Cardiac CaMKII δ and Wenxin Keli Prevents Ang II-Induced Cardiomyocyte Hypertrophy by Modulating CnA-NFATc4 and Inflammatory Signaling Pathways in H9c2 Cells

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Previous studies have demonstrated that calcium-/calmodulin-dependent protein kinase II (CaMKII) and calcineurin A-nuclear factor of activated T-cell (CnA-NFAT) signaling pathways play key roles in cardiac hypertrophy (CH). However, the interaction between CaMKII and CnA-NFAT signaling remains unclear. H9c2 cells were cultured and treated with angiotensin II (AngII) with or without silenced CaMKII δ (siCaMKII) and cyclosporine A (CsA, a calcineurin inhibitor) and subsequently treated with Wenxin Keli (WXKL). Patch clamp recording was conducted to assess L-type Ca 2+ current (I\text{Ca-L}), and the expression of proteins involved in signaling pathways was measured by western blotting. Myocardial cytoskeletal protein and nuclear translocation of target proteins were assessed by immunofluorescence. The results indicated that siCaMKII suppressed Ang II-induced CH, as evidenced by reduced cell surface area and I\text{Ca-L}. Notably, siCaMKII inhibited Ang II-induced activation of CnA and NFATc4 nuclear transfer. Inflammatory signaling was inhibited by siCaMKII and WXKL. Interestingly, CsA inhibited CnA-NFAT pathway expression but activated CaMKII signaling. In conclusion, siCaMKII may improve CH, possibly by blocking CnA-NFAT and MyD88 signaling, and WXKL has a similar effect. These data suggest that inhibiting CaMKII, but not CnA, may be a promising approach to attenuate CH and arrhythmia progression.

1. Introduction

Cardiomyocyte hypertrophy (CH) is an adaptive response to the pathological stimuli that maintain normal cardiac function. CH is a prerequisite marker of heart failure (HF) and usually occurs after myocardial infarction and stress overload. Although the initially adaptive response can maintain cardiac output, sustained hypertrophic growth can lead to a pathological state that leads to decreased compliance, HF, and sudden death [1–5]. Therefore, CH remains
a major threat in the population, and it is necessary to elucidate the pivotal molecular mechanisms involved in CH that can ameliorate pathological CH responses. Different signaling molecules have been considered as causes of myocardial hypertrophy, including nuclear factor of activated T cells (NFAT), calcium-/calmodulin- (CaM-) dependent protein kinase II (CaMKII), and β-adrenergic receptors [6].

Calcineurin (CaN), a calcium- and CaM-dependent serine/threonine phosphatase, is a well-established mediator of β-adrenergic-induced CH [7, 8]. CaN consists of two subunits: the catalytic subunit (CnA) and a regulatory Ca²⁺-binding subunit (CnB) [9, 10]. It is generally known that CaN plays an influential role in regulating pathological hypertrophy. The constitutive activation of CaN and its downstream target, NFAT, are thought to play an important role in abnormal CH [11, 12]. Moreover, it is known that CaMKII phosphorylates many vital signaling factors that are related to initiating abnormal hypertrophy [13]. Transgenic overexpression of the splice variants CaMKIIβb (located in the nucleus) and CaMKIIδc (located in the cytosol) promotes CH and dilated cardiomyopathy, respectively. However, the complete pathological molecular mechanism is not fully understood, which hinders the development of improved treatments for CH [14, 15]. Ca²⁺-dependent signaling through CaMKII and CaN has been suggested to contribute to adverse CH [16]. However, the interaction between CaMKII and CnA-NFAT signaling for CH remains unclear.

Wenxin Keli (WXKL) is the first Chinese medicine approved by the Chinese state for its antiarrhythmic effects; the medicine can tonify qi, supply yin, promote blood circulation, and remove blood stasis according to the theory of traditional Chinese medicine. The main components of WXKL consist of Nardostachys jatamansi (D. Don) DC (Gansong), Codonopsis pilosula (Franch.) Nannf (Dangshen), Panax notoginseng (Burkill) F. H. Chen (Sanqi), Succinum (Hupuo), and Polygonatum cyrtonema Hua (Huangjing). According to the national pharmacopoeia [17], WXKL mainly contains notoginseng saponin R1 (C₄₇H₈₀O₁₈), ginseng saponin Rg1 (C₄₂H₇₂O₁₄), and ginseng saponin Rb1 (C₅₄H₉₈O₂₃). Figure 1 displays the HPLC chromatograms of the chemical reference substances, WXKL, and negative samples of Panax notoginseng and Codonopsis pilosula. Previous studies have shown that WXKL may inhibit HF and arrhythmia by regulating the CaMKII signaling pathway [19–23]. WXKL in the treatment of patients with HF or arrhythmia can improve the exercise tolerance and is beneficial for the recovery of cardiac function [24–26]. However, to the best of our knowledge, whether WXKL improves CH via regulating the CaMKII and CnA-NFAT signaling pathways has not been investigated.

Given the pivotal roles of the CaMKII and CaN signaling pathways in the regulation of abnormal hypertrophy, we selected H9c2 cells induced by angiotensin II (Ang II) and used CaMKII silencing and treatment with cyclosporine A (CsA, a CaN inhibitor) to explore the correlation between the CaMKII and CnA-NFAT signaling pathways. Additionally, we sought to determine whether WXKL could improve HF by regulating CaMKII and CnA-NFAT.

2. Materials and Methods

2.1. Construction of Silenced CaMKIIδ in H9c2-1632 Cells. H9c2 rat embryonic cardiomyocyte cells were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Cell Resource Center, IBMS, CAMS/ PUMC, China). First, RNA interference at the site of the target gene, CaMKIIδ (Rat), was performed. According to the target gene sequence site, four sequences with the target gene were inserted into the vector pCDNA6.2 emGFP, and miRNA reverse primer was used as the sequencing primer (Table 1). Sequencing results verified that the correct sequence was inserted. Transfection and preparation of plasmids, blasticidin screening, and determination of the concentration of H9c2 cells, and finally cell transfection and screening were performed. Real-time PCR results showed that the relative mRNA expression of H9c2-1141 and H9c2-1632 sites was significantly reduced after RNA interference (Figure S2A), and the inhibition rate of relative mRNA expression after H9c2-1632 site interference was slightly lower than that of H9c2-1141, but the difference was not statistically significant (Table 2). CaMKIIδ protein expression was determined by western blot analysis, and the results showed that, after performing RNA interference at four sites, CaMKIIδ protein expression was decreased, with the greatest decrease at the H9c2-1632 site (Figure S2B). Combined with real-time PCR, the H9c2-1632 site was finally selected as a target to construct a CaMKIIδ (Rat) RNA interference cell line. The H9c2-1632 cells were cultured in an incubator containing 5% CO₂ in high-sugar Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C.

2.2. Drugs and Solutions. Ang II (A9525, Sigma Co., St. Louis, MO, USA) was dissolved in deionized water at a concentration of 10⁻⁷ mol/L; 20 μL of this solution was added to the culture medium (2 mL), reaching a final concentration of 10⁻⁷ mol/L. CaS (Sigma Co.) was dissolved in ethyl alcohol at a concentration of 10⁻⁶ mol/L; 20 μL of this solution was added to the culture medium (2 mL) at a final concentration of 10⁻⁶ mol/L. WXKL (1910051, Shandong Buchang Pharmaceuticals Co., Ltd., Shandong, China) was dissolved in saline at a concentration of 5 g/L.

In HPLC, 1 g of finely ground WXKL was added to a conical flask with a stopper, 50 mL of water-saturated n-butanol was added, and the flask was tightly stoppered and weighed. After soaking for 12 hours and ultrasonic treatment (power 300 W, frequency 40 kHz) for 1 h, the flask was weighed again after cooling, water-saturated n-butanol was added to make up for the lost weight, and the flask was shaken well. After filtration, 25 mL of the filtrate was collected. The n-butanol solution was evaporated to dryness, and methanol solution was added to the residue to 10 mL and shaken. According to the proportion of prescription Chinese medicine, notoginseng and Codonopsis were removed separately to make negative samples of Panax notoginseng and Codonopsis pilosula, and the negative control solution was prepared according to the previously mentioned methods.
Methanol was added to prepare reference substances containing notoginsenoside R1 0.247 mg, ginsenoside Rg1 0.422 mg, ginsenoside Rd 0.042 mg, ginsenoside Rb1 0.847 mg, and ginsenoside Rd 0.255 mg per 1 mL.

2.3. Chromatographic Conditions. Analyses were performed on an Agilent 1260 HPLC system consisting of a quaternary delivery system, an autosampler, and a DAD detector. All the separations were carried out on Amethyst C18 column (4.6 × 250 mm, 4 μm). The gradient elution used acetonitrile (A) and water (B) as a mobile phase at a flow rate of 1 mL/min. The gradient program was as follows: 0–14 min, 22%–30%; 14–35 min, 30%–50%; 35–45 min, 38%–38%; 45–47 min, 38%–95%; 47–62 min, 95%–95%; 62–65 min, 95%–22%. The column temperature was maintained at 27°C, and the chromatogram was monitored at a wavelength of 210 nm.

Table 1: CaMKIIδ (Rat) (NM_012519.2) RNAi site.

| Initiation site | RNAi site sequence |
|-----------------|---------------------|
| 1# 408          | TAGAATCTGGCGGTCTCTTGAA |
| 2# 754          | CCTGGATCTCTTTCAGAA |
| 3# 1141         | ACTATGCTGGCTACGAAAT |
| 4# 1632         | GTACATGACATTGGAAAT |

Table 2: Inhibition rate of CaMKIIδ (rat) H9c2-1141 and H9c2-1632 sites after the RNA interference.

| Cells        | Relative expression | Inhibition rate of CaMKIIδ (rat) |
|--------------|---------------------|---------------------------------|
| H9c2-NEG     | 1                   | 0                               |
| H9c2-1141    | 0.1765726           | 82.34%                          |
| H9c2-1632    | 0.1890273           | 81.10%                          |

Methanol was added to prepare reference substances containing notoginsenoside R1 0.247 mg, ginsenoside Rg1 0.422 mg, ginsenoside Rd 0.042 mg, ginsenoside Rb1 0.847 mg, and ginsenoside Rd 0.255 mg per 1 mL.

2.4. Cell Grouping and Drug Administration. H9c2 rat embryonic cardiomyocytes were split into 13 different treatment groups: (1) negative control group: only secondary antibody was added to H9c2 cells with no primary antibody; (2) control group: H9c2 cells were cultured for 72 h; (3) control + Ang II group: H9c2 cells were pretreated with Ang II for 48 h and cultured for another 24 h; (4) control + Ang II + WXKL group: H9c2 cells were pretreated with Ang II for 48 h, treated with WXKL, and cultured for another 24 h; (5) control + WXKL group: H9c2 cells were cultured for 48 h, treated with WXKL, and cultured for another 24 h; (6) siCaMKII group: H9c2-1632 cells were cultured for 72 h; (7) siCaMKII + Ang II group: H9c2-1632 cells were pretreated with Ang II for 48 h and cultured for another 24 h; (8) siCaMKII + Ang II + WXKL group: H9c2-1632 cells were pretreated with Ang II for 48 h, treated with WXKL, and cultured for another 24 h; (9) siCaMKII + WXKL group: H9c2-1632 cells were pretreated with Ang II for 48 h, treated with WXKL, and cultured for another 24 h; (10) CsA + Ang II group: H9c2 cells were pretreated with CsA and Ang II for 48 h and cultured for another 24 h; (11) CsA + Ang II + WXKL group: H9c2 cells were pretreated with CsA and Ang II for 48 h, treated with WXKL, and cultured for another 24 h; (12) CsA + WXKL group: H9c2 cells were pretreated with CsA for 48 h, treated with WXKL, and cultured for another 24 h.

2.5. Western Blot Analysis. Proteins were extracted from H9c2 cells, subsequently lysed with lysis buffer containing phenylmethylsulfonyl fluoride in ice for 40 min,

Figure 1: The HPLC chromatograms of WXKL. (a) Chemical reference substances, (b) WXKL, (c) negative samples of Panax notoginseng, and (d) Codonopsis pilosula. (1) notoginsenoside R1; (2) ginsenoside Rg1; (3) obetyolin; (4) ginsenoside Rb1; (5) ginsenoside Rd.
and shaken once every 8 min during this period. The lysates were centrifuged at 1200 rpm for 20 min at 4°C, and the supernatants were collected. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked in 5% bovine serum albumin (BSA) or milk, and then incubated with primary antibodies at 4°C overnight. Subsequently, the membranes were washed three times with tris-buffered saline/Tween 20 at the specified time intervals and finally incubated with secondary antibody at room temperature. ECL visualization was performed, and the resulting images were captured using the GeneGnome Gel Imaging System (Syngene Co., Bangalore, India). The gel images were analyzed using ImageJ software (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA). The antibodies used in the present study are listed in Table S1.

2.6. Confocal Imaging. For the cytoskeletal assay, H9c2 cells were cultured in a confocal laser culture dish until reaching a moderate density. Upon reaching 70–80% confluence, the cells were washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min, and washed with PBS three times for 5 min each. Cells were incubated with 0.1% Triton X-100 penetrating fluid at room temperature for 5 min and washed thrice with PBS for 5 min each. Rhodamine-phalloidin was diluted with PBS (2.5 μL) and added to 200 μL of the working fluid, fixed for 20 min, and washed with PBS three times for 5 min each. Cells were subjected to confocal laser microscopy. The cells of each group were removed and washed with cold PBS (precooled at 4°C) twice, and the residual PBS was aspirated. Each group was washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min, and washed with PBS three times for 5 min each. Cells were incubated with 0.1% Triton X-100 penetrating fluid at room temperature for 5 min and washed thrice with PBS for 5 min each. Rhodamine-phalloidin was diluted with PBS (2.5 μL) and added to 200 μL of the working fluid, fixed for 20 min, and washed with PBS three times for 5 min each. Cells were subjected to confocal laser microscopy. The cells of each group were removed and washed with cold PBS (precooled at 4°C) twice, and the residual PBS was aspirated. Each group was washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 15 min, and washed with PBS three times for 5 min each. Cells were incubated with 0.1% Triton X-100 penetrating fluid at room temperature for 5 min and washed thrice with PBS for 5 min each. Rhodamine-phalloidin was diluted with PBS (2.5 μL) and added to 200 μL of the working fluid, fixed for 20 min, and washed with PBS three times for 5 min each. Cells were incubated with DAPI for nuclear staining; finally, an antifluorescence quencher was added. Later, a transparent nail polish seal was used, and cells were observed using a confocal microscope.

For measuring the nuclear translocation of NFATc4, the cell processing method is basically the same as previously mentioned. Images were analyzed using Image-Pro Plus Analysis Software. First, color images were converted to gray images. The ratio of IOD to the area represented by NFATc4 fluorescence was measured. NFATc4 translocation was quantified as the ratio between the number of cells containing nucleus-localized NFATc4 (NFATc4-positive), and the total number of cells was counted.

2.7. Electrophysiological Recording. Using the whole-cell patch clamp technique, the whole-cell Ca2+ current was recorded by an Axon-700B amplifier (Axon Instruments, San Jose, CA, USA) with the pCLAMP 9.2 software (Axon Instruments). Borosilicate glass microelectrodes had tip resistances of 3.0–5.0 MΩ, which adjusts the three-dimensional manipulator for G41 scaling and breaks the membrane absorption in the whole-cell recording mode. The membrane capacitance and I_{Ca,L} current were recorded after stabilization. To eliminate the errors resulting from cell size, the I value was expressed as the current density (pA/pF). Cells were superfused with extracellular fluid containing (all in mmol/L): 125 NaCl, 10.8 BaCl2, 1 MgCl2, 5.4 CsCl, 10 HEPES, and 10 glucose (pH 7.35, adjusted with NaOH). A pipette solution was used containing (mmol/L): 120 CsCl, 3 MgCl2, 5 Na2ATP, 10 EGTA, and 5 HEPES (pH 7.3, adjusted with CsOH).

2.8. Statistical Analysis. Data were expressed as mean ± SD. One-way ANOVA was used to compare multiple groups with a normal distribution. Statistical analysis was performed using the SPSS program (version 20.0). P < 0.05 was considered statistically significant, and P < 0.01 was considered highly statistically significant. Data acquisition and analysis were performed using pCLAMP 9.2 software (Axon Instruments), Origin 6.1 software (MicroCal Software, Northampton, MA, USA), and GraphPad Prism 5 (GraphPad Software Incorporate, La Jolla, CA, USA).

3. Results

We explored the interaction between CaMKII and CnA-NFAT signaling in CH induced by Ang II and the effect of WXKL. Therefore, we sought to characterize the role of CaMKII and CnA-NFAT signaling in cardiomyocytes using siRNA-mediated silencing (siCaMKII) and CsA. We analyzed the results of cytoskeletal enlargement; L-type Ca2+ current (I_{Ca,L}); expression of CaMKII, CnA-NFAT, and inflammatory signaling pathways in cardiomyocytes induced by Ang II; and the protein expression and nuclear transfer of NFATc4.

3.1. Effects of SiCaMKII and CsA on Myocardial Cytoskeletal Protein. We used fluorescence confocal microscopy to observe cytoskeletal enlargement (a direct indicator of hypertrophy in myocardial cells) to assess whether the CH model had been successfully established and ensure the feasibility of the experiment. Immunofluorescence staining showed that Ang II effectively induced CH, as evidenced by an increase in cell width and length (Figure 2). CsA and siCaMKII prevented an Ang II-stimulated increase in cell size, and the effect was similar in cells treated with WXKL (Figure 2). Taken together, the results revealed that siCaMKII and CsA may inhibit the hypertrophic response to Ang II in H9c2 cells. Moreover, WXKL improved Ang II-induced CH.
Figure 2: SiCaMKII decreases Ang II-induced cell surface area enlargement in H9c2 cells. H9c2 cells were treated with Ang II (10^{-7} M for 48 h). CaMKII was silenced or CsA (10^{-6} M) was added to the culture medium prior to Ang II administration. After administering Ang II, WXKL (5 g/L) was added, and the culture medium was incubated for 24 h. The control cells received no treatment. (a) Representative images of immunofluorescence staining for phalloidin following treatment (n = 10 cells per group). Scale bar: 30 μm. (b) Mean cell length measurement (n = 10 cells per group). (c) Mean cell width measurement (n = 10 cells per group). Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. (*) P < 0.05 and ** P < 0.01 vs. the control group. (#) P < 0.05 and ## P < 0.01 vs. the Ang II group. (▲) P < 0.05 and ▲▲ P < 0.01; the control group in SiCaMKII or CsA vs. the control group in normal. (▲▲) P < 0.05 and ▲▲▲ P < 0.01; the Ang II group in SiCaMKII or CsA vs. the Ang II group in normal.
3.2. $I_{Ca-L}$ Significantly Reduced after CaMKIIδ Silencing.

$I_{Ca-L}$ is present in many cardiomyocytes, and it plays a key role in the formation of an action potential plateau, intracellular Ca$^{2+}$ elevation, and muscle contraction. CaM, as a Ca$^{2+}$ receptor, plays a major role in Ca$^{2+}$-dependent inactivation and facilitation of $I_{Ca-L}$.

Results revealed that compared with control treatment, Ang II significantly increased the amplitude of $I_{Ca-L}$ within the range of −20 mV to +10 mV. Pretreatment with siCaMKII reduced the elevated $I_{Ca-L}$ amplitude. Similarly, WXKL decreased the Ang II-induced elevated amplitude of $I_{Ca-L}$ (Figures 3(a) and 3(b)). Interestingly, in the CaS group (Figure 3), $I_{Ca-L}$ was significantly increased, and its steady-state activation and inactivation were increased (Figures 3(c) and 3(d)). These results suggest that siCaMKII and WXKL inhibit the calcium current and impede inactivation. Furthermore, treatment with CaS led to the opposite results. Therefore, we suspected that CaN inhibition may alter other pathways and cause an increase in calcium current.

3.3. Treatment with CsA Activates the CaMKII Signaling Pathway.

To further investigate Ang II-induced CaMKII expression in H9c2 cells and the effect of drug intervention on CaMKII protein expression, we measured CaMKII protein levels using laser scanning confocal microscopy. Additionally, CaMKII signaling pathway proteins were subjected to western blotting to explore the mechanism.

The results revealed that Ang II treatment significantly increased the fluorescence intensity of CaMKII in H9c2 cells compared with control treatment (Figure 4(a)). Pretreatment with siCaMKII reduced the Ang II-induced elevation in the fluorescence intensity of CaMKII, and WXKL led to a similar decrease. However, the fluorescence intensity corresponding to the CaMKII protein level was significantly higher in the CaS group than in the normal group (Figure 4(b)) and was observed to decrease after WXKL treatment. Therefore, the results showed that the fluorescence intensity indicating CaMKII protein expression increased after stimulation with Ang II, whereas WXKL treatment significantly reduced this increase. However, treatment with CsA did not inhibit this effect.

The expression of CaMKII, p-CaMKII, RyR2, p-RyR2, PLB, and p-PLB in H9c2 cells after stimulation with Ang II for 48 h was evaluated using western blot analysis. Exposure of H9c2 cells to Ang II resulted in increased expression of CaMKII pathway proteins (Figure 5(a)). Pretreatment with siCaMKII significantly decreased the Ang II-induced elevated expression of p-RyR2 and p-PLB ($P < 0.01$, Figures 5(e) and 5(f)) and reduced that of CaMKII and p-CaMKII ($P < 0.05$, Figures 5(b) and 5(c)). Interestingly, pretreatment with CsA led to significantly upregulated CaMKII expression levels ($P < 0.01$, Figure 5(b)). After treatment with WXKL for 24 h, protein expression was decreased in each group. Therefore, the CaN-NFAT signaling pathway was blocked by silencing CaMKII expression, and WXKL played a role in improving the expression of various proteins in CH.

3.4. CaMKII Controls the CaN-NFAT Pathway.

Next, we elucidated the effects of siCaMKII, CsA, and WXKL on the CaN-NFAT signaling pathway.

Treatment with Ang II significantly increased the fluorescence intensity of CaN in both the nucleus and the cytoplasm (Figure S1). The siCaMKII cells showed downregulation of CaN protein levels and lower fluorescence intensities. After treatment with WXKL for 24 h, Ang II-induced CaN protein expression was inhibited, and its fluorescence intensity and nuclear transfer were reduced (Figure S1). These results revealed that the fluorescence intensity and nuclear transfer of CaN in the Ang II-treated group were significantly higher; however, CaMKII silencing as well as treatment with CsA and WXKL suppressed this effect.

As shown in Figure 6(a), the expression of upstream and downstream proteins, including CaN, p-CaN, NFATc4, p-NFATc4, GATA4, p-GATA4, ANP, and BNP, in the CaN-NFAT signaling pathway was detected by western blotting. Exposure of H9c2 cells to Ang II resulted in increased expression of CaN-NFAT signaling pathway proteins (Figure 6). Pretreatment with siCaMKII markedly decreased the Ang II-induced elevated expression of p-CaN ($P < 0.01$, Figure 6(c)) and reduced that of GATA4 and BNP ($P < 0.05$, Figures 6(f) and 6(i)). Interestingly, pretreatment with CsA led to significantly upregulated ANP expression levels ($P < 0.01$, Figure 6(h)). After treatment with WXKL for 24 h, protein expression was decreased in each group. Therefore, the CaN-NFAT signaling pathway was blocked by silencing CaMKII expression, and WXKL played a role in improving the expression of various proteins in CH.

3.5. SiCaMKII Inhibits Nuclear Transfer of NFATc4 in Hypertrophic Cardiomyocytes.

Subsequently, we further analyzed CaN-NFAT signaling, and immunofluorescence and western blots were performed to detect NFATc4 nuclear translocation.

The results demonstrated that NFATc4 was translocated to the nucleus in response to Ang II stimulation (Figure 7), whereas pretreatment with siCaMKII or CsA inhibited Ang II-induced nuclear translocation of NFATc4; furthermore, such transfer was inhibited after treatment with WXKL. The data presented in Figures 6 and 7 suggest that inhibition of hypertrophy by siCaMKII and WXKL was mediated, at least in part, by CaMKII and CaN-NFATc4 signaling.

3.6. Effects of SiCaMKII and CsA on the Inflammatory Signaling Pathway (MyD88–TLR4).

Finally, to further elucidate the interaction between the CaMKII and CaN-NFAT signaling pathways, western blotting was used to measure the expression of MyD88, NF-kB, p-NF-kB, TLR2, and TLR4 in different groups (Figure 8).

The results demonstrated that protein expression was increased in H9c2 cells after Ang II treatment for 48 h. A statistically significant difference was observed in the expression of MyD88, NF-kB, and p-NF-kB ($P < 0.01$, Figures 8(b), 8(c), and 8(d)). Compared with that after Ang II treatment in normal H9c2 group, inflammation-related
Figure 3: Continued.
protein expression decreased after that in the siCaMKII group. In the CsA group, NF-κB expression was slightly decreased whereas that of other proteins was increased. After treatment with Ang II for 48 h, the rate of increase of MyD88 and TLR2 was higher in the CsA group than that in H9c2 cells. After administering WXKL, protein expression decreased in each group. The previously mentioned findings indicated that, in the siCaMKII group, protein expression reduced in Ang II-induced cardiomyocytes and the inflammatory pathway was inhibited, but the opposite result was observed in the CsA group. Furthermore, the effect of WXKL was similar to that of siCaMKII.

4. Discussion

In the present study, we found that [1] Ang II activated the CaMKII, CnA-NFAT, and MyD88 inflammatory pathways in H9c2 cells and caused myocardial hypertrophy [2]. The siRNA-mediated silencing of CaMKII inhibited protein expression in the CaMKII pathway, further attenuated protein expression in the CnA-NFAT signaling pathway, and inhibited the reduction in NFATc4 nuclear transfer. Moreover, CaMKII silencing played a role in improving Ang II-induced myocardial hypertrophy, and WXKL had a similar effect [3]. CsA, a CaN inhibitor, inhibited expression in the CnA-NFAT pathway but activated the CaMKII and MyD88 signaling pathways.

Both physiological and pathological CH are related to the increase in cardiomyocyte Ca\(^{2+}\) levels. Ca\(^{2+}\) regulates the activity of various Ca\(^{2+}\)-dependent signaling pathways, including those of CaMKII and CnA-NFAT [6] (Figure 9). It is known that CaMKII phosphorylates class II histone deacetylases (HDACs), particularly HDAC4 and HDAC5, by boosting the export of these molecules from the nucleus, resulting in the disinhibition of MEF-2-mediated gene expression and CH [27–30]. CnA can maintain NFAT activity through a noncatalytic mechanism by associating with NFAT, blocking its nuclear export sequence and thereby maintaining its nuclear localization [31]. Once activated by sustained elevation of intracellular calcium, CaN dephosphorylates NFAT, enabling its translocation to the nucleus and consequently activating prohypertrophic target genes [32]. Our data showed that Ang II stimulation results in NFATc4 translocated to the nucleus, whereas pretreatment with siCaMKII or CsA inhibited Ang II-induced nuclear translocation of NFATc4; furthermore, transfer was inhibited after treatment with WXKL.

Previous research has shown that CaMKII inhibition can decrease Ang II-induced cardiac fibroblast proliferation and the secretion of TGF-β1 and TNF-α. In addition, CaMKII inhibition reversed the upregulation of MMP-1, 2, and 9 and collagen I and III after Ang II intervention [33]. A previous study revealed that treatment with KN-93 (a CaMKII inhibitor) significantly reduced the expression of CH-related proteins, including NFATc3, p-HDAC4, p-HDAC5, GATA-4, and the hypertrophy marker BNP. Furthermore, the combined inhibition of the CaMKII and CaN signaling pathways may obviously relieve CH responses [13]. The results of the current study revealed that siCaMKII inhibited
protein expression in the CaMKII pathway and further reduced that in the CnA-NFAT signal pathway, thereby improving AngII-induced hypertrophic cardiomyocytes. This was similar to the results of previous studies that demonstrated that, in the absence of CaMKII signals, CaN does not seem to contribute to abnormal cardiac remodeling, thus highlighting CaMKII and not CaN as a promising drug target to combat HF [16].

Figure 4: Effects of siCaMKII and CsA on the CaMKII signaling cascade. (a) Double immunofluorescence staining to observe the effects of siCaMKII, CsA, and WXKL on CaMKII expression in each group. Scale bar: 50 μm. (b) Mean density of CaMKII in each group. Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. * P < 0.05 and ** P < 0.01 vs. the control group. # P < 0.05 and ## P < 0.01 vs. the Ang II group. ▲ P < 0.05 and ▲▲ P < 0.01; the control group in SiCaMKII or CsA vs. the control group in normal. △ P < 0.05 and △△ P < 0.01; the Ang II group in SiCaMKII or CsA vs. the Ang II group in normal.
Excess CaMKII activity leads to phosphorylation of the L-type Ca²⁺ channel (LTCC), sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase, ryanodine receptor 2 (RyR2), and PLB proteins. CaMKII phosphorylates the LTCC that leads to increased I_{Ca-L} and forms a Ca²⁺ overload in the cell, which causes early afterdepolarizations and arrhythmia. Hyperphosphorylation of the sarcoplasmic reticulum (SR) results in the consumption of SR Ca²⁺ stores, leading to damaged cytosolic Ca²⁺ transients, which in turn induces systolic and diastolic dysfunction. Moreover, hyperphosphorylation events at RyR2 cause abnormal re-

Figure 5: Effects of siCaMKII and CsA on Ang II-induced activation of CaMKII signaling in H9c2 cells. (a) Representative western blotting images. Densitometric analysis of (b) CaMKII, (c) p-CaMKII, (d) RyR2, (e) p-RyR2, (f) PLB, and (g) p-PLB expression levels (n = 3 cells per group). Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. *P < 0.05 and **P < 0.01 vs. the control group. #P < 0.05 and ##P < 0.01 vs. the Ang II group. △P < 0.05 and △△P < 0.01; the Ang II group in SiCaMKII or CsA vs. the Ang II group in normal.
Figure 6: Effects of siCaMKII and CsA on Ang II-induced activation of CnA-NFAT signaling in H9c2 cells. (a) Representative western blotting images. Densitometric analysis of (b) CnA, (c) p-CnA, (d) NFATc4, (e) p-NFATc4, (f) GATA4, (g) p-GATA4, (h) ANP, and (i) BNP expression levels (n = 3 cells per group). Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. *P < 0.05 and **P < 0.01 vs. the control group. †P < 0.05 and ‡P < 0.01 vs. the Ang II group. ▲P < 0.05 and △△P < 0.01; the control group in SiCaMKII or CsA vs. the control group in normal. ∗P < 0.05 and ∗∗P < 0.01; the Ang II group in SiCaMKII or CsA vs. the Ang II group in normal.
exchanger (NCX), which can cause delayed after-
depolarizations [34]. Interestingly, our study revealed that
CnA expression was gradually reduced after treatment with
CsA, but the CaMKII signaling pathway tended to be ac-
tivated. This result was consistent with few studies that
found neither effect on hypertrophy with CsA or FK506 [35, 36]. Conversely, previous studies have
suggested that CaN inhibitors attenuate hypertrophy in a
variety of models [37]. These discrepancies may be explained
by the inherent differences between the different models. In
addition, the inherent nonspecific effects associated with
CsA and FK506 are to be considered. FK506 can also directly
alter ryanodine receptor function through its effects on
FKBP12 and FKBP12.6, while CsA may influence the leakage of
calcium from the sarcoplasmic reticulum through the
lipid bilayer [38, 39]. Acute treatment with CsA causes al-
terations of LTCC activity in dissociated human car-
diomyocytes [40]. The current study demonstrated that
administration of CsA activated the CaMKII signaling
pathway and increased the degree of CH. This is possibly
because the specificity of actions by CsA may be associated
with their selective interactions with specific CaN subtypes
or because of the presence of cell-specific CaN substrates
[41].

![Representative images of immunofluorescence staining of NFATc4. Blue: DAPI staining; red: NFATc4. Scale bar: 50 μm. Yellow arrow: nuclear translocation; white arrow: no nuclear translocation or nuclear translocation was decreased.](a)

![Semiquantitative analysis of NFATc4 nuclear translocation. Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. ∗ P < 0.05 and ∗∗ P < 0.01 vs. the control group. # P < 0.05 and ## P < 0.01 vs. the Ang II group. ▲ P < 0.05 and ▲▲ P < 0.01; the control group in siCaMKII or CsA vs. the control group in normal. Δ P < 0.05 and ΔΔ P < 0.01; the Ang II group in siCaMKII or CsA vs. the Ang II group in normal.](b)

Figure 7: Ang II-induced NFATc4 nuclear translocation is inhibited by siCaMKII. H9c2 cells were treated with Ang II (10^{-7} M) for 48 h. CaMKII was silenced or CsA (10^{-6} M) was added to the culture medium prior to Ang II administration. WXKL (5 g/L) was added for 24 h after Ang II administration. The control cells received no treatment. (a) Representative images of immunofluorescence staining of NFATc4. Blue: DAPI staining; red: NFATc4. Scale bar: 50 μm. Yellow arrow: nuclear translocation; white arrow: no nuclear translocation or nuclear translocation was decreased. (b) Semiquantitative analysis of NFATc4 nuclear translocation. Data are presented as the mean ± SD. Statistical significance was determined by one-way ANOVA. ∗ P < 0.05 and ∗∗ P < 0.01 vs. the control group. # P < 0.05 and ## P < 0.01 vs. the Ang II group. ▲ P < 0.05 and ▲▲ P < 0.01; the control group in siCaMKII or CsA vs. the control group in normal. Δ P < 0.05 and ΔΔ P < 0.01; the Ang II group in siCaMKII or CsA vs. the Ang II group in normal.
Three different CaN subtypes are found in mammals: CaNa and CaNβ, which are universally expressed, and CaNaγ [9]. Furthermore, it has been reported that CaNβ1 may have a cardioprotective action that decreases inflammation and scar formation [9]. Moreover, mice overexpressing constitutively active CaN show decreased apoptosis after ischemia/reperfusion, whereas deletion of the phosphatase-encoding exon of CaNβ leads to increased cell death and reduced cardiac function [42, 43]. In contrast, transgenic mice overexpressing an artificially truncated, constitutively active form of CaNa lacking the autoinhibitory domain show strong CH and develop HF within the first few weeks of life, which is a response phenocopied by overexpression of a constitutively active form of NFAT [32]. Other studies have suggested that knockout mice lacking the phosphatase domain of CaNβ show smaller hearts at baseline and exhibit reduced hypertrophy in response to pressure overload, Ang II, or isoproterenol [44]. Notably, in the study by Zhang et al. [36], CsA augmented hypertension but did not prevent CH in spontaneously hypertensive rats. CsA is known to cause numerous unwanted side effects. It has been shown that CsA increases Ang II receptors independently from CaN inhibition, which causes vasoconstriction and systemic hypertension and can promote CH [35, 45–47]. This may be due to CsA alleviating the cardioprotective action of CaNβ.

There have been some studies on the mechanism of CaMKII and inflammatory pathway regulation [48–53]. Increasing evidence has shown that as a multipurpose kinase, CaMKII plays a pivotal role in many cardiac pathophysiological conditions.
involving inflammation [54]. Previous studies have shown that TLR4 mediates endothelial inflammation, activation, and dysfunction induced by CaN inhibitors, and according to previous observations, MyD88 silencing in endothelial cells prevented the induction of proinflammatory and endothelial activation markers by CsA and tacrolimus [55]. Our data indicate that, after CsA treatment, the expression of MyD88 and TLR2 obviously increased. The activation of MyD88, NF-κB, TLR2, and TLR4 inflammatory response pathways in pathological states induces an increase in CaMKII protein expression, causes calcium homeostasis, and promotes the occurrence of potentially malignant arrhythmias [56, 57].

WXKL is the first antiarrhythmic Chinese medicine to be approved by the state. Our previous studies indicated that WXKL treatment considerably preserves cardiac function and inhibits arrhythmia by modulating the CaMKII signaling pathway [22, 23]. Moreover, WXKL treats CH and arrhythmia through a mechanism that possibly involves LTCC regulation [20]. We propose a new role for WXKL that may inhibit CH by regulating pathological autophagy [21]. Notably, treatment with WXKL significantly inhibited Ang II-induced hypertrophy in the present study. In addition, WXKL suppressed Ang II-induced elevated expression of CaMKII, CaN, and NFATc4 and prevented Ang II-induced nuclear translocation of NFATc4, suggesting that WXKL attenuated CH by inhibiting the CaMKII and CaN-NFAT signaling pathways. Therefore, owing to its numerous therapeutic benefits, WXKL may be considered as a promising choice for the treatment of CH.

In conclusion, the present study demonstrated that siCaMKII attenuates Ang II-induced CH, which may be partially associated with the downregulation of the CaN-NFAT and MyD88 signaling pathways, and WXKL had a similar effect. Furthermore, siCaMKII may be a promising approach to attenuate the progression of CH and arrhythmia. Our studies define the crosstalk between the CaMKII and CaN-NFAT signaling pathways in vitro, so numerous biological studies are needed for further research in vivo. This will improve the understanding of the mechanisms underlying hypertrophy and may provide evidence for drug application in the treatment of CH.

**Abbreviations**

- CaM: Calcium/calmodulin
- CaMKII: Calcium-/calmodulin-dependent protein kinase II
- L-type Ca2+ current: I_{Ca-L}
Data Availability

The data and materials used in the study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

XYW and GYH designed the work. AN wrote the article. CY contributed to cell experiment. XYF and WHH responsible for critical revision of the article. GXY and CHW searched a lot of literature and revised the manuscript. SK and LY contributed to the results interpretation. LXY, YF, and PXD analyzed the data. HXF, WX, and LY revised the manuscript.

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Supplementary Materials

Table S1: list of antibodies. Figure S1: effects of siCaMKII and CsA on the CnA-NAFT signaling cascade; double immunofluorescent staining to observe the effects of siCaMKII, CsA and WXKL on expression of CnA in each group. Figure S2: H9c2-1632: validation of CaMKIIδ (Rat) 1632 site cell line after RNA interference. (Supplementary Materials)

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