*Article*

# Calcineurin suppresses cardiomyocyte-protective autophagy under chronic intermittent hypoxia by downregulating the AMPK pathway

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**Abstract:** Calcineurin plays a key role in cardiovascular pathogenesis by exerting pro-apoptotic effects in cardiomyocytes; however, its involvement in the regulation of cardiomyocyte autophagy under chronic intermittent hypoxia (CIH) remains largely unknown. Here we showed that CIH induced calcineurin activity in H9C2 cells, resulting in the attenuation of adenosine monophosphate-activated protein kinase (AMPK) signaling and inhibition of H9C2 cell autophagy. Autophagy, LC3-II levels, and AMPK phosphorylation were significantly elevated in response to CIH in H9C2 cells by day 3; however, these effects were reversed, and calcineurin activity and apoptosis were significantly increased by day 5. The calcineurin inhibitor, FK506, restored AMPK activation and LC3 protein levels, and reduced CIH-induced H9C2 cell apoptosis, while calcineurin overexpression significantly attenuated the increase in LC3 levels and enhanced H9C2 cell apoptosis. Calcineurin inhibition failed to induce autophagy or alleviate apoptosis in H9C2 cells expressing a dominant negative K45R AMPK mutant. Autophagy downregulation abrogated the protective effects of FK506-mediated calcineurin inhibition. These results indicated that calcineurin suppressed adaptive autophagy during CIH by downregulating AMPK activation. Our findings showed the underlying mechanisms of calcineurin and autophagy regulation during H9C2 cell survival in response to CIH, and suggested a new strategy for preventing CIH-induced cardiomyocyte damage.

**Keywords:** chronic intermittent hypoxia; autophagy; apoptosis; cardiomyocyte damage; calcineurin

## 1. Introduction

Obstructive sleep apnea syndrome (OSAS) is recognized as an emerging yet prevalent disease worldwide. It is associated with multiple complications, including cardiovascular events that are responsible for the majority of OSAS-related deaths [1]. Chronic intermittent hypoxia (CIH) in OSAS is a typical pathological factor induced by the repeated collapse of the airways during sleep, triggering systemic inflammation and oxidative stress, leading to severe cardiac damage, and resulting in abnormal cardiomyocyte apoptosis and myocardial fibrosis [2]. Oxidative stress also induces autophagy in numerous organs, leading to their malfunction. The ability of the heart to maintain physiological homeostasis is particularly dependent on autophagy; however, whether insufficient or excessive abnormal cardiomyocyte autophagy could result in significant cardiac damage, or even heart failure, remains unknown.

Autophagy is a highly conserved cellular process that could be described as "self-eating". During this process, damaged cytosolic organelles and misfolded proteins are degraded into their basic components and then recycled for other cellular processes to enhance survival [3]. Cardiac autophagy plays a critical role in cardiomyocyte development and survival [4], in both pathological and normal physiological states, by regu-

lating cardiomyocyte metabolism and protein levels [5]. Excessive autophagy results in cardiomyocyte death [6], while impaired autophagy leads to the intracardiac disturbances of energy and protein metabolism [7]. Even though autophagy causes cell death under certain physiological conditions, it also promotes cell survival [8].

Adenosine monophosphate-activated protein kinase (AMPK) is a conserved serine/threonine kinase that plays a critical role as an energy sensor in mammalian cells. Furthermore, it is an essential autophagy regulator that maintains myocardial morphology and function by mediating autophagy in response to caloric restriction [9] and oxidative stress [10]. Calcium/calmodulin-dependent protein phosphatase 2B (calcineurin) is an enzyme that modulates multiple signaling cascades in cardiovascular diseases, including apoptosis [11]. It has been shown that calcineurin inhibition enhances AMPK activation, suggesting a potential role of calcineurin in the regulation of autophagy [12]; however, the connection between CIH, AMPK, and autophagy has not been examined yet. Here we investigated the role of calcineurin in the regulation of autophagy during CIH, as well as the underlying molecular mechanisms of CIH.

## 2. Materials and Methods

### *2.1. Cell culture and treatment*

Rat H9C2 cardiomyoblasts were cultured in Dulbecco's modified Eagle's medium containing glucose (2.25 g/L), 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 units/mL) (all from Gibco, Grand Island, NY, USA) at 37 °C in a sustained humidified atmosphere with 5% $CO_2$. Once the cells reached 80% confluence, CIH treatment was initiated using an established protocol as previously described [13,14]. During normoxia (21% $O_2$, 5% $CO_2$, and 74% $N_2$) and CIH (repeated cycles of 21% $O_2$, 5% $CO_2$, 74% $N_2$ for 25 min, followed by 5% $O_2$, 5% $CO_2$, 90% $N_2$ for 35 min) treatments, cells were cultured for specified periods of time in the culture medium containing FK506, a well-characterized calcineurin inhibitor (tacrolimus, 1 μmol/L), Z-VAD-FMK, a caspase inhibitor (40 μmol/L), or rapamycin, a mammalian target of rapamycin (mTOR) inhibitor (100 nmol/L) in dimethyl sulfoxide (all from Sigma-Aldrich, St. Louis, MO, USA), as well as the autophagy inhibitor, 3-methyladenine (3-MA; 10 mmol/L; Selleckchem, Houston, TX, USA) as previously described [13,14].

### *2.2. Cell viability assay*

H9C2 cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were washed and treated as indicated and then incubated with MTT (20 μL, 0.5 mg/mL; Sigma-Aldrich) for 4 h. The culture medium was removed and dimethyl sulfoxide (200 μL) was added to the wells to dissolve formazan by incubating the plates for 10 min on a rocking shaker. Absorbance at 570 nm was then measured using a microplate spectrophotometer (BD Biosciences, Franklin Lakes, NJ, USA).

### *2.3. Autophagic flux measurement*

H9C2 cells were transfected with the Ad-mRFP(monomeric red fluorescent protein)-GFP(green fluorescent protein)-LC3 adenovirus (multiplicity of infection = 50) and cultured for 24 h at 37 °C under 5% $CO_2$. Cells were fixed in 4% paraformaldehyde for at least 10 min and then washed 3 × 5 min with phosphate-buffered saline. Next, the cells were imaged under a fluorescence microscope (Zeiss LSM, Oberkochen, Germany) after adding antifade mounting medium. Yellow puncta (GFP + mRFP) represent autophagosomes, while red puncta (mRFP only) represent autolysosomes, since GFP is quenched in an acidic environment. When autophagy is activated, more autolysosomes (red puncta), rather than autophagosomes (yellow puncta) will be formed in the cells. When autophagy is impaired/blocked, autophagosomes (yellow puncta) will be predominant.

### *2.4. Calcineurin activity assay*

H9C2 cells were lysed in the lysis buffer supplied with the Calcineurin Cellular Assay kit (ENZO Life Science, Farmingdale, NY, USA). Total phosphatase activity was

determined in extracts mixed with RII phosphopeptide in N'-tetraacetic acid buffer at 30 °C; phosphatase activity without calcineurin was determined in the same extracts in the assay buffer. For colorimetric phosphate quantification, 100 μL of BIOMOL Green (Sigma-Aldrich) was added to the wells and samples were incubated for 20 min. The absorbance of the released green phosphate complexes was measured at 620 nm using a microplate spectrophotometer.

*2.5. Western blotting*

H9C2 cells were cultured as previously described [13,14]. Next, cells were collected, washed three times with cold phosphate-buffered saline, and then lysed in radioimmunoprecipitation assay buffer for 30 min. Proteins (50 μg) were resolved via 10% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes. Nonspecific binding was blocked with 5% fat-free milk in Tris-buffered saline. The membranes were then incubated with primary antibodies at 4 °C overnight, washed with Tris-buffered saline, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; cat. no. BS13278. Bioworld Technology, Minneapolis, MN, USA) for 1 h Primary antibodies against caspase-3, cleaved caspase-3, AMPK, phosphorylated (p)-AMPK, LC3-I/II, p-4E-binding protein 1 (4E-BP1), p-p70 ribosomal protein S6 kinase (p70S6K), and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against Akt, p-Akt, mTOR, and p-mTOR were purchased from R&D Systems (Minneapolis, MN, USA). Antibody binding was detected using enhanced chemiluminescence kits (Thermo Fisher Scientific, Waltham, MA, USA). Signal intensities were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The results were normalized to their respective loading control (β-actin).

*2.6. RNA interference*

Small interfering (si) RNA against *Atg7* (5′-GCGA ATT CAA GAA ATA ATG GCG GCA GC-3′) and the negative control RNA were purchased from GenePharma (Shanghai, China). H9C2 cells were transfected with siRNA (100 nM) targeting *Atg7*, or the control negative siRNA duplex.

*2.7. Plasmid transfection*

The GFP-mRFP-LC3 plasmid was obtained from Addgene (21074, Cambridge, MA, USA). The plasmid that encoded the dominant negative mutant of AMPK (DN-AMPK) was obtained from Addgene (15992). The plasmid that encoded the constitutively active form of calcineurin A (CnA) was provided by Dr. Huiguo Liu (Wuhan, China). The control vector pEGFP-C3 was obtained from Clontech (Palo Alto, CA, USA) and the control vector pcDNA3.0 was obtained from Invitrogen (San Diego, CA, USA). H9C2 cells at 70–80% confluence were transfected with 1–2 μg plasmid using Xtreme GENE HP (Roche). The cardiomyocytes were subjected to different treatments after 36 h of transfection.

*2.8. Statistical analyses*

All experiments were repeated at least three times. Data are presented as means ± standard deviation. The significance of differences between groups was evaluated by a one-way analysis of variance followed by the Student–Newman–Keuls test. All data were analyzed using the SPSS software (IBM, Chicago, IL, USA), and $p < 0.05$ was considered significant.

## 3. Results

*3.1. CIH induces calcineurin activity and autophagy, and decreases cell viability in H9C2 cells*

Oxidative damage in H9C2 cells in response to CIH was assessed as changes in autophagy, apoptosis, and viability over time. As shown in Figure 1a, LC3-II/I ratio, an index of autophagy, was significantly increased in response to CIH for the first 3 days and then decreased by day 5. The changes in the phosphorylation levels of AMPK, a key au-

tophagy regulator in cardiomyocytes, were similar in response to CIH. Hypoxia significantly and time-dependently increased the levels of cleaved caspase-3, as well as the ratio of pro-caspase-3 to cleaved caspase-3, a critical indicator of cardiomyocyte apoptosis (Figure 1b). The MTT assay showed that CIH treatment significantly inhibited cell viability, while Z-VAD-FMK, a pan caspase inhibitor, reversed this effect (Figure 1c). The activity of calcineurin, an important upstream factor that plays a role in cell autophagy and apoptosis, was significantly reduced in response to CIH treatment by day 3, and then significantly elevated by day 5 (Figure 1d). The autophagic flux assay demonstrated that the number of autophagosomes initially increased in response to CIH, indicating upregulated autophagy. However, 5 days of CIH significantly decreased the number of autophagosomes (Figure 2), suggesting a considerable reduction of autophagic activity.

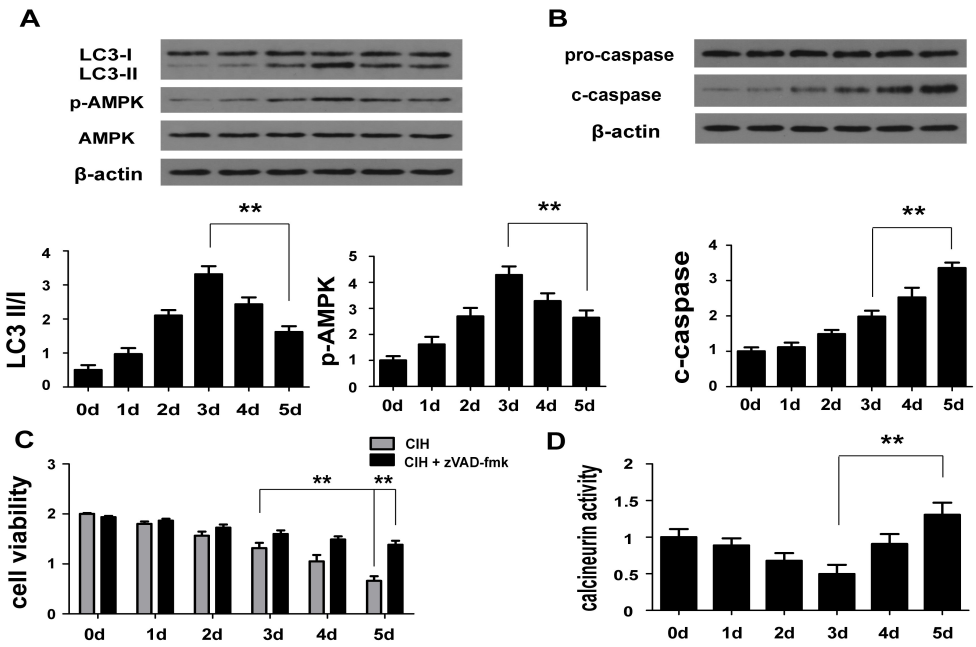


**Figure 1.** Effects of chronic intermittent hypoxia on H9C2 cells cultured for 1–5 days. (**a**) Quantification of lipidated LC3 (an autophagy indicator), AMPK, and p-AMPK (an upstream autophagy regulator) proteins at various time points; representative bolts are shown. (b) Cleaved caspase-3 levels during H9C2 cell apoptosis at various time points; (c) MTT assay evaluating viability of cells cultured with or without ZVAD-FMK, and (d) calcineurin activity at indicated time points. Data are expressed as means ± SD of five independent experiments. **$p < 0.001$. Abbreviations: c-caspase, cleaved caspase-3; CIH, chronic intermittent hypoxia; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase.

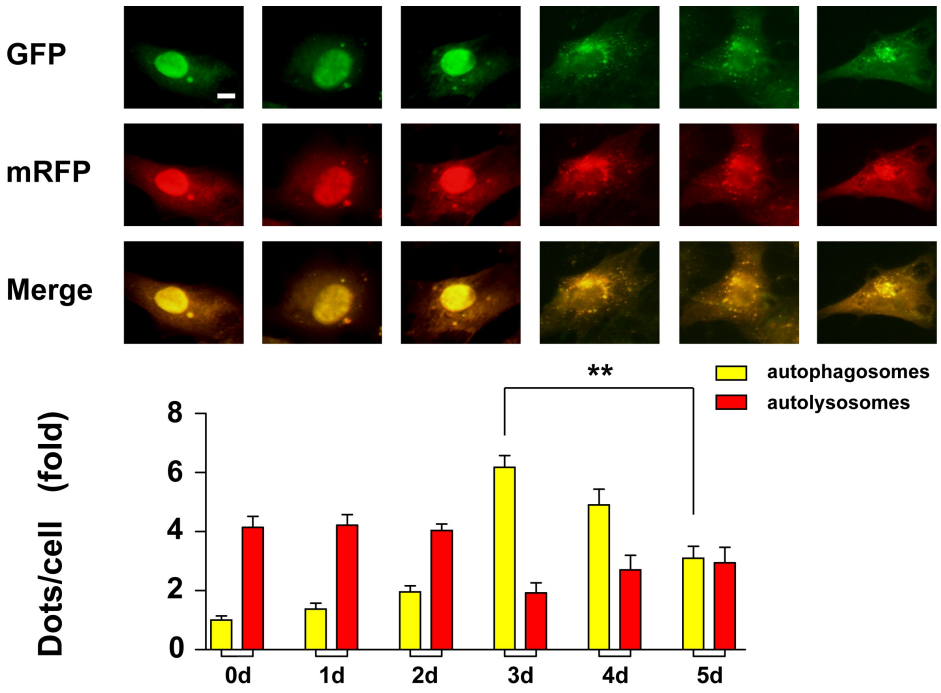


**Figure 2.** Chronic intermittent hypoxia (CIH) reduces autophagic flux in H9C2 cells. H9C2 cells were transfected with GFP-mRFP-LC3 for 24 h, incubated under CIH for 0–5 days, photographed, and mRFP and GFP puncta (dots) were counted. Representative images are shown. Data are expressed as means ± SD of five independent experiments. **p < 0.001. Abbreviations: GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein. Bar: 10 μm.

*3.2. Inhibition of calcineurin induces autophagy and alleviates CIH-induced apoptosis of H9C2 cells*

Next, we evaluated the role of calcineurin in CIH-induced H9C2 cell autophagy by treating cells with FK506, the calcineurin inhibitor, for 5 days of CIH. LC3-II/I ratio was significantly elevated in the FK506 groups regardless of CIH (Figure 3a). Furthermore, FK506 significantly enhanced H9C2 cell survival and decreased apoptosis in the CIH group (Figure 3b,c).

The inhibition of calcineurin by FK506 not only upregulated the LC3-II/I ratio, but also induced dynamic autophagic flux, as shown by a significant increase in the number of mRFP and GFP puncta (Figure 4). These results suggested that calcineurin was involved in the induction of H9C2 cell apoptosis and suppression of protective autophagy in response to CIH.

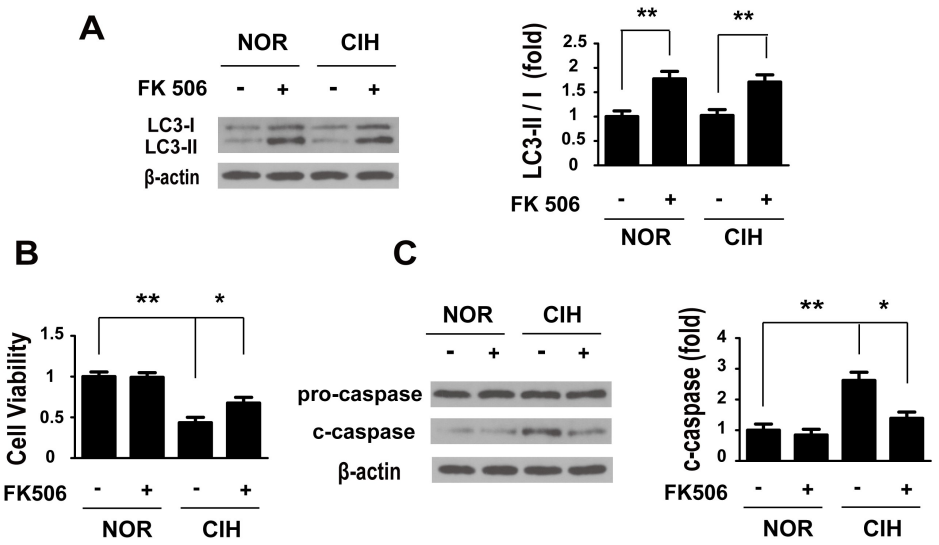


**Figure 3.** FK506 alleviates chronic intermittent hypoxia (CIH)-induced H9C2 cell apoptosis and induces autophagy. H9C2 cells were incubated with or without CIH and 1 μM FK506 (a calcineurin inhibitor) for 5 days and then analyzed by western blotting and MTT assays. (**a**) LC3 protein levels and quantification (panel on the right); representative blots are shown. (**b**) H9C2 cell viability was evaluated by the MTT assay. (**c**) Pro-caspase and c-caspase expression were analyzed by western blotting; the quantification of western blots is shown in the panel on the right. Data are expressed as means ± SD of five independent experiments. *$p < 0.05$, **$p < 0.001$. Abbreviations: c-caspase, cleaved caspase-3; CIH, chronic intermittent hypoxia; NOR, normoxia.

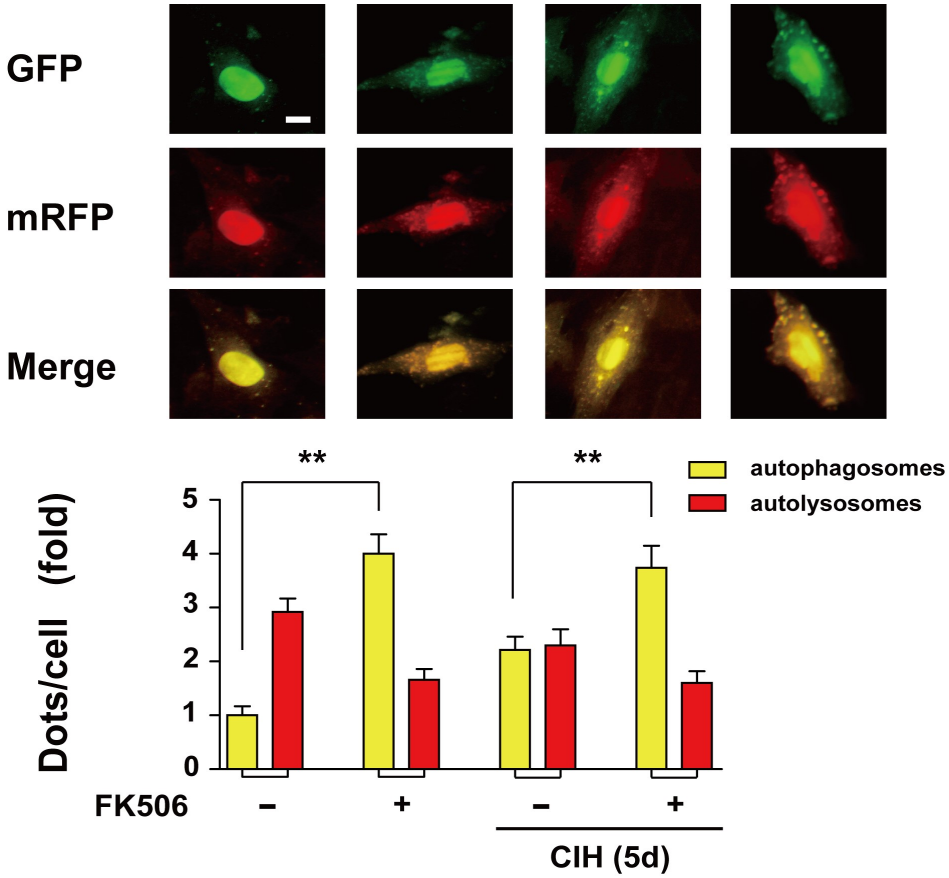


**Figure 4.** FK506 induces autophagic flux. H9C2 cells were transfected with GFP-mRFP-LC3 for 24 h and treated as described in Figure 3. Both mRFP and GFP puncta (dots) were counted. Data are expressed as means ± SD of five independent experiments; five cells/experiment were measured. **p < 0.001. Abbreviations: CIH, chronic intermittent hypoxia; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein. Bar: 10 μm.

*3.3. Overexpression of calcineurin inhibits cardiomyocyte autophagy and exacerbates CIH-induced apoptosis*

To confirm the role of calcineurin in the regulation of autophagy, H9C2 cells were transfected with a construct expressing the constitutively active form of CnA. H9C2 cells were then subjected to normoxia or CIH for 3 days, the time point characterized by the induction of autophagy and low-level apoptosis. The results showed that calcineurin overexpression reduced LC3-II levels in both normoxia- and CIH-treated cells (Figure 5a). Furthermore, CnA overexpression decreased H9C2 cell viability and significantly increased caspase-3 activation in response to CIH for 3 days. (Figure 5b,c). These results suggested that calcineurin decreased protective autophagy and promoted apoptosis in H9C2 cells during CIH.

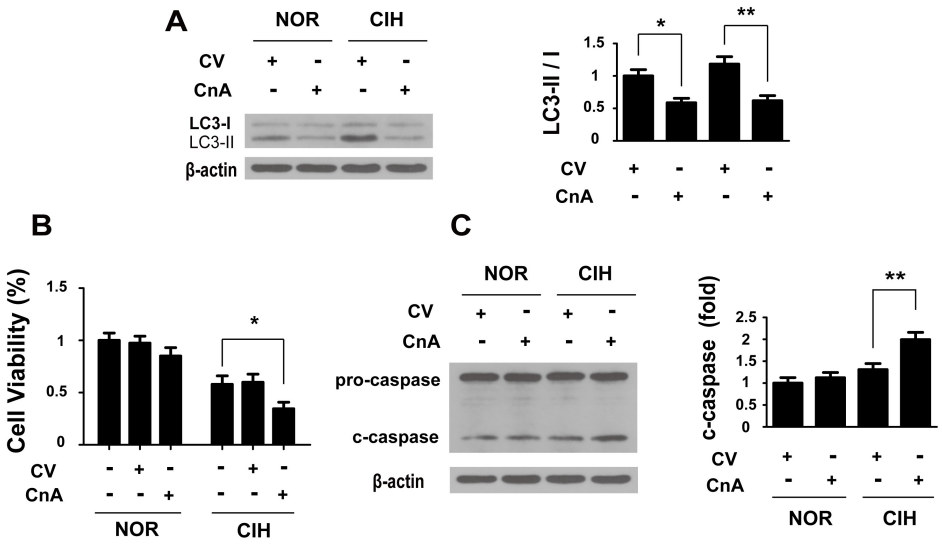


**Figure 5.** Calcineurin overexpression inhibits autophagy and increases chronic intermittent hypoxia (CIH)-induced apoptosis in H9C2 cells. H9C2 cells were transfected with vector containing calcineurin A (CnA) or control vector (CV) for 24 h, incubated with or without CIH for 3 days, and then analyzed by western blotting and MTT assays. (**a**) LC3 protein levels and quantification (panel on the right); representative blots are shown. (**b**) H9C2 cell viability was determined by the MTT assay. (**c**) Pro-caspase and c-caspase expression was evaluated by western blotting. Abbreviations: c-caspase, cleaved caspase-3; CIH, chronic intermittent hypoxia; CnA, calcineurin A; CV, control vector; NOR, normoxia; pro-casp3, pro-caspase-3. *$p < 0.05$. **$p < 0.001$.

*3.4. Calcineurin regulates H9C2 cell autophagy and survival through the AMPK/mTOR pathway*

AMPK and mTOR play key roles in the regulation of autophagy: AMPK activates autophagy, while mTOR inhibits it. Therefore, we evaluated the effect of calcineurin overexpression and inhibition on the activation of the AMPK pathway. The inhibition of calcineurin using FK506 increased the phosphorylation of AMPK (Figure 6a); however, the phosphorylation of mTOR, as well as its downstream targets p70S6K and 4E-BP1, was decreased. In contrast, the overexpression of calcineurin attenuated AMPK phosphorylation and enhanced mTOR signaling in response to CIH and normoxia (Figure 6b).

Calcineurin has been shown to dephosphorylate Akt, another regulator of mTOR [15]. Therefore, next, we examined whether Akt was involved in the modulation of mTOR activity by calcineurin. The inhibition of calcineurin significantly increased Akt phosphorylation under normoxic, but not CIH, conditions (Figure 6c). Moreover, Akt phosphorylation was decreased in response to the overexpression of calcineurin during normoxia, while it was not affected during CIH (Figure 6d). Next, we transfected cells either with the control plasmids or plasmids with DN-AMPK. Calcineurin inhibition (FK506 treatment) increased LC3-II levels in control cells, while it had no effect on LC3-II levels in cells expressing DN-AMPK (Figure 6e). Furthermore, FK506 treatment increased AMPK phosphorylation and decreased mTOR phosphorylation in control vector-transfected cells, while both AMPK and mTOR phosphorylation were not affected in DN-AMPK-transfected cells (Figure 6e,f). Cleaved caspase-3 levels were also decreased in control vector-transfected H9C2 cells treated with the calcineurin inhibitor, while this effect was not detected in cells expressing the DN-AMPK mutant (Figure 6g).

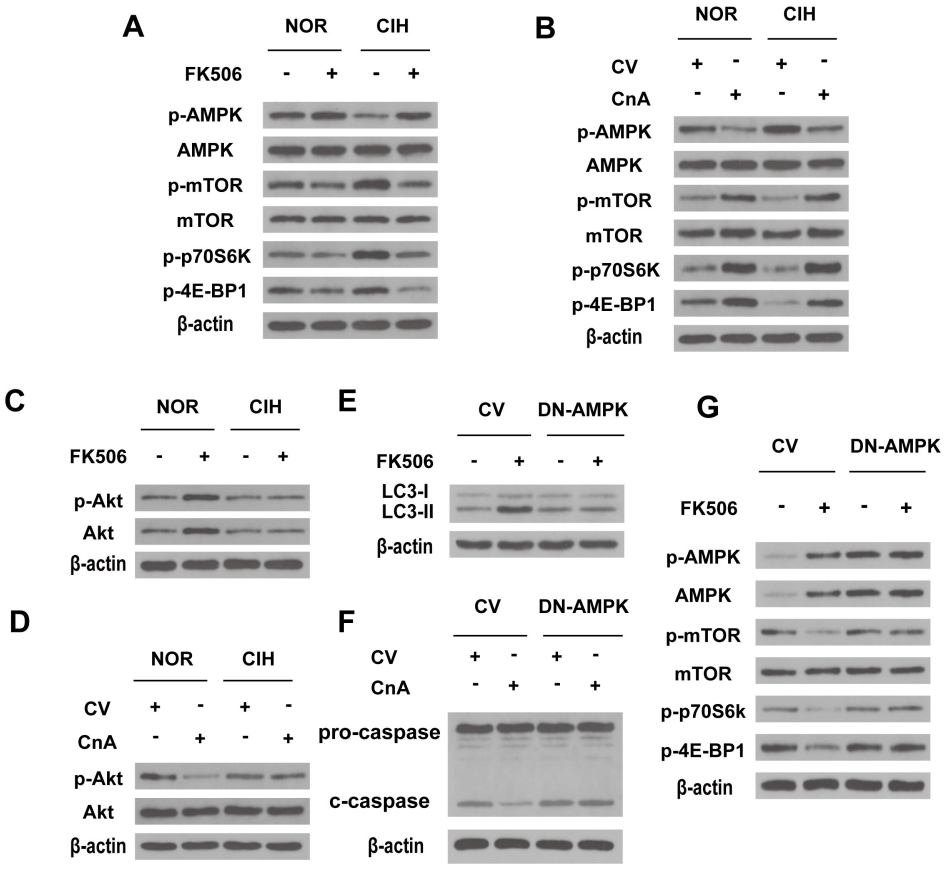


**Figure 6.** Calcineurin mediates H9C2 cell autophagy and survival during chronic inter mittent hypoxia (CIH) treatment through the AMPK/mTOR pathway. (**a**) H9C2 cells were cultured with or without CIH and FK506 as described in the Materials and Methods section. The protein levels of AMPK/p-AMPK, mTOR/p-mTOR, p-p70S6K, and p-4E-BP1 were determined by western blotting. (**b**) H9C2 cells were transfected with control vector (CV) or calcineurin A (CnA) and cultured with or without CIH as described in the Materials and Methods section. The protein levels of AMPK/p-AMPK, mTOR/p-mTOR, p-p70S6K, and p-4E-BP1 were determined by western blotting. (**c**) Cells were cultured as described in (**a**) with or without FK506 under normoxia or CIH, and the protein levels of Akt/p-Akt were determined by western blotting. (**d**) Cells were cultured as described in (**b**) and the protein levels of Akt/p-Akt with CV or CnA under normoxia or CIH were determined by western blotting. (**e**) Cells were cultured with or without FK506 with CV or DN-AMPK and the protein levels of LC3 were evaluated by western blotting. (**f**) Cells were cultured with CV or CnA, with or without DN-AMPK, and protein levels of pro-caspase or c-caspase were determined by western blotting. (**g**) Cells were cultured with or without FK506, with or without DN-AMPK, and the protein levels of AMPK/p-AMPK, mTOR/p-mTOR, p-p70S6K, and p-4E-BP1 were determined by western blotting; β-actin was used as loading control.

### *3.5. Calcineurin induces apoptosis by suppressing autophagy in response to CIH*

To investigate the role of autophagy in H9C2 cell survival during CIH, we used 3-MA and rapamycin to inhibit PI3K and mTOR, respectively. The inhibition of mTOR activation using rapamycin significantly attenuated caspase-3 activation and H9C2 cell apoptosis, and induced autophagy; however, 3-MA reversed the protective effects of FK506 (Figure 7a,b). To validate these results, we used an siRNA to silence one of the key autophagy genes, *Atg7*. As expected, silencing *Atg7* significantly decreased LC3-II levels.

Furthermore, *Atg7* downregulation not only reduced CIH-induced apoptosis, but also efficiently blocked the effects of FK506 (Figure 7c,d). These results suggested that the activation of autophagy protected H9C2 cells from CIH-induced apoptosis. Furthermore, our findings indicated that the calcineurin pathway was involved in the regulation of autophagy in response to CIH.

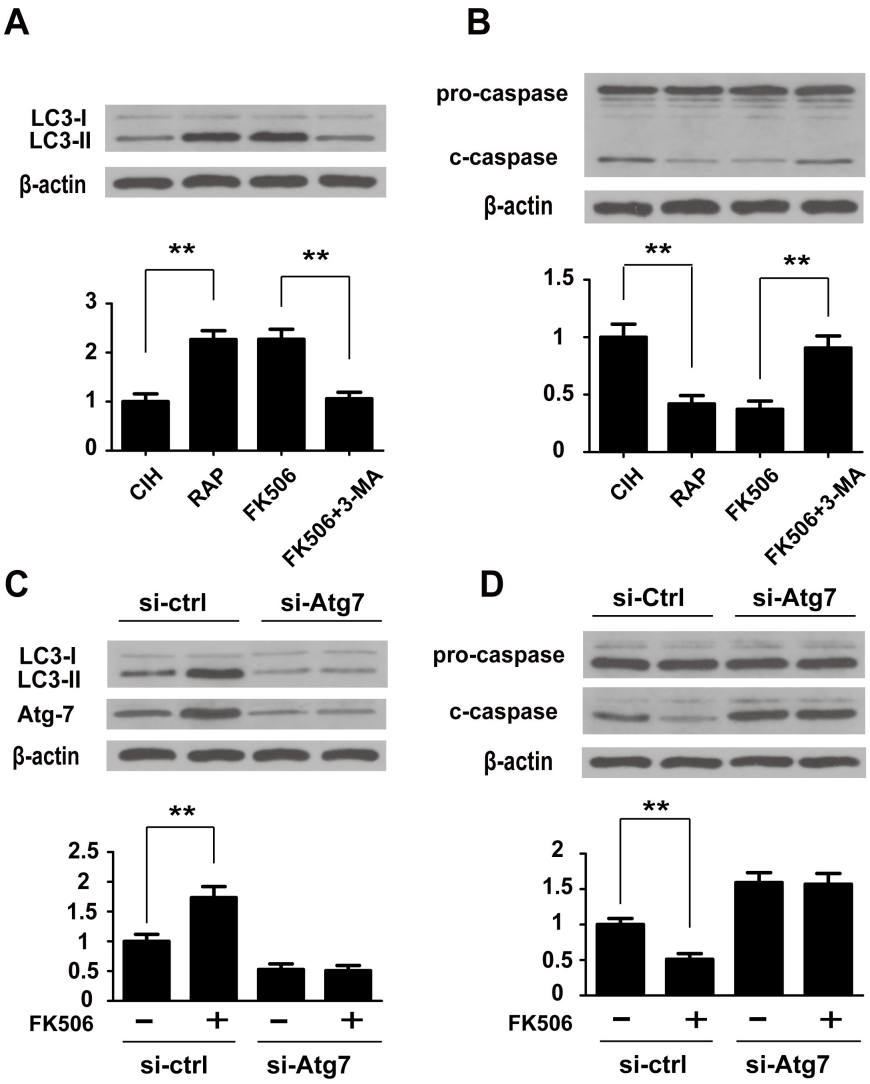


**Figure 7.** Autophagy protects H9C2 cells from chronic intermittent hypoxia (CIH)-induced apoptosis. (**a**) Cells were cultured with RAP, FK506, and FK506+3-MA under CIH, and the protein levels of LC3 were evaluated by western blotting. (**b**) Cells were cultured with RAP, FK506, and FK506+3-MA under CIH, and protein levels of c-caspase and pro-caspase were determined by western blotting. (**c**) Cells were cultured with or without FK506, ci-Ctrl, and si-Atg7, and the protein levels of LC3 were determined by western blotting. (**d**) Cells were cultured with or without FK506, si-Ctrl, and si-Atg7, and protein levels of c-caspase and pro-caspase were determined by western blotting. Data are expressed as means ± SD of five independent experiments. **p < 0.001. Abbreviations: c-caspase, cleaved caspase-3; CIH, chronic intermittent hypoxia; RAP, rapamycin; Si-Atg7, siRNA against *Atg7*; Si-Ctrl, control siRNA; 3-MA, 3-methyladenine.

## 4. Discussion

Our results suggest that autophagy is negatively regulated by calcineurin through the AMPK/mTOR axis. This conclusion is supported by the following key findings: (1)

calcineurin inhibition significantly upregulated AMPK/mTOR signaling, induced autophagy, and partly rescued H9C2 cell apoptosis; (2) calcineurin overexpression attenuated AMPK/mTOR signaling, decreased autophagy, and exacerbated damage induced by CIH in H9C2 cells; (3) the inhibition of AMPK abolished autophagy induced by calcineurin inhibition; and (4) autophagy inhibition reversed the protective effects of calcineurin inhibition in H9C2 cells.

CIH induces excessive reactive oxygen species (ROS) accumulation [16], leading to pathological changes in the cardiovascular system via complex underlying mechanisms. Cardiac ROS accumulation is caused by, among other factors, the dysregulation of hypoxia-inducible factor [17,18]. Excessive ROS induce significant oxidative stress in the mammalian myocardium, suppressing the activity of endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2), a membrane calcium pump that plays a vital role in cardiac calcium homeostasis and is a major factor in myocardial contractility [19]. Moreover, oxidative stress triggers calcium-dependent nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 5 and induces atherosclerosis via cellular calcium overload [20].

Cellular calcium is a critical regulator of calcineurin activation. Lysosomal calcium release through mucolipin-1 activates calcineurin, which, in turn, binds to dephosphorylated transcription factor EB to modulate cellular autophagy [21]. Therefore, intracellular lysosomal calcium signaling modulates autophagy via calcineurin-mediated transcription factor EB dephosphorylation [22]. Moreover, as a calcium-activated phosphatase, calcineurin plays pivotal roles in calcium signaling in response to oxidative stress in various heart diseases [23].

Cardiomyocytes are non-replicating cells with high metabolic activity. The maintenance of cardiomyocyte homeostasis depends mainly on cellular renewal. Since autophagy supports homeostasis and promotes cell survival by recycling cellular waste, cardiomyocytes must maintain autophagic activity in response to insults, such as oxidative stress-induced damage [24].

Oxidative stress causes several types of cell damage, such as mitochondrial destruction and protein misfolding, which are detrimental to normal mitochondrial function, resulting in nutrient and energy depletion [3]. CIH-induced oxidative stress can lead to calcium abnormalities and DNA damage. Given the numerous detrimental effects of oxidative stress-induced damage on autophagy regulation, we hypothesized that several factors were involved in autophagy induction in response to CIH-induced oxidative stress, including hypoxia-inducible factor (HIF) [18] and sequestosome 1 (p62/SQSTM1) [25]; however, additional research is needed to investigate the role of these factors.

Chronic hypoxia causes energy depletion and organelle damage, rendering cells more sensitive to oxidative stress and reducing cardiomyocyte viability [26,27]. As an independent type of programmed cell death, autophagy can regulate apoptosis in numerous cell lines [28]. We previously confirmed that autophagy protects cardiomyocytes under CIH; however, we did not investigate the molecular mechanisms [13,14].

Calcineurin modulates cardiomyocyte apoptosis, as well as autophagy, within the myocardium [29]. The current study showed that the suppression of autophagy regulated by calcineurin activation during CIH was, at least in part, responsible for the increased H9C2 cell apoptosis. Further studies are needed to identify the pathways involved. We also found that autophagosomes rapidly formed during the initial exposure to CIH, and the number of autophagosomes sharply declined thereafter. In addition, the AMPK pathway was attenuated at the later stage of CIH. AMPK, the autophagy regulator, affects autophagosome formation through an mTOR-dependent mechanism. AMPK modulates autophagy via the regulation of autophagosome formation; however, we still cannot rule out the contributions of attenuated AMPK pathway to the decrease of autophagosomes at the later stage.

To confirm the involvement of calcineurin in the regulation of H9C2 cell autophagy via the AMPK pathway, we overexpressed (using CnA) or inhibited (using FK506) calcineurin during CIH. H9C2 cell autophagy and apoptosis were closely associated with CIH, supporting our previous studies [13,14].

It was reported that AMPK inactivation during aging contributes to the dysregulation of intracellular lipid metabolism and reduces mitochondrial function [30]. AMPK and autophagy activation decrease during the chronic, but not acute stages of myocardial infarction [31]. Here, we confirmed that CIH, another contributor to cardiac pathology, inactivates AMPK and decreases autophagy induction in H9C2 cells after an initial adaptive increase.

mTOR, a negative regulator of autophagy located downstream of AMPK, inevitably modulates cardiomyocyte autophagy. For instance, microRNA-mediated mTOR inhibition induces multiple cardiomyopathies by inhibiting autophagy via AMPK [32]. Moreover, 4E-BP1, the factor that inhibits translation initiation and a substrate of multiprotein complex-1 containing mTOR (mTORC1), is involved in mTORC1-mediated cardiomyocyte survival [33]. The interactions between calcineurin, AMPK, and AMPK-regulated autophagy were also demonstrated in a recent study [12]. Our findings support these previous reports; however, we did not observe any significant effects of calcineurin on Akt signaling in response to oxidative stress. Collectively, our results confirmed that calcineurin regulated mTOR in an Akt-independent and AMPK-dependent manner, thus affecting H9C2 cell autophagy by transcriptional modulation, in addition to the regulation of the AMPK/mTOR axis.

Several factors involved in the regulation of autophagy during CIH form a complex interaction network. However, this study did not address all possible underlying mechanisms. Although we investigated the mechanism *in vitro*, these findings were not validated *in vivo*. Nevertheless, we demonstrated that AMPK and autophagy induction are suppressed under prolonged CIH. Our findings emphasize the importance of the protective role of autophagy under CIH and provide a new perspective for treating CIH-induced myocardial injury.

**Data availability statement:**

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**Conflicts of interest:** The authors declare no conflict of interest.

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