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Original Article

Angiotensin receptor-neprilysin inhibitor attenuates ischemia-hypoxia-induced myocardial injury via inhibition of autophagy

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Abstract: Objectives: Angiotensin receptor-neprilysin inhibitor (ARNI) improves cardiac function and protects from an ischemic myocardium. However, the role and mechanism of ARNI on autophagy in cardiac ischemic injury are poorly understood. Here, we investigated the protective effect and underlying mechanisms of ARNI on autophagy in H9c2 cardiomyocytes induced through ischemia and hypoxia (IH) treatment. Methods: The cytotoxicity of IH injury on H9C2 cells with and without ARNI were evaluated using cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) release assays. The effect of ARNI on apoptosis was detected using flow cytometry. The expression of autophagic proteins (LC3-II, Beclin 1, and p62) was detected using western blot. Results: The viability of H9c2 cells was significantly decreased at different IH-treated time points; ARNI pretreatment increased cell viability and inhibited IH injury in a dose-dependent manner. H9c2 cells treated with IH (6 h) significantly increased LDH release, while ARNI dose-dependently improved LDH release, with 20 μmol/L ARNI having the most significant effect. ARNI also ameliorated IH-induced apoptosis. IH treatment increased the protein expression of LC3-II and Beclin 1 and decreased the expression of p62, which were reversed by ARNI pretreatment. Furthermore, autophagy was further increased after pretreatment with rapamycin in IH-induced H9c2 cells, which abrogated the protective effect of ARNI. Conclusions: Our study shows that ARNI has a protective effect on IH-induced cardiomyocyte injury, which may be related to the inhibition of autophagy.

Keywords: Heart failure, ischemia/hypoxia, angiotensin receptor neprilysin inhibitor, cardioprotection, autophagy

Introduction

Heart failure (HF) caused by acute myocardial infarction (AMI) remains a serious public health problem throughout the world [1]. The *China Cardiovascular Health and Disease Report 2020* pointed out that there were 11.39 million people with coronary heart disease and 8.9 million with HF in China alone [2]. HF is a complex clinical syndrome, and its pathological mechanism is closely related to ventricular remodeling caused by abnormal expression of neuroendocrine hormones [3]. Early changes in HF play a compensatory protective role. Still, in persistent pathological conditions, HF causes decompensated changes in the heart and further deterioration of the disease, and it is a severe disease that threatens human health [4]. AMI is considered to be one of the most prominent causes of death from cardiovascular disease worldwide, and the incidence of HF after AMI is approximately 25% [5, 6]. Heart failure mortality rates can reach 50% within five years, and the hospitalization burden and economic cost are also high [7]. Therefore, it is necessary to further investigate therapeutic drugs and their mechanism of action for the treatment of HF.

Angiotensin receptor-neprilysin inhibitor (ARNI) is a new double-effect compound preparation developed recently to treat HF. Its unique mechanism of action may have a better improvement effect on HF than conventional drugs [8]. The

results of our previous study not only revealed that ARNI had an impact on natriuretic diuresis and vasodilation, as well as prevention and reversal of myocardial remodeling but also confirmed that ARNI played a critical role in improving HF, increasing myocardial contractility, inhibiting cardiomyocyte hypertrophy and fibrotic changes after AMI. This indicated that ARNI had a protective effect on an ischemic myocardium [9, 10]. Although there is clinical evidence for the application of ARNI in the treatment of HF, the mechanism of ARNI in myocardial ischemia injury is still unclear. Under physiological conditions, autophagy is maintained at a deficient baseline level. It can be activated by cell ischemia, hypoxia and nutritional deficiency; prolonged hypoxia can induce the autophagic death of cardiomyocytes [11, 12]. Previous studies on the mechanism of autophagy have mainly focused on cardiovascular disease processes such as myocardial hypertrophy [13], myocardial fibrosis [14], atherosclerosis [15], and cardiac remodeling [16]. However, whether ARNI is involved in regulating autophagy in cardiomyocyte injury has not yet been elucidated.

For this reason, we aimed to investigate whether ARNI is involved in regulating cardiac ischemic injury and in elucidating the underlying mechanisms of this process. We proposed the following scientific hypothesis: ARNI improves myocardial damage by mediating autophagy, thereby alleviating HF. In the current study, we constructed an HF model of H9c2 cardiomyocytes *in vitro* through ischemia and hypoxia (IH) treatment to explore whether ARNI can improve myocardial remodeling and cardiac function. The objective of this study was to determine the beneficial effect of ARNI on H9c2 cardiomyocytes which were exposed to IH injury; then, an *in vitro* cell model of IH-induced H9c2 cell injury was established by using an anaerobic gas production bag.

Materials and methods

Materials

ARNI (LCZ696) and rapamycin (an autophagy inducer) were purchased from MedChemExpress (MCE, New Jersey, USA). Fetal bovine serum was obtained from (Royacel, Lanzhou, China). Glucose-free Dulbecco's Modified Eagle Medium was purchased from Gibco (CA, USA). Other culture reagents, all sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and western blotting substrate reagents were purchased from Boster Biological Technology (Wuhan, China). Round-bottomed vertical anaerobic culture bags and anaerobic gas production bags were purchased from hopebio (Qingdao, China).

Cell culture

H9c2 cells were obtained from the Cell Bank at Shanghai Institute for Biological Sciences and cultured in DMEM medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin solution. The cells were then incubated at 37°C in the incubator with 5% CO_2 and 95% oxygen. H9c2 cells were passaged when cells were grown to about 90% confluence. Cells were passaged every 2 to 3 days and inoculated into the corresponding culture plates during the logarithmic growth phase.

Establishment and experimental grouping of IH model of H9c2 cells

To construct a model of IH *in vitro*, H9c2 cells were subjected to IH treatment. H9c2 cells were divided into control, IH model, IH + ARNI, IH + ARNI + Rapamycin (IH + ARNI + Rapa), and IH + Rapamycin (IH + Rapa) group. H9c2 cells were treated with IH to induce *in vitro* HF model. Before modeling, H9c2 cells were first treated with different concentrations (10, 20, 40, 80, 120, 160, and 200 μmol/L) of ARNI for different IH times (3, 6, 12, 24 h) to ascertain the optimal therapeutic concentration of ARNI and the optimal intervention time for IH. When modeling, H9c2 cells were pretreated with a culture medium containing determined concentrations of ARNI and rapamycin (100 nmol/L). Subsequently, the cell culture medium was replaced with a serum-free medium. The culture plate was cultured in a round-bottomed vertical anaerobic culture bag with an anaerobic gas production bag.

Cell viability assay

H9c2 cells were seeded in a 96-well plate with 5000 cells/well, and a blank group with only a culture medium was set up. Plates were then placed in a 37°C incubator to allow the cells to adhere stably. Subsequently, cells were incubated with a gradient of ARNI concentrations

(10, 20, 40, 80, 120, 160, and 200 μmol/L) for 3, 6, 12 h, and 24 h to observe cell growth. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (Absin, Shanghai, China). After adding 10 μL of CCK-8 reagent to each well, the cells were incubated at 37°C for 1.5 h, and the optical density (OD) value was detected at a wavelength of 450 nm. Cell viability (%) = (OD value of model group - OD value of blank group)/(OD value of normal group - OD value of blank group) × 100%.

Detection of LDH leakage rate

A lactate dehydrogenase (LDH) release assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to detect the cardiomyocyte LDH leakage rate in each group of cell culture medium.

Flow cytometric analysis of apoptosis

H9c2 cells were harvested by dissociation with trypsin-EDTA, washed twice with cold phosphate-buffered saline (PBS), and resuspended in a 1 × binding buffer. Next, 100 μL of the solution (1 × 10^5 cells) was transferred into a 5-mL culture tube. After adding 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI), the cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Finally, 400 μL of 1 × binding buffer was added to each tube, and cells were analyzed by flow cytometry within 1 h.

Western blotting

H9c2 cells were washed twice with pre-cold PBS buffer and immediately lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails. The extract protein concentration was determined using a bicinchoninic acid (BCA) kit according to the manufacturer's instructions. SDS-PAGE was used to separate equal amounts of protein (30 μg), which were then electrotransferred onto a nitrocellulose membrane. The membrane was blocked for 2 h with 5% skimmed milk in 10 mM Tris-HCl (pH 7.4) buffer containing 0.05% Tween-20 at room temperature. The membrane was incubated overnight at 4°C with anti-Beclin 1 antibody (Beclin 1, 1:2000 dilution; Abcam, UK), anti-LC3B antibody (LC3B, 1:2000 dilution; Abcam, UK), anti-p62 antibody (p62, 1:2000 dilution; Abcam, UK), and anti-β-actin (1:1000 dilution; Abcam, UK). The next day, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:8000 dilution; Boster, China) for two hours at room temperature. Chemiluminescent blot signals were detected through enhanced chemiluminescence (Boster, Wuhan, China) and captured using a chemiluminescence imaging system (Bio-Rad, USA). Protein bands were normalized to the control sample and the expression level of the proteins was quantified relative to that of β-actin. Finally, the bands were analyzed using Image LabTM analysis software.

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the SPSS 23.0 software (SPSS Inc., USA). The comparisons between groups were performed by one-way analysis of variance (ANOVA). $P < 0.05$ was set as the threshold for statistical significance.

Results

ARNI protected H9c2 cells from ischemia and hypoxia

To determine whether ARNI has a protective effect on IH-injured cardiomyocytes, we performed a CCK-8 assay to investigate the impact of ARNI on H9c2 cells under hypoxic conditions. The cell viability was highest at 20 μmol/L but gradually decreased with increasing concentrations of ARNI in the range of 40-200 μmol/L (**Figure 1A-D**). We also evaluated the effect of varying IH pretreatment times on the viability of cardiomyocytes. The cell viability was highest at 6 h but decreased at 3 h, 12 h, and 24 h (**Figure 1E**). It can be seen from **Figure 1** that ARNI dose-dependently and time-dependently increased IH-induced cell viability, which eventually significantly decreased after 6 h-IH treatment; pretreatment with 20 μmol/L of ARNI for 1 h prior to IH markedly ameliorated the reduced cell viability. These results indicate that ARNI treatment enhances IH-induced cell viability.

ARNI reduced lactate dehydrogenase release in H9c2 cardiomyocytes

LDH is rapidly released into the cell culture supernatant when the plasma membrane is

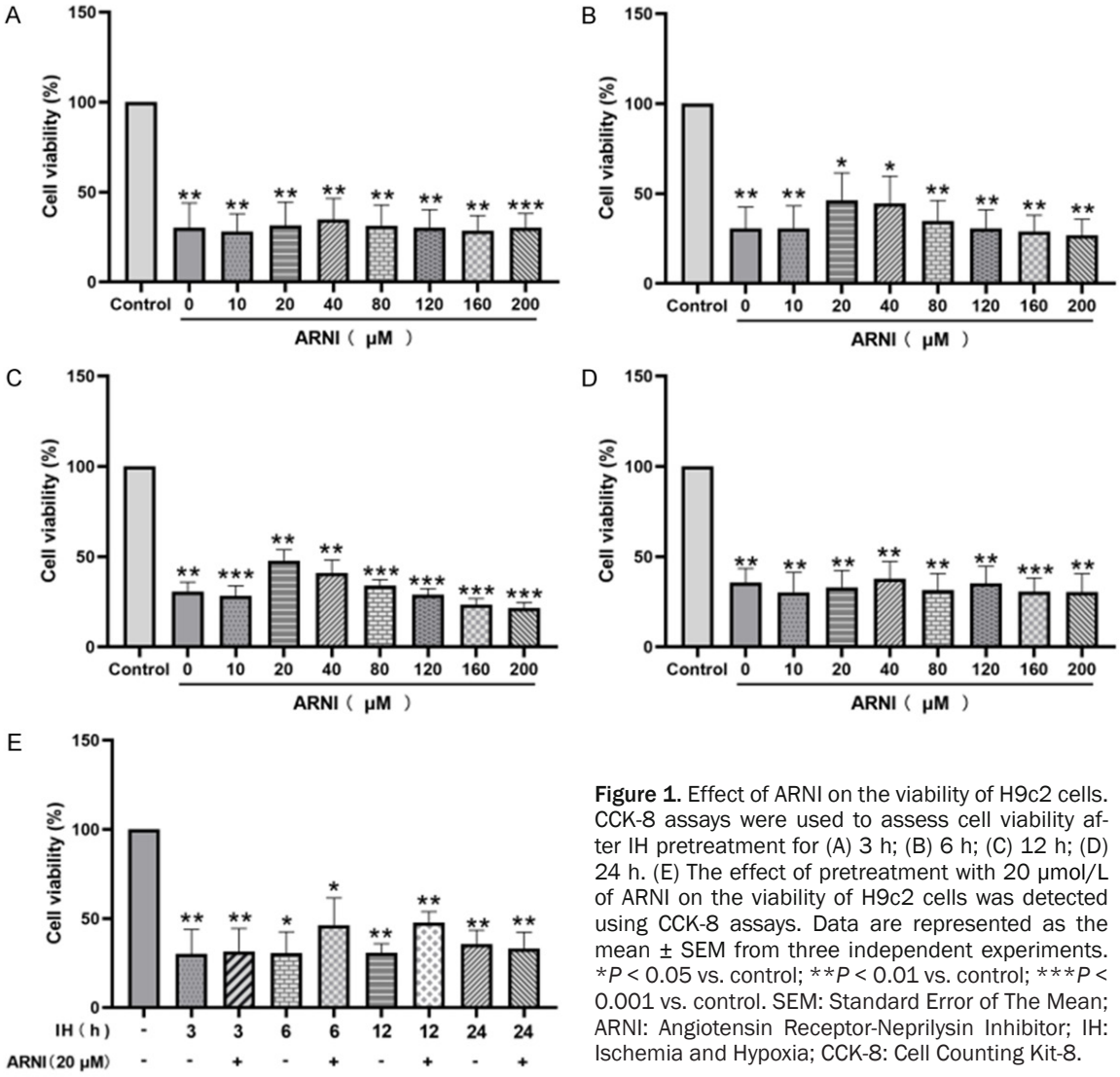

Figure 1. Effect of ARNI on the viability of H9c2 cells. CCK-8 assays were used to assess cell viability after IH pretreatment for (A) 3 h; (B) 6 h; (C) 12 h; (D) 24 h. (E) The effect of pretreatment with 20 μmol/L of ARNI on the viability of H9c2 cells was detected using CCK-8 assays. Data are represented as the mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.001$ vs. control. SEM: Standard Error of The Mean; ARNI: Angiotensin Receptor-Neprilysin Inhibitor; IH: Ischemia and Hypoxia; CCK-8: Cell Counting Kit-8.

damaged, a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage [17]. Following IH treatment, the lactate content of H9c2 cardiomyocytes was measured using the LDH detection kit. In contrast to the control group, the release of LDH in H9c2 cells was significantly increased after IH treatment and gradually increased with the prolongation of IH time in the IH group (**Figure 2A**). Moreover, in contrast to the IH group, the content of LDH in H9c2 cells in the 20 μmol/L of ARNI pretreated group was reduced. As shown in **Figure 2B**, compared with the IH group, the results demonstrated that IH treatment induced LDH overproduction in H9c2 cells, which ARNI could dose-dependently inhibit. These results suggest that 20 μmol/L of ARNI has the most significant inhibitory effect on high LDH content induced by IH.

ARNI inhibited IH-induced apoptosis in H9c2 cardiomyocytes

We further examined the effects of ARNI on H9c2 cardiomyocytes following IH injury by flow cytometry. The H9c2 cardiomyocytes were first pretreated with 20 μmol/L of ARNI and then subjected to IH for 6 h. The flow cytometry results indicated that compared with that in the control group, the apoptosis rate of the IH group was significantly increased (**Figure 3**). Additionally, although lacking a statistically significant difference, the apoptosis rate of the ARNI group was lower than that of the IH group, which indicated that ARNI inhibited H9c2 cell

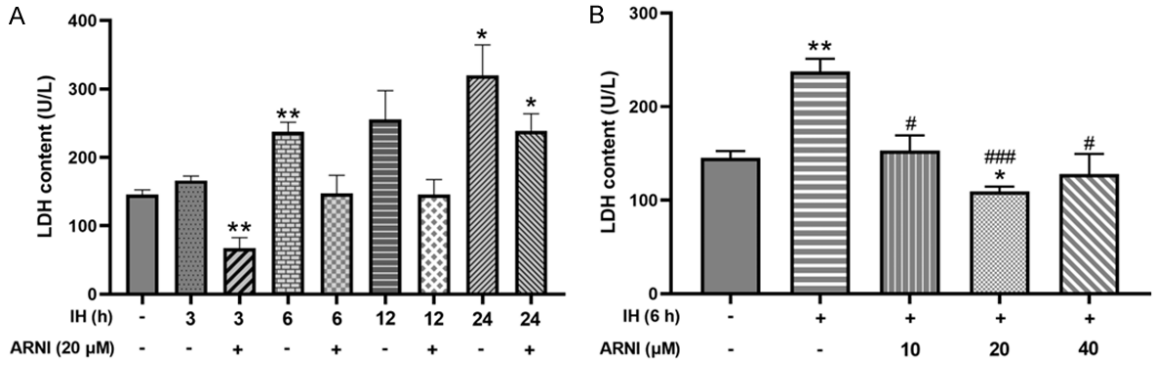

Figure 2. Evaluation of LDH levels in the supernatant of cell culture medium. A: LDH levels in the supernatant after intervention with ARNI for various time periods. B: LDH levels in the supernatant after intervention with different concentrations of ARNI. Data are represented as the mean ± SEM from three independent experiments. **P* < 0.05 vs. control; ***P* < 0.01 vs. control; #*P* < 0.05 vs. IH; ###*P* < 0.001 vs. IH. SEM: Standard Error of The Mean; ARNI: Angiotensin Receptor-Neprilysin Inhibitor; IH: Ischemia and Hypoxia; LDH: Lactate Dehydrogenase.

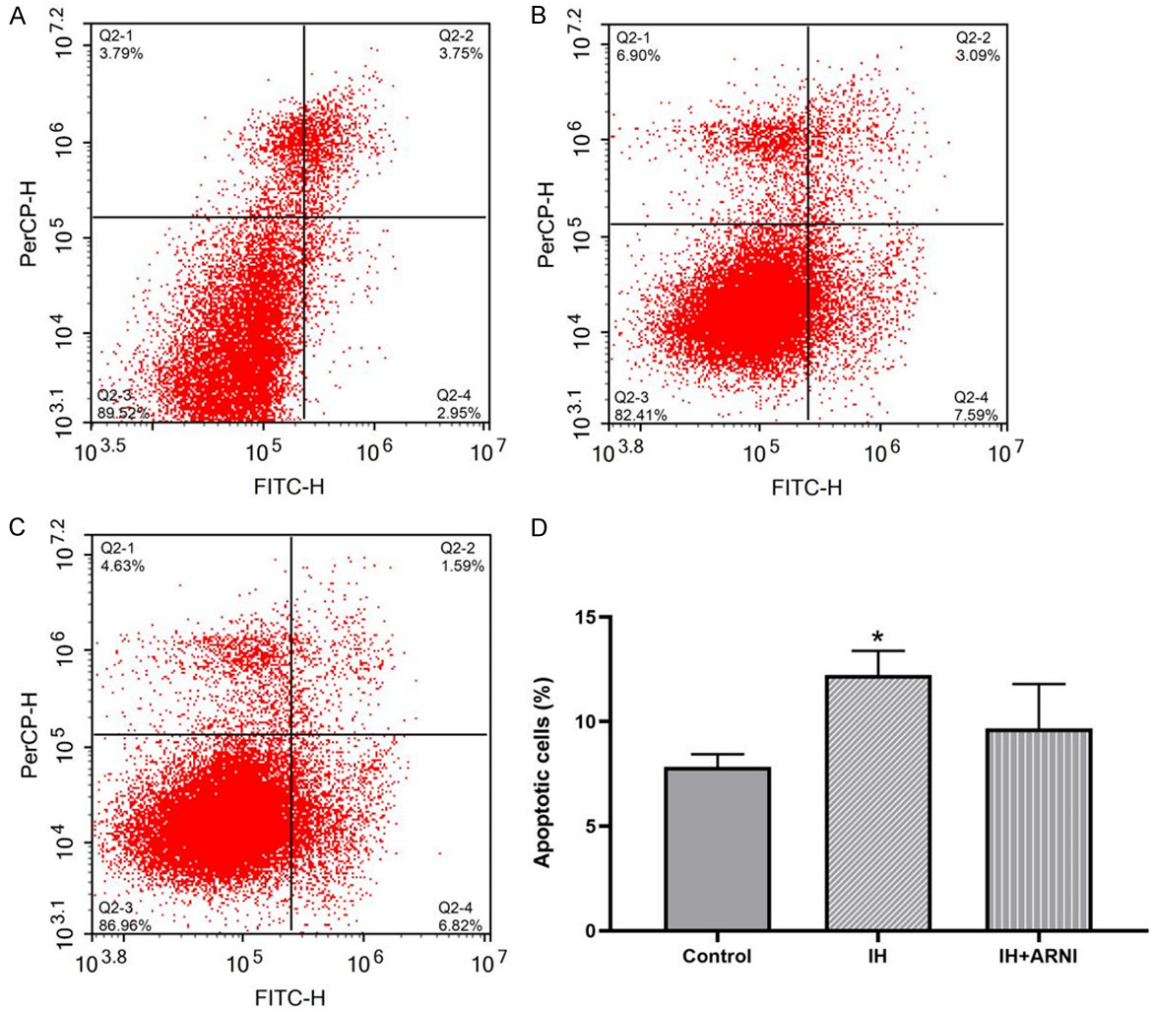

Figure 3. ARNI inhibited IH-induced apoptosis in H9c2 cardiomyocytes. A: Control group. B: IH (6 h) group. C: IH (6 h) + ARNI (20 μmol/L) group. D: Cell apoptosis rates of different groups were analyzed using flow cytometry. Data are represented as the mean ± SEM from three independent experiments. **P* < 0.05 vs. control. SEM: Standard Error of The Mean; ARNI: Angiotensin Receptor-Neprilysin Inhibitor; IH: Ischemia and Hypoxia.

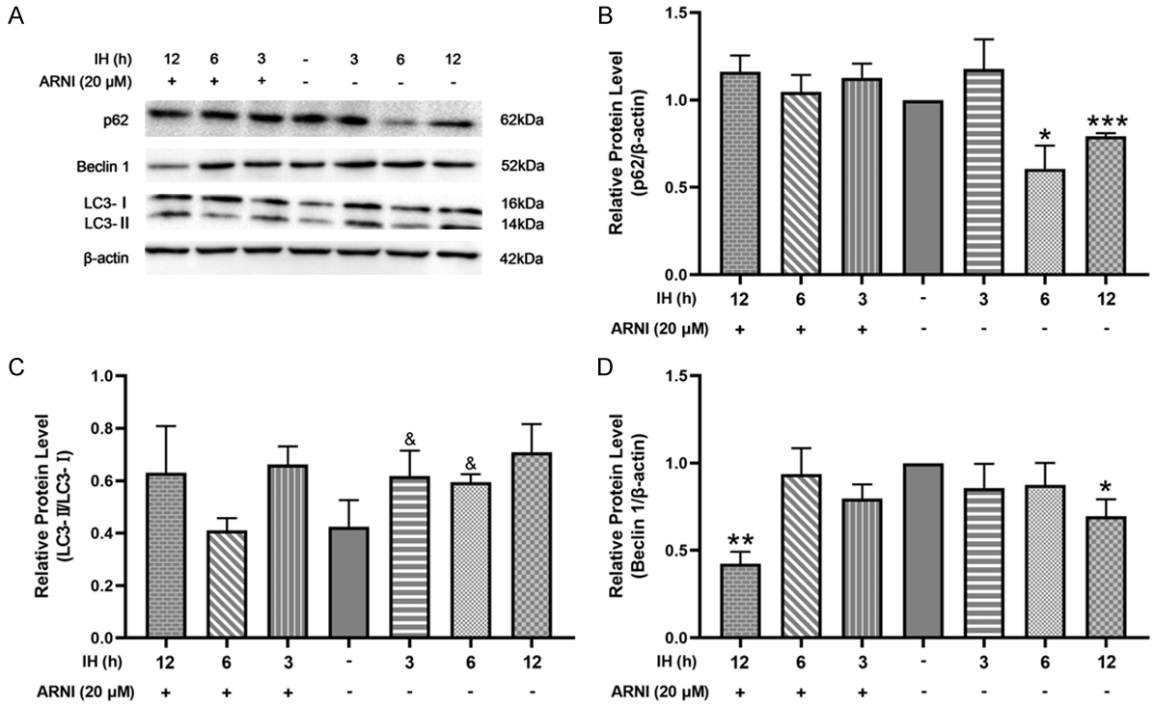

Figure 4. ARNI attenuated autophagy in IH-injured H9c2 cardiomyocytes in a time-dependent manner. A-D: Protein expression levels of LC3-II, Beclin 1, and p62 in H9c2 cardiomyocytes treated with different IH times. Data are represented as the mean ± SEM from three independent experiments. $*P < 0.05$ vs. control; $**P < 0.01$ vs. control; $***P < 0.001$ vs. control; &$P < 0.05$ vs. IH (6 h) + ARNI (20 μM). SEM: Standard Error of The Mean; ARNI: Angiotensin Receptor-Neprilysin Inhibitor; IH: Ischemia and Hypoxia.

apoptosis induced by IH injury. These results suggest that ARNI may be associated with IH-induced apoptotic activity in H9c2 cells.

ARNI attenuated IH-induced autophagy in a time-dependent manner

Our previous research confirmed that ARNI had a protective effect on ischemic myocardium in AMI rats. To explore whether ARNI alleviates cardiac ischemia by affecting autophagy in cardiomyocytes, we examined the protein expression of autophagy marker proteins (LC3-II, Beclin 1, and p62) in H9c2 cardiomyocytes treated with different IH treatment time using western blotting. As shown in **Figure 4A**, **4B**, compared with the control group, the protein expression levels of p62 were markedly down-regulated in H9c2 cardiomyocytes following IH treatment for 6 h and 12 h. In contrast, the expression of p62 was increased in H9c2 cardiomyocytes pretreated with 20 μmol/L of ARNI. Compared with the IH (6 h) group, the protein expression level of LC3-II was significantly down-regulated in IH (6 h) + ARNI group, suggesting that pretreatment with 20 μmol/L of ARNI significantly reduced the IH-induced increased expression of LC3-II (**Figure 4C**). Moreover, compared with the control group, IH treatment for 12 h increased the expression of Beclin 1, which was significantly reduced by preconditioning with ARNI (**Figure 4D**). The data revealed that ARNI attenuated IH-induced cell autophagy in cardiomyocytes compared to control cells.

ARNI attenuated IH-induced autophagy in a dose-dependent manner

To confirm that ARNI can time-dependently alleviate IH-induced autophagy, we further detected the expression levels of LC3-II, Beclin 1, and p62 in H9c2 cardiomyocytes pretreated with different concentrations of ARNI (10, 20, 40 μmol/L) using western blotting. ARNI dose-dependently reduced autophagy in IH-induced H9c2 cardiomyocytes, as confirmed by the decreased expression of LC3-II and Beclin 1 and increased expression of p62 (**Figure 5A-D**). Furthermore, it is worth noting that 20 μmol/L of ARNI reinforced p62 expression and reduced LC3-II expression, and 40 μmol/L of ARNI

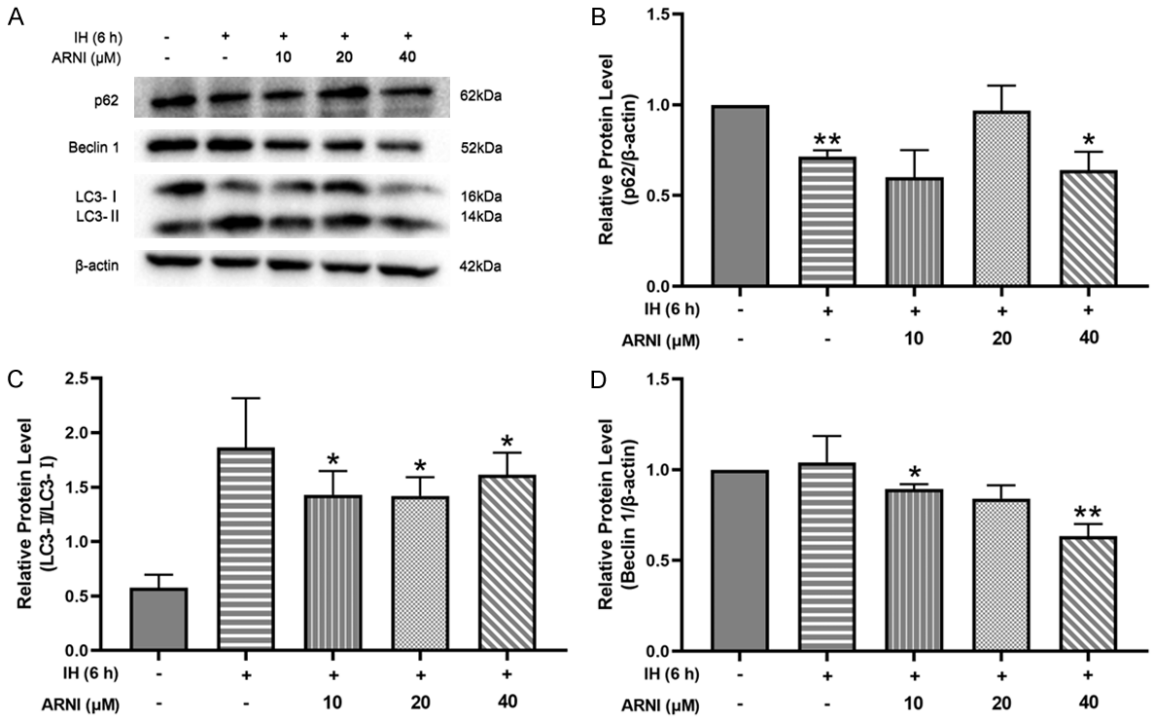

Figure 5. ARNI attenuated autophagy in IH-injured H9c2 cardiomyocytes in a dose-dependent manner. A-D: Protein expression levels of LC3-II, Beclin 1, and p62 in H9c2 cardiomyocytes treated with different concentrations of ARNI. Data are represented as the mean ± SEM from three independent experiments. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control. SEM: Standard Error of The Mean; IH: Ischemia and Hypoxia; ARNI: Angiotensin Receptor-Neprilysin Inhibitor.

improved the increased expression of Beclin 1, which suggests that ARNI dose-dependently inhibited IH-induced cell injury in H9c2 cardiomyocytes.

Rapamycin partially reversed the inhibitory effect of ARNI on autophagy in IH-induced H9c2 cardiomyocytes

To further elucidate the protective role of ARNI in IH-injured H9c2 cardiomyocytes, IH-induced H9c2 cardiomyocytes were co-treated with ARNI and rapamycin (a potent inducer of autophagy). We then detected the protein expression of LC3-II, Beclin 1, and p62 using western blotting. Our measurement of the protein expression levels showed that ARNI enhanced p62 expression in IH-induced H9c2 cells, which could be reduced via co-incubation with rapamycin (**Figure 6A**, **6B**). Autophagy was further promoted in IH-induced H9c2 cardiomyocytes upon treatment with rapamycin, which conversely demonstrated that ARNI worked as a protective role in IH-induced autophagy damage. In addition, we noticed that compared with the IH group, rapamycin did not increase the protein expression of LC3-II and Beclin 1 in IH-induced H9c2 cardiomyocytes (**Figure 6C**, **6D**). These results suggested that ARNI treatment reduced IH-induced autophagy, which could be reversed to some extent by rapamycin.

Discussion

In this study, our data showed that ARNI dose-dependently and time-dependently attenuated ischemic injury in H9c2 cardiomyocytes exposed to IH by inhibiting autophagy. When the IH induction time was 6 h and the concentration of ARNI was 20 μmol/L, IH and ARNI had the most significant effect on the viability of H9c2 cardiomyocytes. Therefore, ARNI can play a protective role and reverse the decline of H9c2 cell viability induced by IH at the same concentration. LDH release was significantly elevated in patients with chronic myocardial ischemia compared to that in age-matched healthy subjects [18]. Similarly, ARNI dose-dependently and time-dependently ameliorated the IH-induced cytotoxicity as confirmed by

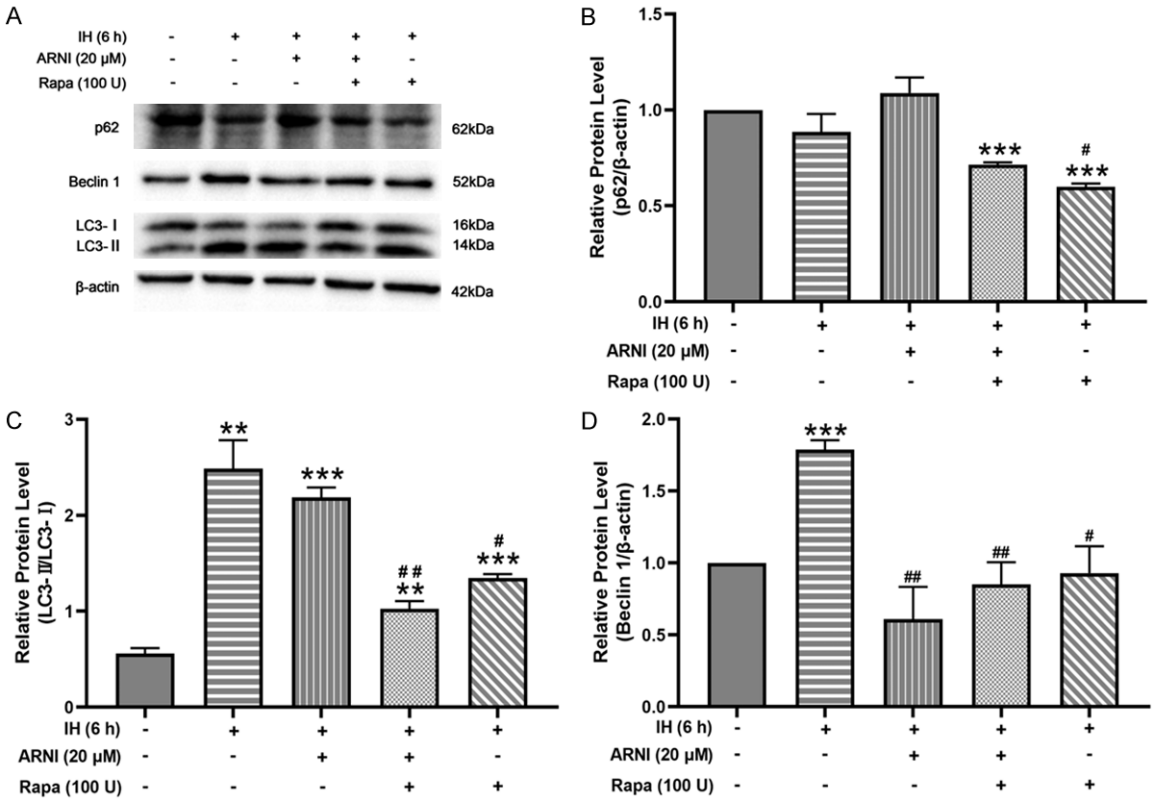

Figure 6. Rapamycin partially reversed the inhibitory effect of ARNI on autophagy in IH-treated H9c2 cardiomyocytes. A-D: Protein expression levels of LC3-II, Beclin 1, and p62 in H9c2 cardiomyocytes treated with an autophagy agonists (rapamycin). Data are represented as the mean ± SEM from three independent experiments. **P* < 0.05 vs. control; ***P* < 0.01 vs. control; ****P* < 0.001 vs. control; #*P* < 0.05 vs. IH; ##*P* < 0.01 vs. IH. SEM: Standard Error of The Mean; IH: Ischemia and Hypoxia; ARNI: Angiotensin Receptor-Neprilysin Inhibitor; Rapa: Rapamycin.

the rescued IH-induced increase in LDH level. These results are consistent with our previous study showing that ARNI has a protective effect on ischemic myocardium [9]. Meanwhile, 20 μmol/L ARNI best alleviated the IH-induced cell injury and apoptosis of H9c2 cells.

AMI is the most common cause of death worldwide, which tends to cause severe heart failure; the proportion of the population suffering from AMI is gradually becoming younger [19]. Myocardial ischemia is the most common form of cardiovascular disease and the leading cause of morbidity and mortality [20]. A large clinical trial on the treatment of HF showed that ARNI has a clear advantage in reducing the risk of cardiovascular mortality and hospitalization for HF compared with angiotensin-converting enzyme inhibitors (ACEI) [21, 22]. Meanwhile, the ARNI treatment group had better tolerance and safety. Moreover, a study in which ARNI was used to treat diabetic cardiomyopathy indicated that ARNI attenuated inflammation and apoptosis in diabetic mice and H9C2 cardiomyocytes and ameliorated cardiac function and oxidative stress *in vivo* and *in vitro* [23]. Our study proved for the first time that ARNI could mitigate apoptosis and dose-dependently and time-dependently reduce LDH overproduction, thereby inhibiting IH-induced cell damage in H9c2 cardiomyocytes. Hypoxia is a common pathophysiological mechanism that can lead to myocardial injury and reduce cell viability [24], which confirms our preliminary findings exploring the specific role of ARNI in ischemic injury.

Autophagy is not only a core molecular pathway for preserving cellular and organismal homeostasis, but also a typical manifestation of IH injury in cardiomyocytes [25]. LC3-II, Beclin 1, and p62 are autophagy marker molecules [26]. Our current results show that ARNI increased the expression of LC3-II and Beclin 1 in a dose-

dependent manner in IH-exposed H9c2 cells, but decreased the expression of p62 in a dose-dependent manner and also alleviated the progress of autophagy in a time-dependent manner. This directly indicated that the changes of ARNI pretreatment affected autophagy in H9c2 cardiomyocytes. We further verified whether ARNI is involved in IH-induced autophagy in H9c2 cardiomyocytes using rapamycin, which promotes autophagy and increases the expression of autophagy-related proteins. Therefore, when ARNI is used with rapamycin, the inhibitory effect of ARNI on autophagy was expected to be weakened, with decreased expression of autophagy proteins. However, although the addition of rapamycin significantly reduced the expression of p62, it did not increase the expression of LC3-II and Beclin 1. We speculate that this may be because rapamycin did not fully play its role in promoting autophagy in the early stage of hypoxia. Overall, rapamycin abolished a protective effect of ARNI on IH induction. In addition, although our study showed that ARNI could improve IH injury by inhibiting apoptosis and autophagy, the impact of ARNI on these processes at different stages of IH injury and its specific mechanism still needs to be further studied.

In conclusion, the protective effect of ARNI in H9c2 cardiomyocytes exposed to IH may provide new insights into ARNI in the treatment of ischemic heart injury disease. However, the specific regulatory mechanism of ARNI involved in autophagy remains to be elucidated, and these findings still require further follow-up studies. This study is the first to demonstrate that ARNI protects H9c2 cardiomyocytes from IH-induced cellular damage by inhibiting autophagy, thereby ameliorating HF and providing insights related to autophagy.

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Disclosure of conflict of interest

None.

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