Heart

Growth Arrest–Specific 6 Exacerbates Pressure Overload–Induced Cardiac Hypertrophy

Yi-fan Zhao,* Da-chun Xu,* Guo-fu Zhu, Meng-yun Zhu, Kai Tang, Wei-ming Li, Ya-wei Xu

Abstract—Growth arrest–specific 6 (GAS6) is a member of the vitamin K–dependent protein family that is involved in the regulation of the cardiovascular system, including vascular remodeling, homeostasis, and atherosclerosis. However, there is still no study that systemically elucidates the role of GAS6 in cardiac hypertrophy. Here, we found that GAS6 was upregulated in human dilated cardiomyopathic hearts, hypertrophic murine hearts, and angiotensin II–treated cardiomyocytes. Next, we examined the influence of GAS6 expression in response to a cardiac stress by inducing chronic pressure overload with aortic banding in wild-type and GAS6-knockout mice or cardiac-specific GAS6 overexpressing mice. Under basal conditions, the GAS6-knockout mice had normal left ventricular structure and function but after aortic banding, the mice demonstrated less hypertrophy, fibrosis, and contractile dysfunction when compared with wild-type mice. Conversely, cardiac-specific overexpression of GAS6 exacerbated aortic banding–induced cardiac hypertrophy, fibrosis, and dysfunction. Furthermore, we demonstrated that GAS6 activated the mitogen-activated protein kinase kinase 1/2–extracellular signal-regulated kinase 1/2 pathway during pressure overload–induced cardiac hypertrophy, and the pharmacological mitogen-activated protein kinase kinase 1/2 inhibitor U0126 almost completely reversed GAS6 overexpression–induced cardiac hypertrophy and fibrosis, resulting in improved cardiac function. Collectively, our data support the notion that GAS6 impairs ventricular adaptation to chronic pressure overload by activating mitogen-activated protein kinase kinase 1/2–extracellular signal-regulated kinase 1/2 signaling. Our findings suggest that strategies to reduce GAS6 activity in cardiac tissue may be a novel approach to attenuate the development of congestive heart failure. (Hypertension. 2016;67:118-129. DOI: 10.1161/HYPERTENSIONAHA.115.06254.)

Key Words: cardiomegaly ■ cardiomyopathies ■ ERK1-2 pathway ■ growth arrest-specific protein 6 ■ mice, knockout

Cardiac hypertrophy, which is commonly observed in hypertension, ischemia, and valvular heart disease, is an adaptive response to long-standing biomechanical pressure or volume overload.1 It is a complex dynamic process that involves a variety of signaling pathways and regulators. Despite mounting studies, the underlying molecular mechanism of pathological cardiac hypertrophy is still poorly understood. Some extracellular stimuli, such as angiotensin II (Ang II), endothelin, catecholamine and transforming growth factor-β can coordinate the hypertrophy of cardiomyocytes by altering cardiac hypertrophy–related gene expression in the nucleus.2 In recent studies, cardiac hypertrophy has been shown to be related to various signaling pathways involving the mitogen-activated protein kinase (MAPK), phosphoinositide 3 kinase/AKT, protein kinase C, nuclear factor-κB, cAMP-response element binding protein, transforming growth factor-β/Smad, and calcineurin/nuclear factor of activated T cells pathways.2,3 Therefore, molecules that selectively affect these signaling pathways may have therapeutic potential in pathological cardiac hypertrophy.

Growth arrest–specific 6 (GAS6) was identified as a member of the vitamin K–dependent protein family that shares 42% structural similarity with protein S, a vitamin K–dependent anticoagulant protein.4,5 It is ubiquitously expressed in most organs including uterus, ovary, kidney, and heart.4 GAS6 is the ligand of the TAM receptor tyrosine kinase family, which is composed of 3 members: Tyro3, Axl, and Mer,6 and has the highest affinity with Axl.7,8 Binding with the receptor induces their dimerization and subsequent kinase activation, which activates the intrinsic signaling molecules to exert various biological functions.9 GAS6 is involved in cell viability,10 immune regulation,11 vascular hemostasis, remodeling, and atherosclerosis via the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), phosphoinositide 3 kinase-AKT, and other signaling pathways.12-15 It has been well established that both ERK1/2 and

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phosphoinositide 3 kinase-AKT signaling play important roles in congestive heart failure. Of note, GAS6 deficiency can attenuate deoxycorticosterone acetate–induced cardiac hypertrophy and fibrosis. However, it still lacks a comprehensive understanding of whether and how GAS6 affects the development of pressure overload–induced cardiac hypertrophy and congestive heart failure.

In the present study, we identified the upregulation of GAS6 in both human dilated cardiomyopathic hearts and hypertrophic murine hearts induced by pressure overload. Cardiomyocyte-specific GAS6 overexpression in mice resulted in a more severe phenotype of cardiac hypertrophy, whereas GAS6 deficiency demonstrated a protective effect. Similar results were also demonstrated in cultured neonatal rat cardiomyocytes treated with Ang II. Furthermore, we found that GAS6 enhanced the activity of mitogen-activated protein kinase kinase 1/2 (MEK1/2)–ERK1/2 signaling both in vivo and in vitro. Blockage of MEK1/2–ERK1/2 activity tremendously reversed the GAS6 overexpression–induced phenotype of cardiac hypertrophy. Altogether, we identified for the first time that GAS6 is a novel positive regulator of pathological cardiac hypertrophy by promoting MEK1/2–ERK1/2 signaling.

**Materials and Methods**

All of the procedures involving human heart samples obey the principle outlined in the Declaration of Helsinki and were approved by the Ethics Committee at Tongji University. Written informed consent was gained from each DCM patient and the relatives of the heart donors. All of the animal procedures were approved by the Animal Care and Use Committees of Shanghai Tenth People’s Hospital. A detailed Materials and Methods is available in the online-only Data Supplement.

**Generation of GAS6-Knockout Mice**

Clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 technique was used to generation the GAS6-knockout (GAS6-KO) mice, which has been described elsewhere. In brief, a pair of sgRNA and Cas9 mRNA was transcribed and microinjected to 1-cell embryos to gain F0 offsprings. Then, we mated F0 offspring to get F1 and F2 offspring.

**Generation of Cardiac-Specific GAS6 Transgenic Mice**

The detailed method to generate cardiac-specific GAS6 transgenic mice was described elsewhere and also in the online-only Data Supplement. Briefly, a construct of pCAG-loxP-CAT-loxP-GAS6-A was constructed and microinjected to the murine embryos. Then, the mice were mated with transgenic mice that carried Cre genes under the control of the α-myosin heavy chain (MHC) gene promoter (Jackson Laboratory, 005650) to generate CAG-CAT-mGAS6/MHC-Cre double transgenic mice.

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**Figure 1.** Growth arrest–specific 6 (GAS6) is upregulated in human dilated cardiomyopathy (DCM) hearts, hypertrophic murine hearts, and cardiomyocytes. A, Representative Western blot results of atrial natriuretic peptide (ANP), β-myosin heavy chain (MHC), and GAS6 expression in both donor human hearts (n=6) and DCM human hearts (n=6; n indicates the number of samples used in each experiment). B, Representative Western blot analysis of ANP, β-MHC, and GAS6 expression in sham operation, 4 wk after aortic banding (AB) and 8 wk after AB (n=6 mice for each experimental group). C, Western blot analysis of ANP, β-MHC, and GAS6 expression in cultured neonatal rat cardiomyocytes treated with PBS or angiotensin II (Ang II) for 24 and 48 h (n=3 independent experiments). Left, Representative Western blot; (right) bar graphs of the quantitative results. All of the data are presented as the mean±SD.
Figure 2. Growth arrest–specific 6 (GAS6) cardiac-specific transgenic mice had detrimental effects in pressure overload–induced cardiac hypertrophy. A, The ratios of heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) determined in CGMC and GAS6-TG mice 4 wk after sham or aortic banding (AB) treatment (n=12 mice per experimental group). B, Left, Histological analysis of hematoxylin and eosin (H&E) staining and wheat germ agglutinin (WGA) staining in CGMC and GAS6-TG mice at 4 wk after the AB surgery or sham operation (n=5–6 mice per experimental group; scale bars, 50 μm). (continued)
Results

GAS6 Expression Is Increased in Hypertrophic Hearts and Cardiomyocytes

To identify the role of GAS6 in pathological cardiac hypertrophy, we first measured the expression level of GAS6 in human samples. Western blot analysis showed that GAS6 protein levels were significantly upregulated, accompanied by increased atrial natriuretic peptide and β-MHC, in human DCM hearts when compared with those in donor hearts (Figure 1A). Subsequently, we examined GAS6 expression in hypertrophic murine hearts 4 and 8 weeks after AB. Similar to the results in human samples, hypertrophic murine hearts had a much higher level of GAS6 than control hearts (Figure 1B). Finally, neonatal rat cardiomyocytes were cultured with Ang II for 24 and 48 hours to generate cardiomyocyte hypertrophy. Not surprisingly, GAS6 levels were also elevated 24 hours after Ang II administration and maintained at a high level after 48 hours of Ang II treatment when compared with PBS-treated groups (Figure 1C). Taken together, these results indicated that GAS6 can be upregulated by hypertrophic stimulation.

GAS6 Cardiomyocyte-Specific Overexpression Aggravates Pressure Overload–Induced Cardiac Hypertrophy

As expression of GAS6 is markedly elevated in hypertrophic hearts and cardiomyocytes, it is essential to study whether changes in GAS6 level can regulate cardiac hypertrophy. First, we generated transgenic mice overexpressing GAS6 targeted to cardiomyocytes using Cre-LoxP techniques (GAS6-TG; Figure S1A in the online-only Data Supplement, left). Four independent lines were established (TG1, TG2, TG3, and TG4). We selected the highest expressing line, TG2, to perform the following experiments (Figure S1A, right). The GAS6-TG mice are normally born with no abnormality in heart morphology and function (data not shown). Nevertheless, 4 weeks after AB, the GAS6-TG mice manifested a more pronounced cardiac hypertrophy than the CAG-CAT-mGAS6/MHC-Cre mice without tamoxifen injection (CGMC) controls, as indicated by heart weight (HW)/body weight (BW), lung weight/BW and HW/tibia length ratios (Figure 2A). Next, hematoxylin-eosin and wheat germ agglutinin staining showed that, compared with CGMC mice, the cross-sectional area was significantly increased in the GAS6-TG group 4 weeks after AB surgery (Figure 2B). In accordance with the increased cell size, worsening cardiac hypertrophy and function were demonstrated in the GAS6-TG mice compared with CGMCs by echocardiography as indicated by fractional shortening, left ventricular end-systolic diameters, and LV end-dilated diameters (Figure 2C). Moreover, cardiac fibrosis, a hallmark of pathological cardiac hypertrophy, was detected by picrosirius red staining. GAS6-TG mice had a more severe fibrosis in both the interstitial and the perivascular areas (Figure 2D), along with an elevation of the fibrotic markers collagen Iα, collagen III, and connective tissue growth factor (CTGF) in CGMC and GAS6-TG mice at 4 wk after AB or sham operation. *P<0.05 vs CGMC/sham; #P<0.05 vs CGMC/AB. All of the data are presented as the means±SD.

GAS6 Deficiency Protects Against Pressure Overload–Induced Cardiac Hypertrophy

Given that GAS6 overexpression deteriorates cardiac hypertrophy, we hypothesized that GAS6 loss-of-function may attenuate cardiac hypertrophy and function. To verify this hypothesis, we generated global GAS6-KO mice using CRISPR-Cas9 techniques (Figure S2A–S2E). Under basal conditions, GAS6-KO mice were normal in cardiac morphology and function after birth. However, GAS6-KO mice had a lower level of HW/BW, lung weight/BW, and HW/tibia length than their wild-type (WT) littermates 4 weeks after AB (Figure 3A). Similarly, the cross-sectional area of GAS6-KO mice was smaller than that of the WT mice 4 weeks after the AB operation (Figure 3B). Echocardiography analysis indicated attenuated cardiac hypertrophy and a preserved contractile function in GAS6-KO mice (Figure 3C). Subsequently, cardiac fibrosis was evaluated. As expected, fibrosis in the interstitial and perivascular space was consistently decreased in the GAS6-KO mice when compared with WT mice accompanied with decreased mRNA level of the fibrotic markers collagen Iα, collagen III, and connective tissue growth factor (Figure 3D and 3E). Thus, these data illustrated that GAS6 deficiency attenuated pressure overload–induced cardiac hypertrophy.

GAS6 Aggravates Cardiomyocyte Hypertrophy In Vitro

Considering the detrimental role of GAS6 in pressure overload–induced cardiac hypertrophy and the results were obtained with global GAS6-deficient mice, not cardiomyocyte-specific. It is necessary to validate the regulatory role of GAS6 specifically in cardiomyocytes. Thus, neonatal rat cardiomyocytes was infected with either AdGAS6 or AdshGAS6 to generate gain-of-function and loss-of-function cell models, respectively (Figure 4A). With PBS treatment, no significant difference in cell size was observed between AdGAS, AdshGAS6, and their respective control groups (Figure 4B–4D). However, after Ang II incubation for 48 hours, we observed obviously larger cardiomyocytes in the AdGAS6 group and much smaller cells in the AdshGAS6 group than in the AdGFP or AdshRNA groups, respectively, as indicated by the cell surface area (Figure 4B–4D).
Figure 3. Growth arrest–specific 6 (GAS6) deficiency ameliorates aortic banding-induced cardiac hypertrophy. A, heart weight (HW)/body weight (BW), lung weight (LW)/BW, and HW/tibia length (TL) determined in wild-type (WT) and GAS6-knockout (KO) mice 4 wk after sham or aortic banding (AB) treatment (n=12 mice per experimental group). B, Left, Hematoxylin-eosin (H&E) staining and wheat germ agglutinin (WGA) staining in WT and GAS6-KO mice at 4 wk after the AB surgery or sham operation (n=5–6 mice per experimental group; scale bars, 50 μm). Right, Statistical results for the calculated cross-sectional area in the indicated groups (n≥100 cells per experimental group). C, Echocardiography results for WT and GAS6-KO mice at 4 wk of treatment after AB or sham operation (n=6–8 per experimental group). (continued)
Hypertrophic markers such as atrial natriuretic peptide, brain natriuretic peptide, α-MHC, and β-MHC were then analyzed by real-time polymerase chain reaction. In accordance with the results of cell surface area, these hypertrophic markers were markedly increased in the AdGAS6 group when compared with the AdGFP group treated with Ang II, whereas the AdhGAS6 group had a lower mRNA level of these markers than the AdshRNA group (Figure 4E and 4F). These data implicate GAS6 as a deteriorative factor of Ang II–induced cardiomyocyte hypertrophy.

GAS6 Is an Activator of MEK1/2–ERK1/2 Signaling During Cardiac Hypertrophy In Vivo and In Vitro

Considering that GAS6 is a potent positive regulator of pathological cardiac hypertrophy, we next investigated the underlying mechanism involved. Given the critical role of the MAPK pathway in the process of cardiac hypertrophy and the ability of GAS6 to regulate ERK activation, we first evaluated the expression level of MAPK signaling components in the heart. As seen in Figure 5A and 5C, the phosphorylated MEK1/2 and ERK1/2 were dramatically upregulated in GAS6-TG mice when compared with their CGMC littermates subjected to AB, whereas c-Jun N-terminal kinase and P38 activity were not different between these 2 groups. In contrast, GAS6 deficiency led to a robust inhibition of MEK1/2 and ERK1/2 without affecting the activity of c-Jun N-terminal kinase and P38 (Figure 5B and 5D). To further confirm the MEK1/2–ERK1/2 activator role of GAS6, in vitro neonatal rat cardiomyocytes were cultured and treated with Ang II. Consistent with the in vivo observation, Western blot analysis demonstrated an elevation of MEK1/2 and ERK1/2 phosphorylation in the AdGAS6 groups and a decline in the AdshGAS6 groups when compared with the respective control group (Figure 5E–5H). Collectively, these data suggest that GAS6 activates MEK1/2–ERK1/2 signaling in cardiomyocytes during pathological cardiac hypertrophy in vivo and vitro.

Blockage of MEK1/2–ERK1/2 Signalin Is Sufficient to Reverse Cardiac Hypertrophy in GAS6-TG Mice

Because of our observation that GAS6 can activate MEK1/2–ERK1/2 signaling under pressure overload and Ang II stimuli, it was important to elucidate whether inhibition of MEK1/2–ERK1/2 could reverse the GAS6-TG–induced hypertrophic phenotype. Therefore, mice were treated with a widely used pharmacological MEK1/2 inhibitor (U0126) after aortic banding. The protein level of phosphorylated MEK1/2 and phosphorylated ERK1/2 was dramatically diminished 4 weeks after AB when compared with DMSO-treated mice (Figure 6A). Notably, the HW/BW, lung weight/BW, and HW/tibia length ratios were all obviously reduced (Figure 6B) accompanied with decreased cross-sectional area (Figure 6C) in U0126-treated mice when compared with DMSO-treated mice. Further echocardiographic analysis confirmed an ameliorative hypertrophic phenotype with improved cardiac function after U0126 injection (Figure 6D). Cardiac fibrosis was also rescued in the U0126-treated group when compared with DMSO-treated mice (Figure 6E). Altogether, the results indicate that inhibition of MEK1/2–ERK1/2 signaling rescued the GAS6 overexpression–induced detrimental phenotype of cardiac hypertrophy and function.

Discussion

Pathological cardiac hypertrophy is a complicated dynamic process that ultimately leads to heart failure. At present, we still have few effective methods to reverse pathological cardiac hypertrophy. Because the cardiovascular mortality increased 6- to 10-fold because of the development of pathological cardiac hypertrophy, it is of great importance to elucidate the underlying molecular mechanism of cardiac hypertrophy and discover potential target molecules to regulate pathological cardiac hypertrophy. Using genetic loss-of-function and gain-of-function animal models, the present study provides the systemic direct evidence that selective inhibition of GAS6 may be a therapeutic target for treating cardiac hypertrophy and congestive heart failure.

Mechanical stress and neurohormone stimuli are the 2 major causes for pathological cardiac hypertrophy. Both of these initially cause number of extracellular molecules to bind with their receptors and subsequently activate intrinsic signaling pathways. Numerous receptors have been shown to be involved in the process of pathological cardiac hypertrophy, including receptor tyrosine kinase, G-protein–coupled receptors, and integrins. Among them, receptor tyrosine kinases can bind with epidermal growth factor, fibroblast growth factors, and neuregulin to activate several prohypertrophic signaling pathways, such as MAPK, AKT, and Ca2+ dependent signaling. Axl, a receptor of GAS6, is a member of the receptor tyrosine kinase family and has the highest expression level in heart and skeletal muscle, which implies that it may play an important role in the pathophysiological process of heart disease. Intriguingly, the protein level of Axl was significantly higher in both myocardial biopsies and serum in patients with end-stage heart failure than in control heart donors. Meanwhile, the biological function of Axl is mainly exerted by binding with GAS6. These phenomena suggest that the expression of GAS6 is likely to increase during heart failure. In the present study, consistent with this speculation, we observed that the expression of GAS6 was obviously increased in hypertrophic hearts and cardiomyocytes. In an AB-induced mouse hypertrophy model, GAS6 began to increase at 4
Figure 4. Growth arrest-specific 6 (GAS6) aggravates angiotensin II (Ang II)–induced cardiomyocyte hypertrophy in vitro. **A**. Left, Western blot analysis to identify the GAS6 expression in cardiomyocytes infected with AdGFP, AdGAS6, AdshRNA, and AdshGAS6. Right, Bar graph of quantitative results of Western blot analysis (n=3 independent experiment). **B**, The immunofluorescence results for cardiomyocytes infected with AdGAS6 or AdshGAS6 (AdGFP and AdshRNA as control, respectively) and 48-h treatment of PBS or Ang II (blue: nuclear and green: α-actinin; scale bar, 20 μm). **C** and **D**, Bar graphs of the cell surface area in the indicated group after 48-h treatment with PBS or Ang II (n>50 cells per experimental group; *P<0.05 vs AdGFP/PBS [C] or AdshRNA/PBS [D]; #P<0.05 vs AdGFP/Ang II [C] or AdshRNA/Ang II [D]). **E** and **F**, Real-time polymerase chain reaction analysis of hypertrophic biomarkers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), α-myosin heavy chain (MHC), and β-MHC in the indicated group after 48 h of treatment with PBS or Ang II (n=4 independent experiments; *P<0.05 vs AdGFP/PBS [E] or AdshRNA/PBS [F]; #P<0.05 vs AdGFP/Ang II [E] or AdshRNA/Ang II [F]). All of the data are presented as the mean±SD.
Figure 5. Growth arrest–specific 6 (GAS6) activates mitogen-activated protein kinase kinase 1/2 (MEK1/2)–extracellular signal-regulated kinase ½ (ERK1/2) during pathological cardiac hypertrophy in vivo and in vitro. A and B, Representative Western blot analysis of phosphorylated and total MEK1/2, ERK1/2, c-Jun N-terminal kinase (JNK), and P38 level in CGMC and GAS6-TG mice (A) or in wild-type (WT) and GAS6-knockout (KO) mice (B) 4 wk after sham or aortic banding (AB) operation (n=6 for each experimental group). C and D, Quantitative results of Western blot of phosphorylated MEK1/2 and ERK1/2 levels in CGMC and GAS6-TG mice (C) or in WT and GAS6-KO mice (D) 4 wk after they were subjected to sham or AB operation. E and F, Representative Western blot analysis of phosphorylated and total MEK1/2, ERK1/2, JNK, and P38 levels in AdGFP and AdGAS6 cardiomyocytes (E) or in AdshRNA and AdshGAS6 cardiomyocytes (F) treated with PBS or Ang II (n=4 independent experiments). G and H, Quantitative results of Western blot of phosphorylated MEK1/2 and ERK1/2 levels in AdGFP and AdGAS6 cardiomyocytes (G) or in AdshRNA and AdshGAS6 cardiomyocytes (H) treated with PBS or Ang II. All of the data are presented as the mean±SD.
Figure 6. Blockage of mitogen-activated protein kinase kinase 1/2 (MEK1/2)–extracellular signal-regulated kinase 1/2 (ERK1/2) signaling reverses GAS6-TG–induced detrimental phenotype of cardiac hypertrophy. (continued)
weeks after AB and continuously grew to a higher level at 8 weeks after AB. As the elevation of GAS6 is synchronous with the progression of heart failure, GAS6 is speculated as a deteriorative factor in the progression from compensated hypertrophy to heart failure. In support of this hypothesis, GAS6-TG mice were generated and demonstrated an acceleration in cardiac hypertrophy and function. Notably, the ratio of lung weight:BW, as an indicator of CHF, is strongly elevated in GAS6-TG mice after AB surgery, whereas GAS6 KO has only a slight effect on this parameter. These data indicate GAS6 as a crucial factor in the regulation of pathological cardiac hypertrophy and the progression from compensated hypertrophy to heart failure.

MAPK is one of the best-characterized signal transduction pathways in the regulation of cardiac hypertrophy, consisting of sequentially functioning kinases. ERK1/2, c-Jun N-terminal kinase, and P38 are the 3 major MAP kinases that are relevant to cardiac hypertrophy. Among the 3 MAPKs, phosphorylated ERK1/2 can lead to the activation of some key prohypertrophic transcription factors, such as GATA4. Thus, it was thought to be a prohypertrophic factor at first. However, with increasing numbers of studies using genetic models, the effect of ERK1/2 in cardiac hypertrophy becomes complicated. Studies using overexpression or knockout animal model of MEK–ERK signaling both demonstrated deteriorative phenotype of cardiac hypertrophy, which suggested that excess ERK activation or complete deletion of ERK is both detrimental to cardiac hypertrophy. In consistent with this hypothesis, some recent studies have demonstrated that an excessive activation of ERK1/2 leads to more severe cardiac hypertrophy and function. Notably, many studies have indicated that Axl/GAS6 binding can exert biological functions via the activation of MEK1/2–ERK1/2 signaling. Goruppi et al demonstrated that GAS6 has mitogenic and prosurvival activities in NIH3T3 fibroblasts, and the activity is dependent on the activation of ERK1/2. Sainaghi et al discovered that the AXL/GAS6 interaction has mitogenic activity in prostate cancer cell lines via MEK1/2 phosphorylation. Thus, it is reasonable that GAS6 could affect cardiac hypertrophy through MEK–ERK1/2 signaling. Our results indicated that the GAS6 aberrantly activates MEK1/2–ERK1/2 signaling under pressure overload and Ang II stimuli, whereas pharmacological blockade of MEK1/2 by U0126 attenuated cardiac hypertrophy and preserved cardiac contractile function in GAS6-TG mice. Therefore, we can conclude that GAS6 aggravates cardiac hypertrophy mostly via activating MEK1/2–ERK1/2 signaling. Whether there are other mechanisms involved still needs further studies.

As a potential therapeutic target for hypertension-related cardiac hypertrophy, inhibition of GAS6 may have advantages over MEK–ERK inhibition, albeit both of them can attenuate pathological cardiac hypertrophy. First, GAS6 is an extracellular protein whose concentration could be evaluated by blood test. Because of different genetic background, treatment, living environment, even had the same disease, the GAS6 level could be strongly fluctuated. Therefore, we can first test the plasma concentration of GAS6 and specifically inhibits GAS6 when the concentration is really high during pressure overload–induced cardiac hypertrophy. In contrast, MEK1/2 and ERK1/2 are intracellular proteins, which seem difficult to be detected in peripheral blood. Moreover, intravenous administration might be more efficient to inhibit GAS6 rather than MEK1/2 and ERK1/2 because of their different distribution (plasma versus cytoplasm). Second, experiments from animal model indicated that ERK1/2 deficiency in murine hearts led to deteriorative cardiac function under physiological condition, whereas, in the present study, we observed normal cardiac morphology and function in GAS6-KO mice (Figure 3) without stimuli. Hence, in terms of hearts, it seems that GAS6 inhibition is safer than ERK1/2 inhibition. Moreover, hypertension can not only cause cardiac hypertrophy but also increases the incidence of coronary heart disease, which is a common but fatal disease in clinics. According to a previous study, GAS6 is upregulated in human and murine atherosclerotic plaques. GAS6 deficiency leads to stabilization of atherosclerotic plaque in apoE−/− mice, which indicated that inhibition of GAS6 may also reduce the risk of myocardial infarction. However, ERK is well defined as a protective factor in ischemia-reperfusion injury because of its prosurvival effects. Therefore, inhibition of GAS6 may have additional cardioprotective role in atherosclerosis and myocardial infarction accompanied by hypertension-related cardiac hypertrophy.

In conclusion, this is the first study to identify GAS6 as a novel deteriorative factor in the regulation of pathological cardiac hypertrophy in vitro and in vivo. Moreover, the prohypertrophic effect of GAS6 is predominantly because of the activation of MEK1/2–ERK1/2 signaling. Thus, GAS6 could be a new target for the prevention and therapy of cardiac hypertrophy and heart failure.

Perspectives

Through using genetic loss-of-function and gain-of-function mice, we performed a comprehensive description of the detrimental effects of GAS6 in pathological cardiac hypertrophy and identified that activation of the MEK1/2–ERK1/2 pathways is the vital mechanism involved. Therefore, targeting
and inhibiting the activity or expression of GAS6 could be a promising method for the prevention and therapy of pathological cardiac hypertrophy.

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Disclosures
None.

References


Novelty and Significance

What Is New?

• Growth arrest–specific 6 (GAS6) is upregulated in failing human hearts, pressure overload–induced murine hypertrophic hearts, and angiotensin II–treated hypertrophic cardiomyocytes.

• For the first time, GAS6 is shown to accelerate the development of pressure overload–induced cardiac hypertrophy, fibrosis, and heart failure.

• GAS6 enhances the activity of mitogen-activated protein kinase kinase 1/2 (MEK1/2)–extracellular signal-regulated kinase 1/2 (ERK1/2) signaling to promote pathological cardiac hypertrophy.

What Is Relevant?

• Receptor tyrosine kinases are important regulators in cardiac hypertrophy, and therefore finding novel ligands for receptor tyrosine kinases is essential to further understand cardiac hypertrophy.

• The GAS6 expression level could be a new marker to estimate the extent of cardiac hypertrophy and heart failure. Inhibition of GAS6 may be a new target for the prevention and therapy of pathological cardiac hypertrophy.

Summary

The present study illustrated that GAS6 loss-of-function protects pathological cardiac hypertrophy, fibrosis, and function, whereas GAS6 overexpression deteriorates the hypertrophic phenotype by activating the MEK1/2–extracellular signal-regulated kinase 1/2 (ERK1/2) cascades. Our work indicates that GAS6 might be a promising molecular in the assessment, prevention, and therapy of cardiac hypertrophy and heart failure.
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Growth arrest-specific 6 exacerbates pressure overload-induced cardiac hypertrophy
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Running title: GAS6 and cardiac hypertrophy

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Materials and Methods

Reagents
The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA): ANP (sc20158 1:200), β-MHC (sc53090 1:200), GATA4 (sc9053 1:200), p-GATA4Ser262 (sc32823 1:200). The antibodies against GAPDH (#2118 1:1000), phospho-MEK1/2Ser217/221 (#9154 1:1000), total-MEK1/2 (#9122 1:1000), phospho-ERK1/2Thr202/204 (#4370 1:1000), total-ERK1/2 (#4695 1:1000), phospho-JNK1/2 (#4668 1:1000), total-JNK1/2 (#9258 1:1000), phospho-P38Thr180/182 (#4511 1:1000), total-P38 (#9212 1:1000) and Axl (#4566 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Fetal calf serum (FCS) was ordered from HyClone (Waltham, MA, USA). The reagents for cell culture were purchased from Sigma (St. Louis, MO, USA).

Human heart samples
All of the procedures involving human heart samples obey the principle outlined in the Declaration of Helsinki and were approved by the Ethics Committee at Tongji University. The failing human hearts were obtained from dilated cardiomyopathy patients who underwent heart transplantation. The control samples were obtained from heart donors who were died due to non-cardiac events and were unsuitable for heart transplantation for technical reasons. Written informed consent was gained from each DCM patient and the relatives of the heart donors.

Experimental Mouse Models
All of the animal procedures were approved by the Animal Care and Use Committees of Shanghai Tenth People’s Hospital.

Generation of cardiac-specific GAS6 transgenic mice
To generate GAS6 cardiac-specific transgenic mice (C57BL/6J background), we generated a construct of pCAG-loxP-CAT-loxP-GAS6-A (designated as CAG (chicken beta-actin)-CAT (chloramphenicol acetyltransferase)-GAS6-A). The construct was linearized and purified with the QIAquick Gel Extraction Kit (Qigagen, 28704) following the manufacturer’s instruction. Then, the vector containing this construct was microinjected into fertilized murine embryos to obtain the transgenic mice. Founder transgenic mice were identified by polymerase chain reaction (PCR) using tail DNA and were bred with C57BL/6J mice. The primer for detection was CAG-forward (5’-CCCCCTGAACCTGAAACATA-3’) and GAS6-Reverse (5’-CAGGCTGCTATTTTCTTC-3’) and yielded a product of 588bp. To induce GAS6 expression specifically in the cardiomyocytes, the CAG-loxP-CAT-loxP-GAS6-A transgenic mice were crossed with transgenic mice that carried Cre genes under the control of the α-MHC gene promoter (Jackson Laboratory, 005650) to generate CAG-CAT-mGAS6/MHC-Cre double transgenic mice. Cre-mediated recombination of floxed alleles was induced by intraperitoneal injection of tamoxifen (80 mg/kg/day, Sigma, T-5648) for 5 consecutive days at 6 weeks old to obtain cardiac-specific GAS6 conditional transgenic mice. Four independent transgenic lines were successfully generated. The CAG-CAT-mGAS6/α-MHC-Cre mice without tamoxifen injection (CGMC) were used as a control group. The protein level of GAS6 was evaluated by Western blot for...
Generation of GAS6 knockout mice

The CRISPR-Cas9 technique was used to generate GAS6 knockout mice. First, the online CRISPR design tool (http://crispr.mit.edu) was utilized to predict the guide sequences of the target site for the mouse GAS6 gene (Figure 4A). Then, two pairs of oligomers: oligomers 1 (oligo1: TAGGAGCACACAGGTCAATAC and oligo2: AAACGTATTGACCTTGTGCT) oligomers 2 (oligo1: TAGGGTAGAAACGTATCCCTTGAGT and oligo2: AAACACTCAAGGATACGCTTTCTAC) were annealed and cloned into the pUC57-sgRNA expression vector (Addgene 51132). Primers spanning the T7 promoter and sgRNA sequences (Forward primer: GATCCCTAATACGACTCACTATAG Reverse primer: AAAAAAGGCACCGACTCGGT) was used to amplify the DNA by PCR. Subsequently, sgRNA was transcribed and purified using the MEGAscript Kit (Ambion, AM1354) and miRNeasy Micro Kit (QiaGen, 217084), respectively. The Cas9 plasmid (Addgene 44758) was transcribed in vitro using the T7 Ultra Kit (Ambion, Am1345). Purification of mRNA by the RNeasy Mini Kit (QiaGen, 74104) was performed following the manufacturer’s instructions. Cas9 and sgRNA mRNA were injected to one-cell embryos using the FemtoJet 5247 microinjection system. Mouse tail genomic DNA was extracted by phenol-chloroform and alcohol precipitation to identify the F0 offspring (Figure 4B). PCR analysis was performed with the primers: GAS6-F (5’-GAAGCAGGCACAGCTTATCC-3’) and GAS6-R (5’-TTCCCCTCATGAATCCACTC-3’). A 2255-bp DNA fragment overlaps the sgRNA target site was amplified. Purified PCR product was analyzed by 3.0% agarose gel. The wild type allele contained a 2255bp amplicon and the mutant allele contained a 630bp amplicon (Figure 4D). Finally, the protein level of GAS6 was analyzed by Western blot (Figure 4E).

Animal Surgery

The aortic banding (AB) operation has been previously described elsewhere1-3. In brief, 8-10 week old male mice (body weight ranging from 24-27 g) were operated on using an AB procedure. Before the operation, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Sigma-Aldrich). Then, the left chest of each mouse was opened along the second and third intercostal space to identify and dissect the thoracic aorta carefully. A 26/27-gauge needle was ligated together with the descending aorta utilizing 7-0 silk sutures for approximately 70% aortic constriction. After aorta ligation, the needle was removed swiftly and then the thoracic cavity was closed. Successful constriction of the aorta was identified and confirmed by Doppler echocardiogram. Sham-operated animals underwent the same surgical procedure without partial aorta ligation. At the indicated time points (4 and 8 weeks after AB or sham operation), the mice were euthanized and the hearts, lungs, and tibia were
collected and weighed. The ratios of heart weight (HW)/body weight (BW) (mg/g), HW/tibia length (TL) (mg/mm), and lung weight (LW)/BW (mg/g) were measured.

**In vivo inhibition experiment**
A MEK1/2 inhibitor, U0126 (#9903, Cell Signaling Technology, Beverly, MA) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 0.1 mg/ml. Then, the U0126 suspension was administered at a constant volume of 1 ml per 100 g of body weight by intraperitoneal injection once every 3 days (1 mg/kg/3 days). The same volume of DMSO mixed with PBS was injected into the abdominal cavity in the control group.

**Echocardiography**
Echocardiography was performed with a MyLab 30CV ultrasound (Biosound Esaote Inc.) with a 10-MHz linear array ultrasound transducer. After anesthetization, the mouse left ventricle (LV) was assessed in both a parasternal short-axis and a long-axis view at a frame rate of 120 Hz. The end-systole and end-diastole were defined as the phase in which the smallest or largest LV area was obtained, respectively. LV end-systolic diameter (LVESd) and LV end-diastolic diameter (LVEDd) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

**Histological Analysis**
Hearts were excised, then instantly placed in a 10% potassium chloride solution to ensure that they were arrested in diastole. Then, the mouse hearts were fixed with 10% formalin, dehydrated, and embedded in paraffin. Subsequently, the hearts were sectioned transversely close to the apex to visualize the left and right ventricles at 5 µm. Several sections of each heart were prepared and stained with hematoxylin–eosin (HE) or with picrosirius red (PSR) following standard procedures to evaluate the cross-sectional area or collagen deposition, respectively, visualized by light microscopy. To further confirm the cell size observed in the HE stain, heart sections were stained with FITC-conjugated wheat germ agglutinin (WGA, Invitrogen Corp) to visualize the cell membrane and with DAPI to observe the nuclei. After the HE and PSR staining, more than 100 LV cardiomyocytes’ cross-sectional areas and more than 25 fields were measured in each group, respectively. All of the images were measured with a quantitative digital image analysis system (Image-Pro Plus 6.0).

**Neonatal rat Cardiomyocyte (NRCM) culture and infection with recombinant adenoviral vectors**

**Cell culture and infection**
NRCMs were prepared followed methods that have been previously described1-3. In brief, 1- to 2-day old Sprague-Dawley rats were euthanized by swift decapitation according to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Then, the hearts were excised and extracted. Subsequently, the hearts were minced and digested with PBS containing 0.03% trypsin and 0.04% collagenase type II. The NRCMs were then seeded at a density of 1×10^6 cells/well in six-well culture plates coated with gelatin. The nutrient medium consisted of DMEM/F12 medium supplemented with 20% fetal calf serum, BrdU (0.1 mmol/L, to inhibit the proliferation of cardiac fibroblast), and
penicillin/streptomycin. After 48 h, the culture medium was replaced with serum-free DMEM/F12 for 12 h to synchronize the NRCMs. Finally, the NRCMs were treated with PBS and angiotensin II (Ang II, 1 μmol/L) for 24 and 48 h to induce cardiomyocyte hypertrophy.

To overexpress GAS6, the rat GAS6 gene under the control of the cytomegalovirus promoter was inserted into replication-defective adenoviral vectors. A similar adenoviral vector encoding the GFP gene was used as a control. To reduce GAS6 expression, rat shGAS6 constructs were used to inhibit the expression of GAS6. AdshRNA was used as a control for AdshGAS6. The NRCMs were infected with AdGFP, AdGAS6, AdshRNA and AdshGAS6 in diluted media at a multiplicity of infection of 100 for 24 h.

**Immunofluorescence**

After the NRCMs were infected with adenovirus and treated with PBS or Ang II (1 μmol/L) for 48 h, the cardiomyocytes were fixed with 100% methanol for 20 minutes at room temperature to quench the GFP signal, and then NRCMs were permeabilized with 0.1% Triton X-100 in PBS for 40 minutes and stained with α-actinin (Sigma-Aldrich, A7811, 1:100 dilution) following standard immunofluorescence staining procedures. The NRCMs were visualized using a fluorescence microscope (Olympus, Tokyo, Japan) and measured with a quantitative digital image analysis system (Image-Pro Plus 6.0). More than 50 cells were visualized in each experimental group.

**Quantitative real-time RT-PCR**

Total mRNA was extract from mice ventricular tissue or NRCMs by TRIzol (15596-026, Invitrogen). cDNA was cloned from oligo (dT) primers using reverse transcription polymerase chain reaction (RT-PCR) by the Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche, Basel, Switzerland). Selected gene expressions were tested thorough quantitative real-time PCR using SYBR Green (04887352001, Roche); the results were normalized as ratio of target gene/GAPDH gene expression.

**Western blotting**

Total proteins were extracted from ventricular tissue and NRCMs in lysis buffer (720ul RIPA, 20ul PMSF, 100ul Complete, 100ul Phos-stop, 50ul NaF, 10ul Na3VO4 in 1 ml lysis buffer); the protein concentrations were measured using the Pierce® BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA). Twenty micrograms of protein were separated by SDS-PAGE (NP0301BOX, Invitrogen, Carlsbad, CA, USA) for each sample and electrically transferred to PVDF membranes (IPVH00010, Millipore Corporation). The PVDF membranes were subsequently blocked in TBST containing 5% skimmed milk powder for 90 minutes at room temperature and incubated with different primary antibodies overnight at 4°C. The next day, the membranes were incubated with secondary antibodies, and the signals were visualized with a FluorChem E imager (Cell Biosciences, Santa Clara, CA, USA). Each protein expression level was normalized as ratio of target protein/GAPDH that had been transferred to the same PVDF membrane.

**Statistical Analysis**
Normality was tested with the Shapiro–Wilk test. The data are presented as the mean ± standard deviation (SD) as all the groups were confirmed to be normal distribution. Comparisons between two groups were performed using a two-tailed Student's t-test. Differences among more than 2 groups were assessed using one-way analysis of variance (ANOVA) followed by Bonferroni test (assuming equal variances) or Tamhane’s T2 test (without the assumption of equal variances). A value of P<0.05 was considered to indicate a statistically significant difference.

References:
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Supplemental Figures

S1

Schematic diagram of the construction of cardiac specific-GAS6 overexpression mice

**Left,** Schematic construction of cardiac-specific GAS6 transgenic mice **Right top,** Western blot analysis to identify the expression of GAS6 in CAG-CAT-GAS6/MHC-Cre mice without tamoxifen administration (CGMC) and GAS6-transgenic (TG) mice (n=4 per experimental group). **Right bottom,** Bar graph of quantitative results of the Western blot.

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S2

**A**

3'...tttttaaggtgtgCATAGCTGAAACACAGGGtttttt...5'  
5'...tttttcaggaAGTAGAAAGGTCTTGGAGTTggctccac...3'

**B**

[Image of a gel with bands indicating DNA fragments of different sizes.]

**C**

5'...agaattcacgattgaccttgtgtgacctc...3'  
3'...tttttaaggtgtgCATAGCTGAAACACAGGG...5'  
#2-5 ggattgatggatatcct...ctag...  
#2-2 5'...agaattcacgattgaccttgtgtgacctc...3'

**D**

[Image of a gel with bands indicating DNA fragments of different sizes.]

**E**

[Image of a gel showing protein bands for WT and GAS6 KO.]
S2 Schematic diagram of the construction of GAS6 knockout mice using CRISPR-Cas9 techniques

A, Two single guiding RNA (sgRNA) chains were designed and constructed in the upstream region of the GAS6 gene exon 1 and downstream of exon 2. B, PCR results indicated that two out of the five mice contained cleavage products after microinjection. C, The mutant mice labeled #2-2 and #2-5 were sequenced, both the #2-2 and the #2-5 mice had a deletion of exon 1 and exon 2. D, F1 generation was obtained by mating founder #2-5 mice with a C57BL/6J background mouse. Then, heterozygous F1 offspring were interbred to establish the GAS6-KO mouse. E, Western blot analysis of GAS6 expression in the heart in both wild type and knockout mice (n=4 per experimental group).