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Autophagy Maintains the Survival of Microglia by Modulating ROS Production under Serum Deprivation Condition

La Autofagia Mantiene la Supervivencia de la Microglia Modulando la Producción de ROS en Condiciones de Privación de Suero

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SUMMARY: Autophagy plays a protective role in maintaining cellular homeostasis under physiological conditions and in helping cells to resist starvation under nutrient deprivation. Cells can excessively produce reactive oxygen species (ROS) under nutrient starvation, which can lead to organelle damage and protein misfolding, thus promoting cell death. However, it is not clear whether autophagy interacts with ROS during this process. In this study, we explored whether autophagy is involved in the protective effect of serum deprivation-induced cell apoptosis and excessive production of ROS in BV-2 cells with Atg5 knockdown. In addition, we examined the autophagy level in cells treated with N-acetylcysteine (NAC). We found that the cells grew slowly and aged, and the levels of ROS and autophagy increased after 24 h of serum deprivation. Autophagy defects significantly increased ROS levels and cell death. Compared with the starvation group, the NAC treatment group reduced the levels of ROS and autophagy ($p<0.05$), and decreased cell death. On conclusion, autophagy and low levels of ROS can promote microglia survival compared with autophagy defects or high levels of ROS under starvation conditions, Therefore, autophagy and moderate ROS are beneficial to maintain cellular homeostasis under pathological conditions.

KEY WORDS: Autophagy; Serum starvation; ROS; Microglia; IL-6; TNF-α.

INTRODUCTION

Autophagy plays an important role in maintaining cellular homeostasis and regulating the inflammatory responses induced by central nervous system injury (Ma *et al*., 2020). There are four types of autophagy, including macroautophagy, microautophagy, chaperone-mediated autophagy, and xenophagy (Anozie & Dalhaimer, 2017). Autophagy depends on lysosomes to degrade intracellular substances, it plays a key role in responding to various cellular stresses such as starvation, hypoxia, and infection (Mizushima & Levine, 2010). It is currently unclear if autophagy is a bona fide lethal mechanism or if it is a protective mechanism against cell death (Galluzzi *et al*., 2008; Dubinsky *et al*., 2014; Kaur & Debnath, 2015; Shpilka *et al*., 2015). Autophagy seems to be a protective mechanism under nutrient deprivation.

Microglia are innate immune cells of the central nervous system, and are the main cells for eliminating bacterial infection in the brain. When programmed cell death occurs, or when the central nervous system is damaged or pathologically damaged during brain development, microglia can act as brain macrophages to remove damaged cells. Our previous study found (Ma *et al*., 2020) that autophagy played a role in regulating the inflammatory state of microglial cells under the pathological conditions of infection. Excessive activation of autophagy was not conducive to the survival of microglial cells, and autophagy played a non-protective role. Some studies have confirmed that autophagy plays a protective role under nutrient deprivation (Kaur & Debnath, 2015; He *et al*., 2018). However, there is no report on the starvation stimulation of microglia. In order to evaluate the effects of autophagy and reactive oxygen species (ROS) production on microglia survival under starvation conditions, we designed and performed in vitro experiments.

The complexity of ROS and autophagy pathways should never be underestimated. It is found that ROS production is involved in autophagy, and autophagy has been shown to be strongly induced by ROS (Li *et al*., 2015).

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Excessive production of ROS causes mitochondrial damage and induces mitophagy and mitochondrial dysfunction (Kurihara *et al*., 2012; Sakellariou *et al*., 2016). In addition, studies have found that inflammation can activate autophagy, meanwhile, ROS are involved in inflammation (Wang *et al*., 2013; Harijith *et al*., 2014). Some studies have shown that ROS can regulate autophagy through multiple pathways, such as AMPK/mTOR pathway (Underwood *et al*., 2010; Mungai *et al*., 2011; Alers *et al*., 2012). ROS production is a hallmark of oxidative damage in many pathological conditions including bacterial infection and can lead to mitochondrial dysfunction, cellular aging, and apoptosis (Murphy, 2009). ROS generation also appears to be linked to the autophagy, a major catabolic pathway in eukaryotic cells that degrades and recycles cellular components in autophagosomes during conditions of starvation. The degraded products of autophagy can be used as components to keep cells alive (Rubinsztein, 2006; Levine & Kroemer, 2008; Mizushima *et al*., 2008; Mizushima *et al*., 2010).

Whether ROS and autophagy interacts with each other to maintain cellular homeostasis under starvation conditions is currently controversial and not fully understood. In this study, we investigated the relationship between ROS and autophagy using autophagy-deficient microglia cells. We found that ROS-induced cell death and ROS levels were increased in autophagy-deficient cells. Interestingly, NAC (ROS inhibitor) decreased ROS-induced cell death and autophagy levels during serum starvation.

MATERIAL AND METHOD

Reagents: The mouse microglia BV2 cell line was a generous gift from Central Laboratory of Shandong University Medical College (Shandong, China). Dulbecco's modified Eagle's medium and NAC were purchased from Gibco/BRL (Gaithersburg, USA). Penicillin-streptomycin and 0.25 % trypsin were purchased from Hyclone (Logan, USA). Rabbit anti-mouse P62 and rabbit anti-mouse Atg5 were purchased from Affinity Biosciences (Cincinnati, OH, USA). Goat anti-rabbit Atg5, anti-mouse LC3, and anti-mouse Lamp-1 were purchased from Sigma (St Louis, USA). Annexin V-FITC apoptosis detection kit and ELISA cytokine detection kit were purchased from BD Bioscience (Franklin Lakes, USA). Horseradish peroxidase-conjugated secondary antibodies and ECL detection system were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Lipofectamine 2000 was purchased from Invitrogen (Waltham, USA). Rabbit anti-mouse LC3 and Goat anti-mouse Lamp-1 were purchased from Merck (Woodbridge, USA). Atg5 small interfering RNA (siRNA) and scrambled siRNA (negative control) were synthesized by Hanbio Biotech (Guangzhou, China).

Cells and Treatment: The mouse microglia BV2 cell line was cultured in Dulbecco's modified Eagle's medium(low-glucose) containing 10 % fetal bovine serum and penicillin-streptomycin (100 U/ml; 0.1 mg/ml) at 37 °C in 5 % CO_2-humidified incubator. Cells in logarithmic phase were trypsinized with 0.25 % trypsin/EDTA to obtain single cell suspensions, which were subsequently seeded into 6-well plates (100 μl/well; 2×10^5 cells/well). The cells were divided into Control group, Starvation group, Starvation + Atg5KO group, and Starvation + NAC (1mM) group and then incubated for 24 h.

SiRNA and Transfection. The Atg5 siRNA was transfected into BV2 cells to inhibit its expression. Briefly, BV2 cell culture medium was replaced with fresh complete medium 2 h before transfection. The Atg5 siRNA and scrambled siRNA plasmids were subsequently transfected into BV2 cells using Lipofectamine 2000. After 48 h, cells were collected and analyzed using Western blot analysis to verify Atg5 expression.

Western Blot Analysis. Starvation-treated BV2 cells were plated in 6-well plates at a density of 2×10^5 cells per well and cultured for 24 h. Stimulated BV2 cells were harvested and then lysed with RIPA buffer (Sigma, USA) containing a protease inhibitor on ice to obtain a total cell lysate. The protein concentration of the cell lysate was determined using BCA assay. Equal amounts of protein (40 μg) were subjected to 10 % SDS-PAGE and then transferred onto nitrocellulose membranes (Millipore, MA, USA). After blocking with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween 20 for 2 h at room temperature, the membranes were incubated separately with anti-mouse LC3, rabbit anti-mouse P62, anti-mouse Lamp-1, rabbit anti-mouse Atg5, and anti-mouse GAPDH primary antibodies at 4 °C overnight followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibodies for 1 h at room temperature. Following washing, the membranes were developed using the ECL detection system (Merck, USA) and were analyzed using Image J software.

ROS Measurement. Intracellular ROS generation was measured by flow cytometry after staining with a fluorescent probe 2´,7´-dichlorofluorescein-diacetate (DCFH-DA). DCFH-DA could be oxidized by ROS into fluorescent product 2¢,7¢-dichlorofluorescein (DCF). BV2 cells were seeded into 6-well plates and pretreated with various inhibitors. Following starvation treatment, BV2 cells were incubated with 10 mM DCFH-DA at 37 °C for 30 min. After washing with cold PBS, the cells were examined by flow cytometry.

Immunofluorescence Imaging. After various treatments, BV2 cells were fixed with 4 % paraformaldehyde for 10 min at room temperature, washed twice with PBS, and then blocked with 5 % normal goat serum. The cells were then incubated with 0.05 % Tween 20 for 1 h to reduce nonspecific binding before incubation with anti-LC3B and anti-LAMP-1 primary antibodies (St Louis, USA) for 1 h at room temperature. Subsequently, incubation with secondary antibody was performed for 1 h at 37°C. For negative controls, cells were incubated under similar conditions without primary antibodies. All images were captured with a LSM780 fluorescence microscope (Zeiss, Germany).

ELISA. The levels of proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 6(IL-6) secreted into the culture supernatants were examined. Cells were treated as previously described. Levels of cytokines in cell culture supernatants were detected using corresponding ELISA cytokine detection kits, according to the manufacturer's instructions. Absorbance values were obtained at 450 nm using a microplate reader (Bio-Rad, USA).

Flow Cytometry Analysis of Apoptosis. Starvation treated cells were cultured for 24 h and then harvested in ice-cold PBS at 4°C for flow cytometry analysis of apoptosis. Annexin V-FITC/PI staining solution (10 μl) was added to 100 μl of a 2×10^5/ml cell suspension in binding buffer and incubated at room temperature for 20 min in the dark. After adding 400 μl of PBS, the cells were analyzed using a flow cytometer (BD, USA).

Statistical Analysis. Statistical analysis of the data was carried out using SPSS version 17.0 software (IBM SPSS Inc., Chicago, IL, USA). Data were shown as the mean ± SEM (standard error of mean) from three independent experiments. Comparisons between two groups were performed using the t-test, and comparisons among more than two groups were performed using one-way ANOVA. A $p < 0.05$ was considered significant.

RESULTS

SiRNA knocks down the expression of ATG5. To knock down ATG5 expression in BV2 cells, siRNA ATG5 transfection was performed. Western blot analysis was conducted to assess the expression of ATG5 protein in the BV2 cells after siRNA treatment for 48 h, with GAPDH as the loading control (Fig. 1A). Atg5 siRNA almost completely blocked the expression of ATG5 in BV2 (Fig. 1B) (**$p < 0.01$). Moreover, immunofluorescence (Fig. 1C) found that higher ATG5 expression was observed in the starvation group but not in the ATG5-KO group compared to the control group.

Autophagy is strongly associated with ROS Levels. ROS are reported to interact with autophagy in serum-deprived cells. Herein, the cellular ROS levels in ATG5-KO cells were measured after staining with DCFH-DA. The expression of autophagy proteins LC3I/II and p62 were detected using Western blot analysis at 24 h. Furthermore, immunofluorescence was used to observe the number of GFP-LC3 autophagic plaques. We found that ROS levels were increased and autophagy was enhanced in the serum starvation group compared with the normal nutritive group (Figs. 2A,B). Autophagy was barely observed in ATG5-KO cells, while ROS level was significantly increased compared to the control group (***$p < 0.001$) and starvation group (**$p<0.01$). In addition, to determine the effect of ROS level on autophagy, NAC was added to inhibit ROS, and the low level of autophagy was confirmed by Western blot and immunofluorescence (Fig. 2B-D). GFP-LC3 immunofluorescence assay showed that autophagy was at high level in the serum starvation group compared with the control group, while autophagy was not observed in Atg5-KO group (Figs. 2D,E). Subsequently, we found that autophagy level was also decreased in starved cells after adding NAC. Therefore, these results demonstrate that starvation induces autophagy, and autophagy deficiency can lead to further increase of ROS levels. Furthermore, inhibiting ROS levels can reduce autophagy, which confirms the close relationship between autophagy and ROS.

Autophagy and moderate ROS levels are beneficial to the survival of nutrient-deficient cells. In order to analyze the effects of autophagy and ROS on cell survival, cell growth status was observed and apoptosis was analyzed flow cytometry. We found that there was more cell shrinkage and cell size reduction in the starvation group than the normal group. In addition, we found more apoptosis of cells in the Atg5-KO group compared with the starvation group (Fig. 3A). Moreover, the levels of TNF-α and IL-6 in each group were detected with ELISA. The results showed that TNF-α and IL-6 levels were increased in the ATG5-KO group compared with that in the control group, while their levels were decreased in the NAC treated group compared with the starvation group (Figs. 3B,C). In addition, the BV2 cells in the starvation group treated with NAC had a better cellular growth, and the apoptosis rate was lower than that in the starvation group (Figs. 3D,E). Thus, our results showed that cell death increased under starvation and further increased after blocking autophagy, while cell death decreased after NAC treatment. These results suggest that autophagy and moderate ROS level may contribute to microglia survival under serum starvation.

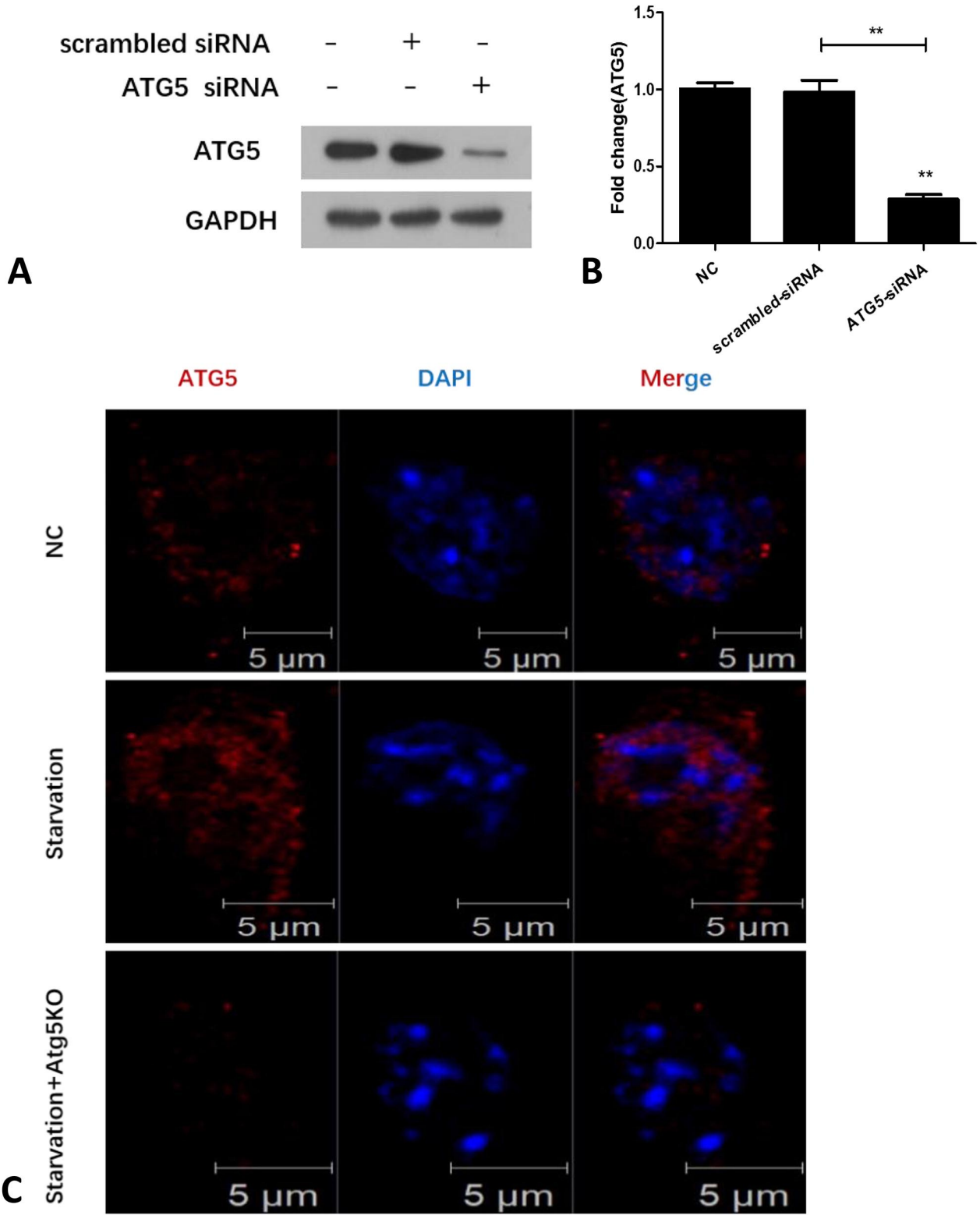


Fig. 1. Atg5-siRNA inhibits the expression of ATG5. A&B Expression of ATG5 protein in BV2 cells transfected with the Atg5-siRNA and scrambled siRNA for 48 h by Western blot. C Immunofluorescence analysis of ATG5 protein in microglia at the 48 h. In the images, the nucleus staining is shown in blue, ATG5 staining is shown in red, and the signals of colocalization are shown in the merged images. The scale bar is 5 mM. Data are presented as mean ± SD; n = 3; **p < 0.01 vs. control.

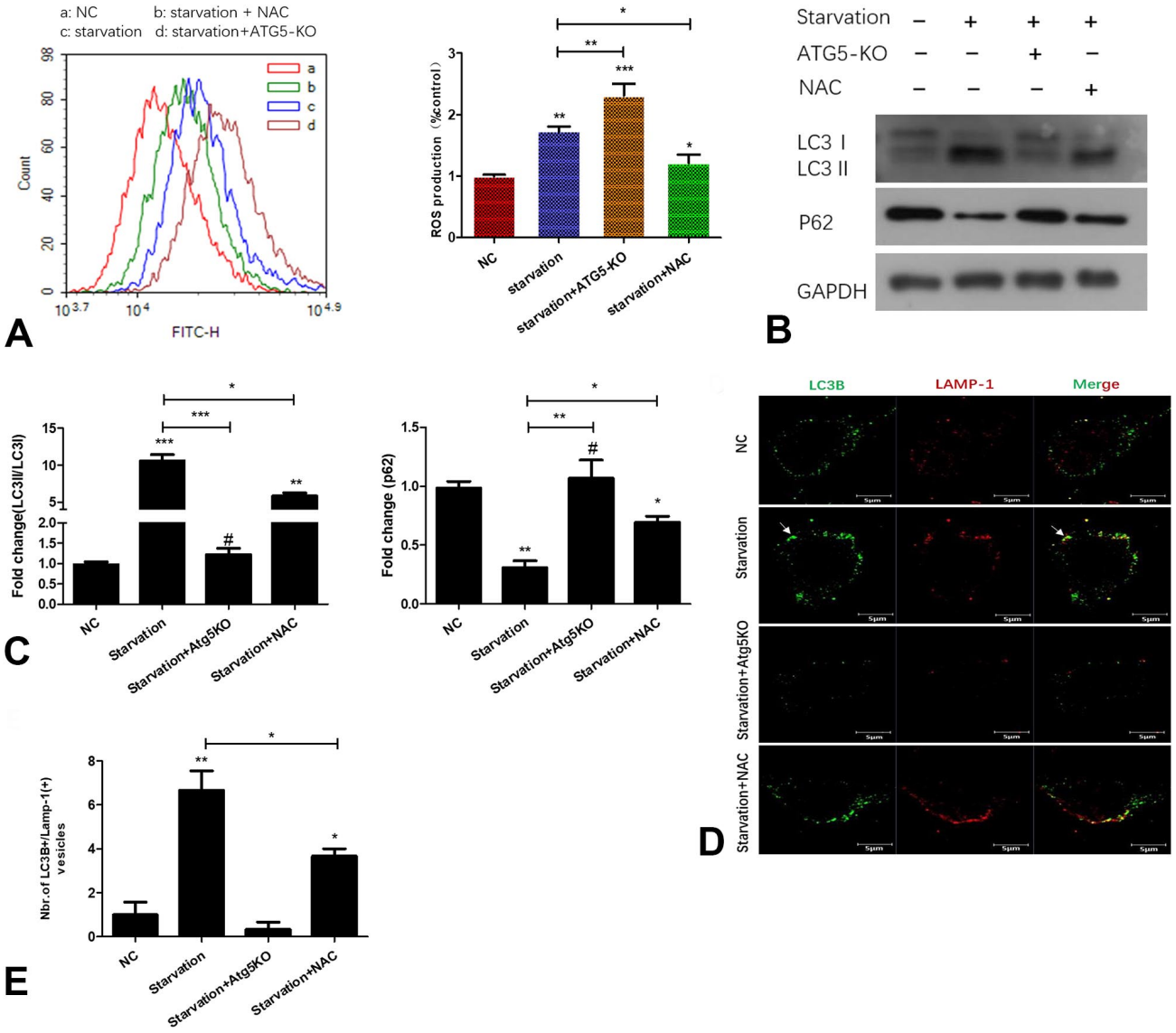
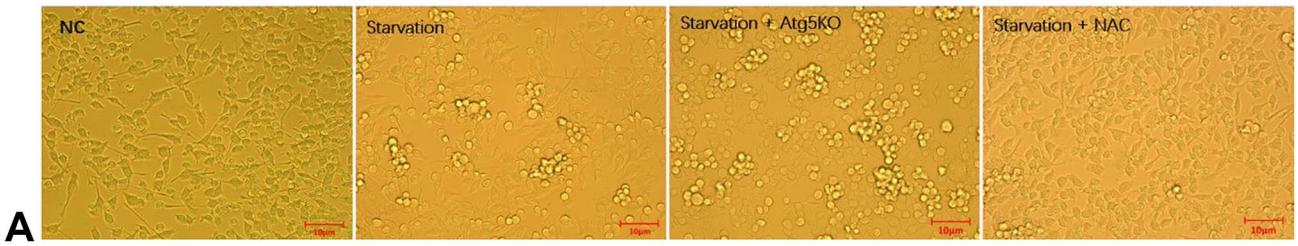
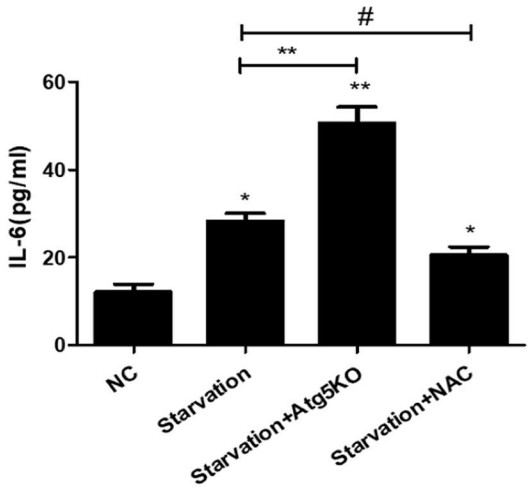


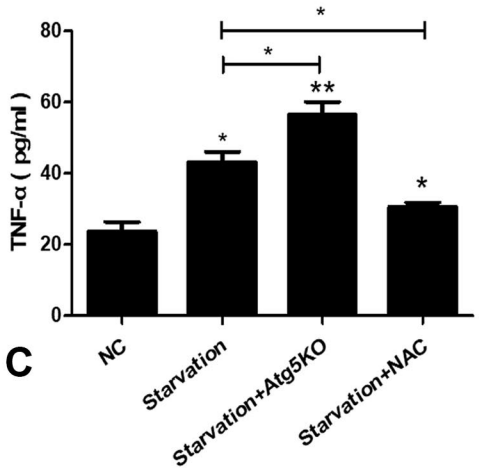
Fig. 2. The relationship between ROS and autophagy in microglia under starvation conditions. A ROS level in BV2 cells assessed by DCFH-DA. B&C Western blot detected the expression of LC3I/II and P62 proteins in BV2 cells under starvation for 24 h and after treatment with Atg5-siRNA or NAC. D&E Immunofluorescence analysis of LC3B and LAMP-1 co-localization at 24 h and the histogram showed the number of labeled autophagosomes. Data are presented as the mean ± SD; n = 3; ***p<0.001; **p < 0.01; *p < 0.05; # p > 0.05 vs. control.

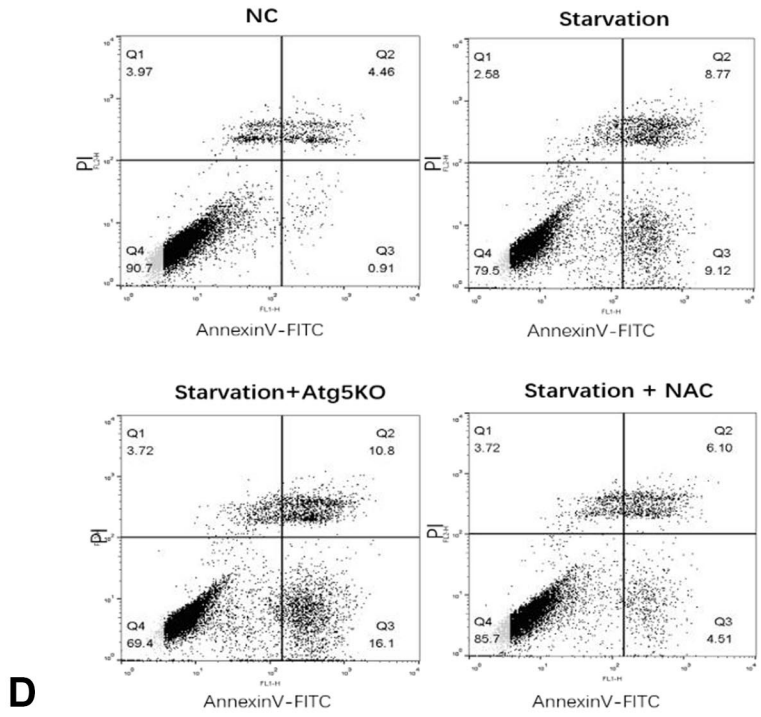
DISCUSSION

Our study showed that autophagy was conducive to the survival of microglia cells under nutrient deficiency, and autophagy was upregulated under cell starvation. Moreover, the production of intracellular ROS increased which may disrupt the homeostasis of cell redox and may be not conducive to cell survival. In our study, autophagy was blocked by knocking down ATG5 (Fig. 1), and it was found that autophagy deficiency enhanced ROS levels and promoted cell death under nutritional deficiency. Under the same conditions, NAC was found to be conducive to cell redox homeostasis and cell survival by downregulating ROS levels (Figs. 2 and 3). Our study showed that both NAC and autophagy could reduce ROS, which is conducive to microglia cell survival.









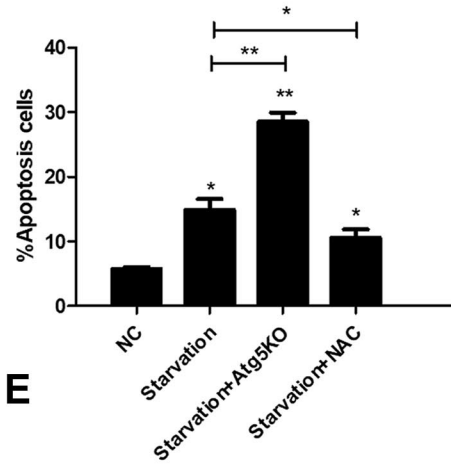


Fig. 3. Autophagy and ROS effects on the survial of cells. A The survival of cells under starvation for 24 h and after treatment with Atg5-siRNA or NAC. B&C ELISA analysis of TNF-a and IL-6. D&E The levels of apoptosis were detected by flow cytometry. Data are presented as the mean ± SD; n = 3; **p < 0.01; *p < 0.05; # p > 0.05 vs. control.

One study found that the expression of ATG5, as an autophagy target gene, could interfere the level of autophagy and cell survival by the expression of ROS involved. The balance between ROS production and clearance has important roles in signal transduction. The physiological role of ROS is two-sided, i.e. ROS not only participate in the normal cellular events, such as signal transduction, gene expression, and enzyme reaction, but also involve in some pathological diseases (Gao *et al*., 2020). In our study, we blocked the autophagy by knocking down ATG5, and inhibited ROS level by using NAC to study the effects of ROS and autophagy level on cell survival.

Autophagy can be enhanced through serum starvation or glucose deprivation, while the level of ROS is also increased. The strong association between autophagy and ROS has been confirmed. For example, Scherz-Shouval *et al.* (2003) clarified that ROS as a signal molecule was involved in starvation induced autophagy . Under starvation conditions, the tumor cells are more likely to produce ROS and H_2O_2. This oxidation process directly affects the formation of autophagosomes. ROS can promote the expression of ATG genes and Beclin-1 via activating p53 and p38, leading to the induction of autophagy. Thus, the interaction between autophagy and ROS contributes to the oxidative cost of cell growth and survival (He *et al*., 2018). It was found that treatment with ROS scavengers could suppress autophagy as well as ROS production (Shin & Cho, 2019; Li *et al*., 2020), suggesting that there is a strong association between ROS and autophagy. In our study, we found that under the condition of serum deprivation, the ROS level of microglia cells increased, and autophagy was also significantly enhanced. After treatment with NAC, the level of ROS decreased, and the autophagy level was also decreased compared with the starvation group (Fig. 2). Therefore, our study indicates that ROS may affect autophagy through a certain pathway.

ROS have multifaceted properties as they are necessary for the functioning of cells, both healthy and diseased (Forrester *et al*., 2018). ROS are decisive for cells to maintain their pathophysiological state in setting of diseases like diabetes, atherosclerosis, stroke, etc. (Kaneto *et al*., 2010). In the early stages of autophagosome formation, ROS play a very important role in signal transduction (Filomeni *et al*., 2010). Although autophagy can be induced by oxidative stress, autophagy can also reduce ROS levels (West & Sweeney, 2012). In our study, autophagy of microglia was blocked by ATG5 knockdown. Compared with the serum starvation group, ROS levels changed significantly, and ROS production and cell apoptosis increased significantly in the ATG5-KO group (Fig. 3). These findings indicate that autophagy can regulate ROS levels. Autophagy can modulate ROS levels through a series of signal pathways, such as the P62 transmission pathway, the mitophagy pathway, and the chaperone-mediated autophagy pathway (Li *et al*., 2015). Typically, ROS can participate in the interaction between autophagy and apoptosis because they can mediate redox signaling pathways (Scherz-Shouval & Elazar, 2007; Circu & Aw, 2010). However, when the autophagy function is disrupted, it will aggravate the oxidative stress response. Oxidative stress leads to the elevated ROS production, which ultimately leads to apoptosis.

One study found that under glucose starvation or hypoxic conditions, the ATM/CHK2/Beclin 1 axis promoted autophagy by sensing ROS, regulating excessive ROS accumulation, eliminating damaged mitochondria, and inhibiting apoptosis (Guo *et al*., 2020). This is consistent with the results of our study. After serum deprivation, autophagy of microglia cells was enhanced, and ROS level was increased compared with the normal control. In ATG5-KO cells, autophagy was not observed, but ROS level was significantly increased, and a large amount of ROS promoted cell apoptosis. In addition, NAC was added to inhibit ROS levels in starvation culture, and the results showed that autophagy level and apoptosis rate were also decreased. Thereby, our study demonstrates the strong association between autophagy and ROS. Autophagy is beneficial to the survival of microglia under starvation by regulating ROS levels, and ROS can also be involved in autophagy.

CONCLUSIONS

On conclusion, our study demonstrates that ROS and autophagy are closely linked, and play similar functions during cell survival and death. Under starvation, ROS and autophagy levels are upregulated, which is required for cell redox homeostasis. Autophagy deficiency is not conducive to cell survival. Autophagy and low levels of ROS are conducive to the survival of microglia. Antioxidant strategies may provide a way to treat nerve cell damage disease caused by accumulation of ROS under nutritional deficiency, which deserves more research attention. However, more studies should be conducted to analyze the effect of antioxidants on autophagy. Additionally, we used a cell model, not an animal model. Further research is needed.

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GUO, J. & MA, K. La autofagia mantiene la supervivencia de la microglia modulando la producción de ROS en condiciones de privación de suero. *Int. J. Morphol., 42(6)*:1567-1575, 2024.

RESUMEN: La autofagia desempeña un papel protector en el mantenimiento de la homeostasis celular en condiciones fisiológicas y en ayudar a las células a resistir la inanición en condiciones de privación de nutrientes. Las células pueden producir especies reactivas de oxígeno (ROS) en exceso en condiciones de privación de nutrientes, lo que puede provocar daño a los orgánulos y mal plegamiento de proteínas, promoviendo así la muerte celular. Sin embargo, no está claro si la autofagia interactúa con las ROS durante este proceso. En este estudio, exploramos si la autofagia está involucrada en el efecto protector de la apoptosis celular inducida por la privación de suero y la producción excesiva de ROS en células BV-2 con inactivación de Atg5. Además, examinamos el nivel de autofagia en células tratadas con N-acetilcisteína (NAC). Descubrimos que las células crecieron lentamente y envejecieron, y que los niveles de ROS y autofagia aumentaron después de 24 h de privación de suero. Los defectos de autofagia aumentaron significativamente los niveles de ROS y la muerte celular. En comparación con el grupo de inanición, el grupo de tratamiento con NAC redujo los niveles de ROS y autofagia ($p < 0,05$) y disminuyó la muerte celular. En conclusión, la autofagia y los niveles bajos de ROS pueden promover la supervivencia de la microglía en comparación con los defectos de autofagia o los niveles altos de ROS en condiciones de inanición. Por lo tanto, la autofagia y los niveles moderados de ROS son beneficiosos para mantener la homeostasis celular en condiciones patológicas.

PALABRAS CLAVE: Autofagia; Inanición de suero; ROS; Microglía; IL-6; TNF-α.

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