Life Sciences 238 (2019) 116976

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Histone deacetylase 6 inhibitor ACY1215 offers a protective effect through the autophagy pathway in acute liver failure

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ARTICLE INFO

Keywords:
Acute liver failure
Histone deacetylase 6 inhibitor
ACY1215
Autophagy

ABSTRACT

Aim: The purpose of the present study was to elucidate the protective effect of histone deacetylase 6 inhibitor ACY1215 on autophagy pathway in acute liver failure (ALF).

Main methods: Lipopolysaccharide (LPS) and D-galactosamine (D-Gal) were used to induce ALF model in C57BL/6 mice. D-Gal and tumor necrosis factor alpha (TNF-α) were applied in L02 cell. Autophagy inhibitor 3-MA and ACY1215 were conducted to induce 3-MA group, ACY1215 group and ACY1215 + 3-MA group.

Results: ACY1215 improved liver histological and functional changes in ALF mice model, whereas the autophagy inhibitor 3-MA aggravated liver tissue pathological and functional damage in ALF mice model group. The apoptotic levels (including apoptotic index/rate and apoptotic proteins) in ALF mice and L02 cell were ameliorated with treatment ACY1215. 3-MA accentuated the apoptotic levels in ACY1215 group. D-Gal/TNF-α could reduce L02 cell mitochondrial membrane potential (ΔΨm) in control group. ACY1215 increased the ΔΨm in ALF model. 3-MA also further reduced the ΔΨm in ACY1215 group. ACY1215 could induce autophagy in ALF mice and cell model group accompanied with an increase in expression of LC3-II and beclin-1 proteins and down-regulation of p62 protein. Moreover, the expression of LC3-II and beclin1 proteins were greatly reduced and the expression of p62 protein was ascended after intervention with 3-MA in ACY1215 group.

Significance: Histone deacetylase 6 inhibitor ACY1215 could protect acute liver failure mice and L02 cell by inhibiting apoptosis pathway through enhancing autophagy way.

1. Introduction

Acute liver failure as characterized by inflammation-mediated hepatocyte damage, always accompanies by hepatocyte apoptosis and necrosis. It is caused by plentiful factors such as hepatitis virus, hepatotoxic drugs and hepatic ischemia-reperfusion injury. It clinically manifests as critical condition, rapid development, high mortality which its exact pathogenesis is not much clear [1]. Recent research finds that all kinds of liver diseases including viral hepatitis, alcoholic hepatitis and acute liver failure are associated with autophagy which can take control of hepatocellular injury and repair [2–4].

Autophagy widely exists in eukaryotic cells within reusing the degradation products dependent on lysosome, which is an essential approach for maintaining cell energy balance. In physiological condition, autophagy maintain in the baseline [5]. Autophagy will be activated when the cells are confronted with the various biological events such as cellular remodeling during development and differentiation, adaptation to stress conditions and extension of lifespan [6]. Autophagy, as a double-edged sword, can exert positive effects on hepatocyte to some extent and may lead to negative effects, which is insufficient or excessive in liver disease [7]. Emerging evidence has demonstrated that the degree and duration of autophagy is pivotal for the development in many diseases [8,9]. Many studies have found that the autophagy is involved in ALF animal model [10].

Apoptosis has a tight connection with autophagy regulating cell aging and death synergistically [11]. Apoptosis is a programmed cell death. There are two main pathways. A pathway is mediated by cell death receptor and another pathway is mediated by mitochondrion [12]. Both pathways are important pathogenic factors in various liver diseases [13]. The apoptosis factor cytochrome c (Cytc) in mitochondria will be released to cytoplasm and activation of caspases cascade, when mitochondrial-mediated apoptosis pathway is initiated [14]. In addition, Bcl-2 family members are extremely vital manipulators. They regulate the release cytochrome c into the cytoplasm [15]. Autophagy and apoptosis are regulated by processes involving common transcription factors and signaling pathways such as nuclear factor-kappa B (NF-κB) and tumor protein p53 [16]. Previous study has shown that the autophagy may precede the occurrence of apoptosis and regulate

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https://doi.org/10.1016/j.lfs.2019.116976
Received 2 September 2019; Received in revised form 8 October 2019; Accepted 15 October 2019
Available online 18 October 2019
0024-3205/

apoptosis at the early stage [17].

Histone deacetylase inhibitor (HDACi) is a burgeoning anticancer agent. It can participate in regulating the cell differentiation, growth arrest, apoptosis and autophagy [18–20]. Our previous study shows that histone deacetylase inhibitor CAY10683 can offer a protective effect in ALF by inhibiting mitochondrial apoptosis pathway [18]. In addition, the broad spectrum HDAC inhibitor TSA is verified to provide new strategies for the treatment of esophageal cancer by adjusting the autophagy pathway [19]. And histone deacetylase inhibitor could also reduce myocardial injury caused by ischemia-reperfusion via activating autophagy pathway [20]. Our previous studies have proved that ACY1215 could exert protective effects on ALF, which could regulate the TLR4-MAPK/NF-κB pathway [21] and regulate the mitochondrial-mediated oxidative stress [22]. However, the effect of HDACi in ALF via the cellular significance of the connection between autophagy and apoptosis remains unclear.

This study adopted the LPS/D-Gal-induced ALF mice model and D-Gal/TNF-α-induced injury in the L02 cell line. Histone deacetylase 6 inhibitor ACY1215 was selected as intervention agent and co-administration of the autophagy inhibitor 3-MA to investigate the protective effect of ACY1215 regulation on the autophagy pathway in ALF.

2. Materials and methods

2.1. Reagents and antibodies

TNF-α (purity of 99%), LPS (purity of 99%) and D-Gal (purity of 98%) were obtained from Sigma (St. Louis, USA). ACY1215 and 3-MA were obtained from Selleck (Houston, USA). Fetal bovine serum (FBS) and dulbecco's modified eagle medium (DMEM) basic were purchased from Gibco (NY, USA). Rabbit anti-mice/human beclin1, LC3-II/I, p62, Bcl-2, Bax and Cytc were purchased from Cell Signaling Technology (Boston, USA). GAPDH was purchased from Proteintech (Wuhan, China). Secondary antibodies applied were the goat anti-rabbit fluorescent antibody purchased from LI-COR Biosciences, Inc. (Lincoln, USA). Mitochondrial membrane potential (ΔΨm) assay kit with JC-1 was obtained Beyotime (Wuhan, China).

2.2. Animal groups

Male C57BL/6 mice (n = 30) specific pathogen-free (SPF) (20–25 g) were purchased from Experimental Animal Center of Wuhan University. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (WDRM20181018). All animals were acclimatized for one week before experimentation. All animals were allowed access to food and water freely throughout the acclimatization and experimental periods. They were kept in temperature (22 ± 2 °C) with a 12 h light/dark cycle. The mice were randomly divided into five groups: control group, ALF model group, 3-MA group, ACY1215 group, ACY1215 + 3-MA group. Except for control group, all mice were modeled by intraperitoneal injection with D-Gal (400 mg/kg) combined with LPS (100 μg/kg). The control group mice were given an equal volume of saline. 3-MA (10 mg/kg) was given by intraperitoneal injection in 3-MA group 2 h before ALF model protocol. ACY1215 (25 mg/kg) was applied by intraperitoneal injection 2 h before given D-Gal/LPS in ACY1215 group. ACY1215 (25 mg/kg) and 3-MA (10 mg/kg) were used concurrently 2 h before ALF model implemented in ACY1215 + 3-MA group. All mice were sacrificed under anesthesia after 24 h when ALF model protocol.

2.3. Tissue collection, histopathological analysis, and serum aminotransferase tests

10% neutral-buffered formalin was applied to fix fresh liver specimens for 2 days. And then it was embedded in paraffin, processed for sectioning and staining by hematoxylin–eosin (HE). Liver sections were assessed under BX 51 light microscope (Olympus, Japan). Hepatocellular apoptosis index was evaluated by TUNEL assay kit (Roche Applied Science) according to the manufacturer's instructions and then analyzed under a fluorescence microscope. The 200 × high magnification was counted in the fields. And the algorithm of apoptosis index of each group was same with our previously report [23]. The serum ALT and AST levels in each group were tested by automated Aeroset chemistry analyzer (Abbott Co. Ltd., USA).

2.4. Cell culture and chemical treatment

The human embryonic liver cell line L02 was grown in DMEM medium mixed with 10% FBS in an incubator at 37 °C, 5% CO_2, and saturated humidity. The intervention groups were divided into control group, model group, 3-MA group, ACY1215 group and 3-MA + ACY1215 group. The cells were passed in 6-well plates and cultured to 70% density. Following the previously report [18], D-Gal (44 μg/mL) and TNF-α (100 ng/mL) were applied to induce model group excluding the control group. 3-MA (10 mM) [24] and or ACY1215 (2.5 μM) was added into 3-MA group, ACY1215 group and ACY1215 + 3-MA group 2 h before ALF model group establishment. After 24 h, the cells were harvested.

2.5. Apoptosis rate detection of L02 cell

Annexin V-PE/7AAD apoptosis kit (BD, USA) was applied to detect L02 cell apoptosis rate. At first, the 1×10^5 cells were cultured in a 6-well plate. After chemical treatment, the cells in each group were collected for staining. Then they were washed twice with PBS. The cells were orderly added with 400 μL buffer solution, 5 μL Annexin V-PE, and 5 μL 7AAD, then incubated for 15 min at 37 °C away from light. The apoptosis rate in the early and late stages was analyzed by flow cytometry (BD, USA) within 1 h.

2.6. L02 cell mitochondrial membrane potential (ΔΨm) detection

The L02 cell mitochondrial membrane potential was measured using the JC-1 MMP assay kit. The culture solution was aspirated and 1 ml of JC-1 staining solution was added. After incubation at 37 °C for 20 min the supernatants were aspirated and washed twice with JC-1 staining buffer (1 ×). It was observed under a fluorescence microscope (200 ×). The fluorescence intensity was detected with a multimode plate reader (PerkinElmer, Finland). 490 nm and 530 nm as the excitation and emission wavelengths were used for detection of monomeric form of JC-1, which is shown as green fluorescence. 525 nm and 590 nm were used to detect aggregation of JC-1, which is shown as red fluorescence. The ΔΨm was represented by red/green fluorescence ratio.

2.7. Western blot analysis for expression of autophagy and apoptosis proteins

Proteins from the liver of mice and L02 cell in each group were separated by to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Millipore, USA). The membranes were incubated with different primary antibodies and secondary antibody. Finally, expression of each protein was detected by Odyssey infrared imaging system. The dilutions of the primary antibodies were as follows: LC3II/I, 1:1000; beclin1, 1:1,000; p62, 1:1000; Bcl-2, 1:1,000; Bax, 1:1,000; Cytc, 1:1,000; and GAPDH, 1:2,000. Membranes were also probed for GAPDH as additional loading controls.

2.8. Statistical analysis

All statistical analyses were performed using SPSS 16.0. Data were

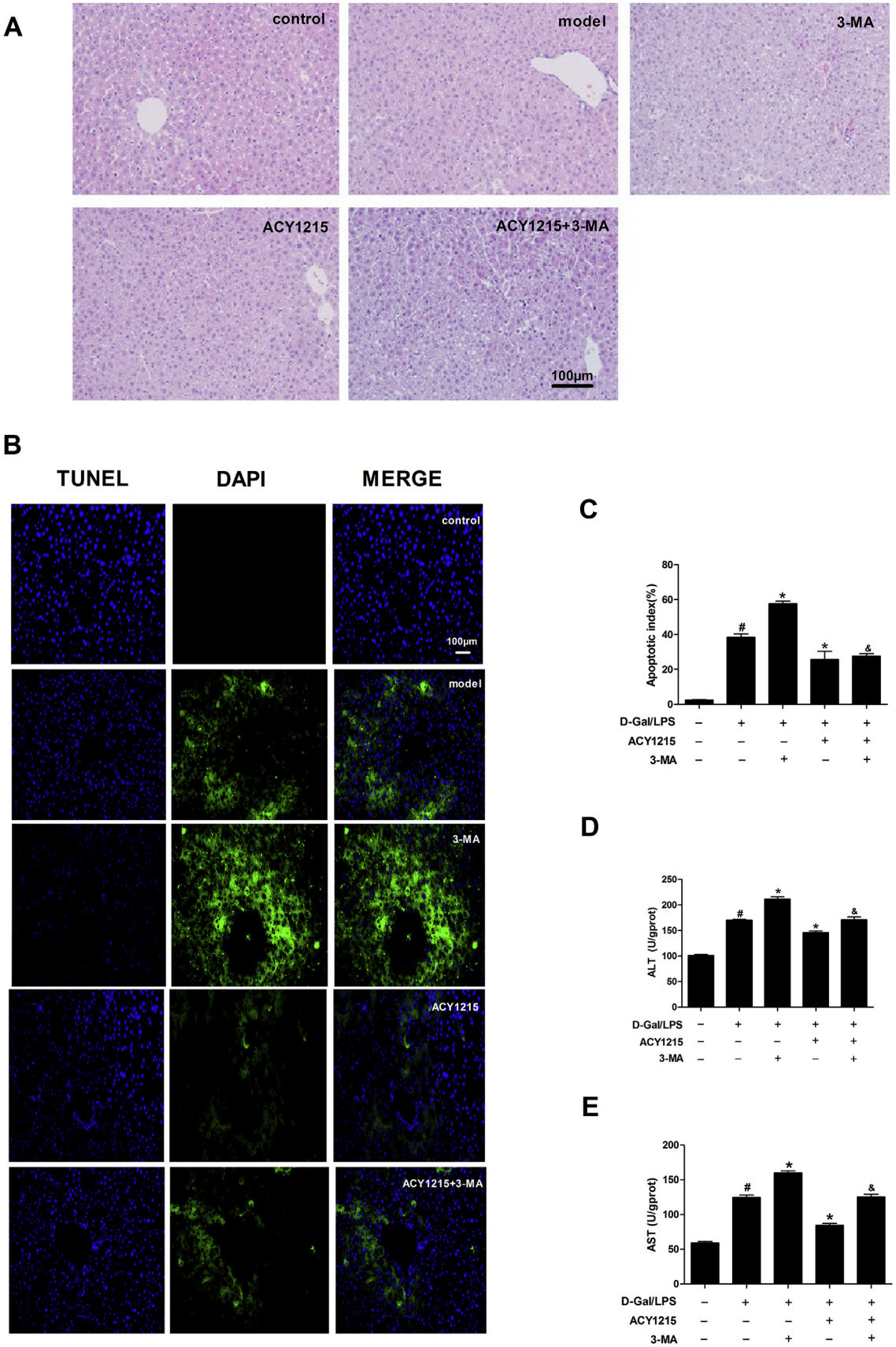

Fig. 1. (A) The liver tissues were stained with HE (200 ×). (B–C) Cell apoptosis was detected by TUNEL method in liver tissues (200 ×). (D–E) The activities of ALT and AST were used to evaluate liver function. Compared with control group, $^{\#}P < 0.05$; Compared with ALF group, $^{*}P < 0.05$; Compared with ACY1215 group, $^{\&}P < 0.05$.

presented as mean ± standard deviation. One-way analysis of variance (ANOVA) or Student's *t*-test was applied to examine the differences between groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. ACY1215 could alleviate the hepatic histological changes and serum biochemical indicators in ALF mice

Liver pathological changes and serum biochemical indicators were essential to evaluate. As shown in Fig. 1 A, the radial arrangement of hepatocytes around central veins was neat, which without infiltration of inflammatory cells. In the ALF model group, the liver lobular structure ruined, and a mass of hepatocytes were necrotic surrounded by inflammatory cell infiltration. However, the autophagy inhibitor 3-MA could further aggravate the liver pathological damage in ALF group. The hepatic lobule structure in ACY1215 group mice was clearer than that in the ALF model group, and the infiltration of inflammatory cells and necrotic hepatocytes were also reduced. The autophagy inhibitor 3-MA could also aggravate the liver pathological damage in ACY1215 group. As shown in Fig. 1B–C, TUNEL staining showed that the apoptotic index in the 3-MA group was visibly increased, when compared with the ALF model group ($P < 0.05$). ACY1215 could significant decrease the apoptotic index in ALF group and in 3-MA group ($P < 0.05$). As shown in Fig. 1D–E, the serum ALT and AST in ALF mice were distinctly increased than in controls ($P < 0.05$). Compared with the ALF model group, the ALT and AST levels were greatly lowered in ACY1215 group ($P < 0.05$). 3-MA increased the serum ALT and AST levels in ACY1215 group ($P < 0.05$).

3.2. ACY1215 reduced the apoptosis rate in ALF L02 cell

The L02 cell apoptosis rate in each group was detect by flow cytometry. As shown in Fig. 2A–B, the results indicated that the apoptosis rate of ALF group was higher than the control group and ACY1215 could relieve the L02 cell apoptosis after D-Gal/TNF-α disposing. 3-MA could further aggravate the L02 cell apoptosis in the pretreatment of ACY1215 in model group ($P < 0.05$).

3.3. ACY1215 enhanced the mitochondrial membrane potential of the ALF L02 cell

The JC-1 staining was utilized to detect ΔΨm. The decline of ΔΨm was marked as an event during early cell apoptosis. The change in ΔΨm was displayed as shown in Fig. 3A–B. D-Gal/TNF-α treatment reduced the ΔΨm compared with the control ($P < 0.05$). ACY1215 pretreatment could increase the ΔΨm in ALF group ($P < 0.05$). However, autophagy inhibitor 3-MA reduced the protective effect of ACY1215 by decreasing the ΔΨm in ALF group ($P < 0.05$).

3.4. ACY1215 activated autophagy pathway in ALF mice and in L02 cell

The autophagy-related protein levels were detected to investigate the mechanism of ACY1215 on alleviating D-Gal/LPS-induced liver injury and D-Gal/TNF-α-induced L02 cell injury. As shown in Fig. 4A–B and Fig. 5A–B, compared with control group, the protein levels of beclin1 and LC3-II were significant decreased and the protein level of p62 was greatly increased in ALF group ($P < 0.05$). However, ACY1215 pretreatment could up-regulate the beclin1 and LC3-II protein levels and down-regulate the p62 protein level in ALF group ($P < 0\ 05$). Moreover, autophagy inhibitor 3-MA could reduce the protective effect of ACY1215 by down-regulating the expression of beclin1 and LC3-II protein and elevating the p62 protein level which further indicates the potential mechanism of ACY1215 protect against ALF with the autophagy activation ($P < 0\ 05$).

3.5. ACY1215 improved the expression of apoptosis-related protein in ALF mice and in L02 cell

As shown in Fig. 4C–D and Fig. 5C–D, the expression of Bax and

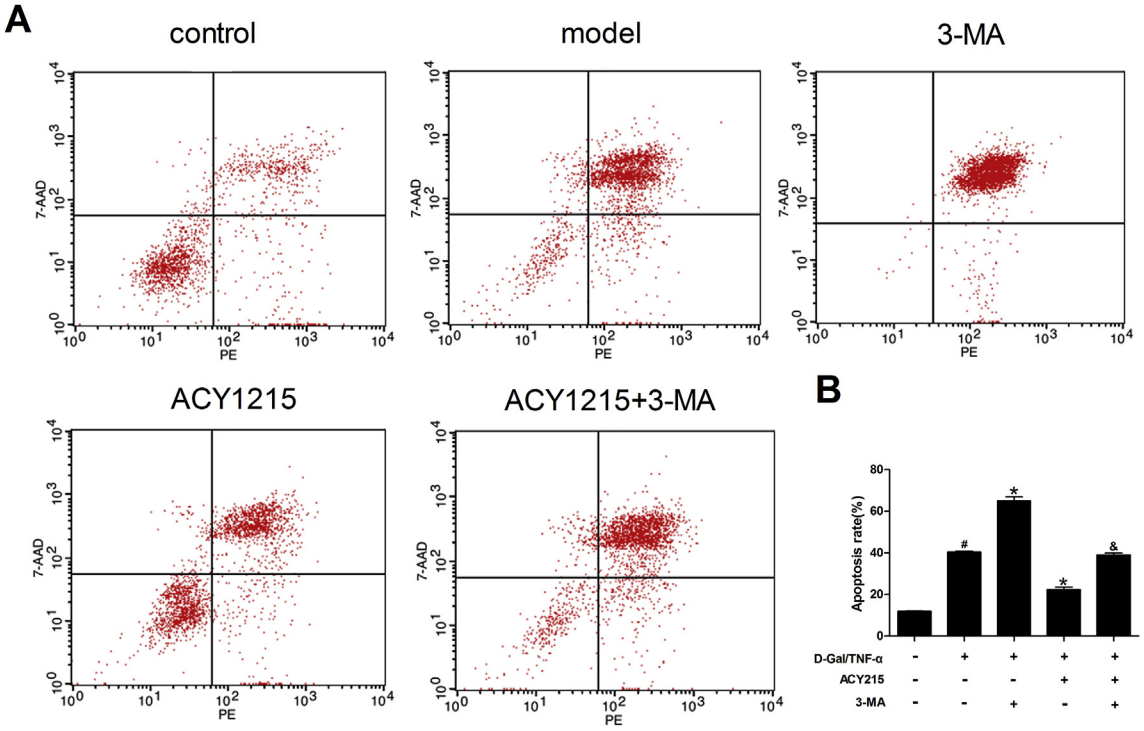

Fig. 2. (A–B) The L02 cell apoptosis rate from each group was detected by flow cytometry. The apoptosis rate of ALF group is higher than the control group ($^{\#}P < 0.05$). ACY1215 can relieve the L02 cell apoptosis in ALF group ($^{*}P < 0.05$) and 3-MA can increase the L02 cell apoptosis in ALF group ($^{*}P < 0.05$). Besides, 3-MA can further aggravate the L02 cell apoptosis in the pretreatment of ACY1215 group ($^{\&}P < 0.05$).

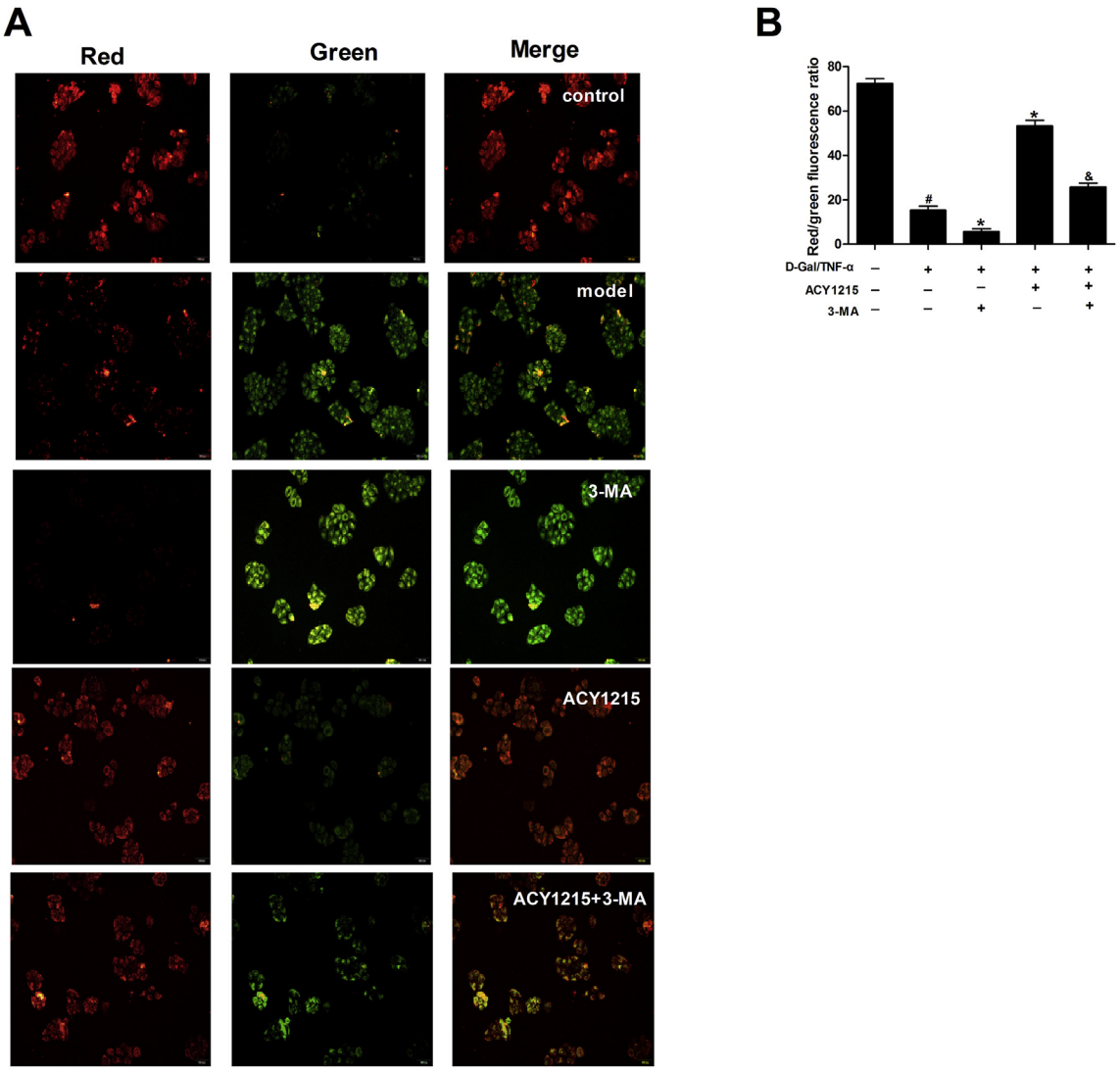

Fig. 3. (A–B) The JC-1 staining was carried out to detect the mitochondrial membrane potential (ΔΨm) in L02 cell. Red fluorescence indicated intact mitochondrial membrane potential. Green fluorescence indicated dissipation of ΔΨm. The ΔΨm was reflected by red/green fluorescence ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Cytc was visibly elevated in ALF mice and L02 cell model group than the control ($P < 0.05$). However, ACY1215 could down-regulate the levels of Bax and Cytc in ALF group ($P < 0.05$). Autophagy inhibitor 3-MA reduced the protective effect of ACY1215 by up-regulating the expression of Bax and Cytc in ALF group ($P < 0.05$). It was an adverse characteristic in the expression of Bcl-2 protein ($P < 0.05$).

4. Discussion

Acute liver failure (ALF) is a serious liver disease with a high mortality, which is caused by a variety of factors. Other than liver transplantation, there is currently no effective therapy for the end stage of the disease [1]. Hence, further research is urgently required to distinguish novel therapeutic agents and develop more potent combination strategies for ALF treatment. A number of studies have shown that there is a dynamic profile of autophagy in the progression of ALF animal models, as well autophagy plays a vital role in the occurrence and development of ALF [4,7]. Besides, it's verified that autophagy activation represents an effective strategy to ameliorate ALF pathology [7].

Autophagy is a highly conserved degradation process which mainly to eliminate long life protein and damaged organelles. Furthermore, it may produce free amino acids and fatty acids for the body to update using which to keep the steady state [6]. Autophagy is regulated by a variety of autophagy-related kinase complexes [25]. The one of most important formation of complexes provides a site for microtubule-associated protein 1 light chain 3 (LC3) which is a marker protein on the autophagosome membrane. There are two forms of LC3 proteins in cells: LC3-I and LC3-II. After the synthesis of LC3, the c-terminal of LC3 protein is cleaved by Atg4 protease to LC3-I. Then LC3-I is scattered in the cytoplasm. When autophagosomes are formed, LC3-I and phosphatidylethanolamine are coupled to form LC3-II and localize to the autophagy inner membrane and outer membrane. LC3-II is stably retained on the autophagosome membrane until it fuses with lysosomes. Therefore, it is used as a marker for autophagosomes, and the level of LC3-II reflects the number of autophagosomes to some extent [26]. The p62 protein can bind directly to LC3.When cellular autophagy pathway is inhibited, p62 protein will be accumulated. However, the level of p62 protein will be lessened when autophagy is induced. The degradation of p62 protein is by autophagy as well. It can unite ubiquitinated proteins to the autolysosome to assure their degradation in the lysosome [27]. Beclin1 is the first self-identifying marker gene in mammals, and represents the activity of autophagy. Current research indicates that Beclin1 plays an essential role in the initial stage of autophagy. It is also participated in the maturation stage of autophagosomes [25]. The

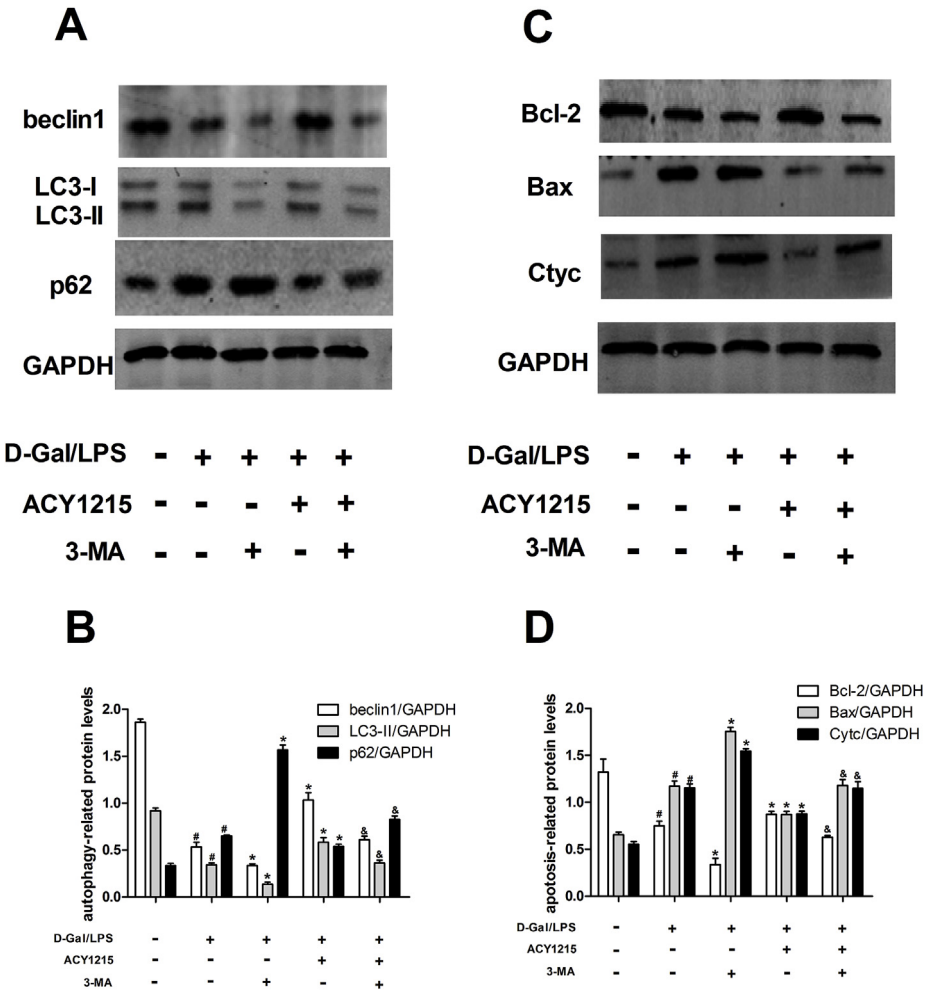

Fig. 4. (A–D) The levels of autophagy protein and apoptosis-related protein expression in liver tissues from different groups. Compared with control group, $^{\#}P < 0.05$; Compared with ALF group, $^{*}P < 0.05$; Compared with ACY1215 group, $^{\&}P < 0.05$.

expressions of LC3-II and beclin1 proteins are positively correlated with the level of autophagy.

Mitochondrial apoptosis pathway is a momentous characteristic in the pathogenesis of many liver diseases [28]. The mitochondrial apoptosis pathway usually companies with the swelling of mitochondria, reduced ΔΨm and increased contents released from mitochondria into cytoplasm. The depletion of mitochondrial capacity will eventually be induced [29,30]. Bcl-2 family proteins are found to regulate the occurrence of such apoptotic pathways. They are mainly divided into as the two-opposite protein: anti-apoptotic protein (like Bcl-2) and pro-apoptotic protein (like Bax). Cytc is a vital apoptosis factor to activate caspase-9/3-dependent cascades when mitochondrial pathway of apoptosis is initiated. In addition, hepatic apoptosis is regulated by autophagic activity [11]. Moreover, autophagy can specifically control the mitochondrial degradation and then influence the mitochondrial recycle [16]. As a key autophagy molecule, beclin1 is essential for the recruitment of other autophagy proteins involved in the expansion step and can interact with anti-apoptotic proteins of the Bcl-2 family. For example, beclin1 can regulate the apoptosis process of cells through its BH3 structure interacting with Bcl-2 protein [31]. Knockdown of autophagy proteins beclin-1 or LC3 will inhibit cigarette smoke extract-induced apoptosis, suggesting that an enhanced autophagy is associated with apoptotic death in epithelial cells [26]. An abundance of autophagy protein p62 can stimulate the production of reactive oxygen species (ROS) which activates mitochondrial apoptosis pathway [32].

Previous studies have proved that ACY1215 as a leading HDAC6 inhibitor, can kill tumor cells via multitudinous mechanisms, including regulate DNA damage response, cell cycle checkpoint disruption and the induction of apoptosis. In addition, ACY1215 has been tested in advanced clinical trials of hematological cancer [33]. In addition, recent reports reveal that HDAC6 inhibitor ACY1215 also can adjust cell growth, metastasis, autophagy and apoptosis [34]. The HDAC6 inhibitor tubastatin can induce autophagy pathway to ameliorate pulmonary fibrosis [35]. Our previous study has demonstrated that ACY1215 protected acute liver failure through regulating the TLR4-MAPK/NF-κB pathway [21]. Inhibiting NF-κB pathway not only improved the survival rate of mice with liver failure, but also decreased the levels of inflammatory cytokines including TNF-α, IL-1β, and IL-6. In our previous study, ACY1215 could inhibit necrosis of hepatocytes in ALF mice through regulating the mitochondrial-mediated oxidative stress. The quantitative proteomic analysis showed that ACY1215 restrained oxidative phosphorylation, normalized the mitochondria function respiratory electron-transport chain and inhibited the generation of reactive oxygen species (ROS) [22]. They were two different key protective mechanisms of ACY1215 for ALF. Additionally, the protective mechanisms of HDAC6 inhibitor ACY1215 in ALF may via regulating autophagy pathway is not yet been studied. It is completely meaningful to continue to explore the other potential protective mechanism of ACY1215 in ALF. Therefore, the present experiments were performed.

The specific HDAC6 inhibitor ACY1215 was used *in vitro* study. ACY1215 treatment significantly increased the proteins level of beclin1, LC3-II and Bcl-2 in L02 cell and decreased the proteins level of p62, Bax and Cytc in the ALF group. The apoptosis rate was significantly

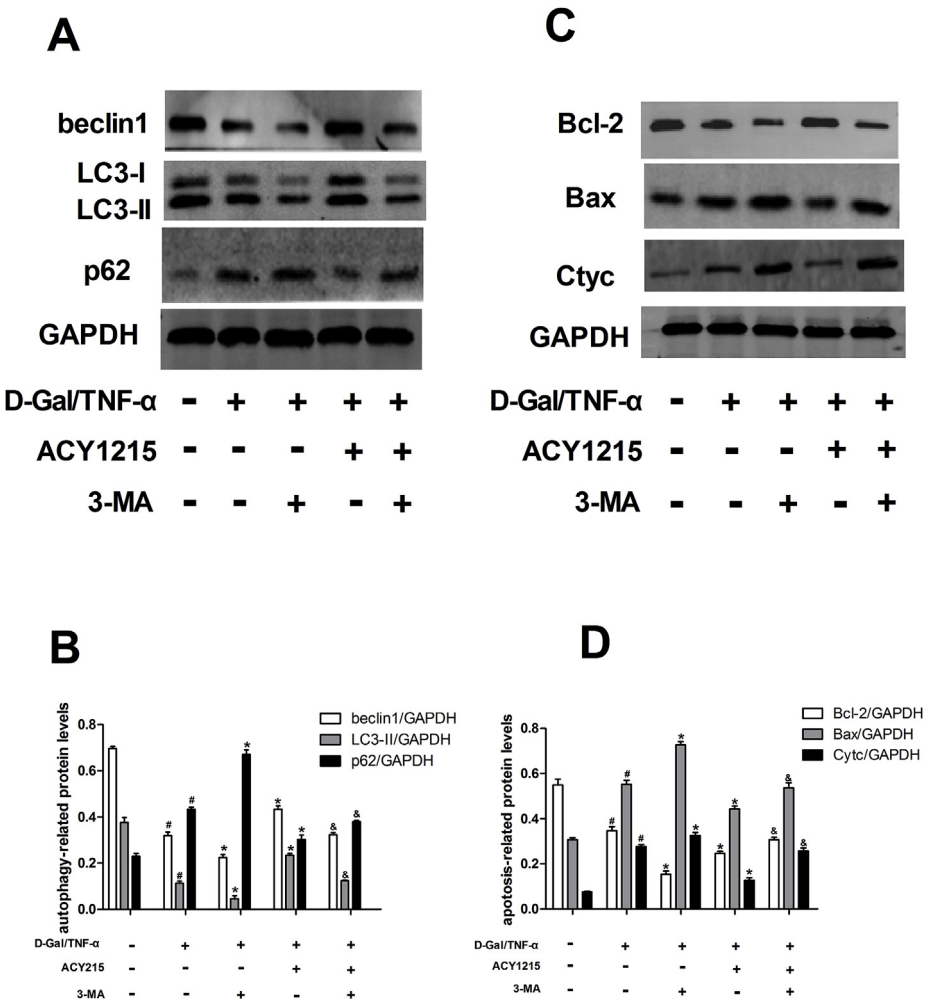

Fig. 5. (A–D) The levels of autophagy protein and apoptosis-related protein expression in L02 cell from different groups. Compared with control group, $^{\#}P < 0.05$; Compared with ALF group, $^{*}P < 0.05$; Compared with ACY1215 group, $^{\&}P < 0.05$.

decreased and mitochondrial membrane potential was obvious increased in the ACY1215 group than ALF group. To further observe the effects of modulations of ACY1215 in autophagy pathway, the autophagy inhibitor 3-MA [36] which based on their inhibitory effect on class III PI3K activity was performed. Our data revealed that 3-MA reversed the autophagy level and aggravated the apoptosis level both in ALF group and ACY1215 group. *In vivo* experiments, the ALF mice model was successfully established by D-Gal/LPS. Then the liver pathological changes and serum biochemical indicators in ALF mice were explored to study the protective effect of ACY1215. TUNEL staining revealed that the ACY1215 could distinctly reduce apoptotic index in the ALF group. Likewise, the expression level of autophagy-related proteins and apoptosis-related proteins had similar tendency with the *in vitro* study. However, the autophagy inhibitor 3-MA not only reduced the level of autophagy and increased the apoptosis level in ALF mice, but also further worse liver pathological changes and serum biochemical indicators.

These experimental results showed that autophagy level was down-regulated and apoptosis level was up-regulated in ALF. HDAC6 inhibitor ACY1215 could up-regulate autophagy pathway and then inhibit the apoptosis pathway in ALF. The results proved that the autophagy pathway plays a crucial role in ALF as well. However, the protective effect of ACY1215 on ALF by up-regulating the autophagy pathway could be suppressed by the autophagy inhibitor 3-MA. It was further verified that the protective mechanism of ACY1215 on ALF may also activate autophagy pathway.

5. Conclusion

Collectively, this study manifested that ACY1215 possessed a protective effect on ALF *in vitro* and *in vivo*, which is consistent with our previous studies [34,35]. Especially, a proteomic analysis of the differentially expressed pivotal autophagy and autophagy protein profiles confirmed that they were associated with the pathogenesis of ALF. Our results demonstrated that apoptosis levels of ALF mice and L02 cell were improved by ACY1215 via the activation of the autophagy pathway. These findings would contribute to understand the different molecular hepato-protective effect of ACY1215. It provides a strongly favorable reason for reasonable use of this drug for hepato-protection. In addition, autophagy will likely become a new target for the treatment of ALF. The development of drugs that can target the autophagy pathway of different cell types will find new key points for treatment. Further preclinical studies on autophagy-inducing therapies are expected as well.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Abbreviations

ALF	acute liver failure
LPS	lipopolysaccharide

D-Gal	D-galactosamine
TNF-α	tumor necrosis factor alpha
HDACi	histone deacetylase inhibitor
LC3	microtubule-associated protein 1 light chain 3
Bcl-2	B-cell lymphoma 2
Bax	Bcl-2 associated X protein
Cytc	cytochrome c

Authors' contributions

ZJG contributed to study conceptualization, supervision, and original draft. QC conceived and designed the experiments. QC, YW and FZJ performed the experiments. QC and CXS analyzed the data. QC, YW and FZJ contributed reagents, materials and analysis tools. QC wrote the paper. ZJG reviewed and edited the manuscript. All authors approved the final version of the manuscript.

Funding

This work was supported by the Natural Science Foundation of China (81870413). The authors would like to thank the central laboratory at Renmin Hospital of Wuhan University (Wuhan, Hubei, China) for their technical supports.

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