Cardiac-Specific Deletion of Mkk4 Reveals Its Role in Pathological Hypertrophic Remodeling but Not in Physiological Cardiac Growth

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Abstract—Mitogen-activated protein kinase kinase (M KK4) is a critical member of the mitogen-activated protein kinase family. It is able to activate the c-Jun NH2-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase in response to environmental stresses. JNK and p38 are strongly implicated in pathological cardiac hypertrophy and heart failure; however, the regulatory mechanism whereby the upstream kinase M KK4 activates these signaling cascades in the heart is unknown. To elucidate the biological function of M KK4, we generated mice with a cardiac myocyte-specific deletion of mkk4 (M KK4-/- mice). In response to pressure overload or chronic β-adrenergic stimulation, upregulated NFAT (nuclear factor of activated T-cell) transcriptional activity associated with exacerbated cardiac hypertrophy and the appearance of apoptotic cardiomyocytes were observed in M KK4-/- mice. However, when subjected to swimming exercise, M KK4-/- mice displayed a similar level of physiological cardiac hypertrophy compared to controls (M KK4+/+). In addition, we also discovered that M KK4 expression was significantly reduced in heart failure patients. In conclusion, this study demonstrates for the first time that M KK4 is a key mediator which prevents the transition from an adaptive response to maladaptive cardiac hypertrophy likely involving the regulation of the NFAT signaling pathway. (Circ Res. 2009;104:905-914.)

Key Words: cardiac hypertrophy ■ signal transduction ■ genetically modified mice

Pathological cardiac hypertrophy presents as a complex of cardiac remodeling, characterized by fetal gene reactivation, interstitial fibrosis, myocyte apoptosis, and contractile dysfunction. It occurs in patients with hypertension, ischemic infarction, or valvular heart diseases. Although pathological hypertrophy is initially likely to be an adaptive response, sustained stress inevitably leads to heart failure. In contrast to pathological hypertrophy, cardiac growth during normal postnatal development or the hypertrophy induced by exercise and by pregnancy is referred to as “physiological” and is characterized by more forceful ejection of blood.1,2 Although these 2 forms of hypertrophy have been well described, the regulation mechanisms determining the molecular differences between physiological and pathological hypertrophy, and the signaling pathways involved in the induction of pathological hypertrophy, are largely unknown and, in many cases, remain controversial.

c-Jun NH2-terminal protein kinase (JNK) and p38 belong to the stress-activated mitogen-activated protein kinase (MAPK) family.3,4 Because of their involvement in regulating cardiac hypertrophy and heart failure,5-7 the role of JNK and p38 MAPK in the heart has been the focus of much research. The analyses of transgenic and conventional gene-targeting knockout mouse models revealed that JNK, as well as p38, buffer the magnitude of pathological cardiac hypertrophy through inhibition of calcineurin-NFAT (nuclear factor of activated T-cell) signaling.8-9 However, a recent study of cardiac-specific p38α knockout mice demonstrated that p38 was required for cardiomyocyte survival but not for pathological cardiac hypertrophy.10 Additionally, p38 was also shown to regulate physiological hypertrophy by suppressing Ser/Thr protein kinase (PK)B (also known as AKT) activity.11 These observations and discrepancies reflect the complexity of the MAPK cascades and highlight the need for further study of the signaling regulation of JNK and p38 in the heart.

JNKs are phosphorylated and activated by M KK4 and M KK7 on tyrosine 185 and threonine 183, respectively.12,13 Differing from M KK7, M KK4 was reported to activate p38 in some cell types.14,15 The targeted deletion of the mkk4 or mkk7 gene leads to embryonic lethality, providing genetic evidence that M KK4 and M KK7 have nonredundant roles in

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vivo. This concept is underscored by the observation in mice with brain-specific ablation of mkk4 that decreased JNK activity causes a defect in neuronal migration and premature death.\(^{15}\)

MKK4 is proposed to be involved in cardiac hypertrophy, however, previous studies provided conflicting results, and the role of MKK4 in the heart is still far from conclusive. For example, overexpression of dominant-negative MKK4 in either cultured cardiomyocytes or the adult rat heart attenuated stress-induced cardiac hypertrophy\(^{16,17}\); conversely, diminished JNK activation with enhanced hypertrophy was detected in mkk1\(^{+/−}\) mice (MEKK1 is the upstream kinase of MKK4) subjected to pressure overload.\(^{18}\) Together, these controversial data prompted this study, in which we proposed to determine whether MKK4 is a nodal kinase activating JNK and p38 in the heart, and whether MKK4 regulates physiological or pathological cardiac hypertrophy. Analysis of cardiac-specific MKK4 knockout mice subjected to pressure overload or chronic β-adrenergic stimulation revealed that reduced JNK activity was associated with exacerbated cardiac hypertrophy and a significant increase in the NFAT transcription activity. Moreover, apoptotic cardiomyocytes were evident in hypertrophic MKK4\(^{cko}\) mice. Following swimming exercise, the mutant mice displayed a similar level of activity in cardiac hypertrophy as the control group. These data suggest that the MKK4/JNK signaling pathway is specifically required for pathological cardiac hypertrophy/remodeling, but not for physiological cardiac hypertrophy.

### Materials and Methods

An expanded Materials and Methods section describing generation of MKK4 mutant mice, osmotic minipump infusion, in vivo physiology analysis, histological analysis, immunoblot analysis, quantitative real-time PCR, small interfering (si)RNA transfection, and luciferase reporter assay is available in the online data supplement at http://circres.ahajournals.org.

#### Transverse Aortic Constriction

Eight-week-old male MKK4\(^{f/f}\) and MKK4\(^{cko}\) mice were subjected to transverse aortic constriction (TAC) or a sham operation under isoflurane anesthesia, 0.1 mg/kg buprenorphine, and artificial ventilation as previously described.\(^{19}\) Hearts were analyzed for cardiac hypertrophy at different time points following TAC.

#### Swimming Exercise

Eight-week-old male MKK4\(^{f/f}\) and MKK4\(^{cko}\) mice were subjected to a swimming program. Mice swam for a period of up to 90 minutes twice a day in tanks containing water at 30°C to avoid thermal stress. Constant monitoring was given to ensure the safety of the mice. After the 28-day swimming program, the hearts were analyzed to evaluate hypertrophic growth.

#### Human Tissue Samples

Myocardial tissue samples from heart failure patients and normal subjects were obtained from Asterand (Herts, United Kingdom). Informed consent for the use of human tissue samples was obtained by Asterand and approved by the United Kingdom Human Tissue Authority.

#### Data Analysis

Data are expressed as means±SEM and were analyzed using the paired Student’s t test or 2-way ANOVA followed by post hoc multiple comparison test where appropriate, with a value of \(P<0.05\) considered to be statistically significant.

### Results

#### Characterization of MKK4\(^{cko}\) Mice

To study the functional importance of MKK4 in the heart, we generated mutant mice with a cardiac-specific deletion of mkk4 (MKK4\(^{cko}\)) using the Cre-LoxP system. MKK4\(^{cko}\) mice were born at the expected Mendelian ratio; they developed to term and appeared normal. Immunoblot analysis of ventricular extracts from MKK4\(^{cko}\) mice at 8 weeks of age showed an almost complete ablation of MKK4 protein (Figure 1A). This result was confirmed by immunostaining of isolated single cardiomyocytes (Figure 1B). It was evident that excision of MKK4 was specific to the heart because protein levels of MKK4 were equivalent in brain, liver, and skeletal muscle from both MKK4\(^{cko}\) and MKK4\(^{f/f}\) mice (Figure 1A).

The absence of MKK4 in the heart did not cause any compensatory changes in the protein levels of MKK7, JNK, and p38 (Figure 1A). To determine whether MKK4 is able to activate JNK and p38 in vivo, ventricular tissues from 2 groups were collected 30, 60, and 90 minutes after isoproterenol treatment (10 mg/kg). Immunoblot analysis showed a rapid increase in JNK activity in controls 30 minutes after the treatment, whereas we barely detected any activation in the mutant hearts (Figure 1C). Consistent with this, MKK4 activity in controls was also increased 30 minutes after isoproterenol treatment (Figure 1 in the online data supplement). In contrast, the activation of p38 in both groups escalated to a similar level at 30 minutes after the treatment, sustained to 60 minutes, and then gradually declined (Figure 1C). This is confirmed by results obtained from in vitro kinase assays (supplemental Figure II). JNK reached only 25% of the level of activation seen in the controls following isoproterenol treatment, whereas p38 activity in both groups was comparable. These results suggest that MKK4 is the specific activator for JNK in the heart. The disruption of MKK4 in cardiomyocytes does not affect p38 activity.

#### Pressure Overload Caused Exacerbated Cardiac Hypertrophic Remodeling in MKK4\(^{cko}\) Mice

At baseline, there was no difference in the ratio of heart weight to tibia length (HW/TL) in MKK4\(^{cko}\) mice compared to controls, and cardiac function, as measured by echocardiography, was not altered (supplemental Table I). To determine whether MKK4 is essential for pressure overload-induced cardiac hypertrophy, MKK4\(^{f/f}\) and MKK4\(^{cko}\) mice were subjected to TAC for 1 or 5 weeks.

After 1 week of TAC, MKK4\(^{cko}\) mice showed an increased response to the hypertrophic stimulus demonstrated by enhanced HW/TL (58.3% [MKK4\(^{cko}\)] versus 18.3% [MKK4\(^{f/f}\)]) (Figure 2B) and greater cross-sectional area of cardiomyocytes (346.5±3.26 μm\(^2\) [MKK4\(^{cko}\)] versus 252.4±6.88 μm\(^2\) [MKK4\(^{f/f}\)]) (Figure 2A and 2C). Echocardiography showed a decline in contractility in the mutant hearts (supplemental Figure III); however, no apparent interstitial fibrosis and apoptosis was associated with the hypertrophy at this time point (data not shown). The transcripts of hypertrophic gene markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and regulator of calcineurin 1.4 (RCA1L4) (a marker for calcineurin/NFAT activity) were significantly
upregulated in MKK4cko-TAC mice compared to the controls (Figure 2D).

When subjected to prolonged TAC treatment (5 weeks), an adaptive hypertrophic response was seen in MKK4f/f mice characterized by preserved cardiac function (dP/dt max: 5125 ± 480 mm Hg/sec; dP/dt min: 552 mm Hg/sec; fractional shortening: 35.19 ± 1.07%) (Figure 3E and 3F); however, the extent of hypertrophic remodeling in MKK4cko hearts was much more pronounced, which was evident by a conspicuous increase in HW/TL (103.92% [MKK4cko] versus 26.545 ± 55.2% [MKK4f/f]) (Figure 3D). These structural changes were accompanied by a detrimental change in cardiac function in MKK4cko mice (dP/dt max: 3384 ± 254 mm Hg/sec; dP/dt min: -2868 ± 187 mm Hg/sec; fractional shortening: 10.05 ± 2.03%) (Figure 3E and 3F). As expected, the transcripts of hypertrophic gene markers ANP, BNP, and Myh7 (myosin heavy polypeptide 7 cardiac muscle β); the fibrosis maker genes procollagen type I (Col1a1), and procollagen type III, α1 (Col3a1); as well as RCAN1.4 were significantly upregulated in MKK4cko-TAC mice compared to the controls (supplemental Figure IV).

After 5 weeks of TAC, we observed pronounced apoptosis in MKK4cko cardiomyocytes (the number of TUNEL-positive nuclei was 6.3 times of that in controls), along with an increased level in activated caspase 3 (Figure 4A and 4B). In addition, MKK4cko-TAC mice showed an augmented protein level of Bax (proapoptotic Bcl-2 family member), with a higher ratio of Bax/Bcl-2 (1.81 times higher compared to controls), suggesting the Bcl family members are involved in hypertrophy-induced apoptosis in MKK4cko hearts (Figure 4B).

To further investigate the mechanisms involved in this increased hypertrophic response, we examined the activation of both pro- and antihypertrophic pathways after 1 and 5 weeks of TAC. The activity of the prohypertrophic genes extracellular signal-regulated kinase (ERK)1/2, ERK5, and PKB in the 2 genotypes was comparable. GSK3β and JunD, which are known to prevent hypertrophy, also showed similar levels of activity in the 2 groups (supplemental Figures V and VI). In parallel with these data, we also detected that after TAC, the upregulation of JNK activity seen in MKK4f/f mice was not evident in MKK4cko hearts. The activation of p38 MAPK and MKK7 was comparable in both groups (supplemental Figure VII).

The Reduction of MKK4 Enhanced the NFAT Transcriptional Activity

Led by the result that the transcript level of RCAN1.4 was upregulated in MKK4cko-TAC hearts, we further examined whether the NFAT transcriptional activity was regulated by MKK4. Using an siRNA method, we specifically suppressed MKK4 expression by 66% in neonatal rat cardiomyocytes; this reduction of MKK4 led to attenuated JNK activity following isoproterenol treatment (Figure 5C). This finding suggests that the MKK4/JNK pathway regulates cardiac hypertrophy most likely through NFAT activity.
Chronic β-Adrenergic Stimulation Enhances the Cardiac Hypertrophic Response in MKK4cko Mice

We next investigated the requirement of MKK4 in isoproterenol-induced hypertrophy. After 7 days of administration of isoproterenol (10 mg/kg per day), MKK4cko mice developed advanced hypertrophy reflected by a 56.31% increase in HW/TL, compared to controls (32.54%). Consistently, enlarged cross-sectional area of cardiomyocytes and ventricular fibrosis were more notable in the mutant mice (supplemental Figures VIII and IX). However, cardiac function in MKK4cko mice remained relatively normal (supplemental Figure IX). Furthermore, the transcriptional levels of ANP, BNP, Myh7, Col1a2, Col3α1, and RCAN1.4 were found to be remarkably elevated in the MKK4cko hearts, compared to controls (supplemental Figure X).

MKK4cko and MKK4f/f Mice Exhibited a Similar Level of Physiological Cardiac Growth in Response to Swimming Exercise

Next, we explored whether MKK4 was necessary for physiological cardiac hypertrophy. Subjected to a 28-day swimming program, both groups displayed a comparable level of hypertrophic growth, with no significant difference in the HW/TL ratio and the cross-sectional area of cardiomyocytes (Figure 6A and 6B). Ventricular fibrosis was not detected by histological analysis, and echocardiography presented unperturbed cardiac function in both genotypes (data not shown). Because p38 and PKB have been implicated in the regulation of physiological hypertrophy,10,20 we examined the activation of JNK, p38, and PKB following swimming. We noted a similar rise in both p38 and PKB activity 30 minutes after swimming, whereas swimming exercise did not induce JNK or MKK4 activation within this time frame (supplemental Figures XI and I, B). Together, our data suggest that the MKK4/JNK signaling pathway is not required for the development of physiological hypertrophy.

Analysis of MKK4 Expression and Activity Following Hypertrophic Stress and in Human Failing Heart

To determine the response of MKK4 to hypertrophic stresses, we analyzed the protein level and activity of
MKK4 following TAC, or swimming exercise in genetically normal myocardium (C57BL/6:129Sv mice). Intriguingly, both expression and activity of MKK4 were markedly upregulated after 5 weeks of TAC, but after 12 weeks of TAC, no detectable MKK4 activation was seen, and there was a trend toward a decrease in MKK4 protein level (86.5% of MKK4 expressed in sham groups, \( P=0.16 \)) (Figure 7A). Following 28 days of swimming exercise, no detectable MKK4 activation was seen, and there was a trend toward a decrease in MKK4 protein level (86.5% of MKK4 expressed in sham groups, \( P=0.16 \)) (Figure 7A). Following 28 days of swimming exercise,
MKK4 expression and activity were unchanged (data not shown). We also examined the expression and phosphorylation of MKK4 in heart failure patients and in normal subjects. Interestingly, a significant reduction of expression (around 60%) associated with diminished activity of MKK4 was observed in heart failure patients (Figure 7B).

**Discussion**

Data presented here depict a clear picture of the signaling regulation underlying 2 distinct forms of cardiac hypertrophy. The major finding of this study is that MKK4 acts as a modulator to protect the heart from pathological hypertrophy likely through the JNK-regulated NFAT activity, but it is not required for the development of physiological cardiac hypertrophy. Data obtained from heart failure patients showing a remarkable decrease in MKK4 expression strengthens such a notion and, for the first time, provides evidence for the functional importance of MKK4 in the human heart.

Pathological cardiac hypertrophy is a multifaceted process affecting myocardial growth, cardiomyocyte survival, cardiac pumping capacity, and fetal gene reactivation. The calcineurin-NFAT signaling pathway is believed to be a major character mediating pathological hypertrophy. It is reported that JNK antagonizes myocardial growth by directly phosphorylating NFAT and inhibiting its transcriptional activity. In line with this paradigm, our data showed that the transcript level of RCAN1.4 was significantly increased in TAC- or isoproterenol-treated MKK4^cko hearts. Because the transcription of RCAN1.4 (also known as MCIP1.4) is tightly modulated by NFAT transcription factors, our observation implicates an increase in the NFAT activity in MKK4^cko hearts. In addition, no change in the transcription of RCAN1.4 was found in swimming-activated MKK4^cko hearts (data not shown), which is consistent with a previous study showing that the calcineurin-NFAT signaling pathway participated only in maladaptive hypertrophy and heart failure. Furthermore, in response to isoproterenol stimulation the NFAT activity was found to be elevated in rat cardiomyocytes in which MKK4 was exclusively knocked down, establishing the direct evidence that MKK4 is engaged in regulating the NFAT signaling. In contrast, De Windt et al reported that calcineurin-mediated hypertrophy was attenuated by overexpression of dominant negative MKK4 adenovirus in neonatal rat cardiomyocytes (NRCMs). Such a discrepancy can be explained by the use of different experimental models. Neonatal cardiomyocytes, which lack mature sarcomeric organization, are not regarded as an ideal model for studying pathological hypertrophic response in adults. Moreover, overexpression of dnMKK4 in NRCMs may cause some unwanted effects, thereby masking the true phenotypes; however, in our study, NRCMs with a specific knockdown of MKK4 closely resemble MKK4^cko cardiomyocytes, making...

![Figure 4. MKK4 is required for cardiomyocyte survival in response to hypertrophic stress. A, Increased apoptosis in MKK4^cko ventricular myocardium after 5 weeks of TAC was detected by TUNEL assay. Triple staining was performed: TUNEL staining (green), DAPI marking nuclear chromatin (blue), α-actinin marking cardiomyocytes (red). Arrows indicate TUNEL-positive nuclei. Scale bar=20 μm. The bar graph summarizes the number of apoptotic nuclei in MKK4^cko hearts compared to that in control hearts after 5 weeks of TAC. B, Immunoblot analysis of protein levels of active caspase 3, the proapoptotic Bcl-2 family member Bax, and the antiapoptotic protein Bcl-2. Tubulin expression is the protein loading control. The increased ratios of active caspase 3 to tubulin and Bax/Bcl-2 in the mutant hearts are represented by the bar graphs (graphs). Data are means±SEM (n=4 per group).](http://circres.ahajournals.org/)}
this a suitable experimental model for measuring the NFAT activity by luciferase assay.

In parallel, we also investigated the activation of a number of hypertrophic regulators following TAC stress, including ERK1/2, ERK5, and PKB, known as promoters for hypertrophy, and GSK3β and JunD, which have inhibitory functions on hypertrophic growth. However, we did not observe any difference in their activities between the 2 genotypes after either 1 or 5 weeks of TAC. This strongly suggests that the effect of MKK4 in preventing pathological hypertrophy is likely to be mediated via specific regulation of the NFAT activity and not through crosstalk with other hypertrophic regulators.

Apart from hypertrophic changes, we also detected a much higher prevalence of apoptotic cardiomyocytes with increased Bax protein level in TAC-treated mutant hearts, which clearly indicates that MKK4 protects against cardiomyocyte loss in response to hypertrophic stress by attenuating the mitochondrion-dependent apoptotic pathway. However, previous studies provided data to dispute the role of MKK4 in cardiac apoptosis in response to distinct stimuli of various durations.

MKK4cko mice displayed detrimental cardiac contractility following 5 weeks of TAC, which was possibly related to enhanced apoptosis and massive ventricular fibrosis. Of note, in 1-week TAC-treated mutant hearts, the contractility was moderately blunted but no maladaptive remodeling was seen, implying that the loss of MKK4 may directly affect cardiac contractility. However, cardiac function in MKK4cko mice at baseline was normal, and there were no changes in the expression levels of Ca²⁺ handling proteins in MKK4cko hearts, such as SERCA2 (sarcomplasmic reticulum Ca²⁺-ATPase 2), Na⁺/Ca²⁺ exchanger, and phospholamban (data not shown). It is therefore uncertain whether MKK4 primarily regulates intracellular Ca²⁺ transients following hypertrophic stress.

Figure 5. MKK4 is involved in regulating the NFAT activity in cardiomyocytes. A, Neonatal rat cardiomyocytes (NRCMs) were transfected with MKK4 siRNA (100 nmol/L) or negative control siRNA. Cells were cultured in serum-free medium for 72 hours before preparation of protein lysates for analysis of MKK4 protein level by immunoblotting. MKK7 protein level was also determined to examine the specificity of siRNA-mediated knockdown of MKK4. The ratio of MKK4 expression to MKK7 is presented as a bar graph. B, siRNA-transfected NRCMs were treated with isoproterenol (10 μmol/L) for 30 minutes before detecting JNK phosphorylation by immunoblotting. The ratio of phosphorylation to expression of JNK is presented as a bar graph. C, siRNA-transfected NRCMs were infected with Ad-NFAT-Luc (25 multiplicities of infection) for 24 hours, followed by stimulation with isoproterenol (10 μmol/L). The NFAT-dependent transcriptional activity was measured by the luciferase reporter assay system. Data are means±SEM (n=3 per group).
PKB activity, \textsuperscript{10} ASK1 is a mitogen-activated protein kinase kinase kinase lying upstream of MKK4,\textsuperscript{35} which leads us to hypothesize whether the MKK4/JNK pathway has a dual function in regulating not only pathological hypertrophy/remodeling, but also physiological hypertrophy. Although our results do not support this hypothesis, this study provides valuable information clarifying the signaling determinant underlying physiological hypertrophy.

In this study, we also examined the expression and activity of MKK4 in wild-type mice. Interestingly, phosphorylation and expression of MKK4 were significantly upregulated after 5 weeks of TAC, whereas no increase in MKK4 expression was after 12 weeks of TAC. This result suggests that increased MKK4 may initially act as a brake to slow down hypertrophic growth; however, when sustained stress is applied, MKK4 recedes, thereby contributing to advanced...
hypertrophic growth (HW/TL: 38% [12 weeks of TAC] versus 23% [5 weeks of TAC]). These data are in line with our findings in mkk4^−/− mice and are further underpinned by the observation that MKK4 is markedly reduced in heart failure patients.

Previous studies showed that MKK4 was able to phosphorylate p38 MAPK in both in vitro and in vivo systems. However, in this study, under β-adrenergic stimulation or pressure overload, we consistently discerned that only JNK activation was abolished in MKK4-deficient myocardium, whereas p38 activity was unimpaired. This result is in agreement with a previous finding that overexpression of dominant-negative MKK4 blocked endothelin-1–induced hypertrophy in NRCMs via the inhibition of JNK activity, whereas p38 activity was unimpaired. This result is in agreement with a previous finding that overexpression of dominant-negative MKK4 blocked endothelin-1–induced hypertrophy in NRCMs via the inhibition of JNK activity, without affecting activation of p38 MAPK. Such discrepancies reflect the complexity of the MKK4-dependent signaling regulation in different cell types or tissues. It would be of great interest to further dissect the role of MKK4 in regulating JNK and/or p38 MAPK activation in various pathophysiological settings. Based on this study, MKK4 is the major, yet specific activator for JNK in hypertrophic remodeling. This further suggests that MKK7, another JNK upstream activator, may have distinct functions differing from those of MKK4 in the heart. This notion is supported by the analysis of a mouse model overexpressing dominant-active MKK7 in ventricular myocytes. It was revealed that MKK7 interacted with the transforming growth factor-β signaling pathway affecting extracellular matrix composition; also, MKK7 was involved in regulating ventricular electric conduction. It is known that MKK4 and MKK7 preferentially phosphorylate the Tyr and Thr residues within the T-X-Y motif of JNK; however, whether it is the molecular determinant underlying the different effects of MKK4 and MKK7 in the heart requires further investigation. Meanwhile, to distinguish the distinct roles of MKK4 and MKK7 in myocardium, a cardiac-specific MKK7 knockout mouse model is certainly needed.

In conclusion, this study provides convincing evidence to clearly address the in vivo role of MKK4 in the heart. MKK4 modulates the progression of pathological hypertrophy likely by regulating the NFAT transcription factor. The recognition of the functional importance of MKK4 in preventing maladaptive cardiac hypertrophy and ventricular remodeling and the fact that it does not interfere with physiological hypertrophy may shed light on the development of new therapeutic targets modulating the heart failure process.

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Disclosures

None.

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Materials and Methods

Generation of MKK4 Mutant Mice

The mkk4-flox mice (referred to as MKK4<sup>f/f</sup>) were previously generated and characterised<sup>1</sup>. MKK4<sup>f/f</sup> mice were mated with mice expressing Cre under a myosin light chain (MLC2v) promoter to generate cardiac-specific MKK4 knockout mice (referred to as MKK4<sup>cko</sup>). The MLC2v-Cre line (kindly provided by Dr. KR Chien, Massachusetts General Hospital, USA) is a well established model that provides ventricular specific and efficient Cre recombinase activity and is known not to cause any abnormality in cardiac morphology and function following cardiac stress<sup>2</sup>. All mice used in this study were maintained in a pathogen-free facility at the University of Manchester. The animal studies were performed in accordance with the Home Office and institutional guidelines.

Osmotic Minipump Infusion of Isoproterenol

To generate β-adrenergic-induced cardiac hypertrophy, isoproterenol (Iso, Sigma-Aldrich) (10mg/kg/day) or vehicle (ddH<sub>2</sub>O) was administrated to 10 week old male MKK4<sup>f/f</sup> and MKK4<sup>cko</sup> mice via osmotic mini-pumps (Alzet) implanted subcutaneously. Hearts were isolated and analysed for cardiac hypertrophy following 7 days of Iso infusion.

In vivo Physiology Analysis

In vivo hemodynamic analysis was performed using a pressure volume system (Millar Instruments). We used a 1.4F pressure-volume catheter (SPR-839) following a protocol described previously<sup>3</sup>. Maximal derived pressures were obtained during systole (dP/dt<sub>max</sub>)
and diastole (dP/dt_{min}) as indices representing cardiac contractile functions. For cardiac morphological analysis, transthoracic two dimensional and M-mode echocardiography were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously\(^2\). Parameters of intraventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and fractional shortening (FS) were obtained.

**Immunostaining of Single Cardiomyocytes**

Hearts from 8-week old mice were perfused via the aorta using a modified collagenase and protease digestion method\(^4\). Dissociated cardiomyocytes were plated and fixed in 4% paraformaldehyde prior to being incubated with antibodies specific to MKK4 (1:100, BD Pharmingen) or to α-actinin (1:500, Sigma). The secondary anti-mouse antibody conjugated to Alexa Fluoro 568 (1:500, Invitrogen), or conjugated to Alexa Fluoro 488 (1:500, Invitrogen) was applied respectively to detect the immune signals, respectively. Fluorescence images were viewed with a Nikon upright confocal microscope.

**Histology and TUNEL Assay**

Freshly dissected heart tissue was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 5-7μm thick sections were cut and stained with hematoxylin & eosin or Masson’s trichrome method as described\(^5\). To calculate the mean cross-sectional area approximately 150 randomly selected cardiomyocytes were measured. 45 randomly chosen frames from Masson’s trichrome stained sections were quantified to assess the degree of myocardial fibrosis using Image J software. TUNEL assay to detect apoptosis was performed on paraffin-embedded heart sections using the *in situ* Cell Death
Detection kit (Roche). Triple staining with DAPI, anti-α-actinin antibody (Sigma), and TUNEL was performed to confirm apoptotic morphology in cardiac nuclei.

**Preparation of Lysates and Immunoblot Analysis**

Proteins were homogenised and extracted from tissues in Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin). Protein extracts (20 µg) were subjected to Western blot analysis with antibodies against MKK4, MKK7 (BD Pharmingen); JNK, p38 MAPK, JunD, Bax (Santa Cruz); PKB, active caspase-3, Bcl-2, phospho-PKB (Ser 473), phospho-MKK4, phospho-MKK7, phospho-JNK, phospho-p38 MAPK, GSK3β, phospho-GSK3β, phosphor-cJun/JunD (Ser 73) (Cell Signalling); ERK5 (Upstate); phospho-ERK5 (Invitrogen); and tubulin (Sigma). Immunocomplexes were detected by enhanced chemiluminescence with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

**Protein Kinase Assay**

JNK and p38 MAPK activities were measured in ventricular tissue lysates following precipitation with glutathione S-transferase (GST)-c-Jun (the substrate for JNK) and glutathione-Sepharose beads (Amersham), or with a polyclonal antibody to p38 MAPK (Santa Cruz) and protein A agarose beads (Sigma), respectively, for 2 hours at 4°C. The kinase reactions were performed at 30°C for 20 minutes in kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 0.1% orthovanadate) containing 50 µM [γ-32P] ATP (10 Ci:mmol) and 1 µg of GST-transcription factor 2 (ATF2) for p38 MAPK assays. The reactions were terminated by
the addition of Laemmli sample buffer. Phosphorylated substrates were examined and quantified after SDS-PAGE by phosphorImager analysis (Fuji FLA 3000).

**Quantitative Real-time PCR**

Total RNA was prepared from ventricular tissue using Trizol reagent, followed by the synthesis of cDNA. Real-time quantitative PCRs were performed using the SYBR-green I Core Kit (Eurogentec). The primers used for ANP, BNP, Myh7, Col1α21 and Col3α1 were obtained from Qiagen. The primers used for RCAN1.4 were as follows: forward primer, 5'-AGCTCCCTGATTGCCTGTGT-3', reverse primer, 5'-TTTGCCCTGGTCTCCTTTT-3'. PCR products were detected in the ABI-PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the 2-∆∆CT method. The level of expression of mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**siRNA Transfection**

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described. NRCMs were transfected with siRNA (100nM) using Lipofectamine Plus reagent according to the manufacturer’s instructions (Invitrogen). Rat MKK4 siRNA (gene ID # 287398; si genome SMART pool) was purchased from Dharmacon, siRNA negative control (Si Neg) was obtained from Eurogenetec. To assess the specific effect of MKK4 siRNA on silencing MKK4 expression, the protein levels of MKK4 and MKK7 were detected by immunoblot analysis 72h post-transfection. In addition, JNK activation upon isoproterenol stimulation (10µM) was determined in siRNA-treated NRCMs by immunoblot analysis.
Luciferase Reporter Assay

48h post-transfection of siRNA, NRCMs were infected with recombinant adenovirus encoding NFAT-luciferase reporter gene (AdNFAT-luc) at 25 MOI in serum-free medium. At 24h after infection, the virus was removed. Following isoproterenol treatment (10µM), aliquots of NRCM lysates were assayed for the NFAT luciferase activity using a luciferase assay kit (Promega). NFAT-Luc reporter cDNA was obtained from Clontech, AdNFAT-luc was generated using the AdEasy™ adenoviral vector system (Stratagene).

Figure legends

Online Table I. Echocardiographic assessment of 8-week old MKK4^{ff} and MKK4^{cko} mice at baseline. All measurements are mean ± SEM. N indicates the number of mice analysed in each group. HW/TL, the ratio of heart weight and tibia length; dIVS, end-diastolic interventricular septal wall thickness; dPW, end-diastolic left ventricular posterior wall thickness; LVEDD, diastolic left ventricular internal dimension; LVESD, systolic left ventricular internal dimension; FS, fraction shortening.

Online Figure I. Isoproterenol (A) or swimming exercise (B) induced activation of MKK4 in control hearts was examined by immunoblot analysis at various time points. The ratios of phosphorylation to expression of MKK4 are shown in the bar graphs. Data correspond to the mean ± SEM (n=3 per group). * no difference found between two groups.
Online Figure II. The deletion of MKK4 in myocardium severely blunts JNK activity. *In vitro* kinase assay was performed to measure JNK and p38 activities after 30 minutes of isoproterenol stimulation. Phosphorylated GST-cJun or GST-ATF2, used for measuring JNK or p38 activity respectively were examined and quantified after SDS-PAGE by phosphorImager analysis. Black bar: control, grey bar: 30 mins after isoproterenol treatment. n=3, * no difference between two groups. Data are presented as mean ± SEM.

Online Figure III. Echocardiography assessment (EF%, FS%) demonstrate moderately decreased contractility in MKK4<sup>cko</sup> mice (n=4). Data are presented as mean ± SEM.

Online Figure IV. Quantitative real-time PCR analyses of gene markers associated with hypertrophy and fibrosis following 5 weeks TAC. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content and expressed relative to the mRNA extracted from sham treated-MKK4<sup>f/f</sup> mice. Data are the mean ± SEM, n=3. *ANP*, atrial natriuretic peptide; *BNP*, brain natriuretic peptide; *Myh7*, β-myosin heavy chain; *Col1α2*, procollagen type 1 alpha 2; *Col3α1*, procollagen type III alpha 1, *RCAN1.4*, regulator of calcineurin 1.4 isoform.

Online Figure V. Analysis of hypertrophic regulators in MKK4<sup>cko</sup> hearts in response to TAC for 1 week. Protein extracts from MKK4<sup>f/f</sup> and MKK4<sup>cko</sup> hearts subjected to TAC or sham operation were examined by immunoblotting for total ERK1/2, ERK5, PKB, GSK3β and JunD expression, as well as their phosphorylation levels using specific
antibodies. The ratios of phosphorylation to total expression of each protein are shown (right panel). Data are mean ± SEM, n=3, * no differences between two groups.

Online Figure VI. Analysis of hypertrophic regulators in MKK4<sup>cko</sup> hearts in response to TAC for 5 weeks. Protein extracts from MKK4<sup>f/f</sup> and MKK4<sup>cko</sup> hearts subjected to TAC or sham operation were examined by immunoblotting for total ERK1/2, ERK5, PKB, GSK3β and JunD expression as well as their phosphorylation levels using specific antibodies. Bar graphs summarize the ratios of phosphorylation to total expression of each protein (right panel). Data are the mean ± SEM, n=3, * no differences found between two groups.

Online Figure VII. Immunoblot analyses of the phosphorylation levels of JNK, p38 and MKK7 together with their expression levels in MKK4<sup>f/f</sup> and MKK4<sup>cko</sup> hearts in response to 1 week TAC (A), or 5 weeks TAC (B). MKK4<sup>cko</sup> mice showed a substantial reduction in p-JNK compared to controls in both 1 week and 5 week TAC groups. Both p38 and MKK7 were activated by TAC treatment, and levels of p-p38 and p-MKK7 were equivalent in these two groups. Bar graphs summarize the ratios of phosphorylation to total expression of each protein (right panel). Data are the mean ± SEM, n=3, * no differences are found between two groups.

Online Figure VIII. The loss of MKK4 promotes isoproterenol induced cardiac hypertrophy. (A) MKK4<sup>cko</sup> mice display an enhanced cardiac hypertrophy (upper panel, scale bar: 2mm) following isoproterenol treatment compared to MKK4<sup>f/f</sup> control mice.
Hematoxylin & eosin staining of heart cross-sections indicates enlarged cardiomyocytes in MKK4\textsuperscript{cko} mice (bottom panel, scale bar: 20µm). (B) HW/TL ratios of MKK4\textsuperscript{f/f} and MKK4\textsuperscript{cko} mice were evaluated after 7-day isoproterenol treatment. Both MKK4\textsuperscript{f/f} and MKK4\textsuperscript{cko} mice develop increased HW/TL ratio following treatment with isoproterenol compared the vehicle treated mice. MKK4\textsuperscript{cko} mice display enhanced HW/TL indicative of cardiac hypertrophy compared to MKK4\textsuperscript{f/f} controls. Data are presented as mean ± SEM, (n=8). (C) Mean cross-sectional areas of cardiomyocytes in MKK4\textsuperscript{f/f} and MKK4\textsuperscript{cko} mice are calculated. Data correspond to the mean ± SEM (n=8).

Online Figure IX. Characterization of hypertrophic remodeling in MKK4\textsuperscript{cko} mice treated with isoproterenol. (A) Ventricular interstitial fibrosis is apparent in MKK4\textsuperscript{cko} hearts shown by Masson’s trichrome-staining of cross-sections (left panel) (arrows are pointed to fibrosis areas, scale bar: 50µm). Quantification of the relative fibrosis area is expressed as percentage of the fibrosis area in the microscope views (right panel, data are the mean ± SEM, n=8). (B) \textit{In vivo} hemodynamic analysis of dP/dt\textsubscript{max} (contractile response) and dP/dt\textsubscript{min} (lusitropic response) and (C) echocardiographic assessment of fractional shortening (FS) are indices of cardiac function. Data are mean ± SEM, n=4 for \textit{in vivo} hemodynamic analysis, n=8 for echocardiography. * no difference between the two groups.

Online Figure X. Quantitative RT-PCR analyses of marker genes for isoproterenol induced cardiac hypertrophy and ventricular remodeling. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content and expressed relative to the mRNA extracted from MKK4\textsuperscript{f/f} mice treated with
vehicle. Hypertrophic marker genes: ANP, BNP, Myh7; fibrosis marker genes: Col1α2, Col3α1 and RCAN1.4. Data are the mean ± SEM, n=3.

Online Figure XI. JNK is not activated by swimming. Ventricular tissues prepared from M KK4^{ff} and M KK4^{cko} hearts after swimming were analyzed for expression and phosphorylation levels of JNK, p38 and PKB. Ratios of phosphorylation to expression levels are shown in graphs (n=3). Data are presented as mean ± SEM.

References


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<th>Genotype</th>
<th>CTL (n=8)</th>
<th>KO (n=8)</th>
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<td>5.81±0.15</td>
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<td>Heart Rate (bpm)</td>
<td>414±7</td>
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<td>dPW (mm)</td>
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<td>LVEDD (mm)</td>
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<td>LVESD (mm)</td>
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<td>FS (%)</td>
<td>35.5±0.8</td>
<td>34.0±1.9</td>
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Echocardiographic assessment of adult MKK4<sup>+/−</sup> and MKK4<sup>cko</sup> mice at baseline
Online Figure I

A

B
Online Figure II

MKK4^{f/f}  |  MKK4^{CKO}  
---|---
0 | 30 (min)

MKK4^{f/f}  |  MKK4^{CKO}  
---|---
0 | 30 (min)

**c-Jun**

**ATF2**

**JNK activity (\% of maximum)**

**p38 activity (\% of maximum)**

\( P < 0.001 \)

* *
Online Figure III

Ejection fraction (%)

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P<0.01

Fractional shortening (%)

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P<0.01
Online Figure IV

**ANP**

- mRNA levels for ANP with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.

**BNP**

- mRNA levels for BNP with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.

**Myh7**

- mRNA levels for Myh7 with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.

**Col1a2**

- mRNA levels for Col1a2 with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.

**Col3a1**

- mRNA levels for Col3a1 with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.

**RCAN1.4**

- mRNA levels for RCAN1.4 with sham and TAC treatments for MKK4^WT^ and MKK4^CKO^ mice.
- Significant differences indicated by *P < 0.001*.
Online Figure V

**P/T** ERK1/2 ratio (arbitrary units)
**P/T ERK5 ratio (arbitrary units)**
**P/T** JunD ratio (arbitrary units)

**P/T GSK3β ratio (arbitrary units)**
**P/T** PKB ratio (arbitrary units)

**P/T** JUN ratio (arbitrary units)
Online Figure VIII

A

MKK4<sup>fl/fl</sup>   MKK4<sup>CKO</sup>  

vehicle iso  vehicle iso

B

HW/TL (mg/mm)

vehicle iso vehicle iso

MKK4<sup>fl/fl</sup> MKK4<sup>CKO</sup>

C

Myocyte cross-sectional area (µm<sup>2</sup>)

vehicle iso vehicle iso

MKK4<sup>fl/fl</sup> MKK4<sup>CKO</sup>
Online Figure IX

A

Interstitial myocardial fibrosis (percentage %)

B

Fractional shortening (%)

C

Online Figure IX

MKK4^{f/f} vs. MKK4^{CKO} comparison of interstitial myocardial fibrosis and fractional shortening under iso and vehicle conditions.

MKK4^{f/f} vs. MKK4^{CKO} comparison of dP/dt max and dP/dt min under iso and vehicle conditions.

MKK4^{f/f} vs. MKK4^{CKO} comparison of fractional shortening under iso and vehicle conditions.
Online Figure X

**ANP**

**BNP**

**Myh7**

**Col1a2**

**Col3a1**

**RCAN1.4**
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**Graphs:**

- **P/T JNK ratio (arbitrary units):**
  - MKK4^f/f: Black diamonds
  - MKK4^CKO: Black squares

- **P/T p38 ratio (arbitrary units):**
  - MKK4^f/f: Black diamonds
  - MKK4^CKO: Black squares

- **P/T PKB ratio (arbitrary units):**
  - MKK4^f/f: Black diamonds
  - MKK4^CKO: Black squares