Decreased KCNE2 Expression Participates in the Development of Cardiac Hypertrophy by Regulation of Calcineurin–NFAT (Nuclear Factor of Activated T Cells) and Mitogen-Activated Protein Kinase Pathways

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Background—KCNE2 is a promiscuous auxiliary subunit of voltage-gated cation channels. A recent work demonstrated that KCNE2 regulates L-type Ca\(^{2+}\) channels. Given the important roles of altered Ca\(^{2+}\) signaling in structural and functional remodeling in diseased hearts, this study investigated whether KCNE2 participates in the development of pathological hypertrophy.

Methods and Results—We found that cardiac KCNE2 expression was significantly decreased in phenylephrine-induced cardiomyocyte hypertrophy in neonatal rat ventricular myocytes and in transverse aortic constriction–induced cardiac hypertrophy in mice, as well as in dilated cardiomyopathy in human. Knockdown of KCNE2 in neonatal rat ventricular myocytes reproduced hypertrophy by increasing the expression of ANP (atrial natriuretic peptide) and β-MHC (β-myosin heavy chain), and cell surface area, whereas overexpression of KCNE2 attenuated phenylephrine-induced cardiomyocyte hypertrophy. Knockdown of KCNE2 increased intracellular Ca\(^{2+}\) transient, calcineurin activity, and nuclear NFAT (nuclear factor of activated T cells) protein levels, and pretreatment with inhibitor of L-type Ca\(^{2+}\) channel (nifedipine) or calcineurin (FK506) attenuated the activation of calcineurin–NFAT pathway and cardiomyocyte hypertrophy. Meanwhile, the phosphorylation levels of p58, extracellular signal-regulated kinase 1/2, and e-Jun N-terminal kinase were increased, and inhibiting the 3 cascades of mitogen-activated protein kinase reduced cardiomyocyte hypertrophy induced by KCNE2 knockdown. Overexpression of KCNE2 in heart by ultrasound-microbubble–mediated gene transfer suppressed the development of hypertrophy and activation of calcineurin–NFAT and mitogen-activated protein kinase pathways in transverse aortic constriction mice.

Conclusions—This study demonstrates that cardiac KCNE2 expression is decreased and contributes to the development of hypertrophy via activation of calcineurin–NFAT and mitogen-activated protein kinase pathways. Targeting KCNE2 is a potential therapeutic strategy for the treatment of hypertrophy. (Circ Heart Fail. 2017;10:e003960. DOI: 10.1161/CIRCHEARTFAILURE.117.003960.)

Key Words: animals ■ calcineurin ■ calcium ■ hypertrophy ■ mice

Cardiac hypertrophy is an adaptive response to pathological stimuli to preserve contractility and cardiac function. However, prolonged hypertrophy leads to maladaptive structural and functional remodeling and is associated with a significant increase in the risk for serious conditions, including arrhythmias, sudden death, and heart failure (HF).1,2 The pathological hypertrophic development involves multiple intracellular signaling pathways, including enhancement of calcium-regulated signaling pathways.3-5 Among them, the most extensively studied is calcineurin transcriptional NFAT (nuclear factor of activated T cells) pathway. Calcineurin, a serine threonine protein phosphatase, is activated by calcium/calmodulin under various prohypertrophic stimuli, including α- and β-adrenergic agonists, angiotensin II, and endothelin-1. On dephosphorylation by calcineurin, NFAT translocates to the nucleus of the cardiomyocyte where it mediates the transcription of numerous targets involved...
in hypertrophic growth. Interestingly, calcineurin–NFAT signals are intertwined with various hypertrophic signaling components. It has been suggested that calcineurin–NFAT pathway is associated with stimulation of many protein kinase C isoforms and mitogen-activated protein kinase (MAPK) cascades.

KCNE2 is a member of the small auxiliary subunits of voltage-gated cation channels encoded by KCNE family gene. KCNE2 has the most promiscuity of function which can coassemble with multiple α-subunits of voltage-dependent cation channels, including the human Ether-á-go-go-Related Gene (hERG), KCNQ1–3, Kv3.1, Kv3.2, Kv4.2, Kv4.3, Kv1.5, and Hyperpolarization-activated, cyclic nucleotide-gated (HCN) (pacemaker), and modulate their gating, conductance, and pharmacology. It is well established that KCNE2 plays an important role in maintaining the cardiac electric stability. Downregulation in KCNE2 expression has been observed in HF and linked to cardiac arrhythmia. However, it remains unknown whether KCNE2 participates in structural and functional (besides electric) remodeling in diseased hearts. Our recent work demonstrated that KCNE2 modulates cardiac L-type Ca²⁺ current by regulating the function of N-terminal inhibitory module of Cav1.2. Furthermore, deletion or knockdown of KCNE2 decreases the magnitude of I₁, and decreased I₉ can facilitate the development of cardiac hypertrophy by prolonging action potential duration and a window for Ca²⁺ entry. All the evidence suggests that alteration in KCNE2 may contribute to hypertrophic remodeling in diseased heart.

Hence, we examined KCNE2 expression in cardiac hypertrophy and explored the causal relationship between alteration in KCNE2 and development of cardiac hypertrophy. Our data revealed that KCNE2 expression was decreased in cardiac hypertrophy. Knockdown of KCNE2 induced cardiomyocyte hypertrophy by activation of calcineurin–NFAT and MAPK pathways. Furthermore, we found that increasing cardiac KCNE2 expression inhibited cardiac hypertrophy.

### Materials and Methods

#### Human Heart Samples

All human studies were performed in accordance with the principles of the Declaration of Helsinki. Human heart samples were obtained via protocols approved by University of Iowa Institutional Review Boards on Human Subjects. Advanced cardiomyopathy subjects donating tissue were consented under an approved Institutional Review Board (IRB) protocol. At the time of left ventricular (LV) assist device implantation or heart transplantation, tissues were procured, raffinized and rehydrated, and endogenous peroxidase was inhibited with 0.3% H₂O₂ methanol. For antigen retrieval, slides were boiled in 0.01 mol/L, pH 6.0 sodium citrate buffer for 15 minutes in a microwave oven. After being blocked with the 5% normal goat serum, primary anti-KCNE2 polyclonal antibody (1:200; Alomone) or anti-Flag polyclonal antibody (1:400; Cell signaling technology) in blocking buffer (1:50) was applied and the slides were incubated at 4°C overnight. After incubation with secondary antibody, the visualization signal was developed with 3, 3-diaminobenzidine tetrachloride.

#### Immunohistochemistry

For immunofluorescence, the heart tissue sections (4 μm) were deparaffinized and rehydrated, and endogenous peroxidase was inhibited with 0.3% H₂O₂ methanol. For antigen retrieval, slides were boiled in 0.01 mol/L, pH 6.0 sodium citrate buffer for 15 minutes in a microwave oven. After being blocked with the 5% normal goat serum, primary anti-KCNE2 polyclonal antibody (1:200; Alomone) or anti-Flag polyclonal antibody (1:400; Cell signaling technology) in blocking buffer (1:50) was applied and the slides were incubated at 4°C overnight. After incubation with secondary antibody, the visualization signal was developed with 3, 3-diaminobenzidine tetrachloride.

#### Measurements of Cell Apoptosis

To quantify the apoptosis in transverse aortic constriction (TAC) mice heart, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed in heart tissue sections using the FragEL DNA Fragmentation Detection Kit (Calbiochem) according to the protocols of the manufacturer. To determine the number of apoptotic cells, TUNEL-positive and TUNEL-negative cells were counted. Results are expressed as number of TUNEL-positive cells/total cells×100%.

#### Animal Model of Hypertrophy With TAC

C57BL/6 male mice (9–10 weeks, 18–22 g) were randomly divided into 4 groups: sham and TAC with or without KCNE2 overexpression, 6 in each group. The TAC model was produced as described previously.

Animals received gene therapy with doxycycline-inducible KCNE2 or empty vectors mediated by an ultrasound-microbubble technique, as described previously.

#### Statistical Analysis

The data were presented as means±SE. The Kolmogorov–Smirnov test and the Levene test were used to assess the normality of distribution and homogeneity for each variable within group. For a variable following normal distribution, the Student or Welch t test was performed when variances were equal or unequal between groups, respectively. For comparisons of multiple groups, 1-way ANOVA was performed followed by Tukey–Kramer correction. For correlated observations within animal, the general linear model (univariate) was adopted with animal as a random effect. Values of P <0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics18.0 software (IBM Corporation, Armonk, NY).

### Results

#### KCNE2 Protein Expression Was Decreased in Cardiac Hypertrophy

We first explored the alteration of cardiac KCNE2 expression under hypertrophic stimulation. Cardiomyocyte hypertrophy prepared with the BLOCK-iT Adenoviral RNA interference Expression system (Invitrogen). The sequences of the oligonucleotides for KCNE2 RNA interference were as follows: forward, CACCGGATTGGCAG CAGAAGTATAGCGCAAACTATACTTGTGCAATTC; reverse, AAAGGGATTTGGCAGCAGAGTATAGGATTTGCTATACTTCTG CTGCAATTC. Adenoviral vectors containing a scrambled shRNA sequence at the same multiplicity of infection served as the control (Ctrl-shRNA). To overexpress KCNE2, NRVMs were infected with recombinant Ad-KCNE2 (adenovirus vectors carrying human KCNE2 gene) at a multiplicity of infection of 5, with Ad-GFP (adenovirus carrying the gene encoding green fluorescent protein) applied at the same multiplicity of infection as the control.

#### Histological Analysis of Collagen Fibrosis

Mid-left ventricular short-axis heart sections were prepared and stained with Masson trichrome blue. Percentage of fibrosis was determined using ImageJ software to quantify blue (fibrotic) versus nonblue (nonfibrotic) pixels.
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in vitro was produced by stimulating cultured rat ventricular myocytes (NRVMs) with phenylephrine (20 μmol/L) for 72 hours, as previously described. Almost all the cells show the expression of α-actinin (Figure I in the Data Supplement), indicating that there is little contamination of fibroblasts and other cells in the cultured NRVMs. Phenylephrine stimulation significantly increased the cell size indexed by the cell surface area and the protein levels of ANP (atrial natriuretic peptide) and β-MHC (β-myosin heavy chain), indicating successful production of cardiomyocyte hypertrophy (Figure 1A and 1B). Pressure overload–induced cardiac hypertrophy was produced by TAC in mice, where the expression of ANP and β-MHC was significantly increased (Figure 1C). We found that KCNE2 expression was significantly decreased in cardiac hypertrophy both in vitro and in vivo, where KCNE2 protein levels were decreased by 66% and 57% in phenylephrine-stimulated cells and in TAC hearts, respectively, when compared with control (Figure 1B and 1C). Furthermore, we compared KCNE2 protein levels in human hearts from adult donors (nonfailing) and patients with idiopathic dilated cardiomyopathy and ischemic HF. The result shows that the expressions of KCNE2 in idiopathic dilated cardiomyopathy and ischemic HF are significantly lower than that in nonfailing hearts (Figure 1D).

Knockdown of KCNE2 Induced Cardiomyocyte Hypertrophy

We next explored whether decreased KCNE2 participates in the pathogenesis of hypertrophy by knockdown of KCNE2 expression with RNA interference in cardiomyocytes. We found that a 66% decrease in KCNE2 caused a significant increase in the expressions of both ANP and β-MHC (Figure 2A). Meanwhile, the cell surface area of NRVMs was markedly increased (Figure 2B). The results indicate that decreased KCNE2 induces cardiac hypertrophy.

Increasing KCNE2 Expression Inhibited Phenylephrine-Induced Cardiomyocyte Hypertrophy

To further confirm that decreased KCNE2 contributes to the development of pathological hypertrophy, we examined the role of increasing KCNE2 expression in phenylephrine-stimulated hypertrophy. We found that increasing KCNE2 expression by 110% by transfection of cardiomyocytes with adenovirus carrying KCNE2 gene significantly inhibited phenylephrine-induced increase in cell surface area and ANP and β-MHC protein levels (Figure 3A and 3B). The results indicate a causal relationship between downregulation of KCNE2 and pathogenesis of cardiac hypertrophy.

Figure 1. KCNE2 expression was decreased in cardiac hypertrophy. A, Representative images of cultured neonatal rat ventricular myocytes (NRVMs) immunostained with sarcomeric α-actinin (green), and statistics of cell surface area of NRVMs in Control (n=138 cells from 6 animals) and phenylephrine (PE)-stimulated cells (PE, n=205 cells from 6 animals). The results based on the mean of each animal were analyzed using an unpaired Student $t$ test. B and C, Expression of KCNE2, ANP (atrial natriuretic peptide), and β-MHC (β-myosin heavy chain) proteins in Control and PE-stimulated cells (B, n=5 per group) and in hearts from sham and transverse aortic binding (TAC)–induced hypertrophic mice (C, n=4 per group). D, Representative immunoblots and statistical data of KCNE2 protein levels in human hearts from adult donors (nonfailing, NF) and patients with idiopathic dilated cardiomyopathy (IDC) and ischemic heart failure (HF; n=4, 5, 5 in NF, IDC, and ischemic HF group, respectively). β-Actin or β-catenin was used as an internal control. Unpaired Student $t$ tests were used in B–D. *$P<0.05$, **$P<0.01$ vs Control or Sham or NF.
hypertrophy and implicate the therapeutic potential of targeting KCNE2 in the treatment of pathological hypertrophy.

**Decrease in KCNE2 Activated Ca\(^{2+}\)-Dependent Calcineurin–NFAT Signaling Pathway**

We next investigated the mechanisms underlying the development of hypertrophy induced by downregulation of KCNE2. Previous study indicates that knockdown of KCNE2 increased L-type Ca\(^{2+}\) channel.\(^{17}\) We thus examined whether Ca\(^{2+}\)-dependent calcineurin–NFAT signaling pathway was activated by KCNE2 knockdown. In agreement with previous study, KCNE2 knockdown increased systolic Ca\(^{2+}\) transient, where the amplitude of Ca\(^{2+}\) transient (ΔF/F₀) was increased by 31.27% (Figure 4A and 4B). Concomitantly, knockdown of KCNE2 increased the activity of calcineurin without changing its expression (Figure 4C; Figure II in the Data Supplement). Meanwhile, NFAT was transferred into nuclear indexed by the increase of nuclear NFAT protein level (Figure 4D and 4E).

The causal relationship between the activation of Ca\(^{2+}\)-activated calcineurin–NFAT signaling pathway and the development of hypertrophy by KCNE2 knockdown was investigated by blocking L-type Ca\(^{2+}\) channel with nifedipine and inhibition of calcineurin with FK506, respectively. We found that pretreatment of the cells with nifedipine (20 µmol/L) significantly

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**Figure 2.** Knockdown of KCNE2 induced cardiomyocyte hypertrophy. A, Expression of KCNE2, ANP (atrial natriuretic peptide), β-MHC (β-myosin heavy chain), and β-actin proteins in control (Ctrl-shRNA, CSh) and KCNE2 knockdown (KCNE2-shRNA, KSh) groups (n=5 per group). Unpaired Student t test was used. B, Representative images and cell surface area analysis of neonatal rat ventricular myocytes immunostained with sarcomeric α-actinin (green) in CSh (n=169 cells from 6 animals) and KSh (n=243 cells from 6 animals) groups. The results based on the mean of each animal were analyzed using an unpaired Student t test; **P<0.01 vs CSh.

**Figure 3.** Increasing KCNE2 expression inhibited phenylephrine (PE)-induced cardiomyocyte hypertrophy. A, Representative immunoblots and average data of KCNE2, ANP (atrial natriuretic peptide), and β-MHC (β-myosin heavy chain) proteins in control (Ad-GFP [green fluorescent protein adenovirus]), KCNE2 overexpression (Ad-KCNE2 [adenovirus vectors carrying human KCNE2]), control plus PE stimulation (Ad-GFP+PE) and KCNE2 overexpression plus PE stimulation (Ad-KCNE2+PE) groups (n=5 per group). One-way ANOVA, Tukey honest significance difference (HSD) tests. B, Representative images and statistics of cell surface area of neonatal rat ventricular myocytes immunostained with sarcomeric α-actinin (green) in Ad-GFP, Ad-KCNE2, Ad-GFP+PE, and Ad-KCNE2+PE groups. n=150 to 180 cells from 7 animals per groups. The results based on the mean of each animal were analyzed using 1-way ANOVA, followed by Tukey HSD tests. *P<0.05, **P<0.01 vs Ad-GFP. *P<0.05, **P<0.01 vs Ad-GFP+PE.
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Ca²⁺ transient (Figure 4C through 4E). Furthermore, nifedipine or FK506 decreased the cell surface area and the protein level of ANP and β-MHC increased by KCNE2 knockdown (Figure 4F and 4G). The results collectively indicate that activation of the Ca²⁺-activated calcineurin–NFAT (nuclear factor of activated T cells) signaling pathway by KCNE2 downregulation activated Ca²⁺ transient (Figure 4A and 4B), calcineurin activity (Figure 4C), and the protein level of nuclear NFAT (Figure 4D and 4E) in KCNE2 knockdown (KSh) cells with or without pretreatment of nifedipine (Nife) or FK506 (FK).
of Ca\(^{2+}\)-dependent calcineurin–NFAT signaling pathway contributes to decreased KCNE2-induced hypertrophy.

It has been suggested that the NFAT pathway can be regulated by Ca\(^{2+}\)-independent AMP-activated protein kinase.\(^{24,25}\) We thus examined the effect of KCNE2 knockdown on AMP-activated protein kinase phosphorylation. The result shows that the phosphorylated AMP-activated protein kinase was unaffected by KCNE2 knockdown (Figure III in the Data Supplement), indicating that the activation of calcineurin–NFAT pathway is unrelated to AMP-activated protein kinase activation.

**Knockdown of KCNE2 Increased the Phosphorylation of p38, Extracellular Signal-Regulated Kinase, and Jun Kinase**

We also investigated whether other hypertrophy-related signaling pathways participated in KCNE2 knockdown-induced cardiomyocyte hypertrophy. Previous studies documented that hypertrophic stimuli activate kinases, such as MAPK cascades and Akt phosphatidyl inositol 3-kinase, and mediate hypertrophic responses.\(^{26-28}\) Figure 5 shows that KCNE2 knockdown increased the phosphorylation levels of extracellular signal-regulated kinase (ERK), Jun kinase (JNK), and p38, but not Akt (Figure IV in the Data Supplement). Furthermore, inhibiting ERK, JNK, and p38 by pretreatment of cells with U0126 (10 \(\mu\)mol/L), SP600125 (10 \(\mu\)mol/L), and SB203580 (20 \(\mu\)mol/L), respectively, decreased the expression of ANP and \(\beta\)-MHC increased by KCNE2 knockdown. The results indicate that decreased KCNE2-mediated cardiome hypertrophy is related to activation of MAPK pathway.

**Overexpression of KCNE2 Attenuated Pressure Overload–Induced Cardiac Hypertrophy**

The important role of decreased KCNE2 in the development of pathological hypertrophy and the potential therapeutic effect of manipulating KCNE2 expression were further examined in a mouse model of LV hypertrophy, which was induced by pressure overload with TAC. Cardiac-specific overexpression of KCNE2 was achieved by ultrasound-microbubble–mediated gene transfer of the inducible KCNE2 (flag-tagged). The expression of exogenous KCNE2 was examined by immunohistochemistry detection of the flag protein in heart tissue sections. Ultrasound treatment resulted in higher levels of KCNE2 gene transfer and KCNE2 expression as demonstrated by the staining of flag protein in almost all cardiomyocytes (Figure 6A). Furthermore, the expression of inducible KCNE2 was detected by Western blotting of flag protein and increased level of KCNE2 protein (Figure 6B). At 6 and 8 weeks post-TAC, the thickness of systolic and diastolic LV posterior wall was significantly enlarged (Figure 7A through 7C). The systolic and diastolic LV internal diameter was decreased at 6 weeks but slightly increased at 8 weeks post-TAC (Figure 7A, 7D, and 7E), suggesting that there is a compensation of cardiac function at 6 weeks but decompensation at 8 weeks after TAC. The data that LV fractional shortening and ejection fraction were significantly increased at 6 weeks post-TAC, but decreased at 8 weeks post-TAC (Figure V in the Data Supplement), also indicate a cardiac functional transition from compensation to decompensation. Furthermore, the heart weight/body weight ratio and the wet lung weight/body weight ratio were increased in TAC mice. Mice with KCNE2 overexpression demonstrate a blunted response to TAC (Figure 7F through 7H). The hematoxylin and eosin staining of coronal sections of hearts shows an increase of ventricular wall thickness after TAC, which was attenuated by exogenous KCNE2 overexpression (Figure 7I). The protein levels of ANP and \(\beta\)-MHC exhibit a similar pattern in response to TAC with or without KCNE2 intervention (Figure 7J). These results collectively indicate the contribution of KCNE2 in the pathogenesis of hypertrophy. Meanwhile, our data also suggest that increasing KCNE2 expression can also...
attenuate TAC-induced cardiac fibrosis (Figure 7K and 7L) and myocyte apoptosis (Figure 7M and 7N).

Overexpression of KCNE2 Inhibited Calcineurin–NFAT and MAPK Pathways in TAC Mice

We next examined the possible involvement of calcineurin–NFAT and MAPK pathways in KCNE2-mediated attenuation of cardiac hypertrophic response. Consistent with previous studies,26,29,30 calcineurin–NFAT and MAPK pathways were activated in TAC hearts, where calcineurin activity and nuclear NFAT protein content (Figure 8A and 8B) and the phosphorylation levels of p38, ERK, and JNK were increased (Figure 8C). Overexpression of KCNE2 had no significant effect on calcineurin–NFAT and MAPK pathways at baseline, except that it slightly decreased the phosphorylation level of p38 in sham hearts (Figure 8A through 8C). However, overexpression of KCNE2 remarkably decreased calcineurin activity and nuclear NFAT protein level in TAC hearts (Figure 8A and 8B). Meanwhile, the phosphorylation levels of ERK, JNK, and p38 were also remarkably decreased by exogenous KCNE2 expression (Figure 8C).

Discussion

The major findings of this study are as follows: (1) KCNE2 expression was decreased in cardiac hypertrophy, which is a contributing factor in the development of hypertrophy; (2) overexpression of KCNE2 attenuated the development of phenylephrine-induced cardiomyocyte hypertrophy in vitro and pressure overload–induced cardiac hypertrophy in vivo; and (3) Ca²⁺-dependent calcineurin–NFAT and MAPK pathways participate in KCNE2-mediated hypertrophy. Together, these data uncover an important pathological role of KCNE2 in hypertrophic remodeling.

The pathological hypertrophic reprogramming represents a well-choreographed multiplicity of intracellular signaling pathways resulting in the hypertrophic phenotype. On hypertrophic stimuli, intracellular Ca²⁺ is increased to compensate for increased wall stress and facilitates systolic performance, which consequently activates hypertrophy-related signaling pathways and thus results in the development of pathological hypertrophy.31 In this study, we found that decreased KCNE2 induced cardiac hypertrophy by activating Ca²⁺/CaM-dependent calcineurin–NFAT signals. This was supported by the following findings: (1) knockdown of KCNE2 increased intracellular Ca²⁺ transients, calcineurin activity, and NFAT translocation to nuclear; (2) blocking intracellular Ca²⁺ increase with nifedipine or inhibiting calcineurin activity with FK506 attenuated the development of cardiomyocyte hypertrophy induced by knockdown of KCNE2; and (3) overexpression of KCNE2 in heart inhibited the activation of calcineurin–NFAT pathway in pressure overload–induced hypertrophy. Our previous study reported that KCNE2 regulates L-type Ca²⁺ channels by interacting with N-terminal inhibitory module of Ca v1.2.17 Knockdown of KCNE2 increases L-type Ca²⁺ current and thus intracellular Ca²⁺ level, which provides an explanation for the activation of calcineurin–NFAT pathway and consequently the development of hypertrophy.

In addition, our data suggest that a decrease in KCNE2 expression leads to activation of MAPK hypertrophic signaling pathway. It is known that 3 branches of the MAPK pathway, including MEK/ERK, JNK, or p38, seem to control distinct aspects of cardiomyocyte events. The MEK/ERK pathway has been shown to mediate compensatory hypertrophy without pathological features. Activation of JNK and p38 is involved in cardiac myocyte apoptosis and other pathological alterations, leading to dilated cardiomyopathy and HF. Interestingly, we found that knockdown of KCNE2 activated all 3 branches of MAPK pathway, and inhibiting each cascade attenuated cardiomyocyte hypertrophy. These data indicate that KCNE2-mediated cardiac hypertrophy...
Figure 7. Overexpression of KCNE2 attenuated transverse aortic constriction (TAC)–induced cardiac dysfunction. A, Representative M-mode tracings of echocardiography of mice at 6 and 8 wk of TAC or sham operation, with or without KCNE2 overexpression. B and C, Quantification of the thickness of left ventricular posterior wall at systole (LVPWs, B) and diastole (LVPWd, C). D and E, Quantification of systolic left ventricular internal dimension (LVIDs, D) and diastolic left ventricular internal dimension (LVIDd, E). n=10 to 12 mice per group. One-way ANOVA, Tukey honest significance difference (HSD) test was used. *P<0.05, **P<0.01 vs Sham. *P<0.05, **P<0.01 vs TAC. For analyzing the data at 6 and 8 wk, the general linear model (univariate) was adopted with animal as a random effect. &P<0.05, &&P<0.01 vs 6 wk in each group. F, Representative image of hearts in sham or TAC group with or without KCNE2 overexpression. G and H, Statistics of heart weight/body weight (HW/BW) ratio (G) and wet lung weight/body weight (LW/BW) ratio (H) in 4 groups (n=6–8 per group). I, Haematoxylin and Eosin (HE) staining of coronal sections of hearts from sham or TAC mice with or without KCNE2 overexpression. J, Representative Western blots and statistics of ANP (atrial natriuretic peptide) and β-MHC (β-myosin heavy chain) protein levels in 4 groups (n=6–8 mice in each group). One-way ANOVA, Tukey HSD test was used (Continued)
Role of KCNE2 in Cardiac Hypertrophy

Hypertrophy involves activation of MAPK pathway. It has been suggested that the 3 cellular signaling pathways, Ca\(^{2+}\), calcineurin, and MAPKs, share some degree commonality or interaction in the development of hypertrophy. MAPK may be activated by both \(\alpha\)- and \(\beta\)-adrenergic agonists via regulation of Ca\(^{2+}\) mobilization. Many studies have demonstrated that calcineurin–NFAT and MAPK pathways are interdependent, though the results are somewhat contradictory. For example, De Windt et al. have shown that calcineurin hypertrophic signaling is associated with stimulation of JNK, but not p38 and ERK1/2, whereas Sanna et al. have demonstrated that calcineurin–NFAT and MEK1/ERK1/2 signaling pathways are interdependent in cardiomyocytes, in which they directly coregulate the hypertrophic growth response. Presently, we have no direct evidence to support whether activation of MAPKs by decreased KCNE2 is related to increased intracellular Ca\(^{2+}\) or activation of calcineurin–NFAT or the direct effect of KCNE2 on MAPK cascades. These cellular processes might interweave and jointly contribute to KCNE2-mediated cardiac hypertrophy.

Finally, the observation that overexpression of KCNE2 attenuated the development of cardiac hypertrophy and inhibited calcineurin–NFAT and MAPK pathways provides further supporting evidence for the involvement of calcineurin–NFAT and MAPK pathways in decreased KCNE2-induced cardiac hypertrophy.

It is well established that KCNE2 plays an important role in maintaining the cardiac electric stability. Genetic mutations of KCNE2 have been linked to ventricular arrhythmia relating to long-QT syndrome. Previous study has demonstrated that decreased KCNE2 expression contributes to cardiac arrhythmia in infarcted cardiomyopathy in dog. Cardiac hypertrophy is an independent risk for arrhythmia. We postulate that change in KCNE2 in hypertrophy predisposes the heart to arrhythmia and overexpression of KCNE2 may also have an antiarrhythmic benefit. Further experiments are warranted to testify this notion.

In summary, this study uncovered a novel pathological role of KCNE2 in cardiac hypertrophic remodeling, where cardiac KCNE2 expression was decreased by hypertrophic stimuli.

**Figure 8.** Effects of overexpression of KCNE2 on calcineurin–NFAT (nuclear factor of activated T cells) and mitogen-activated protein kinase (MAPK) pathways in transverse aortic constriction (TAC) mice. A, Measurement of calcineurin activity in sham or TAC mice with or without KCNE2 overexpression. B, Representative Western blots and quantification of nucleus and cytosolic NFAT3 protein levels. C, Representative Western blots and quantification of extracellular signal-regulated kinase (ERK) and phosphorylated ERK, Jun kinase (JNK) and phosphorylated JNK, and p38 and phosphorylated p38. n=6 to 8 in each group. One-way ANOVA, Tukey honest significance difference (HSD) test. *P<0.05, **P<0.01 vs Sham; #P<0.05, ##P<0.01 vs TAC.
and consequently led to the development of hypertrophy via activation of calcineurin–NFAT and MAPK pathways. The ability of overexpression of KCNE2 to attenuate the development of hypertrophy underscores a novel therapeutic strategy for the treatment of cardiac hypertrophy by manipulation of KCNE2 expression.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Cardiac hypertrophy is an adaptive response most commonly to hypertension. It is an independent risk factor for serious conditions, including arrhythmias, heart failure, and sudden death. Although there has been progress in understanding the mechanisms underlying the development of hypertrophy, gaps still remain, and the therapeutic strategies are limited. KCNE2 is a promiscuous auxiliary subunit of voltage-gated cation channels and plays an important role in maintaining cardiac electric stability. Our previous work has established that KCNE2 modulates cardiac L-type Ca\(^{2+}\) current, suggesting its potential role in Ca\(^{2+}\)-related structural and functional remodeling in diseased hearts. Here, we demonstrate that the expression of cardiac KCNE2 is decreased in phenylephrine-mediated cardiomyocyte hypertrophy and transverse aortic constriction–induced mouse cardiac hypertrophy, and in human with dilated cardiomyopathy. Knockdown of KCNE2 reproduces cardiomyocyte hypertrophy by activating Ca\(^{2+}\)-dependent calcineurin–NFAT (nuclear factor of activated T cells) and mitogen-activated protein kinase signaling pathways. Particularly, adenoviral-mediated KCNE2 overexpression in cardiomyocytes suppressed phenylephrine-induced cardiomyocyte hypertrophy, and ultrasound-microbubble–mediated KCNE2 overexpression in mouse heart attenuated transverse aortic constriction–induced cardiac hypertrophy. Together these data indicate a critical role of KCNE2 in the development of cardiac hypertrophy and reinforce the therapeutic potential of targeting KCNE2 in the treatment of cardiac hypertrophy.
Decreased KCNE2 Expression Participates in the Development of Cardiac Hypertrophy by Regulation of Calcineurin–NFAT (Nuclear Factor of Activated T Cells) and Mitogen-Activated Protein Kinase Pathways

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SUPPLEMENTAL MATERIAL

Materials and Methods

Culture of neonatal rat ventricular myocytes (NRVMs) and treatment protocol

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-3-day-old Sprague-Dawley rats and cultured as previously described. Briefly, hearts were obtained following decapitation and immersed in PBS and minced with scissors. The small pieces of heart tissue were digested with 0.25% Trypsin–EDTA in PBS at 37 °C. The isolated cells were put in fetal bovine serum (FBS) and pelleted by centrifugation at 1000 rpm for 5 min. The pelleted cells were resuspended in warm DMEM containing 5% FBS, 1% penicillin–streptomycin and then preplated for 30 min at 37 °C to allow fibroblasts to adhere to the plate. The un-adhered cells were pelleted again and resuspended in DMEM containing 5% FBS, 1% penicillin–streptomycin and bromodeoxyuridine (1:100, to inhibit fibroblast growth), which were finally plated at a concentration of about 1 million cells /35mm plate. To produce hypertrophy, NRVMs were treated with 100 μM phenylephrine (PE, Sigma) for 24 h after 24 h of serum starvation.

Measurement of cell surface area

The surface area of a single myocyte was measured with Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, MD). Quantification of cell surface area was performed by measuring 50 random cells from three experiments, and the average value was used for analysis.

For cell size analysis, NRBCM were cultured on glass coverslips treated with 0.1% gelatin. After the indicated treatment, cells were washed once with PBS and fixed in ice cold methanol for 10 min and then blocked with 1% goat serum for 30 min at room temperature. Cardiomyocytes were labelled with sarcomeric α-actinin (Sigma) followed by incubation with goat anti-mouse FITC-conjugated secondary. DAPI was used as a nuclear counterstain, and images were captured with the Zeiss LSM780. The surface area of a single myocyte was measured with Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, MD). Quantification of cell surface area was performed by measuring 80 random cells from three experiments, and the average value was used for analysis.

Western Blot Analysis

Total cellular extracts from NRVMs were lysed in cell lysis buffer. The supernatant was
ultracentrifuged and equal amount of proteins were loaded, separated on 10% SDS-PAGE before being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Nuclear protein extraction was performed using a Nuclear and Cytoplasmic Protein Extraction kit according to the manufacturer’s instruction (Pierce, Thermo Scientific). Ventricular tissue proteins were obtained by homogenizing with lysis buffer. Target proteins were reacted with their respective antibodies, ANP (Santa Cruz Biotechnology), β-MHC (Santa Cruz Biotechnology), calcineurin (Abcam), LaminA/C, NFAT3, AMPK, p-AMPK, ERK, p-ERK, JNK, p-JNK, p38, p-p38 (Cell Signaling Technology), and then incubated with respective secondary antibody. β-Actin was used as a loading control for ANP and calcineurin proteins, and LaminA/C as a loading control for NFAT3 nuclear protein. Bound antibodies were visualized using the enhanced chemiluminescence (ECL) detection kit (Invitrogen).

**Measurement of Ca^{2+} transient**

Cardiomyocytes loaded with Ca^{2+} indicator fluo-4 AM (5 mmol/l, for 8 min) (Invitrogen) were placed in a recording chamber. Confocal line-scan imaging was carried out in cells at 488 nm excitation and 505 nm collection with a Zeiss 710 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany) with 40 X oil immersion lens (NA 1.3). After the myocytes were stimulated with field stimulation (1 Hz) to reach a steady state, confocal line-scan imaging was acquired at a sampling rate of 3.84 ms per line.

**Measurement of calcineurin activity**

Calcineurin activity was determined in cardiomyocyte extracts using a colorimetric Calcineurin Cellular Activity Assay Kit following the manufacturer’s instructions (Enzo Life Sciences). Calcineurin activity was determined as nanomoles phosphate released at 620 nm using a SpectraMax 5 (Molecular Devices, Sunnyvale, CA) plate reader.

**Animal Model of Hypertrophy with Transverse aortic constriction (TAC)**

C57BL/6 male mice (9-10wk, 18-22 g) were randomly divided into four groups: sham and TAC with or without KCNE2 overexpression, 12 in each group. The TAC model was produced as described previously. In brief, the animal was endotracheally intubated under anesthesia with a mixture of pentobarbital sodium (50 mg/kg ip). The chest cavity was entered in the second intercostal space at the left upper sternal border, and the transverse aorta between the carotid arteries was isolated and constricted by a 7-0 silk suture ligature tied firmly against a 26-gauge
needle. The latter was promptly removed to yield a constriction of 0.45 mm in diameter. Sham-operated mice underwent a similar surgical procedure without constriction of the aorta.

**Two-dimensional guided M-mode echocardiography**

Two-dimensional (2-D) guided M-mode echocardiography was performed in anesthetized mice (with 1.5% isoflurane) using a Vevo 2100 system (VisualSonics, Toronto, Ontario, Canada). The heart was imaged in the 2-D mode in the parasternal short-axis view. From this view, the following parameters were measured: percentage of left ventricular (LV) fractional shortening (LVFS), LV ejection fraction (EF), LV internal dimensions at both diastole and systole (LVIDd and LVIDs, respectively), and LV posterior wall dimensions at both diastole and systole (LVPWd and LVPWs, respectively). All measurements were done from leading edge to leading edge according to the American Society of Echocardiography guidelines. The percentage of LVFS (%) was calculated as \( \frac{(\text{LVIDd} - \text{LVIDs})}{\text{LVIDd}} \times 100 \), and EF (%) was calculated as \( \frac{(\text{LVIDd}^2 - \text{LVIDs}^2)}{\text{LVIDd}^2} \times 100 \).

**Ultrasound-microbubble-mediated gene transfer of the inducible KCNE2**

Following the protocol as previously described, a mixture of doxycycline-regulated pTRE2-Flag-M2-KCNE2-expressing plasmids was prepared and transferred into the mouse heart using the non-invasive ultrasound-microbubble-mediated technique. Briefly, pTRE2-Flag-M2-KCNE2 and Tet-on plasmids (100 mg/mouse) were mixed with Sonovue (Bracco Diagnostics, Princeton, NJ, USA) in a 1:1 ratio (volume:volume). Then the mixture (400 mL) was injected into mice via tail vein, followed by ultrasound treatment (2 W/cm²) by placing the ultrasound probe on the chest skin over the heart with a plus-wave output for a total of 5 min with 30 s intervals. After ultrasound treatment, 200 mg/mL of doxycycline (Sigma, St Louis, MO, USA) was injected intraperitoneally, followed by the addition of doxycycline in the daily drinking water (200 mg/mL) for the entire experimental period. Control animals had the same protocol but received the Tet-on/pTRE2 empty vectors without KCNE2. KCNE2 gene transfer was at the same time of TAC model operation and repeated on day 14.
Fig. S1

α-actinin Merge DAPI

100 µm
A

C<sub>Sh</sub> K<sub>Sh</sub>

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<th>K&lt;sub&gt;Sh&lt;/sub&gt;</th>
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<tr>
<td>α-AMPK</td>
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B

p-α-AMPK/α-AMPK protein abundance

C<sub>Sh</sub> K<sub>Sh</sub>

Fig.S3
Fig S4

A

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<tr>
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B

![Bar Chart](#)

p-Akt/Akt protein abundance

CSh

KSh

Fig. S4
Fig. S5

A

B

FS (%)  

LVEF (%)

Sham  

TAC  

KCNE2  

KCNE2+TAC

0% 10% 20% 30% 40% 50% 60%

6W 8W

0% 10% 20% 30% 40% 50% 60%

6W 8W

Fig. S5
Figure legends

**Figure S1.** Representative images of cultured neonatal rat ventricular myocytes (NRVM) immunostained with sarcomeric α-actinin (green). DAPI (blue) was used as a nuclear counterstain.

**Figure S2.** Effect of KCNE2 knockdown on the expression of calcineurin. A) and B) Representative immunoblots (A) and average data of calcineurin (B) in control (CSh) and KCNE2 knockdown (KSh) cells with or without pretreatment of nifedipine or FK506 (n=4/group).

**Figure S3.** Effect of KCNE2 knockdown on AMPK phosphorylation. A) and B) Representative images (A) of the proteins of α-AMPK and phosphorylated α-AMPK (p-α-AMPK), and statistics (B) of the ratio of P-α-AMPK to α-AMPK (n=4/group).

**Figure S4.** Effect of KCNE2 knockdown on Akt phosphorylation. A) and B) Representative images (A) of the proteins of Akt and phosphorylated Akt at Thr308 (P-Akt308), and statistics (B) of the ratio of p-Akt to Akt (n=4).

**Figure S5.** Effects of overexpression of KCNE2 on cardiac function in sham and transverse aortic constriction (TAC)-induced hypertrophy. A) and B) quantification of left ventricular fraction shortening (FS, A) and left ventricular ejection fraction (LVEF, B). n=6-8 mice in each group. Data were analyzed with one-way ANOVA, followed by Tukey’s HSD test. **P < 0.01 vs. Sham. For analyzing the data at 6w and 8w, General Linear Model (univariate) was adopted with animal as a random effect. ^&^P < 0.01 vs. 6w in each group.