Exacerbating Pressure Overload–Induced Cardiac Hypertrophy

Novel Role of Adaptor Molecule Src Homology 2-B3

Xuehai Zhu, Jing Fang, Ding-Sheng Jiang, Peng Zhang, Guang-Nian Zhao, Xueyong Zhu, Ling Yang, Xiang Wei, Hongliang Li

Abstract—The adaptor protein Src homology 2-B3 (SH2B3), which belongs to a subfamily of Src homology 2 proteins, is a broad inhibitor of growth factors and cytokine signaling in hematopoietic cells. However, the role of SH2B3 in nonhematopoietic systems, particularly cardiomyocytes, has not been defined. In this study, we observed noticeable increase in SH2B3 protein expression during pathological cardiac remodeling in both humans and rodents. Follow-up in vitro gain- and loss-of-function studies suggested that SH2B3 promotes the cardiomyocyte hypertrophy response. Consistent with the cell phenotype, SH2B3 knockout (SH2B3−/−) mice exhibited attenuated cardiac remodeling with preserved cardiac function after chronic pressure overload. Conversely, cardiac-specific SH2B3 overexpression aggravated pressure overload–triggered cardiac hypertrophy, fibrosis, and dysfunction. Mechanistically, SH2B3 accelerates and exacerbates cardiac remodeling through the activation of focal adhesion kinase, which, in turn, activates the prohypertrophic downstream phosphoinositide 3-kinase-AKT-mammalian target of rapamycin/glycogen synthase kinase 3β signaling pathway. Finally, we generated a novel SH2B3 knockout rat line and further confirmed the protective effects of SH2B3 deficiency on cardiac remodeling across species. Collectively, our data indicate that SH2B3 functions as a novel and effective modulator of cardiac remodeling and failure. (Hypertension. 2015;66:571-581. DOI: 10.1161/HYPERTENSIONAHA.115.05183.) ● Online Data Supplement

Key Words: aortic banding ■ cardiomegaly ■ knockout rats ■ LNK protein, mouse ■ TALEN

Pathological cardiac remodeling is the process by which a compensatory response is transformed to a maladaptive response on reacting to mechanical load (eg, valvular regurgitation or stenosis) or humoral factors (eg, catecholamine) and is the strongest predictor of heart failure, arrhythmia, and sudden death. In the early stage of this pathological process, to enhance cardiac pump function, the heart undergoes hypertrophic growth via the enlargement of individual myocytes without effects on cardiac function. However, continued insult would ultimately leads to cardiac chamber enlargement and fibrosis with perturbed contractile performance. In addition to histological and functional deterioration, cardiac remodeling is a composite of cellular and molecular changes, including protein synthesis, sarcomeric organization, fibrosis, cell death, and energy metabolism. Although the pathogenesis and development of pathological hypertrophy have been studied extensively, the complete molecular network has not been sufficiently dissected. Moreover, we have recently demonstrated that a number of protein molecules traditionally regarded as immunologic molecules exert key regulatory effects on this process. Given the prominent and omnipresent role of the immune network in cell biology, the identification of new immune regulators in cardiac hypertrophy will provide additional insights into the mechanism and treatment of this life-threatening disease.

Since its discovery, adaptor protein Src homology 2-B3 (SH2B3) has been highlighted as a nonredundant negative modulator in hematopoietic and B-cell development. Adaptor proteins, which function as molecular scaffolds to orchestrate cellular signals, possess multiple functions, including organization and control of protein translocation, mediation of protein–protein interactions, and recruitment of required enzyme substrates. Based on structural similarities, SH2B3 (also referred to as Lnk) belongs to the SH2B family...
of adaptor proteins, which also contains SH2B1 and SH2B2 (APS). Full-length SH2B comprises 5 structurally important domains: the carboxyl-terminal SH2 domain, which is responsible for binding to the phosphotyrosine residue; the pleckstrin homology domain, which is essential for the recognition of phosphoinositides and the translocation of SH2B3 to the cell membrane; a dimerization domain, which mediates homodimerization and heterodimerization between members of the SH2B family; a proline-rich region and a conserved C-terminal CBL recognition motif. SH2B3 functions as an adaptor molecule downstream of various tyrosine kinases and lacks enzymatic activity. SH2B3 also exerts pleiotropic effects on cardiovascular diseases independent of its immune activities. Recent genome-wide association studies have indicated that variants of SH2B3 single-nucleotide mutations are associated with increased susceptibility to hypertension, myocardial infarction, and vascular disease, including retinal vessels and peripheral arteries. However, the regulatory effects and potential mechanisms of action of SH2B3 in these diseases are largely unknown. These clinical discoveries motivated us to investigate the potential role of SH2B3 in cardiovascular systems.

Herein, we observed a striking increase in SH2B3 expression in human samples of cardiac remodeling. The same phenomenon was observed in a mouse model of pressure overload–induced hypertrophy, which permitted us to investigate the potential role of SH2B3 in cardiac remodeling. The prohypertrophic effect of SH2B3 was first examined in in vitro cell studies. Consistent with the cell investigations, aortic banding (AB)–induced hypertrophy was greatly ameliorated by SH2B3 inactivation in SH2B3 knockout (SH2B3−/−) mice. By contrast, gain-of-function experiments in which SH2B3 overexpression was targeted to the hearts exaggerated cardiac remodeling. Thus, we have identified a causal role of SH2B3 in pathological hypertrophy. The responsible downstream signaling cascade of SH2B3 is not to the mitogen-activated protein kinase (MAPK) pathway, as typically observed for the immune system, but rather the focal adhesion kinase (FAK)-phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR)/glycogen synthase kinase 3β (GSK3β) signaling pathway. In addition, a novel SH2B3−/− rat strain was successfully generated using a transcription activator–like effector nuclease (TALEN) approach and was applied to further ascertain the requirement of SH2B3 in cardiac remodeling.

Materials and Methods

All animal experiments were conducted in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology. All experimental procedures involving human samples were approved by the Human Research Ethics Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology, and written informed consent was obtained before heart sample collection. A expanded Methods section is available in the online-only Data Supplement, which includes detailed methods of the following: reagents, human heart samples, mice used in experiments, generation of SH2B3 knockout rats, animal models of pressure overload–induced cardiac remodeling, echocardiography and hemodynamic measurements, histological analysis, primary cardiomyocyte culture, recombinant adenoviral infection and immunofluorescence, quantitative real-time polymerase chain reaction, western blot analysis, and statistical analysis.

Results

Upregulation of SH2B3 Protein Expression in Failing Hearts Suggests a Role in Cardiac Remodeling

The potential role of SH2B3 in cardiac hypertrophy was implicated from failing hearts explanted from patients undergoing heart transplantation. As shown in Figure 1A to 1D, compared with normal donor heart, the abundance of SH2B3 were dramatically elevated in the left ventricles (LVs) of both dilated cardiomyopathy and hypertrophic cardiomyopathy, along with increases in the hypertrophic markers atrial natriuretic peptide and β-myosin heavy chain. The results of the animal experiments were consistent with these findings. At 4 weeks after the C57BL/6 mice underwent AB surgery, the expression of SH2B3 was increased, accompanied by increased expression of hypertrophic markers (Figure 1E–1G). However, SH2B3 expression levels at 8 weeks after surgery, although still above basal levels, were slightly decreased compared with 4 weeks after surgery (Figure 1E and 1F). Immunohistochemical staining of SH2B3 on heart tissue sections demonstrated that most of SH2B3 were localized in the cytoplasm of cardiomyocytes (Figure 1G). Collectively, these results raise the possibility that SH2B3 influence cardiac remodeling.

SH2B3 Is a Potent Regulator in Angiotensin-II–Induced Cardiomyocyte Hypertrophy In Vitro

To further consolidate our hypothesis, we performed gain- and loss-of-function assays on neonatal rat cardiomyocytes, a well-controlled in vitro experimental system. Increasing and decreasing SH2B3 expression levels of neonatal rat cardiomyocytes were approached by adenoviral SH2B3 and adenoviral shSH2B3 transfection, respectively (Figure 2A and 2B). Hypertrophy was subsequently induced in these cells via angiotensin-II stimulation for 48 hours. In the control adenoviral green fluorescent protein– and nonsense adenoviral shRNA–transfected groups, cardiomyocytes exhibited increased individual cell size (Figure 2C–2E) and mRNA levels of hypertrophic markers (Figure 2F and 2G) after stress. However, the hypertrophic response was blocked in adenoviral shSH2B3–treated cells (Figure 2C, 2D, and 2F). In sharp contrast, increasing SH2B3 expression robustly promoted cardiomyocyte hypertrophy (Figure 2C, 2E, and 2G). These results indicate that SH2B3 is indispensable for the promotion of cardiomyocyte hypertrophy in vitro.

Loss of SH2B3 Exerts a Strong Protective Effect Against AB-Induced Cardiac Hypertrophy In Vivo

We then extended the aforementioned explorations to the studies of SH2B3 knockout (SH2B3−/−) mice. The loss of SH2B3 expression in SH2B3−/− mice was confirmed by western blot analysis of heart lysates (Figure S1A in the online-only Data Supplement). These mice were subsequently challenged with AB surgery, and examinations were performed 4 weeks later.
Hemodynamic analysis showed that AB induced significant and comparable blood pressure elevation in SH2B3+/+ and SH2B3−/− mice (Figure S1D). The increment in heart size was first estimated by echocardiography in live mice. Under isoflurane anesthesia, no difference in heart rates was observed between groups (Figure S1B). The LV end-systolic diameter and LV end-diastolic diameter, which reflect heart chamber dimensions in vivo, increased more pronouncedly after 4 weeks of pressure overload in the control mice (Figure 3A and 3B). Importantly, cardiac contractive function (reflected by the ejection fraction % and fractional shortening %) deterioration was alleviated in SH2B3−/− mice (Figure 3C). Consistently, hemodynamic comparison conducted by cardiac catheterization revealed that both systolic function (assessed by ejection fraction % and maximal dP/dt; Figure S1E and S1F) and diastolic function (assessed by minimal dP/dt; Figure S1G) were largely preserved in SH2B3−/− mice.

The ameliorated hypertrophic response in SH2B3−/− mice was further confirmed by morphological examinations. Macroscopically, SH2B3 inactivation greatly alleviated the hypertrophic response, as evidenced by smaller whole-heart transversal sectional area (Figure 3D, top). Microscopically, individual cardiomyocyte hypertrophy was blunted after SH2B3 deficiency (Figure 3D, middle and bottom) as assessed by decrease in the cardiomyocyte cross-sectional area (Figure 3E). The hypertrophic phenotype was also supported by tibia length and body weight–corrected heart mass measurement (Figure 3F and 3G). The ratio of lung weight/body weight, which indicates the development of pulmonary congestion, was also decreased after SH2B3 inactivation (Figure 3H). Fibrosis, another characteristic of pathological hypertrophy, was greatly mitigated in SH2B3−/− mice as displayed by less perivascular and interstitial collagen deposition (Figure 3I and 3J). It should be noted that the loss of SH2B3 had no effect on nonstressed hearts.
Cardiac-Specific SH2B3 Overexpression Potentiates the Progression of Pressure Overload–Induced Cardiac Hypertrophy

We then asked whether SH2B3 overexpression may promote cardiomyocyte hypertrophy in vivo. We selectively overexpressed SH2B3 in cardiac myocytes under the control of the α-myosin heavy chain promoter. Four founders were established that exhibited 1.4- to 3.7-fold SH2B3 overexpression relative to the wild-type control (Figure S2A and S2B). The most robust overexpression founder, which has 3.7-fold SH2B3 expression relative to control, was used for the in vivo gain-of-function experiments. This strain was fertile and exhibited no apparent abnormalities under basal conditions.

Concordant with our hypothesis, cardiac-targeted SH2B3 overexpression resulted in a disastrous hypertrophic response to pressure overload for 4 weeks. The exacerbated LV dilation was first identified by echocardiographic examinations, which exhibited increased LV end-diastolic diameter and LV end-systolic diameter under similar heart rates (Figure 4A and 4B; Figure S2C). Both echocardiographic and hemodynamic analyses indicated that the enlarged heart chambers were accompanied by more severe impairments of cardiac function in SH2B3 transgenic mice (Figure 4C; Figure S2D–S2F). However, there was an increase in dP/dT (maximum) and dP/dT (minimum) (Figure S2G and S2H). These results were caused by increased end-diastolic volume and end-systolic volume (Figure S2I and S2J). In agreement with the functional conclusions, the morphological examinations revealed that both the whole-heart enlargement and individual myocyte hypertrophy were more pronounced in SH2B3 transgenic mice (Figure 4D and 4E). Similarly, SH2B3 overexpression dramatically increased both heart and lung weight after pressure overload (Figure 4F–4H). Compared with the picrosirius red staining slices of the control mice, the LVs of the
SH2B3 transgenic mice exhibited devastating fibrosis and a 3-fold increase in the LV collagen volume (Figure 4I and 4J). Collectively, these in vivo experiments indicate that SH2B3 overexpression rendered the heart more susceptible to maladaptive cardiac hypertrophy.

Alternations in the FAK-PI3K-AKT-mTOR/GSK3β Signaling Pathway Underlie the SH2B3-Mediated Hypertrophic Response In Vivo and In Vitro

Copious evidence have proved that the MAPK signaling pathways play a fundamental role in the development of pathological cardiac hypertrophy. Moreover, extracellular signal–regulated kinase 1/2 (ERK1/2) signaling is also regulated by SH2B3 in hematopoietic cells. Thus, we tested whether the regulatory effect on ERK1/2 signaling might underlie the mechanism of SH2B3 in promoting cardiac hypertrophy. In agreement with previous studies, ERK1/2 phosphorylation was substantially increased in hypertrophic hearts (Figure S3A and S3B). However, the activation of neither ERK1/2 nor the upstream MAPK kinase 1/2 was affected by SH2B3 expression levels in different mouse strains (Figure S3A and S3B). We also compared the other 2 branches of the MAPK superfamily, namely, the c-Jun N-terminal kinase and p38, which have also been implicated in SH2B3-regulated cell biology. As displayed in Figure S3A and S3B, although both pathways were activated in hypertrophy, we did not observe perceptible difference among the SH2B3−/− mice, the SH2B3 transgenic mice, and their controls. Our findings unexpectedly demonstrated that the involvement of SH2B3 in cardiac remodeling was independent of the MAPK pathway, which suggests that additional mechanisms may be responsible for SH2B3-regulated pathological hypertrophic responses.

To gain broader insight into SH2B3 and AKT signaling, we tracked several classic activators upstream of AKT. Persad et al. demonstrated that the serine kinase integrin-linked kinase (ILK) could directly phosphorylate AKT on serine 343. However, ILK was similarly activated in all mouse strains after pressure overload (Figure 6A–6D). PI3K, a protein kinase and lipid enzyme, has long been recognized as another AKT upstream signal. Indeed, the phosphorylation of the key p85 subunit in PI3K was decreased in SH2B3 knockout hearts and, conversely, increased in SH2B3-overexpressing hearts (Figure S3A and S3B). However, it remains to be decided the specific kinase that catalyzes the activation of PI3K, because SH2B3 have no enzymatic activities. Among the known candidates that may directly activate PI3K, the FAK was recently identified as a prohypertrophic signal transducer. In fact,
the activation state of FAK was greatly influenced by SH2B3 expression levels. As shown in Figure 6A and 6B, FAK phosphorylation is reduced in SH2B3−/− heart tissues after stress. By contrast, SH2B3 overexpression leads to increased pressure overload–induced FAK activation (Figure 6C and 6D).

These observations suggest that SH2B3 may act through FAK to exert its effect. To further substantiate these findings, we confirmed the in vivo results in ex vivo cardiomyocytes. Neonatal rat cardiomyocytes were infected with the indicated adenovirus as described above to artificially set the SH2B3 expression levels. After stressed with angiotensin-II for 60 minutes, FAK phosphorylation was robustly elevated in control adenoviral shRNA– and adenoviral green fluorescent protein–infected cells (Figure 6E–6H). Consistent with the in vivo studies, SH2B3 reduction by adenoviral shSH2B3 greatly abrogated angiotensin-II–induced FAK activation (Figure 6E and 6F). By contrast, SH2B3 gain-of-function promoted angiotensin-II–induced FAK activation (Figure 6G and 6H). Moreover, activation of downstream AKT, mTOR, and GSK3β varied depending on altered SH2B3 expression and FAK activation (Figure 6E–6H). In conclusion, the FAK-PI3K-AKT-mTOR/GSK3β axis is a key target of SH2B3 in defining prohypertrophic activity.

Cardiac Hypertrophy Is Blunted in SH2B3 Knockout Rats After Pressure Overload

Nearly the same hypertrophic phenotype occurred in the rat cardiomyocytes, which prompted us to investigate whether the regulatory effects of SH2B3 were generic across species. Indeed, in wild-type Sprague-Dawley rats challenged with abdominal AB surgery, SH2B3 expression was increased in LVs (Figure 7A and 7B), consistent with observations in humans and mice. We subsequently generated SH2B3 knockout rats using the TALEN technique as noted in the methods. A TALEN pair was designed to target SH2B3 exon1 as outlined in Figure S4A. Then, the in vitro transcribed TALEN SH2B3 mRNAs were injected into fertilized rat oocytes to splice SH2B3. Three founders of 8 live births with cleavage products were chosen and sequenced to verify the precise mutation of the indels (Figure S4B and S4C). Afterward, founder numbers 4 to 8 were selected for sibling mating into homozygosity. The presence of mutant and wild-type alleles was verified by polymerase chain reaction analysis as illustrated in Figure S4D. The absence of intact protein in the presence of the SH2B3 null allele was ascertained by western blot (Figure S4E). The homozygous SH2B3−/− rats were viable, fertile, normal in size, and exhibited no phenotypic abnormalities.

These rats were subsequently stressed with pressure overload by AB surgery for 4 weeks. As illustrated in Figures 7C–7E and S4G, echocardiographic examinations revealed that SH2B3 inactivation ameliorated heart chamber dilation accompanied by preserved cardiac function in live rats. The heart and lung mass increments were all reduced in SH2B3−/− rats (Figure 7F–7H). Morphologically, pressure overload–triggered cardiomyocyte enlargement, and, perivascular and interstitial fibrosis were all attenuated by SH2B3 deficiency (Figure 7I–7K). At the molecule level, SH2B3 deficiency
offset the AB-mediated stimulation of FAK and downstream AKT-mTOR/GSK3β activation (Figure 7L and 7M), which also occurred in mice and ex vivo cells. Collectively, SH2B3 knockout in rats attenuated cardiac hypertrophy via a mechanism similar to that in mice.

**Discussion**

SH2B3 was initially cloned from a rat lymph node cDNA library and recognized as an essential transducer in T-cell receptor signaling. Since its discovery, the highest recognition of SH2B3 is its functional significance in immune cells and hematopoietic homeostasis. SH2B3 is essential for normal peripheral B-cell expansion and bone marrow progenitor cell accumulation of both erythroid and megakaryocyte lineage. Molecule studies have revealed that SH2B3 functions as a negative regulator in multiple cytokine signals, including stem cell factor, thrombopoietin, erythropoietin, and macrophage colony–stimulating factor. SH2B3 also promotes self-renewal in hematopoietic progenitor cells. However, the role of SH2B3 in nonhematopoietic systems, particularly heart biology, remains poorly investigated. The implication of SH2B3 in hypertrophic biology was inspired by human samples. Although minimally expressed under basal conditions, SH2B3 expression is strikingly elevated in human failing hearts and mouse and rat hypertrophic hearts. Gain- and loss-of-function assays in neonatal rat cardiomyocytes suggested a causal role of SH2B3 in promoting myocyte hypertrophy. Consistent with the cell culture studies, animal studies demonstrated that SH2B3 inactivation blunted cardiac hypertrophy, whereas forced SH2B3 expression predisposed the heart vulnerable to hypertrophy. The altered hypertrophic response was not solely demonstrated by heart size but also supported by fibrosis and cardiac function analysis. It should be noted that under basal conditions, neither SH2B3 knockout nor SH2B3 overexpression affected cardiac structure and function. Thus, using genetic approaches, we identified SH2B3 as a novel regulator in the process of pressure overload–induced hypertrophy and as a stress sensor only under stressed conditions will SH2B3 take effect.

In an effort to better understand the molecular mechanisms by which SH2B3 functions, we first examined the MAPK signaling pathway. Various prohypertrophic stimuli function via the activation of ERKs, c-Jun N-terminal kinases, and p38, which trigger numerous intracellular targets and, in turn, launch the hypertrophy process. In terms of ERK1/2, studies performed in different systems have yielded contradictory conclusions: SH2B3 is a negative modulator of the ERK1/2 signaling pathway downstream of c-kit, EpoR, c-Mpl, and FLT3 on engagement with their specific ligands. Whereas in macrophages, SH2B3 deficiency diminishes ERK1/2 activation when stimulated with M-CSF. Moreover, there are evidences that p38 and c-Jun N-terminal kinase are also involved in the signaling downstream of SH2B3. Nonetheless, we obtained no evidence for differences in all 3 branches of MAPK activation among SH2B3−/− mice, transgenic mice, and wild-type mice. This inconformity might be explained by the fact that SH2B3 plays specific roles via binding to diverse partners to activate or suppress downstream signaling pathways in cell type–or stimulation-specific manners.

We then demonstrated that the altered hypertrophic phenotype affected by SH2B3 expression is attributable to AKT

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**Figure 5.** AKT signaling acts downstream of Src homology 2-B3 (SH2B3). A–D, At 4 weeks after aortic banding (AB), the phosphorylated and total protein levels of AKT, mammalian target of rapamycin (mTOR), and glycogen synthase kinase 3β (GSK3β) in SH2B3 knockout (SH2B3−/−), wild-type (SH2B3+/+), SH2B3 transgenic (TG) and nontransgenic (NTG) groups were compared by western blot analysis. The statistical results of 6 hearts per group are shown in (B; *P<0.05 vs the SH2B3+/+ sham group; #P<0.05 vs the SH2B3+/+ AB [4W] group) and (D; *P<0.05 vs the SH2B3 NTG sham group; #P<0.05 vs the SH2B3 NTG AB [4W] group).
Figure 6. Focal adhesion kinase (FAK) acts upstream of AKT and is responsible for Src homology 2-B3 (SH2B3)–mediated cardiac hypertrophy. **A–D.** At 4 weeks after aortic banding (AB) surgery, the phosphorylation of integrin-linked kinase (ILK), phosphoinositide 3-kinase (PI3K) and FAK and their basal volumes in the SH2B3 knockout (SH2B3−/−), wild-type (SH2B3+/+), SH2B3 transgenic (TG), and nontransgenic (NTG) groups were examined by western blot analysis. Representative results are shown in (**A** and **C**). The statistical results for 6 hearts per group are shown in (**B**; *P<0.05 vs the SH2B3+/+ sham group; #P<0.05 vs the SH2B3+/+ AB [4W] group) and (**D**; *P<0.05 vs the SH2B3 NTG sham group; #P<0.05 vs the SH2B3 NTG AB [4W] group). **E–H.** Neonatal rat cardiomyocytes were infected with adenoviral short hairpin SH2B3 (AdshSH2B3) or adenoviral short hairpin RNA (AdshRNA; **E**), adenoviral SH2B3 (AdSH2B3) or adenoviral green fluorescent protein (AdGFP; **G**) for 24 hours. After the addition of Ang-II (1 μmol/L) or PBS for 60 minutes, the cell lysates were assayed for phosphorylated and total levels of FAK, AKT, the mammalian target of rapamycin (mTOR), and glycogen synthase kinase 3 beta (GSK3β) via western blot. The quantitative results for 6 samples per group are shown in (**F**; *P<0.05 vs the AdshRNA PBS group; #P<0.05 vs the AdshRNA Ang-II group) and (**H**; *P<0.05 versus the AdGFP PBS group; #P<0.05 versus the SH2B3 AdGFP Ang-II group).
signaling. Acting at the intersection of multiple influent and efferent signals, AKT influences nearly every aspect of cardiac biology. AKT is a serine/threonine protein kinase; following activation, it phosphorylates GSK3β and mTOR and, in turn, initiates the hypertrophic process. AKT phosphorylation at Thr308 generates its active form. However, phosphorylation of Ser473 is required for its full activation. The ILK molecule is an essential hub that propagates hypertrophic signaling at the cytoplasmic tails of integrins. As the name implies, initial studies indicated that ILK possesses serine/threonine kinase activity and directly phosphorylates AKT at Ser473 to complete its full activation. Moreover, ILK also possesses kinase activity to phosphorylate GSK3β and inhibit its activity. A recent report by Devallière et al demonstrated that ILK is a molecular partner of SH2B3 in the regulation of AKT and GSK3β phosphorylation; thus, it is our first choice to select ILK as a candidate partner in SH2B3-mediated hypertrophic response. However, in our study, despite the increased phosphorylation of AKT and GSK3β in transgenic mice after hypertrophy, ILK phosphorylation was unaltered and vice versa in SH2B3 KO mice. This finding concordant with the results of studies demonstrating that phosphorylation of AKT and GSK3β is separable from increased ILK levels in the failing hearts. This is also supported by recent comments suggesting that ILK might function as an adaptor rather than a kinase in integrin signaling.

In addition to ILK, AKT could also be directly activated by phosphoinositide-dependent kinase, which is the downstream effector of PI3K. FAK is a nonreceptor tyrosine kinase and plays a major role in the mediation of signaling initiated from integrins and growth and hormonal factors. When phosphorylated, FAK directly binds to and phosphorylates the p85 subunit of PI3K. Emerging data have revealed a critical role of FAK in the cardiac hypertrophic response. However,
previous studies from different groups have provided conflicting conclusions using cardiac-specific FAK null mice.24 Most recently, Clemente et al38 using cardiac-specific FAK transgenic mice demonstrated that increased FAK activity causes cardiac hypertrophy under basal conditions and augments hypertrophy in response to pressure overload. Importantly, the increased FAK activity selectively upregulated the PI3K/ AKT/mTOR pathway but not the Src/ERK1/2 pathway.39 These results are consistent with our conclusions.

Although FAK is a major mediator of integrin signaling, we did not obtain evidence that SH2B3 regulates the FAK in the integrin cascade. However, previous studies have suggested a role for SH2B3 as an important regulator in integrin signaling. Takizawa et al demonstrated that SH2B3 was important for the regulation of VCAM-1–initiated integrin signaling in hematopoietic stem cells. In platelets, SH2B3 is an essential contributor to the out-in signaling of integrin αIIbβ3. Specifically in endothelial cells, SH2B3 promotes the phosphorylation of FAK and paxillin in the integrin signaling, which subsequently impairs FA disassembly and decreases endothelial migration. Based on these findings, we postulate that SH2B3 may function in integrin signaling to regulate cardiomyocyte hypertrophy.

Perspectives

A better understanding of the molecular network that underlies cardiac hypertrophic growth will provide new possibilities for the clinical management of heart failure. Although SH2B3 has long been recognized as a prototypical immunologic molecule, studies on the role of SH2B3 in cardiovascular diseases are now emerging. The unanticipated role of SH2B3 in hypertrophy identified here will provide further knowledge of the regulation of nonimmune cell activities by immunologic molecules. Our results suggest that SH2B3 will be a promising therapeutic target in drug investigations for the treatment of heart failure progression.
Novelty and Significance

What Is New?

- Src homology 2-B3 (SH2B3) is upregulated in human, mouse, and rat hearts after cardiac remodeling.
- SH2B3 promotes pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. By contrary, loss of SH2B3 exerts a strong protective effect against pressure overload–induced pathological cardiac remodeling through the inhibition of the FAK-P IKK-akt-mTOR/GSK3β pathway.

What Is Relevant?

- Knowledge of how immune network regulates cardiac remodeling has been limited.
- The role of SH2B3 in heart biology has not been addressed.
- These findings extend the regulatory network of cardiac remodeling and introduce an immune molecule to the understanding of heart failure disease.

Summary

This work demonstrates that the overexpression of the adaptor protein SH2B3 promotes pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. By contrary, loss of SH2B3 exerts a strong protective effect against pressure overload–induced pathological cardiac remodeling through the inhibition of the FAK-P IKK-akt-mTOR/GSK3β signaling cascade. The causal role of SH2B3 in cardiac remodeling suggests that the immune network controls this life-threatening process and may provide promising therapeutic targets for heart failure.
Exacerbating Pressure Overload–Induced Cardiac Hypertrophy: Novel Role of Adaptor Molecule Src Homology 2-B3
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Hypertension. 2015;66:571-581; originally published online June 22, 2015;
doi: 10.1161/HYPERTENSIONAHA.115.05183

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/66/3/571

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Exacerbating Pressure overload-Induced Cardiac Hypertrophy: the Novel Role of Adaptor Molecule SH2B3

Xuehai Zhu¹,²,³, Jing Fang¹,²,³, Ding-Sheng Jiang⁴,⁵, Peng Zhang⁴,⁵, Guang-Nian Zhao⁴,⁵, Xueyong Zhu⁴,⁵, Ling Yang⁴,⁵, Xiang Wei¹,²,³#, Hongliang Li⁴,⁵#

¹ Division of Cardiothoracic and Vascular Surgery, ²Heart-Lung Transplantation Center, ³Sino-Swiss Heart-Lung Transplantation Institute, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; ⁴Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, China; ⁵Cardiovascular Research Institute of Wuhan University, Wuhan 430060, China

Short title: SH2B3 promotes cardiac hypertrophy;

#Correspondence should be addressed to
Hongliang Li, MD, PhD
Department of Cardiology
Renmin Hospital of Wuhan University;
Cardiovascular Research Institute, Wuhan University,
Jiefang Road 238, Wuhan 430060, PR China
Tel/Fax: 86-027-6875-9302
E-mail: lihl@whu.edu.cn

Xiang Wei, MD, PhD
Division of Cardiothoracic and Vascular Surgery
Heart-Lung Transplantation Center
Sino-Swiss Heart-Lung Transplantation Institute
Tongji Hospital
Tongji Medical College
Huazhong University of Science and Technology
1095 Jiefang Ave., Whuan 430030, China
Tel/Fax: 86-027-8366-5290
Email: xiangwee@126.com
SUPPLEMENTARY MATERIALS

Methods and Materials

Reagents
Antibodies against SH2B3 (sc7222), Myh7 (sc53090) and ANP (sc20158) were purchased from Santa Cruz Biotechnology. The β-tubulin antibody (T0023) was a product of Affinity Biologics Company. Antibodies against mTOR (#2983), phospho-mTORSer2448 (#2971), phospho-AKTSer473 (#4060), AKT (#4691), GSK3β (#9315), phospho-GSK3βSer9 (#9322), ERK1/2 (#4695), phospho-ERK1/2 (#4370), MEK1/2 (#9122), phospho-MEK1/2 (#9154), JNK1/2 (#9258) phospho-JNK1/2 (#4668), p38 (#9212), phospho-p38 (#4511), PI3Kp85α (#4257), phospho-PI3Kp85α (#4228) and GAPDH (#2118) were obtained from Cell Signaling Technology. The FAK (BS3583) and phospho-FAK (BS4718) antibodies were purchased from Bioworld Technology. Antibodies against ILK (ab76468) and phospho-ILK (AP3679a) were purchased from Abcam and Abgent Company, respectively.

Human heart samples
Human heart samples of cardiac remodeling were collected from the left ventricles of dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) patients undergoing heart transplantation. Normal left ventricle tissues were obtained from brain dead donors who passed away in accidents and whose hearts were unsuitable for heart transplantation for non-cardiac reasons. The human experiments conformed to the principles of the Declaration of Helsinki. Written informed consent was obtained prior to heart sample collection, and all experimental procedures involving human samples were approved by the Human Research Ethics Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology.

Mice used in experiments
Cardiac-specific transgenic (TG) mice (C57BL/6J background) were generated by our laboratory. Briefly, full-length mouse SH2B3 cDNA was cloned downstream of the cardiac α-myosin heavy chain (α-MHC) promoter. The linearized α-MHC-SH2B3 plasmid was then microinjected into fertilized mouse embryos. The genotypes of the offspring were determined via polymerase chain reaction (PCR) analysis of tail genomic DNA. Genotyping was performed using the following primers:
forward: 5'-ATCTCCCCCATAAGAGTTGAGTC-3’;
reverse: 5'-GGTGTCGATGTCATTGGTGT-3’.
Four independent TG lines were established, and the highest cardiac-specific SH2B3-expressing line was used for subsequent experiments. SH2B3 knockout mice (SH2B3-/-, B6.129S4-Sh2b3tm1Rmp/Rbre) were purchased from RIKEN BioResource Center (RBRC00993) and backcrossed with C57BL/6J mice for 10 generations. All genetically modified mice were fertile, viable and phenotypically indistinguishable from wild-type mice. All mice were housed in a specific-pathogen-free (SPF) animal facility.
with controlled light (12 h light/dark), temperature and humidity, with food and water available ad libitum. Only male mice, aged 8 to 10 weeks with body weight of 24-27g were subjected for experiment use. All the treatments were performed in a blind fashion. All animal experiments were conducted in accordance with NIH guidelines and were approved by the Animal Care and Use Committees of Renmin Hospital of Wuhan University and Tongji Hospital of Huazhong University of Science and Technology.

Generation of SH2B3 knockout rats
A transcription activator-like effector (TALE) nuclease targeting exon1 of the Sh2b3 gene was designed using the targeter designer (https://tale-nt.cac.cornell.edu/node/add/talen-old). Repeat variable di-residue (RVD) arrays containing HD, NG, NI and NN monomers were assembled following “Unit Assembly” as described. The TALEN expression plasmids were linearized with PmeI (NEB, R0560L) and transcribed and tailed using the mMessage mMachine T7 Ultra Kit (Ambion, AM1345). Mature mRNA was subsequently purified using the RNeasy Mini Kit (Qiagen, 74104) following the manufacturer’s instructions. These purified mRNAs were mixed with injection buffer (10 mM Tris-HCl/0.1 mM EDTA, pH 7.4) to a final concentration of 10 ng/μl mRNA per TALEN arm. A 2-pl aliquot of the mixture was injected into the cytoplasm of Sprague-Dawley (SD) rat one cell-stage embryos under standard conditions. The injected zygotes were transferred into a pseudo-pregnant female rat, and viable pups were obtained.

A fragment of the SH2B3 gene spanning the TALEN target site was amplified by PCR using the following primers:
forward: 5’-ACACTGCTGTCTCCTCAGGT -3’;
reverse: 5’- TACCTTGGCTCTGCCCTGTA -3’.
The PCR products were purified and then denatured/re-annealed prior to treatment with T7 endonuclease 1, which cuts at base mismatches to highlight insertions, deletions and substitutions. The resulting fragments were subsequently separated by gel electrophoresis for analysis, and editing events were identified based on differences in the sizes of the fragments. In addition, the PCR products of the founders were cloned into the plasmid pMD-19T, and the individual plasmids were sequenced, which allowed the heterozygous and mosaic editing events to be independently analyzed. To F1 and F2, offsprings were genotyped using the following primers:
SH2B3-155-forward: 5’- ACCAGCCTCTTCCCTACAG-3’;
SH2B3-155-reverse: 5’- ATGTCAAAGGACACCCAGAG-3.
The PCR products were then resolved by 3.0% agarose gel electrophoresis. The rats homozygous for SH2B3 deficiency that weighed 200-250 g at 40 days of age were used for the experimental.

Animal models of pressure overload-induced cardiac remodeling
The pressure overload-induced cardiac remodeling mouse model was established via AB
surgery as previously described. Briefly, the mice were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Sigma-Aldrich). The left chest was then opened and a blunting dissection was performed at the second intercostal space to access the thoracic aorta. A 7-0 silk suture was introduced to ligate the descending aorta (thoracic aorta) against a 26/27-gauge needle. After ligation, the needle was quickly removed and the thoracic cavity was closed. Appropriate constriction of the aorta was examined by Doppler analysis. The sham-operated group underwent the same surgical procedure without AB.

The cardiac remodeling rat model was conducted as previously described. In brief, after anesthetization with chloral hydrate (300 mg/kg, Sigma-Aldrich), the rat was subjected to a midline abdominal incision to access the abdominal aorta, which was then banded against a 22-gauge needle using a 7-0 silk suture. The needle was removed prior to closing the abdominal cavity. Doppler flow analysis was used to confirm appropriate aortic constriction. Similar procedures without aortic constriction were carried out in sham group rats.

**Echocardiography and hemodynamic measurements**

For echocardiography and hemodynamic examinations, animals were anesthetized with 2% inhaled isoflurane. A MyLab 30CV ultrasound system (Biosound Esaote Inc.) equipped with a 15-MHz transducer was used for the echocardiographic measurements at the indicated time points, similar to previous studies. Short-axis views at the papillary muscle level and parasternal long-axis views were recorded. End-systole and end-diastole were defined as the phases in which the smallest or largest left ventricle (LV) area was obtained, respectively. The LV end-systolic diameter (LVESd), LV end-diastolic diameter (LVEDd) and LV wall thickness were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level. The LV fractional shortening (FS, %) was calculated using the formula: \((\text{LVEDd-LVESd})/\text{LVEDd} \times 100\). Echocardiographic measurements were performed in triplicate on more than 4 individual mice or rats per group.

For invasive hemodynamic analysis, a 1.4-F Millar micro-tip pressure transducer (SPR-839; Millar Instruments, Houston, Texas) was cannulated into the right carotid artery and advanced into the left ventricle. The heart rates, pressures, and volume signals were continuously recorded using a pressure-volume conductance system (MPVS-300 Signal Conditioner, Millar Instruments, Inc.) after stabilization for 15 minutes. The results were analyzed using Chart 5.0 software.

**Histological Analysis**

Immediately after echocardiography and hemodynamic examinations, animals were arrested with 1mol/L KCl, and the hearts were removed and fixed in 10% formalin, dehydrated, and paraffin-embedded. After deparaffination and re-hydration, 5 µm-thick transverse sections were prepared. The sections were stained with hematoxylin and eosin
(H&E) or picrosirius red (PSR) to visualize histopathology and collagen deposition, respectively. FITC-conjugated wheat germ agglutinin (WGA, Invitrogen) staining was performed to determine the myocyte cross-sectional area. DAPI was used to label nuclei. At least 100 circular to oval LV myocytes from 5 different mice or rats per group were traced. The myocyte cross-sectional area was measured using Image-Pro Plus (version 6.0) with captured images.

**Primary cardiomyocyte culture, recombinant adenoviral infection and immunofluorescence**

Primary neonatal rat cardiomyocytes (NRCMs) were isolated from the hearts of 1- to 2-day-old SD rat pups and cultured as previously described.\(^1\) To overexpress SH2B3, the entire coding region of the rat SH2B3 gene was subcloned into replication-defective adenoviral vectors under the control of a cytomegalovirus promoter to construct the adenoviral SH2B3 (AdSH2B3). A similar adenoviral vector encoding the GFP gene was used as a control. To downregulate SH2B3 expression, rat shSH2B3 constructs (SABiosciences, KR45310G) were cloned into adenovirus. Non-targeting AdshRNA was used as the control. All cells were infected with the indicated virus at a multiplicity of infection (MOI) of 100. After 24h of adenoviral infection, the cells were cultured in DMEM/F12 medium containing 20% FCS, BrdU (to inhibit fibroblast proliferation), and penicillin/streptomycin for 48 hours. The medium was then replaced by DMEM/F12 medium containing 1% FCS for an additional 12 h to synchronize the cardiomyocytes. Subsequently, these cells were stimulated with angiotensin II (Ang II, 1 μmol/L) or PBS in DMEM/F12 medium containing 1% FCS for 48 h.

For immunofluorescence staining, the cells were cultured on cover slips as described previously. To quench the GFP signal, the cardiomyocytes were fixed in prewarmed (37°C) 100% methanol for 20 min at room temperature. After three times wash, the cells were permeabilized in 0.1% Triton X-100 in PBS for 40 min and stained with α-actinin (Sigma-Aldrich, A7811, 1:100 dilution) using standard immunofluorescence staining techniques.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed as previously described.\(^1\) Briefly, RNA was extracted from primary cells using TRIzol reagent (Invitrogen) and used in a reverse transcriptase (RT) reaction to synthesize cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s protocol. The expression levels of ANP and Myh7 were determined by quantitative real-time PCR using the SYBR Green PCR Master Mix (Roche) with the following primers:

- rat ANP forward: 5’-AAAGCAAACCTGAGGGCCTTGCTCG-3’
- rat ANP reverse: 5’-TTCCGTAACCGGAAGCTTGTTGCA-3’
- rat Myh7 forward: 5’-TCTGGACAGCTCCCCATTCT-3’
- rat Myh7 reverse: 5’-CAAGGCTAACCCTGGAGAAGATG-3’
The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

**Western blot analysis**
The total protein was extracted from left ventricle tissues or cultured cardiac myocytes, separated by SDS-PAGE (Invitrogen), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and subsequently probed with the indicated primary antibodies at 4°C overnight. The membranes were then incubated with a secondary IRDye 800CW-conjugated antibody (Li-Cor Biosciences, at 1:10000 dilution), and the signals were detected and analyzed with an Odyssey Imaging System (Li-Cor Bioscience). Some membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. Prior to visualization using the ChemiDoc™ XRS+ (Bio-Rad) system, the membranes were reacted with ECL reagents (Bio-Rad). Specific protein expression levels were normalized to β-tubulin or GAPDH on the same PVDF membrane to confirm equal loading before comparisons between groups.

**Statistical analysis**
The data are presented as the mean ± standard deviation (SD). Student's two-tailed *t*-tests were used for comparisons between two groups. Differences among three or more groups were compared using one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-test. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) software, version 13.0. A *P* value less than 0.05 was considered significant.
References:
Supplementary Figures

Figure S1 Src homology 2-B3 (SH2B3) inactivation protects mouse hearts against aortic banding (AB) induced hypertrophy. **A**, The loss of SH2B3 in SH2B3−/− mouse hearts was confirmed by western blot analysis as compared with SH2B3+/+ mice. **B** and **C**, Heart rates (B) and ejection fraction (C) were measured by echocardiographic analysis at 4 weeks after aortic banding (AB) surgery (HR: heart rates; bpm: beats per minute; EF: ejection fraction. n=6 or 7 mice per group). **D-G**, End-systolic pressure (D), EF % (E), $dP/dt_{\text{max}}$ (F) and $dP/dt_{\text{min}}$ (G) were evaluated by invasive hemodynamic examinations at 4 weeks after AB ($dP/dt_{\text{max}}$ indicates the maximum rate of pressure increase; $dP/dt_{\text{min}}$ indicates the minimum rate of pressure increase; n=4-7 mice per group). *$P<0.05$ versus the SH2B3+/+ sham group; #$P<0.05$ versus the SH2B3+/+ AB (4W) group.
Figure S2 Cardiac-specific Src homology 2-B3 (SH2B3) overexpression exacerbates pressure overload-induced hypertrophy. A and B, Cardiac SH2B3 expression in different transgenic (TG) lines versus non-transgenic (NTG) controls was examined by western blot assays. C and D, Heart rates (C) and ejection fraction (D) were measured by echocardiographic analysis at 4 weeks after aortic banding (AB) surgery (HR: heart rates; bpm: beats per minute; EF: ejection fraction. n=6 or 7 mice per group). E-J, Heart functional parameters were determined by hemodynamic examination 4 weeks after AB (EF: ejection fraction; dP/dt_max: maximum rate of pressure increase; dP/dt_min: minimum rate of pressure increase; n=4-6 mice per group). *P<0.05 versus the SH2B3 NTG sham group; #P<0.05 versus the SH2B3 NTG AB (4W) group.
Figure S3 Mitogen-activated protein kinase (MAPK) signaling was not responsible for Src homology 2-B3 (SH2B3) mediated hypertrophic response. A-B, At four weeks after aortic banding (AB) surgery, the phosphorylated and basal protein levels of extracellular signal-regulated kinase 1/2 (ERK1/2), MAPK kinase 1/2 (MEK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38 of MAPK signaling in the indicated experimental groups were examined by western blot analysis (representative of two independent experiments).
Figure S4 Src homology 2-B3 (SH2B3) deficiency in rats blunted aortic banding (AB)-induced cardiac hypertrophy. A, The transcription activator-like effector nuclease (TALEN) was designed to target the rat SH2B3 exon1. B, T7 endonuclease 1 assays were used to screen 8 live births of F0 rats. Cleavage products were identified for founders #4-1, #4-5 and #4-8. C, The polymerase chain reaction (PCR) products of the three mutant founders were TA cloned and sequenced to verify the precise mutation of the indels. D, All F1 and F2 offspring of founder #4-8 were genotyped by PCR and 3.0% agarose gel electrophoresis. The 155-bp band represents the WT allele, and the 144-bp band represents the mutant allele. E, The loss of SH2B3 in SD rat hearts was validated by western blot analysis. F-G, Echocardiographic examinations of heart dimensions and function at 4 weeks after surgery (HR: heart rates; bpm: beats per minutes; EF: ejection fraction). *P<0.05 versus the SH2B3 +/+ sham group; #P<0.05 versus the SH2B3 +/+ AB (4W) group.