Cardiac Hypertrophy

Pivotal Role of Regulator of G-protein Signaling 12 in Pathological Cardiac Hypertrophy

Jia Huang, Lijuan Chen, Yuyu Yao, Chengchun Tang, Jiandong Ding, Cong Fu, Hongliang Li, Genshan Ma

Abstract—Cardiac hypertrophy is a major predictor of heart failure and is regulated by diverse signaling pathways. As a typical multi-domain member of the regulator of G-protein signaling (RGS) family, RGS12 plays a regulatory role in various signaling pathways. However, the precise effect of RGS12 on cardiac hypertrophy remains largely unknown. In this study, we observed increased expression of RGS12 in the development of pathological cardiac hypertrophy and heart failure. We then generated genetically engineered mice and neonatal rat cardiomyocytes to investigate the effects of RGS12 during this pathological process. Four weeks after aortic banding, RGS12-deficient hearts showed decreased cardiomyocyte cross area (374.7±43.2 μm² versus 487.1±47.9 μm² in controls; P<0.05) with preserved fractional shortening (43.0±3.4% versus 28.4±2.2% in controls; P<0.05), whereas RGS12-overexpressing hearts exhibited increased cardiomyocyte cross area (582.4±46.7 μm² versus 474.8±40.0 μm² in controls; P<0.05) and reduced fractional shortening (20.8±4.1% versus 28.6±3.2% in controls; P<0.05). RGS12 also contributed to angiotensin II–induced hypertrophy in isolated cardiomyocytes. Mechanistically, our data indicated that the activation of MEK1/2–ERK1/2 signaling may be responsible for the prohypertrophic action of RGS12. In addition, the requirement of the MEK1/2–ERK1/2 signaling for RGS12-mediated cardiac hypertrophy was confirmed in rescue experiments using the MEK1/2-specific inhibitor U0126. In conclusion, our findings provide a novel diagnostic and therapeutic target for pathological cardiac hypertrophy and heart failure. (Hypertension. 2016;67:1228-1236. DOI: 10.1161/HYPERTENSIONAHA.115.06877.)

Key Words: amino acids ■ angiotensin II ■ dilated cardiomyopathy ■ heart failure ■ signal transduction

Cardiac hypertrophy is initially an adaptive response to cardiac stresses, including mechanical overload, ischemia, and endocrine disorders.1,2 At the beginning, it elicits beneficial effects by reducing ventricular wall stress and maintaining cardiac output without adverse pathology. However, sustained hypertrophy of the myocardium can progress into a decompensated state that is characterized by fetal gene expression, contractile dysfunction, and extracellular remodeling, eventually leading to heart failure.3,4 During this complicated process, specific signaling pathways mediate the critical transition from compensated hypertrophy to decompensated hypertrophy and heart failure.5,6 Therefore, a better understanding of the molecular mechanisms regulating the hypertrophy-related signaling pathways is important for the development of new strategies to treat pathological cardiac hypertrophy and heart failure.

Regulator of G-protein signaling (RGS) proteins represent a family of proteins originally defined by the presence of a semiconserved region of ~120 amino acids called the RGS domain. They function as GTPase-activating proteins for Gα proteins to repress G-protein–coupled receptor signaling.7,8 To date, several members of the RGS family have been found to participate in pathological cardiac hypertrophy.9-13 Recently, a growing number of studies demonstrated that RGS proteins could regulate cardiac hypertrophy independent of GTPase-activating protein activity. Among them, RGS3, RGS5, and RGS10 protect against pressure overload–induced hypertrophic response and improve cardiac function by inhibiting MEK1/2–ERK1/2 signaling.11,13,14 RGS12 is the largest, typical multidomain member of the RGS family.15 In addition to a central RGS domain, RGS12 contains a PDZ (PSD-95/disclarge/ZO-1 homology) domain, with putative CXXC2-binding properties, and a phosphotyrosine-binding domain at the N terminus, whereas a tandem repeat of Ras-binding domains and a Gai/o-Loco motif are located at the C terminus of RGS12.16-18 This multidomain architecture contributes to the regulatory role of RGS12 in diverse signaling pathways.18 Huang et al19 reported that RGS12 inactivates phosphorylated Goi to ameliorate the inhibition of cAMP formation and thus facilitates muscle relaxation. In addition, Willard et al20 found that RGS12 serves as a scaffold and organizes...
a signal transduction complex with TrkA, Ras, Raf, MEK, and ERK to promote glial cell differentiation. Furthermore, RGS12 is essential for osteoclast differentiation through its effects on the RANKL-PLCγ-[-Ca2+]i oscillations–nuclear factor of activated T cells (NFAT) 2 signaling. Considering the involvement of mitogen-activated protein kinase (MAPK) signaling and NFAT2 in cardiac hypertrophy has been well documented, it is reasonable to hypothesize the regulatory effects of RGS12 on cardiac hypertrophy. Interestingly, the mRNA of RGS12 is exclusively expressed in ventricular but not in atrial cardiomyocytes. 

This study was designed to elucidate the precise role of RGS12 in pathological cardiac hypertrophy. Initially, alternations in RGS12 expression were observed in myocardium from human patients with dilated cardiomyopathy or hypertrophic cardiomyopathy and mice that underwent aortic banding (AB). Moreover, AB-induced hypertrophic response was attenuated in RGS12-deficient mice, whereas cardiac-specific RGS12 overexpression exaggerated cardiac hypertrophy, dysfunction, and heart failure on chronic pressure overload. Mechanistically, we demonstrated that RGS12 contributes to pathological cardiac hypertrophy by promoting MEK1/2–ERK1/2 signaling. To the best of our knowledge, we provide the first evidence for the positive regulatory effect of RGS12 in the development of pathological cardiac hypertrophy and subsequent heart failure.

Materials and Methods
All experiments conducted on animals were approved by the Animal Care and Use Committee of the Zhongda Hospital of Southeast University, China. An expanded Materials and Methods section is available in the online-only Data Supplement, which includes reagents, human ventricular samples, study animals, AB, echocardiography measurements, histological analysis, cultured neonatal rat cardiomyocytes (NRCMs) and recombinant adenoviral vectors, quantitative real-time polymerase chain reaction, and Western blotting.

Statistical Analysis
The data are presented as the mean±SD. Unpaired Student t tests were performed to analyze the differences between the 2 groups, whereas comparisons among >2 groups were analyzed by 1-way analysis of variance followed by the Bonferroni post hoc test (assuming equal variances) or the Tamhane T2 post hoc test (without the assumption of equal variances). All statistical analyses were carried out using SPSS software (version 19.0), and P<0.05 was considered statistically significant.

Results
RGS12 Expression Is Increased in the Development of Pathological Cardiac Hypertrophy and Heart Failure
To explore the potential involvement of RGS12 in the development of pathological cardiac hypertrophy and heart failure, we first determined whether RGS12 expression was altered in failing human hearts. Western blots showed that the RGS12 protein level was significantly increased in failing hearts from dilated cardiomyopathy and hypertrophic cardiomyopathy patients, and this occurred in conjunction with elevated levels of cardiac hypertrophic markers, including atrial natriuretic peptide and β-MHC. RGS12 expression was dramatically upregulated in pressure overload–induced hypertrophied mouse hearts to ≈168% after 4 weeks of AB and to ≈231% after 8 weeks of AB compared with sham-operated controls (Figure 1C and 1D). Consistent with in vivo experiments, the significant increases in RGS12 expression and hypertrophic markers were also observed in cultured NRCMs administered with angiotensin II (Ang II) for 24 or 48 hours (Figure 1E and 1F). Collectively, the enhanced RGS12 expression in hypertrophied hearts and NRCMs strongly suggested the involvement of RGS12 in the pathogenesis of cardiac hypertrophy.

Absence of RGS12 Attenuates Pathological Cardiac Hypertrophy Induced by Pressure Overload
The upregulated levels of RGS12 observed in failing human hearts and hypertrophied mouse hearts prompted us to hypothesize the causation between the elevated level of RGS12 and the cardiac hypertrophy. Therefore, we generated RGS12 global knockout mice to investigate the effect of RGS12 deficiency on cardiac hypertrophy and heart failure in vivo (Figure S2A–S2E). Compared with wild-type (WT) mice, RGS12-deficient mice seemed normal, fertile, and had no apparent abnormalities in cardiac structure and function at baseline (data not shown). After 4 weeks of AB, RGS12-deficient mice exhibited a remarkable amelioration of cardiac hypertrophy compared with their littermate controls, as evidenced by reductions in the ratio of heart weight/body weight (HW/BW), the ratio of heart weight/tibia length (HW/TL; Figure 2A), the gross size of the heart (Figure 2B), the cardiomyocyte cross area (Figure 2C), as well as the mRNA levels of hypertrophic markers, including atrial natriuretic peptide, B-type natriuretic peptide (BNP), and β-MHC (Figure S2F).

Meanwhile, picrosirius red staining was used to determine the extent of fibrosis in both the interstitial and the perivascular regions. Compared with WT mice, RGS12 deficiency significantly suppressed pressure overload–mediated cardiac fibrosis (Figure 2B and 2D). Correspondingly, RGS12-deficient hearts exhibited lower mRNA levels of fibrotic markers (collagen I, collagen III, and connective tissue growth factor) than their WT littermates (Figure S2G). In addition, RGS12 depletion compromised ventricular dilation triggered by AB, as evidenced by the downregulated left ventricular end-diastolic dimension and left ventricular end-systolic dimension compared with WT mice (Figure 2E). Furthermore, lower ratios of lung weight/body weight (LW/BW) (Figure 2A), a critical hallmark of the development of pulmonary congestion, and upregulation of fractional shortening were observed in RGS12-deficient mice compared with their WT littermates (Figure 2E), implying better cardiac contractility. Taken together, deletion of RGS12 did not lead to pathological alterations at baseline but exerted a cardioprotective role during cardiac hypertrophy and heart failure on pressure overload.

Cardiac-Specific Overexpression of RGS12 Exaggerates Pathological Cardiac Hypertrophy Induced by Pressure Overload
To further confirm the effect of RGS12 in the development of cardiac hypertrophy and heart failure, we generated 4 independent lines of transgenic mice (transgenic 1, transgenic 2,
transgenic 3, and transgenic 4) with cardiac-specific RGS12 overexpression (Figure S3A). The overexpression of RGS12 in hearts from each line was verified by Western blot (Figure S3B and S3C). At baseline, all lines of RGS12-transgenic mice were healthy and fertile, and there were no apparent abnormalities in their cardiac structure and function compared with their CAG-CAT-RGS12/\(\alpha\)-MHC-MerCreMer littermates without tamoxifen administration (CRMC; data not shown). The transgenic 2 line with the highest RGS12 expression levels in the heart was selected for the following experiments. RGS12 overexpression enhanced the susceptibility of hearts to AB-induced cardiac hypertrophy compared with CRMC mice, as evidenced by higher HW/BW ratio, HW/TL ratio (Figure 3A), larger gross size of the heart (Figure 3B), larger cardiomyocyte cross area (Figure 3C), and higher mRNA levels of hypertrophic markers (Figure S3D). In addition, chronic pressure overload led to aggravated fibrosis in both the interstitial and the perivascular regions in RGS12-overexpressing hearts (Figure 3D). Consistently, in response to AB, indices of collagen synthesis were significantly augmented in CRMC hearts and were further enhanced in RGS12-overexpressing hearts (Figure S3E). Therefore, RGS12 may function in the heart as a positive regulator of pathological cardiac hypertrophy.

RGS12 Exacerbates Cardiomyocyte Hypertrophy In Vitro

Given the potent effect of RGS12 on the development of cardiac hypertrophy and heart failure, which may be influenced by the mutual effects of RGS12 between different cell types, we further analyzed whether RGS12 directly modulate cardiomyocytes on prohypertrophic stresses. To this end, NRCMs with either decreased or increased expression of RGS12 were generated by infection with the adenovirus harboring the RGS12 shRNA sequence (AdshRGS12) or RGS12 cDNA (AdRGS12; Figure S4A). After challenge with Ang II (1 \(\mu\)mol/L) or phosphate-buffered saline (PBS) for 48 hours, NRCMs were immunostained with \(\alpha\)-actinin to measure the cell surface area (Figure 4A). There were no remarkable distinctions in cardiomyocyte morphology or cell surface area between the groups with PBS administration. However, compared with AdshRNA-infected controls, RGS12 deficiency alleviated Ang II–induced cardiomyocyte hypertrophy, as evidenced by the downregulation of cell surface area and hypertrophic markers (Figure 4B; Figure S4B). Conversely, AdRGS12 infection–mediated RGS12 overexpression markedly aggravated cardiomyocyte enlargement and upregulated mRNA levels of atrial natriuretic peptide, BNP, and \(\beta\)-MHC compared with AdGFP-infected controls (Figure 4C; Figure S4C). Endothelin-1, another hypertrophic stimulus, showed the similar results (Figure S4D–S4F). Thus, these in vitro data support the concept that RGS12 exerts prohypertrophic action on isolated cardiomyocytes on prohypertrophic stimuli.

RGS12 Promotes the Activation of MEK1/2–ERK1/2 Signaling Induced by Prohypertrophic Stimuli

To elucidate the molecular mechanisms by which RGS12 plays a deleterious role in pathological cardiac hypertrophy, we determined the states of the MAPKs, including MEK1/2, ERK1/2, JNK1/2, and P38, which are well-established...
regulators of cardiac hypertrophy. On chronic pressure overload, although the total expression of MAPKs was not altered among the tested groups, the pressure overload–induced phosphorylation of MEK1/2 and ERK1/2 was attenuated in RGS12-deficient hearts but was further augmented by RGS12 overexpression (Figure 5A and 5B). However, neither deletion nor forced expression of RGS12 in hearts influenced the phosphorylation of P38 and JNK after AB (Figure 5A and 5B). Consistent with the in vivo results, RGS12 deficiency suppressed MEK1/2 and ERK1/2 phosphorylation in NRCMs with Ang II administration, whereas RGS12-overexpressing NRCMs exhibited elevated phosphorylation levels of MEK1/2 and ERK1/2 (Figure S5A and S5B). To identify whether RGS12 directly regulates MEK1/2, isolated NRCMs treated with PBS or Ang II are used to perform coimmunoprecipitation experiments. Our data showed that RGS12 could interact with MEK1/2 weakly at baseline (PBS), but this interaction was significantly enhanced by Ang II administration (Figure S5C and S5D). These results suggested that MEK1/2–ERK1/2 signaling may be responsible for the prohypertrophic effect of RGS12.

Activation of MEK1/2–ERK1/2 Signaling Pathway Is Essential for RGS12-Exaggerated Cardiac Hypertrophy

To verify the causal linkage between MEK1/2–ERK1/2 signaling and RGS12-mediated cardiac hypertrophy, CRMC and RGS12-transgenic mice were injected with the MEK1/2-specific inhibitor U0126 for 4 weeks after AB surgery. As shown in Figure S6, pressure overload–induced phosphorylation of MEK1/2–ERK1/2 cascades was largely blocked by U0126. As expected, U0126 offset the hypertrophic growth and subsequent adverse remodeling, including fibrosis and cardiac dysfunction, induced by AB surgery in both CRMC and RGS12-transgenic hearts (Figure 6A–6E). More importantly, the following indices of pathological hypertrophic response were comparable between U0126-treated CRMC and U0126-treated RGS12-transgenic mice: (1) the HW/BW, the HW/TL (Figure 6A), the gross size of the heart and the cardiomyocyte cross area (Figure 6B and 6C); (2) the interstitial and perivascular fibrosis in the hearts (Figure 6B and 6D); and (3) the cardiac function indices (Figure 6A and 6E). Taken together, these data provided convincing evidence that RGS12 exerts a prohypertrophic effect largely by promotion of MEK1/2–ERK1/2 signaling.

Discussion

Heart failure is the final stage of diverse cardiovascular diseases. Even with advances in therapies, the prognosis of patients with heart failure remains poor. As an early hallmark of heart failure, cardiac hypertrophy has attracted much attention, and an increasing number of studies have been performed in the past decade to elucidate the precise molecular mechanisms underlying its initiation and progression. Here, we provide the first evidence that RGS12 functions as a positive regulator of cardiac hypertrophy in response to pathological stimuli. Because RGS12 expression was upregulated during cardiac hypertrophy and heart failure, we performed
loss-of-function studies to investigate the cause-and-effect relationship between increased RGS12 and pathological cardiac hypertrophy. As predicted, RGS12 deficiency ameliorated pressure overload–induced hypertrophic growth. In contrast, exaggerated hypertrophic response and cardiac dysfunction was observed in RGS12-overexpressing mice subjected to AB surgery. Consistently, similar results were observed in genetically engineered NRCMs on prohypertrophic stimuli. Further analyses of the signaling pathways revealed that the prohypertrophic effect of RGS12 is largely mediated by promotion of MEK1/2–ERK1/2 signaling. Collectively, these data indicate that RGS12 plays a pivotal role in the development of pathological cardiac hypertrophy and heart failure.

RGS12 belongs to a protein family whose members modulate G-protein–coupled receptor signaling through their GTPase-activating protein activity. In addition to negative regulation of G-protein signaling, a growing number of studies revealed GTPase-activating proteins–independent regulatory effects of RGS members on cardiac hypertrophy. A recent study found that cardiac hypertrophy and activation of MEK1/2–ERK1/2 signaling was exaggerated in RGS10-knockout hearts but alleviated in RGS10-transgenic hearts. Considering the multiple signaling regulatory domains in RGS12, it will be interesting to determine whether RGS12 plays a pivotal role in the development of pathological cardiac hypertrophy and heart failure.

Because RGS12 expression was augmented in hypertrophied hearts, we speculated that the increase in RGS12 may contribute to pathological stimuli–induced adverse cardiac remodeling. Our hypothesis was strengthened by the observation that systemic RGS12-knockout mice displayed attenuated hypertrophic response on chronic pressure overload. Given that systemic absence of RGS12 may affect cardiac cells other than cardiomyocytes, cardiac-specific RGS12-overexpressing mice and genetically engineered NRCMs were used to confirm the prohypertrophic effect of RGS12 on cardiomyocytes. On the basis of the in vivo and in vitro results, even we cannot exclude the involvement of other types of cells in the prohypertrophic action of RGS12; we can conclude that RGS12 contributes to pathological cardiac hypertrophy, at least partly, by regulating cardiomyocytes.

To explore the molecular mechanisms responsible for RGS12-mediated hypertrophy, we analyzed the MAPK regulatory mechanisms of RGS12 expression were seldom reported in previous studies. In parallel with mechanical tension-triggered RGS12 expression in hearts, enhanced RGS12 levels were detected in human myometrial smooth muscle cells during labor. In addition, because increased expression of several RGS proteins were observed in cardiomyocytes stimulated with G-protein–coupled receptor agonists (including endothelin-1, phenylephrine, and isoproterenol), we reasoned that the expression of RGS12 may be regulated by similar mechanisms. However, further studies are required to decipher how RGS12 expression is regulated by prohypertrophic stimuli.
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cascades in genetically engineered mice and NRCMs subjected to pathological stimuli. Intriguingly, RGS12 deficiency compromised the prohypertrophic stimuli-induced activation of MEK1/2–ERK1/2 signaling, whereas RGS12 overexpression resulted in the opposite phenotype. In addition, we observed the interaction between RGS12 and MEK1/2 in cardiomyocytes, which was enhanced by hypertrophic stimuli. These results were in agreement with the previous report that RGS12 serves as a Ras/Raf/MEK scaffold to accelerate ERK1/2 activation during neuronal differentiation.21 Furthermore, the requirement of the MEK1/2–ERK1/2 signaling for RGS12-exacerbated cardiac hypertrophy and heart failure was validated by the rescue experiments using the MEK1/2-specific inhibitor U0126. On one hand, activated ERK1/2 translocate to the nucleus and thus phosphorylate transcription factors, such as Elk-1 and GATA4, resulting in the hypertrophic gene expression.30,31 On the other hand, activated ERK1/2 phosphorylate their downstream kinases, including tuberous sclerosis complex 2 and MAPK-interacting kinases 1/2, in the cytoplasm. Although phosphorylated
tuberous sclerosis complex 2 activated mTOR signaling, activated MAPK-interacting kinases 1/2 were reported to facilitate eukaryotic initiation factor 4E phosphorylation directly. Eventually, both of the downstream cascades of ERK1/2 in the cytoplasm converge on the activation of protein synthesis. Therefore, RGS12-mediated ERK1/2 activation may exert a prohypertrophic role via effects on both transcription and translation. Given the well-established association between cardiac hypertrophy and heart failure, activated ERK1/2 may contribute to heart failure by similar manners. Hasegawa et al reported the requirement of GATA4 in MHC isoform changes triggered by pressure overload. Because the MHC isoform composition shift from the α-MHC isoform with higher ATPase activity and actin filament sliding velocity to the β-MHC isoform with reduced power output and shortening velocity, phosphorylation of GATA4 account, at least partly, for the MEK1/2-ERK1/2-mediated systolic dysfunction and heart failure. Meanwhile, cytoplasmic downstream kinases of ERK1/2, promote protein synthesis and accumulation in cardiomyocyte, which would result in the thickness of the cardiac wall following by an exacerbation in chamber stiffness and diastolic dysfunction.

However, the prohypertrophic role of ERK1/2 was challenged by loss-of-function studies in which deletion or inhibition of cardiac ERK1/2 potentiates cellular apoptosis and heart failure. This discrepancy may result from the divergent role of ERK1/2 in the regulation of antiapoptotic function and hypertrophic response in cardiomyocytes. Appropriate ERK1/2 activity is essential for the survival of cardiomyocytes in response to pathological stimuli. In contrast, excess and sustained activation of ERK1/2 would result in cardiac hypertrophy. Interestingly, our results indicated that RGS12 deficiency dramatically suppressed MEK1/2-ERK1/2-mediated cardiac hypertrophy without predisposing the heart to adverse remodeling and failure. The effect of MEK1/2—ERK1/2 signaling in cardiac hypertrophy is determined by the duration and intensity of the signaling, as well as their upstream effectors. Willard et al reported that RGS12 is essential for the prolonged activation of ERK1/2 but has no influence on transient ERK1/2 activation. As the central role of prolonged ERK1/2 activation in cardiac hypertrophy, this phenomenon may provide a potential explanation for the antihypertrophic action of RGS12 deficiency without detrimental side effects. Alternatively, RGS12 was reported to serve as a scaffold to coordinate specific signals upstream of MEK1/2—ERK1/2 (B-Raf, but not C-Raf), suggesting that only a fraction of MEK1/2—ERK1/2 signaling is regulated by RGS12. Specifically, although both B-Raf and C-Raf are prohypertrophic upstream of MEK1/2—ERK1/2 signaling, compared with B-Raf, C-Raf has a more complex function with antiapoptotic effects in response to pathological stimuli. Thus, inhibition of RGS12 likely suppresses a specific fraction of MEK1/2—ERK1/2 signaling, such as the B-Raf-dependent fraction, so that the prohypertrophic effect is abolished but the antiapoptotic function is preserved. However, additional studies are required to verify our hypotheses. Furthermore, there were no apparent abnormalities in structure and function of
RGS12-deficient or RGS12-overexpressing hearts, suggesting that RGS12 does not participate in the development and physiological function of the heart. Taken together, our data indicate that RGS12 represents a promising therapeutic target for pathological cardiac hypertrophy.

This study demonstrates that chronic stress–induced pathological cardiac hypertrophy seem to be result, at least partly, from the upregulation of RGS12. Mechanistically, increased RGS12 elicits cardiodetrimental effects by fostering MEK1/2–ERK1/2 signaling. Therefore, our findings not only advance our understanding of the molecular mechanisms underlying cardiac hypertrophy and heart failure but also provide a potential diagnostic and therapeutic strategy for pathological cardiac hypertrophy.

**Perspectives**

In this study, our findings provide the first evidence that RGS12 contributes to pathological cardiac hypertrophy by augmenting MEK1/2–ERK1/2 signaling. These findings extend our comprehension of the molecular mechanisms underlying cardiac hypertrophy and indicate that RGS12 may be a novel target for prevention and treatment of pathological cardiac hypertrophy.

**Acknowledgments**

We thank Jun Gong, Rui Zhang, Yan Zhang, Xue-Yong Zhu, Ling Huang, and Xin Zhang for providing experimental technological assistance.

**Sources of Funding**

This work was supported by grants from the National Natural Science Foundation of China (No. 51102043, 81270203, 81300160, and 81470400).

**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

- Regulator of G-protein signaling (RGS) 12 levels was upregulated in failing human hearts, hypertrophied mouse hearts, and cultured neonatal rat cardiomyocytes incubated with angiotensin II.
- RGS12 exaggerated both prohypertrophic stimuli-induced cardiac hypertrophy in vivo and cardiomyocyte enlargement in vitro.
- RGS12 exerted a prohypertrophic effect by promoting MEK1/2–ERK1/2 signaling.

What Is Relevant?

- RGS12 was reported to participate in various pathological processes, but whether it is involved in cardiac hypertrophy is unknown.
- The precise molecular mechanisms underlying the initiation and progression of cardiac hypertrophy are not fully elucidated.

- This study provides new insights into the molecular mechanisms of cardiac hypertrophy and suggests a novel therapeutic target for pathological cardiac hypertrophy.

Summary

RGS12 expression is increased in the development of pathological cardiac hypertrophy and heart failure. RGS12 deficiency alleviated pressure overload–induced hypertrophic growth, fibrosis and cardiac dysfunction by suppressing MEK1/2–ERK1/2 signaling, whereas RGS12-overexpressing hearts exhibited a deteriorated hypertrophic response on chronic pressure overload. Our results suggest that RGS12 might be a novel diagnostic and therapeutic target for pathological cardiac hypertrophy and heart failure.
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*Hypertension.* 2016;67:1228-1236; originally published online April 18, 2016; doi: 10.1161/HYPERTENSIONAHA.115.06877

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/67/6/1228

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Pivotal Role of Regulator of G-protein Signaling 12 in Pathological Cardiac Hypertrophy

Jia Huang¹, Lijuan Chen¹, Yuyu Yao¹, Chengchun Tang¹, Jiandong Ding¹, Cong Fu¹, Hongliang Li²,³, Genshan Ma¹#

(Running Title: RGS12 promotes adverse cardiac remodeling)

¹Department of Cardiology, Zhongda Hospital Affiliated to Southeast University, Nanjing, Jiangsu 210009, PR China; ²Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China; ³Cardiovascular Research Institute of Wuhan University, Wuhan 430060, PR China.

Jia Huang is the first author.
Genshan Ma is the corresponding author.

Corresponding author to
Genshan Ma, MD, PhD
Department of Cardiology
Zhongda Hospital Affiliated to Southeast University
No. 87 Dingjiaqiao, Nanjing, Jiangsu, 210009, PR China
Tel/Fax: +86-13002580569
E-mail: magenshan@hotmail.com
Supplemental Materials and Methods

Reagents
Antibody against RGS12 (ab14258) was from abcam. Antibodies against ANP (sc20158), β-MHC (sc53090) and were from Santa Cruz Biotechnology. Antibodies against P-MEK1/2\(^{\text{Ser217/221}}\) (9154), T-MEK1/2 (9122), P-ERK1/2\(^{\text{Thr202/204}}\) (4370), T-ERK1/2 (4695), P-JNK1/2\(^{\text{Thr183/Tyr185}}\) (4668), T-JNK1/2 (9258), P-P38\(^{\text{Thr180/182}}\) (4511), T-P38 (9212) and GAPDH (2118) were from Cell Signaling Technology. The BCA protein assay kit was from Pierce. Fetal calf serum was from Hyclone. Cell culture reagents and all other reagents were from Sigma.

Human ventricular samples
All of the procedures requiring human samples complied with the principles of the Declaration of Helsinki and were approved by the Ethics Committee at the Zhongda Hospital of Southeast University in Nanjing, China. Left ventricle (LV) samples were obtained from dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) patients who underwent heart transplantation for end-stage heart failure (New York Heart Association Class IV). The controls normal hearts were obtained from donors who had died in accidents unrelated to cardiac disease but whose hearts were unsuitable for transplantation for technical reasons. We obtained written informed consent from patients who underwent heart transplantation and from the immediate family members of the heart donors.

Study animals
All experiments conducted on animals were approved by the Animal Care and Use Committee of the Zhongda Hospital of Southeast University. The investigation complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

For the production of RGS12 global knockout (KO) mice: The RGS12 protein includes two predominant variants. To disrupt both of them, we predicted the guide sequences for targeting the common genetic site of the two variants in the mouse genome using the online CRISPR design tool (http://crispr.mit.edu) and inserted the sequences into the BsaI restriction site of the pUC57-sgRNA expression vector (Addgene, 51132). After being purified, sgRNA was transcribed with the MEGA shortscript Kit (Ambion, AM1354) and purified with the miRNeasy Micro Kit (Qiagen, 217084). A plasmid (Addgene 44758) harboring Cas9 was linearized by PmeI and subsequently used as the template for \textit{in vitro} transcription with the T7 Ultra Kit (Ambion, AM1345), and then purified using the RNeasy Mini Kit (Qiagen, 74104). Both Cas9 mRNA and sgRNA mRNA injections for one-cell embryos were performed using the FemtoJet 5247 microinjection system. Genomic DNA was extracted from mouse tails using phenol-chloroform and alcohol precipitation. A DNA fragment including the sgRNA targeting site was amplified by PCR with the following primers: RGS12-forward (5’-GGCTAGGTTCAGGTGCAGT-3’) and RGS12-reverse (5’-TGTAACGCTACCTACCCCA-3’). After PCR, the amplified products were directly sequenced to identify founders harboring mutant alleles.
Additionally, T-A colonies cloned from PCR products of the founders were sequenced. F1 and F2 offspring were also sequenced to identify mice harboring mutant alleles. Finally, the expression level of RGS12 was evaluated by western blot.

For the production of transgenic (TG) mice: To acquire the transgene vector pCAG-CAT-RGS12, lacZ in pCAG-loxP-CAT-loxP-lacZ was replaced by mouse RGS12 cDNA. The production was linearized and purified with the QIAquick Gel Extraction Kit (Qiagen, 28704) and was subsequently used in pronuclear microinjection. Founder transgenic mice were identified by tail DNA amplification and were then bred with C57BL/6J mice. The primers for PCR were: CAG gene promoter-forward (5'-CCCCCTGAACCTGAAACATA-3') and RGS12-reverse (5'-GCAGGACTCCAGAGCATTTC-3'). These primers yielded a 561-bp product. CAG-CAT-RGS12 mice were then bred with α-MHC-MerCreMer (Jackson Laboratory, 005650) to generate CAG-CAT-RGS12/α-MHC-MerCreMer double transgenic mice. At 6 weeks old, the double transgenic mice were injected with Tamoxifen (80 mg/kg/day) for 5 days to generate cardiac-specific conditional RGS12-TG mice. CAG-CAT-RGS12/α-MHC-MerCreMer mice without Tamoxifen administration (CRMC) were used as the control group. The expression level of RGS12 from each line was evaluated by western blot.

**Aortic banding**
Pressure overload-induced cardiac hypertrophy models were generated by performing aortic banding (AB) surgery on the mice.1,2 Male mice aged 8-10 weeks and weighing 24-27 g were subjected to AB surgery. Before surgery, the mice were anesthetized using pentobarbital sodium (50 mg/kg, i.p., Sigma). When the toe-pinch reflex disappeared, we opened the left chest of the mice to locate the thoracic aorta. Then, AB was performed by banding the thoracic aorta using a 27-gauge needle with 7-0 silk sutures. Finally, the needle was removed before closing the thoracic cavity. After surgery, Doppler echocardiography was used to confirm the extent of aortic constriction. Meanwhile, sham-operated mice were subjected to the same surgical procedure without aorta constriction. The specific MEK1/2 inhibitor U0126 (1 mg/kg/3 days, CST, 9903) was injected intraperitoneally every 3 days following AB. A series of preliminary experiments were performed to determine the dose of U0126 (0.1–1 mg/kg/day). The dose used resulted in significant inhibition of ERK1/2 activation in the hearts. The same volume of DMSO was used in these experiments as the control experiments. The experiments were blinded to the experimental conditions for all experiments and subsequent analyses.

**Echocardiography measurements**
Echocardiography measurements were performed at the indicated time by using a MyLab 30CV ultrasound (Biosound Esaote Inc.) with a 15-MHz linear array ultrasound transducer. The left ventricle (LV) measurements were analyzed in both the parasternal long-axis and short-axis views at a frame rate of 120 Hz. The end-systole or end-diastole was defined as the phase when the areas of the LV were the smallest or largest, respectively. M-mode tracing was used to assess the LV end-systolic diameter.
(LVESD) and the LV end-diastolic diameter (LVEDD) at the mid-papillary muscle level with a sweep speed of 50 mm/s. The percentage of left ventricular fractional shortening (FS) was defined as (LVEDD-LVESD)/LVEDD×100%. At least eight mice in each group were assessed by M-mode tracing, and the analyses were performed in triplicate.

**Histological analysis**

Hearts, lungs, and tibiae were excised from the sacrificed mice to be weighed and analyzed for the ratios of heart weight to body weight (HW/BW) (mg/g), lung weight to body weight (LW/BW) (mg/g) and heart weight to tibia length (HW/TL) (mg/mm). After excision, hearts were stopped in diastole using 10% potassium chloride solution, washed with saline solution and fixed in 10% formalin. They were then embedded in paraffin. Subsequently, hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of individual hearts (5-µm thick) were obtained from the mid-papillary muscle level. In addition, each section was stained with hematoxylin-eosin (H&E) or picrosirius red (PSR) to evaluate histopathology or collagen deposition, respectively. Furthermore, each section was stained with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen) to assess the cardiomyocyte cross area. Images of cardiomyocytes and collagen deposition were captured by microscopy to measure cardiomyocyte cross area and left ventricular collagen volume using a quantitative digital analysis imaging system (Image-Pro Plus 6.0). More than 100 eligible cardiomyocytes in sections from at least four mice were measured in each group.

**Cultured neonatal rat cardiomyocytes (NRCMs) and recombinant adenoviral vectors**

One- to two-day-old Sprague-Dawley rats were sacrificed by swift decapitation following the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The hearts were then excised, minced and then digested in a phosphate-buffered saline (PBS) solution supplied with 0.03% trypsin and 0.04% collagenase type II. Subsequently, the NRCMs were isolated and seeded in six-well culture plates coated with gelatin in DMEM/F12 supplemented with 20% FCS, BrdU, and penicillin/streptomycin, at a density of 3×10^5 cells/well. After 48 hours, the culture medium was replaced by serum-free DMEM/F12 for 12 hours. Then, the NRCMs were incubated with PBS, angiotensin II (Ang II, 1 µmol/L) or endothelin-1 (ET-1; 100 nmol/L).

Three rat shRGS12 constructs from SABiosciences (KR44486G) were used to establish AdshRGS12 adenoviruses and knockdown RGS12 expression in NRCMs. The construct that suppressed RGS12 expression to the greatest extent was used in the following experiments. A non-targeting short hairpin RNA (AdshRNA) was used as a control. To overexpress RGS12, the entire coding region of the rat RGS12 gene controlled by the cytomegalovirus promoter was inserted into replication-defective adenoviral vectors. Meanwhile, similar adenoviral vectors containing the green fluorescent protein gene (AdGFP) were used as controls. NCRMs were infected with
different adenoviruses in diluted media at a multiplicity of infection of 10 for 24 hours.

**Quantitative real-time PCR and western blotting**

We extracted total RNA from left ventricular tissues and cultured cardiomyocytes using TRIzol reagent (Invitrogen). Then, cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems), and each PCR reaction was repeated in triplicate. The results of each PCR reaction are presented as the average of the relative gene expression normalized to the corresponding GAPDH gene expression.

For western blotting, both left ventricular tissues and cultured cardiomyocytes were lysed in RIPA lysis buffer (720 µl RIPA, 20 µl PMSF, 100 µl Complete, 100 µl Phos-stop, 50 µl NaF, and 10 µl Na3VO4). After centrifugation of the samples, the protein concentrations were evaluated using the Pierce® BCA Protein Assay Kit (Pierce). The lysates with equal amounts of proteins were run on SDS-PAGE (Invitrogen), and then proteins were transferred to polyvinylidene difluoride membranes (Millipore). After blocking with 5% non-fat milk for 1 hour at room temperature, the membranes were probed with primary antibodies overnight at 4°C. After incubation with the secondary peroxidase-conjugated antibodies for 1 hour at room temperature, immunoblots were visualized using the Bio-Rad ChemiDoc™ XRS+ (Bio-Rad). The expression levels of specific proteins were normalized to the corresponding GAPDH expression levels.

**Immunoprecipitation**

Cultured NRCMs were collected and lysed in IP buffer containing 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP-40, 1 mmol/L EDTA and a protease inhibitor cocktail (Roche). Soluble extracts were incubated on ice for 20 minutes, followed by centrifugation (14000 g) at 4 °C for 15 min. After being pre-cleared with normal mouse or rabbit immunoglobulin G, the cell lysates were incubated with protein A agarose beads and the indicated antibodies for 3 hours at 4 °C. The immunocomplex was collected, washed five to six times with cold IP buffer and blotted using the indicated primary antibodies.

**References**


Supplemental Figure S1. RGS12 expression is upregulated in HCM human hearts. Representative western blots (left) and quantitative results (right) of RGS12, ANP and β-MHC expression levels in hearts from normal donors or patients with HCM (n=4 hearts per experimental group; *P<0.05 vs. donor hearts).
Supplemental Figure S2. Absence of RGS12 attenuates pathological cardiac hypertrophy induced by pressure overload. (A) A region in the common genetic site of the two variants in the RGS12 mouse gene was targeted by one single guide RNA (sgRNA). (B) Representative results of DNA sequencing from the founders and the double peak traces in the sequencing chromatogram indicated an indel in heterozygous mutants. (C) Sequencing results of the RGS12 mutant allele in four founder mice. The numbers of nucleotides of deletions (Δ) are shown to the right of each allele. (D) DNA sequences of WT and RGS12-KO mice showing the 7-bp deletion in the RGS12-KO sample. (E) Representative western blots of RGS12 protein levels from WT and global RGS12-KO hearts (n=4 mice per experimental group; GAPDH was used as a loading control). (F) Real-time PCR assays were performed to determine the relative mRNA levels of ANP, BNP and β-MHC in each group (n=4 mice per experimental group). (G) Real-time PCR assays were performed to determine the relative mRNA levels of collagen I, collagen III, and CTGF in each group (n=4 mice per experimental group). *P<0.05 vs. WT/sham; #P<0.05 vs. WT/AB.

Supplemental Figure S3. Cardiac-specific overexpression of RGS12 exaggerates pathological cardiac hypertrophy induced by pressure overload. (A) Schematic diagram of the generation of transgenic (TG) mice harboring a full-length mouse RGS12 cDNA under the control of the α-MHC promoter. (B, C) B, Representative western blots; C, Quantitative results of RGS12 expression levels in myocardium
from four TG lines and their CAG-CAT-RGS12/α-MHC-MerCreMer littermates without tamoxifen administration (CRMC) (n=3 independent experiments; GAPDH was used as a loading control). (D) Real-time PCR assays were performed to determine the relative mRNA levels of ANP, BNP and β-MHC in each group (n=4 mice per experimental group). (E) Real-time PCR assays were performed to determine the relative mRNA levels of collagen I, collagen III and CTGF in each group (n=4 mice per experimental group). *P<0.05 vs. CRMC/sham; #P<0.05 vs. CRMC/AB.

Supplemental Figure S4. RGS12 exacerbates cardiomyocyte hypertrophy in vitro. (A) Representative western blots (left) and quantitative results (right) of RGS12 expression levels in NRCMs infected with AdshRNA, AdshRGS12, AdGFP or
AdRGS12 (n=3 independent experiments; GAPDH was used as a loading control). (B) Real-time PCR assays were performed to determine the relative mRNA levels of ANP, BNP and β-MHC in NRCMs infected with AdshRNA or AdshRGS12 and treated with PBS or Ang II (1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdshRNA/PBS; #P<0.05 vs. AdshRNA/Ang II). (C) Real-time PCR assays were performed to determine the relative mRNA levels of ANP, BNP and β-MHC in NRCMs infected with AdGFP or AdRGS12 and treated with PBS or Ang II (1 μmol/L) for 48 hours (n=3 independent experiments; *P<0.05 vs. AdGFP/PBS; #P<0.05 vs. AdGFP/Ang II). (D) Representative images of NRCMs infected with AdshRNA, AdshRGS12, AdGFP or AdRGS12 and treated with ET-1 (100 nmol/L) for 48 hours (n=3 independent experiments; scale bar, 20 μm). (E) Quantitative results of the cell surface area of NRCMs infected with AdshRNA or AdshRGS12 and treated with PBS or ET-1 (100 nmol/L) for 48 hours (n≥50 cells per experimental group; *P<0.05 vs. AdshRNA/PBS; #P<0.05 vs. AdshRNA/ET-1). (F) Quantitative results of the cell surface area of NRCMs infected with AdGFP or AdRGS12 and treated with PBS or ET-1 (100 nmol/L) for 48 hours (n≥50 cells per experimental group; *P<0.05 vs. AdGFP/PBS; #P<0.05 vs. AdGFP/ET-1).
Supplemental Figure S5. RGS12 promotes the activation of MEK1/2-ERK1/2 signaling induced by pro-hypertrophic stimuli. (A) Representative western blots (top) and quantitative results (bottom) of phosphorylated and total MAPK levels in NRCMs infected with AdshRNA or AdshRGS12 60 minutes after PBS or Ang II administration (n=3 independent experiments; *P<0.05 vs. AdshRNA/PBS; #P<0.05 vs. AdshRNA/Ang II; n.s. indicates no significant difference vs. AdshRNA/Ang II). (B) Representative western blots (top) and quantitative results (bottom) of phosphorylated and total MAPK levels in NRCMs infected with AdGFP or AdRGS12 60 minutes after PBS or Ang II administration (n=3 independent experiments; *P<0.05 vs. AdGFP/PBS; #P<0.05 vs. AdGFP/Ang II; n.s. indicates no significant difference vs. AdGFP/Ang II). (C) Western blots with RGS12 or MEK1/2 antibody after co-IP of RGS12 or MEK1/2 from NRCMs lysates using RGS12 or MEK1/2 antibody after PBS administration. (D) Western blots with RGS12 or MEK1/2 antibody after co-IP of RGS12 or MEK1/2 from NRCMs lysates using RGS12 or MEK1/2 antibody after Ang II administration. GAPDH was used as a loading control.
Supplemental Figure S6. Activation of MEK1/2-ERK1/2 signaling pathway is essential for RGS12-exaggerated cardiac hypertrophy. Representative western blots (left) and quantitative results (right) of phosphorylated and total MEK1/2-ERK1/2 levels in hearts from DMSO or U0126 treated RGS12-TG mice four weeks after AB surgery (n=4 mice per experimental group; GAPDH was used as a loading control). *P<0.05 vs. RGS12-TG/AB/DMSO.