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ClC-3 chloride channel is involved in isoprenaline-induced cardiac hypertrophy☆

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ABSTRACT

Isoprenaline, an activator of β-adrenergic receptor, has been found to induce cardiac hypertrophy *in vivo* and *in vitro*, but the exact mechanism is still unclear. ClC-3 is a member of the chloride channel family and is highly expressed in mammalian myocardium. In the present study, the role of ClC-3 in isopronaline-induced cardiac hypertrophy was investigated. We found that ClC-3 expression was reduced in isoprenaline-induced hypertrophic H9c2 cells, primary rat neonatal cardiomyocytes and myocardium of C57/BL/6 mice, and this reduction was prevented by the pretreatment of propranolol. Adeno-associated virus 9 (AAV9)-mediated ClC-3 expression in myocardium decreased heart mass index, thinned interventricular septum and left ventricular wall and lowered the mRNA expression of natriuretic peptide type A (ANF) and β-myosin heavy chain (β-MHC). Our results showed that ClC-3 played an important role in β-adrenergic cardiac hypertrophy which could be associated with ANF and β-MHC, and all these findings suggested that ClC-3 may be a novel therapeutic target for the prevention or treatment of myocardiac hypertrophy.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death in the world. Data from WHO showed that an estimated 17.5 million people died from CVDs in 2012. Cardiac hypertrophy, primarily characterized by increased cardiomyocyte size, is considered as a major risk factor that promotes many CVDs, such as hypertension, arrhythmia, dilated cardiomyopathy, myocardial ischemia and myocardial infarction (Accornero et al., 2011; Aggarwal et al., 2014; Ai et al., 2010). Therefore, further understanding of cardiac hypertrophy underlying mechanisms may contribute to improve the prevention or treatments of CVDs.

Although the mechanisms of cardiac hypertrophy are complicated, β-adrenergic receptors are thought as an important part of sympathetic activation which is tightly associated with heart function (Fu et al., 2012). Isoprenaline, an agonist of β-adrenergic receptor, could mimics sustained adrenergic stimulation and develop maladaptive cardiac hypertrophy, accompanied by reactivation of Atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) (Chen et al., 2012; Mansier et al., 1993; Odashiro et al., 2002; Szabo et al., 1975). ANF and β-MHC are fetal cardiac genes, and their expression levels are down-regulated after birth. However, strongly increased expression levels of the cardiac ANF and β-MHC have been detected during hypertrophy and heart failure (Hong et al., 2017). ANF and β-MHC are considered to be marker genes for cardiac hypertrophy (Palazzesi et al., 2006; Saadane et al., 1999).

ClC-3 is a member of the voltage-gated chloride channel superfamily and has many roles in cell proliferation, apoptosis, cell cycle

Abbreviations: AAV9, adeno-associated virus 9; ANF, natriuretic peptide type A; CVDs, cardiovascular diseases; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; BSA, bovine serum albumin; IP, intraperitoneal injections; LV, left ventricle; IVS,d, end-diastolic interventricular septum; IVD,s, end-systolic interventricular septum; LVPW,d, diastolic left ventricular posterior wall; LVPW,s, systolic left ventricular posterior wall; EF, ejection fraction; FS, fractional shortening; H&E, hematoxylin and eosin; S.E.M., standard error of the mean

☆ The authors declare here that they do not have any direct financial relation with the commercial identities mentioned in the paper that might lead to conflicts of interest.

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Table 1
Primer sets used for real-time PCR analysis.

Gene	Organism	Forward primer	Reverse primer	Product's size, bp	Accession number
ClC-3	Rat	AGACATTGCTGCTGACTGGA	CCCTCTCTTCAAACGTCGTC	116	NM_053363.2
ANF	Rat	GGAAGTCAACCCGTCTCA	AGCCCTCAGTTTGCTTTT	97	NM_012612.2
β-MHC	Rat	GGGTATCCGCATCTGTAGGA	CCTTTCCGGCTATCAATGAA	123	NM_017240
GAPDH	Rat	AGGAGTAAGAAACCCTGGAC	CTGGGATGGAATTGTGAG	132	NM_017008.4
ClC-3	Mice	GGTCAGGATGGCTTGTTGTT	ACAATGCACTGAGGCAGATG	120	NM_007711.3
ANF	Mice	TAGGAGACAGTGACGGACAA	GAAGAAGCCCAGGGTGAT	130	NM_008725.3
β-MHC	Mice	CAGCAGTTCTTCAACCACCA	TCTCGATGAGGTCAATGCAG	121	NM_080728
GAPDH	Mice	GGCATGGACTGTGGTCATGA	TTCACCACCATGGAGAAGGC	237	NM_001289726.1

PCR, polymerase chain reaction; ClC-3, chloride channel, voltage-sensitive 3; ANF, natriuretic peptide type A; β-MHC, β-myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

progression and so on (Dai et al., 2005). A growing number of studies showed that ClC-3 encoded a volume-regulated Cl^- channel ($I_{Cl,Vol}$) in heart, that could contribute to the regulation of the cardiomyocyte volume (Nagasaki et al., 2000). Activation of ClC-3-related volume-sensitive outwardly-rectifying anion channels displayed the protection of myocardium from infarction through regulating cardiac volume decrease when cell swells during ischemia and reperfusion (Bozeat et al., 2011; Yu et al., 2016). Recent studies demonstrated that cardiac-specific inducible ClC-3 gene deletion eliminated native $I_{Cl,Vol}$ and promoted myocardial hypertrophy in adult mice (Duan, 2011; Xiong et al., 2010). Isoprenaline was found to consistently reduce $I_{Cl,vol}$ in Ca^{2+}-free hypotonic bath solutions with strong intracellular Ca^{2+} buffering in canine atrial myocytes (Nagasaki et al., 2000). All these results suggested that ClC-3 may play a role in cardiac hypertrophy induced by isoprenaline.

Therefore, our present study intends to study whether ClC-3 is associated with cardiac hypertrophy induced by isoprenaline. Rat cardiomyoblast cell line H9c2 cells, rat primary neonatal cardiomyocyte cells, wild C57/BL/6 mice and mice treated with adeno-associated virus 9 (AAV9) carrying ClC-3 were used as models *in vitro* and *in vivo* respectively. Cell volume, heart mass index, histological analysis and echocardiography were detected to evaluate the cardiac hypertrophy of models. Changes in the expression levels of ANF, β-MHC and ClC-3 in the models in the absence or presence of propanolol, an antagonist β-adrenergic receptor, were assessed by real-time PCR, immunofluorescence techniques, western blot and immunohistochemical staining.

2. Materials and methods

2.1. Cell culture and treatment

Rat cardiomyoblast cell line H9c2 cells were obtained from Sun Yat-Sen University. Primary rat neonatal cardiomyocytes were isolated from the hearts of 1- or 2-day-old Sprague-Dawley rats and cultured as described previously (Kawaguchi-Manabe et al., 2007). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose supplement (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C with a humidified atmosphere of 5% CO_2. After being serum-starved overnight, cells were incubated with vehicle or 10 μmol/L isoprenaline for 48 h. The concentration of isoprenaline was selected according to the literature (Chen et al., 2012).

2.2. Determination of cell volume

Cell volume was calculated by the diameter of the cell. After different treatment as described above, the cells were digested with trypsin solution and then counted with a hematocytometer. The diameter of the cells was measured by Countstar Automated Cell Counter (Shanghai Ruiyu Biotech Co. Ltd., Shanghai, China). For each group, three observation fields were captured, and 10 cells in each field were selected for measurement of their average diameter. Three replicates were used in each experimental group and five separate experiments were performed.

2.3. Real-time PCR analysis

Real-time PCR analysis was used to measure the mRNA expression levels of genes. Firstly, total RNA was extracted from individual samples by TRIzol reagent according to the manufacturer's instructions (TaKaRa Biotechnology Co., Ltd., Dalian, China). Secondly, 1 μg of RNA was reverse transcribed into cDNA using the RevertAid™ First-Strand cDNA synthesis kit (Fermentas, Lithuania). Lastly, real-time PCR reaction was performed with SYBR GreePremix Ex Taq™ (ToYoBo, Japan) according to the manufacturer's instructions. All primers were purchased from Sangon Biotech (Shanghai, China; Table 1). mRNA expression levels of related genes were normalized to an internal standard GAPDH.

2.4. Immunofluorescent staining

Immunofluorescent analysis was used to determine ClC-3 protein expression in vitro. Cells treated with or without isoprenaline were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.1% Triton X-100/PBS for 10 min. After being blocked with 1% bovine serum albumin (BSA) for 60 min at room temperature, cells were incubated with the anti-ClC-3 rabbit polyclonal antibody solution (1:100; Abcam, Cambridge, UK) at 4 °C overnight. Then cells were rinsed three times in PBS and incubated with Dylight™ 488-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoRearch Laboratories, Inc., Pennsylvania, USA) for 60 min and also counterstained with DAPI (0.5 μg/mL) for 5 min. Images were visualized by a fluorescence confocal microscope (Olympus, Osaka, Japan) and were analysed in 3 fields.

2.5. Western blot analysis

Western blot analysis was used to measure the protein levels of ClC-3. Briefly, heart tissues cut into pieces or cells from different groups were washed with PBS and incubated with lysis buffer containing protease inhibitors (Thermo Scientific, USA). Subsequently, protein lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-rad, USA), followed by incubation with rabbit anti-ClC-3 primary antibody (Abcam, UK) and mouse anti-α-tubulin primary (Beyotime Biotech., Nantong, China) overnight at 4 °C. After being blocked with 5% BSA and washed, the membrane was incubated with respective secondary antibody (Beyotime Biotech., Nantong, China). Finally, immunoblots were visualized with an ECL chemiluminescent assay kit (Thermo Scientific, USA). Western blot bands were quantified by NIH Image J software. α-Tubulin was used as an internal control to correct

for differences in quantity and quality between protein samples, and the expression level of ClC-3 was calculated as a ratio to α-tubulin.

2.6. Animals and experimental treatment

Some animals were treatment with isoprenaline alone or in combination with propranolol. Six-week-old male C57/BL/6 mice were purchased from Guangdong Provincial Medical Laboratory Animal Center and maintained in a pathogen-free environment. The animal experimental protocol was conducted under the animal license issued by the Health Department of Government of China and the Animal Subjects Ethics Subcommittee of Guangdong Pharmaceutical University. After a week for acclimation, 27 mice were randomly divided into three groups (each with 9 mice): Control group was treated with intraperitoneal injections (IP) of normal saline, ISO group was treated with isoprenaline (7.5 mg/kg/day, IP), and ISO + Pro group was treated with propranolol (10 mg/kg/day, IP) before 1 h of treatment with isoprenaline. The time schedules and dosages of isoprenaline and propranolol were chosen based on previous results from our laboratory and others (Patrizio et al., 2008). After treatment for 7 days, mice were anesthetized for transthoracic echocardiography and subsequently weighted and euthanized. Heart tissues were collected and weighted, and heart mass index was calculated as a ratio of heart weight to body weight. During the whole experimental period, all mice had free access to food and double-distilled water at controlled room temperature.

Some animals were administrated with adeno-associated virus 9 (AAV9) carrying ClC-3 or control vector followed by isoprenaline. Adeno-associated virus type 9 (AAV9) vector has been reported as a good candidate for intravascular gene delivery. The AAV9 vector carrying mouse CIC-3 cDNA was generated by Hanbio Biotechnology Co. Ltd (Shanghai, China). 5×10^{11} AAV9 virus particles containing ClC-3 (AAV9-ClC-3) or control vector (AAV9) were injected respectively into 9 mice by the tail vein in a total volume of 0.1 mL. For *in vivo* control, 0.1 mL normal saline was injected into 18 age-match C57/BL/6 mice. The mice recovered quickly from the injection without loss of mobility. After 3 weeks, 27 mice (including 9 mice received AAV9-ClC-3 injection, 9 mice received AAV9-vector injection and 9 mice received normal saline injection) were subjected to the treatment with isoprenaline (7.5 mg/kg/day, IP), and 9 mice received normal saline injection were treated with IP normal saline as the normal control. After one week of treatment with isoprenaline or normal saline, mice were anesthetized for echocardiography and subsequently weighted and euthanized. Heart tissues were weighted and collected, and heart mass index was calculated as a ratio of heart weight to body weight.

2.7. Echocardiography assay

Transthoracic echocardiography was performed by using a high resolution ultrasound imaging system with a MS-400 transducer with a frequency of 30 MHz (VEVO2100, Visual Sonics, Toronto, Canada). Briefly, mice were anesthetized with isofluorane inhalation (2.0% isofluorane for induction and 0.5% for maintenance) and the chest hair was shaved. Then the transducer was placed on the left hemithorax, and two dimensionally guided left ventricle (LV) M-mode images at the papillary muscle level were obtained from the parasternal short axis view. End-diastolic interventricular septum (IVS,d), end-systolic interventricular septum (IVD,s), diastolic left ventricular posterior wall (LVPW,d), systolic left ventricular posterior wall (LVPW,s), ejection fraction (EF) and fractional shortening (FS) were measured. All parameters of cardiac function were determined from the mean of three successive cardiac cycles and analyzed by an experienced technician who was blinded for the treatment status.

2.8. Histopathological changes

Histopathological changes were observed by hematoxylin and eosin (H&E) staining. Briefly, heart tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 3 μm thickness. Then the sections were deparaffinized, rehydrated and stained using H&E staining according to standard procedures (Kang et al., 2015). Histological images were taken under × 200 magnification.

2.9. Immunohistochemical staining

Immunohistochemical staining was chosen to determine the protein expression of ClC-3 in myocardium. After being retrieved for antigen, the sections were washed with PBS and blocked with 1% bovine serum albumin (BSA). Then the heart sections were incubated with anti-ClC-3 antibody (1:100) overnight at 4 °C and treated with secondary antibody (rabbit anti-mouse IgG) for 1 h at room temperature. Images were observed with × 100 magnification under a microscope (OLYMPUS, Tokyo, Japan).

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Data were analyzed by one-way analysis of variance followed by multi-range tests using the statistical software, GraphPad Prism 6 Demo for Windows (San Diego, CA). A significant level of $P < 0.05$ was used for all comparisons.

3. Results

3.1. Increase of cell volume and down-regulation of ClC-3 expression in H9c2 cells treated with isoprenaline

H9c2 cells line, initially derived from the ventricular tissue of a BDIX rat heart, was usually used to investigate the cellular and molecular changes that occur during hypertrophic response (Watkins et al., 2011). The treatment with 10 μmol/L isoprenaline for 48 h in H9c2 cells could obviously enlarge cell volume (Fig. 1A), up-regulated the mRNA expression levels of the hypertrophic marker gene β-MHC (Fig. 1B) and down-regulate the mRNA (Fig. 1B) and protein expression levels (Fig. 1C and D) of ClC-3. But there is no significant difference in the mRNA expression of another hypertrophic marker gene ANF between isoprenaline treated group and untreated control group (Fig. 1B).

3.2. Down-regulation of ClC-3 protein in hypertrophic primary neonatal rat cardiomyocytes induced by isoprenaline

The primary neonatal cardiomyocytes, isolated from rat neonatal heart, were widely used as an in vitro model of cardiac hypertrophy (Ohba et al., 2007). After being treated with isoprenaline (10 μmol/L) for 48 h, primary neonatal cardiomyocytes were found with visible increases in cell volume (Fig. 2A), accompanied by up-regulation of ANF and β-MHC mRNA expression level (Fig. 2B) and down-regulation of ClC-3 expression at the mRNA (Fig. 2B) and protein (Fig. 2C and D) levels.

3.3. ClC-3 expression was down-regulated in myocardium of C57/BL/6 mice with cardiac hypertrophy induced by isoprenaline

In C57/BL/6 mice, H&E and immunohistochemical staining were selected to estimate the changes in histopathology and expression levels of ClC-3 in myocardium of mice treated with isoprenaline. As compared with the control mice, an increase in the size of cardiomyocytes (Fig. 3A) and an obvious decrease in the protein expression of ClC-3 (Fig. 3B) in the myocardium were observed in isoprenaline-treated mice under the light microscope level.

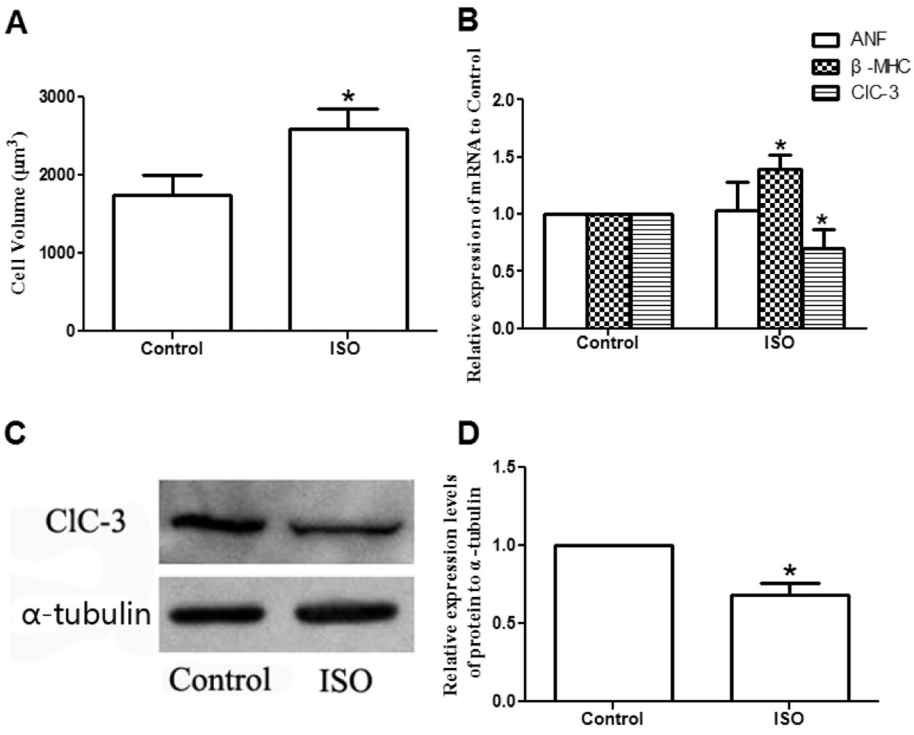

Fig. 1. The expression of ClC-3 were reduced in isoprenaline-induced hypertrophy in H9c2 cells. (A) Cell volume. Data were calculated by the diameter of the cell by Countstar Automated Cell Counter. (B) mRNA expression levels of ANF, β-MHC and ClC-3 by real-time PCR. GAPDH was used as an internal control. Data were expressed as percentage of control. (C) Protein expression levels of ClC-3 by Western-blot. α-Tubulin was used as a loading control. (D) Densitometry of protein level. After treatment with or without isoprenaline (10 μmol/L) for 48 h, cells were harvested for the check of hypertrophic model and lysed for detection of expression levels of ANF, β-MHC and ClC-3. Data were normalized to control. Data are expressed as mean ± SEM (n = 7). $^{*}P < 0.05$ represented significant difference when compared with the control group (treatment without isoprenaline).

3.4. Up-regulation of mice myocardial ClC-3 expression alleviated myocardial hypertrophy induced by isoprenaline

Since the expression of ClC-3 was reduced in isoprenaline-induced myocardial hypertrophy in mice, the effect of AAV9-mediated myocardial ClC-3 on isoprenaline-induced myocardial hypertrophy was observed. As shown in Fig. 4A, the infusion of AAV9-ClC-3 markedly stimulated myocardial ClC-3 expression in mice as compared with that of AAV9-vector (ISO + AAV9-ClC-3 *vs* ISO + AAV). AAV-vector (ISO + AAV) administration to isoprenaline-treated mice gave similar effect as observed in ISO mice, including an increased heart mass index, a thickened interventricular septum and left ventricular posterior wall and elevated ANF and β-MHC mRNA expression (Table 2 and Fig. 4B, C and D). Otherwise, pretreatment of AAV9-ClC-3, compared with that of

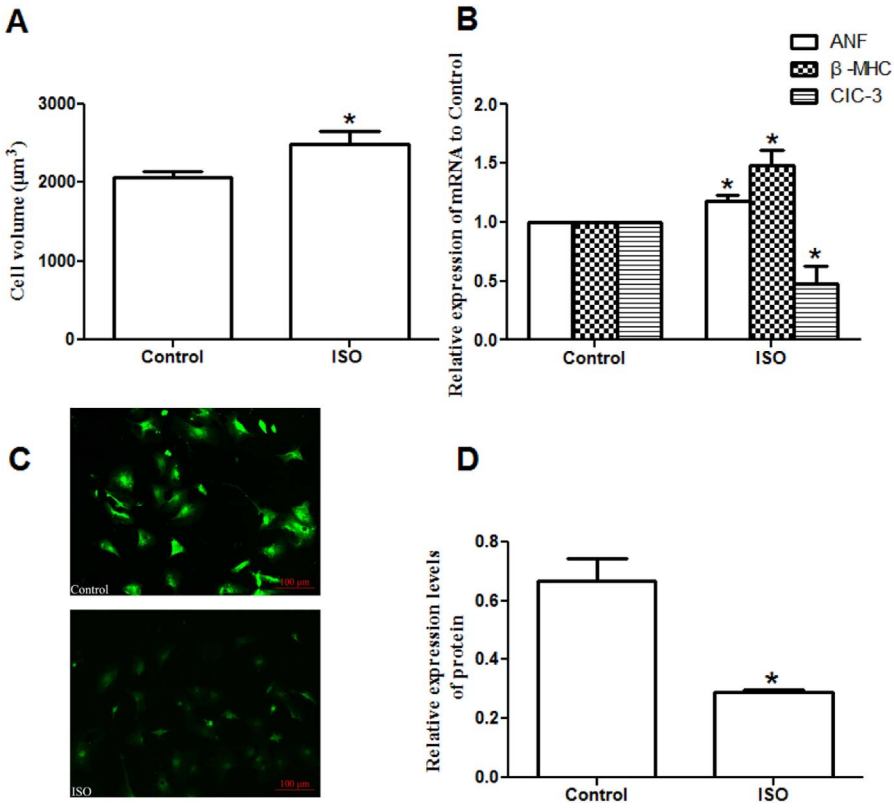

Fig. 2. The expression of ClC-3 was reduced in isoprenaline-induced hypertrophy in primary rat neonatal cardiomyocytes. (A) Cell volume calculated by the diameter of the cell by Countstar Automated Cell Counter. (B) mRNA expression levels of ANF, β-MHC and ClC-3 by real-time PCR. GAPDH was used as an internal control. Data were expressed as percentage of control. (C) Protein expression levels of ClC-3 by immunofluorescence technology. (D) Fluorescence intensity of protein level. After treatment with or without isoprenaline (10 μmol/L) for 48 h, cells were harvested for the check of hypertrophic model and lysed for detection of expression levels of ANF, β-MHC and ClC-3. Data were normalized to control. Data are expressed as mean ± SEM (n = 7). $^{*}P < 0.05$ represented significant difference when compared with the control group (treatment without isoprenaline).

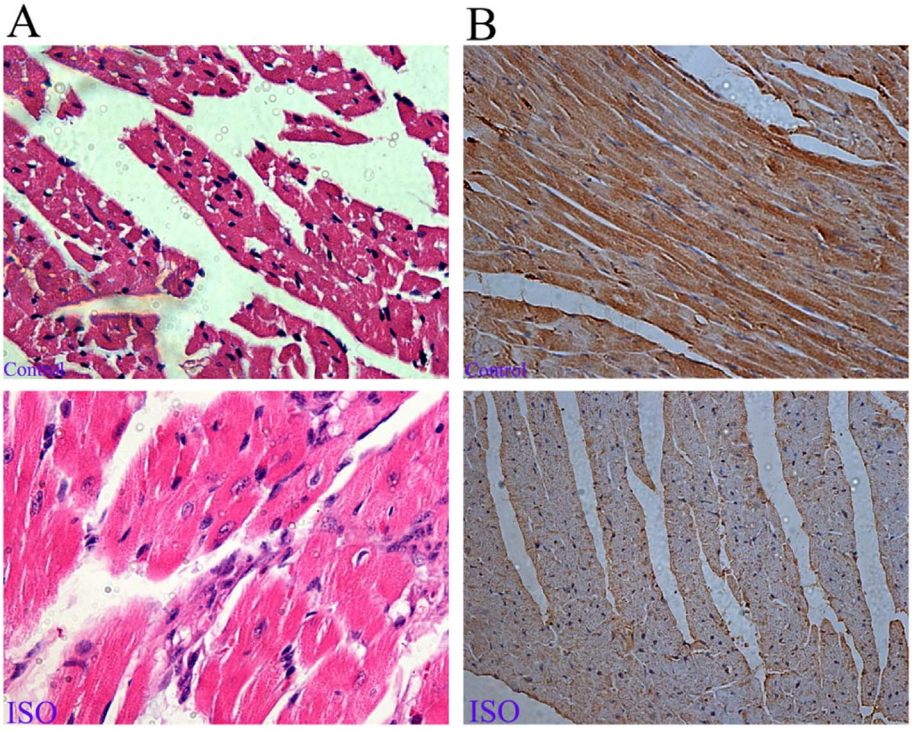

Fig. 3. The expression levels of ClC-3 were reduced in isoprenaline-induced hypertrophy in C57/BL/6 mice (n = 9). (A) Histological changes by H&E staining. (B) Protein expression levels of ClC-3 by immunohistochemical staining. After treatment with or without isoprenaline (7.5 mg/kg/day, IP) for 7 days, mice were sacrificed and myocardial tissues were separated, fixed, embedded, sectioned and stained.

AAV9-vector, could significantly reverse the cardiac hypertrophy and ANF and β-MHC mRNA expression induced by isoprenaline (Table 2 and Fig. 4B, C and D).

3.5. Blockade of β-adrenergic receptor by propranolol prevented down-regulation of myocardial ClC-3 in cardiac hypertrophic mice induced by isoprenaline

As shown in Table 3, Fig 5A and B, a higher the heart mass index (heart weight/body weight, $P < 0.01$), a thicker interventricular septum (IVS,s and IVS,d, both $P < 0.05$) and left ventricular posterior wall (LVPW,d, $P < 0.05$) were observed in ISO mice than those in control mice. As expected, treatment of isoprenaline combined with propranolol almost fully transform these negative effect induced by isoprenaline alone, and displayed no differences compared with normal saline ($P > 0.05$). Apart from those, there were no significant differences in ejection fraction (EF) and fractional shortening (FS) in all these three groups. On the other hand, there was a marked decrease in ClC-3 expression (mRNA expression $P < 0.001$; protein expression, $P < 0.01$, Fig. 5C and D) and an obvious increase in ANF and β-MHC expression (mRNA expression, both $P < 0.001$, Fig. 5B) in ISO mice as compared with those in control mice. Interestingly, co-administration of propranolol in isoprenaline-treated mice could significantly inhibit the reduction of ClC-3 (mRNA expression $P < 0.001$; protein

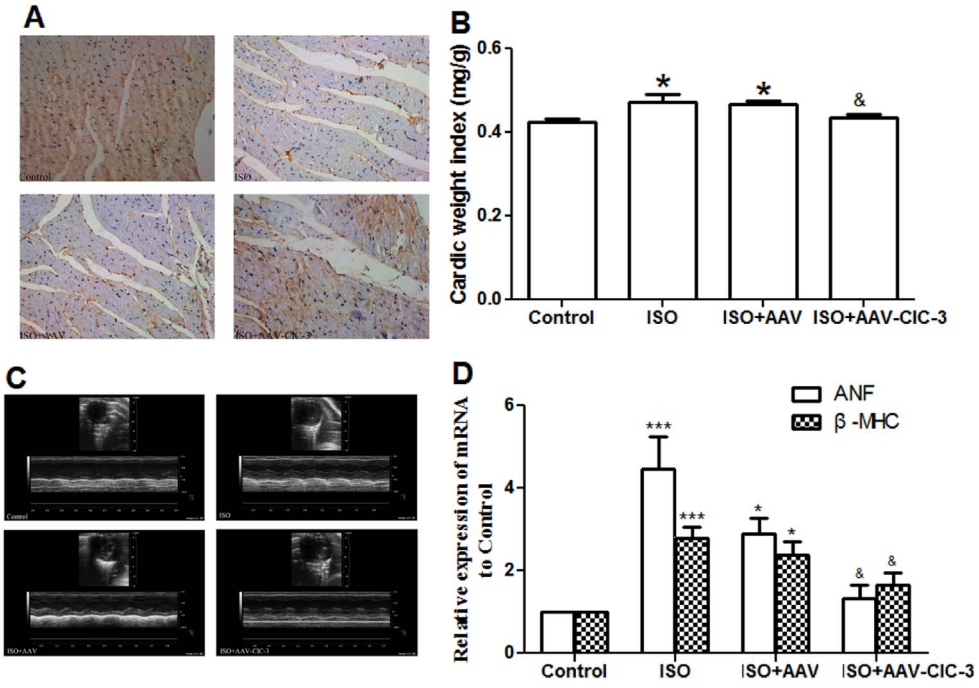

Fig. 4. AAV9-mediated ClC-3 expression improved myocardial hypertrophy induced by isoprenaline in C57/BL/6 mice (n = 9). (A) Protein expression levels of ClC-3 by immunohistochemical staining. (B) Heart mass index (a radio of heart weight to body weight. (C) Echocardiography images by VEVO 2100 from Visual Sonics Inc. (D) mRNA expression levels of ANF and β-MHC by real-time PCR. GAPDH was used as an internal control. Data were expressed as a ratio of control. After 3 weeks of treatment with normal saline, AAV9-vector or AAV9-ClC-3 (5×10^{11}), C57/BL/6 mice were subjected to the administration of isoprenaline (7.5 mg/kg/day, ip) or normal saline for 1 week. Mice were anesthetized for echocardiography. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ represented significant difference when compared with the control group (with treatment with normal saline), and $^{\&}P < 0.05$, represented significant difference when compared with the ISO + AAV group (with treatment with isoprenaline and AAV9-vector).

Table 2
Myocardial hypertrophy induced by isoprenaline were alleviated by AAV9-mediated ClC-3 expression in C57BL/6 mice (n = 9).

	Control	ISO	ISO + AAV	ISO + AAV-ClC-3
IVS,s (mm)	1.11 ± 0.04	1.43 ± 0.05**	1.56 ± 0.08***	1.23 ± 0.05&
IVS,d (mm)	0.7 ± 0.02	1.01 ± 0.02***	1.06 ± 0.04***	0.88 ± 0.06&
LVPW,s (mm)	1.07 ± 0.05	1.33 ± 0.03**	1.36 ± 0.06***	1.18 ± 0.02&
LVPW,d (mm)	0.78 ± 0.03	0.98 ± 0.03**	1.00 ± 0.03**	0.87 ± 0.04&
EF	63.87 ± 3.93	69.85 ± 0.45	67.77 ± 2.88	64.69 ± 2.40
FS	34.19 ± 2.92	38.06 ± 0.46	36.78 ± 2.32	34.66 ± 1.71

IVS,d, end-diastolic interventricular septum; IVD,s, end-systolic interventricular septum; LVPW,d, diastolic left ventricular internal diameter; LVPW,s, systolic left ventricular posterior wall; EF, ejection fraction; FS fractional shortening. After 3 weeks of treatment with normal saline, AAV-vector or AAV-ClC-3 (5×10^{11}), C57/BL/6 mice were subjected to the administration with isoprenaline (7.5 mg/kg/day, ip) or normal saline again as normal control for 1 week. Mice were anesthetized for echocardiography. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ represented significant difference when compared with the control group (with treatment with normal saline), and $^{\&}P < 0.05$ represented significant difference when compared with the ISO + AAV9 group (with treatment with isoprenaline and AAV9-vector).

Table 3
Myocardial hypertrophy induced by isoprenaline were rescued by the treatment with propranolol in C57/BL/6 mice (n = 9).

	Control	ISO	ISO + Pro
Heart weight (HW, mg)	95.22 ± 9.24	105.23 ± 5.99	88.59 ± 7.39###
Body weight (BW, mg)	20.43 ± 1.81	20.32 ± 1.37	18.57 ± 1.34
Heart mass index (mg/g)	4.61 ± 0.25	5.19 ± 0.35**	4.77 ± 0.17#
IVS,s (mm)	1.29 ± 0.13	1.38 ± 0.07*	1.25 ± 0.10#
IVS,d (mm)	0.82 ± 0.09	1.01 ± 0.04*	0.88 ± 0.07#
LVPW,s (mm)	1.25 ± 0.20	1.30 ± 0.09	0.99 ± 0.04#
LVPW, d (mm)	0.86 ± 0.04	0.95 ± 0.08*	0.83 ± 0.06#
EF	60.21 ± 5.48	71.15 ± 1.82	65.96 ± 1.88
FS	31.52 ± 3.61	39.33 ± 1.47	35.20 ± 1.41

IVS,d, end-diastolic interventricular septum; IVD,s, end-systolic interventricular septum; LVPW,d, diastolic left ventricular internal diameter; LVPW,s, systolic left ventricular posterior wall; EF, ejection fraction; FS fractional shortening. After treatment with normal saline or isoprenaline (7.5 mg/kg/day, IP) alone or combination with propranolol (10 mg/kg/day, IP) for 7 days, mice were anesthetized for echocardiography, then sacrificed and collected and weighted heart tissue. Heart mass index was calculated as a radio of heart weight to body weight. Data were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ represented significant difference when compared with the control group (with treatment with normal saline), and $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ represented significant difference when compared with the ISO group (with treatment with isoprenalin).

expression, $P < 0.01$, Fig. 5C and D) and the elevation of ANF and β-MHC (mRNA expression, $P < 0.001$, Fig. 5B).

4. Discussion

Cardiac hypertrophy is characterized by an increased cardiomyocyte size, excess protein synthesis, and thickened ventricular walls. Although myocardiac hypertrophy initially might be adaptive and compensatory, a sustained pathological hypertrophic response is deleterious and eventually develops with heart failure, such as dilated cardiomyopathy and myocardial infarction (Accornero et al., 2011; Aggarwal et al., 2014; Ai et al., 2010). Isoprenaline, a good activator of β-adrenergic receptors in heart, is believed to evoke myocardial hypertrophy in many studies *in vitro* and *in vivo* (Chen et al., 2012; Odashiro et al., 2002; Szabo et al., 1975). In line with these findings, our present study demonstrated that isoprenaline could significantly induce an increase in cell size of H9c2 cells, primary rat neonatal cardiomyocytes and cardiomyocytes of C57/BL/6 mice, an augment of heart mass index and a thickening of interventricular septum and left ventricular posterior wall in mice, associated with a rise in ANF and β-MHC mRNA expression in primary rat neonatal cardiomyocytes and C57/BL/6 mice. Meanwhile, our results showed that isoprenaline down-regulated ClC-3 expression in hypertrophic cardiomyocytes and mice myocardium, and this down-regulation of ClC-3 expression elicited by isoprenaline could be prevented by the pretreatment with propranolol.

ClC-3, a large gene family of chloride channels, was initially found to be expressed in rat brain neuronal cells as a neuronal anion channel (Kawasaki et al., 1994). A large number of studies has documented that ClC-3 is critical for adaptive remodeling in response to physiological and pathophysiological stress correlated with cell volume perturbations in different cell types, such as vascular smooth muscle cells (Ganapathi et al., 2013; Guan et al., 2006), human nonpigmented ciliary epithelial cells (Coca-Prados et al., 1996), human prostate cancer epithelial cells (Lemonnier et al., 2004), nasopharyngeal carcinoma cells (Huang et al., 2014; Mao et al., 2008), Hela cells (Hermoso et al., 2002). However, the functional role of ClC-3 associated with cell volume in cardiomyocytes is currently little known. Nagasaki, M et al. found that chloride currents activated by changes in cell volume (volume-activated chloride currents, $I_{Cl,vol}$) obviously increased in cardiac ClC-3 expression, and thought that ClC-3 might be a ubiquitous volume-regulated chloride channel in myocardium (Nagasaki et al., 2000). Hiramatsu et al. observed that ClC-3 mRNA levels in rat hypertrophic heart induced by banding the abdominal aorta decreased in the early phase after banding, whereas increased in the late phase (Hiramatsu et al., 2002). Xiong et al. found that cardiac-specific inducible ClC-3 knock-out mice exhibited myocardial hypertrophy and heart failure (Xiong et al., 2010). In the present study, we presented several lines of evidence *in vitro* and *in vivo* to support the idea that the mRNA and protein expression levels of ClC-3 were reduced in hypertrophic cardiomyocytes or cardiac hypertrophy mice induced by isoprenaline, and this reduction could be reversed by propranolol-pretreatment. These suggest that the down-regulation of ClC-3 mRNA and protein expression induced by isoprenaline may be involved in cardiac hypertrophy through reducing volume-activated chloride currents. However, volume-activated chloride currents are double regulated by the levels of ClC-3 protein expression and the activity of single ClC-3 chloride channel (Gong et al., 2004; Guo et al., 2016). In addition to reducing the expression of ClC-3 mRNA and protein expression, does isoprenaline also affect the activity of ClC-3 chloride channels? Further study is needed.

Propranolol, initially discovered in 1964, is first and most widely studied as a non-selective beta-blocker. Propranolol through β-adrenergic receptor blockade has reduced heart rate and blood pressure and have anti-arrhythmogenic and anti-ischemic effects, and been proposed as an effective strategy for many CVDs, such as hypertension, cardiac arrhythmias, and angina pectoris (Al-Majed et al., 2017). It is therefore not surprising here to observe that the administration of propranolol resulted in the relatively low level of the heart mass, index interventricular septum and left ventricular posterior wall in mice stimulated by isoprenaline, and rescued cardiac function, which coincides with many previous studies (Marano et al., 2002; Pantos et al., 2000). At the same time, we found for the first time that the treatment with propranolol could prevent the decrease of the expression of ClC-3 in the heart of mice induced by isoprenaline, suggested that ClC-3 maybe play a role in cardiac hypertrophy stimulated by the activation of β-adrenergic receptor. Nevertheless, a propranolol dosage of 10 mg/kg/day for experiments in vivo may not completely block the β-adrenergic

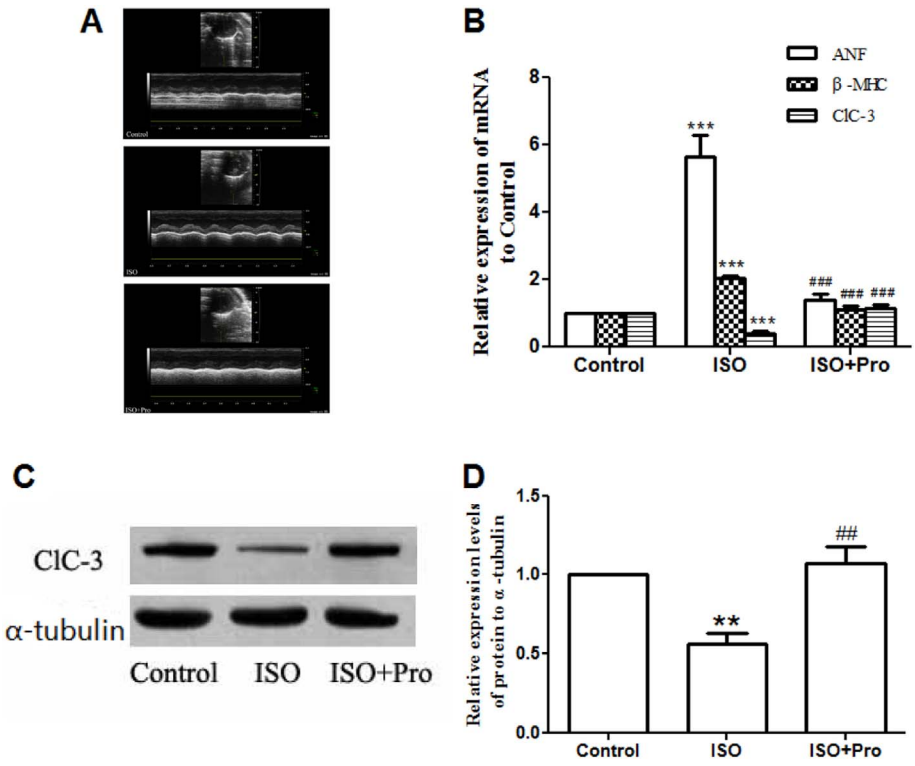

Fig. 5. The reduced expression of ClC-3 induced by isoprenaline were rescued by the treatment with propranolol in C57/BL/6 mice (n = 9). (A) Echocardiography images by VEVO 2100 from Visual Sonics Inc. (B) mRNA expression levels of ANF, β-MHC and ClC-3 by real-time PCR. GAPDH was used as an internal control. Data were expressed as percentage of control. (C) Protein expression levels of ClC-3 by Western-blot. α-Tubulin was used as a loading control. (D) Densitometry of protein level. After treatment with normal saline or isoprenaline (7.5 mg/kg/day, IP) alone or in combination with propranolol (10 mg/kg/day, IP) for 7 days, mice were anesthetized for echocardiography, followed by being sacrificed. Myocardial tissues were collected for further experiment. Data were normalized to control. Data were expressed as mean ± SEM (n = 9). $^{**}P < 0.01$, $^{***}P < 0.001$ represented significant difference when compared with the control group (with treatment with normal saline), and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ represented significant difference when compared with the ISO group (with treatment with isoprenaline).

receptor. Our results show that treatment of isoprenaline combined with propranolol almost fully transform these negative effect induced by isoprenaline alone. Another possible mechanism can interpret this phenomenon. In addition to mainly block the β-adrenergic receptor propranolol may also directly up-regulate C1C-3 expression or activate ClC-3 chloride channel.

To confirm the protective effects of ClC-3 on myocardiac hypertrophy induced by isoprenaline, we pretreated isoprenaline-stimulated mice with adeno-associated virus (AAV9) carrying ClC-3. Adeno-associated virus (AAV) is a small naked, single stranded virus first discovered in 1965(Atchison et al., 1965). Up to now, more than 12 different serotypes and hundreds of capsid variants have been found (Duan, 2016). Due to its safety and the ability to elicit robust and long-term transgene expression in animals and humans, AAV has garnered the growing interest and become the most preferred vector for gene therapy over last two decades (Clement and Grieger, 2016; Duan, 2016). AAV9 mediated gene therapy was thought as a good candidate for cardiomyopathy (Duan, 2016). In our current study, pretreatment of AAV9-mediated ClC-3 expression in myocardium in isoprenaline-induced hypertrophic mice showed minor heart mass indexes, thinner interventricular septums and left ventricular walls, lower the mRNA expression of ANF and β-MHC than that of AAV9-vector control, and displayed the protective effect on myocardiac hypertrophy.

Atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC), as familiar molecular markers of hypertrophy, are produced and released by cardiac myocytes as a compensatory mechanism to positively modulate the cardiac functions against cardiac hypertrophy (Hong et al., 2017). Many studies have demonstrated that ligands of adrenergic receptors such as isoprenaline used in our present investigation could stimulate ANF and β-MHC expression with the increased CVD risk (Palazzesi et al., 2006; Saadane et al., 1999), but it's not well known about the underlying mechanisms. Several studies found that ANF and β-MHC expression levels were influenced by the elevation of cytosolic calcium and the activation of PKC, ERKs, p38 MAP kinase, NF-kappa-B, PI3K/AKT or JNKs (Hong et al., 2017; Sergeeva and Christoffels, 2013; Sun et al., 2016). Li et al. found that miR-145 down-regulated ANF and β-MHC expression and displayed the protection against cardiomyocyte hypertrophy induced by isoprenaline through targeting the expression and localization of GATA6 (Li et al., 2013). In the present study, we found that isoprenaline stimulated ANF and β-MHC mRNA expression accompanied by the reduction of ClC-3 expression in primary rat neonatal cardiomyocytes and C57/BL/6 mice, and that could be inhibited by propranolol in C57/BL/6 mice. Interestingly, pretreatment of AAV9-mediated ClC-3 expression in myocardium could down-regulate ANF and β-MHC mRNA expression and rescue the in cardiac hypertrophy induced by isoprenaline.

In summary, we have shown that the expression of ClC-3 was reduced in hypertrophic H9c2 cells, primary rat neonatal cardiomyocytes and myocardium of C57/BL/6 mice, and this down-regulation of ClC-3 expression induced by isoprenaline could be prevented in propranolol-pretreated mice. Adeno-associated virus 9 (AAV9)-mediated ClC-3 expression in myocardium decreased heart mass index, thinned interventricular septum and left ventricular wall and lowered the mRNA expression of natriuretic peptide type A (ANF) and β-myosin heavy chain (β-MHC). Our results showed that ClC-3 played an important role in β-adrenergic cardiac hypertrophy which could be attributed to its down-regulation of ANF and β-MHC, and all these findings suggested that ClC-3 may be a novel therapeutic target for the prevention or treatment of myocardiac hypertrophy.

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