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Bone morphogenetic protein-4 contributes to the down-regulation of Kv4.3 K^+ channels in pathological cardiac hypertrophy

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ABSTRACT

Kv4.3 K^+ channels contributing to Ito are involved in the repolarization of cardiac action potential. Kv4.3 K^+ channels decrease in pathological cardiac hypertrophy, but the mechanism remains unclear. Our previous study found that the expression of bone morphogenetic protein 4 (BMP4) increased in pressure-overload and Ang II constant infusion induced cardiac hypertrophy. Since the downregulation of Kv4.3 K^+ channels and the upregulation of BMP4 simultaneously occur in pathological cardiac hypertrophy, we hypothesize that the up-regulated BMP4 would contribute to the downregulation of Kv4.3 K^+ channels in cardiac hypertrophy. We found that BMP4 treatment reduced Kv4.3 but not Kv4.2 and Kv1.4 K^+ channel protein expression, and BMP4-induced decrease of Kv4.3 K^+ channel protein expression was reversed by BMP4 inhibitor noggin and DMH1 in cultured cardiomyocytes *in vitro*. BMP4-induced decrease of Kv4.3 K^+ channel protein expression was also reversed by the NADPH oxidase inhibitor apocynin and the radical scavenger tempol. In *in vivo* transverse aortic constriction (TAC)-induced cardiac hypertrophy, constant infusion of DMH1 completely rescued TAC-induced down-regulation of Kv4.3 K^+ channel protein expression. We conclude that BMP4 contributes to the downregulation of Kv4.3 K^+ channels in pathological cardiac hypertrophy and the underlying mechanism might be through increasing ROS production.

1. Introduction

Multiple pathological stimuli such as hypertension and aortic valvular stenosis cause pathological heart hypertrophy. Chronic pathological cardiac hypertrophy finally progresses to cardiac arrhythmias and heart failure [1]. Pathological cardiac hypertrophy is generally accompanied with cardiac electrical remodeling which in turn worsens the hypertrophy progression [2]. In pathological cardiac hypertrophy, multiple inward and outward currents are disturbed [2]. Among these channels, Kv4.3 K^+ channels are always diminished in different stimuli-induced pathological cardiac hypertrophy such as pressure-overload, PE, angiotensin-II or ET-1 treatment [3–6]. Downregulation of Kv4.3 K^+ channels has pathological significance because that *in vivo* gene transfer of Kv4.3 restores the downregulation of Ito and abrogates the hypertrophic response [7]. However, the mechanism underlying the downregulation of Kv4.3 K^+ channels in pathological cardiac hypertrophy remains unclear.

Our previous study found that the expression of bone morphogenetic protein 4 (BMP4), a mechanosensitive and pro-inflammatory gene, was significantly increased in pressure overload and Ang II constant infusion induced cardiac hypertrophy; in turn, BMP4 induced cardiomyocyte hypertrophy and apoptosis and the underlying mechanism was through increasing NADPH oxidase 4 (NOX4) expression and ROS production [8]. Since the downregulation of Kv4.3 and the upregulation of BMP4 simultaneously occur in pathological cardiac hypertrophy and it is reported that activation of NOX destabilizes cardiomyocyte Kv4.3 mRNA and decreases Kv4.3 protein expression [6], we hypothesize that the up-regulated BMP4 would contribute to the downregulation of Kv4.3 K^+ channels in cardiac hypertrophy.

2. Materials and methods

2.1. Materials

Recombinant human BMP-4 and recombinant human noggin were purchased from R&D Systems. DMH1 was purchased from

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Sigma (Sigma–Aldrich); tempol was purchased from Santa Cruz; Anti-BMP-4 antibodies were from Santa Cruz; Anti-Kv1.4, -Kv4.2, and -Kv4.3 antibodies were from Alomone Labs.

2.2. Pressure-overload induced cardiac hypertrophy in vivo

The pressure-overload heart hypertrophy models were obtained by subjecting the animals (Kunming mice, male, body weight 22–24 g) to transverse aortic constriction (TAC) as described in our previous works [8]. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Harbin Medical University, PR China.

2.3. Preparation of primary rat cardiomyocytes

Cardiomyocytes were prepared by dissociation of 10-day-old neonatal rat (Wistar) hearts and were differentially plated to remove fibroblasts. Because the transient outward current developmentally increased from the day-1 neonate to the adult [9], we used the 10-day-old neonatal rat hearts to obtain affluent Kv4.3 expression. Cardiomyocytes were treated with BMP-4 (50 ng/ml), noggin (100 ng/ml) or DMH1 (10 μM) for 48 h. The culture media containing different drugs was renewed every 24 h.

2.4. BMP4 inhibitor DMH1 treatment in vivo

The detail information was described in our previous work [8]. In brief, after TAC operation, 1-cm incision was prepared in the mid-scapular region of mice and an osmotic mini-pump (Alzet model 2004; Alza) containing DMH1 (Sigma–Aldrich, USA) was implanted. The constant infusion rate of DMH1 was 2 mg/kg per day. After 4 weeks, mice were sacrificed and hearts were harvested for analysis.

2.5. Western blot

The detail information was described in our previous work [8].

2.6. Real-time PCR analysis

One microgram of total RNA from each sample was used to generate cDNA by using M-MLV reverse transcriptase per manufacturer's specifications (Promega Corporation, USA). Real-time PCR was cycled in 95 °C/15 s, 60 °C/30 s and 72 °C/30 s for 40 cycles, after an initial denaturation step at 95 °C for 10 min using SYBR Green PCR Master Mix purchased from Applied Biosystems (USA). Amplification was performed by using 7500 fast real-time PCR systems (Applied Biosystems, USA) and the products were routinely checked by using dissociation curve software. Transcript quantities were compared by using the relative quantitive method, where the amount of detected mRNA normalized to the amount of endogenous control (GAPDH). The relative value to the control sample was given by $2^{-\Delta\Delta CT}$. The real-time PCR primer sequences for Kv4.3 (NM_031739.2, rat) were: forward primer (5′-3′) TTGGCTCCATCTGCTCCCTAA and reverse primer (5′-3′) CTTGCCC ATGTGCTCCTCTTC.

2.7. Data analysis

Data were presented as mean ± SEM. Significance was determined by using Student t test or one-way ANOVA, followed by Tukey post test. $P < 0.05$ was considered significant.

3. Results and discussion

We established pressure-overload cardiac hypertrophy by transverse aortic constriction (TAC) in mice. Our recent work found that pressure-overload for four weeks induced significant cardiac hypertrophy and induced significant increase of BMP4 expression in protein level [8]. In this pressure-overload induced cardiac hypertrophy, BMP4 protein expression increased and Kv4.3 protein expression decreased (Fig. 1A and B). Next, we exposed cultured cardiomyocytes to BMP4 in the presence and absence of BMP-4 inhibitor noggin and DMH1. BMP4 (50 ng/ml) induced significant cardiomyocyte hypertrophy which was inhibited by noggin and DMH1 treatment [8]. We evaluated the effects of BMP4 on Kv4.2 and Kv1.4 K^+ channel protein expression and found that BMP4 (50 ng/ml) treatment had no effect on Kv4.2 and Kv1.4 K^+ channel protein expression (Fig. 2A and B). However, BMP4 (50 ng/ml) treatment reduced Kv4.3 K^+ channel protein expression and the reduction was reversed by noggin and DMH1 treatment (Fig. 2C). Thus, we further examined the effects of BMP4 on Kv4.3 mRNA expression and the results showed that BMP4 (50 ng/ml) treatment reduced Kv4.3 mRNA expression and the reduction was reversed by noggin and DMH1 treatment (Fig. 2D).

Our previous work found that BMP4 treatment increased ROS production and NOX4 mRNA and protein expression in cardiomyocytes, furthermore, NADPH oxidase inhibitor apocynin and the radical scavenger tempol inhibited BMP4-induced cardiomyocyte hypertrophy [8]. It has been reported that activation of NOX destabilizes cardiomyocyte Kv4.3 mRNA and decreases Kv4.3 protein expression [6], therefore, we speculate that the apocynin and tempol could ameliorate BMP4-induced down-regulation of Kv4.3 K^+ channels. As shown in Fig. 3, apocynin and tempol treatment rescued BMP4-induced downregulation of Kv4.3 protein expression.

We further investigated whether BMP4 inhibitor could prevent downregulation of Kv4.3 K^+ channel protein expression in pathological cardiac hypertrophy *in vivo*. Alzet pump containing the small molecule chemical DMH1 was implanted subcutaneously in mice after TAC operation for 4 weeks. As shown in our previous work [8], administration of DMH1 completely suppressed TAC-induced cardiac hypertrophy, as demonstrated by the decreased HW/BW index, LVW/BW index, ANP, BNP, β-MHC mRNA expressions, and myocyte cross section area. Administration of DMH1 also inhibited TAC-induced increase of BMP4, NOX4 and ox-CaMKII protein expressions [8]. Meanwhile, administration of BMP4 inhibitor DMH1 completely rescued TAC-induced down-regulation of Kv4.3 K^+ channel protein expression (Fig. 4A). We also measured Kv1.4 protein expression in this pressure-overload cardiac

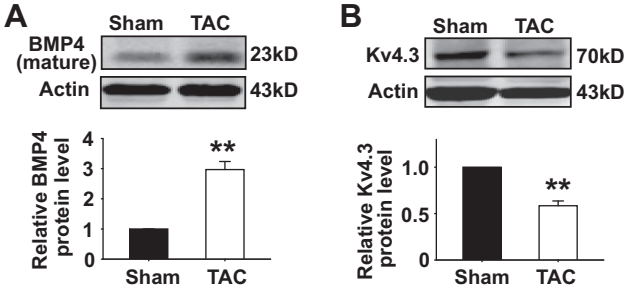

Fig. 1. Upregulation of BMP4 and downregulation of Kv4.3 in pressure-overload cardiac hypertrophy. (A) The representative Western blot and summarized data for BMP4 protein expression in pressure-overload cardiac hypertrophy. $^{**}P < 0.01$, vs. sham. $n = 5$ hearts in each group. (B) The representative Western blot and summarized data for Kv4.3 protein expression in pressure-overload cardiac hypertrophy. $^{**}P < 0.01$, vs. sham. $n = 5$ hearts in each group.

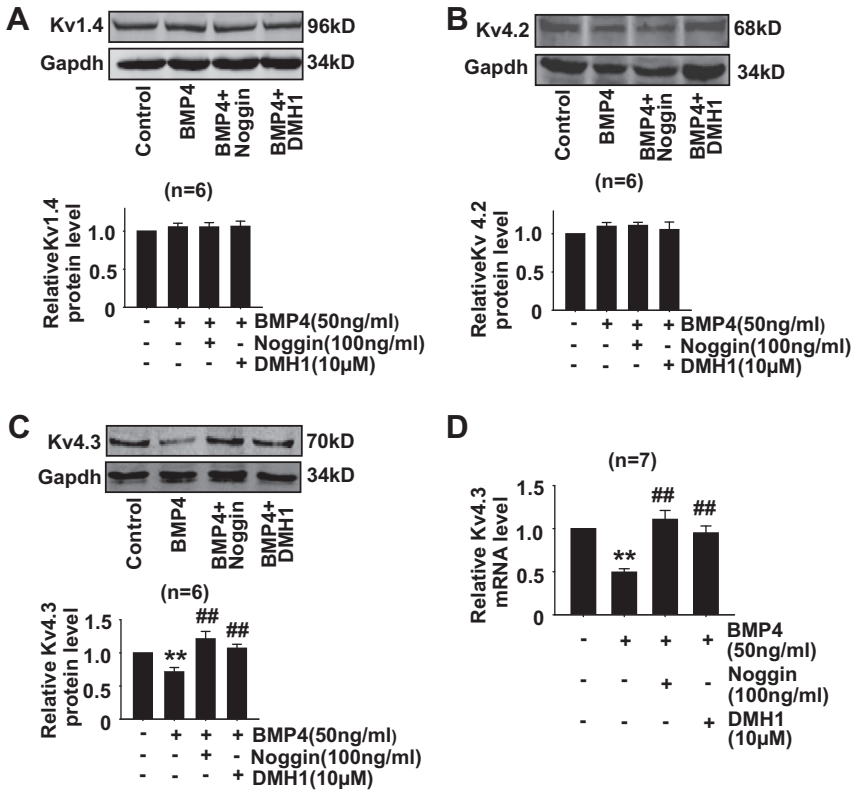

Fig. 2. BMP4-induced downregulation of Kv4.3 protein expression was rescued by BMP4 inhibitors noggin and DMH1. (A and B) The representative Western blot and summarized data for Kv1.4 and Kv4.2 protein expression in cardiomyocytes. (C) The representative Western blot and summarized data for Kv4.3 protein expression in cardiomyocytes. $^{**}P < 0.01$, vs. control; $^{\#\#}P < 0.01$ vs. BMP4. (D) The effects of BMP4 on Kv4.3 mRNA expression in cardiomyocytes. $^{**}P < 0.01$, vs. control; $^{\#\#}P < 0.01$ vs. BMP4.

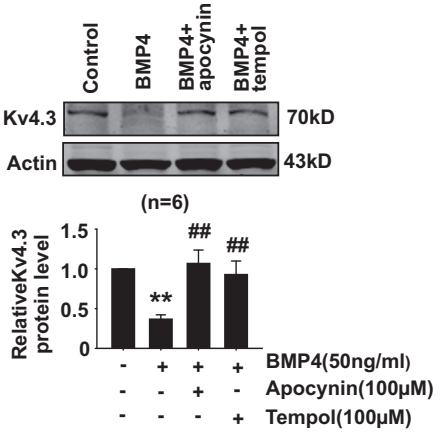

Fig. 3. BMP4-induced downregulation of Kv4.3 protein expression was rescued by NADPH oxidase inhibitor apocynin and the radical scavenger tempol. $^{**}P < 0.01$, vs. control; $^{\#\#}P < 0.01$ vs. BMP4.

hypertrophy model, and found that Kv1.4 K^+ expression increased and the increase was inhibited by DMH1 administration (Fig. 4B).

Here, we reported for the first time that the upregulated BMP4 contributed to the downregulation of Kv4.3 K^+ channels in cardiac hypertrophy, providing new understanding of electrical remodeling in cardiac hypertrophy. Kv4.3 K^+ channels are proteins encoded by the KCND3 gene, contributing to the cardiac Ito which is responsible for the repolarizing phase of action potential. Kv4.3 K^+ channels underly significant fraction of the Ito in canine and human cardiomyocytes and contribute partly to Ito in mice, rat cardiomyocytes [9]. Kv4.3 K^+ channels are impaired in different stimuli induced cardiac hypertrophy [3–6], however, the mechanism of down-regulation of Kv4.3 K^+ channel in cardiac hypertrophy is less reported.

NADPH oxidase family enzymes (NOXs) are the major sources of reactive oxygen species (ROS) that are implicated in the pathophysiology of many cardiovascular diseases. Zhou et al. (2006) reported that Ang II treatment or stretch specifically destabilized cardiac myocyte Kv4.3 channel mRNA by activating NADPH oxidase [6], but they did not identify the subtype of NOXs involved in down-regulating Kv4.3. Our previous work [8] showed that: (1) BMP4 increased ROS production in cardiomyocytes; (2) BMP4 increased NOX4 mRNA and protein expression but not NOX2; (3) In *in vivo* pathological and physiological cardiac hypertrophy models, the fold increase of NOX4 in TAC-and Ang II-induced cardiac hypertrophy was much higher than that in swimming-exercise induced cardiac hypertrophy. These results indicate that NOX4 play a more important role in pathological cardiac hypertrophy. NOX4 mainly locates mitochondrial membrane and has been reported to be the major sources of oxidative stress in pressure-overloaded heart [10], together with the present results that NOX inhibitor apocynin and the radical scavenger tempol inhibit BMP4-induced Kv4.3 downregulation (Fig. 3), we speculate that NOX4 contributes BMP4-induced downregulation of Kv4.3 K^+ channels.

Zhang et al. (2001) previously showed that AngII and phenylephrine (PE) decreased Kv4.3 expression via distinct mechanisms [11], indicating that the down-regulation of Ito in heart hypertrophy involves multiple pathways in addition to BMP4 pathway. They found that Ang II decreased Kv4.3 mRNA expression and PE inhibited Kv4.3 promoter activity [11]. Our present data show that BMP4 reduces Kv4.3 mRNA expression, which is similar to the effects of Ang II.

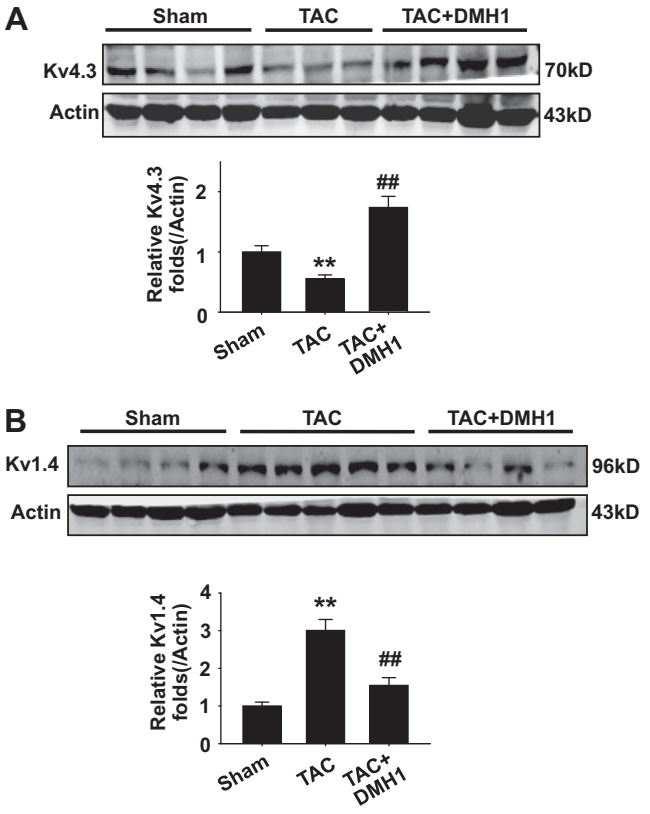

Fig. 4. BMP4 inhibitor DMH1 prevented downregulation of Kv4.3 protein expression in TAC-induced cardiac hypertrophy *in vivo* (A) Representative westernblot for cardiac Kv4.3 protein expression in left ventricle tissue and the summarized data. $^{**}P < 0.01$, vs. sham; $^{\#\#}P < 0.01$ vs.TAC $n = 7$ hearts in each group. (B) Representative Western blots for cardiac left ventricle tissue and the summarized data. $^{**}P < 0.01$, vs. sham; $^{\#\#}P < 0.01$ vs.TAC. $n = 7$ hearts in each group.

Kv1.4 is another component of Ito and shows different change pattern from Kv4.3 in cardiac hypertrophy, so we measure it as comparison. Most of studies reported that Kv1.4 was increased in cardiac hypertrophy [5,12,13]. In the present study, we found that Kv1.4 protein expression increased in pressure-overload cardiac hypertrophy *in vivo*, and the increment was suppressed by DMH1 administration (Fig. 4B). However, the *in vitro* experiments showed that BMP4 had no direct effect on Kv1.4 expression (Fig. 2A), indicating that the upregulation of Kv1.4 might be directly related to cardiac hypertrophy, independently of BMP4 signaling pathway.

We further performed the patch-clamp experiments to evaluate the effects of BMP4 on Kv4.3 K^+ currents in cardiomyocytes. We recorded two typical types of Ito currents in 30 recordings of the normal cultured cardiomyocytes, one type showed a transient outward property with peak current (Supplementary Fig. 1A), another type showed no significant peak currents (Supplementary Fig. 1B). In BMP4-treated cardiomyocytes, we also observed the similar Ito property. The amplitude of Ito component was too small relatively to the sustained component (as shown in Supplementary Fig. 1A). Moreover, in the Ito component, Kv1.4 and Kv4.2 would definitely contaminate Kv4.3 analysis. We considered that it was unreliable to evaluate the effects of BMP4 on Kv4.3K^+ currents in cultured cardiomyocytes by using patch-clamp techniques in the present conditions; therefore, we did not compare the functional data of Kv4.3K^+ channels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.113.

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