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Mitofusin 2 Inhibits Angiotensin II-Induced Myocardial Hypertrophy

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Abstract

Background and Objectives: Myocardial hypertrophy is a common clinical finding leading to heart failure and sudden death. Mitofusin 2 (Mfn2), a hyperplasia suppressor protein, is downregulated in hypertrophic heart. This study examined the role of Mfn2 in myocardial hypertrophy and its potential signal pathway. **Methods and Results:** In in vitro studies, neonatal cardiac myocytes were isolated and cultured. Incubation of cultured cardiomycytes with angiotensin II (Ang II) inhibited gene expression of Mfn2; induced cell hypertrophy and protein synthesis; and activated protein kinase Akt. Pretreatment of cells with AdMfn2—a replication-deficient adenoviral vector encoding rat *Mfn2* gene—upregulated *Mfn2* expression and subsequently attenuated Ang II-induced cell hypertrophy; protein synthesis; and Akt activation. In in vivo studies, direct gene delivery of AdMfn2 into myocardium decreased the infusion of Ang II-induced atrial natriuretic factor (ANF, a hypertrophic marker) expression and cardiomyocyte cross-sectional area. Consistently, upregulation of Mfn2 in myocardium decreased the thicknesses of anterior and posterior walls of left ventricle (LV) and the ratio of LV mass/body weight in Ang II-treated rats. Of note, AdGFP (control for AdMfn2) did not affect the effects of Ang II in vitro or in vivo. **Conclusions:** Upregulation of Mfn2 inhibits Ang II-induced myocardial hypertrophy. In this process, inhibition of Akt activation seems to play a significant role. These findings indicate Mfn2 is a critical protein in modulating myocyte hypertrophy.

Keywords

cardiac remodeling, myocyte hypertrophy, mitofusin 2

Introduction

Cardiac remodeling is a common consequence of different heart diseases, including hypertension, myocardial infarction, and diabetic cardiomyopathy, ultimately leading to heart failure. Proliferation of cardiac fibroblasts,[1] deposition of extracellular collagen,[2] and hypertrophy/loss of cardiomyocytes[3] are critical pathological findings in this process. Studies[4,5] have shown that angiotensin II (Ang II) plays an important role in cell proliferation, hypertrophy, and cell death. Its effects are mediated by Ras-ERK1/2 signaling pathway.[6]

Mitofusin 2 (Mfn2) plays a key role in the maintenance of the mitochondrial network and normal function in mammalian cells.[7] Our previous studies have demonstrated that Mfn2 acts as an endogenous Ras inhibitor and regulates vascular proliferative processes.[8] Overexpression of Mfn2 profoundly suppresses the mitogenic stimulus-evoked proliferation of vascular smooth muscle cells via inhibiting the Ras-ERK1/2 signaling pathway.[8] Recent study has shown that Mfn2 was downregulated in various conditions of cardiac hypertrophy both in vitro and in vivo.[9] Overexpression of Mfn2 in cardiomyocytes attenuates phenyephrine-induced protein synthesis and cell surface area increase.[10]

In this study, we used adenovirus-Mfn2 delivery to modulate Mfn2 expression in cultured cardiomyocytes. We examined the role of Mfn2 in hypertrophy and potential intracellular signaling in responsive to Ang II in cultured cardiomyocytes. The effects of Mfn2 on hypertrophy were further documented in animals by delivery of AdMfn2 into the myocardium.

Methods

Materials

Ang II (Sigma, St. Louis, MO); and [^{3}H] leucine (PerkinElmer Boston, MA); antibodies to Akt and phosphorylated-Akt (p-Akt, Ser473), cell signaling technology (Beverly, MA); antibody to rat Mfn2 (Santa Cruz Biotech, Santa Cruz, CA) were used.

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Cell Isolation and Culture

Neonatal cardiac cardiomyocytes were isolated from Sprague Dawley (SD) rats, as described.[11-13] Cardiomyocytes were plated at a density of 5×10^4 cells/mL into culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μmol/L bromodeoxyuridine (BrdU), and antibiotics for the first 48 hours. We obtained cell cultures in which more than 90% of cells were myocytes, as assessed by immunohistochemical analysis with a monoclonal antibody against sarcomeric α-actinin (Santa Cruz). The cultured cells were a pooled cell population from 30 to 40 neonatal rat ventricles of each experiment and at least 4 independent experiments in triplicate were performed for each condition.

Quantification of Protein Synthesis

[^{3}H] leucine incorporation was used to assess the protein synthesis of cardiomyocytes. Cells (2×10^4 per well) were treated with adenovirus, AngII, and 1 μCi/mL of [^{3}H] leucine, as described earlier. Data of incorporation were normalized on the basis of protein concentration of cell lysates of each well.

Western Blot for Akt Phosphorylation Activity

Protein was extracted from cell lysis. Western blot analysis was performed as described by Guo et al.[14] Antiphospho-specific Akt antibody and antitotal Akt antibody were probed on the same membranes successively. The ratio of phosphorylated proteins versus total Akt proteins was used to assess Akt activation. The data were expressed as the ratio of experimental group to control group (replication-deficient adenoviral vectors encoding recombinant green fluorescent protein [AdGFP] alone).

Real-Time Quantitative Polymerase Chain Reaction

Tissue RNA was extracted from the cardiac tissue using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and 1 μg of total RNA was converted into complementary DNA (cDNA) with the use of reverse transcriptase and oligo (dT) (Promega Madison, MI) as a primer. Real-time quantitative polymerase chain reaction (PCR) was performed using the TransStart SYBR Green qPCR SuperMix Kit (TransGen Biotech, Beijing, China) on an Opticon TM detection system (MJ Research Waltham, MA), and the results were analyzed with the Opticon Monitor analysis software. Polymerase chain reaction was performed by standard methods using synthetic gene-specific primer. The primers sequences were Mfn2: 5′-ATGATAGACGGCTTGAA-3′ (F), 5′-CGACTCC CTCTTTGTGA-3′ (R); atrial natriuretic factor (ANF): 5′-AG TGCGGTGTCCAACACAGAT-3′ (F), 5′-TTCT CCTCCAG GTGGTCTAGCA-3′ (R); and 18s ribosomal RNA: 5′-TAC-CACATCCAAGGAAGGCAGCA-3′ (F), 5′-TGGAATTACC GCGGCTGCTGGCA-3′ (R). Transcription abundance was expressed as fold increase over the 18s ribosomal RNA value from the same RNA samples calculated by $2^{-\Delta Ct}$ method.

Animals

Male SD rats (8-10 weeks) were randomly assigned to each group (n = 9). Rats were fed normal chow and water ad libitum in standard cages on a 12: 12-h light–dark cycle. The study was conducted according to the Principles of Laboratory Animal Care (NIH publication no 85-23, revised 1996) in the Care and Use of Animals, and the protocol was approved by the Animal Care Committee, Peking University Health Science Center, China.

Adenoviral Vectors and Gene Transfer

Replication-deficient adenoviral vectors encoding recombinant green fluorescent protein (AdGFP) or rat Mfn2 (AdMfn2) were described previously.[8] Viruses were multiplied and purified using ViraBind Adenovirus Purification Kit (Cell Biolabs, USA). A thoracotomy was performed over the third intercostal space of the left thorax to expose the heart of rat. AdMfn2 or AdGFP (titer at 1×10^{10} PFU/ml in 100 μl Elution Buffer / 10% glycerol) was slowly injected into multi-points of the left ventricular (LV) free wall. The chest was closed and extubation after negative pleural pressure reestablished. Animals were given antibiotics via intramuscular injection. At day 7 after gene was transferred, Mfn2 expression and echocardiography was evaluated.[15,16]

Animal Model and Echocardiography

Micro-osmotic pumps (model 1002, Alzet, Alza Corporation, Palo Alto, CA) releasing Ang II (0.7 mg/kg per d in 0.9% NaCl/0.01N acetic acid)[17-19] or saline were implanted subcutaneously into the 2 group rats. Myocardial hypertrophy was assessed by echocardiography with Vevo770 ultrasound system (Visual Sonics Inc, Canada) equipped with a 17.5-MHz microprobe. Ventricular measurements in M mode were taken 21 days after pump implantation, with 3 readings per rat. Left ventricular wall thickness was assessed by echocardiography as described before.[20] The observers were blinded to the treatment of rats.

Myocardial Tissue Sampling and Staining

At day 7 after direct injection of viruses, animals were anesthetized for echocardiogram and then sacrificed for tissue sampling by overdose of pentobarbital sodium. Hearts were rapidly removed and lavaged through coronary vessels with phosphate-buffered saline (PBS), then LVs were weighed for calculating LV mass. Some myocardial tissues were used to extract total messenger RNA (mRNA) for real-time PCR of ANF, as described earlier. Some myocardial tissues were cut in a cross-section just below the level of the papillary muscle, fixed in 4% paraformaldehyde solution, and embedded in paraffin for tissue sections. Hematoxylin and eosin (H&E) staining was performed to facilitate quantification of cardiomyocyte

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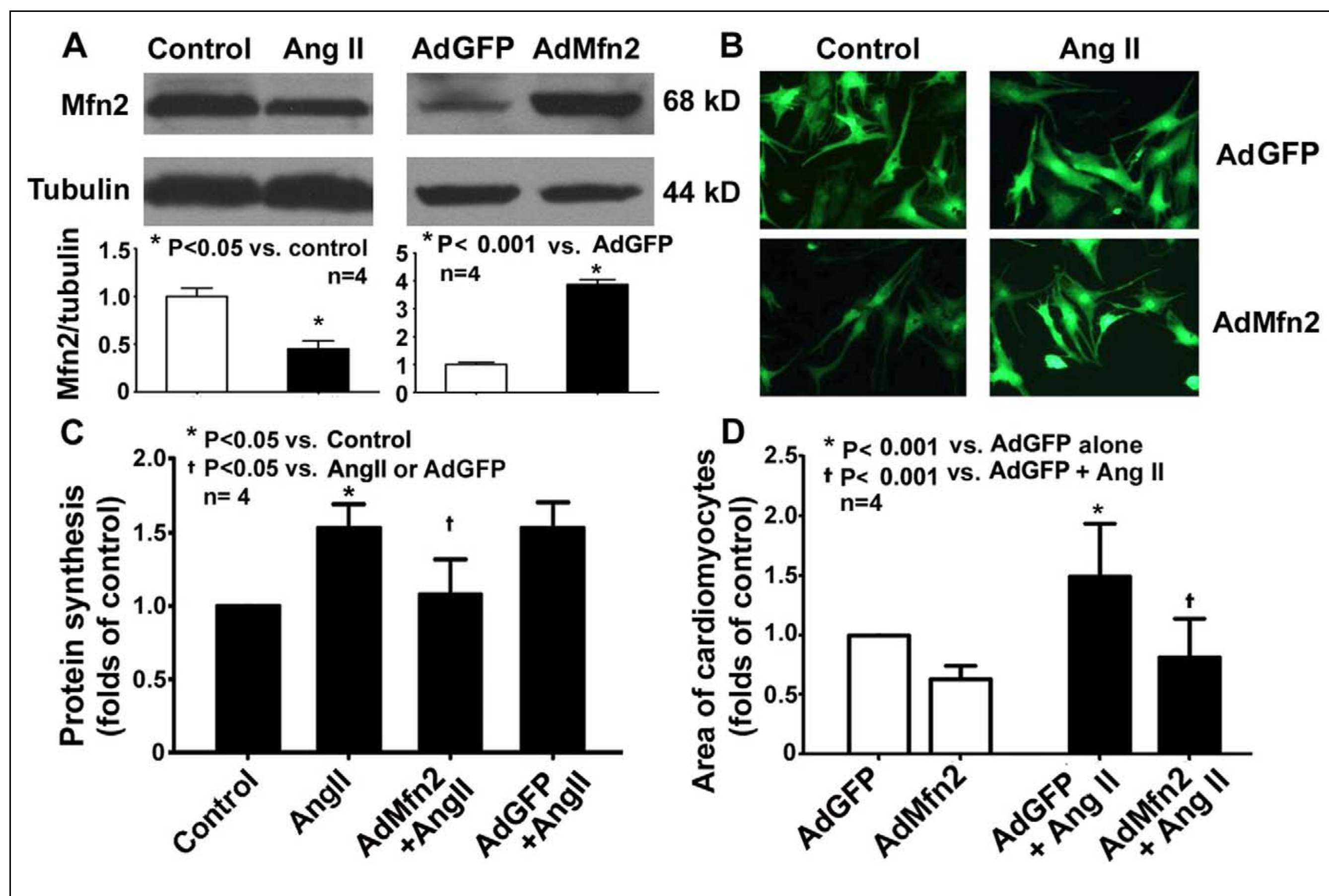

Figure 1. Mfn2-inhibited hypertrophy of cardiomyocytes. A, Incubation of cultured cardiomyocytes with Ang II reduced Mfn2 protein expression (left panel). Pretreatment of cardiomyocytes with AdMfn2 increased protein expression of Mfn2 (right panel). B, Cultured cardiomyocytes transfected with AdGFP or AdMfn2 with GFP. Ang II stimulated cell hypertrophy. Upregulation of Mfn2 inhibited the effect of Ang II on cell hypertrophy. Summarized data were shown in D. C, Pretreatment of cardiomyocytes with Ang II increased protein synthesis. The increase in Mfn2 reduced protein synthesis in response to Ang II. Pretreatment of cardiomyocytes with AdGFP as a control did not affect size and protein synthesis of cells. Ang II indicates angiotensin II; Mfn2, mitofusin 2; AdGFP, replication-deficient adenoviral vectors encoding recombinant green fluorescent protein.

size. Hypertrophy was assessed by measuring cross-sectional area of 100 cardiomyocytes per section in 10 randomly selected fields in the LV free wall.[21]

Statistical Analysis

Data were expressed as mean $\pm$ SD. Groups were compared through Student *t* test and 1-way analysis of variance (ANOVA), followed by Newman-Keuls test. Multiple comparisons among 3 or more groups were performed by 2-way ANOVA and Fisher exact test for post hoc analyses. $P < .05$ was considered a statistical difference.

Results

Modulation of Mfn2 and Hypertrophy of Cardiomyocytes In Vitro

Incubation of cultured cardiomyocytes with Ang II (10^{-6} mol/L) for 48 hours markedly reduced gene expression of Mfn2 ($P < .05$ vs control, n = 4; Figure 1A left panel). Pretreatment of cardiomyocytes with AdMfn2 for 24 hours significantly increased protein expression of Mfn2 in cardiomyocytes ($P < .05$ vs AdGFP alone, n = 4, Figure 1A right panel). As shown in Figure 1B and C, pretreatment of cardiomyocytes with Ang II markedly increased cell size and protein synthesis, respectively. Importantly, the increase in Mfn2 significantly reduced cell size and their protein synthesis in response to Ang II. In parallel experiments, pretreatment of cardiomyocytes with AdGFP for 24 hours did not affect cell size and protein synthesis of cells. Figure 1B is a representative of cells transfected by AdMfn2 or AdGFP with GFP. Figure 1D is the summarized data of cell size from Figure 1C.

Effects of Mfn2 and Activation of Akt in Cultured Cardiomyocytes

To study the potential intracellular signaling pathway of Mfn2, we studied the activation of Akt in response to Ang II in

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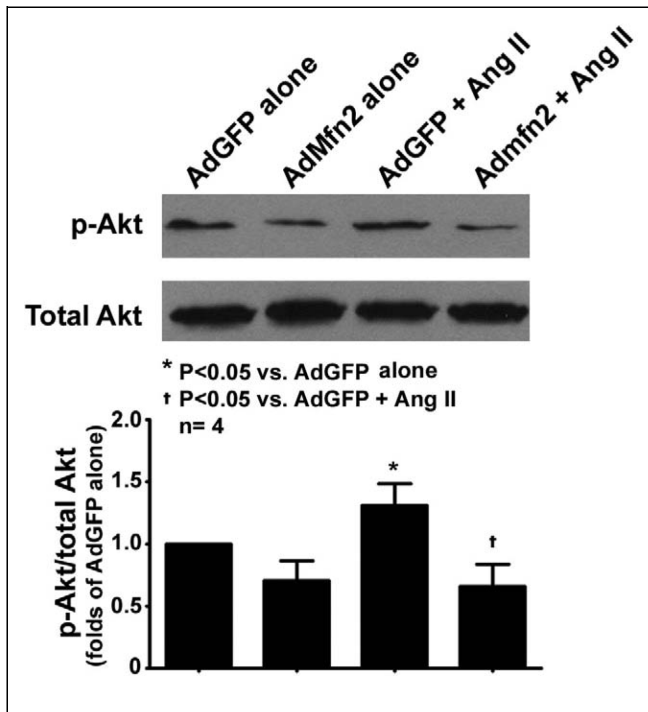

Figure 2. Mfn2 and activation of Akt in cultured cardiomyocytes. Ang II induced phosphorylation of Akt compared to control. Upregulation of Mfn2 suppressed phosphorylation of Akt in response to Ang II. AdGFP had no effect on Akt activation. The top panel is a representative from 4 separate experiments. That the ratio of activated Akt over total Akt was normalized by control group treated with AdGFP alone were shown in the bottom panel. Ang II indicates angiotensin II; Mfn2, mitofusin 2; AdGFP, replication-deficient adenoviral vectors encoding recombinant green fluorescent protein.

cardiomyocytes. We found that Ang II induced phosphorylation of Akt in cardiomyocytes. Importantly, upregulation of Mfn2 significantly depressed phosphorylation of Akt in response to Ang II in cardiomyocytes (Figure 2).

Gene Delivery of Mfn2 Expression In Vivo

Messenger RNA and protein expression of Mfn2 was significantly reduced at day 14 in the myocardium of rats treated with continuous infusion of Ang II (all $P < .05$ vs sham control group). It is noted that direct injection successfully delivered AdMfn2 to myocardium since protein and mRNA expression of Mfn2 was significantly upregulated in myocardium at day 7 after injection with AdMfn2 compared to control group injected with AdGFP (Figure 3).

Mfn2 Expression and Myocardial Hypertrophy in Rats

There was an increase in thicknesses of the anterior and posterior walls and the ratio of LV mass/body weight in Ang II-treated rats (all *P*s < .05 vs control). Notably, we found that upregulation of Mfn2 significantly inhibited Ang II-induced thickness of LV walls and LV mass (all *P*s < .05 vs only Ang II-treated group). In parallel experiments, AdGFP as a control had no effect on Ang II-induced myocardial hypertrophy (Figure 4).

Further myocardial tissue study showed that Ang II increased in the cross-sectional area of the cardiomyocytes in rats compared to control ($P < .05$, n = 9). This effect was significantly inhibited by upregulation of Mfn2. Again, AdGFP as a control had no effect. Fetal gene *ANF* is a marker for myocardial hypertrophy. It was significantly high in myocardium treated with Ang II compared to control ($P < .05$). Upregulation of Mfn2 suppressed gene expression of ANF compared to group-treated with Ang II ($P < .05$). This effect of Ang II was not affected by AdGFP delivery (Figure 5).

Discussion

Increasing evidence indicate that Mfn2 may play a critical role in restraining myocardial hypertrophy. In the current study, we demonstrated that upregulation of Mfn2 significantly inhibited Ang II-induced hypertrophy and protein synthesis in cultured cardiomyocytes. Activation of Akt might play an important role in this process. Further study documented that upregulation of Mfn2 attenuated a hypertrophic gene *ANF* expression and myocardial hypertrophy in response to Ang II in rats.

Mfn2 and Hypertrophy of Cardiac Myocytes

Mfn2 is a ubiquitously expressed and well-conserved gene.[8] It is highly expressed in cells with high-energy requirement, such as cardiomyocytes[10,22] or smooth muscle cells.[8,14] Mfn2 has been proposed as an important mitochondrial protein in maintaining mitochondrial network and bioenergetics.[7] Studies[22] have demonstrated that Mfn2 is a major determinant of oxidative stress-mediated heart muscle cell apoptosis. A study by Chen et al[10] showed that phenyephrine increases ANF mRNA level and cell surface area in cultured neonatal cardiomyocytes. Upregulation of Mfn2 mRNA and protein significantly attenuated these effects of phenyephrine on expression of ANF and size of cell surface compared with control group. Other studies[23,24] showed that Mfn2 was downregulated spontaneously in hypertensive rats.

It is well known that Ang II is a critical factor in cardiovascular disease including hypertrophic cardiomyopathy and heart failure. In the current study, we first demonstrated that role of Mfn2 in Ang II-mediated pathophysiological effects in cardiac myocytes. We found that incubation of cardiomyocytes with Ang II increased the size of cardiomyocytes and their protein synthesis. Upregulation of Mfn2 inhibited Ang II-induced hypertrophy of cardiomyocytes. These findings were further confirmed in rats. Continuous infusion of Ang II in rats resulted in myocardial hypertrophy which was indicated by measurement of thickness of anterior and posterior walls and ratio of LV mass over body weight. Ex-vivo study also showed an increase in the size of myocytes and a hypertrophic marker ANF. Notably, upregulation of Mfn2 expression in vitro and in vivo significantly inhibited these effects of Ang II. These

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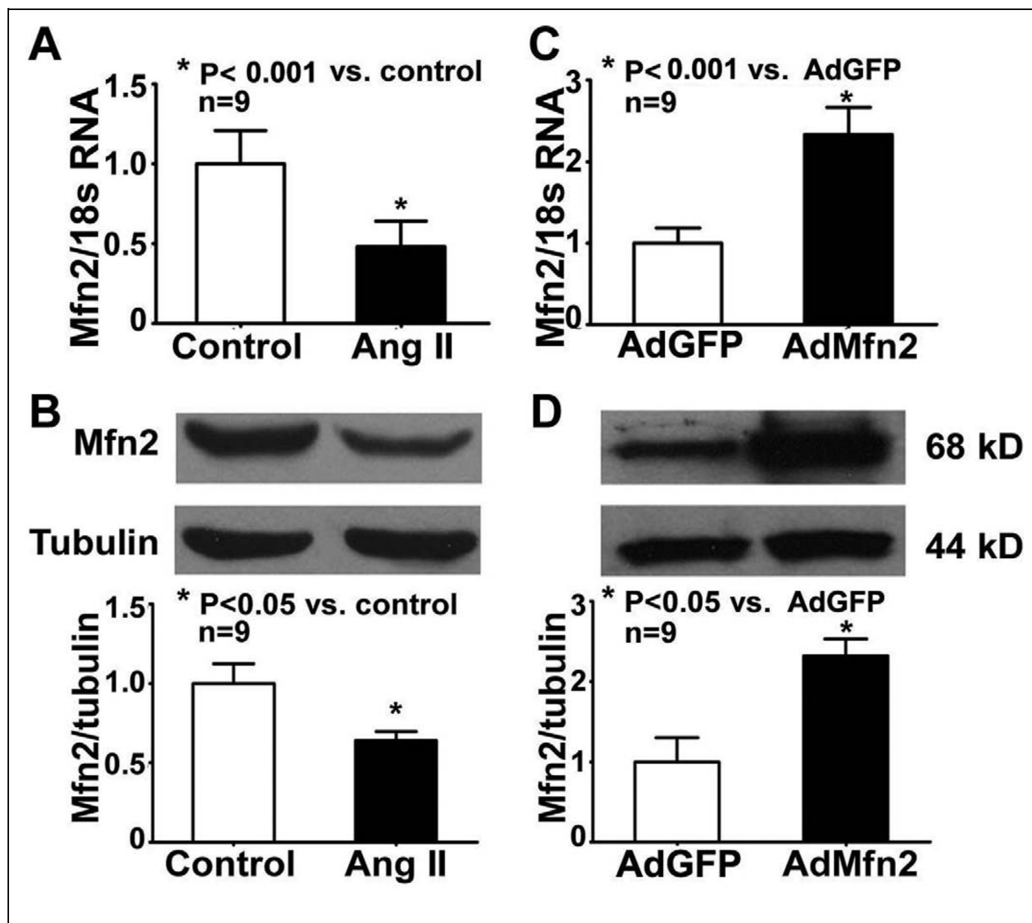

Figure 3. Gene delivery of Mfn2 expression in vivo: A, mRNA expression of Mfn2 determined by real-time PCR was reduced at day 14 in the myocardium of rats treated with continuous infusion of Ang II compared to control. C, Direct injection of AdMfn2 into myocardium increased mRNA expression of Mfn2. B, Protein expression of Mfn2 was reduced at day 14 in the myocardium of rats treated Ang II. D, Delivery of AdMfn2 into myocardium increased protein expression of Mfn2. AdGFP had no effect. One representative is shown on the top panel of B and D and summarized data are shown on the bottom of B and D. Ang II indicates angiotensin II; Mfn2, mitofusin 2; AdGFP, (replication-deficient adenoviral vectors encoding recombinant green fluorescent protein; mRNA, messenger RNA; PCR, polymerase chain reaction.

observations suggest Mfn2 is an important gene in modulating hypertrophy of myocardium. Imbalance of Mfn2 expression may be a crucial pathogenic element and a potential therapeutic target for hypertrophic cardiomyopathy and heart failure.

Mfn2 and Its Intracellular Mechanisms

Ang II exerts its hyperplastic effects by activating a number of intracellular signal transduction pathways through AT1 receptor.[25] The activation of ERK1/2-induced by Ang II plays an important role in the enhancement of DNA synthesis in cardiac fibroblasts.[26-30] Chen et al[8] had demonstrated that Mfn2 has a p^{21}ras signature motif that binds to Ras and subsequently inhibits ERK1/2 signaling pathway in cultured vascular smooth muscle cells. Guo et al[31] demonstrated that Mfn2 triggered apoptosis of vascular smooth muscle cells at least partly through inhibition of PI3K-Akt. Other study[22] showed that Mfn2 is a major determinant of oxidative stress-mediated cardiomyocytes apoptosis through inhibition of Akt activation. In the current study, we found that Ang II inhibited Mfn2 expression and activated Akt in cultured cardiomyocytes and increased protein synthesis and a marker of hypertrophy ANF in myocardium. Importantly, upregulation of Mfn2 inhibited Ang II-induced activation of Akt in cardiomyocytes. These results indicate that increase in cardiac Mfn2 expression is both necessary and important to modulate signaling pathway and myocardial hypertrophy.

In summary, upregulation of Mfn2 inhibits Ang II-induced downregulation of *Mfn2* gene expression and increases protein synthesis and hypertrophy in cardiomycytes. In this process, inhibition of Akt activation may play an important role. These findings indicate that Mfn2 may be a key factor in cardiac remodeling.

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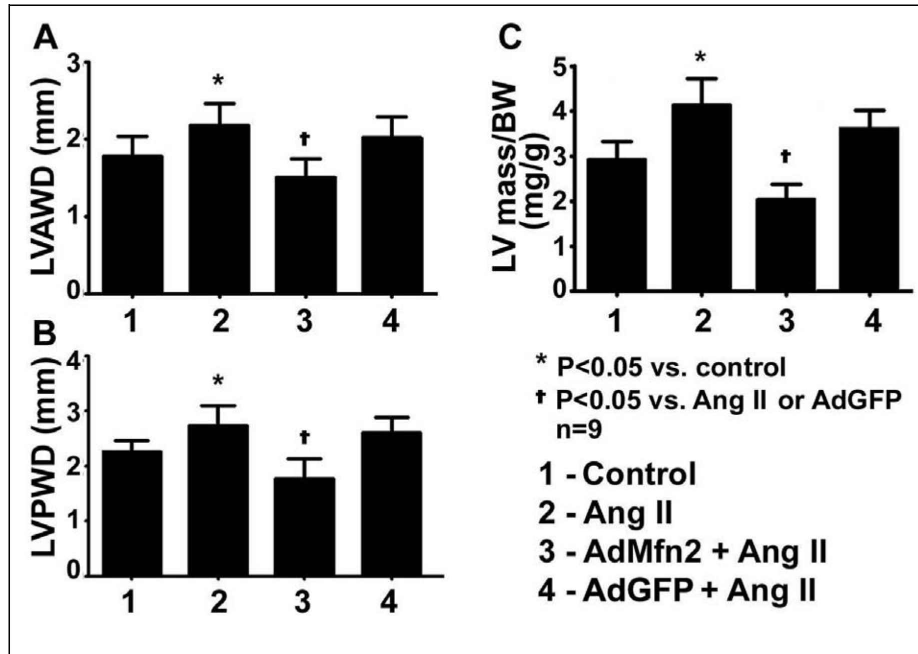

Figure 4. Mfn2 expression and myocardial hypertrophy in rats. There was an increase in the thicknesses of the anterior (A) and posterior walls (B) and the ratio of LV mass/body weight (C) in Ang II-treated rats. Notably, upregulation of Mfn2 significantly inhibited Ang II-induced thickness of LV walls and LV mass compared to Ang II-alone-treated group. AdGFP as a control had no effect on Ang II-induced myocardial hypertrophy. Ang II indicates angiotensin II; Mfn2, mitofusin 2; AdGFP, (replication-deficient adenoviral vectors encoding recombinant green fluorescent protein; LV, left ventricle.

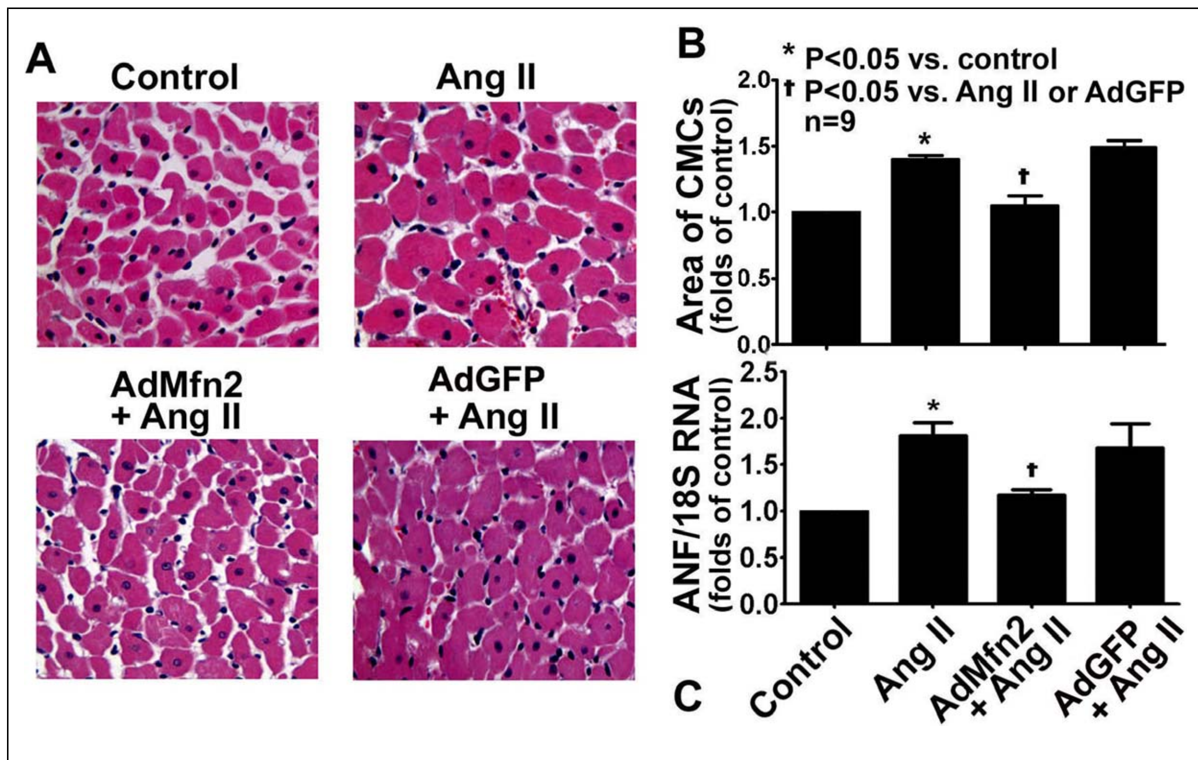

Figure 5. A. Ang II enlarged the cross-sectional area of the cardiomyocytes in rats compared to control. This effect was inhibited by upregulation of Mfn2. Again, AdGFP as a control had no effect. B, Summarized data from 9 different experiments. C, Fetal gene atrial natriuretic factor (ANF), a marker for myocardial hypertrophy, was upregulated in myocardium-treated with Ang II compared to control. Upregulation of Mfn2 attenuated gene expression of ANF compared to Ang II-treated group. This effect of Ang II was not affected by AdGFP delivery. Ang II indicates angiotensin II; Mfn2, mitofusin 2; AdGFP, (replication-deficient adenoviral vectors encoding recombinant green fluorescent protein.

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Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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