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Regulator of G Protein Signaling 3 Protects Against Cardiac Hypertrophy in Mice

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ABSTRACT

Regulator of G protein signaling 3 (RGS3) is a negative regulator of G protein-mediated signaling. RGS3 has previously been shown to be expressed among various cell types within the mature heart. Basic and clinical studies have reported abnormal expressions of RGS3 in hypertrophic hearts and in the failing myocardium. However, the role of RGS3 in cardiac remodeling remains unclear. In this study, we investigated the effect of cardiac overexpression of human RGS3 on cardiac hypertrophy induced by aortic banding (AB) in RGS3 transgenic mice and wild-type littermates. The extent of cardiac hypertrophy was evaluated by echocardiography as well as pathological and molecular analyses of heart samples. RGS3 overexpression in the heart markedly reduced the extent of cardiac hypertrophy, fibrosis, and left ventricular dysfunction in response to AB. These beneficial effects were associated with the inhibition of MEK-ERK1/2 signaling. In vitro studies performed in cultured neonatal rat cardiomyocytes confirmed that RGS3 overexpression inhibits hypertrophic growth induced by angiotensin II, which was associated with the attenuation of MEK-ERK1/2 signaling. Therefore, cardiac overexpression of RGS3 inhibits maladaptive hypertrophy and fibrosis and improves cardiac function by blocking MEK-ERK1/2 signaling. J. Cell. Biochem. 115: 977–986, 2014.

KEY WORDS: HYPERTROPHY; SIGNAL TRANSDUCTION; RGS3; ERK1/2

Cardiac hypertrophy is a common response of the myocardium to a variety of pathological stimuli including hypertension, valve disease, myocardial ischemia, and genetic mutations, characterized by an increase in the size of individual cardiac myocytes and whole-organ enlargement [Li et al., 2010; Jiang et al., 2013]. Although cardiac hypertrophy may initially be a compensatory response, its continued presence often leads to poor clinical outcomes, including heart failure, arrhythmia and sudden death [Lorell and Carabello, 2000]. Many researchers in recent decades have attempted to elucidate the underlying mechanisms of pathological hypertrophy and have sought to reverse its maladaptive consequences. However, effective anti-hypertrophic targets have not been clearly defined to date. Thus, it is important to define and modulate the specific signaling mechanism activated by each hypertrophic stimulus to enable the discovery of novel molecular targets for suppressing maladaptive hypertrophy.

G protein-coupled receptors (GPCRs) belong to one of the largest superfamilies of cell membrane receptors and are involved in the regulation of a wide variety of physiological processes. GPCRs interact with G-proteins to activate multiple downstream intracellular cascades and in turn modulate subsets of effector proteins. The activation of GPCR-mediated signaling pathways is a critical feature of many cardiovascular diseases including cardiac hypertrophy and heart failure [Heineke and Molkentin, 2006; Belmonte and Blaxall, 2011]. Regulators of G-protein signaling (RGS) proteins are negative regulators of G protein-mediated signaling that serve as GTPase-activating protein for heterotrimeric G proteins and thereby inactivate G protein-coupled receptor signaling pathways [Wieland et al., 2007; Zhang and Mende, 2011]. Previous studies have demonstrated that several RGS proteins, including RGS2, RGS4, and RGS5, play important roles in the regulation of cardiac hypertrophy [Tokudome et al., 2008; Takimoto et al., 2009; Li et al., 2010]. One

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member of the RGS protein superfamily, RGS3, is one of the predominant RGS proteins expressed in the human heart [Zhang and Mende, 2011]. RGS3 has been reported to inhibit the Gαi- and Gαq-mediated signaling pathways in the cardiovascular system, including those acting via the cardiovascular signaling molecules angiotensin II (Ang II), sphingosine 1-phosphate and endothelin-1 [Cho et al., 2003]. Basic and clinical studies have reported an abnormal expression of RGS3 in hypertrophic hearts and failing myocardium [Zhang et al., 1998; Chakir et al., 2011]. However, the role of RGS3 as a regulator of cardiac remodeling and heart failure has not been previously elucidated. In the present study, we demonstrate that the cardiac constitutive expression of human RGS3 protects against cardiac hypertrophy by blocking the MEK-ERK1/2 signaling pathway. Our findings suggest that RGS3 is a critical modulator of cardiac hypertrophy and heart failure.

MATERIALS AND METHODS

ANIMALS AND ANIMAL MODELS

All protocols were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). A human RGS3 cDNA construct that contained full-length human RGS3 cDNA was cloned downstream of the human cardiac α-myosin heavy chain (α-MHC) promoter. Transgenic mice were produced by microinjecting the α-MHC-RGS3 construct into fertilized mouse embryos. Transgenic mice were identified by polymerase chain reaction (PCR) and Western blot assays. Mice were housed with free access to food and water and exposed to 12-h light/dark cycles. Wild-type C57BL/6 and cardiac-specific RGS3 transgenic male mice, whose body weights ranged from 23.5 to 27.5 g and whose ages ranged from 8 to 10 weeks, were divided into the sham and aortic banding (AB) groups. AB was performed as previously described [Li et al., 2010].

ECHOCARDIOGRAPHY AND HEMODYNAMIC MEASUREMENTS

At 4 weeks after AB, echocardiography and hemodynamics evaluations were to test the morphology and function of the mice. Echocardiography was performed using a Mylab30CV (Esaote S.p.A.) instrument with a 10 MHz linear array ultrasound transducer. The tissue Doppler images of the left ventricle (LV) were assessed in the parasternal short-axis view at a frame rate of 50 Hz. End-systole and end-diastole measurements defined as the phase with the smallest and largest area of the LV, respectively, were obtained. The left ventricular end diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD) were measured from the LV-M-mode tracing with a sweep speed of 10 mm/s at the papillary muscle level. Subsequently, the percentage of left ventricular fractional shortening (LVFS) was calculated as (LVEDD-LVESD)/LVEDD × 100%.

For hemodynamic measurements, a microtip catheter transducer was inserted into the right carotid artery and advanced into the left ventricle. The signals were continuously recorded using a Millar Pressure-Volume System (MPVS-400, Millar Instruments, Houston, TX), and the data were processed by PVAN data analysis software.

At the end of the experiment, the hearts and lungs of the sacrificed mice were dissected and weighed to compare the heart weight to body weight (HW/BW, in mg/g), lung weight to body weight (LW/BW, in mg/g), and heart weight to tibia length (HW/TL, in mg/cm) ratios.

HISTOLOGICAL ANALYSIS

Hearts were excised, washed with saline solution and placed in 10% formalin. Hearts were transversely cut close to the apex to visualize the left and right ventricles. Several sections of heart (5 μm thick) were prepared and stained with hematoxylin and eosin (H&E) for histopathology or picrosirius red (PSR) for collagen deposition and then visualized by light microscopy. To obtain the myocyte cross-sectional area (CSA), the sections were stained for membranes using FITC-conjugated WGA (Invitrogen) and for nuclei using 6-diamidino-2-phenylindole dihydrochloride (DAPI). Single myocytes were measured using a quantitative digital image analysis system (Image Pro-Plus, version 6.0). The outlines of no less than 100 myocytes were traced in each group.

RECOMBINANT ADENOVIRAL VECTORS AND CULTURED NEONATAL RAT CARDIOMYOCYTES

To overexpress RGS3, we used replication-defective adenoviral vectors that encoded for the entire coding region of the RGS3 gene. A similar adenoviral vector encoding the GFP gene was used as a control. To knock down RGS3 expression, we generated AdshRGS3 adenoviruses, which produced a significant decrease in RGS3 levels. Ad-shRNA was used as a control.

Primary neonatal rat cardiomyocytes (NRCMs) were cultured as previously described [Li et al., 2010]. Briefly, neonatal hearts of 1- to 2-day-old Sprague-Dawley rats were removed from the thoracic cavities after euthanization. PBS containing 0.03% trypsin and 0.04% collagenase type II was used to digest finely minced heart tissue. NRCMs were enriched by differential pre-plating for 2 h; seeded at a density of 1×10^5 cells/well onto gelatin-coated, six-well culture dishes; and cultured in media consisting of DMEM/F12 medium, 20% FCS, BrdU (0.1 mM), and penicillin/streptomycin at 37°C for 48 h. Subsequently, culture media were changed to serum-free DMEM/F12 for 12 h before adenoviral infection and/or Ang II, or U0126 treatment. Protein synthesis in cultured cells was evaluated by Western blotting. For the CSA measurement, cardiomyocytes were stained with a-actin and DAPI. Microscopic images were captured, and the myocyte surface area was measured using a quantitative digital image analysis system.

QUANTITATIVE REAL-TIME PCR

The tissues were frozen in liquid nitrogen for RNA and protein analyses. For quantitative real-time PCR, the total RNA was extracted from the left ventricle using TRIzol. Real-time polymerase chain reaction analyses (RT-PCR) were used to detect the mRNA expression of hypertrophy and fibrosis markers from hearts in different groups, including atrial natriuretic peptide (ANP) (forward: 5′-ACCTGCTAGAC-CACCTGGAG-3′ and reverse: 5′-CCTTGGCTGTTATCTTCGGTACCGG-3′), B-type natriuretic peptide (BNP) (forward: 5′-GAGGTCACTCC-TATCCTCTGG-3′ and reverse: 5′-GCCATTTCCTCCGACTTTTCTC-3′), β-myosin heavy chain (β-MHC) (forward: 5′-TTCATCCGAATC-CATTTTGGGG-3′ and reverse: 5′-GCATAATCGTAGGGGTTGTTGG-3′),

procollagen type Iα1 (Col1α1) (forward: 5′-AGGCTTCAGTGGTTTG-GATG-3′ and reverse: 5′-CACCAACAGCACCATCGTTA-3′), procollagen type III α1 (Col3α1) (forward: 5′-CCCAACCCAGAGATCCCATT-3′ and reverse: 5′-GAAGCACAGGAGCAGGTGTAGA-3′), and connective tissue growth factor (CTGF) (forward: 5′-TGACCCCTGCGACCCACA-3′ and reverse: 5′-TACACCGACCCACCGAAGACACAG-3′). GAPDH (forward: 5′-ACTCCACTCACGGCAAATTC-3′ and reverse: 5′-TCTCCATGGTGGT-GAAGACA-3′) mRNA was used for normalization.

WESTERN BLOTTING

Cardiac tissue and cultured NRCMs were lysed in RIPA lysis buffer. Protein was measured using a protein assay kit (Thermo), and equal amounts of samples(50 μg per lane) were separated using PAGE with 4–12% Bis–Tris gels (Invirogen), and subsequently transferred to PVDF membranes. Immunoblots were incubated overnight at 4°C with the antibody (CST, Santa crus, Bioword). After washing, the immunoblots were incubated with secondary IgG antibodies (LI-COR). Immunoblots were scanned using the Odyssey Infrared Imaging System (LI-COR), and all determined values were normalized to GAPDH.

STATISTICAL ANALYSES

The SPSS 13.0 statistical software package was used. Data were expressed as the mean ± SE. Differences among the groups were tested using a one-way ANOVA followed by a post hoc LSD test or Tamhane's T2 test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

THE EXPRESSION OF RGS3 IS DOWN-REGULATED IN EXPERIMENTAL HYPERTROPHIC MODELS

To explore the potential role of RGS3 in cardiac hypertrophy, we first examined RGS3 expression in heart samples collected from mice that had been subjected to AB. Our data revealed that RGS3 expression in the murine heart was gradually and significantly down-regulated from 4 to 8 weeks after the AB operation (Fig. 1A). Similarly, the expression of endogenous RGS3 was strikingly reduced in NRCMs in response to Ang II stimulation (Fig. 1B). These results suggest that RGS3 may be involved in the development of cardiac hypertrophy.

CHARACTERISTICS OF CARDIAC-SPECIFIC HUMAN RGS3 OVEREXPRESSION MICE

To examine the in vivo role of endogenous RGS3 in the regulation of cardiac hypertrophy, we generated TG mouse lines with cardiac-specific overexpression of human RGS3 (i.e., TG mice) using the α-MHC promoter. The four lines of TG mice were confirmed by PCR and Western blot analysis. All of the lines were viable and exhibited normal reproductive rates and sex distributions. Among the four established lines of TG mice, the line that expressed the highest levels of human RGS3 protein in the heart was used in further experiments (Fig. 2A). We analyzed the RGS3 protein levels in various tissues of TG mice by Western blot analyses using a human-specific anti-RGS3 antibody. A high level of RGS3 expression was observed in the heart but not in the other organs, indicating the specificity of the TG expression (Fig. 2B). Under basal conditions, the cardiac-specific overexpression of RGS3 had no significant effect on the HW/BW or LW/BW relative to the WT littermates (Fig. 3A), suggesting that RGS3 plays a negligible role in cardiac development or function under basal conditions.

RGS3 OVEREXPRESSION REPRESSES CARDIAC HYPERTROPHY IN VIVO

To investigate the role of RGS3 in the heart in response to chronic pressure overload, WT littermates and TG mice were subjected to either AB surgery or a sham operation. Cardiac morphology and

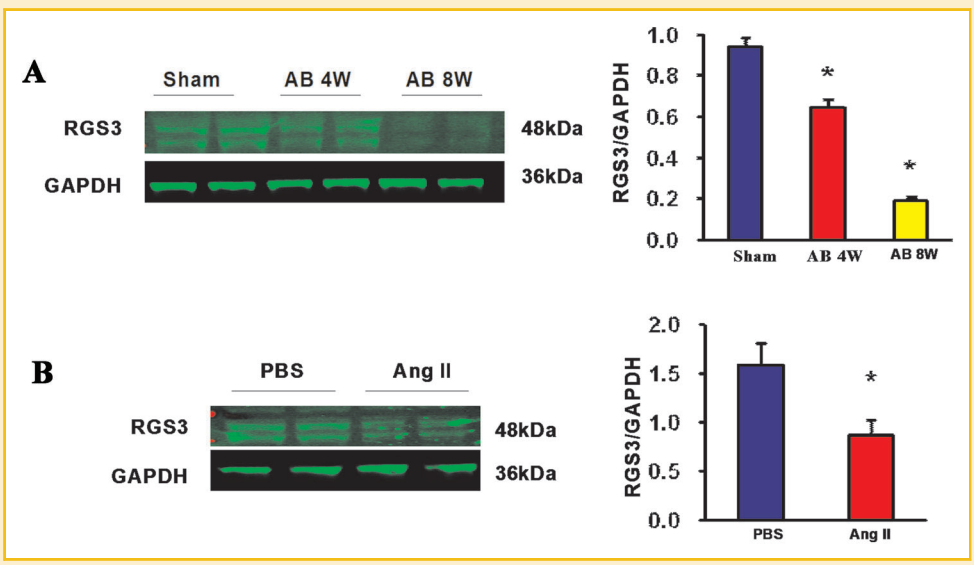

Fig. 1. RGS3 expression is down-regulated in experimental hypertrophic models. RGS3 protein levels in left ventricular samples from (A) wild-type mice at the indicated times after sham or AB operation; and (B) NRCMs treated with Ang II for 48 h (n = 3 independent experiments, * $P < 0.05$ vs. sham or PBS). Left: representative blots; Right: quantitative results.

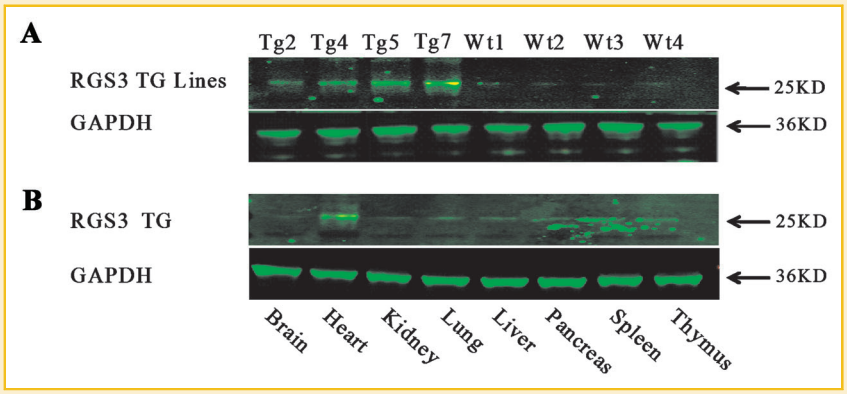

Fig. 2. Characterization of human RGS3 transgenic mice. A: Representative Western blots of the human RGS3 protein in heart tissues from four lines of TG and WT mice. B: Representative Western blots of human RGS3 protein from various tissues of TG mice, as indicated.

function were examined after 4 weeks of AB. Compared with WT mice, TG mice exhibited a significant attenuation of hypertrophy, as evidenced by improvements in HW/BW, HW/TL, and LW/BW ratios as well as the size of the CSA (Fig. 3A). No significant differences were observed in the sham operation TG and WT mice. We subsequently examined the cardiac function of TG and WT mice using echocardiography. Gross hearts and H&E-stained sections further confirmed the inhibitory effect of RGS3 on cardiac remodeling after AB (Fig. 3B). We compared the expression level of several cardiac hypertrophy markers in TG and WT mice after 4 weeks of AB and found that the induction of hypertrophic markers ANP, BNP, and β-MHC were significantly increased in WT mice after AB, and these increases were markedly suppressed in TG mice (Fig. 3C). These results indicate that cardiac RGS3 plays a critical protective role against pressure overload-induced cardiac hypertrophy in vivo. RGS3 overexpression also significantly alleviated AB-triggered cardiac dilation and dysfunction compared with controls, as determined by echocardiographic and hemodynamic analysis (Fig. 3D,E). Thus, RGS3 not only inhibited the development of cardiac hypertrophy but also improved ventricular function in vivo.

RGS3 OVEREXPRESSION ATTENUATES HYPERTROPHIC GROWTH IN VITRO

To examine the in vitro role of RGS3 in hypertrophy, we performed gain- and loss-of-function studies using cultured NRCMs. Cells were infected with Ad-RGS3 or Ad-shRGS3 and then treated with 1 μM Ang II for 48 hours. Ad-RGS3 infection resulted in a substantial increase in the level of RGS3 protein, whereas Ad-shRGS3 markedly inhibited the expression of RGS3 in NRCMs (Fig. 4A). Further studies showed that Ang II-mediated hypertrophy was alleviated by infection with Ad-RGS3 and enhanced by infection with Ad-shRGS3, as determined by CSA measurements (Fig. 4B,C). These data suggest an inhibitory effect of RGS3 on hypertrophy in vitro.

RGS3 OVEREXPRESSION BLOCKS MEK-ERK1/2 SIGNALING IN VIVO AND IN VITRO

To explore the molecular mechanisms underlying the RGS3 attenuation of the hypertrophic response, we examined the activation status of MAPKs in TG and WT hearts induced by pressure overloads. Our data revealed that AB caused significant activations of MEK1/2, ERK1/2, JNK1/2, and p38 in WT mice. However, the increased levels of MEK1/2 and ERK1/2 were almost completely blocked in TG hearts, whereas p38 and JNK1/2 were similarly activated in the two groups (Fig. 5A). The reason for the selective inhibition of ERK1/2 activity is not clear and warrants further investigation. AKT is another important signaling pathway involved in cardiac hypertrophy. We examined the activation state of AKT and its downstream targets GSK3β. However, we did not observe any differences in the AKT signal activation between WT and TG mice (Fig. 5B). To further confirm the inhibitory effect of RGS3 on MEK1/2 and ERK1/2 activation, we exposed cultured NRCMs infected with Ad-RGS3 to 1 μM Ang II. The results of this experiment demonstrated that Ang II-stimulated MEK1/2 and ERK1/2 phosphorylation was attenuated by infection with Ad-RGS3 (Fig. 5C). Taken together, our findings suggest that RGS3 inhibits MEK-ERK1/2 signaling in vivo and in vitro in response to hypertrophic stimuli.

The aforementioned experimental results suggested that ERK1/2 inactivation would rescue the accelerative effects of RGS3 knockdown on hypertrophy. To test this hypothesis, we exposed cultured NRCMs infected with Ad-shRGS3 to ERK1/2 inhibitor U0126, followed by the addition of Ang II for 48h. Our results of cell surface area analysis show that the accelerative effects of RGS3 knockdown on Ang II-induced cell hypertrophy were dramatically dampened down by U0126 (Fig. 5D). These data indicate that regulatory role of RGS3 in pathological cardiac hypertrophy is dependent, at least partly, on the inhibition of MEK-ERK1/2 signaling.

RGS3 OVEREXPRESSION INHIBITS FIBROSIS IN RESPONSE TO PRESSURE OVERLOAD

Pathological cardiac hypertrophy is associated with increased fibrosis. To further investigate the mechanisms by which RGS3 suppresses maladaptive remodeling in response to hypertrophic stimuli, we examined the effect of RGS3 on fibrosis. The extent of fibrosis in the heart was determined by PSR staining. Marked perivascular and interstitial collagen deposition were observed in WT mice subjected to AB, although the levels of deposition were markedly reduced in TG mice (Fig. 6A). Quantitative analyses also showed an increased volume

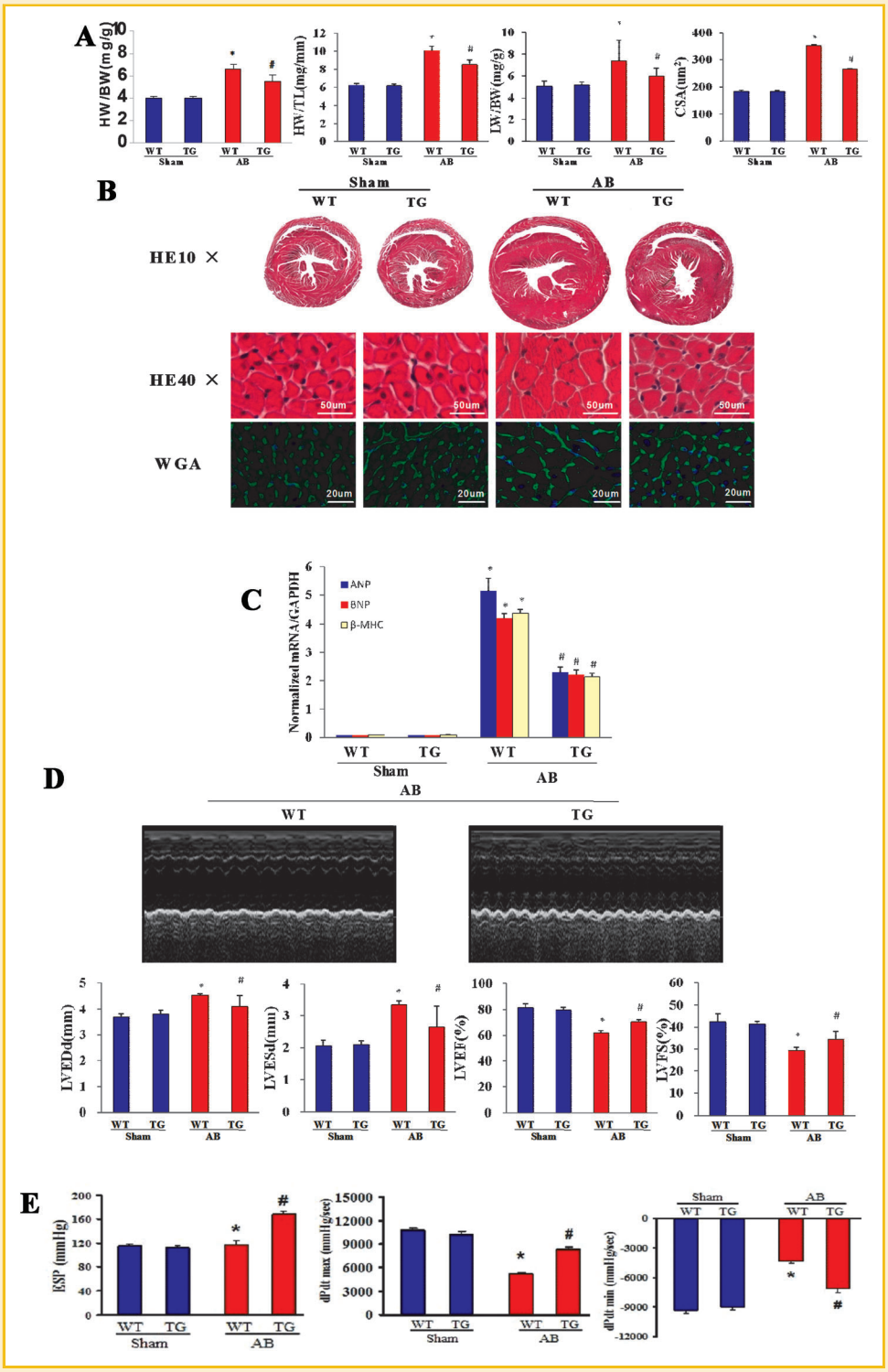

Fig. 3. The effects of RGS3 on cardiac hypertrophy and cardiac performance in vivo. A: Statistical analyses of the HW/BW, HW/TL, and LW/BW ratios as well as myocyte CSA (n = 100+ cells) 4 weeks after AB in TG and WT mice (n = 11–13). B: Gross hearts and WGA-FITC and H&E staining 4 weeks after AB in TG and WT mice (n = 5). C: The expression of hypertrophic markers induced by AB was determined by real-time PCR analysis in TG and WT mice (n = 4). D: The upper panel displays M-mode echocardiography images at 4 weeks after AB in WT and TG mice. The lower panel shows the statistical results for the LVEDD, LVESD, LVEF and LVFS in TG and WT mice (n = 6–7). E: Hemodynamic parameters for WT and TG mice at 4 weeks after AB (n = 6–7). $P < 0.05$ for WT/sham; $^{\#}P < 0.05$ for WT/AB.

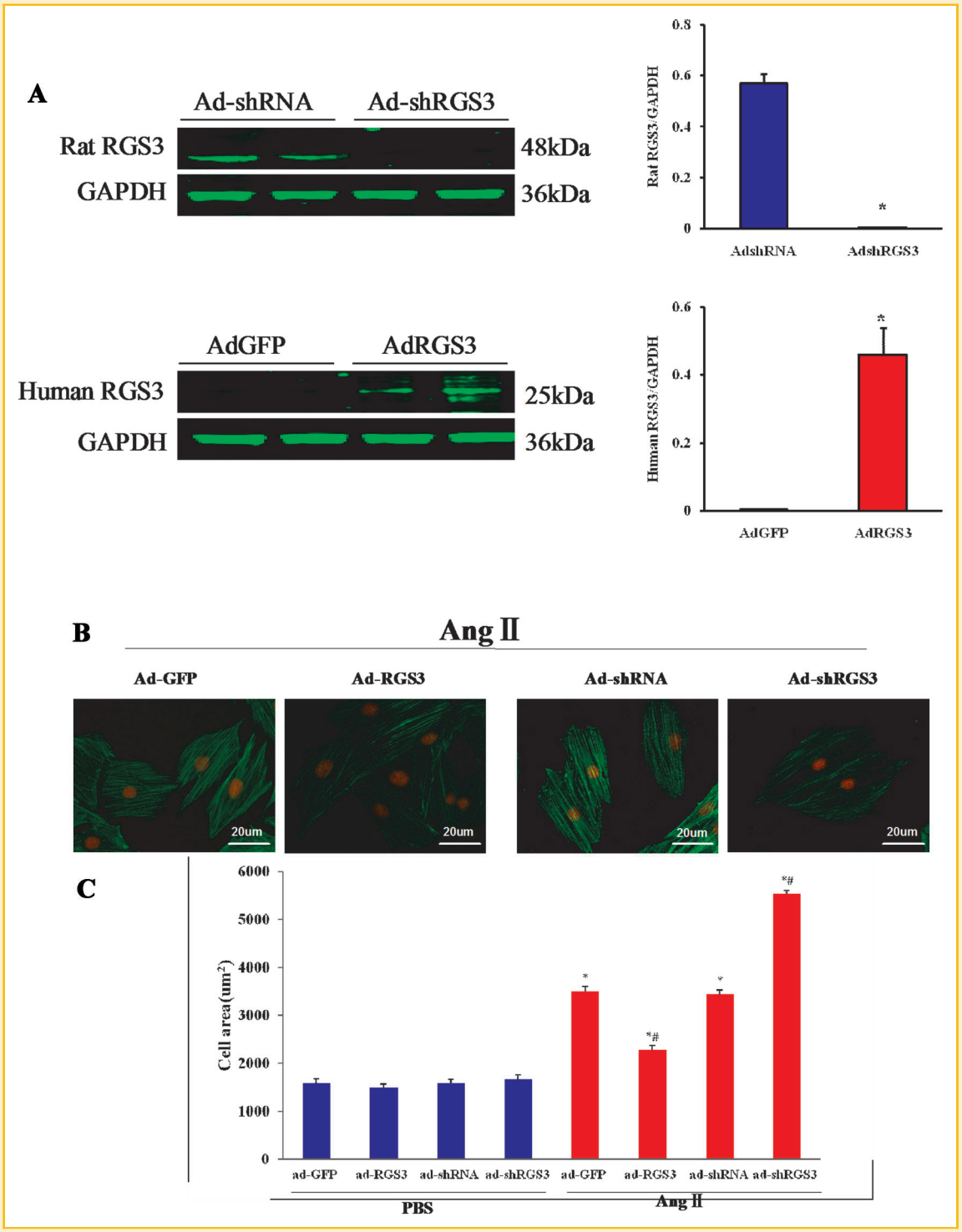

Fig. 4. The effects of RGS3 on hypertrophy in vitro. A: RGS3 was knocked down in Ad-shRGS3-infected cardiomyocytes, whereas RGS3 was increased in Ad-RGS3-infected myocytes. Left: representative blots. Right: quantitative results. B: Representative images of cultured NRCMs infected with Ad-shRNA, Ad-shRGS3, Ad-GFP, or Ad-RGS3 in response to Ang II. C: Quantitative results of the cell surface area showing that overespression of RGS3 significantly attenuated Ang II-triggered hypertrophy, compared to GFP-cells, whereas knockdown of RGS3 greatly increased Ang II-triggered hypertrophy, compared to control shRNA-cells. $^{*}P < 0.05$ for PBS, respectively; $^{\#}P < 0.05$ for Ad-GFP/Ang II or Ad-shRNA/Ang II.

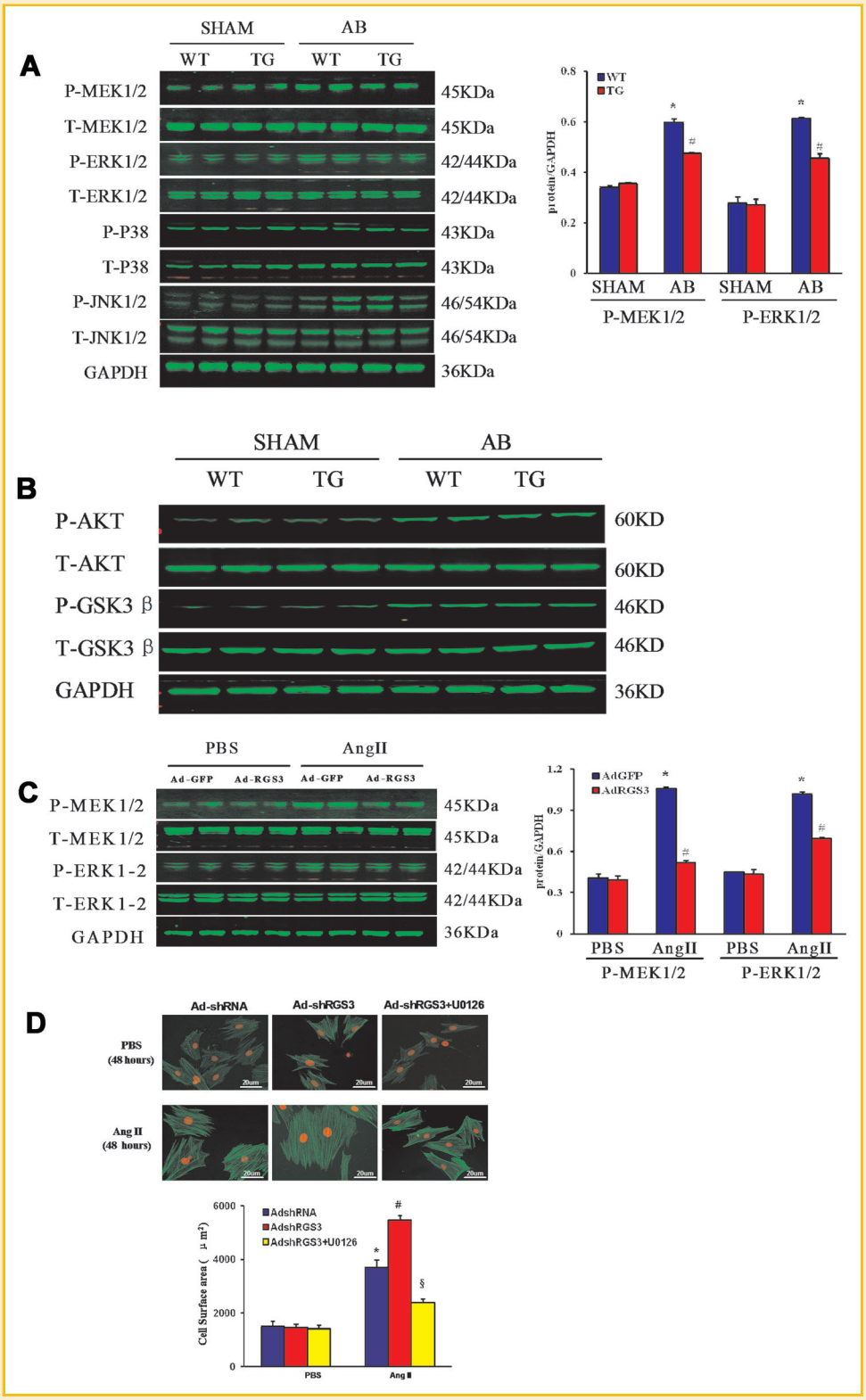

Fig. 5. The effects of RGS3 on MEK–ERK1/2 and AKT signaling pathway in vivo and in vitro. A: Left: Representative blots of MEK1/2, ERK1/2, P38, and JNK 1/2 phosphorylation and their total protein expression 4 weeks after AB surgery in RGS3 transgenic mice and WT mice. Right: Quantitative results of MEK1/2, ERK1/2, P38, and JNK 1/2 phosphorylation and their total protein in TG mice 4 weeks after AB ($n = 4$). B: Representative blots of AKT and GSK3β phosphorylation and their total protein expression 4 weeks after AB surgery in RGS3 transgenic mice and WT mice ($n = 4$). The results were reproduced in three separate experiments. $^{*}P < 0.05$ for WT/sham; $^{\#}P < 0.05$ for WT/AB. C: Left: Representative blots of MEK1/2 and ERK1/2 activation after treatment with Ang II in cultured NRCMs infected with Ad-GFP or Ad-RGS3. Right: Quantitative results of MEK1/2 and ERK1/2 phosphorylation and total levels ($n = 4$). The results were reproduced in three separate experiments $^{*}P < 0.05$ for Ad-GFP/PBS; $^{\#}P < 0.05$ for Ad-GFP/Ang II. D: Upper panel: Representative images of cultured NRCMs infected with Ad-shRGS3 in response to Ang II stimulation in the presence or absence of U0126. Lower panel: Quantitative results of the cell surface area showing that U0126 treatment dramatically suppressed RGS3 knockdown-induced accelerative effects on cell hypertrophy. $^{*}P < 0.05$ for PBS; $^{\#}P < 0.05$ for AdshRNA/Ang II; $^{\S}P < 0.05$ for AdshRGS3/Ang II.

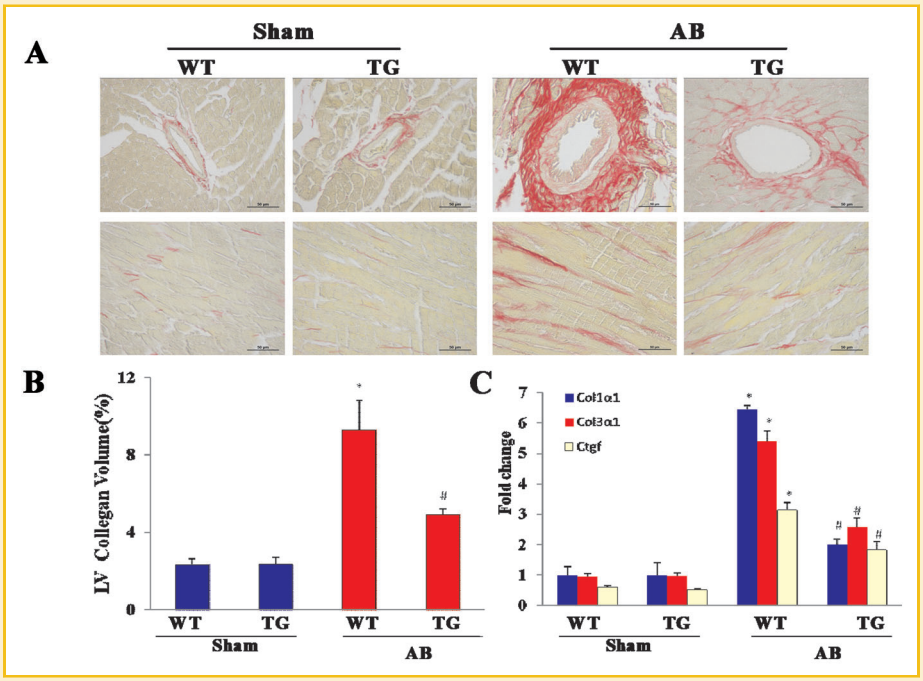

Fig. 6. The effects of RGS3 on cardiac fibrosis. A: PSR staining of histological sections of the LV was performed for the indicated groups at 4 weeks after AB ($n = 5$). B: Collagen volume fraction from histological sections was quantified using a digital image analysis system ($n = 5$). Five or six fields were randomly selected in each animal. Collagen volume fraction was calculated for the heart as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas. C: Real-time PCR analyses of Col1α1, Col3α1, and CTGF were performed to determine the mRNA expression levels in the indicated mice ($n = 4$). $^{*}P < 0.05$ for WT/sham; $^{\#}P < 0.05$ for WT/AB.

of collagen in the myocardium of WT mice relative to with TG mice (Fig. 6B). Subsequent analyses of mRNA levels of known mediators of fibrosis, including Col1α1, Col3α1, and CTGF, revealed a suppressed fibrotic response in TG mice (Fig. 6C). Collectively, these data suggest that RGS3 has an anti-fibrotic effect in the heart.

DISCUSSION

The present study demonstrates that the cardiac-specific expression of RGS3 protects against maladaptive hypertrophy, dilatation, and fibrosis in response to chronic pressure overload by attenuating MEK-ERK1/2 signaling. These novel findings suggest that RGS3 is a critical anti-hypertrophic factor that protects against maladaptive hypertrophy and the transition to heart failure. To our knowledge, these data provide the first direct evidence demonstrating the crucial role of RGS3 in the regulation of cardiac hypertrophy and fibrosis.

RGS3 expression was significantly down-regulated in failing human hearts [Chakir et al., 2011]. Our study found that the transgenic overexpression of RGS3 in the heart attenuated AB-induced hypertrophy and fibrosis. These findings strongly suggest that RGS3 plays an important role in protecting the heart against maladaptive cardiac remodeling in response to stress. This finding is supported by recent basic and clinical studies that have shown cardiac resynchronization therapy to improve rest and β-adrenergic-stimulated myocyte function as well as calcium handling in part by upregulating the RGS3 expression in the failing heart [Chakir et al., 2009, 2011].

The molecular mechanisms by which RGS3 exerts its antihypertrophic effects remain elusive. Although pathological cardiac hypertrophy is initially an adaptive process that maintains cardiac output, sustained hypertrophy can ultimately result in profound changes in gene expression, extracellular remodeling, and cardiac dysfunction [Berk et al., 2007]. However, despite extensive studies, the molecular mechanisms that mediate the transition from compensated hypertrophy to decompensated heart failure are not fully understood. There is growing evidence that GPCR-mediating signaling pathways play an important role in promoting cardiac hypertrophy and fibrosis [Heineke and Molkentin, 2006; Belmonte and Blaxall, 2011]. GPCRs comprise seven transmembrane spanning receptors coupled to heterotrimeric G proteins of the Gαi and Gαq/Gα11 families. GPCRs can transduce signals from a large variety of extracellular stimuli into cellular responses via classical mechanisms of G-protein signaling. The activation of GPCRs by Ang II, endothelin-1, and adrenergic agonists can promote the growth of cardiomyocytes and the accumulation of extracellular matrix in the heart [Heineke and Molkentin, 2006]. Previous studies have demonstrated that the Gαq-mediated signaling pathway is both necessary and sufficient for mediating cardiac hypertrophy [Dorn and Force, 2005]. Gαq protein overexpression has been shown to contribute to the development of cardiac hypertrophy, whereas the inhibition of Gq-mediated signaling prevented pressure overload induced cardiac hypertrophy [Mende et al., 1998; Wettschureck et al., 2001]. Although the exact mechanism has not been identified, the activation of Gαq/α11 is thought to be a potent inducer of MAPK signaling in cardiac myocytes [Minamino et al., 2002]. Accumulating

evidence suggest that the MAPK signaling pathways play critical roles in promoting cardiac hypertrophy [Rose et al., 2010]. The MAPK signaling cascade consists of a series of successively acting protein kinases, including ERKs, JNKs, and p38. These kinases are often activated in cardiac myocytes in response to various extracellular stresses and phosphorylate a variety of intracellular effectors including numerous transcription factors that induce the reprogramming of cardiac gene expression [Heineke and Molkentin, 2006]. RGS3 is a negative regulator of G protein-mediated signaling, which inactivates the Gα(q) and Gα(i) proteins [Zhang and Mende, 2011]. RGS3 has been shown previously to inhibit ERK signaling in vitro [Nishiura et al., 2009]. In an attempt to elucidate the signaling mechanisms underlying the protective effect of RGS3 on cardiac hypertrophy, we examined the status of MAPKs signaling in the present study. The results demonstrated that MEK-ERK1/2 activation was almost completely blocked by cardiac-specific RGS3 overexpression in response to chronic pressure overload. However, the phosphorylation levels of JNK and p38 signaling were not affected by RGS3. In addition, the effect of RGS3 on MEK-ERK1/2 signaling was further confirmed in our in vitro study. Furthermore, rescue experiments demonstrated that inhibition of MEK-ERK1/2 signaling suppressed the accelerative effects of RGS3 knockdown on Ang II-induced cell hypertrophy. Therefore, MEK-ERK1/2 signaling is a crucial pathway through which RGS3 protects against cardiomyocyte hypertrophy. Previous studies demonstrated that activation of ERK 1/2 subjected to GPCR-mediated signaling is regulated by RGS proteins [Anger et al., 2008]. Our previous study revealed that RGS5 protects against cardiac hypertrophy and fibrosis during biomechanical stress of pressure overload through inhibition of MEK-ERK1/2 signaling [Li et al., 2010]. Nunn et al. [2010] reported that RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy partly through blockade of ERK1/2 activation. It seems that regulation of MEK-ERK1/2 signaling is a generic property of the R4 subfamily of RGS proteins. However, G-protein coupled ERK 1/2 activation is subjected to specific RGS proteins upon different stimulations, suggesting that a specific network of endogenously expressed RGS proteins is involved in the activation of ERK 1/2 signaling [Anger et al., 2008].

Fibrosis, an established morphological feature in structural myocardial remodeling, involves a disproportionate accumulation of collagen [Li et al., 2007; Bian et al., 2010; Huang et al., 2010]. Another major finding of this study is that RGS3 attenuates fibrosis in vivo. It is widely recognized that myocardial fibrosis is associated with an increased accumulation of type I and III collagen within the adventitia of coronary arteries (perivascular fibrosis), which progressively extends into the neighboring interstitial spaces (interstitial fibrosis) in response to pressure overload [Pandya et al., 2006]. RGS3 has previously been shown to regulate transforming growth factor beta (TGF-β) signaling via its interaction with Smads and by interfering with Smad heteromerization, suggesting that RGS3 may play an important role in the development of cardiac fibrosis [Yau et al., 2008]. In the present study, we observed increased collagen deposition in the hearts of WT mice after AB and demonstrated that cardiac-specific RGS3 overexpression inhibits collagen synthesis, as evidenced by down-regulated mRNA levels of collagen I and III. CTGF has been shown to be an important downstream mediator of the profibrotic effects of TGF-β [Leask, 2010]. Our study demonstrates that RGS3 overexpression attenuates the increase in CTGF expression after AB. Thus, RGS3 overexpression can protect against cardiac remodeling by regulating collagen deposition and the pro-fibrotic mediator.

In conclusion, our data clearly demonstrate the crucial role of RGS3 in protecting against cardiac hypertrophy and fibrosis in response to hypertrophic stimuli through the inhibition of the MEK-ERK1/2 signaling pathway. These observations may have significant implications in the development of novel strategies for the treatment of cardiac hypertrophy through RGS3 targeting.

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