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CaMKIIγ advances chronic intermittent hypoxia-induced cardiomyocyte apoptosis via HIF-1 signaling pathway

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Abstract

Background Our previous study have demonstrated chronic intermittent hypoxia (CIH) induced cardiomyocyte apoptosis and cardiac dysfunction. However, the molecular mechanisms are complicated and varied. In this study, we first investigated the CaMKIIγ expression and signaling pathway in the pathogenesis of cardiomyocyte apoptosis after CIH.

Methods Rats were separated into CIH and Normoxia groups, and H9c2 cells were divided into Control and CIH + 8 h groups. Rat body weight (BW) was markedly gained from two to six weeks. Furthermore, CIH decreased cardiac dysfunction, damaged cellular structure, induced myocardial fibrosis, and promoted cardiomyocyte apoptosis by HE, masson, sirius-red, and TUNEL staining. Western blot, immunohistochemical, immunofluorescence, double immunofluorescence staining were performed to investigate CaMKIIγ, Bcl-2, Bax, Caspase 3, HIF-1 protein expression.

Results Heart weight (HW) and HW/BW ratio in CIH group was markedly gained compared with the Normoxia group. CaMKIIγ expression was notably increased after CIH, and mainly expressed in the cytoplasm in vivo and vitro. The results of HIF-1 expression have the same trend of CaMKIIγ expression and cardiomyocyte apoptosis. In addition, the co-localizations of CaMKIIγ with Caspase 3, and CaMKIIγ with HIF-1 were observed by double immunofluorescence staining.

Conclusions These results indicated increased CaMKIIγ expression advances CIH-induced cardiomyocyte apoptosis via HIF-1 signaling pathway, which afford a new insight and provide a potential therapy for OSA patients.

Keywords Chronic intermittent hypoxia · CaMKIIγ · Apoptosis · HIF-1 · Cardiomyocyte · H9c2 cells

Introduction

Obstructive sleep apnea (OSA) is a highly prevalent sleep disorder, caused by complete or partial occlusion of the upper airway collapses during sleep, which is leading to chronic intermittent hypoxia (CIH), hypercapnia, sleep fragmentation, and sympathetic activity [1, 2]. Furthermore, OSA impacts between 9% and 38% of the population, affects nearly a billion adults aged 30–69 years worldwide [3]. Nearly half were classified as moderate to severe OSA, which required treatment [4]. However, closing to 82% of men and 93% of women have not been clinically diagnosed and untreated, especially in many developing countries [5]. Notably, patients with OSA results in multisystem dysfunction, which seriously affects the life quality and life safety [2, 3]. The high prevalence of OSA has a significant impact on the occurrence and progression of cardiovascular disease [6]. OSA elevates cardiovascular risk, including cardiac arrhythmia, hypertension, and stroke [6, 7]. CIH is a unique pathological mechanism of OSA, which is considered to be an independent risk factor for atrial fibrillation (AF) [6]. CIH has been shown to induce cardiomyocytes apoptosis, leading to cardiac injury [7, 8]. Therefore, improving cardiomyocyte survival, protecting cardiac function, and exploring new molecular mechanisms or signaling pathways are crucial for the development of impactful therapies in OSA patients. In our previous study, according to a CIH

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rat model by our group, which presents the characteristics of OSA to mimicked OSA diseases, we have demonstrated that CIH induces atrial remodeling, increases AF inducibility, improves sympathetic nerve excitability, raises blood pressure, advances cardiomyocyte apoptosis, and alters many protein expression [9–11]. However, the molecular mechanisms are complicated and varied. In this study, we first investigated the CaMKIIγ expression and signaling pathway in the pathogenesis of cardiomyocyte apoptosis after CIH.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is an enzymatically active and a ubiquitously expressed multifunctional serine/threonine kinase, with four isoforms (CaMKIIα, CaMKIIβ, CaMKIIγ, and CaMKIIδ), which increases in Ca^{2+} to activation of cell apoptosis, gene transcription, and ion channels [12]. CaMKII consists of twelve subunits, each containing of three structural domains [13]. CaMKII is a prominent regulator in cardiomyocytes for regulating various biological functions of the heart, by regulating myocardial cell membrane excitability, maintaining cell Ca^{2+} homeostasis and regulating the cell cycle [14–16]. CaMKII is heterogeneously distributed in cardiomyocyte, muscle, nerve, and immune tissues. CaMKIIγ and CaMKIIδ are mainly manifested in the cardiovascular system, and are associated with the cardiovascular diseases such as cardiac arrhythmia [17–19]. As all we know, CaMKII isoforms have different targets and effects on cardiac pathophysiology [17]. CaMKII is implicated in the regulation of apoptosis. CaMKII low expression protects against endoplasmic reticulum stress-induced cardiomyocyte apoptosis [20], but overexpression enhances renal tubular epithelial cell apoptosis [21].

Apoptosis, called programmed cell death, was related to the pathogenesis of cardiac diseases such as arrhythmia [10, 22]. Although many studies on CaMKII in cardiovascular disease, however, whether CaMKIIγ isoform is omplicated in the pathogenesis of cardiomyocyte apoptosis in CIH and by which signaling ways remain unclear. In addition, reducing cardiomyocyte apoptosis and improving cardiomyocyte survival after CIH exposure is imminent for finding potential cardioprotective targets and searching for effective therapies in patients with OSA.

Thus, the aim of our study was to understand the mechanisms and signaling ways of CaMKIIγ in CIH induced cardiomyocyte apoptosis. The results of our study afford new insights for ameliorating cardiomyocyte apoptosis, and provide potential therapeutics for OSA patients.

Materials and methods

CIH model in vivo

The OSA model was performed according to our previous study [9–11], CIH rat model was performed to repeats hypoxia events of OSA patients by exposing experimental animals to the hypoxia/reoxygenation. In this study, twenty male and female SD rats (initial weight 200 ± 20 g; 9 weeks old) were used as the experimental subjects and housed in individual cages in a temperature-controlled (22–24 °C) room with a 12-hour light-dark cycle, and all the experimental manipulations met the experimental requirements of the Jiangsu Provincial Animal Care Ethics Committee. Water and food were available ad libitum in the cages. The rats were randomly divided into the CIH or Normoxia group. CIH rats were exposed to intermittent hypoxia chambers, and the hypoxic conditions underwent cyclic changes (2 min) 8 h per day (from 8 a.m. to 4 p.m.) for 6 weeks. In each cycle, oxygen concentration was decreased to 6% in the first 30 s and remained for 50 s, then increased to 21% in 40 s. Ten hearts from each group were examined by different methods. And each experiment consisted of at least three replicates per condition.

Echocardiography and Weight analysis

We evaluated the heart weight (HW), body weight (BW) and HW/BW ratio of all rats. In addition, the cardiac function of all rats were scanned by ultrasound system according our previous studies [9, 23].

Histochemical analysis

Heart tissues were harvested from rats after CIH, immersed in 10% formalin, embedded in paraffin, sectioned at 7 μm, then dewaxed and hydrated. Hematoxylin and eosin (HE) staining was performed according our previous study [24, 25]. Subsequently, Masson trichrome staining and Sirius-red staining were performed according to the manufacturer's instructions in order to assess heart fibrosis. Furthermore, the TUNEL assay was carried out to study the cardiomyocyte apoptosis [10]. All photographs were collected directly and assessed blindly by a certified pathologist.

CIH model in vitro

CIH model in vitro was performed according our previous study [11]. Briefly, H9c2 cells were randomly separated into the CIH + 8 h and Control group. H9c2 cells of CIH + 8 h group were hypoxic exposured ($1\%O_2$) for one hour in a controlled hypoxic plastic chamber, then were reoxygenated

(95%O2) for 1 h in a normoxic incubator. The whole procedure was repeated 4 cycles for 8 h. And each experiment consisted of at least three replicates per condition.

Western blot analysis

According to our previous study, western blotting analysis was carried out [24, 26]. Briefly, the tissues were examined with the following antibodies: CaMKIIγ (1:1000), β-Tubulin (1:10000), GAPDH (1:1000), and HIF-1 (1:1000).

Immunohistochemical staining

Depending on our previous studies [10, 24], the immunohistochemistry was successfully examined in the heart tissues of two groups with the following antibodies CaMKIIγ (1:200), Bax (1:200), and Bcl-2 (1:200).

Immunofluorescence staining

According to previous articles [11, 23], immunofluorescence staining and double Immunofluorescence staining were performed to investigate the temporal expression and cellular localization of CaMKIIγ (1:50), HIF-1(1:100).

Statistical analysis

All dates were analyzed by GraphPad Prism or SPSS 19.0. All values are mean ± SEM. T-test was used to analyze the experimental data and results of $P < 0.05$ were considered as statistically significant. Each trial consisted of at least three replicates per condition.

Results

The weight changes of rat after CIH

The BW of rats in the CIH and Normoxia group were markedly gained (Fig. 1A&B). The BW of two group rats were substantially similar in the first week ($P = 0.0816$), but the growth rate slowed in the CIH group began in the second week ($P = 0.0007$). Compared with the CIH group, BW of Normoxia group was markedly gained from two to six weeks. The average BW in two groups at the end of study was significant difference ($P = 0.0000$). To further investigate the study of cardiac changes of rats after CIH, the HW and HW/BW ratio at six week were studied (Fig. 1C&D). HW of CIH group was markedly gained compared to the Normoxia group ($P = 0.0108$). The HW/BW ratio in the CIH group was also markedly gained compared with the Normoxia group ($P = 0.0000$). These date indicated the BW and HW of rats were affected by CIH.

Cardiac alterations of rat exposed to CIH

To examine the possible cardiac alterations of rat exposed to CIH 6 weeks, we performed a series of histopathological studies of cardiomyocytes by echocardiography, HE staining, masson staining, and sirius-red staining. Representative photographs of rat heart slice are shown in Fig. 2 (original magnification ×20). Compared to the Normoxia group, disordered cardiomyocytes arrangement, increased interstitial space, oedema, necrosis, and abnormal architecture were observed in the CIH group (Fig. 2A). Results showed cardiomyocytes injuries after CIH exposed. The echocardiographic parameters are shown in Table 1. The echocardiography (Fig. 2B) in CIH rats all showed significant decreased in ejection fraction (EF) and fractional shortening (FS). However, the left ventricular internal diameter in systole (LVIDs) and diastole (LVIDd), left ventricular posterior wall in systole (LVPWs) and diastole (LVPWd), and left ventricular (LV) mass were significant increased in the CIH group. Echocardiography demonstrated CIH decreased EF and FS, but caused left ventricle (LV) hypertrophy and remodeling, resulting in cardiac dysfunction. To examine the possible fibrosis in cardiomyocytes after 6 weeks of CIH, masson staining and sirius-red staining were performed to study collagen fibers in physiology and pathology. Masson staining (Fig. 2C) showed the muscle fibers were red, and the collagenous fibers were blue. Apparently, compared with the Normoxia group, the collagenous fibers were considerably increased expression in the CIH group. Sirius-red staining (Fig. 2D) showed the muscle fibers were yellow, and the collagenous fibers were red. The results of sirius-red staining have the same trend as masson staining. These date showed that CIH decreased EF and FS, damaged cardiomyocytes, precipitated left ventricle (LV) hypertrophy and remodeling, leading to myocardial fibrosis and cardiac dysfunction.

CaMKIIγ expression was notably upregulated in vivo

Western blot was performed to investigate the temporal expression pattern of CaMKIIγ protein in vivo. The bands corresponding to total CaMKIIγ protein are shown in Fig. 3A. We found the CaMKIIγ protein expression was low in the Normoxia group. Compared with the Normoxia group, the CaMKIIγ protein was notably upregulated in the CIH group (Fig. 3A). Quantitative CaMKIIγ protein analysis was performed as described in Fig. 3B. To further investigate the CaMKIIγ expression and the cellular localization, we performed immunohistochemistry staining experiments

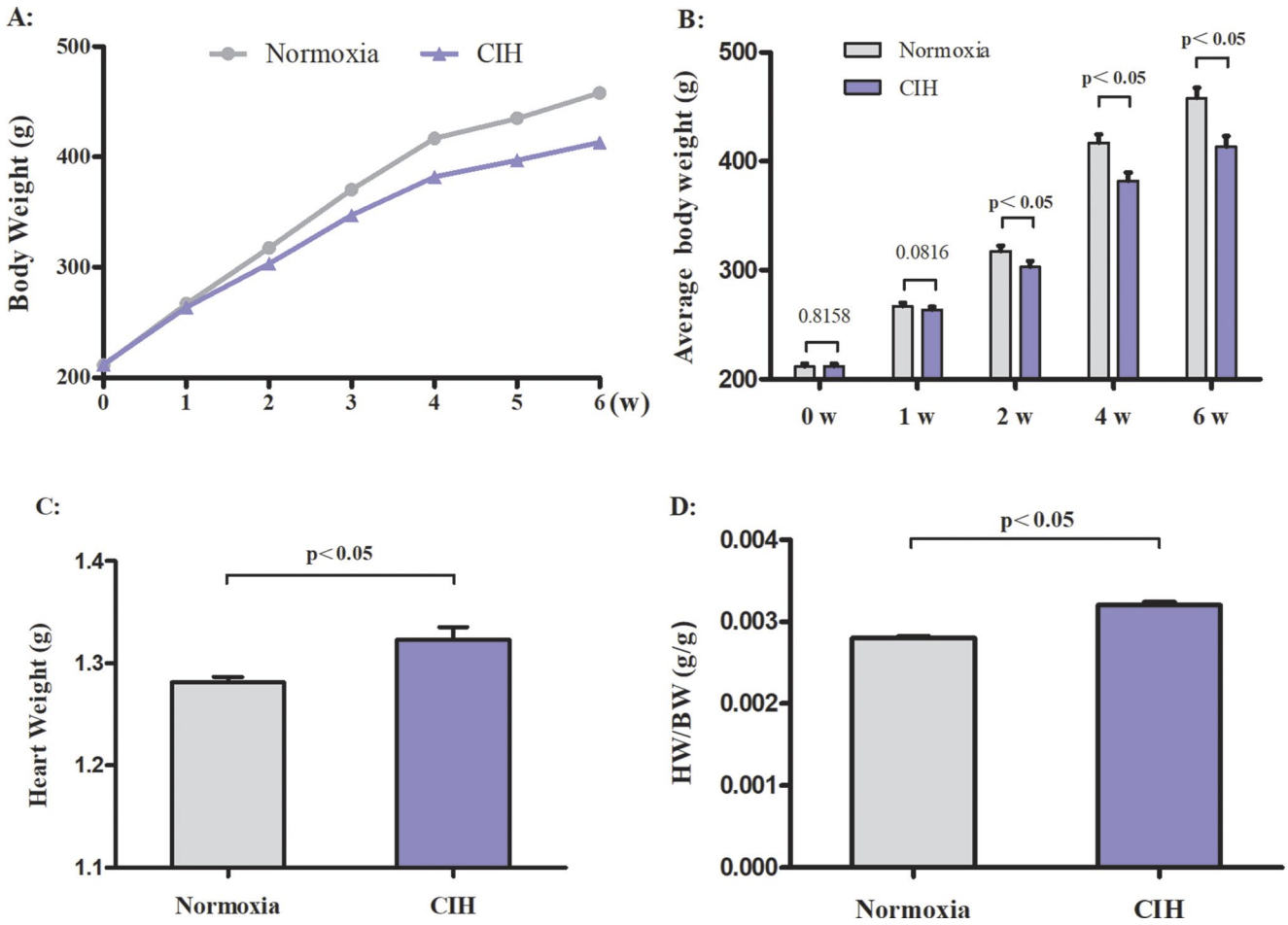

Fig. 1 (**A**) The changes of rats BW in the Normoxia group and CIH group. (**B**) The average BW of rats in different time point. (**C**) The HW of rats in the Norxiama and CIH group. (**D**) The HW/BW ratio of two groups

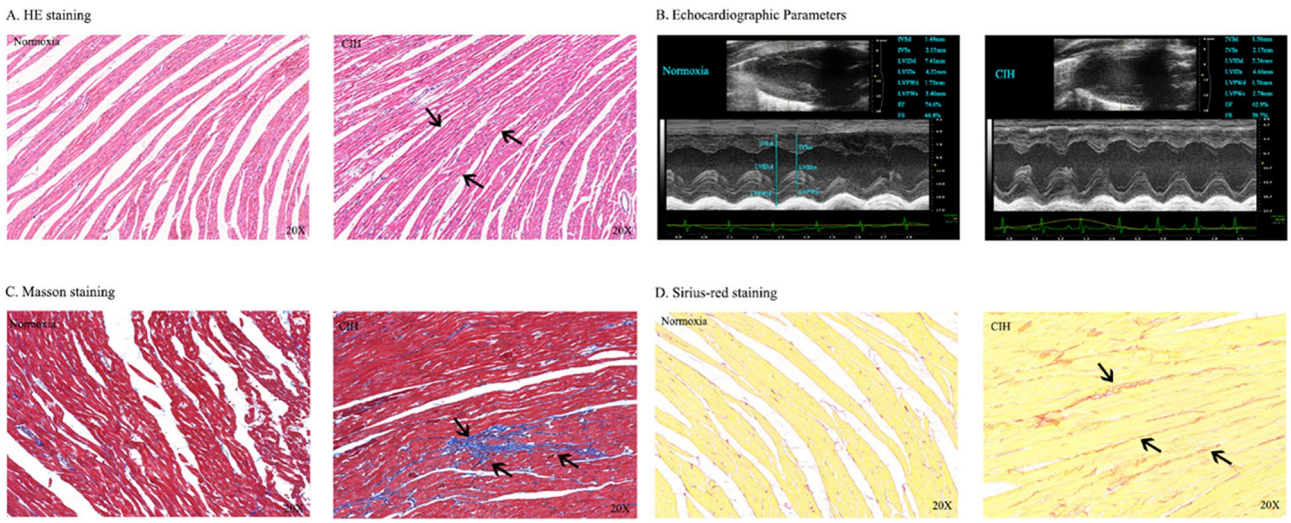

Fig. 2 (**A**) HE staining in the CIH group and Normoxia group. (**B**) The echocardiography of rats in two groups. (**C**) Masson staining showed the muscle fibers were red, and the collagenous fibers were blue. (**D**) Sirius-red staining showed the muscle fibers were yellow, and the collagenous fibers were red. Representative photographs of rat heart slice (original magnification ×20)

Table 1 Echocardiographic parameters

Parameters	Normoxia	CIH
IVS;d (mm)	1.502±0.011	1.505±0.017
IVS;s (mm)	2.169±0.112	2.170±0.136
RVID;d (mm)	2.203±0.072	2.211±0.064
RVAW (mm)	0.531±0.006	0.528±0.007
LVID;d (mm)	7.425±0.175	7.729±0.213*
LVPW;d (mm)	1.728±0.013	1.785±0.008*
LVID;s (mm)	4.228±0.189	4.673±0.261**
LVPW;S (mm)	2. 403±0.075	2.783±0.1572**
EF (%)	75.179±1.723	62.931±2.748***
FS (%)	49.851±2.361	40.064±1. 824***
LV Mass (mg)	857.32±16.89	948.92±20.36***

All values are means ± SD. *p<0.05, **p<0.01, ***p<0.001 vs. Normoxia group

in rats. Representative examples were shown in Fig. 3C. The date showed that CaMKIIγ was mainly expressed in the cytoplasm of cardiomyocytes. The CaMKIIγ expression in the CIH group was markedly increased compared with the Normoxia group. Statistical results of the number of CaMKIIγ positive cells are shown in Fig. 3D. Apparently, the results of the immunohistochemistry staining were similar to the western blot dates. We further determined the localization and expression of CaMKIIγ cells by immunofluorescence staining. Immunofluorescence also revealed CaMKIIγ expression was considerably increased in the CIH group, and it was mainly expressed in the cytoplasm of cardiomyocytes (Fig. 3E&F). These results proved that CIH notably enhanced the CaMKIIγ expression in the cardiomyocytes.

CaMKIIγ expression was markedly increased in vitro

Results in vivo has been confirmed that CIH markedly enhanced the CaMKIIγ expression in the cardiomyocytes. In addition, our previous study have demonstrated CaMKIIγ expression in H9c2 cells was markedly suppressed after connexin 43 overexpression [11]. To further confirm the CaMKIIγ expression in H9c2 cells, the western blot and immunofluorescent staining were performed, respectively. As western blot date shown in Fig. 4A, the CaMKIIγ expression in the CIH + 8 h group was markedly increased compared with the Control group. Quantification of the total CaMKIIγ expression presented in Fig. 4B. Immunofluorescent staining indicated that CaMKIIγ expression mainly expressed in the cytoplasm of H9c2 cells (Fig. 4C), and the expression of CaMKIIγ in the CIH + 8 h group were markedly upregulated than Control group (Fig. 4D). The results of immunofluorescence staining were consistent with western blot. These dates confirmed the expression of CaMKIIγ was markedly increased in vitro.

The increased expression of CaMKIIγ advanced apoptosis

Previous study by our team have demonstrated the apoptosis of cardiomyocytes was acknowledged after CIH [10, 11]. Specifically, connexin 43 overexpression remarkably suppressed the expression of CaMKIIγ and inhibited myocardial apoptosis [11]. In this study, we tested the Bcl-2 and Bax expression by immunohistochemistry staining in vivo. Representative pictures of Bcl-2 and Bax are shown in Fig. 5. Immunohistochemistry staining shown the level of Bcl-2 at CIH group are lower, compared to the Normoxia group (Fig. 5A). Number of Bcl-2 positive cells are shown in Fig. 5B. However, the level of Bax are higher at the CIH group than the Normoxia group (Fig. 5C). Number of Bax positive cells was performed as described in Fig. 5D. To further investigate the apoptosis of rat cardiomyocytes after CIH, the TUNEL assay was carried out. Representative examples of cardiomyocytes apoptosis by TUNEL assay are shown in Fig. 5E. TUNEL assay showed the cardiomyocytes apoptosis were mainly occurred in the nucleus. The TUNEL positive cells are markedly increased in the CIH group compared with the Normoxia group. Statistical results of positive cells are shown in Fig. 5F. Furthermore, the TUNEL staining was performed in H9c2 cells (Fig. 6A&B). TUNEL positive H9c2 cells in the CIH + 8 h group are significant higher, compared with the Control group (Fig. 6A&B). Apparently, the occurrence of apoptosis in vivo and vitro are very common after CIH. These results further corroborate our previous study, which CIH lead to myocardial apoptosis. In addition, we have found CIH markedly enhanced the CaMKIIγ expression in the cardiomyocytes and H9c2 cells. To understand the relationship between CaMKIIγ and cardiomyocytes apoptosis, double labeling immunofluorescent staining was performed to examined the co-localization of CaMKIIγ and Caspase 3 in H9c2 cells. The co-localizations (yellow) of CaMKIIγ (green) with Caspase 3 (red) in H9c2 cells were observed (Fig. 6C). Expression of CaMKIIγ was positively correlated with cardiomyocytes apoptosis. Taken them all, these results indicated that the increased expression of CaMKIIγ advanced cardiomyocytes apoptosis after CIH.

CaMKIIγ regulated HIF-1 signaling pathway

To investigate the possible and effective signaling pathways of CaMKIIγ after CIH, we researched the HIF-1 signaling pathway. The HIF-1 protein expression was detected by western blot. HIF-1 protein expression in the CIH + 8 h group was notably increased compared with the Control group (Fig. 7A). Quantification of the total HIF-1 protein expression presented in Fig. 7B. To further demonstrate the expression trend of HIF-1 and its cellular localization, the fluorescent staining was performed. As representative examples are shown in Fig. 7C,

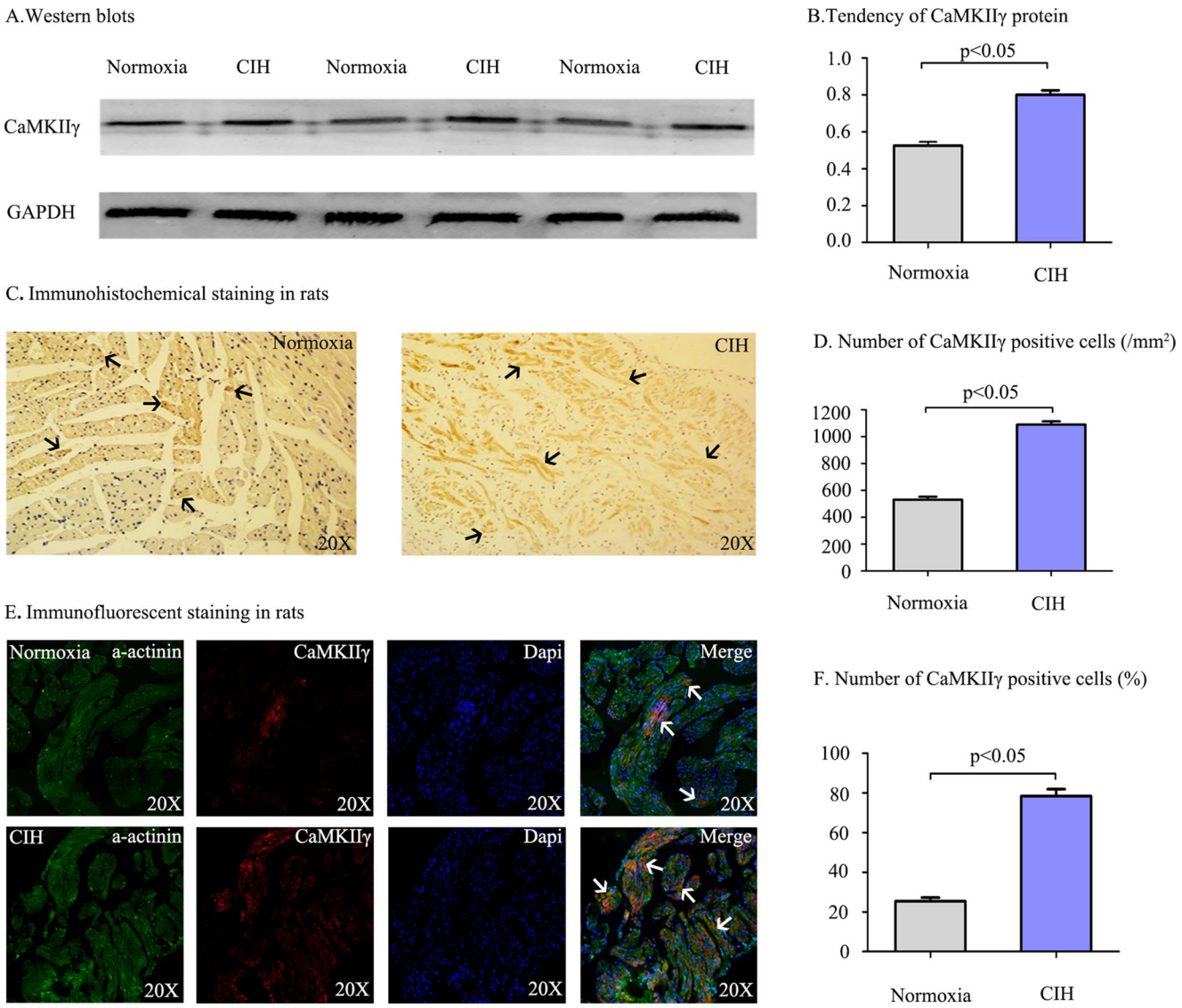

Fig. 3 (**A**) The expression of CaMKIIγ protein in the Normoxia group and CIH group were detected by western blots. CaMKIIγ protein expression was significantly increased in the CIH group. (**B**) Quantitative CaMKIIγ protein analysis in two groups. (**C**) Representative immunohistochemical staining of CaMKIIγ in rats. Black arrows indicate CaMKIIγ expression in cardiomyocytes (Original magnification ×20). (**D**) Quantification of the CaMKIIγ positive cells in the Normoxia group and CIH group. (**E**) Representative immunofluorescence staining photographs among α-actinin (green), CaMKIIγ (red), and Dapi (blue), (original magnification×20). (**F**) Number of CaMKIIγ positive cells

the HIF-1 expression in the CIH+8 h group was markedly increased compared with the Control group. Statistical results are shown in Fig. 7D. To understand whether CaMKIIγ regulated HIF-1 signaling pathway, the co-localization of CaMKIIγ and HIF-1in H9c2 cells were examined by double labeling immunofluorescent staining with the following cell-specific makers: CaMKIIγ, HIF-1, and Dapi. Obviously, the co-localization of CaMKIIγ and HIF-1 in H9c2 cells were observed (Fig. 7E). These results confirmed that CaMKIIγ regulated HIF-1 signaling pathway.

Discussion

OSA is a common sleep and breath disease, impacts between 9% and 38% of the population, affects nearly a billion adults aged 30–69 years worldwide, results in hypercapnia, sleep fragmentation, sympathetic activity, and CIH, the later was distinct pathological mechanism of OSA [1–3, 6]. CIH was considered to be an independent risk factor for cardiovascular pathogenesis, and was closely associated with increased mortality cardiovascular diseases [6]. In addition, CIH has been shown to induce cardiomyocytes apoptosis, leading to cardiac injury [7, 8]. Thus, using a CIH rat model to mimic

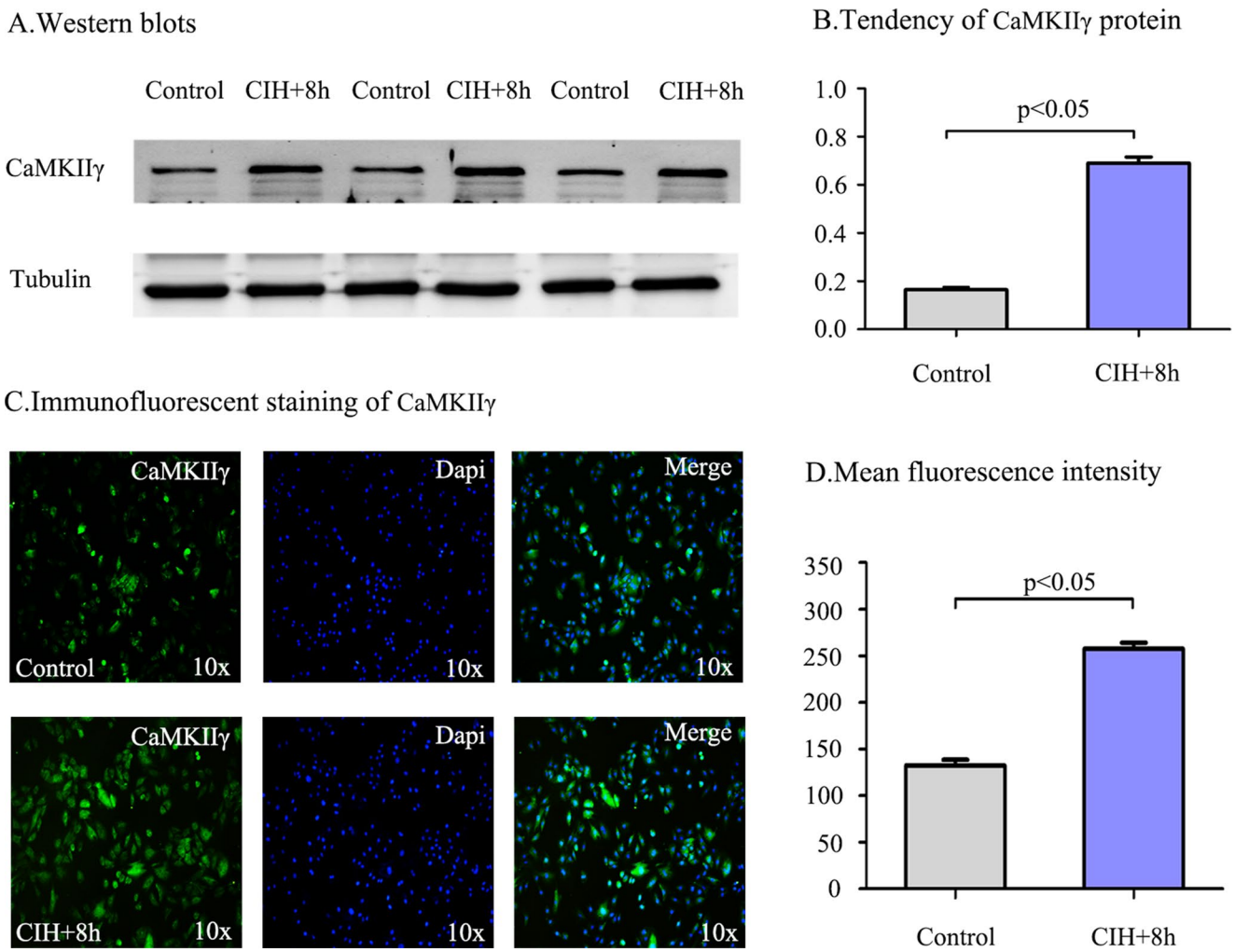

Fig. 4 The expression of CaMKIIγ in H9c2 cells were detected by western blots and immunofluorescence staining. (**A**) Compared with the Control group, the CaMKIIγ expression in the CIH + 8 h group was markedly increased. (**B**) Tendency of CaMKIIγ protein expression in the Control group and CIH + 8 h group. (**C**) Representative immunofluorescence staining of CaMKIIγ (green) and mean fluorescence intensity analysis (**D**) in the Control group and CIH + 8 h group (original magnification×10)

the pathophysiological mechanisms and cardiovascular consequences of OSA was important and urgent. According to our previous study [9, 10], CIH rat model was performed to repeats hypoxia events of OSA patients by exposing experimental animals to the hypoxia/reoxygenation. CIH causes cardiac dysfunction by multiple complex mechanisms, such as increased oxidative stress [27], induced cardiac inflammation [28], aggravated cardiomyocyte apoptosis [29]. Our group have demonstrated that CIH induces atrial remodeling, increases AF inducibility, improves sympathetic nerve excitability, raises blood pressure, advances cardiomyocyte apoptosis, and alters many protein expression, then resulting in cardiac dysfunction [9]. In this study, we found that the HW of CIH group was significantly increased compared to the Normoxia group, and the HW/BW ratio was also significantly increased (Fig. 1). These date indicated the BW and HW of rats were affected after CIH. In addition, we observed disordered cardiomyocytes arrangement, increased interstitial space, oedema, necrosis, and abnormal architecture in the CIH group by HE staining (Fig. 2A). Echocardiography demonstrated CIH decreased EF and FS, and caused LV hypertrophy and remodeling, resulting in cardiac dysfunction (Fig. 2B). Furthermore, masson staining and sirius-red staining found significant cardiomyocyte fibrosis (Fig. 2C&D), and TUNEL staining showed significant cardiomyocyte apoptosis in the CIH group (Fig. 5C) and in the CIH + 8 h group (Fig. 5D). To further investigate the apoptosis of rat cardiomyocytes after CIH, we examined the pro-apoptotic Bax protein and anti-apoptotic member Bcl-2 expression by immunohistochemistry staining. Immunohistochemistry staining shown the level of Bcl-2 at CIH group are lower, compared to the Normoxia group (Fig. 5A). However, the level of Bax at CIH group are higher than Normoxia group (Fig. 5C). Furthermore, the TUNEL staining was performed in rats (Fig. 5E) and H9c2 cells (Fig. 6A). Apparently, the occurrence

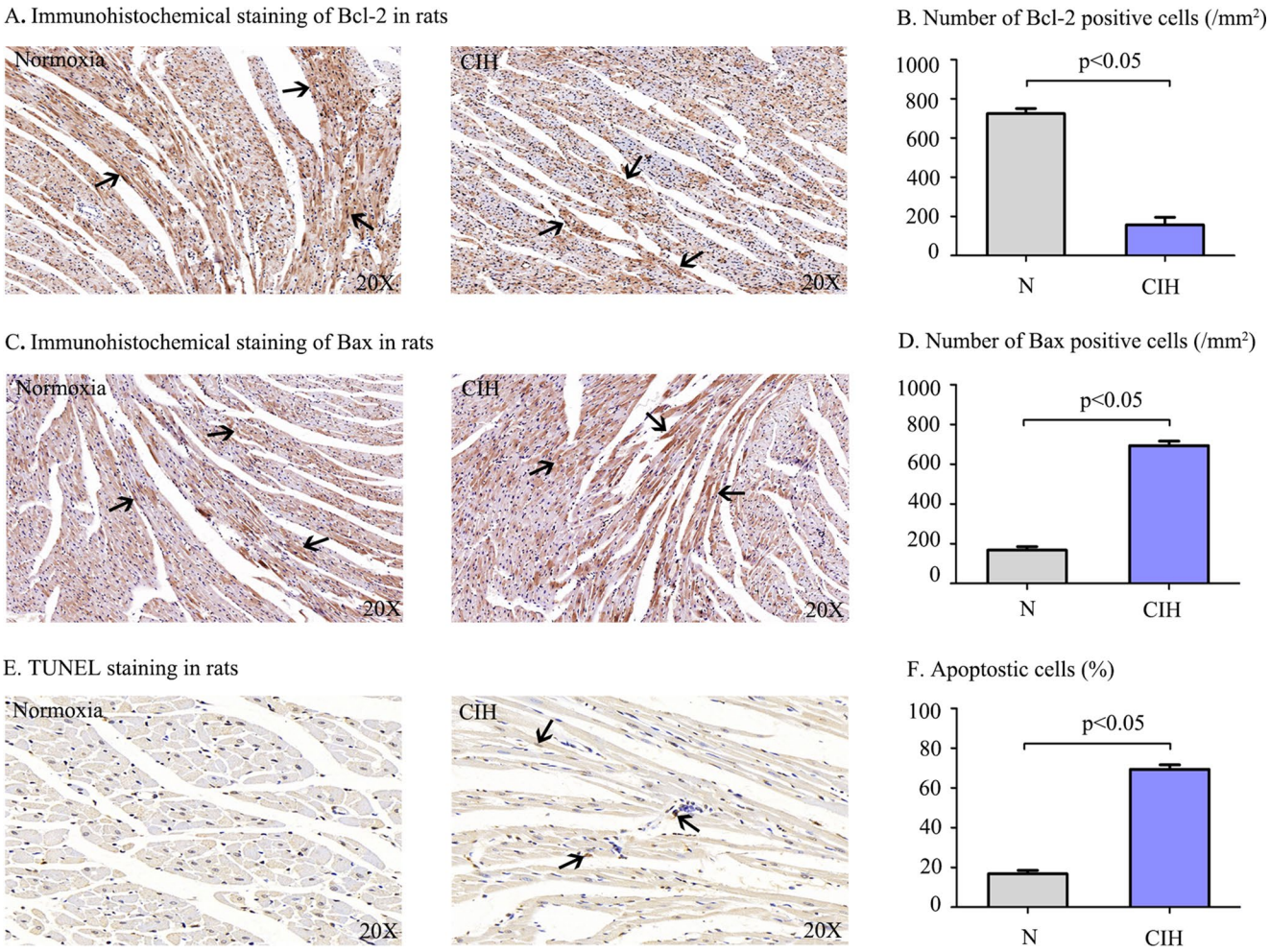

Fig. 5 The apoptosis of cardiomyocytes in vivo after CIH. The immunohistochemical staining of Bcl-2 (**A**) and Bax (**C**) in rats (Original magnification×20). Compared with the Normoxia group, the Bcl-2 (**B**) expression in the CIH group was markedly decreased, however, the Bax (**D**) expression was notably increased. (**E**) TUNEL assay showed the cardiomyocytes apoptosis were mainly occurred in the nucleus. (**F**) Statistical results of positive cells. Black arrow indicate TUNEL-positive cells (Original magnification×20)

of apoptosis in vivo and vitro are very common after CIH. The expression of apoptotic protein indicated that CIH caused cardiomyocytes apoptosis. In sum, our findings have the same conclusion as prior results of CIH, which CIH caused cellular structural changes, cardiomyocyte apoptosis, and cardiac dysfunction. However, the molecular mechanisms are complicated and varied. Importantly, there is no suitable treatments. Thus, it is crucial to look for new alternative molecular mechanisms involved in CIH-induced cardiomyocyte apoptosis.

CaMKII is a serine and threonine kinase with four subtypes: CaMKIIα, CaMKIIβ, CaMKIIγ, and CaMKIIδ, belongs to Ca^{2+}/calmodulin (CaM)-dependent kinases (CaMKs) large family of protein kinases [13, 30, 31]. CaMKII as an important signaling molecule, is heterogeneously distributed in cardiomyocyte, muscle, nerve, immune tissues, and plays a critical role in diseases development [12, 31]. CaMKII are associated with the cardiovascular diseases such as cardiac arrhythmia, myocardial ischemia/reperfusion injury, heart failure, myocardial infarction, and sudden death through triggering inflammation, causing arrhythmia, activating Ca^{2+}, disrupting DNA repair, affecting metabolic reprogramming, augmenting myocardial cell death, and other mechanisms [17, 31]. CaMKII low expression protects against endoplasmic reticulum stress-induced cardiomyocyte apoptosis [20], but overexpression enhances renal tubular epithelial cell apoptosis [21]. Previous study also suggested that CaMKIIγ activates ASK1 to induce the apoptosis of spinal astrocytes under oxygen–glucose deprivation [32]. Our group also demonstrated that Cx43 overexpression inhibits the expression of CaMKIIγ and reduces the incidence of atrial fibrillation [11]. However, the expression and the signaling pathways of CaMKIIγ in the pathogenesis of cardiomyocyte apoptosis in CIH remains unknown. In this study, we examined the temporal expression pattern of CaMKIIγ protein in vivo and vitro by western blot, immunohistochemistry staining and immunofluorescent staining. Compared with the Normoxia group, the CaMKIIγ was notably upregulated in

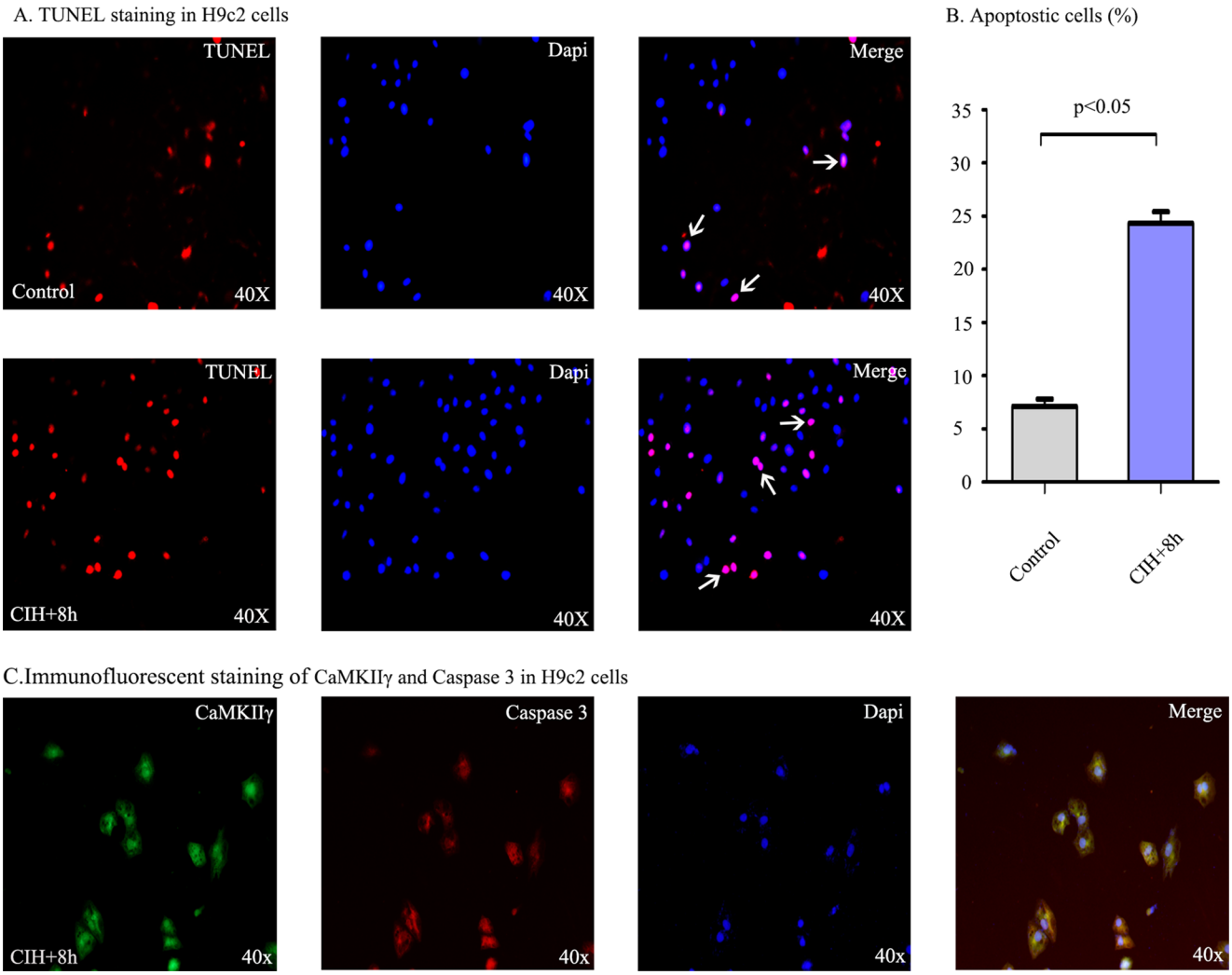

Fig. 6 CaMKIIγ advanced cardiomyocytes apoptosis. (**A**) Representative examples of TUNEL staining in H9c2 cells (Original magnification×40). White arrow indicate TUNEL-positive cells in H9c2 cells. (**B**) TUNEL positive H9c2 cells in the CIH+8 h group are significant higher, compared with the Control group. (**C**) Representative examples of CaMKIIγ (green) with Caspase 3 (red) and Dapi (blue) staining. The co-localizations (yellow) of CaMKIIγ with Caspase 3 in H9c2 cells were observed (Original magnification ×40)

CIH group and mainly expressed in the cytoplasm of cardiomyocytes (Fig. 3). To further confirm the CaMKIIγ expression in H9c2 cells, the western blot and immunofluorescent staining were performed, respectively. As date shown in Fig. 4, the CaMKIIγ expression in the CIH+8 h group was markedly increased compared with the Control group. These date indicated CIH markedly enhanced the CaMKIIγ expression in vivo and vitro. Whether increased expression of CaMKIIγ is involved in the pathogenesis of cardiomyocyte apoptosis in CIH, the co-localizations of CaMKIIγ and active caspase-3 were carried out (Fig. 6). Double immunofluorescent staining confirmed the co-localizations of CaMKIIγ and active caspase-3 in CIH+8 h group. Taken them all, we demonstrated that the increased expression of CaMKIIγ advanced apoptosis after CIH.

Previous study suggested that prolonged-excessive hypoxia induced cardiomyocyte apoptosis directly via inhibiting the myocardial survival pathway mediated by HIF-1-IGFBP-3-dependent signaling pathways [33]. Recent study by Ratul has demonstrated that cardiac-specific overexpression of HIF-1 ameliorates hypoxic cardiomyocyte apoptosis by differential regulation of anti-oxidative and hypoxia-inducible pro-apoptotic genes during acute myocardial infarction [34]. Hypoxia after myocardial infarction leads to HIF1-dependent differential splicing of CaMKIIγ. There are also studies that CaMKIIγ played an important role in Ca^{2+} signaling and transcriptional response to hypoxia [35]. In addition, our group have demonstrated that Cx43 overexpression reduces the myocardial apoptosis and incidence of AF after CIH via the Cx43/CaMKIIγ/HIF-1 axis [11]. However, whether increased CaMKIIγ expression aggravates cardiomyocyte apoptosis via HIF-1 signaling

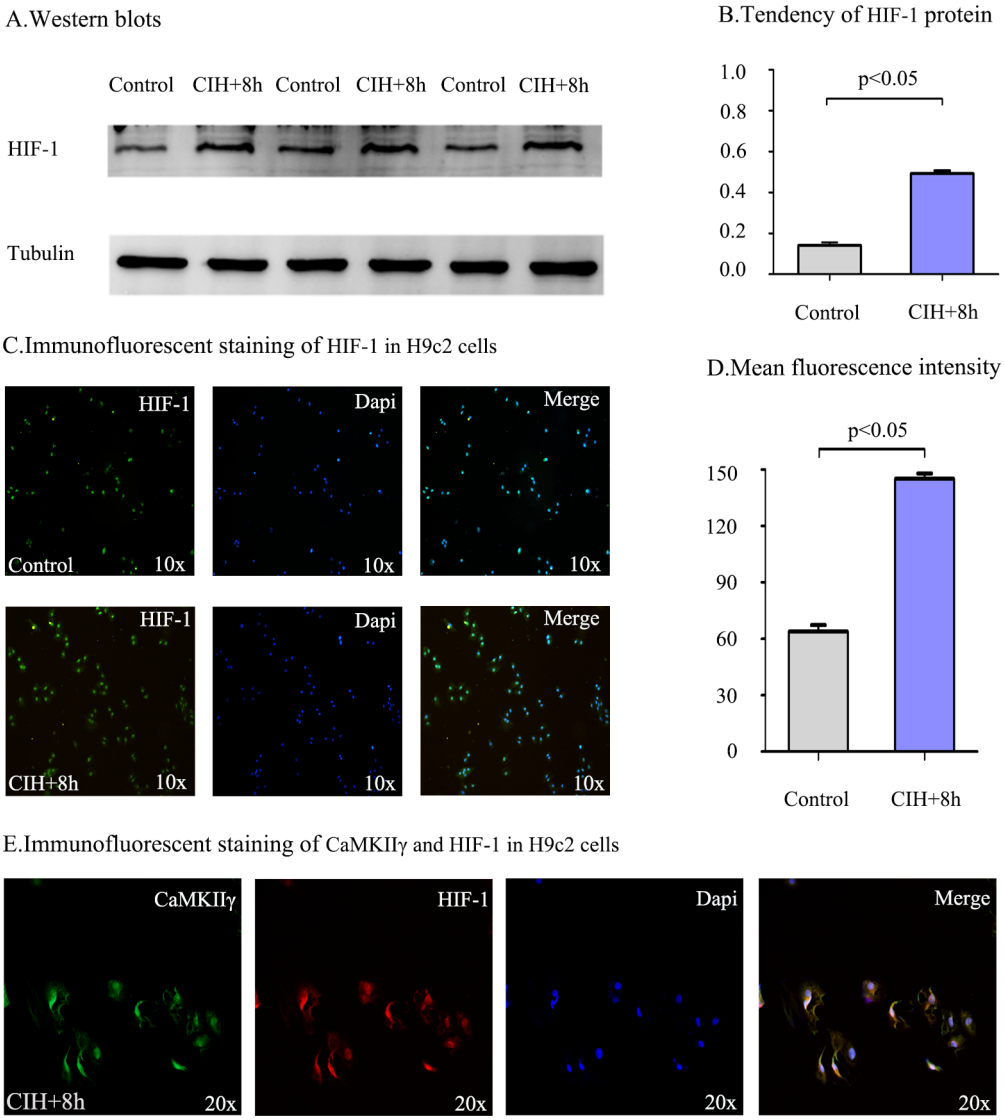

Fig. 7 CaMKIIγ regulated HIF-1 signaling pathway. HIF-1 expression in H9c2 cells were detected by western blots and immunofluorescence staining. (**A**) Western blot showed the HIF-1 expression was notably increased in the CIH + 8 h group. (**B**) Tendency in two groups. (**C**) Representative immunofluorescence staining of HIF-1 (green) and mean fluorescence intensity analysis (**D**) in the Control group and CIH + 8 h group (Original magnification×10). (**E**) Representative examples of CaMKIIγ (green) with HIF-1 (red) and Dapi (blue) staining. The co-localizations of CaMKIIγ with HIF-1 (yellow) were observed in H9c2 cells (Original magnification ×20)

pathway after CIH still remains obscure. Thus, we examined the expression pattern of HIF-1 after CIH in this study. Compared with the Control group, HIF-1 expression in CIH + 8 h group was significantly increased by western blot (Fig. 7A) and by immunofluorescent staining (Fig. 7C). The results of HIF-1 expression have the same trend of CaMKIIγ expression and cardiomyocyte apoptosis, which indicated that CaMKIIγ may affect the HIF-1 expression in CIH induced cardiomyocyte apoptosis. Furthermore, to understand whether CaMKIIγ regulated HIF-1 signaling pathway, the co-localization of CaMKIIγ and HIF-1 in H9c2 cells were examined by double labeling immunofluorescent staining. Obviously, the co-localization of CaMKIIγ and HIF-1in H9c2 cells were observed (Fig. 7E). These results showed that CaMKIIγ regulated HIF-1 signaling pathway in CIH induced cardiomyocyte apoptosis.

In conclusion, this study demonstrated that increased CaMKIIγ expression advances CIH-induced cardiomyocyte apoptosis via HIF-1 signaling pathway, which afford a new insight and provide a potential therapy for OSA patients.

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Data availability The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval The protocol of this study was approved by the Jiangsu Province Animal Care ethics committee and performed in accordance with the Animal Management Rule of the People's Republic of China and the Care and Use of the Laboratory Animals Guide of the Nantong University.

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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