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# NAKα2 inhibits fibrosis formation and protects against cardiomyocyte hypertrophy by suppressing hypertrophy associated molecules and activating LTCC/NCX signaling pathway

S.-S. SONG, G. TANG, L.-X. TANG, L.-Y. SI, W. XIONG

Geriatric Department of The First Hospital Affiliated To AMU, Chongqing, China

**Abstract. – OBJECTIVE: Cardiomyocyte hypertrophy is considered to be a compensatory process of heart suffering from pathological damages. This study aimed to evaluate effects of $Na^+/K^+$ APTaseα2 (NAKα2) on isoprenaline (ISO) induced cardiomyocyte hypertrophy.**

**MATERIALS AND METHODS: Mouse atrial cardiomyocytes were cultured and treated with ISO to establish cardiomyocyte hypertrophy model. NAKα2 over-expression and small interfere RNA (siRNA) plasmids were constructed and transfected to cardiomyocytes. Influx $Ca^{2+}$ ($[Ca^{2+}]$ i) was measured using flow cytometry method. Fibrosis formation was examined with Masson staining. Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining was used to examine apoptosis. Major histocompatibility complex β (β-MHC), atrial natriuretic peptides (ANP), B-type natriuretic peptides (BNP) were evaluated with quantitative Real-time PCR (qRT-PCR). Western blot was used to detect β-MHC, ANP, BNP, $Na^+/Ca^{2+}$ exchanger (NCX) and L-type calcium channel (LTCC).**

**RESULTS: NAKα2 significantly inhibited NCX and LTCC expression compared to that in ISO-treated cardiomyocytes ($p<0.05$). NAKα2 significantly suppressed expression of β-MHC, ANP and BNP compared to that in ISO-treated cardiomyocytes ($p<0.05$). NAKα2 significantly alleviated fibrosis formation and inhibited apoptosis compared to that in ISO-treated cardiomyocytes ($p<0.05$). NAKα2 reduced intracellular calcineurin and activated phosphorylation of calcineurin-nuclear factor of activated T cells (NFAT) compared to ISO-treated cardiomyocytes ($p<0.05$). NAKα2 significantly strengthened effects of Klotho on ISO-induced up-regulation of hypertrophy associated molecules ($p<0.05$) by activating LTCC and NCX. Comparing to ISO-treated cardiomyocytes, NAKα2 combining Klotho treatment exhibited significantly better improvement of $Ca^{2+}$ influx, alleviation of fibrosis and reduction of apoptosis by triggering LTCC/NCX signaling pathway.**

**CONCLUSIONS: Over-expression of NKAα2 suppressed fibrosis formation and protected against cardiomyocyte hypertrophy by inhibiting hypertrophy associated molecules, alleviating apoptosis and activating LTCC/NCX signaling pathway.**

*Key Words*

Cardiomyocyte hypertrophy, NKAÐ2, Fibrosis, NCX, LTCC.

## Introduction

Cardiomyocyte hypertrophy is considered to be a compensatory process of heart that keeps the cardiac output when the cardiomyocytes suffering from the pathological damages, such as the hypertension[1,2]. In clinical, the continuous cardiomyocyte hypertrophy always causes the decompensation and leads to heat failure, and even sudden death eventually[3]. The cardiomyocyte hypertrophy mainly characterizes by the enlargement of the cells involving the pathological hypertrophy and physiological hypertrophy[4]. The pathological cardiomyocyte hypertrophy is always associated with the peri-vascular fibrosis and interstitial fibrosis, and induces the production of brain/B-type natriuretic peptides (BNP) and the atrial natriuretic peptides (ANP)[5]. The initial stage of cardiomyocyte hypertrophy is the adaptive phase that characterizes by the concentric hypertrophy resisting the high afterload[6,7]. Then, following with the cardiac damage, the cell length is increased, which causes the aggravating hypertrophy[8]. Meanwhile, the calcineurin-nuclear factor of activated T cells (NFAT) is well known as a critical mediator for plenty of transduction signaling pathways, which participate in the coordination of the stimulation of pathology[9].

Till now, majority of the anti-hypertrophic drugs mainly target the outside-in signaling path-

*Corresponding Author:* Wei Xiong, MD; e-mail: weijjzh@sina.com

way in the cardiomyocytes, however, the effects exhibit some limitations and with a few side-effects[10]. Therefore, the promising novel therapeutic strategies would be much beneficial if which could target myocardium directly. Discover of the novel mediators that primarily response to the cardiomyocyte hypertrophy is important for developing the effective drugs.

$Na^+/K^+$ APTase (NKA) is a critical $Na^+$ extrusion pathway for consuming the ATP to pump 3 $Na^+$ ions out and exchange 2 $K^+$ ions in, which triggers the $Na^+$ into the cardiomyocytes[11]. The NAK, composed of α1, α2 and β1 subunit in the heart, is coupled functionally to the $Na^+/Ca^{2+}$ exchanger (NCX) in the cardiomyocytes[12]. Especially for the α2 subunit of NAK (NAKα2), which coupled directly with the NCX in the t-tubes and surface sarcolemma[13]. Despa et al[14] reported that the inhibition of NAKα2 increases the transients of $Ca^{2+}$, which suggests that NAKα2 is response to regulate the NCX to modulate the intracellular $Ca^{2+}$ in the cardiomyocytes. Musumeci et al[15] reported that L-type calcium channel (LTCC) plays a critical role in the iron uptake into heart by an iron-overloaded pattern, which is an important cause for the fatal cardiac complications. The inhibition of LTCC could decrease the influx of L type $Ca^{2+}$ (IL-$Ca^{2+}$) and causes the compensatory down-regulation of NCX. Therefore, the IL-$Ca^{2+}$ is also critical for the transients of $Ca^{2+}$ in the cardiomyocytes, which might be associated with the function of NAKα2[16,17]. Therefore, in the present study, we evaluated the effects of $Na^+$ extrusion biomarker, NAKα2, on isoprenaline (ISO) induced cardiomyocyte hypertrophy, and clarified the specific signaling pathways involving in the protective functions.

## Materials and Methods

### Cell Culture

The mouse atrial cardiomyocytes were purchased from ATCC Cell Bank (Manassas, VA, USA). The cardiomyocytes were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, NJ, USA) containing 10% fetal bovine serum (FBS, Gibco BRL. Co. Ltd.) and supplemented with 100 μg/mL streptomycin (Beyotime Biotech., Shanghai, China), 100 U/ml penicillin (Beyotime Biotech.), 0.1 mM norepinephrine (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine (Invitrogen/Life Technologies, Carlsbad, CA, USA), at 37°C in 5% $CO_2$ conditions. When the cardiomyocytes were generated for 10-12 passages, the experiments were conducted. The present study was approved by the Ethics Committee of The First Hospital Affiliated to AMU (Chongqing, China).

### Generation of NAKα2 Over-Expression and Silencing Genes

The NAKα2 complementary DNA (cDNA) was cloned by the PCR from the mouse heart using the sense primer 5'-CATCATGGATTCTGGCCACCATGGGT CGTGGGCAGGGCGTGA-3' and the anti-sense primer 5'-CATCATGAATTCTCAG TAGTACGTCTCCTTCTCCACCCA-3' (the underlines represented the restriction sites). The above fragments were digested by using the *EcoR I* and *BamH I* restriction enzyme (TaKaRa Bio., Dalian, China) and sub-cloned into the pcDNA3.1 (+) plasmid (Cat. No. V79020, Invitrogen/Thermo Scientific Pierce, Carlsbad, CA, USA). The pcDNA3.1 plasmid carrying NAKα2 gene was transfected into cardiomyocytes by using the Lipofectamine 2000 Transfection Reagent (Cat. No. 11668019, Invitrogen/Thermo Scientific Pierce, Carlsbad, CA, USA) according to manufacturer's instruction. A small interfering RNA (siRNA) targeting NAKα2 was targeted to coding sequence 5'-CGGAGAACATCTCCGTGTCATTCAAGAGATGACACGGAGATG TTCTCTTTTTTCCAA-3'. The cardiomyocytes were transfected with NAKα2 siRNA by using the Lipofectamine 2000 Transfection Reagent (Cat. No. 11668019, Invitrogen/Thermo Scientific Pierce, Carlsbad, CA, USA).

### Measurement for Influx $Ca^{2+}$ ($[Ca^{2+}]i$)

The $[Ca^{2+}]i$ was evaluated by using the flow cytometry method according to the previous study[18]. In brief, the 4-hydroxyethyl piperazine ethylsulfonic acid (HEPES)-buffered balanced salt solutions were applied for $[Ca^{2+}]i$ evaluation. The $[Ca^{2+}]i$ was evaluated by using Fluo-3 AM (Beyotime Biotech. Shanghai, China). The fluorescence in the cardiomyocytes was excited by using the argon laser at 488 nm wavelength and examined at 525 nm with the FACS flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). A total of $2\times10^3$ cardiomyocytes were analyzed to evaluate the mean intensity of the emission fluorescence in every sample. The $[Ca^{2+}]i$ value was calculated by the $Mn^{2+}$ quenching according to the previously published study[19].

### Masson Staining

The cardiomyocytes were cultured onto the slides and fixed with 4% paraformaldehyde (Beyotime Biotech. Shanghai, China). The cells were then stained by using Masson staining (Cat. No. DC0032-50, Beijing Leagene Biotech. Co. Ltd., Beijing, China) method according to the previously published study[20]. The stained images were captured with a digital camera system (Olympus, Tokyo, Japan) installed on an Olympus divert microscope (Mode: IX53, Olympus, Tokyo, Japan). The epicardial and perivascular fibrosis areas were excluded by setting interesting regions. The Labworks™ Analysis Software (version: 4.0, Labworks, Upland, CA, USA) was employed to determine the fractions of atrial tissues made up of fibrosis.

### Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL) Staining

The cardiomyocytes were fixed with 4% paraformaldehyde (Beyotime Biotech. Shanghai, China) for 20 min, treated with 3% $H_2O_2$ (Beyotime Biotech.) for 10 min and incubated with 0.1% Triton X-100 (Beyotime Biotech.) for 2 min. Next, the cardiomyocytes were stained by using TUNEL apoptosis detection fluorescent kit (Cat. No. C1091, Beyotime Biotech. Shanghai, China) according to the instruction of manufacturer. Finally, the TUNEL-stained positive cardiomyocytes were imaged and quantified with a microscopy (Mode: BX51, Olympus, Tokyo, Japan) by selecting at least 6 fields in each image.

### Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNAs in cardiomyocytes were extracted with TRIzol, chloroform, isopropanol and absolute ethyl alcohol regents (Beyotime Biotech. Shanghai, China) according to the previous study described[21]. Complementary DNAs (cDNAs) were produced by using Reverse Transcription (RT) Regent (Western Biotech., Chongqing, China). The mRNAs of major histocompatibility complex β (β-MHC), ANP, BNP and β-actin were amplified with Sybr Green I PCR system (Western Biotech.). Primers for the qRT-PCR assay were synthesized by Western Biotech. (Chongqing, China) and listed in Table I. Conditions of qRT-PCR were listed as pre-denaturation at 94°C for 4 min, following with 35 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 30 s, and finally terminated at 72°C for 10 min. Eventually, relative gene expressions were analyzed with a GDS8000 gel scanning system (UVP, Sacramento, CA, USA) and calculated by using $2^{-\Delta\Delta ct}$ method[22].

### Western Blot Assay

The cardiomyocytes were lyzed with Cell Lysis Buffer (Cat. No. P0013, Beyotime Biotech. Shanghai, China) and centrifuged at 12000 r/min at 4°C for 5 min. Concentrations of proteins in samples were evaluated by using bincinchonic acid (BCA) Protein Assay Kit (Cat. No. P0010, Beyotime Biotech.) according to instruction of manufacturer. Then, proteins were separated using the 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Sangon Biotech. Co. Ltd., Shanghai, China) and transferred onto the polyvinylidene difluoride membrane (PVDF, Cat. No. 1620177, Bio-Rad Laboratories, Hercules, CA, USA) by using Trans-Blot SD Semi-Dry Electrophoretic Transfer (Mode: 170-3940, Bio-Rad Laboratories). The rabbit anti-mouse β-MHC polyclonal antibody (1: 2000; Cat. No. ab180779), rabbit anti-mouse ANP polyclonal antibody (1:2000, Cat. No. ab180649), rabbit anti-mouse BNP (1:3000, Cat. No. ab236101), rabbit anti-mouse NCX polyclonal antibody (1:2000, Cat. No. ab151608), rabbit anti-mouse LTCC polyclonal antibody (1:2000., Cat. No. ab154161) and rabbit anti-mouse GAPDH monoclonal antibody (1: 3000; Cat. No. ab181602) were employed to incubate polyvinylidene difluoride (PVDF) membranes at 4°C overnight. All of the above antibodies were purchased from Abcam Biotech. (Cambridge, MA, USA). PVDF membranes were then incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1: 1000, Cat. No. ab6721, Abcam Biotech) for 2 h at room temperature. Finally, PVDF membranes were treated with Pierce ECL Western Blotting Substrate (Cat. No. 32106, Thermo Scientific Pierce, Carlsbad, CA, USA) for 2 min at 37°C in dark. The Western blotting bands were captured and analyzed by using Labworks™ Analysis Software (version: 4.0, Labworks, Upland, CA, USA).

### Statistical Analysis

Data were assigned as mean ± standard deviation (SD) and analyzed using SPSS software 20.0 (SPSS Inc., Armonk, NY, USA). Student's *t*-test was used to compare differences between two groups. Tukey's post-hoc test validated analysis of variance (ANOVA) was used to compare differences among multiple-groups. The experiments or tests were performed for 6 repeats at least. The $p<0.05$ was represented as significant difference.

## Results

### *NAKα2 Interfered Expression of $Ca^{2+}$ Channel Associated Molecules in ISO Treated Cardiomyocytes*

At 48 h post the transfection of pcDNA3.1-NAKα2 and siNAKα2, the expression of NAKα2 was examined in cardiomyocytes. The results indicated that NAKα2 expression in NAKα2 group was significantly higher, in siNAKα2 was significantly lower, compared to that in NC group or pcDNA3.1 group (Figure 1A, $p<0.05$). The Western blot assay also indicated that the $Ca^{2+}$ channel associated molecule, NCX, was significantly increased and LTCC was significantly decreased in ISO treated cells compared to that in cells of NC group (Figure 1B, C, $p<0.05$). The results also showed that the expression of NCX in NC+ISO group was significantly inhibited compared to that in NAKα2+ISO group, and expression of NCX in NC+ISO was significantly activated compared to that in siNAKα2+ISO group (Figure 1B, C, $p<0.05$). Meanwhile, the expression of LTCC in NAKα2+ISO group was significantly activated compared to that in NC+ISO group, and expression of LTCC in siNAKα2+ISO group was significantly suppressed compared to that in NC+ISO group (Figure 1B, D, $p<0.05$).

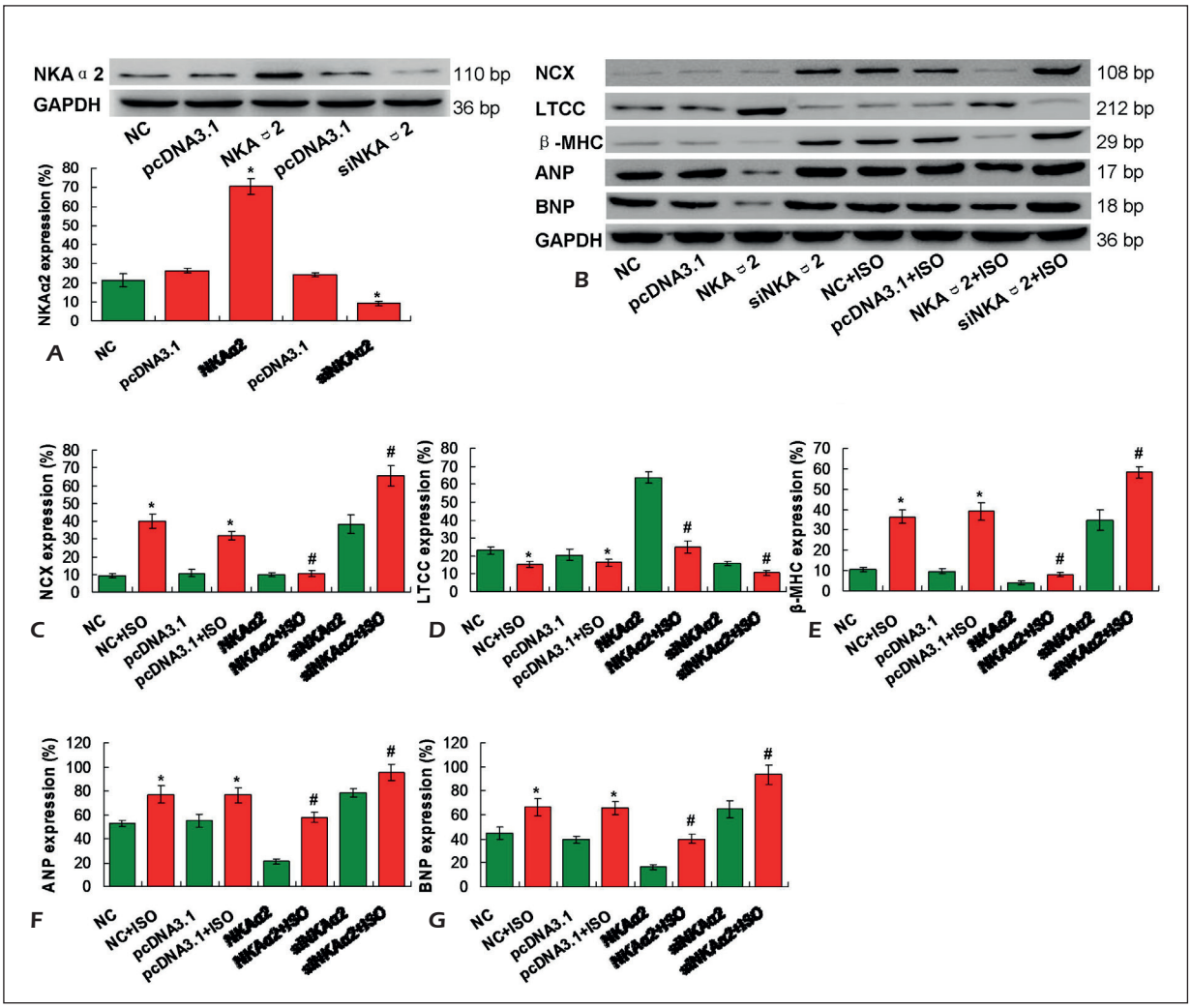


**Figure 1.** Evaluation for the effects of NKAα2 on hypertrophy associated and $Ca^{2+}$ channel associated molecules by using western blot assay. **A**, Western blot assay and statistical analysis for NKAα2 expression. **B**, Western blot assay for hypertrophy associated and $Ca^{2+}$ channel associated molecules. **C**, Statistical analysis for NCX expression. **D**, Statistical analysis for LTCC expression. **E**, Analysis for β-MHC expression. **F**, Statistical analysis for ANP expression. **G**, Statistical analysis for BNP expression. *$p<0.05$ vs. NC group. #$p<0.05$ *vs.* NC+ISO group.

### NAKα2 Inhibited Expression of Hypertrophy Associated Molecules in ISO Treated Cardiomyocytes

According to the Western blot results, the expressions of cardiomyocyte hypertrophy associated molecules, including β-MHC (Figure 1E), ANP (Figure 1F) and BNP (Figure 1G), in NC+ISO were significantly increased compared that in NC group ($p$<0.05). Moreover, the expression of -MHC (Figure 1E), ANP (Figure 1F) and BNP (Figure 1G) in NAKα2+ISO group was significantly lower compared to that in the NC+ISO group ($p$<0.05).

### NAKα2 Reduced $Ca^{2+}$ Influx of ISO Treated Cardiomyocytes

In order to evaluate the effects of NAKα2 on the $Ca^{2+}$ influx of ISO treated cardiomyocytes, the flow cytometry assay was conducted. The results indicated that comparing with the NC group, ISO treatment significantly increased the $Ca^{2+}$ influx ($p$<0.05) and NAKα2 treatment significantly decreased $Ca^{2+}$ influx (Figure 2A, $p$<0.05). Also, NAKα2 treatment (NAKα2+ISO group) significantly reduced the $Ca^{2+}$ influx compared to the NC+ISO group (Figure 2A, $p$<0.05). However, the silencing of the NAKα2 (siNAKα2+ISO group) significantly increased the $Ca^{2+}$ influx compared to the NC+ISO group (Figure 2A, $p$<0.05).

### NAKα2 Alleviated Fibrosis Formation and Inhibited Apoptosis in ISO Treated Cardiomyocytes

The results showed that the ISO treatment induced obvious fibrosis compared to no ISO treatment group (Figure 2B). Meanwhile, the NAKα2 treatment significantly alleviated the formation of fibrosis compared to that of NC+ISO group (Figure 2B). Furthermore, the TUNEL

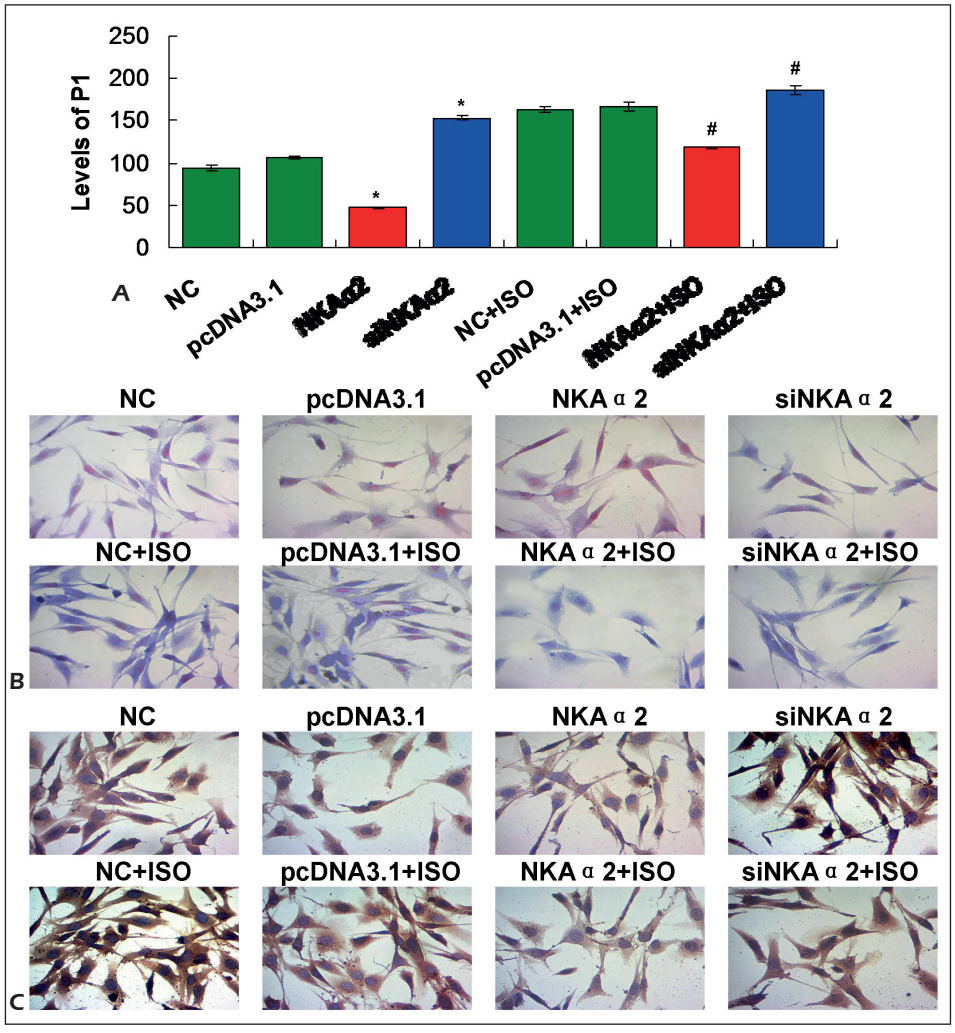


**Figure 2.** Determination for the $Ca^{2+}$ influx, fibrosis formation and apoptosis in ISO-treated cardiomyocytes. **A**, Evaluation for $Ca^{2+}$ influx by using flow cytometry assay. **B**, Fibrosis examination using Masson staining. **C**, Apoptosis evaluation by using TUNEL assay. *$p$<0.05 vs. NC group. #$p$<0.05 *vs.* NC+ISO group.

results indicated that ISO induced the significantly apoptosis compared to no ISO treatment group (NC group, Figure 2C). However, the NAKα2 treatment remarkably reduced the apoptosis compared to the NC+ISO group (Figure 2C). Meanwhile, the silencing of NAKα2 significantly enhanced the apoptosis compared to that in the NC group (Figure 2C).

### NAKα2 Reduced Intracellular Calcineurin Levels and Activated Phosphorylation of NFAT

To verify the calcineurin accumulation and the associated mechanism, the intracellular calcineurin levels and phosphorylation status of NFAT were evaluated by using Western blot assay (Figure 3A). The results demonstrated that ISO induced significantly higher levels of calcineurin (Figure 3B), significantly lower expression of NFAT (Figure 3C) and significantly higher expression of p-NFAT (Figure 3D), compared to that in NC group ($p<0.05$). Moreover, NAKα2 treatment significantly decreased the calcineurin levels (Figure 3B), significantly down-regulated NFAT expression (Figure 3C) and significantly up-regulated p-NFAT expression (Figure 3D), compared to that in the NC+ISO group ($p<0.05$).

### NAKα2 Strengthened Effects of Klotho on ISO-Induced Up-Regulation of Hypertrophy Associated Molecules

Klotho is a key molecule for improving the cell status by suppressing hypertrophy associated molecules expression[23], therefore, we evaluated effects of NAKα2 combining Klotho on ISO-induced up-regulation of BNP, ANP and β-MHC by using Western blot assay (Figure 4A). The results illustrated that NAKα2 combining Klotho (NAKα2+ISO+Klotho group) significantly inhibited BNP (Figure 4B), ANP (Figure 4C) and β-MHC (Figure 4D) expression compared to that in the NC+ISO+Klotho group ($p<0.05$). However, the siNAKα2 treatment significantly weakened the down-regulatory effects of Klotho on the ISO-treated cardiomyocytes compared to the

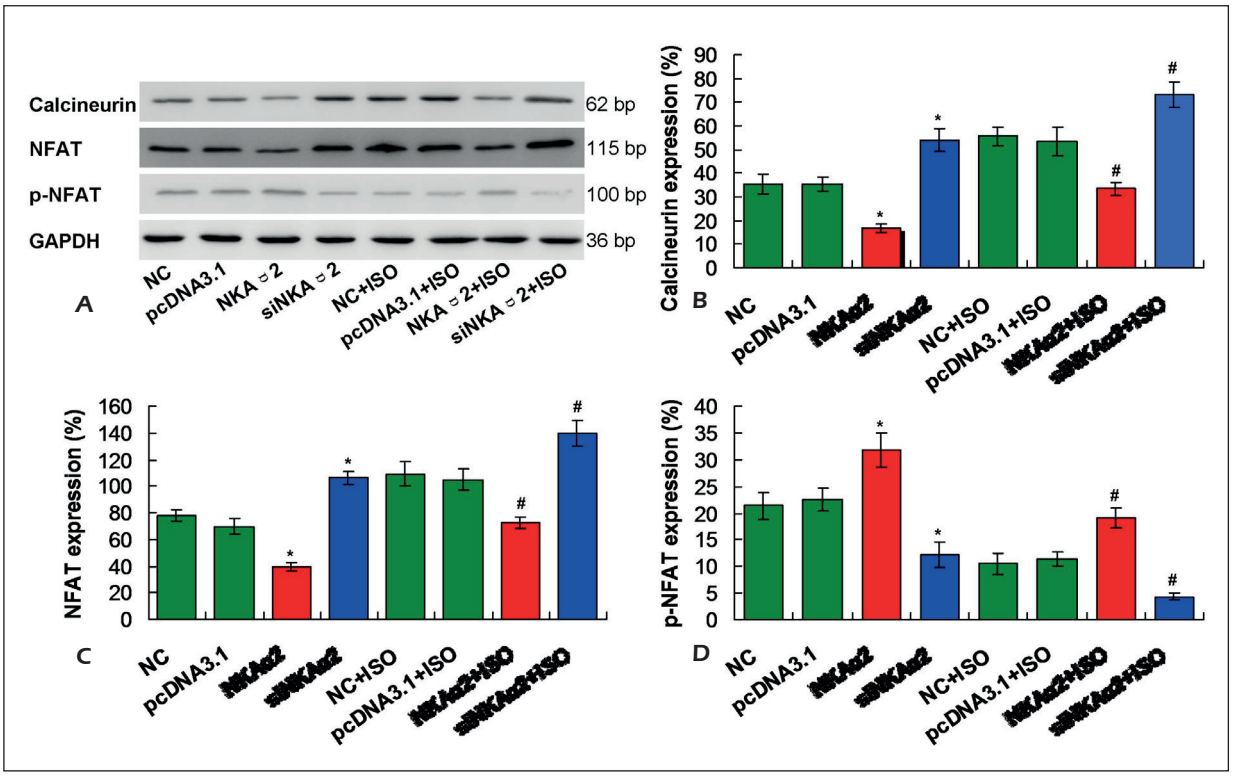


**Figure 3.** Examination for effects of NKAα2 on calcineurin, NFAT and p-NFAT by using Western blot assay. **A**, Western blot assay images. **B**, Statistical analysis for calcineurin expression. **C**, Statistical analysis for NFAT expression. **D**, Statistical analysis for p-NFAT expression. *$p<0.05$ vs. NC group. #$p<0.05$ *vs.* NC+ISO group.

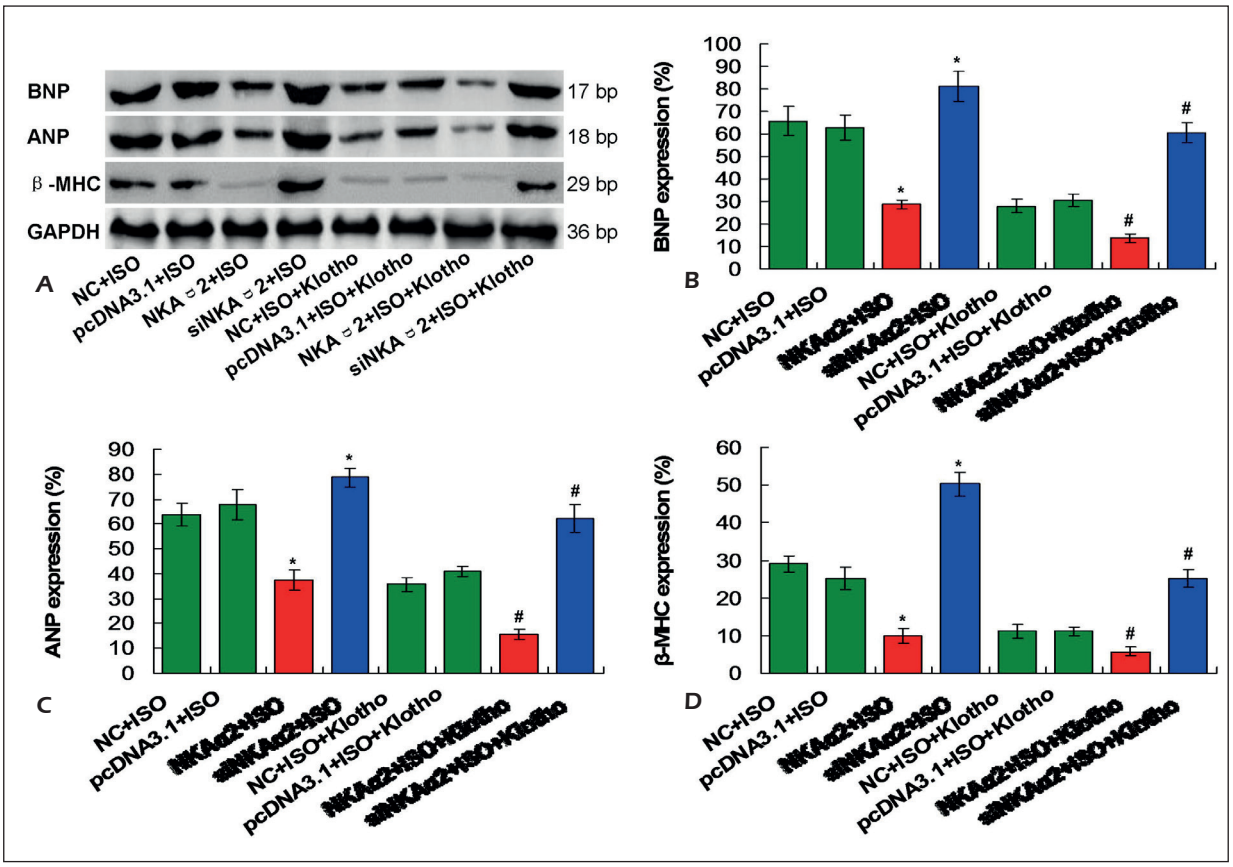


**Figure 4.** Evaluation for levels of hypertrophy associated molecules of in cardiomyocytes undergoing Klotho treatment by using Western blot assay. A. Western blots assay for the hypertrophy associated molecules. **B**, Statistical analysis for BNP expression. **C**, Statistical analysis for ANP expression. **D**, Statistical analysis for β-MHC expression. *$p$<0.05 *vs.* NC+ISO group. #$p$<0.05 *vs.* NC+ISO+Klotho group.

NC+ISO+Klotho group (Figure 4B, C, D, $p$<0.05), including increased BNP, ANP and β-MHC expression.

### NAKα2 Combining Klotho Treatment Exhibited Better Improvement of $Ca^{2+}$ Influx, Alleviation of Fibrosis and Reduction of Apoptosis in ISO Treated Cardiomyocytes

The results showed that NAKα2 combining with Klotho (NAKα2+ISO+Klotho group) significantly decreased $Ca^{2+}$ influx (Figure 5A), remarkably reduced fibrosis formation (Figure 5B) and significantly decreased the apoptosis (Figure 5C), compared to that in the single Klotho treatment group (NC+ISO+Klotho group $p$<0.05). However, the silencing of NAKα2 (siNAKα2+ISO+Klotho group) played the opposite functions on the $Ca^{2+}$ influx, fibrosis formation and apoptosis, compared to the single Klotho treatment group (NC+ISO+Klotho group, Figure 5, $p$<0.05).

### NAKα2 Regulated Hypertrophy Associated Molecules Through Activating LTCC and NCX Pathway

In order to clarify the mechanism for the hypertrophy NAKα2 (or NAKα2 combining with Klotho) triggered decreased $Ca^{2+}$ influx, reduced fibrosis formation and apoptosis, the LTCC inhibitor[24] and NCX reverse mode inhibitor[25] were used to interfere the expression of hypertrophy associated molecules. The data exhibited that NAKα2 administration (NAKα2+ISO+Klotho+LTCC-Inhibitor group) significantly inhibited the enhancement of BNP (Figure 6A), ANP (Figure 6B) and β-MHC (Figure 6C) expression caused by the LTCC inhibitor treatment, compared to that in NC+ISO+Klotho+LTCC-Inhibitor group ($p$<0.05). However, silencing of NAKα2 (siNAKα2+ISO+Klotho+LTCC-Inhibitor group) played the opposite functions on BNP (Figure 6A), ANP (Figure 6B) and β-MHC (Figure 6C) expression, compared to NC+ISO+Klotho+LTCC-Inhibitor group ($p$<0.05).

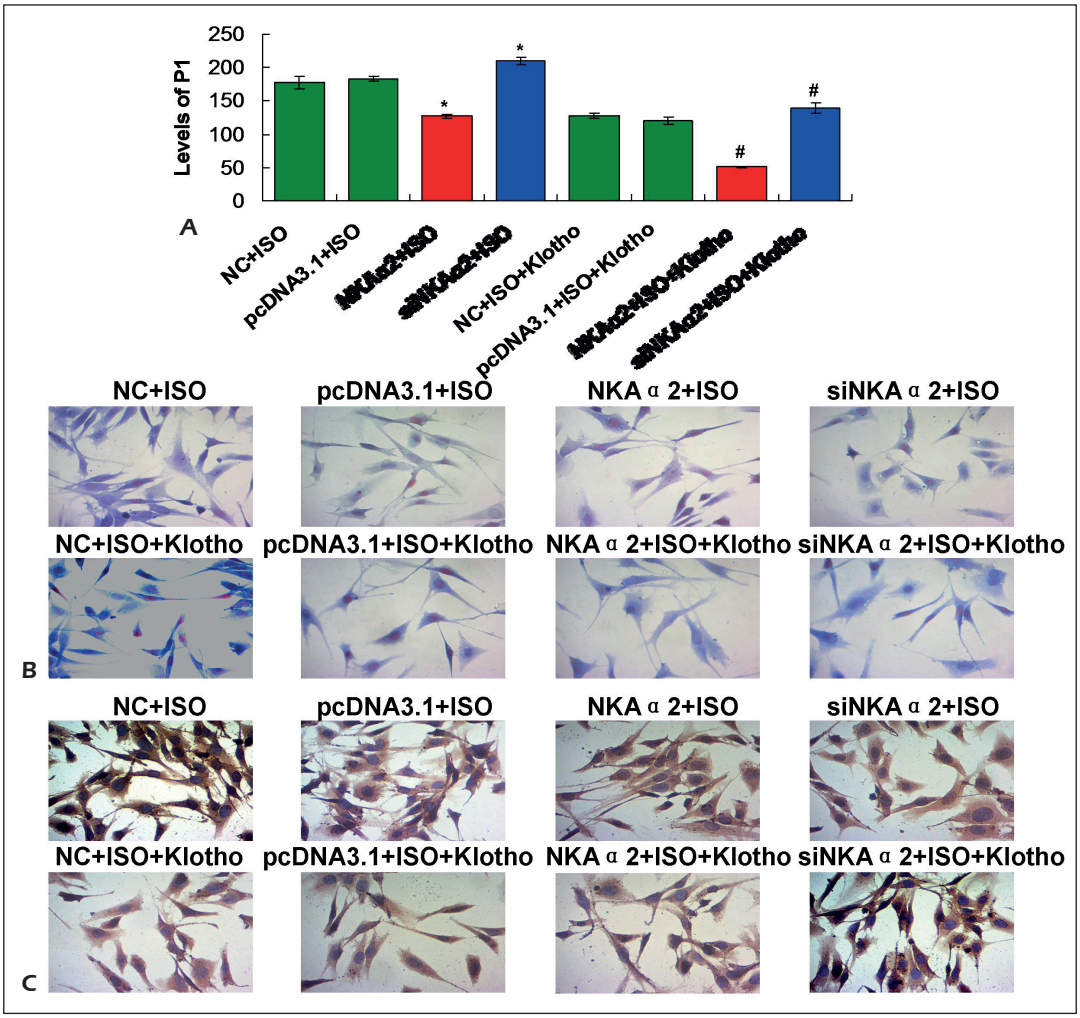


**Figure 5.** Determination for $Ca^{2+}$ influx, fibrosis formation and apoptosis in ISO and Klotho treatment cardiomyocytes. **A**, Statistical analysis for $Ca^{2+}$ influx by using flow cytometry assay. **B**, Fibrosis examination using Masson staining. **C**, Apoptosis evaluation by using TUNEL assay. *$p$<0.05 *vs.* NC group. #$p$<0.05 *vs.* NC+ISO group.

Moreover, NAKα2 administration (NAKα2+ISO+Klotho+NCX-Inhibitor) and siNAKα2 administration (siNAKα2+ISO+Klotho+NCX-Inhibitor) also illustrated the equal effects on the BNP (Figure 6A), ANP (Figure 6B) and β-MHC (Figure 6C) expression.

### *NAKα2 reduced $Ca^{2+}$ Influx, Alleviated Fibrosis and Inhibited Apoptosis Through Activating LTCC/NCX Signaling Pathway*

The results indicated that NAKα2 administration (NAKα2+ISO+ Klotho+LTCC-Inhibitor) significantly reduced $Ca^{2+}$ influx (Figure 7), alleviated the fibrosis (Figure 8A) and inhibited apoptosis (Figure 8B) compared to that in both NC+ISO+Klotho+LTCC-Inhibitor and NAKα2+ISO+Klotho+ NCX-Inhibitor group ($p$<0.05). Furthermore, silencing of NAKα2 (siNAKα2+ISO+Klotho+LTCC- or +NEX-Inhibitor group) acted the opposite roles in $Ca^{2+}$ influx, fibrosis formation and apoptosis, comparing to NC+ISO+Klotho+LTCC- or +NEX-Inhibitor group (Figure 8, $p$<0.05).

## Discussion

Cardiomyocyte hypertrophy is a critical risk factor that causes the cardiovascular mortality and morbidity in clinical[26]. Although the cardiomyocyte hypertrophy is only a compensatory disorder for the mechanical loading initially, the sustainable hypertrophy finally induces to the heart failure or

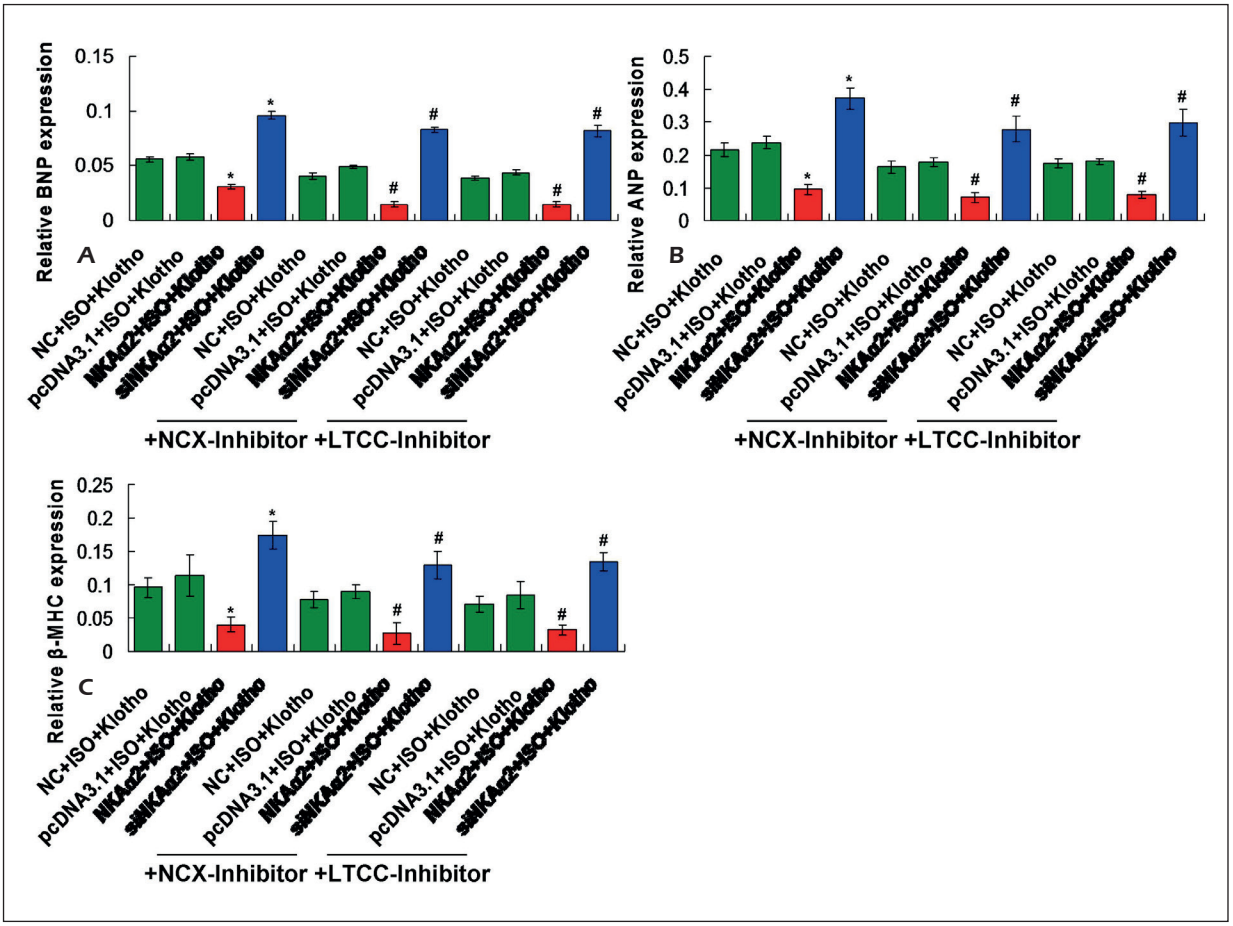


**Figure 6.** Observation for levels of hypertrophy associated molecules of in cardiomyocytes undergoing Klotho and NCX/LTCC inhibitor treatment by using qRT-PCR assay. **A**, Statistical analysis for BNP expression. **B**, Statistical analysis for ANP expression. **C**, Statistical analysis for β-MHC expression. *$p<0.05$ *vs.* NC+ISO+Klotho group. #$p<0.05$ *vs.* NC+ISO+Klotho+NEC inhibitor or +LTCC inhibitor group.

cardiac dysfunction[27]. Therefore, it's critical to discover the therapeutic strategy or negative regulators for blocking the cardiac hypertrophy. Till now, plenty of molecules, including long non-coding RNA[28], microRNAs[29], exosomes[30], sestrin 2[7] have been investigated. However, in the recent years, following with the development of $Na^+/K^+$ APTase, the NAKα2 has attracted more and more attentions. NAKα2 plays critical roles in removing the $Na^+$ from the cytosol of the cardiomyocytes[31] and maintaining $Na^+$ and $K^+$ concentration gradients cross plasma membrane[32], both of which are important for the functions of cardiomyocytes. James et al[33] also reported that the NAKα2 heterozygous gene-knockout mouse is hyper contractile for the function of heart. Therefore, in this study, we identified the evidence demonstrating that NAKα2 played fundamental roles in the hypertrophy of cardiomyocytes.

In this study, NAKα2 affected the expression of $Ca^{2+}$ channel associated molecules, such as NCX, LTCC, in the ISO treated cardiomyocytes. Actually, the NCX always facilitates the effects of local intracellular [$Na^+$] influx on the $Ca^{2+}$ transients and the contractility[34]. The LTCC could modulate the voltage-gated $Ca^{2+}$ channel activity and is important for retaining the balance of $Ca^{2+}$.[35] Our results indicated that ISO treatment induced the increased NCX and decreased LTCC, both of which would damage the cardiomyocytes. Also, the NAKα2 treatment (NAKα2+ISO group) significantly inhibited the NCX expression in the NAKα2+ISO group, and siNAKα2+ISO group significantly activated the NCX. On the contrary, expression of LTCC in NAKα2+ISO group was significantly activated, and LTCC in siNAKα2+ISO group was significantly suppressed, compared to that in NC+ISO group. These results

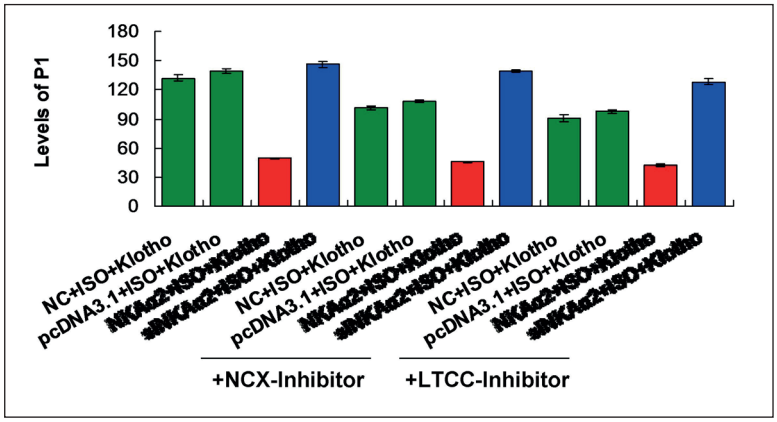


**Figure 7.** Evaluation for $Ca^{2+}$ influx in cardiomyocytes undergoing Klotho and NCX/LTCC inhibitor treatment by using flow cytometry assay. #$p<0.05$ *vs.* NC+ISO+Klotho+NEC inhibitor or +LTCC inhibitor group.

suggest that the NAKα2 regulated the $Ca^{2+}$ channel associated molecules, the effects of which are antagonistic to the ISO administration. Due to the regulation of $Ca^{2+}$ channels by $Ca^{2+}$ NAKα2 treatment, the $Ca^{2+}$ influx was also reduced in the ISO-treated cardiomyocytes, which is critical for keeping the $Ca^{2+}$ balance of cardiomyocytes[36]. Moreover, NAKα2 also kept the $Ca^{2+}$ levels by reducing the intracellular calcineurin levels and activating phosphorylation of NFAT,

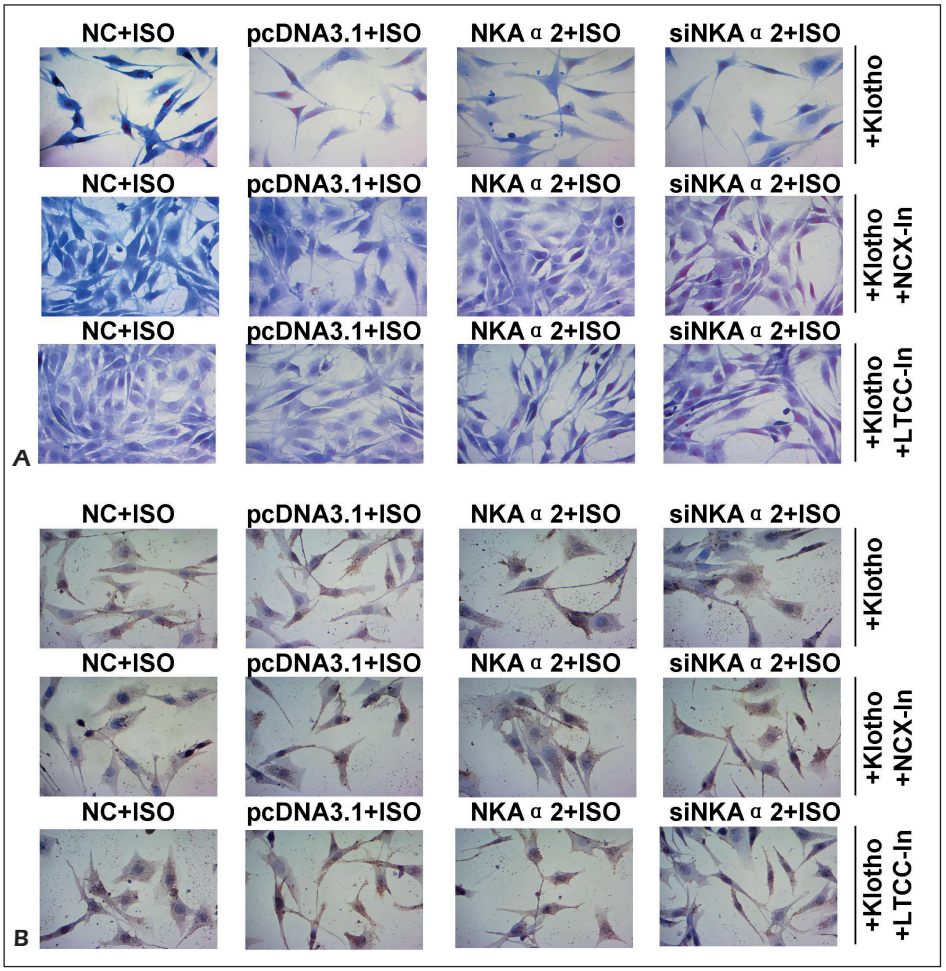


**Figure 8.** Detection for fibrosis formation and apoptosis in cardiomyocytes undergoing Klotho and NCX/LTCC inhibitor treatment. **A**, Fibrosis examination using Masson staining. **B**, Apoptosis evaluation by using TUNEL assay.

both of which play critical roles in the normal physiological function[37].

The previous study[6,38] proved that the β-MHC, BNP and ANP are the key biomarkers for the hypertrophy of cardiomyocytes. The present results showed that β-MHC, ANP and BNP levels in NAKα2+ISO group were significantly decreased compared to that in the NC+ISO group, suggesting that NAKα2 treatment inhibited the hypertrophy of cardiomyocytes by suppressing expression of β-MHC, ANP and BNP. Meanwhile, we also discovered that NAKα2 significantly alleviated the accumulation of the fibrosis (of formation), which is closely associated with the hypertrophy of cardiomyocytes[39,40]. Moreover, NAKα2 also inhibited the apoptosis of ISO treated cardiomyocytes, suggesting NAKα2 inhibits hypertrophy by enhancing the activity of cardiomyocytes.

Klotho is a putative anti-aging gene expressed pre-dominantly in the epithelial cells and protects against the myocardial hypertrophy[41]. Our findings showed that NAKα2 could strengthen the effects of Klotho on ISO-induced up-regulation of hypertrophy associated molecules. Meanwhile, NAKα2 combining with Klotho treatment exhibited better improvement of $Ca^{2+}$ influx, alleviation of fibrosis and reduction of apoptosis of ISO treated cardiomyocytes. These results hint that there is a synergistic action between NAKα2 and Klotho on the inhibition of hypertrophy, which has never be proven in the previous study[42].

In order to confirm the roles of LTCC and NCX molecules in NAKα2 mediated protective effects on hypertrophy, the LTCC inhibitor[11] and NCX reverse mode inhibitor[12] were employed to interfere hypertrophy associated molecules expression. The data showed that NAKα2 regulated hypertrophy associated molecules through activating LTCC and NCX pathway. Moreover, NAKα2 also reduced the $Ca^{2+}$ influx, alleviated the fibrosis and inhibited the apoptosis. Therefore, the above findings suggest that NAKα2 played the protective effects on hypertrophy by activating the LTCC/NCX signaling pathway.

## Conclusions

We provided evidence that over-expression of NKAα2 suppressed fibrosis formation and protected against the cardiomyocyte hypertrophy by inhibiting hypertrophy associated molecules, alleviating apoptosis and activating LTCC/NCX signaling pathway.

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### Conflict of Interests

Authors declare no competing financial or commercial interests in this manuscript.

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