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Calponin1 inhibits dilated cardiomyopathy development in mice through the εPKC pathway ☆

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ABSTRACT

Background: Calponin1 (CNN1) is involved in the regulation of smooth muscle contraction in physiological situation and it also expresses abnormally in a variety of pathological situations. We found that the expression of CNN1 decreased significantly in the heart tissue of a $cTnT^{R141W}$ transgenic dilated cardiomyopathy (DCM) mouse model and an adriamycin (ADR)-induced DCM mouse model, suggesting that CNN1 is involved in the pathogenesis of DCM. However, the role of CNN1 on cardiac function, especially on pathogenesis of DCM, has not been clarified. In this study, we tested whether rescued expression of CNN1 could prevent the development of DCM and investigated its possible mechanisms.

Methods and results: The DCM phenotypes were significantly improved with the transgenic expression of CNN1 in the $cTnT^{R141W} \times$ CNN1 double transgenic (DTG) mice, which was demonstrated by the survival, cardiac geometry and function analyses, as well as microstructural and ultrastructural observations based on echocardiography and histology examination. The expression of CNN1 could also resist the cardiac geometry breakage and dysfunction in the ADR-induced DCM mice model. Meanwhile, the epsilon isoform of protein kinase C (εPKC) activator and inhibitor could reverse the activation of εPKC/ERK/mTOR pathway and DCM phenotypes in the $cTnT^{R141W}$ and $cTnT^{R141W} \times$ CNN1 double transgenic (DTG) mice.

Conclusions: εPKC/ERK/mTOR pathway activation induced by the rescued expression of CNN1 contributed to the improvement of cardiac dysfunction and pathological changes observed in the DTG mice. CNN1 could be a therapeutic target to prevent the development of DCM and heart failure (HF).

1. Introduction

Calponin1 (CNN1, also called basic calponin or calponin h1) is a 34-kDa troponin-like molecule that was originally isolated from the smooth muscles of bovine aortas and chicken gizzards and was indicated later to be present in most vertebrate smooth muscles. CNN1 binds to actin, tropomyosin, and calmodulin and is involved in the regulation of smooth muscle contraction in physiological situation [1–6]. CNN1 expresses abnormally in a variety of pathological situations, including abnormal gastrointestinal motility and multiple tumors [7–13]. The bone formation is increased which associated with enhancement of bone morphogenetic protein responses in mice lacking smooth muscle CNN1 [14]. CNN1 is not only involved in smooth muscle contraction, but also function as a integrating molecule in signal transduction that interacts with the proteins including ERK1/2, p160Rho kinase, protein kinase C (PKC) α and PKC ε [6,15–19]. CNN1 plays a significant role in the regulation of vascular smooth muscle contraction [6,20–23] and may also be a useful target for therapies aiming to disrupt tumor vasculature [12,24–29].

It has been shown that CNN1 is expressed in embryonic cardiac muscle and in smooth muscle cells (SMCs) [30–32]. We found that the expression of CNN1 decreased significantly in the heart tissue of a $cTnT^{R141W}$ transgenic dilated cardiomyopathy (DCM) mouse model and an adriamycin (ADR)-induced DCM mouse model [33–35], suggesting that CNN1 is involved in the pathogenesis of DCM. However, the effect of CNN1 on cardiac function, especially on pathogenesis of DCM, remains largely unknown. In the present study, we generated a cardiac-specific overexpression of CNN1 transgenic mouse to rescue the expression of CNN1 in heart tissue of $cTnT^{R141W}$ transgenic mice *in vivo* to determine whether and by what mechanism this would prevent the development of DCM.

☆ All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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2. Materials and methods

2.1. Generation of the transgenic mice

Full-length human CNN1 cDNA was cloned into an expression plasmid under the α-MHC promoter. The heart-specific CNN1 transgenic mouse was generated by microinjection and was genotyped by PCR with the primers 5′AAGGGCGGAACATCATTGGGCT and 5′CTCGAAGATCTGCCGCTTGGT. For genotyping, a 215-bp fragment of the transgenic gene was amplified with 35 PCR cycles consisting of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s. The expression of CNN1 was screened by western blot analysis using a rabbit monoclonal antibody (Abcam). The α-MHC-cTnT^{R141W} (referred to as cTnT^{R141W}) dilated cardiomyopathy (DCM) transgenic mice were previously generated in our laboratory [33]. The α-MHC-cTnT^{R141W} × CNN1 double transgenic (referred to as DTG) mice were generated through mating of the α-MHC-CNN1 (referred to as CNN1) with the cTnT^{R141W} transgenic mice. All mice used in this study were maintained on a C57BL/6J genetic background and were bred in an AAALAC-accredited facility. The use of animals was approved by the Animal Care and Use Committees of the Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-GC-2012-001).

2.2. ADR-induced DCM mice model

The CNN1 mice and non-transgenic (NTG) littermates at 2 months of age were used for adriamycin (ADR) treatment. In the groups of NTG + ADR and CNN1 + ADR, ADR was injected intraperitoneally at a constant volume of saline at 4 mg/kg every other day for a total of 2 weeks [34,35]. The groups designated as NTG + saline and CNN1 + saline received the same amount of saline. Echocardiography was performed on all mice at 0 day (the day before the treatment of ADR). All of the surviving mice were chosen for follow-up echocardiography at 3 months of age (2 weeks after the cessation of ADR treatment) and were used to analyze the expression of CNN1 in the heart tissues.

2.3. Survival analysis

The cumulative percent mortality in each group of mice was calculated every month, and the data from 1 to 8 months of age were summarized. Upon the death of each mouse, the body was autopsied by a pathologist and the morphological and pathological changes of the heart were recorded. Kaplan–Meier curves for the survival analysis were compared by the log-rank test (SPSS 16.0 software).

2.4. Echocardiography

M-mode echocardiography was performed at 1, 3, 6 and 8 months of age on the transgenic mice and their littermates with the small animal echocardiography analysis system (Vevo770, Canada) as previously described [34,36].

2.5. Histological analysis

For light microscopy, mice were euthanized by cervical dislocation at 6 months of age and cardiac tissue was fixed in 4% formaldehyde and mounted in paraffin blocks, and the sections were stained with H&E or Masson trichrome as previously described [34,36] and analyzed using Aperio ImageScope v8.2.5 software. For transmission electron microscopy (TEM), cardiac tissue was prepared as previously described [34,36]. Myocytes were analyzed by an observer who was blinded to the genotypes of the mice.

2.6. RNA extraction, quantification and RT-PCR

Mice were euthanized by cervical dislocation at 6 months of age, and total RNA was isolated from the heart tissues using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 2 μg of total RNA using random hexamer primers and Superscript III reverse transcriptase according to the manufacturer's protocol (Invitrogen). Procollagen type III α1 (Col3α1) mRNA was detected by RT-PCR using GAPDH for normalization under standard conditions (primers: for Col3α1, forward 5′CTCAAGAGCGGAGAATAC TGG and reverse 5′CAATGTCATAGGGTGCGATA; for GAPDH, forward 5′CAAGGTCAT CCATGACAACTTTG and reverse 5′GTCCACCACCCTGTTGCTGTAG).

2.7. Protein extraction and immunoblotting

Mice were euthanized by cervical dislocation, and total protein lysates from mouse heart tissues were prepared as previously described [34,36]. After performing SDS-PAGE and transferring the bands to nitrocellulose (Millipore), the membranes were incubated overnight with antibodies against CNN1 (Abcam), εPKC, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-mTOR (Ser2448), p44/42 MAPK (Erk1/2) and mTOR. After incubation with the appropriate secondary antibody for 1 h at room temperature, the antibody binding was detected with an HRP-conjugated immunoglobulin G (Santa Cruz) using a chemiluminescence detection system (Santa Cruz). The quantitative analysis of the level of phosphorylated proteins uses corresponding total protein for normalization, others use GAPDH for normalization, and bands were quantified using the ImageJ software.

2.8. Immunohistochemical staining

Mice were euthanized by cervical dislocation, and the sections of the hearts were prepared by a standard pathological procedure. For paraffin-embedded heart tissues, 6-μm sections were dewaxed, rehydrated, unmasked, blocked and then incubated with an anti-CNN1 monoclonal antibody (Abcam) overnight at 4 °C. The sections were washed with PBS and incubated with an anti-rabbit horseradish peroxidase conjugated secondary antibody for 1 h at room temperature, and all slides were counterstained with hematoxylin. The slides were then dehydrated, coverslips were placed on top, and the specimens were photographed. Images of the sections were collected and analyzed using Aperio ImageScope v8.2.5 software.

2.9. Drug treatment

The cTnT^{R141W} mice were treated with an εPKC activator (TOCRIS, FR 236924, CAS Number: 28399-31-7, IUPAC Name: 2-[(2-pentylcyclopropyl)methyl] cyclopropaneoctanoic acid, 0.60 mol/kg weight) [37], and the DTG mice were treated with an εPKC inhibitor (Sigma, PKC isoenzyme inhibitor PKC Y translocation peptide, 0.15 mol/kg) [38] from 6 months of age. In the group of cTnT^{R141W} + activator and DTG + inhibitor mice, the activator or inhibitor was injected intraperitoneally every other day for a total of 3 weeks. The groups of cTnT^{R141W} + saline and DTG + saline received the same amount of saline. Echocardiography was performed on all mice at 0 day (the day before the treatment with the εPKC activator/inhibitor). All surviving mice were chosen for follow-up echocardiography analysis, histological analysis and expression analysis of signaling molecules in the heart tissues at 7 months of age (1 week after cessation of drug treatment).

2.10. Statistical analysis

The data were analyzed by one-way ANOVA for multiple groups followed by Tukey's *post-hoc* analysis. The data are expressed as the means ± SD from individual experiments. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Rescue of CNN1 expression in cTnT^{R141W} mice by heart-specific transgenic expression of CNN1

We tested the expression of CNN1 in the NTG and DCM hearts from cTnT^{R141W} mice and the ADR-treated mice, and the results indicated that CNN1 was expressed in the adult hearts, and its expression was decreased significantly by 54.0% and 59.1%, respectively, in the DCM hearts from cTnT^{R141W} mice and ADR-treated mice (Fig. 1A and B, $n = 3$, $P < 0.01$). To verify whether the rescue of CNN1 expression in the heart tissues of cTnT^{R141W} mice could improve the DCM phenotypes, we generated CNN1 transgenic mice overexpressing CNN1 in the heart tissues (Fig. 1C–E, $n = 3$, $P < 0.01$), which were indistinguishable from their non-transgenic (NTG) littermates at birth and in young mice. The CNN1 mice were then crossed with the cTnT^{R141W} mice to generate the cTnT^{R141W} × CNN1 double transgenic (DTG) mice, which compensated for the loss of CNN1 expression in the heart tissues of the cTnT^{R141W} mice (Fig. 1F).

3.2. Rescue of CNN1 expression increased the survival rate of cTnT^{R141W} mice

Cumulative mouse mortality data of the NTG, CNN1, cTnT^{R141W} and DTG mice were recorded between 1 and 8 months of age. Dilation and mural thrombi were observed in the hearts of mice during the post-mortem examinations. The survival rate was 100% in the NTG group ($n = 67$) and CNN1 group ($n = 34$), while the survival rate was only 81.4% in the cTnT^{R141W} group (Fig. 2, $n = 59$, $P < 0.001$ *versus* NTG group) until 8 months of age. However, the survival rate in the DTG group ($n = 25$) was increased by 14% compared with that of the cTnT^{R141W} group (Fig. 2, $P < 0.01$) due to the rescued expression of CNN1.

3.3. Rescue of CNN1 expression improves cardiac morphology breakage and dysfunction in DCM mice models

Rescue of CNN1 expression increased the survival rate of cTnT^{R141W} mice. Furthermore, we analyzed the cardiac geometry and function in