Small Heterodimer Partner Blocks Cardiac Hypertrophy by Interfering With GATA6 Signaling

Yoon Seok Nam, Yoojung Kim, Hosouk Joung, Duk-Hwa Kwon, Nakwon Choe, Hyun-Ki Min, Yong Sook Kim, Hyung-Seok Kim, Don-Kyu Kim, Young Kuk Cho, Yong-Hoon Kim, Kwang-II Nam, Hyoun Chul Choi, Dong Ho Park, Kyoungho Suk, In-Kyu Lee, Youngkeun Ahn, Chul-Ho Lee, Huens-Sik Choi, Gwang Hyeon Eom, Hyun Kook

Rationale: Small heterodimer partner (SHP; NR0B2) is an atypical orphan nuclear receptor that lacks a conventional DNA-binding domain. Through interactions with other transcription factors, SHP regulates diverse biological events, including glucose metabolism in liver. However, the role of SHP in adult heart diseases has not yet been demonstrated.

Objective: We aimed to investigate the role of SHP in adult heart in association with cardiac hypertrophy.

Methods and Results: The roles of SHP in cardiac hypertrophy were tested in primary cultured cardiomyocytes and in animal models. SHP-null mice showed a hypertrophic phenotype. Hypertrophic stresses repressed the expression of SHP, whereas forced expression of SHP blocked the development of hypertrophy in cardiomyocytes. SHP reduced the protein amount of Gata6 and, by direct physical interaction with Gata6, interfered with the binding of Gata6 to GATA-binding elements in the promoter regions of natriuretic peptide precursor type A. Metformin, an antidiabetic agent, induced SHP and suppressed cardiac hypertrophy. The metformin-induced antihypertrophic effect was attenuated either by SHP small interfering RNA in cardiomyocytes or in SHP-null mice.

Conclusions: These results establish SHP as a novel antihypertrophic regulator that acts by interfering with GATA6 signaling. SHP may participate in the metformin-induced antihypertrophic response. (Circ Res. 2014;115:493-503.)

Key Words: GATA6 transcription factor ■ hypertrophy ■ metformin ■ nuclear receptor subfamily 0, group B, member 2 ■ orphan nuclear receptors

Cardiac hypertrophy is an increase in the individual cellular volume of the heart and is an adaptive response to exogenous stresses. The increase in cellular volume is associated with reactivation of the fetal gene program, which is finely regulated by diverse cardiac transcription factors. Specifically, GATA4, GATA6, myocyte enhancer factor 2C, nuclear factor of activated T cells, NK2 homeobox 5, and serum response factor have been shown to be involved in the development of cardiac hypertrophy. GATA4 and GATA6 are heart-specific transcription factors that regulate pathological cardiac hypertrophy in association with atrial natriuretic factor (ANF) expression. Transgenic mice that overexpress GATA4 in the heart show cardiac hypertrophy, and GATA4 activity is regulated by physical interaction with other transcription factors, such as serum response factor, NK2 homeobox 5, and myocyte enhancer factor.

GATA6 also induces pathological cardiac hypertrophy, which was demonstrated by recent studies of cardiac-specific overexpression of GATA6 in mice. In addition to effects as transcription factors, some of these factors also act as nuclear receptors that provide signal-mediated responsiveness to endogenous or exogenous ligands. For example, peroxisome proliferator-activated receptor-α or steroid receptors respond to a ligand and then mediate diverse cellular events as well as metabolic regulation. Not surprisingly, these nuclear receptors also play important roles in various organs, and therapeutic applications of their modulators are growing. The nuclear receptors are classified depending on their structural similarities. Interestingly, 1 group of nuclear receptors is termed the orphan nuclear receptors. In contrast with the other nuclear receptors that have endogenous ligands.

Original received May 15, 2014; revision received July 9, 2014; accepted July 11, 2014. In June 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15 days.

From the Department of Pharmacology and Medical Research Center for Gene Regulation (Y.S.N., Y.K., H.J., D.-H.K., N.C., H.-K.M., G.H.E., H.K.), Forensic Medicine (H.-S.K.), and Anatomy (K.-I.N.), Chonnam National University Medical School, Gwangju, Korea; Department of Pharmacology, College of Medicine, Yeungnam University, Gyeongsan, Korea (H.C.C.); Departments of Internal Medicine (I.-K.L.), Pharmacology, Brain Science and Engineering Institute (K.S.), and Ophthalmology (D.H.P.), Kyungpook National University School of Medicine, Daegu, Korea; Departments of Cardiology (Y.S.K., Y.A.) and Pediatrics (Y.K.C.), Chonnam National University Hospital, Gwangju, Korea; National Creative Research Initiatives Center for Nuclear Receptor Signals and Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju, Korea (D.-K.K., H.-S.C.); and Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea (Y.H.K., C.-H.L.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.304388/-/DC1.

Correspondence to Hyun Kook, MD, PhD, or Gwang Hyeon Eom, MD, PhD, Department of Pharmacology, Medical Research Center for Gene Regulation Chonnam National University Medical School, 5 Hak-dong, Dong-khu, Gwangju, 501–746, Korea. E-mail kookhyun@chonnam.ac.kr or innueondo@chonnam.ac.kr

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.304388

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to activate or modulate their activity, orphan nuclear receptors do not have endogenous ligands despite their structural similarities with other nuclear receptors. However, these orphan nuclear receptors also have diverse biological roles in development, homeostasis, and diseases.10

Small heterodimer partner (SHP) protein, one of the orphan nuclear receptors, is a unique member of the mammalian nuclear receptor superfamily that lacks a conventional DNA-binding domain but contains a putative ligand-binding domain.11 Previous studies demonstrated that SHP regulates target proteins by direct binding or by interference with other coactivator interaction12–14 and thereby works mainly as a transcriptional repressor. Because many target genes are related to metabolism, until recently, studies regarding SHP were focused mainly on metabolism or endocrine organs.15,16 However, the function of SHP in the adult heart has not yet been fully described. Thus, in this study, we investigated the functional roles of SHP in primary cultured cardiomyocytes and in animal models. We show that SHP is a novel antihypertrophic regulator that acts by interfering with GATA6 signal cascades. In addition, we reveal that SHP is a downstream target of metformin, an antiadipic and antihypertrophic drug.

Methods

Reagents

Antibodies, plasmids, and other reagents are described in the Online Data Supplement.

Animal Model, Histology, and Administration of Metformin

The experimental protocol for animal experiments was approved by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. SHP-knockout (SHP KO) mice and animal models were described previously.17–19

Mouse hearts were cross-sectioned at the level of the papillary muscle. Cross-sectional area measurement, H&E staining, immunohistochemistry, and Masson trichrome staining were described previously.20–22 For administration of metformin, from 1 day after isoproterenol-Alzet pump implantation, mice were dosed in the subcutaneous tissue with either metformin (200 mg/kg per day) or vehicle for 10 days.

Adenoviral Green Fluorescent Protein and SHP

The recombinant adenovirus was described previously.23 SHP adenovirus was prepared as described previously.22 One hundred multiplicity of infection of the virus was used for the experiments. The expression of protein was examined by infection of adenoviral green fluorescent protein.

In Vitro Binding Assay, Gel Shift Assay, and Chromatin Immunoprecipitation

To investigate the SHP-mediated interference of GATA6 binding to GATA-binding element (GATAE), in vitro binding assay,24 gel shift assay,25 and chromatin immunoprecipitation (ChIP) analysis26 were performed as described previously with slight modifications. For the in vitro binding assay, pcDNA3.1-Gata6-V5, pcDNA3-SHP-Flag, or pcDNA3.1 (mock) was separately transfected to HEK293T cells. Cell lysates were prepared, and the lysates of GATA6-transfected cells were mixed with SHP-transfected cell lysates or with mock-transfected cell lysates and then precipitated with biotinylated double-stranded oligonucleotides. DNA-bound proteins were subjected to Western blotting.

Gata6-V5 and SHP-Flag proteins were prepared by use of an in vitro transcription/translation system and were incubated with [32P]-tagged double-stranded GATAE for gel shift assay. Anti-V5 antibody was used to show the supershifting of Gata6. ChIP was performed by use of a commercially available kit. The binding of Gata6 on GATAE was determined by quantitative polymerase chain reaction.

Statistical Analysis

Data are represented as mean±SEM. The data were analyzed by use of the unpaired Student t test. The significance of more than two groups was calculated by analysis of variance with Tukey post hoc. Homogenous of variance was determined by Levene test. Statistical analysis was performed with PASW Statistics 21 (SPSS; IBM Company, Chicago, IL).

Adult rat ventricular cardiomyocyte isolation, Northern blot analysis, and primer sequences are described in the Online Data Supplement.

All other experimental methods such as neonatal rat ventricular cardiomyocyte (NRVC) isolation, cell cultures, promoter analysis, [1H]-leucine incorporation, quantitative real-time polymerase chain reaction, immunocytoschemistry, Western blot analysis, in vitro transcription/translation, and cell size measurements were described previously.19–21,23,26

Results

SHP KO Mouse Hearts Are Hypertrophied

To study the functional role of SHP in the heart, we examined the cardiac phenotypes of SHP KO mice. Compared with wild-type heart, the hearts from SHP KO mice were larger (Figure 1A). By measuring heart weight, we found that both parameters of heart weight-to-tibia length (Figure 1B) and heart weight-to-body weight (Online Figure IA) were significantly increased in SHP KO mice. However, the intracardiac structures of SHP KO mice were well preserved (Online Figure IB), and no clear functional derangements were observed by echocardiographic evaluation (data not shown). The cross-sectional area was measured in H&E-stained heart sections obtained from 12-week-old mice. Significant increases in myocyte bundles were observed (Figure 1C), which suggested that cardiac hypertrophy, rather than hyperplasia, took place in the SHP KO mice. We were not able to see any significant changes in the number of proliferating cell nuclear antigen-positive cells (Online Figure IC) or any obvious fibrosis as seen by Masson trichrome staining (Online Figure ID) in SHP KO mice. Cardiac hypertrophy in SHP KO mice was accompanied by an increase in the expression of ANF (Figure 1D and 1E). SHP KO heart also showed increases in the mRNA amounts of hypertrophy markers such as natriuretic peptide precursor type A (Nppa, encoding ANF), myosin heavy chain 7 (encoding β-myosin heavy chain), and skeletal α-actin (Figure 1F; Online Figure IE).

Hypertrophic Stresses Reduce the Expression of SHP

We next investigated whether exogenous hypertrophic stresses affect the expression of SHP. Transverse aortic constriction (aortic banding) for 7 days induced cardiac hypertrophy (Online Figure IIA and IIB), and these changes were
corroborated by the increase in ANF (Figure 2A and 2B). Aortic banding reduced the expression of SHP in mouse heart (Figure 2A and 2B). In NRVCs, treatment with 100 μmol/L phenylephrine, an α-adrenergic agonist to induce cardiac hypertrophy, reduced the expression of SHP (Figure 2C and 2D), whereas other hypertrophic markers such as ANF, B-type natriuretic peptide, and α-tubulin were significantly increased (Figure 2C; Online Figure IIIA).

Endothelin-1, an alternate hypertrophic agonist, and phenylephrine significantly reduced the mRNA level of SHP (Figure 2E). In promoter analysis with a −2.2 kb human SHP-luciferase construct, both phenylephrine (Figure 2F) and endothelin-1 (Figure 2G) successfully repressed the luciferase activity. These results suggested that hypertrophic stresses repress the transcription of SHP and thereby the protein amounts.

**Forced Expression of SHP Prevents Cardiac Hypertrophy**

We next investigated whether SHP itself affects cardiac hypertrophic phenotypes. Treatment with 100 μmol/L phenylephrine induced rearrangement of sarcomeric α-actinin (Online Figure IIIB) and increased the adult rat ventricular cardiomyocyte size (Figure 3A and 3B). However, infection with Ad-Flag-SHP (Online Figure IIIC) prevented these hypertrophic phenotypes. Likewise, we also observed the antihypertrophic effect of SHP in NRVCs (Figure 3C; Online Figure IIID). The protein synthesis, which can be evaluated by measuring [3H]-leucine incorporation, in NRVCs was significantly increased by treatment with phenylephrine. However, this increase was completely blocked by infection with Ad-Flag-SHP (Figure 3D). Treatment of cardiomyocytes with phenylephrine also increased −3500 myosin heavy chain 7 promoter activity, which was blocked by infection of the cells with adenoviral SHP (Figure 3E). Likewise, phenylephrine-induced activation of −3003 Nppa promoter activity was also blocked by SHP (Online Figure IVA). Transient transfection of mammalian expression vector of pcDNA3-SHP-Flag also inhibited both myosin heavy chain 7 (Figure 3F) and Nppa (Online Figure IVB) promoter activities in a dose-dependent fashion. Endothelin-1 (10 nmol/L) significantly increased the −3500 myosin heavy chain 7 promoter activity, which was also significantly attenuated by infection of the cell with adenoviral SHP (Figure 3G). Even the hypertrophic responses induced by high concentrations of fetal bovine serum were successfully inhibited by adenoviral SHP (Online Figure IVC and IVD). These results consistently demonstrated that SHP works as an antihypertrophic mediator against diverse hypertrophic stresses.

**GATA Transcription Factors Are a Target of SHP**

In the present study, the almost complete attenuation of hypertrophic phenotypes by overexpression of SHP suggested that its action is mediated by the modulation of key transcription factors. We postulated that certain potent prohypertrophic transcription factors would be a target of SHP, and we used several truncated mutant promoters of Nppa to find the SHP-responsive element. When 200 ng pcDNA3-HA-SHP was transfected into cardiomyocytes, basal −3003 Nppa promoter activity was successfully

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**Figure 1. Small heterodimer partner (SHP) knockout (KO) mice show cardiac hypertrophy.** A. Heart obtained from SHP KO mouse was enlarged. Bar, 1 mm. WT indicates wild type. B. Heart weight-to-tibia length ratio of 6- to 8-week-old WT or KO mice. C. Cross-sectional area obtained after H&E staining is shown. D. Protein amounts for both atrial natriuretic factor (ANF) and skeletal α-actin were increased in SHP KO hearts. Each lane shows the result from an individual sample. E. Expression of ANF was increased in SHP KO mice. Bar, 100 μm. F. Transcription amounts of natriuretic precursor protein type A (Nppa) and skeletal α-actin were quantified.
repressed. The repressive effect was also seen with 2 truncated forms of −638 and −366 Nppa promoters, which suggested that the SHP target transcription factor works within this minimal promoter region. Interestingly, however, the inhibition of activity with the −189 Nppa promoter was significantly attenuated. In addition, repression of transcription was completely absent when the −130 Nppa promoter was used (Figure 4A).

Next, the transcription factor-binding elements were scrutinized by use of bioinformatics tools (http://www.genomatics.de), and 2 GATAE located ≈ 300 and 130 bp upstream from the transcription start site drew our interest (Online Figure VA). The GATAE consists of the GATA sequence in the binding element and is recognized by GATA transcription factors. Among GATA1 to GATA6, GATA4 and GATA6 are well known to induce cardiac hypertrophy, and diverse hypertrophic stresses work through these GATA factors to induce eventual fetal gene reprogramming. Therefore, we postulated that the GATA transcription factors could be a target of SHP and that the GATAEs in −302 bp and −145 bp might be involved.

To confirm this postulation, we introduced site-directed mutations in either the proximal or the distal GATAE in the −638 Nppa promoter constructs and examined the responsiveness to SHP. The altered nucleotides are shown in Online Figure VA. When either the distal or the proximal GATAE was mutated, SHP-mediated transcriptional repression was partially recovered. However, in the double mutant in which both GATAEs were disrupted, SHP failed to repress the Nppa promoter activity (Figure 4B). These results suggested that both GATAEs in the minimal promoter region were required for the full repressive effects of SHP.

Because GATA4 and GATA6 share their binding element, we examined whether SHP directly regulates the expression of GATA4 or of GATA6 by infection of NRVCs with Ad-Flag-SHP. As shown in Figure 4C, the expression of Gata6 was most significantly decreased by Ad-Flag-SHP in cardiomyocytes, whereas that of Gata4 was moderately decreased. These changes were quantified as shown in Figure 4D and 4E. Other prohypertrophic transcription factors, such as serum response factor (Figure 4C), NFATc1 (nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1), myocyte enhancer factor 2C, and NK2 homeobox 5 (Online Figure VB), were not significantly repressed by SHP. Transient transfection of pcDNA3-HA-SHP also inhibited the expression of Gata4 and Gata6 in H9c2 cells, a rat cardiomyoblast cell line (Figure 4F; quantification results in Online Figure VC and VD).

To compare the inhibitory effects of SHP on GATA-mediated transcriptional activation, we again used −3003 Nppa promoter luciferase constructs. Transient transfection of pcDNA3-HA-SHP also inhibited the expression of Gata4 and Gata6 in H9c2 cells, a rat cardiomyoblast cell line (Figure 4G and 4H).

Alterations of Gata protein amounts were also examined in SHP KO mouse hearts (Figure 4I and 4J). Gata4 expression was slightly increased in SHP KO mice with no statistical significance. The Gata6 protein amount, however, was significantly increased, which was accompanied by an increase in Gata6 mRNA (Online Figure VIA). We were not able to see significant changes in the protein amounts of myocyte enhancer factor 2C or NK2 homeobox 5 in SHP KO mouse hearts (Online Figure VIB).
SHP Binds to GATA6 and Interferes With its Interaction With GATAE

SHP itself does not have a DNA-binding domain. Instead, its transcriptional repression mechanism is largely dependent on direct binding to other transcription factors. We postulated that SHP might bind to GATA transcription factors. We performed an immunoprecipitation assay by transfection of pcDNA3-HA-SHP and pcDNA3.1-Gata6-V5 in H9c2 cells. Anti-V5 (Gata6) antibody was used for immunoprecipitation, and SHP was detected with anti-HA (SHP) antibody. Gata6 successfully recruited SHP (Figure 5A). In HEK293T cells, HA-tagged SHP pulled Gata6 down (Figure 5B). Inversely, V5-tagged Gata6 successfully recruited HA-tagged SHP in HEK293T cells (Online Figure VIIA). Endogenous Gata6 also directly associated with SHP in H9c2 cells (Figure 5C). We also performed the immunoprecipitation assay with Gata4. In our experimental model, however, Gata4 failed to interact with SHP (Online Figure VIIB).

We next examined whether SHP interferes with binding of GATA6 to GATAE in the Nppa promoter region by use of an in vitro binding assay with biotin-conjugated, double-stranded nucleotides spanning the GATAEs in the Nppa promoter region (Online Figure VIIF). As shown in Figure 5D, SHP failed to directly interact with wild-type (intact) distal GATAE. Distal GATAE was successfully recruited when Gata6 lysates were used, which suggested the binding of Gata6 to GATAE. This binding was attenuated when a mixture of both Gata6 lysates and SHP lysates was used, which suggested that SHP interfered with the binding of Gata6 to the GATAE. In contrast, a mutant form of GATAE failed to recruit Gata6. We repeated the same experiment with proximal GATAE (Online Figure VIIF). We tried to confirm whether SHP interferes with the binding of GATA6 to GATAE by use of both gel shift (Figure 5E) and ChIP (Figure 5F and 5G) assays. Compared with mock, reticulolysates obtained after in vitro transcription/translation of pcDNA3.1-Gata6-V5 retarded the mobility of double-stranded nucleotide probes of proximal GATAE (lower arrows at third lane). Addition of reticulolysates treated with pcDNA3-SHP-Flag reduced the binding of Gata6 to GATAE (fourth lane). Anti-V5 antibody successfully induced supershifting of the Gata6/GATAE complex. ChIP analysis using H9c2 cell lysates further confirmed that transfection of SHP reduced the binding of Gata6 to the distal GATAE (Figure 5F; fourth versus fifth lane). Quantification results of ChIP using the proximal GATAE are shown in Figure 5G. These results suggested that binding of GATA6 to GATAE was responsible for the SHP-mediated transcriptional repression.

Metformin Induces SHP in Cardiomyocytes in an AMP-Activated Protein Kinase–Independent Manner

We next screened chemicals that are known to induce SHP in other organs to see whether they could induce SHP in...
cardiomyocytes. Among the chemicals tested, metformin drew our interest because of its pleiotropic action. Metformin is widely used as an antihyperglycemic drug as a first-line therapy for type 2 diabetes mellitus. Metformin also has an antihypertrophic action through AMP-activated protein kinase (AMPK) activation, which involves mitogen-activated protein kinase 1/2 and mammalian target of rapamycin signaling. In addition to these signaling pathways, we postulated that SHP is also involved in the beneficial action of metformin.

To validate this hypothesis, we first examined whether SHP is induced by metformin in cardiomyocytes. Metformin increased the protein amount of SHP in dose- (Figure 6A) and time-dependent (Figure 6B) manners. Metformin also induced an increase in the SHP mRNA level (Figure 6C). Metformin is known to activate AMPK through an increase in the AMP/ADP ratio in the cell. It has been also reported that metformin induces the transcription of SHP in an AMPK-dependent manner in liver cells. Thus, we checked whether metformin activates AMPK and whether activated AMPK increases SHP in cardiomyocytes. We first showed that metformin successfully induced phosphorylation of AMPK in cardiomyocytes (Figure 6D). We next checked whether activation of AMPK induced SHP. Interestingly, however, A769662, an AMPK activator, failed to increase the amount of SHP in NRVCs.
although it could induce AMPK phosphorylation (Figure 6E). An alternate AMPK activator, \textsuperscript{35} 5-aminoimidazole-4-carboxamide ribonucleotide, did not affect SHP protein amounts (Online Figure VIIIA). Neither A769662 nor compound C, an AMPK inhibitor, altered SHP mRNA levels (Figure 6F). Likewise, constitutively active AMPK (\textit{Ad-AMPK CA}) failed to induce SHP mRNA. In addition, 2 forms of dominant-negative AMPK (\textit{Ad-AMPK DN\textalpha}_{1} and DN\textalpha_{2}) did not reduce SHP mRNA (Figure 6G), although the adenovirus was successfully infected (Online Figure VIIIIB). These results suggest that, unlike in liver cells,\textsuperscript{27} AMPK is not involved in the metformin-induced increase in SHP in cardiomyocytes.

**SHP Mediates Metformin-Induced Antihypertrophic Action in Cardiomyocytes**

We next checked whether metformin-induced SHP mediates antihypertrophic action. Metformin successfully repressed basal ANF expression (Figure 7A). In addition, phentolamine-induced increases in both ANF protein amount determined by Western blot (Figure 7B) and \textit{Nppa} mRNA level determined by Northern blot analysis (Online Figure VIIIC) were blocked by metformin. Protein synthesis was also repressed by metformin in a dose-dependent fashion in cardiomyocytes (Figure 7C).

Next, we checked whether the metformin-induced repression of these hypertrophic phenotypes could be recovered by knockdown of \textit{SHP}. Small interfering RNA for \textit{SHP} successfully repressed the \textit{SHP} mRNA level (Online Figure VIIID). Metformin induced reduction of [\textit{H}] -leucine incorporation stimulated by phenylephrine. Interestingly, this reduction was significantly attenuated when small interfering RNA for \textit{SHP} was transfected (Figure 7D). The phenylephrine-induced increase in \textit{Nppa} promoter activity was repressed by treatment with metformin, which was then recovered by small interfering RNA for \textit{SHP} (Figure 7E). Likewise, the repression of individual cell size was attenuated by knockdown of \textit{SHP} (Figure 7F).
These results suggested the involvement of SHP in the antihypertrophic mechanism of metformin in cardiomyocytes.

**Metformin-Induced Prevention of Cardiac Hypertrophy Is Blunted in SHP KO Mice**

We next extended our results to an in vivo mouse model. First, we introduced cardiac hypertrophy by implantation of an isoproterenol-releasing pump and then checked whether metformin repressed this effect. Isoproterenol-induced cardiac hypertrophy was significantly blunted when metformin was administered (Figure 8A; Online Figure IXA). In this animal model, SHP was significantly reduced by isoproterenol infusion because it was either by aortic banding in mouse or by phenylephrine in cardiomyocytes (Figure 2). This reduction, however, was blunted when metformin was administered (Figure 8B).

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**Figure 6. Metformin induces small heterodimer partner (SHP) in AMP-activated protein kinase (AMPK)-independent manner in cardiomyocytes.** A and B, Metformin increased SHP protein amount in a dose-dependent (A) and time-dependent (B) fashion. C, Metformin increased SHP mRNA. D, Metformin induced phosphorylation of AMPK in cardiomyocytes. E, A769662, an AMPK activator, failed to induce SHP, whereas it phosphorylated AMPK. F, Neither A769662 nor compound C, an AMPK inhibitor, affected the SHP mRNA level (quantitative real-time reverse transcriptase polymerase chain reaction result). G, Adenoviral infection of either constitutively active (CA) or dominant negative (DNα1 and DNα2) AMPK failed to alter SHP mRNA.

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**Figure 7. Small heterodimer partner (SHP) mediates metformin-induced antihypertrophic effects.** A, Metformin reduced the basal expression of atrial natriuretic factor (ANF). B, Metformin repressed phenylephrine (PE)-induced increase in ANF expression. C, Metformin inhibited the increase in [3H]-leucine incorporation induced by PE in cardiomyocytes in a dose-dependent fashion. Metformin-induced attenuations of PE-mediated hypertrophic phenotypes were blunted by knockdown of SHP with small interfering RNA: [3H]-leucine incorporation (D), −3003 Nppa promoter activity (E), individual cell size measurement (F).
By using SHP KO mice, we examined whether SHP mediates metformin-induced antihypertrophic action in vivo. Metformin successfully induced regression of heart size enlargement induced by isoproterenol infusion. Strikingly, however, the metformin-induced regression of heart size was completely abolished in SHP KO mice (Figure 8C). Quantification of the results of heart weight-to-tibia length is shown in Figure 8D. Isoproterenol-induced cardiac hypertrophy (first versus second group) was significantly blocked by metformin administration (second versus third group). In SHP KO mice, however, metformin-induced inhibition of isoproterenol-induced cardiac hypertrophy was absent (fourth versus fifth group). Failure of repression induced by metformin was also demonstrated by measuring the heart weight-to-body weight ratio (Online Figure IXA). Increase in the expression of Nppa in hypertrophied heart was almost completely blocked by metformin in wild-type mice. However, metformin failed to do so in SHP KO mice (Figure 8E).

Loss of the antihypertrophic action of metformin was also demonstrated by Western blot with ANF antibody (Online Figure 1XB). These results suggested that the metformin-induced repression of cardiac hypertrophy was mediated by SHP in the animal model.

Discussion

This study is the first to elucidate the function of SHP as a novel antihypertrophic nuclear protein that acts by interfering with the GATA6 signal transduction system. In addition, we also showed that SHP is involved in the antihypertrophic action of metformin (Figure 8F). We first observed that SHP KO mice showed cardiac hypertrophy and that the SHP expression level was downregulated by diverse hypertrophic stresses in cardiomyocytes and in mice. Forced expression of SHP prevented the hypertrophy and interfered with binding of GATA6 to its target element. Thus, we propose that SHP is a novel antihypertrophic mediator with potential application as a therapeutic agent in cardiac diseases.

SHP KO mice are known to have various phenotypes, such as mild defects in bile acid homeostasis, repress osteoblast differentiation and decreases in bone mass, enhancement of hepatocyte proliferation and transformation, and susceptibility to endotoxin-induced sepsis. However, no clear cardiac phenotypes have been reported. Indeed, we were not able to observe other phenotypes such as changes in blood pressure, heart rate, or overall intracardiac structures. However, the adult hearts were enlarged. We did not see an obvious increase in cell
proliferation or apparent fibrosis. It is possible that sustained and mild exogenous stresses during aging might cause this enlargement of the heart. Indeed, it has been reported that knock-out of histone deacetylase 5, an antihypertrophic gene, does not induce obvious hypertrophic phenotypes before 8 months of age unless other exogenous hypertrophic stimuli are given.\(^3\)

SHP represses transcription factor–mediated transactivation either by inhibiting DNA binding or by competing with coactivators for nuclear receptors. Known binding partners of SHP include various nuclear receptors and transcription factors such as estrogen receptor, glucocorticoid receptor, thyroid hormone receptor, androgen receptor, retinoic acid receptor, and retinoid X receptor, and orphan receptors such as liver receptor homolog-1, hepatocyte nuclear factor-4, Nur77, estrogen-related receptor-γ, constitutive androstane receptor, liver X receptor, and peroxisome proliferator-activated receptor. Thus, we also postulated that SHP would follow those repression mechanisms in the prevention of cardiac hypertrophy. Indeed, we found that strong prohypertrophic GATA transcription factors are a target of SHP-mediated regulation. GATA4-transgenic mice show cardiac hypertrophy.\(^4\) Like GATA4, GATA6 is closely involved in cardiac hypertrophy\(^5\,\,^6\) and is capable of inducing hypertrophy.\(^7\) In the present study, we showed that GATA protein expression is markedly downregulated by SHP (Figure 4) and that physical interaction between GATA6 and SHP impaired the binding of GATA6 to its binding elements (Figure 5). Thus, we propose that GATA6 is an alternate target for the biological role of SHP and that this association plays a role in the development of cardiac hypertrophy.

The most striking observation in this study was the involvement of SHP in metformin action. The antidiabetic mechanism\(^2\,\,^7\) of action of SHP partially overlaps with the mechanism of action of metformin.\(^8\) Indeed, SHP is upregulated by metformin in liver.\(^2\) Wide application of metformin other than in diabetes mellitus was suggested after beneficial outcomes in clinical or experimental conditions. One of these possible therapeutic applications is in cardiac hypertrophy. Beauleoye et al\(^9\) reported that cardiac hypertrophy is inhibited by metformin, and as a mechanism they suggested inactivation of p70S6K through AMPK/mammalian target of rapamycin activation. On the basis of our results, we postulated that metformin also induces SHP in the heart and that it may also participate into the antihypertrophic mechanism of metformin. Indeed, in this study, we clearly demonstrated that SHP is upregulated by metformin in cardiomyocytes (Figure 6) and that knockdown of SHP resulted in the failure of metformin to prevent cardiomyocyte hypertrophy (Figure 7). We further extended this observation to an in vivo model by using SHP KO mice that were resistant to metformin in the relief of cardiac hypertrophy (Figure 8). Thus, we postulate that in addition to the previously known mammalian target of rapamycin/p70S6K\(^10\) or peroxisome proliferator-activated receptor-α\(^11\) signal cascades, the activation of SHP may participate in the antihypertrophic mechanism of metformin. Indeed, in some of our experiments, metformin-induced antihypertrophic action was partially, but not completely, attenuated by SHP small interfering RNA, which may suggest that diverse signals are involved simultaneously.

It has been known that activation of AMPK prevents aortic banding-induced cardiac hypertrophy\(^12\) by enhancement of peroxisome proliferator-activated receptor-α signaling in the heart\(^13\) and that AMPK induces SHP in response to metformin in hepatocytes.\(^22\) In the present study, we also observed that metformin activates AMPK in cardiomyocytes. Interestingly, however, activation of AMPK did not result in the induction of SHP, which suggests that an alternative regulatory mechanism in response to metformin may exist. Nevertheless, considering that GATA6 is one of the master regulators in the development of cardiac hypertrophy, the SHP–GATA6 signal cascades would be novel targets for new therapeutics.

Acknowledgments
We are grateful to Sera Shin for her technical assistance and to Professor Joohun Ha of Kyung Hee University for his critical comments on AMP-activated protein kinase and for kind present of shuttle vectors for generation of adenovirus.

Sources of Funding
This study was supported by a National Research Foundation of Korea grant funded by the Korean government (MEST, #2014–027276), by the Korea Science and Engineering Foundation through the Medical Research Center for Gene Regulation (2011–0030132), and by a grant of the Korean Health Technology R&D Project, Ministry of Health and Welfare (A111345 and A121256).

Disclosures
None.

References
In the absence of SHP, the ability of metformin to prevent cardiac hypertrophy is weakened.

Cardiac hypertrophy accompanies many forms of heart disease, including ischemic disease, myocardial infarction, hypertension, aortic stenosis, and valvular dysfunction. Although cardiac hypertrophy itself is an initial adaptive process, uncorrected continuous stresses often induce the transition to congestive heart failure. Few interventions have proven effective in blocking the hypertrophy or the transition to heart failure. Cardiomyocyte hypertrophy is regulated by heart-specific transcription factors such as GATA4, GATA6, myocyte enhancer factor 2, nuclear factor of activated T cells, and NFκB homeobox 5. The subsequent reactivation of the fetal gene program and repression of adult cardiac genes by these transcription factors are closely linked to deterioration of heart function. Here, we show SHP to be a novel antihypertrophic transcriptional modulator. We found that diverse hypertrophic stresses repressed SHP expression and the lack of SHP resulted in cardiac hypertrophy. SHP bound to GATA6 and interfered with GATA6 binding to the GATA element in the target gene promoter. Metformin induced SHP expression in the heart, which prevented cardiac hypertrophy. Thus, SHP is involved at least in part in the antihypertrophic action of metformin. Our work suggests that the development of modulators of SHP–GATA6 signal pathways could be a novel platform for the development of new therapeutics for cardiac hypertrophy.
Small Heterodimer Partner Blocks Cardiac Hypertrophy by Interfering With GATA6 Signaling

Yoon Seok Nam, Yoojung Kim, Hosouk Joung, Duk-Hwa Kwon, Nakwon Choe, Hyun-Ki Min, Yong Sook Kim, Hyung-Seok Kim, Don-Kyu Kim, Young Kuk Cho, Yong-Hoon Kim, Kwang-II Nam, Hyoung Chul Choi, Dong Ho Park, Kyoungho Suk, In-Kyu Lee, Youngkeun Ahn, Chul-Ho Lee, Hueng-Sik Choi, Gwang Hyeon Eom and Hyun Kook

_Circ Res_. 2014;115:493-503; originally published online July 11, 2014; doi: 10.1161/CIRCRESAHA.115.304388

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/5/493

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/07/11/CIRCRESAHA.115.304388.DC1

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SUPPLEMENTAL MATERIAL

Detailed Methods

Antibodies

Anti-SHP antibody was purchased from Peptron (Peptron, Daejeon, Korea); AMPK, phospho-AMPK (Cell signaling Technology, Inc., Danvers, MA, USA); atrial natriuretic factor was from Meridian (Meridian Life Science, Inc., Saco, ME, USA); GATA4, GATA6, MEF2C, and NNX2.5 were from Abcam (Abcam, Cambridge, UK); V5 was from Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA); hemagglutinin (HA) was from Roche (12CA5, Roche, Grenzacherstrasse, Basel, Switzerland); sarcomeric α-actinin and Flag were from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA); and NFATc1, α-tubulin, BNP, SRF, skeletal α-actin, and Gapdh were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Anti-mouse or anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Sigma (Sigma-Aldrich Corp.). Alexa Fluor 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG secondary antibodies were purchased from Invitrogen.

Reagents

Isoproterenol (ISP), phenylephrine (PE), endothelin-1 (ET-1), and 2,2,2-tribromoethanol, 1,1-dimethylbiguanide hydrochloride (metformin), 6-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-3-(4-pyridyl)pyrazolo[1,5-a]pyrimidine (compound C), and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) were purchased from Sigma (Sigma-Aldrich Corp.). 6,7-dihydro-3-(2'-hydroxy][1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-b]pyridine-1-carbonitrile (A769662) was purchased from Tocris Bioscience (Cambridge, UK). Treatment time of chemicals was overnight or as specifically described in the figures. SHP siRNA was purchased from Dharmacon (Thermo Fisher Scientific Inc., Waltham, MA, USA) and scramble was purchased from Bioneer (Daejeon, Korea). The Alzet® micro-osmotic pump was from DURECT (DURECT Corporation, Cupertino, CA, USA).

Plasmids

Generation and characteristics of plasmids of -2200 human SHP promoter-luciferases, pcDNA3-HA-vector, pcDNA3-HA-SHP, and pcDNA3-mouse SHP-Flag were described previously.1, 2 pcDNA3-HA-GATA4 and pcDNA3.1-GATA6-V5 were kindly provided by Prof. Jonathan A. Epstein (University of Pennsylvania, Philadelphia, PA). The -3003 rat Nppa and -3500 mouse Myh7 promoter-luciferases were kindly provided by Prof. Young Sook Lee (University of Wisconsin Medical School, Madison, WI, USA) and Dr. Fadia Haddad (University of California, Irvine, CA), respectively. GATA binding element (GATAE)-disrupted Nppa promoter luciferase was constructed by CosmoGeneTech (Seoul, Korea). Various length of Nppa promoter luciferase plasmids (-638, -366, -189, and -130) were subcloned into pGL3-luciferase.3 Shuttle vectors for generation of adenovirus expressing AMPK-CA, DNα1, or DNα2 were kindly provided by Prof. Joohun Ha (Kyung Hee University, Seoul, Korea).4 Biotinylated double-stranded oligonucleotides containing rat Nppa promoter flaking putative GATA binding sites were purchased from Bioneer (Daejeon, Korea). All constructs were confirmed by automatic sequence analysis.

Preparation of Recombinant Adenovirus

Briefly, the cDNA encoding hSHP was cloned into pAd-YC2 shuttle vector. For homologous recombination, pAd-YC2 shuttle vector (5 μg) and a rescue vector, pJM17 (5 μg), were cotransfected into 293 cells. The mouse SHP (mSHP) cDNA was cloned by insertion of the BamHI/XbaI digested
fragment into the *BglII/XbaI* site of the pAdTrack-CMV vector. Recombination of the AdTrack-CMVmSHP with adenoviral gene carrier vector was performed by transformation to pretransformed adEasy-BJ21 competent cells. The generation and titration of adenovirus was carried out with a commercially available kit according to the manufacturer's protocol (Agilent Technologies, Inc., Santa Clara, CA, USA) (Online material, Preparation of recombinant adenovirus).

**Adult Rat Ventricular Cardiomyocyte Isolation**

Rat neonatal cardiomyocytes were prepared as described previously. For cell size measurement, cellular sizes of 3 or 5 cells in one field were averaged and counted as one case. Three independent sets of immunocytochemistry staining were performed. For adult cardiomyocytes, ventricular myocytes were isolated from adult SD rat hearts as previously described with minor modifications. Male rats of 8 to 12 weeks of age were used. The heart was quickly removed from the chest and the aorta was retrogradely perfused with calcium-free Tyrode buffer (137 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES [pH 7.4], 10 mM 2,3-butanedione monoxime, and 5 mM taurine). The enzymatic digestion was initiated by adding collagenase type B (0.35 U/ml; Roche) and hyaluronidase (0.1 mg/ml; Worthington) to the perfusion solution. When the heart became swollen after 10 min of digestion, the left ventricle was quickly removed, cut into several chunks, and further digested in a shaker. The supernatant containing the dispersed myocytes was filtered through a cell strainer (100 μm pore size; BD Falcon) and gently centrifuged at 500 rpm for 1 min. Extracellular Ca²⁺ was incrementally added back to 1.25 mM to avoid the Ca²⁺ paradox. This procedure usually yielded ≥80% viable rod-shaped ventricular myocytes with clear sarcomere striations.

**Animal Models**

Eight-week-old adult male CD1 or C57BL/6 mice were purchased from Samtako (Korea) and were housed individually in plastic cages in a temperature-controlled room. All surgical procedures and echocardiography were performed with the animals under anesthesia with 2,2,2-tribromoethanol (300 mg/kg, intraperitoneally).

**SHP KO mice**

The first exon of SHP was replaced with a β-galactosidase/pGKneo cassette by homologous recombination. After confirming germline transmission, heterozygote F1 mice were crossed to generate SHP null mice. The genotype of each mouse was determined both by Southern blot and by PCR.

**Cross-Sectional Area Measurements**

For each experimental group, hearts from 3 animals were mounted after coronal section and stained with hematoxylin-eosin. Nine fields in the LV free wall were randomly chosen, and the cross-sectional areas of more than 10 cardiac muscle bundles per field were measured with Multi Gauge software (Fuji Photo Film Co) in a blind fashion.

**Protein preparation**

For western blot and immunoprecipitation, cell lysates were obtained from cells by use of NP lysis buffer (1% Nonidet-P40, 50 mM Tris pH 8.0, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT) supplemented with protease inhibitor cocktail. For heart tissues, RIPA lysis buffer (1% Nonidet-P40, 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT) was utilized.
In Vitro Binding Assay

*pcDNA3.1-GATA6-V5, pcDNA3-SHP-Flag*, or mock was separately transfected to HEK293T cells. After preparing cell lysates, the lysates of GATA6-transfected cells were mixed with SHP-transfected cell lysates or with mock-transfected cell lysates. Biotinylated double-stranded oligonucleotides was diluted into 10 pM concentration in TE buffer and double-stranded probes were prepared by annealing of both oligomers at 95°C for 10 minutes with water chamber to allow gentle cool-down to the room temperature. Cell lysate with proper combination were mixed with probes then incubated for 4 hours at 4°C with continuous rotation for allowing binding to GATAE. The mixed cell lysates were then pre-cleared with ImmunoPure streptavidin-agarose beads (30 μL/sample) and then incubated for 16 h with 10 pmol of biotinylated double-stranded oligonucleotides containing either 30 bp distal or 31 bp proximal rat Nppa promoter flaking intact (wt: GATA) or disrupted (mt: GTTA), distal oligomer wild type (GATA) or mutant (CTTA). GATA binding sites. DNA-bound proteins were collected with 30 μL immobilized streptavidin-agarose beads for 1 h and subjected to Western blotting with anti-V5 antibodies.

Gel Shift Assay

Proteins were prepared by use of in vitro translational system (Promega Corporation, Madison, WI, USA) and translation was confirmed by western blot analysis. GATAE-containing oligomer was prepared by same method described above and labeled with [γ-32P]ATP by reaction with T4 kinase. In vitro translated protein and the 32P-labeled probes were incubated for 30 minutes in binding buffer [20 mM HEPES pH7.9, 100 mM NaCl, 1 mM EDTA, 6% glycerol, 1 mM DTT, 1 mM PMSF, 0.25 mg/ml BSA, and 2 μg of poly(dI-dC)]. The protein-DNA complexes were separated in 5% nondenaturing polyacrylamide gel in 0.5 X TBE buffer. Electrophoresis was carried out at 180 V for 4 hours. Supershift was made by adding V5 antibodies. The gel was dried and shift band was visualized by exposure to autoradiography film for one (short exposure) or 4 day (long exposure) at -80°C.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were carried out following the manufacturer’s protocol (Millipore Corporation, Billerica, MA, USA). Two days after transfection, H9c2 cells were incubated with 1% formaldehyde for 10 min to induce cross-link. Cell lysates were prepared by SDS-lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1) and sonication was performed to reduce chromatin fragments. Ten microliters of the sonicated-chromatin was utilized for input control and the chromatin was immunoprecipitated with 1 μg of anti-V5 antibodies. The immune complexes were collected by adding protein G-conjugated agarose beads and cross-linking were reversed. Proteins were digested by incubation with proteinase K and the co-immunoprecipitated DNA was harvested via phenol-chloroform extraction method. The binding of GATA6 on GATAE was determined by PCR amplification. Fragments containing GATAE were generated by use of following primers: proximal, 5'-GGAGGGAGGTACTGGAGCTCCTCAT-3' (sense) and 5'-CATTCTGTCATTGCGCGGC-3' (antisense); distal, 5'-GGTCCCCGTGAGCAGAGAGG-3' (forward) and 5'-GATGATTTGCCACTCAGAGGGCC-3' (reverse). 

Primers for quantitative RT-PCR are listed below:

**Mouse**

For *Nppa*: forward 5'-ACTGAGGAAGCAGGGGCGACTTA-3';
reverse 5'-AGCTGCGTACAGCACACACACACCAGGGCT-3';

For *Myh7*: forward 5'-CACCACCAATCTCAGCAGAT-3';
reverse 5'-AAGAGGCCCCGAGTAGTGATAG-3';

For *SHP*: forward 5'-CTGCAGGTCCAGGCACCTT-3';
reverse 5’- TGTCTTGGCTAGGACATCCA-3’;
For GAPDH: forward 5’-GCATGGCCTTCCGTGTTCCCT-3’;
    reverse 5’-CCCTGTTTGCTGATCCCTGTTACAT-3’.
For Skeletal α-actin: forward 5’-ATCGCTGACCACAATGCAGAA -3’;
    reverse 5’-TGCGCTAGAAGCAGATTTTGCGGT -3’;
Rat
For Nppa: forward 5’- TCCTGGGCTTTTGGCCTCCA-3’;
    reverse 5’- CCGAAGCAGCTTGACCTTCCGA-3’;
For SHP: forward 5’-ATGAGCTCCAGCCAATCGGGGTC -3’;
    reverse 5’-TCACCTCAACAAAGCATGTCTTCCG -3’;
For GAPDH: forward 5’-GCATGGCCTTCCGTGTTCCCT-3’;
    reverse 5’-CCCTGTTTGCTGATCCCTGTTACAT-3’.
For AMPK: forward 5’-ATCCGCAGAGGATCCAGAA-3’;
    reverse 5’-CGTCGACTCTCTTTTGCTG-3’.

Cardiomyocytes, H9c2, and 293T cells were maintained as described previously.5

Northern blot

Northern blot analysis was performed in a standard method as described previously.8,9
Online Figure I. **SHP knockout mice show cardiac hypertrophy.** (A) Heart weight to body weight ratio of either wild-type or SHP knockout mice. (B) Cross-sections obtained after H&E staining are shown. (C) Cardiomyocyte proliferation was checked by immunohistochemistry with proliferating cell nuclear antigen (PCNA) antibody. Note that no clear increase in the PCNA-positive cell number was observed. (D) Masson trichrome staining to show fibrosis. No apparent fibrosis was observed in 8-week-old mouse heart section. (E) Expressions of mRNA for *natriuretic precursor protein type A* (*Nppa*) and *myosin heavy chain 7* (*Myh7*) are shown by representative gel pictures.
Online Figure II. Aortic banding (AB) induced cardiac hypertrophy. (A) Heart weight to body weight ratio. Either sham or AB was introduced to CD1 mice. AB for 7 days sufficiently induced cardiac hypertrophy. (B) Heart weight to tibia length ratio.
Online Figure III. Forced expression of SHP prevents stress fiber formation in rat neonatal ventricular cardiomyocytes. (A) Quantitative results of Western blot of SHP after treatment with PE. (B) Confocal microscopic analysis showing the rearrangement of sarcomeric α-actinin in ARVCs. Note that PE induced strong striation, which was attenuated by Ad-SHP. (C) Successful expression of SHP after Ad-SHP infection. (D) Fluorescent microscopic analysis for immunocytochemistry with sarcomeric α-actinin in NRVCs. Three representative pictures for each case are shown.
Online Figure IV. SHP prevents hypertrophic phenotypes. (A) PE-induced increase in -3003 Nppa promoter activity was completely abolished by adenoviral SHP infection. (B) PE-induced Nppa promoter activation was attenuated by transient transfection with pcDNA-SHP-Flag in a dose-dependent fashion. (C-D) Either leucine incorporation (C) or Nppa promoter activity (D) induced by FBS was significantly repressed by adenoviral SHP infection.
Online Figure V. *Nppa* promoter structure showing the two GATA binding elements and the effects of SHP transfection on GATA transcription factors. (A) The location of the two GATAE (either proximal or distal) is shown. Numerals indicate the truncated *Nppa* promoters that were used in this study. (B) Changes in MEF2C, NFAT, and NKX2.5 protein amounts by Ad-SHP infection. Ad-SHP did not repress the expression. (C-D) Quantitative results of Western blot for Gata4 (C) and Gata6 (D) after transfection of pcDNA3.1-SHP-Flag.
Online Figure VI. Changes in Gata6, MEF2C, and NKX2.5 in SHP KO hearts. (A) Quantitative RT-PCR results showing the changes in Gata6 in SHP KO hearts. (B) Changes in expression level of MEF2C and NKX2.5 in SHP KO hearts.
Online Figure VII. **SHP binds to Gata6 to interfere with its binding to GATAE.** (A) Immunoprecipitation results showing the recruitment of SHP by GATA6 in 293T cells. (B) GATA4 failed to interact with SHP. Both *pcDNA3.1-SHP-Flag* and *pcDNA3-HA-Gata4* were transfected to 293T cells and either IgG or anti-HA antibody was used for immunoprecipitation assay. (C) Double stranded nucleotides for in vitro binding assays are shown. Biotinylated double-stranded nucleotides were used for in vitro binding assay. (D) In vitro binding assay showing that SHP interfered with the binding of Gata6 to distal GATAEs in *Nppa* promoter.
Online Figure VIII. Metformin blocks induction of Nppa by PE. (A) AICAR, an AMPK activator, failed to induce SHP. (B) Quantitative RT-PCR results showing successful delivery of adenoviral AMPK modulators. CA: constitutively active, DNα1 and DNα2: dominant negative isoforms 1 and 2. (C) Northern blot analysis showing that metformin successfully inhibits PE-induced Nppa expression. (D) Successful attenuation of SHP mRNA expression by small interfering technology in rat neonatal ventricular cardiomyocytes.
Online Figure IX. **Anti-hypertrophic effect of metformin is abolished in SHP knockout mice.** (A) Heart weight to body weight ratio. Note that metformin failed to repress the heart weight enlargement by isoproterenol (ISP). (B) Representative Western blot analysis showing the changes in Anf.
**Supplemental References**


