating physiological activities in the cell (Feng and Qiu, 2018). In many cell systems, autophagy is

controlled by this pathway. Akt may directly or indirectly phosphorylate mTOR. mTOR (mammalian target of rapamycin) is a major negative feedback regulatory protein of autophagy (Lopiccolo *et al.*, 2008). The activity of mTOR can be inhibited by Rapa, thereby inducing autophagy (Liu *et al.*, 2017). 3MA inhibits autophagy by blocking the class III PI3K pathway, and it can also suppress the formation of autophagosomes and autophagic lysosomal protein degradation (Zhang *et al.*, 2021). Previous studies have shown that inhibiting the PI3K/Akt/mTOR pathway contributes to the induction of autophagy (Tu *et al.*, 2020). The present study observed that compared with controls, the amount of p-Akt and p-mTOR expression significantly decreased in groups of Rapa + *S. agalactiae* and *S. agalactiae*. After adding 3MA to inhibit autophagy, although the expression of p-Akt and p-mTOR did not change significantly, it still showed an upward trend. Studies have demonstrated that after serum stimulation, p-Akt could enter the nucleus (Lorenzini *et al.*, 2002; Mawal-Dewan *et al.*, 2002). In our case, the results of confocal microscope showed that after the addition of Rapa and *S. agalactiae* at the same time, the level of p-Akt in the nucleus decreased significantly,

and the number of LC3 aggregation spots increased significantly compared with the control group and *S. agalactiae* group. Therefore, we speculate that the activation of p-Akt and p-mTOR were inhibited when *S. agalactiae* induced autophagy of bMECs.

An intracellular survival assay was carried out to further explore the function of autophagy on intracellular *S. agalactiae* survival. In particular, when 3MA was added to inhibit autophagy, the number of intracellular *S. agalactiae* reduced significantly, whereas the number of bacteria increased significantly when Rapa was added. This finding may indicate that it was related to the immune escape of intracellular *S. agalactiae*, and the occurrence of autophagy was beneficial to *S. agalactiae* survival.

In conclusion, we have presented evidence that *S. agalactiae* can induce autophagy in bovine mammary epithelial cells. The mechanism involves the PI3K/Akt/mTOR pathway, and the data further suggest that the pathogen utilizes autophagy to ensure its survival within the cell.

Acknowledgements. The project was supported by the National Natural Science Foundation of China (31802259, 31872535), Shandong Key R&D Program (2019GNC106141), Shandong Natural Science Foundation of China (ZR2018MC027), China Postdoctoral Science Foundation (2018M632704, 2019T120601), and Funds of Shandong 'Double Tops' Program.

References

- Almeida A, Alves-Barroco C, Sauvage E, Bexiga R and Glaser P (2016) Persistence of a dominant bovine lineage of group B *Streptococcus* reveals genomic signatures of host adaptation. *Environmental Microbiology* **18**, 4216–4229.
- Babuta M, Furi I, Bala S, Bukong TN, Lowe P, Catalano D, Calenda C, Kodys K and Szabo G (2019) Dysregulated autophagy and lysosome function are linked to exosome production by micro-RNA 155 in alcoholic liver disease. *Hepatology* **70**, 2123–2141.
- Chikte S, Panchal N and Warnes G (2014) Use of LysoTracker dyes: a flow cytometric study of autophagy. *Cytometry Part A* **85**, 169–178.
- De Gaetano GV, Pietrocola G, Romeo L, Galbo R and Beninati C (2018) The *Streptococcus agalactiae* cell wall-anchored protein PbsP mediates adhesion to and invasion of epithelial cells by exploiting the host vitronectin/ α v integrin axis. *Molecular Microbiology* **110**, 82–94.
- Eskelinen EL (2006) Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Molecular Aspects of Medicine* **27**, 495–502.
- Feng FB and Qiu HY (2018) Effects of Artesunate on chondrocyte proliferation, apoptosis and autophagy through the PI3K/AKT/mTOR signaling pathway in rat models with rheumatoid arthritis. *Biomedicine & Pharmacotherapy* **102**, 1209–1220.
- Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M and Nishida E (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308–311.
- Geng N, Liu K, Lu J, Xu Y and Han B (2020a) Autophagy of bovine mammary epithelial cell induced by intracellular *Staphylococcus aureus*. *The Journal of Microbiology* **58**, 320–329.
- Geng N, Wang X, Yu X, Wang R and Liu Y (2020b) *Staphylococcus aureus* avoids autophagy clearance of bovine mammary epithelial cells by impairing lysosomal function. *Frontiers in Immunology* **11**, 746.
- He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J and Sun Q (2012) Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* **481**, 511–515.
- Jiang P and Mizushima N (2015) LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells. *Methods (San Diego, Calif.)* **75**, 13–18.
- Jørgensen HJ, Nordstoga A, Sviland S, Zadoks RN, Sølvørød L, Kvilte B and Mørk T (2015) *Streptococcus agalactiae* in the environment of bovine dairy herds – rewriting the textbooks? *Veterinary Microbiology* **184**, 64–72.
- Kang R, Zeh HJ, Lotze MT and Tang D (2011) The Beclin 1 network regulates autophagy and apoptosis. *Cell Death & Differentiation* **18**, 571–580.
- Liu G, Pei F, Yang F, Li L, Amit A, Liu S, Buchan J and William C (2017) Role of autophagy and apoptosis in non-small-cell lung cancer. *International Journal of Molecular Sciences* **18**, 367.
- Lopiccolo J, Blumenthal GM, Bernstein WB and Dennis PA (2008) Targeting the PI3K/Akt/mTOR pathway: effective combinations and clinical considerations. *Drug Resistance Updates* **11**, 32–50.
- Lorenzini A, Tresini M, Mawal-Dewan M, Frisoni L, Hong Z, Allen RG, Sell C and Cristofalo VJ (2002) Role of the Raf/MEK/ERK and the PI3K/Akt(PKB) pathways in fibroblast senescence. *Experimental Gerontology* **37**, 1149–1156.
- Lu N, Li X, Tan R, An J, Cai Z, Hu X, Wang F, Wang H, Lu C and Lu H (2018) HIF-1 α /Beclin1-mediated autophagy is involved in neuroprotection induced by hypoxic preconditioning. *Journal of Molecular Neuroscience* **66**, 238–250.
- Maejima Y, Kyo S, Zhai P, Tong L and Sadoshima J (2013) Mst1 inhibits autophagy by promoting Beclin1-Bcl-2 interaction. *Nature Medicine* **19**, 1478–1488.
- Martins WK, Santos NF, Rocha CDS, Bacellar IOL and Baptista MS (2018) Parallel damage in mitochondria and lysosomes is an efficient way to photoinduce cell death. *Autophagy* **15**, 259–279.
- Mawal-Dewan M, Lorenzini A, Frisoni L, Zhang H, Cristofalo VJ and Sell C (2002) Regulation of collagenase expression during replicative senescence in human fibroblasts by Akt-forkhead signaling. *Journal of Biological Chemistry* **277**, 7857–7864.
- Mukhopadhyay S, Panda PK, Sinha N, Das DN and Bhutia SK (2014) Autophagy and apoptosis: where do they meet? *Apoptosis* **19**, 555–566.
- Niu H, Zhang H, Wu F, Xiong B, Tong J and Jiang L (2020) Proteomics study on the protective mechanism of soybean isoflavone against inflammation injury of bovine mammary epithelial cells induced by *Streptococcus agalactiae*. *Cell Stress and Chaperones* **26**, 91–101.
- Pang M, Sun L, He T, Bao H, Zhang L, Zhou Y, Zhang H, Wei R, Liu Y and Wang R (2017) Molecular and virulence characterization of highly prevalent *Streptococcus agalactiae* circulated in bovine dairy herds. *Veterinary Research* **48**, 65.
- Pierzyńska-Mach A, Janowski PA and Dobrucki JW (2014) Evaluation of acridine orange, LysoTracker Red, and quinacrine as fluorescent probes for long-term tracking of acidic vesicles. *Cytometry Part A* **85**, 729–737.
- Run W, Wen Z, Lumei W, Na G, Xiaozhou W, Meihua Z and Jianzhu L, Yongxia L and Bo H (2021) Intracellular *Staphylococcus aureus* inhibits autophagy of bovine mammary epithelial cells through activating p38 α . *Journal of Dairy Research* **88**, 293–301.
- Runwal G, Stamatakou E, Siddiqi FH, Puri C and Rubinsztein DC (2019) LC3-positive structures are prominent in autophagy-deficient cells. *Scientific Reports* **9**, 10147.
- Seegers H, Fourichon C and Beaudreau F (2003) Production effects related to mastitis and mastitis economics in dairy cattle herds. *Veterinary Research* **34**, 475–491.
- Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen P, Blanz J, Figura KV and Saftig P (2000) Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* **406**, 902–906.
- Tong J, Sun M, Zhang H, Yang D and Jiang L (2020) Proteomic analysis of bovine mammary epithelial cells after in vitro incubation with *S. agalactiae*: potential biomarkers. *Veterinary Research* **51**, 98.
- Tu QD, Jin J, Hu X, Ren Y, Zhao L and He Q (2020) Curcumin improves the renal autophagy in rat experimental membranous nephropathy via regulating the PI3K/AKT/mTOR and Nrf2/HO-1 signaling pathways. *Biomed Research International* **2020**, 7069052.
- Yang J, Pi C and Wang G (2018) Inhibition of PI3K/Akt/mTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells. *Biomedicine & Pharmacotherapy* **103**, 699–707.
- Zhang A, Song Y, Zhang Z, Jiang S and Ni G (2021) Effects of autophagy inhibitor 3-Methyladenine on ischemic stroke: a protocol for systematic review and meta-analysis. *Medicine* **100**, e23873.
- Zhao Z, Sun C, Chen L, Qin J and Li W (2019) Inorganic nitrite increases the susceptibility of tilapia (*Oreochromis niloticus*) leucocytes to *Streptococcus agalactiae*. *Fish & Shellfish Immunology* **97**, 1–11.
- Zhitomirsky B, Farber H and Assaraf YG (2018) LysoTracker and MitoTracker Red are transport substrates of P-glycoprotein: implications for anticancer drug design evading multidrug resistance. *Journal of Cellular & Molecular Medicine* **22**, 2131–2141.