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The δA isoform of calmodulin kinase II mediates pathological cardiac hypertrophy by interfering with the HDAC4-MEF2 signaling pathway

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ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a new promising target for prevention and treatment of cardiac hypertrophy and heart failure. There are three δ isoforms of CaMKII in the heart and previous studies focused primarily on δB and δC types. Here we report the δA isoform of CaMKII is also critically involved in cardiac hypertrophy. We found that δA was significantly upregulated in pathological cardiac hypertrophy in both neonatal and adult models. Upregulation of δA was accompanied by cell enlargement, sarcomere reorganization and reactivation of various hypertrophic cardiac genes including atrial natriuretic factor (ANF) and β-myocin heavy chain (β-MHC). Studies further indicated the pathological changes were largely blunted by silencing the δA gene and an underlying mechanism indicated selective interference with the HDAC4-MEF2 signaling pathway. These results provide new evidence for selective interfering cardiac hypertrophy and heart failure when CaMKII is considered as a therapeutic target.

1. Introduction

Heart failure remains a leading cause of death and disability in industrialized society and cardiac hypertrophy is a promising target for prevention and treatment of heart failure. A number of signaling pathways have been identified mediating pathological cardiac hypertrophy [1,2]. Among others, dysregulation of various protein kinases and phosphatases is known to be central to hypertrophic remodeling followed by cell death, heart failure and cardiac arrhythmias [3,4]. Many prohypertrophic kinases such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII), protein kinase D1 (PKD1), and Ca^{2+}-activated phosphatase calcineurin (CaN), are involved in cardiac hypertrophy by sensing increased intracellular Ca^{2+}. Downstream, CaN dephosphorylates the nuclear factor of activated T-cell (NFAT), followed by its nuclear entry. In parallel, the class II histone deacetylases (HDACs) were phosphorylated by CaMKII and PKD1, and the phosphorylated HDAC45 disinhibits the inert cardiac genome and activates myocyte enhancer factor-2 (MEF2) [1,2,4]. Activation of the CaN-NFAT and/or HDAC45-MEF2 signaling pathways initiates hypertrophic genomic reprogramming.

The critical role of CaMKII δ-isoforms in cardiac hypertrophy and failure has been well documented, but the major isoforms extensively investigated are δB and δC [5–7]. These two isoforms have a broad spectrum of substrates including phospholamban (PLB), ryanodine receptors, HDACs, and the L-type calcium channels [3,4]. However, CaMKIIδ has an additional isoform termed δA, which is mainly expressed in neonatal cardiac myocytes. Whether δA plays any role in adult cardiac hypertrophy and heart failure remains elusive. A recent study indicated CaMKIIδA is significantly over-expressed along plasma membranes and T-tubules and is required for cardiac hypertrophy in ASF/SF2-deficient animals [8]. These results suggest CaMKIIδA could play an important role in adult cardiac hypertrophy. Here we report CaMKIIδA was significantly upregulated in cardiac hypertrophy in both neonatal and adult models. Upregulation of CaMKIIδA was accompanied by reactivation of fetal cardiac genes such as ANF and β-MHC.

Abbreviations: CaMKII, Ca^{2+}/calmodulin-activated kinase II; HDAC, histone deacetylase; MEF2, myocyte enhancer factor-2; CaN, calcineurin; NFAT, nuclear factor of activated T-cell; PKD, protein kinase D; ANF, atrial natriuretic factor; βMHC, β-myosin heavy chain; NRVM, neonatal rat ventricular myocytes; ARVM, adult rat ventricular myocytes; siRNA, small RNA interference; qPCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISO, isoproterenol.

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These pathological changes were largely blunted by silencing the δA gene and an underlying mechanism indicates selective interference with the HDAC4-MEF2 signaling pathway.

2. Materials and methods

2.1. Isolation and culture of ventricular myocytes

Sprague–Dawley rats were used throughout experiments and purchased from the Military Academy of the Medical Science Laboratory Animal Center (Beijing, China). The principles governing the care and treatment of animals as described by the American Physiological Society were followed at all times during this study. Isolation of adult rat ventricular myocytes (ARVM) was performed by Langendorff perfusion with a buffer containing low Ca^{2+}, collagenase and protease as described in our previous papers [9,10]. Isolation and culture of neonatal rat ventricular myocytes (NRVM) were conducted using the overnight trypsin–collagenase digestion method as described [9,10]. All experiments with NRVMs were performed on 2–4 d cultures when synchronously contracting cells were observed. The purity of the cardiomyocytes was confirmed by anti-α-actin antibody.

2.2. Small interfering RNAs (siRNA)

siRNA was performed using the standard method as described in our recent publication [11]. Two siRNA sequences were designed to target the coding region of CaMKIIδA: 5′-AGCCAACGUGGUAACCAGCTT-3′ and 5′-GCUGGUUACCACGUUGGCUTT-3′. The choice of these sequences was based on high silencing efficacy as verified by reverse transcription-polymerase chain reaction (RT-PCR). A scrambled siRNA 5′-UUCUCCGAACGUGUCACGUTT-3′ was used as negative control (NC). All nucleotides were chemically synthesized and 2′ *O*-methyl modified by GenePharma Co. (Shanghai, China). Transfection efficiency was estimated using FAM-conjugated NC siRNA and defined as the percentage of FAM-positive cells of the propidium iodine (PI) positive cells. For transient transfection, cells were incubated for 6 h in transfection medium comprising serum-free DMEM, 2 mM glutamine supplemented with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were retained in incubation medium containing serum-free DMEM, 2 mM glutamine, 1× ITS Liquid Media Supplement (Sigma–Aldrich) and penicillin/streptomycin for 36 h. This was followed by treatment with N.S. vehicle or isoproterenol (ISO, 10 μM) for 24 h.

2.3. RT-PCR and real-time PCR (qPCR)

Total RNA was isolated from heart tissue or ventricular myocytes using Trizol reagent (Invitrogen). For cDNA synthesis 1.0 μg RNA was used and reactions were carried out using a reverse transcription system (Promega). PCR was performed in a Genemate thermal cycler (Jinge Instr, Hangzhou, China) with the following primer sets for CaMKIIδA: forward, 5′-CGAGAAATTTTTCAGCAGCC-3′; reverse, 5′-ACAGTAGTTTGGGGCTCCAG-3′. 18-S ribosomal RNA (18-S rRNA) was used as an internal control and the primer sets used are: forward, 5′-ACCGCAGCTAGGAATAATGGA-3′; reverse, 5′-GCCTCAGTTCCGAAAACCA-3′. PCR products were subjected to 1.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized with LAS3000.

qPCR was performed using SYBR Green Master Mix (Takara, Japan) in a Bio-Rad IQ5 detection system as described [12]. The primer sets for each gene are as follows: ANF (forward, 5′-GG GGGTAGGATTGACAGGAT-3′; reverse, 5′-CTCCAGGAGGGTATTCA CCA-3′); β-MHC (forward, 5′-CCTCGCAATATCAAGGGAAA-3′; reverse, 5′-TACAGGTGCATCA GCTCCAG-3′); MEF2 (forward, 5′-GA GCAGAGCCCCCTGCTGGAGGACA-3′; reverse, 5′-TAGCAGGCCGC TGGGGCAGGCCCGG-3′). 18-S rRNA was used as internal standard. The cycle threshold (CT) values corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above the baseline emission were automatically determined in triplicates and averaged. Abundances of each target gene was normalized to that of 18-S rRNA using the formula of $2^{-\Delta CT}$, where ΔCT = CT of target genes – CT of 18-S rRNA. The ratios were expressed as –fold changes when compared with saline controls.

2.4. Western blotting

Cells were lysed in RARI buffer (50 mM Tris, pH 7.4, 1.0 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate). Cell lysates were resolved in 10% SDS–PAGE and transferred to PVDF membranes (Millipore) as described previously [9]. HDAC4 and HDAC4-p proteins were reacted with polyclonal anti-HDAC4 or anti-HDAC4-p (Santa cruz) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control with appropriate antibody (Santa cruz). Band density was quantified using ImageJ (The US National Institutes of Health, http://rsb.info.nih.gov/ij/) where required.

2.5. Indirect immunofluorescence

Indirect immunofluorescent staining was conducted essentially the same as described previously [9,11]. Heart cells were grown on laminin-coated glass coverslips and fixed in 4% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. After blocking in 1% BSA-containing PBS, cells were incubated with primary antibody and subsequently with secondary antibodies (Invitrogen). Images were collected and analyzed by TCS-SP confocal laser microscopy (Leica, Germany). For surface area determination ImageJ was used and at least 50 individualized cells were analyzed for each experiment.

2.6. NFAT–luciferase assays

Neonatal myocytes were co-transfected with NFAT reporter plasmid pGL4.30[luc2P/NFAT-RE/Hygro] and control plasmid pGL7.4 expressing *Renilla reniformis* luciferase reporter gene (Promega) in the presence or absence of NC siRNA or CaMKIIδA siRNA. Transient transfection was performed with the electroporation nucleofector kit (AMAXA Biosystems). After 36 h incubation in a 12-well plate, cells were treated with N.S. or 10 μM ISO for further 48 h. Cells were then lysed and a luciferase assay was performed with the dual luciferase kit (Promega) according to the manufacturer's instructions. Luciferase activity was measured using a TR717 microplate luminometer (Applied Biosystems). Data were expressed as fold change (= average relative light units of induced cells/average relative light units of control cells).

2.7. In vivo cardiac hypertrophic model and immunohistochemistry

In vivo cardiac hypertrophy was induced by chronic ISO injection as described [13]. For histology, paraffin-embedded heart tissue sections (4 μm in thickness) were made and stained with standard HE or immunohistochemistry techniques.

2.8. Statistical analysis

Results are expressed as means ± SEM. One-way ANOVA followed by Newman–Keuls test or Student's *t* test was performed as implemented in IgorPro (Wavemetrics, Portland, OR) as described [14]. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. CaMKIIδA is overexpressed in various cardiac hypertrophy models

Previous studies have shown both δB and δC isoforms of CaMKII are critical to cardiac hypertrophy [5,15] but the role of the δA isoform in this process is unclear. To investigate function of the latter we first determined how the three isoforms were expressed in isolated adult rat myocytes (ARVM) and cultured neonatal myocytes (NRVM) using RT-PCR techniques. As shown in Fig. 1A, neonatal cells expressed all three isoforms while adult cells do not have appreciable amounts of CaMKIIδA. Surprisingly, CaMKIIδA was markedly over-expressed in both NRVM and ARVM after incubation with isoproterenol for 24 h, classic *in vitro* cardiac hypertrophy models (Fig. 1B).

We further demonstrated CaMKIIδA was up-regulated in a whole animal hypertrophy model. We induced cardiac hypertrophy by injecting rats with ISO for 5 d and animals were sacrificed on day 7 for examination. Compared to normal saline-injected animals, ISO-treated rats developed typical hypertrophy as evidenced by enlarged heart size (Fig. 1C), over-expression of hypertrophic genes such as ANF and βMHC (see later), and nuclear entry of NFAT (see later). As expected, ISO-treated animal hearts showed significant over-expression of CaMKIIδA (Fig. 1D and E). Thus, CaMKIIδA is upregulated in cardiac hypertrophy of both neonatal and adult models.

3.2. Upregulation of CaMKIIδA is accompanied by reactivation of fetal cardiac genes

A salient feature of pathological cardiac hypertrophy is reactivation of fetal cardiac genes such as ANF and βMHC [2,16]. We investigated the relationship between CaMKIIδA upregulation and fetal cardiac gene reactivation. Using indirect immunofluorescent techniques, we found ISO induced a strong fluorescent signal for ANF along membrane and T-tubule contours in isolated adult myocytes (Fig. 2A). Furthermore, in the ISO-injected animals both ANF and βMHC mRNAs were increased by ~30-fold and ~25-fold, respectively, in the hypertrophied hearts as detected with qPCR (Fig. 2B). These results demonstrate upregulation of CaMKIIδA in adult cardiac hypertrophy models, is accompanied by reactivation of fetal cardiac genes.

We further determined whether these upregulated hypertrophic genes were inhibited by gene silencing of CaMKIIδA. Because isolated adult myocytes and whole animals are not feasible for molecular interference, we chose to use neonatal myocytes as an alternative testing model. Depletion of the δA gene was achieved using siRNA. In our system we could achieve 80% transfection efficiency and δA expression was significantly inhibited even after 72 h. Although <2% cells cultured in regular medium plus NC siRNA for 48 h showed a faint ANF signal as detected with indirect immunofluorescence, nearly 60% cells expressed the protein after incubation with ISO plus NC siRNA for 48 h (Fig. 2C). By contrast, in cells pretreated with siRNA against δA, this effectively prevented the appearance of ANF induced by ISO (Fig. 2C). We further quantified ANF at the mRNA level using qPCR. ISO induced ~6-fold increase in ANF at 48 h and this effect was blunted by pretreatment with siRNA against δA (Fig. 2D). Qualitatively similar results were obtained when βMHC, another well-known hypertrophic marker gene, was compared (Fig. 2D). These results demonstrate reactivation of various fetal cardiac genes requires concomitant CaMKIIδA upregulation.

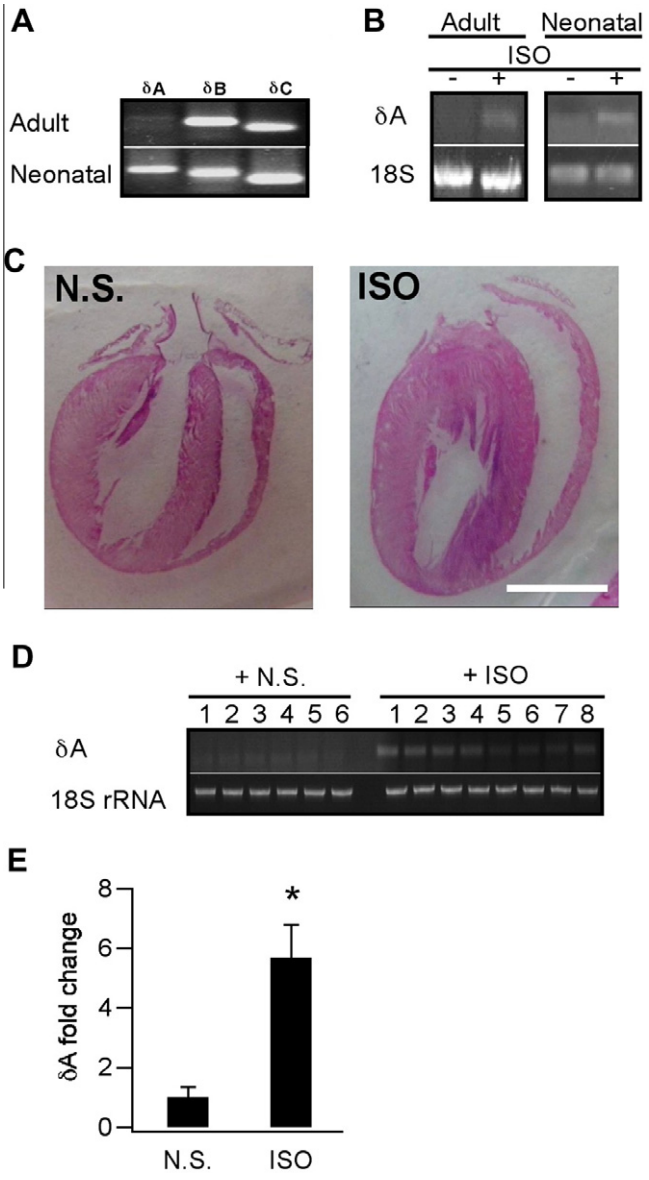

Fig. 1. The δA isoform was upregulated in various cardiac hypertrophy models. (A) mRNAs of three CaMKII δ-isoforms were detected using RT-PCR in isolated adult (upper) and cultured neonatal (lower) myocytes. δA was not detectable in adult cells. (B) CaMKIIδA mRNA in adult and neonatal myocytes was upregulated by ISO (10 μM) treatment for 24 h. 18-S rRNA was used as an internal control. (C) Representative four-chambered heart pictures showing typical cardiac hypertrophy induced by 5-d injection of ISO (5 mg kg^{-1} d^{-1}, i.p.). The hearts were fixed, paraffin-embedded, sectioned and stained by the H&E method. Scale bar = 1 cm. (D) CaMKIIδA mRNA (upper) from ISO-injected animal's heart tissue ($n = 8$) was upregulated compared to that of NS-injected ones ($n = 6$). 18-S rRNA was used as a loading control (lower). (E) Quantitative analysis of data shown in (D). Band density of CaMKIIδA for each sample was determined using ImageJ and normalized to their respective 18-S rRNA values. ($n = 6$–8, $^*p < 0.01$, Student's *t* test).

3.3. Silencing CaMKIIδA prevents cell enlargement

In addition to genomic reprogramming, cardiac hypertrophy is manifested by cell enlargement and sarcomere reorganization

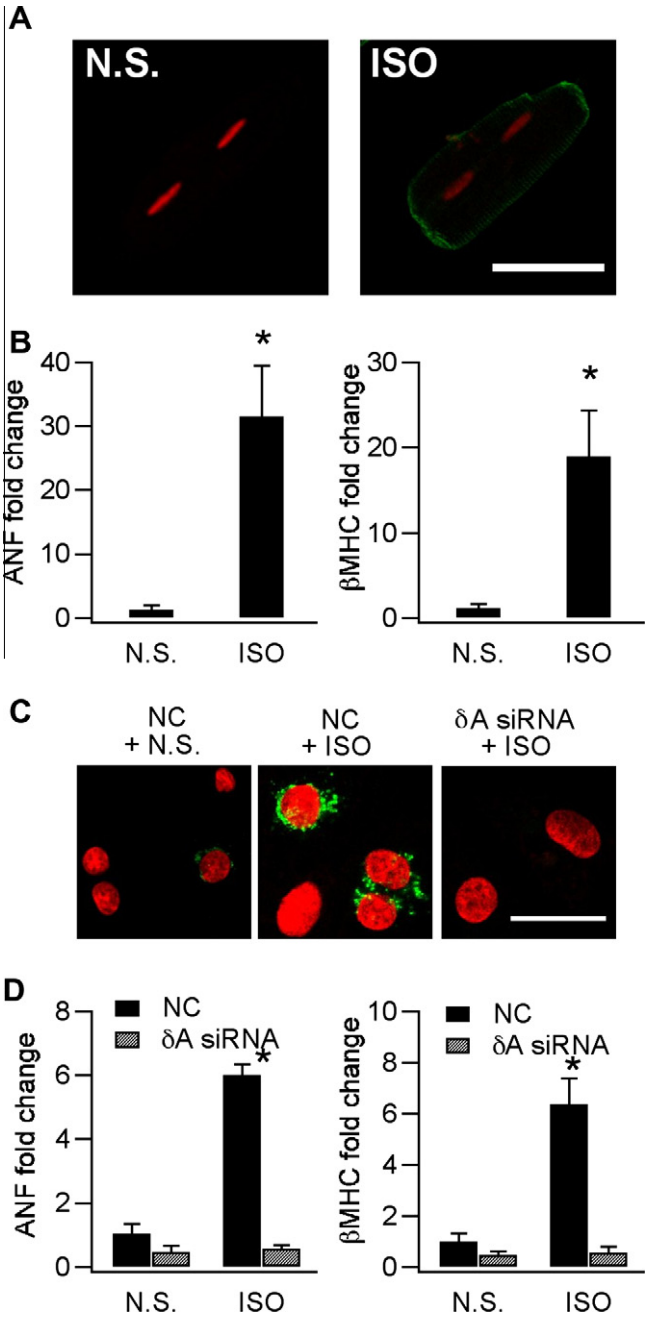

Fig. 2. Reactivation of fetal cardiac genes was blunted by CaMKIIδA silencing. (A) Induction and immunolocalization of ANF protein in isolated adult myocytes treated by ISO (2 μM) for 48 h. For immunofluorescence cells were first incubated with an anti-ANF antibody followed by reaction with Alexa-488 conjugated second antibody (green). Propidium iodine (PI) was used as counterstain for nuclei (red). Scale bar = 50 μm. (B), Upregulation of ANF and βMHC mRNAs in the hearts from ISO-injected animals. mRNAs were analyzed by qPCR and normalized to 18-S rRNA. The mean normalized value for expression of each gene in NS-injected animals is defined as 1. (n = 3–4, $^*p < 0.01$, Student's t-test). (C) Induction and immunolocalization of ANF protein in cultured neonatal myocytes by ISO (10 μM) for 48 h. Note the ANF signal is primarily located in the perinuclear area. Scale bar = 20 μm. (D) qPCR assays showing both ANF and βMHC mRNAs were upregulated by ISO (10 μM) application for 48 h and these effects were blunted by δA siRNA. (for both panels n = 3, $^*p < 0.01$ vs NC/NS group, ANOVA).

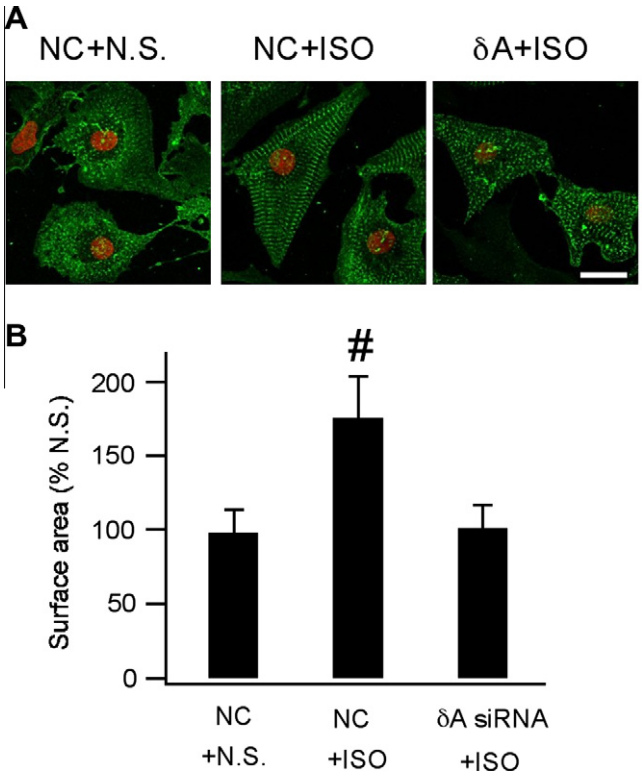

Fig. 3. Morphological alterations were prevented by CaMKIIδA silencing. (A) confocal images of neonatal myocytes with various treatments as indicated. Cells were treated with N.S. or ISO for 48 h and incubated with anti-α-actinin followed by reaction with Alexa-488 conjugated second antibody (green). Propidium iodine was used as counterstain for nuclei (red). Scale bar = 50 μm. Note the large cell size and sarcomere reorganization induced by ISO treatment (middle). These alterations are largely prevented by pretreatment with δA siRNA (right). (B) Quantitative analysis of data shown in (A). At least 100 cells were quantified for each group and experiments were repeated twice. (n = 3, $\#p < 0.05$, ANOVA).

[2]. We next determined how the cell morphology is modulated by manipulation of δA in the neonatal hypertrophic model. We employed α-actinin immunofluorescence as a sarcomere marker and cell surface area as a measure of size. Results from these experiments indicated apart from induction of obvious sarcomere reorganization (Fig. 3A), 48-h incubation with ISO increased the cross-sectional areas by ~75% (Fig. 3A and B). This effect was prevented by pre-treatment with siRNA against δA (Fig. 3A and B). Thus, we reveal that silencing CaMKIIδA is sufficient to prevent ISO-induced myocardial hypertrophy.

3.4. Silencing CaMKIIδA blunts HDAC4-mediated signaling pathway

Finally we investigated how CaMKIIδA participates in the pathogenesis of cardiac hypertrophy. Although many hypertrophic stressors exist that induce cardiac hypertrophy, two general mechanisms seem to operate. The first involves the transcription factor NFAT family [17,18]. These proteins are normally phosphorylated and located in the cytoplasm where it is sequestered by the buffering protein 14-3-3. Upon dephosphorylation by CaN activated by various hypertrophic stimulus, NFAT enters the nucleus and initiates hypertrophic responses by further recruiting other hypertrophic transcription factors such as GATA-4 and Nkx2.5 [19]. In our whole animal hypertrophic model induced by ISO, we found prominent NFATc4 accumulation in the nucleus (Fig. 4A) as detected using immunohistochemistry methods along with up-regulation of ANF and β-MHC (Fig. 2B) at the mRNA level. GATA-4, a hypertrophic gene closely related to NFAT activation was also significantly upregulated (Fig. 4B). However, when a neonatal hypertrophic model was used to test the effect of δA silencing, we noticed δA silencing exacerbated NFAT transcription activity as estimated using a NFAT–luciferase gene reporter assay (Fig. 4C). Thus, we

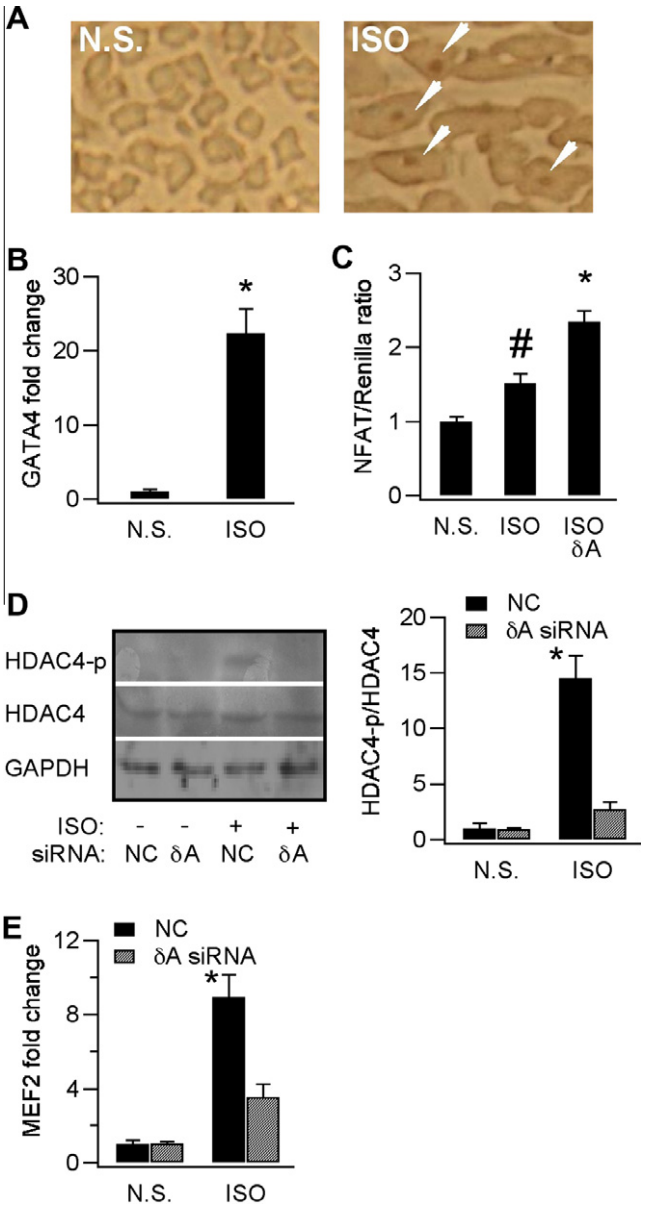

Fig. 4. CaMKIIdA silencing blunted activation of HDAC4-MEF2 signaling. (A) *In situ* detection of nuclear NFATc4 protein (arrows in right panel) using immunohistochemistry in ISO-induced hypertrophy models. In NS-injected animals, no appreciable NFATc4 signal was revealed (left). Note the larger cell size in right panel. (B) qPCR assay showing GATA-4 mRNA was significantly amplified in heart tissues of ISO-treated animals ($n = 6$, $^{*}p < 0.01$, Student's t test). (C) NFAT-luciferase assay showing NFAT-mediated transcription activity was increased by ISO alone and further enhanced by CaMKIIδA silencing in neonatal myocytes. ($n = 3$, $\#p < 0.05$ vs N.S.; $^{*}p < 0.05$ vs ISO alone, ANOVA). (D) Western blot showing profound phosphorylation of HDAC4 by 48-h ISO incubation. CaMKIIδA silencing prevented HDAC4 phosphorylation evoked by ISO (left). Plotted on the right is quantitative analysis of data shown in the left panel. Band density was determined and analyzed using ImageJ. ($n = 4$, $^{*}p < 0.01$ vs NC/NS group, ANOVA). (E) qPCR assay showing MEF2 mRNA was upregulated by ISO incubation for 48 h and this effect was blunted by CaMKIIδA silencing. ($n = 4$, $^{*}p < 0.01$ vs NC/NS group, ANOVA).

conclude the pro-hypertrophic effect of CaMKIIδA may be not mainly by interfering with the NFAT-mediated signaling pathway.

We then turned to the other hypertrophic mechanism involving HDAC-MEF2 signaling [20]. The class II HDACs (4,5,7,9) normally remain associated with the cardiac genome, keeping the latter in an inert state. When phosphorylated by HADC kinases such as CaMKII and PKD1, HDACs exit the nucleus and the relatively inactive cardiac genome becomes transcriptionally active. Activation of MEF2 followed is essential for the HDAC-mediated hypertrophy. Previous studies established that HDAC4 but not HDAC5 is the major isoform of HDACs affected by ISO. As expected, ISO-treated neonatal cells presented a heavily phosphorylated HDAC4 subtype (Fig. 4D) accompanied by significant upregulation of MEF2 mRNA (Fig. 4E). Both of the ISO-induced pro-hypertrophic effects were blunted by pre-silencing treatment with δA siRNA (Fig. 4D and E). These results clearly demonstrate silencing CaMKIIδA prevents cardiac hypertrophy by dominant interference of the HDAC4-MEF2 signaling pathway.

4. Discussion

We have provided clear evidence showing that, in addition to δB and δC, CaMKIIδA is also critically involved in cardiac hypertrophy in both neonatal and adult models. The major immediate target for δB isoform is HDAC4 [21,22] while that for δC remains uncertain because the latter has a broad cytoplasmic distribution [15]. Although we showed here that the pro-hypertrophic effect of CaMKIIδA relies on its predominant interference of HDAC4-MEF2 signaling, the immediate molecular target does not seem to be HDAC itself because δA is chiefly membrane- and T-tubule-located [8]. Because L-type calcium channels are located in T-tubules and are well-known for their critical involvement in cardiac hypertrophy and heart failure [23,24] we suspect the channels may be an immediate target for δA. This possibility requires further investigation.

An unexpected finding in this study was that CaMKIIδA negatively regulates the NFAT signaling pathway. A similar finding was also reported with CaMKII in cardiac hypertrophy models although which particular isoform is responsible for such phenomenon remains uncharacterized [25]. The significance of the negative hypertrophic regulation is unknown but may represent a self-protection mechanism against hypertrophy. However, the self-defense mechanism if any is so weak that it could not overcome the pro-hypertrophic effect exerted by the more powerful HDAC4-MEF2 activation. Thus, non-selective inhibition of pan-CaMKIIδ may be detrimental to cardiac hypertrophy and heart failure.

Since both δB and δC isoforms are constitutively expressed in normal adult hearts [5–7], any therapeutic measure targeting these molecules may come up with serious side-effects. In this regard measures directed at the δA isoform apparently have superiority because the latter is only upregulated in diseased hearts. Nanoparticle-mediated siRNA techniques [26], for example, may be implemented as a novel selective therapy toward cardiac hypertrophy and heart failure.

Acknowledgments

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