

Hydrogen Sulfide Regulates Krüppel-Like Factor 5 Transcription Activity via Specificity Protein 1 S-Sulfhydration at Cys664 to Prevent Myocardial Hypertrophy

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Background—Hydrogen sulfide (H₂S) is a gasotransmitter that regulates multiple cardiovascular functions. Krüppel-like factor 5 (KLF5) exerts diverse functions in the cardiovascular system. Whether and how H₂S regulates KLF5 in myocardial hypertrophy is unknown.

Methods and Results—In our study, hypertrophic myocardial samples in the clinic were collected and underwent histological and molecular biological analysis. Spontaneously hypertensive rats and neonatal rat cardiomyocytes were studied for functional and signaling responses to GYY4137, an H₂S-releasing compound. Expression of cystathionine γ -lyase, a principal enzyme for H₂S generation in heart, decreased in human hypertrophic myocardium, whereas KLF5 expression increased. After GYY4137 administration for 4 weeks, myocardial hypertrophy was inhibited in spontaneously hypertensive rats, as demonstrated by improvement in cardiac structural parameters, heart mass, size of cardiac myocytes, and expression of atrial natriuretic peptide. H₂S diminished expression of KLF5 in myocardium of spontaneously hypertensive rats and in hypertrophic cardiomyocytes. H₂S also inhibits platelet-derived growth factor A promoter activity, decreased recruitment of KLF5 to the platelet-derived growth factor A promoter, and reduced atrial natriuretic peptide expression in angiotensin II-stimulated cardiomyocytes, and these effects are suppressed by KLF5 knockdown. KLF5 promoter activity and KLF5 expression was also reversed by H₂S. H₂S increased the S-sulfhydration on specificity protein 1 in cardiomyocytes. Moreover, H₂S decreased KLF5 promoter activity; reduced KLF5 mRNA expression; attenuated specificity protein 1 binding activity with KLF5 promoter; and inhibited hypertrophy after specificity protein 1 mutated at Cys659, Cys689, and Cys692 but not Cys664 overexpression.

Conclusions—These findings suggest that H₂S regulates KLF5 transcription activity via specificity protein 1 S-sulfhydration at Cys664 to prevent myocardial hypertrophy. (*J Am Heart Assoc.* 2016;5:e004160 doi: 10.1161/JAHA.116.004160)

Key Words: atrial natriuretic peptide • hydrogen sulfide • Krüppel-like factor 5 • myocardial hypertrophy • specificity protein 1 • S-sulfhydration

Hypertension is a common cardiovascular disease and a leading cause of mortality worldwide. Myocardial hypertrophy, structural remodeling, and extracellular matrix

accumulation are prominent in the left ventricle in the context of hypertension, which constitutes an independent risk factor for acute cardiovascular events such as myocardial ischemia

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or infarction and heart failure.¹ To date, however, the pathogenetic mechanisms underlying myocardial hypertrophy in this situation remain incompletely elucidated.

The Krüppel-like factor (KLF) family exerts diverse functions in the cardiovascular system.² It is noted that mice with deficient KLF5 expression (KLF5^{+/-} mice) exhibit decreased inflammatory vascular response and cardiac hypertrophy induced by reduced angiotensin II (Ang II).³

Hydrogen sulfide (H₂S) has long been known as a toxic gas, but the physiological importance of endogenously produced H₂S has become increasingly recognized in recent years, and it is now accepted as the third “gasotransmitter” after nitric oxide and carbon monoxide.⁴ Accumulating evidence suggests that H₂S influences a wide range of physiological and pathological processes, especially in the cardiovascular system, including modulation of blood vessel tone and cardioprotection, for which cystathionine γ -lyase (CSE) is the main enzyme for H₂S generation.^{5,6}

The cardiovascular protective effects of H₂S include homeostatic regulation of blood pressure and decreased production of myocardial reactive oxygen species, suggesting that H₂S may protect against hypertrophy.^{7,8} Several reports suggest that H₂S is able to regulate activity of transcriptional factor to perform its physiological or pathophysiological effects. H₂S inhibited nuclear factor κ B activation in oxidized low-density lipoprotein-stimulated macrophage.⁹ H₂S stabilized hypoxia-inducible factor 1 α and increased hypoxia-responsive gene expression in colon.¹⁰ H₂S also enhanced the nuclear translocation of signal transducer and activator of transcription 3.¹¹ Nevertheless, the pathogenesis of myocardial hypertrophy is complex, and the precise mechanisms by which H₂S may protect against it remain unclear.

To date, no information exists about the potential role of H₂S in modulating activity of transcriptional factor KLF5 in the regulation of myocardial hypertrophy. The aims of the present study were to examine whether H₂S can attenuate myocardial hypertrophy and to elucidate the possible role and detailed mechanism of KLF5 in this protective effect.

Methods and Materials

Collection and Analysis of Human Myocardium and Blood Samples

Human myocardium samples of hypertension were collected during cardiac surgical procedures of cardiac valve replacement and classified into those with no myocardial hypertrophy (n=21, control group) and those with myocardial hypertrophy (n=26, hypertrophy group) according to echocardiographic findings prior to surgery. Blood samples were also collected. Myocardial samples were subjected to histological or

molecular biological analysis. H₂S concentration in plasma and myocardium was also measured. Human study conformed to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the ethics committee of First Affiliated Hospital of Nanjing Medical University (approval no. 2014-SRFA-128). Written informed consent was obtained from all patients.

Measurement of Plasma Ang II

Plasma Ang II from the patients was measured using iodine I 125 Ang II radioimmunoassay kits (North Institute of Biological Technology). Each measurement was performed in duplicate and averaged for statistics analysis. According to the level of Ang II, samples with myocardial hypertrophy were classified into those with normal Ang II (53–115 pg/mL) and high Ang II (>115 pg/mL).

Immunohistochemistry

Human myocardium sections fixed in paraformaldehyde were incubated with anti-KLF5 and anti-CSE antibodies overnight at 4°C, washed 3 times, and incubated with affinity-purified biotinylated IgG for 1 hour at room temperature. Sections incubated with PBS instead of primary antibody served as negative controls. They were washed again and overlaid with streptavidin–biotin–peroxidase complex for 1 hour at room temperature (Maixin Bio). After a final wash, labeling was performed with 3,3'-diaminobenzidine (Maixin Bio). Counterstaining was then performed with Mayer's hematoxylin.

Measurement of H₂S in Plasma and Myocardium

H₂S in plasma was measured using an H₂S-specific microelectrode (ISO-H₂S-2; World Precision Instruments) connected to a free radical analyzer (TBR4100; World Precision Instruments).¹² The sensor was set to the 10-nA range and the poise voltage to +150 mV. Prior to initiation of the experiments, the sensor was polarized and calibrated by adding 4 aliquots of the Na₂S stock solution at final concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 μ mol/L. Concentrations of H₂S in the samples were calculated using a standard curve of Na₂S, and the level in patients was normalized to the level in those without hypertrophy.

H₂S in myocardium was determined in tissue homogenates.⁶ Briefly, the assay mixture (500 μ L) contained 460 μ L tissue homogenate, 20 μ L L-cysteine (10 mmol/L), and 20 μ L pyridoxal 5'-phosphate (2 mmol/L). Incubations were carried out in tightly sealed vials. After incubation (37°C, 30 minutes), concentration of H₂S was measured using an H₂S-specific microelectrode, as described earlier.

Animal Model

Male spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats aged 12 weeks were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, People's Republic of China). Normotensive Wistar-Kyoto rats served as controls (Wistar-Kyoto group, $n=10$), and age-matched SHRs were randomly divided into 4 groups ($n=10$ per group) that were assigned different dosages of GYY4137, a water-soluble H_2S -releasing compound (in mg/kg per day): 0 (SHR group), 10 (GYY10 group), 25 (GYY25 group), or 50 (GYY50 group). GYY4137 was given by intraperitoneal injection once daily over a 4-week period.

Animal experiments were performed in accordance with the US National Institutes of Health Guidelines for Care and Use of Laboratory Animals. This study was approved by the Committee on Animal Care of Nanjing Medical University (approval no. NJMU-ERLAUA-20110112).

Echocardiography

After 4 weeks of treatment, cardiac geometry and function were evaluated after anesthesia under light isoflurane (1–2%) anesthesia using an echocardiography system (Visual Sonics Vevo 2100; VisualSonics) equipped with a 12-MHz linear-array transducer. Two-dimensional images were obtained in the parasternal long- and short-axis views and apical 2- and 4-chamber views. Thicknesses of the interventricular septum and left ventricular (LV) posterior wall and LV end-systolic and end-diastolic diameters were measured from the 2-dimensional targeted M-mode echocardiographic tracings. LV ejection fraction and fractional shortening were derived by goal-directed, diagnostically driven software. Diastolic function was assessed by the ratio between the peak of initial inflow velocity (E wave) and the atrial contraction (A wave) of transmitral flow (E/A).

Recording of Hemodynamic Parameters

After echocardiography, anesthesia was induced using 3% isoflurane in 3 L/min of oxygen and maintained using 2.5% isoflurane per liter of O_2 . Arterial pressures were assessed using a Millar Tip catheter (Millar Instruments Inc) that was inserted into the right carotid artery and was connected to a pressure transducer. After an equilibration period of 30 minutes, systolic blood pressure, diastolic blood pressure, and mean arterial pressure were recorded. Then the catheter was advanced into the LV cavity. LV end-systolic pressure and maximal positive LV pressure development were determined from systolic LV performance. Diastolic LV function was represented by LV end-diastolic pressure, and maximal LV pressure decay was calculated. Arterial and LV pressures were recorded simultaneously using a data acquisition system (MedLab-U/4C501, Nanjing Medeas Science and Technology).

Assessment of Cardiac Index

After completion of hemodynamic measurements, rats were euthanized with an overdose of isoflurane (5% isoflurane with 2 L/min oxygen). Heart weight, body weight, heart mass index (heart weight:body weight ratio), LV weight, LV mass index (LV weight:body weight ratio) and LV weight:tibia length ratio were measured and calculated as indices of cardiac hypertrophy.

Neonatal Cardiomyocyte Culture and Treatment

Sprague-Dawley rats aged 1 to 3 days were euthanized by decapitation. Hearts were removed immediately. The ventricles were separated from the atria, trisected, and digested with 0.25% trypsin (Beyotime), at 37°C for 7 to 10 cycles until completely digested. All supernatants from each cycle except the first were pooled. DMEM (Wisent Inc) with 10% FBS (Wisent Inc) at a volume equal to that of supernatants was added to terminate digestion, cells were pelleted, and the pellet was resuspended in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. Bromodeoxyuridine (0.1 mmol/L; Sigma-Aldrich) was also added to prevent nonmyocyte proliferation. The cell suspension was incubated for 4 hours at 37°C in a humidified 5% CO_2 incubator to separate cardiomyocytes and fibroblasts. The cells from 5 rats were planted in a 6-well plate or other culture plate with equal area. Density of cardiomyocytes was adjusted to 4×10^5 cells/mL.

After neonatal rat cardiomyocytes had been cultured for 2 to 3 days, the medium was changed to DMEM supplemented with 0.5% FBS for 24 hours. Serum-starved cells were then pretreated with different concentrations of GYY4137 (12.5, 25.0, or 50.0 μ mol/L) or NaHS (100 μ mol/L) for 4 hours before being incubated with Ang II (100 nmol/L; Sigma-Aldrich) for a further 24 hours. ZYJ1122 (a structural analogue of GYY4137 lacking sulfur) and “spent” GYY4137 (GYY4137 dissolved for 72 hours with no definitive H_2S release) were used as controls in the experiment.^{6,7,13} After undergoing the various treatments outlined earlier, digested cells were photographed at high magnification using an inverted microscope.

Plasmid or siRNA Transfection

Cultured neonatal rat cardiomyocytes were serum deprived for 2 hours and then transfected with rat KLF5-specific siRNA (5'-AACCCGGAUCUGGAGAAGCGA-3'), nonspecific control siRNA (5'-GCGCGCUUUGUAGGAUUCG-3'), specificity protein 1 (SP-1) siRNA or nonspecific control (Santa Cruz Biotechnology, Dallas, TX); wild-type SP-1 or SP-1 mutated at Cys659, Cys664, Cys689, and Cys692 to Ala (Haibio, Shanghai, China); wild-type CSE plasmid (provided by R.W.) using the Lipofectamine 2000 or Lipofectamine 3000 reagent

(Invitrogen), according to the manufacturer's protocol. After 24 hours, the cells were pretreated with GYY4137 for 4 hours, followed by Ang II (100 nmol/L) for 24 hours.

Luciferase Reporter Assay

Neonatal rat cardiomyocytes were incubated in 12-well plates in DMEM supplemented with 10% FBS for 24 hours. Cells in each dish were transfected at 70% confluence with 1 µg KLF5 or platelet-derived growth factor A (PDGF-A; Zoonbio Biotechnology Co. Ltd) promoter–luciferase fusion plasmid and 0.1 µg of pRL-TK reporter plasmid (control reporter) using Lipofectamine 2000 reagent (Invitrogen). At 24 hours after transfection, cells were treated with GYY4137 (50 µmol/L) or vehicle for 4 hours, followed by Ang II stimulation for 24 hours. Cells were harvested in cell lysis buffer, and the assay was performed with a dual luciferase reporter assay system (Promega), according to the manufacturer's instructions. Firefly luciferase activity was normalized to that of control reporter. The relative luciferase activities compared with the luciferase activities of pRL-TK were determined in triplicate.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed using the Pierce Agarose ChIP Kit (Thermo Fisher Scientific), according to the manufacturer's recommendations. In brief, neonatal rat cardiomyocytes treated as described earlier were cross-linked with 1% formaldehyde for 10 minutes and sonicated 5 to 10 times each for 10 seconds at 4°C in lysis

buffer (150 mmol/L NaCl, 25 mmol/L Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) to reduce the average DNA length to 0.4 to 0.5 kb. The samples were then precleared with protein A agarose/salmon sperm DNA for 30 minutes at 4°C, followed by overnight incubation at 4°C with antibodies specific for KLF5 or SP-1 or normal rabbit IgG (as a negative control). Immune complexes were precipitated with protein A agarose for 1 hour. Precipitated genomic DNA was amplified by real-time polymerase chain reaction (PCR) with primers. Potential KLF5-binding sites on the PDGF-A promoter region were amplified with the primer pair 5'-TGTCTGCAAAACACGGACGGTG-3' (sense) and 5'-TTGATGTA-CAGGTGCATTCC-3' (antisense). Potential SP-1-binding sites on the KLF5 promoter region were amplified with the primer pair 5'-GACCTGCACACCATATAGCA-3' (sense) and 5'-CACTCTCTCCACCGCTCATA-3' (antisense).

Measurement of SP-1 Binding Activity

SP-1 binding activity was measured using an electrophoretic mobility shift assay kit (Thermo Fisher Scientific), following the manufacturer's instructions. In brief, nuclear extracts from cardiomyocytes were prepared using a Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific). Nuclear extracts (8 µg) were incubated with a reaction system (10× binding buffer 2 µL, poly(deoxyinosinic-deoxycytidylic) acid 1 µL, 50% glycerol 1 µL, 1% NP-40 1 µL, KCl 1 µL, MgCl₂ 1 µL). An SP-1 oligonucleotide probe was added to the samples. The nucleotide sequence of the oligonucleotides used for electrophoresis mobility

Table 1. Sequences of Primers Used

Gene	Sense Primer	Antisense Primer
Rat KLF2	5'- ACTTGCACTACACCAACTG-3'	5'- CTGTGACCCGTGTGCTTG-3'
Rat KLF3	5'- TCATGTACACCAGCCACCTG-3'	5'- CATGTACACCAGCCACCTG-3'
Rat KLF4	5'- TCAAGAGCTCATGCCACCGG-3'	5'- CTCGCCTGTGTGAGTTCGCA-3'
Rat KLF5	5'- AGCTCACCTGAGGACTCATA-3'	5'- GTGCGCAGTGCTCAGTTCT-3'
Rat KLF6	5'- CCTTACAGATGCTCTGGGA-3'	5'- GGAGAAACACCTGTCCACAGT-3'
Rat KLF9	5'- TGGCTGTGGAAAGTCTATGG-3'	5'- CTCGTCCGAGCGCGAGAACT-3'
Rat KLF10	5'- TACTGATGTCTTACCTACAG-3'	5'- GTACCACAACATGACCGTG-3'
Rat KLF11	5'-AACAGAATCACCTTAGCAGAG-3'	5'-AACAGCCAGAGACCATGG-3'
Rat KLF15	5'-GATGAGTTGTACGGCACC-3'	5'-CACTGCGCTCAGTTGATGG-3'
Rat ANP	5'-GCT TCC GGG ATG AAA ACG TC-3'	5'-ACC AAT CCA TTG CCG ACT ATG-3'
Rat CSE	5'-GAGGGAAGTCTTGAAATGGC-3'	5'-CGCAACATTTTCATTTCCCG-3'
Human <i>KLF5</i>	5'-CACTACTGCGATTACCCTG-3'	5'-GGTCTGTCAATTGAGGGAG-3'
Human <i>ANP</i>	5'- TCTGCCCTCCTAAAAAGCAA-3'	5'- ATCACAACCTCCATGGCAACA -3'
Human <i>CSE</i>	5'- GACTCTACATGTCCGAATGG -3'	5'- AACCTGTACACTGACGCTTCA-3'
18S	5'- AGTCCCTGCCCTTTGTACACA -3'	5'- CGATCCGAGGGCCTCACTA -3'

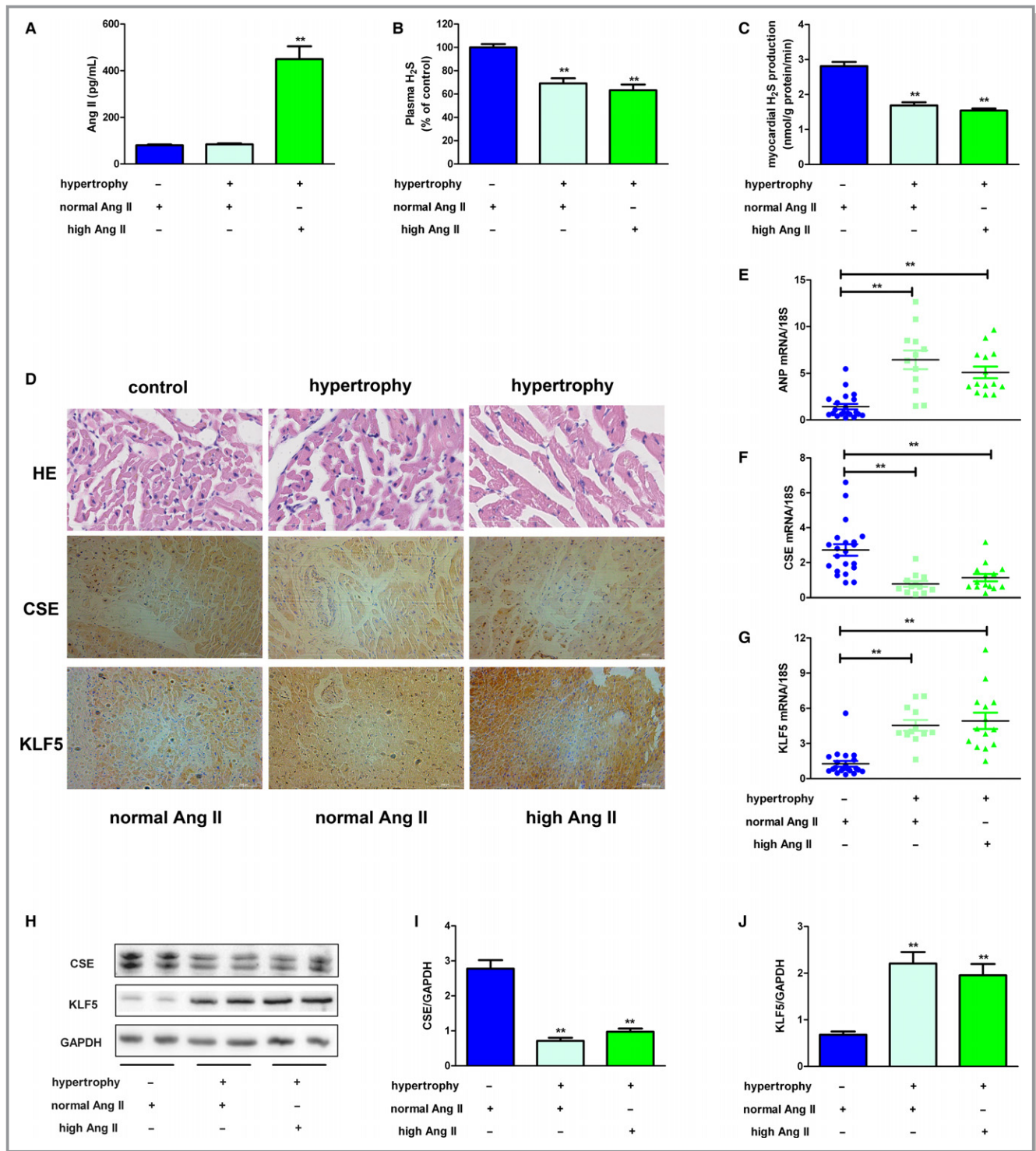


Figure 1. Level of H₂S in human plasma and myocardium and expression of ANP, CSE, and KLF5 in human myocardium. Myocardium or blood samples were collected from patients with hypertension, with or without left ventricular hypertrophy. A, Plasma Ang II concentration. B, H₂S concentration in plasma (as percentage of control). C, H₂S level in myocardium. D, Histological examination of human myocardium by HE staining (bar=100 μm) and measurement of CSE and KLF5 expression by immunohistochemistry staining (bar=50 μm). E, Quantification of ANP mRNA expression by real-time PCR. F–J, Measurement of CSE and KLF5 expression by real-time PCR and Western blotting. Sample size: (A–C, E–G) n=21 in control group, n=12 in the group with hypertrophy with normal Ang II, n=14 in the group with hypertrophy with high Ang II; (D) n=6; (H–J) n=12 in control group, n=6 in the group with hypertrophy with normal Ang II, n=6 in the group with hypertrophy with high Ang II. **P<0.01 vs control (without hypertrophy). Ang II indicates angiotensin II; ANP, atrial natriuretic peptide; CSE, cystathionine γ-lyase; H₂S, hydrogen sulfide; HE, hematoxylin and eosin; KLF5, Krüppel-like factor 5; PCR, polymerase chain reaction.

shift assay was 5'-AATCGATCGGGCGGGGCGAGC-3'. Synthetic double-strand oligonucleotides used in gel mobility shift assays were biotin labeled at the 5' end (Invitrogen). A probe without a biotin label as a nonspecific competitor was added to the mixture, followed by incubation at room temperature for 20 minutes prior to adding the labeled probe. Tubes were incubated for another 20 minutes at room temperature. DNA-protein complexes were resolved on nondenaturing acrylamide gels and transferred to a nylon membrane (Amersham Pharmacia Biotech). The proteins were ultraviolet cross-linked twice for 1 minute each time. Protein bands were visualized by enhanced chemiluminescence.

Quantitative Real-Time PCR

Total RNA was extracted using Trizol reagent (Takara). RNA (500 ng) was added as a template to reverse transcriptase reactions carried out using a PrimeScript RT Master Mix Kit (Takara). Quantitative real-time PCR was carried out with resulting cDNAs using SYBR Green Premix (Takara) and an ABI 7500 Real Time PCR System (Applied Biosystems). Experimental cycle threshold values were normalized to 18S, and relative mRNA expression was calculated versus a reference sample. Each sample was run and analyzed in triplicate (Table 1).

Western Blotting Analysis

Protein samples were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore). After blocking at room temperature in 5% vol/vol nonfat milk with TBST buffer (Tris-HCl 10 mmol/L, NaCl 120 mmol/L, Tween-20 0.1%, pH 7.4) for 2 hours, membranes were incubated overnight with the appropriate primary anti-KLF5, tubulin, and lamin B1 (1:1000; Santa Cruz Biotechnology); anti-SP-1 (1:2000; Abcam); anti-ANP (1:1000; Abcam); anti-CSE (1:1000; Bioworld); and anti-GAPDH (1:5000; Sigma-Aldrich) at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours. Protein bands were visualized by enhanced chemiluminescence.

“Tag-Switch” Method for the Detection of Protein S-Sulfhydration on SP-1

SP-1 S-sulfhydration was detected with the “Tag-Switch” method.¹⁴ Briefly, after treatment, neonatal cardiomyocytes were resuspended in HEN buffer (250 mmol/L HEPES, 50 mmol/L NaCl, 1 mmol/L EDTA, 0.1 mmol/L neocuproine, 1% NP-40) supplemented with protease inhibitors and lysed by 5×20-second cycles of ultrasonication. Cell lysate was mixed

with 50 mmol/L MSBT-A (a water-soluble methylsulfonyl benzothiazole), incubated 1 hour at 37°C, and then desalted on Micro Bio-Spin P-6 gel columns (Bio-Rad Laboratories). The protein of SP-1 was pulled down with immunoprecipitation and then treated with 20 mmol/L biotin-linked cyanoacetate in PBS containing 2.5% SDS for 1 hour at room temperature. The excess of biotin-linked cyanoacetate was removed by passing the samples through Micro Bio-Spin P-6 gel columns. Prior to electrophoresis, samples were mixed with nonreducing Laemmli buffer (Bio-Rad Laboratories) and boiled at 95°C for 1 minute. Proteins were resolved on 10% SDS polyacrylamide gels and then transferred on polyvinylidene fluoride membrane. Membrane was blocked with 5% nonfat milk for 1 hour at room temperature and incubated overnight with horseradish peroxidase-labeled mouse monoclonal anti-biotin antibody. The signal of protein S-sulfhydration was visualized using Pierce ECL reagent.

Statistical Analysis

All data are expressed as mean±SEM. Data with normal distribution were analyzed by *t* test or 1-way analysis of variance followed by the Bonferroni post hoc test, as appropriate. Data without normal distribution were analyzed by Kruskal-Wallis test (Stata 13.0 software; Stata-Corp). Values of *P*<0.05 were considered statistically significant.

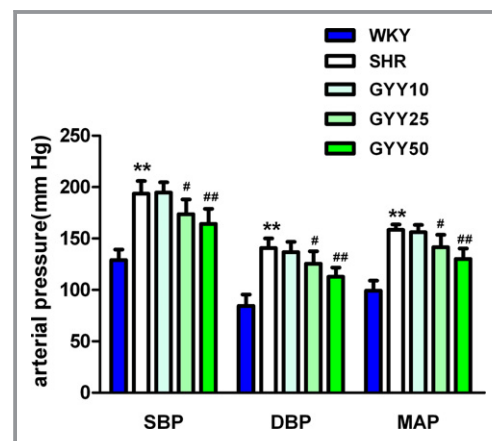


Figure 2. Effect of GYY4137 on blood pressure in SHRs. Male SHRs and WKY rats aged 12 weeks were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg per day (GYY10), 25 mg/kg per day (GYY25), or 50 mg/kg per day (GYY50) for 4 weeks. SBP, DBP, and MAP were measured from the left carotid artery after 4 weeks of treatment. *n*=10. ***P*<0.01 vs WKY rats; #*P*<0.05, ###*P*<0.01 vs SHRs. DBP indicates diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat.

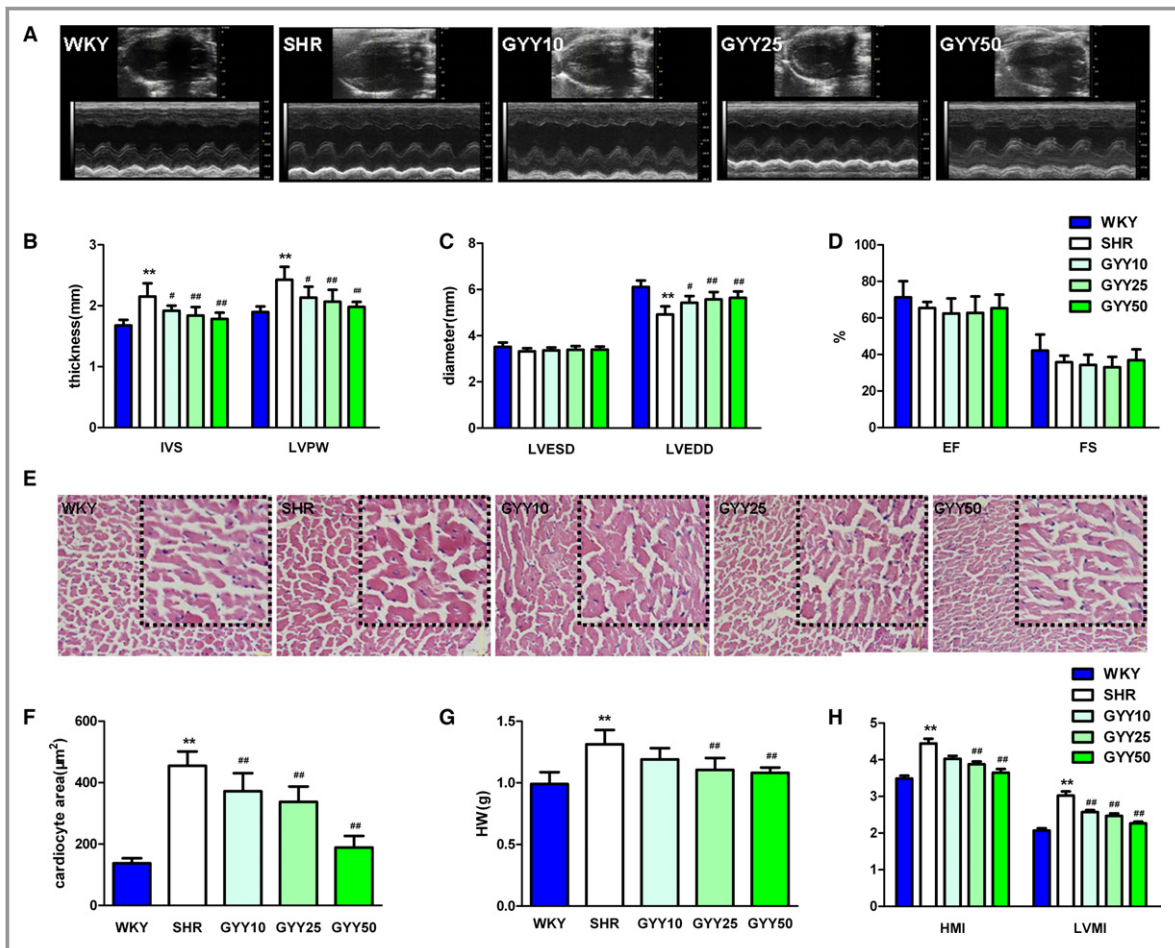


Figure 3. Effect of GYY4137 on myocardial hypertrophy in SHR and Ang II-induced neonatal rat cardiomyocytes. A, Male SHR and WKY rats at 12 weeks of age were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg per day (GYY10), 25 mg/kg per day (GYY25) or 50 mg/kg per day (GYY50) for 4 weeks. Representative 2-D M-mode echocardiograms in rat hearts after 4 weeks' treatment. B through D, Quantitative analysis of IVS and LVPW thickness, LVESD, LVEDD, EF, and FS by echocardiography (n=8). E, Histological examination with hematoxylin and eosin staining of myocardium. Bar=6.3 μm . F, Cardiomyocyte area was then calculated by morphometric analysis (n=10). G–I, HW, HMI, LVMI, and LVW/TL were measured and calculated (n=10). J, Quantification of ANP expression by real-time polymerase chain reaction (n=8). K, Quantification of ANP expression by Western blotting (n=3). L, Neonatal rat cardiomyocytes were pretreated with GYY4137 (12.5, 25, and 50 $\mu\text{mol/L}$) for 4 hours followed by Ang II (100 nmol/L) for a further 24 hours. Cells were digested, and cell surface area was calculated (n=4). M and N, Quantification of ANP mRNA and protein expression (n=4). Untreated cells served as control. For (A–K), ** P <0.01 vs WKY; # P <0.05, ## P <0.01 vs SHR. For (L–N), ** P <0.01 vs con; # P <0.05, ## P <0.01 vs cells treated with Ang II alone. Ang II, angiotensin II; ANP, atrial natriuretic peptide; EF, ejection fraction; FS, fractional shortening; HMI, heart mass index; HW, heart weight; IVS, interventricular septum; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; LVMI, left ventricular mass index; LVPW, left ventricular posterior wall; LVW/TL, left ventricular weight/tibia length ratio; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat.

Results

Hypertrophic Human Myocardium Exhibits Decreased CSE but Enhanced KLF5 Expression

According to the plasma level of Ang II, patients were classified into those with Ang II levels that were normal (53–115 pg/mL) or high (>115 pg/mL) (Figure 1A). H_2S concentrations in both plasma and myocardium were lower in

patients exhibiting myocardial hypertrophy (regardless of Ang II level) than in those without hypertrophy (Figure 1B and 1C). The presence or absence of myocardial hypertrophy according to echocardiograms was further confirmed by cardiomyocyte size (Figure 1D) and level of atrial natriuretic peptide (ANP; as an indicator of myocardial hypertrophy) mRNA expression in myocardium (Figure 1E). All of the hypertrophic myocardium samples, regardless of Ang II level,

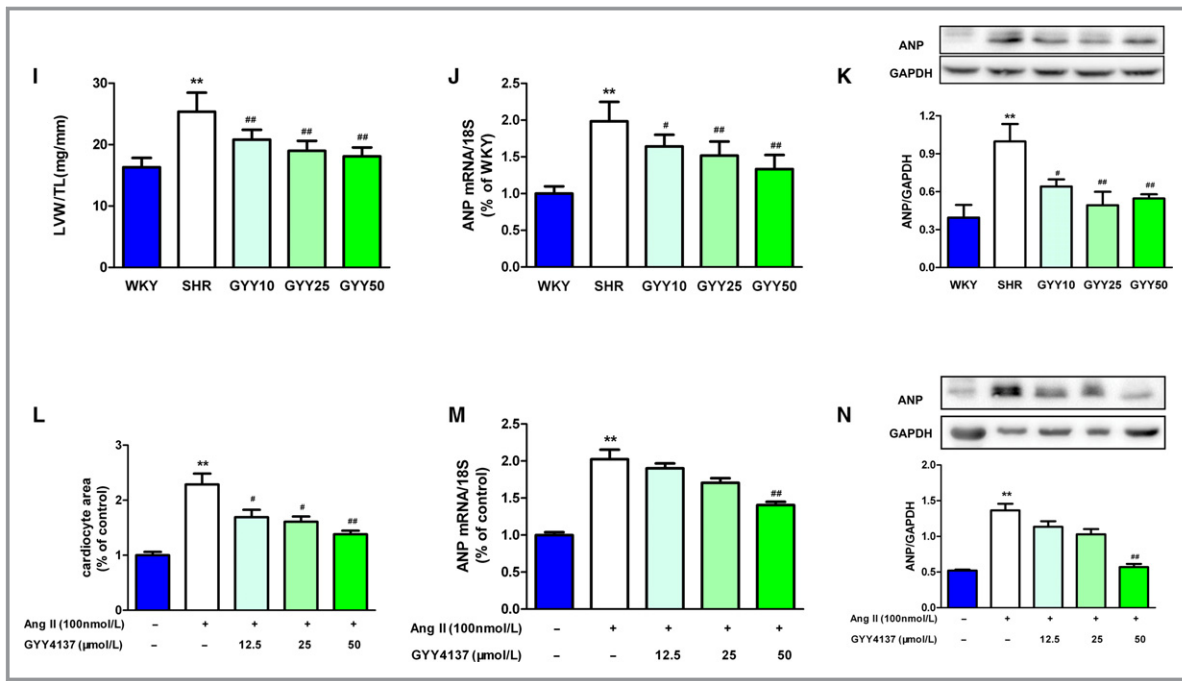


Figure 3. continued.

exhibited higher expression of KLF5 but lower expression of CSE, as assessed by immunohistochemistry, real-time PCR, and Western blotting (Figure 1D, 1F–1J). Consequently, we

investigated the effect of H₂S supplementation on myocardial hypertrophy and the possible involvement of KLF5 in its effect in this regard.

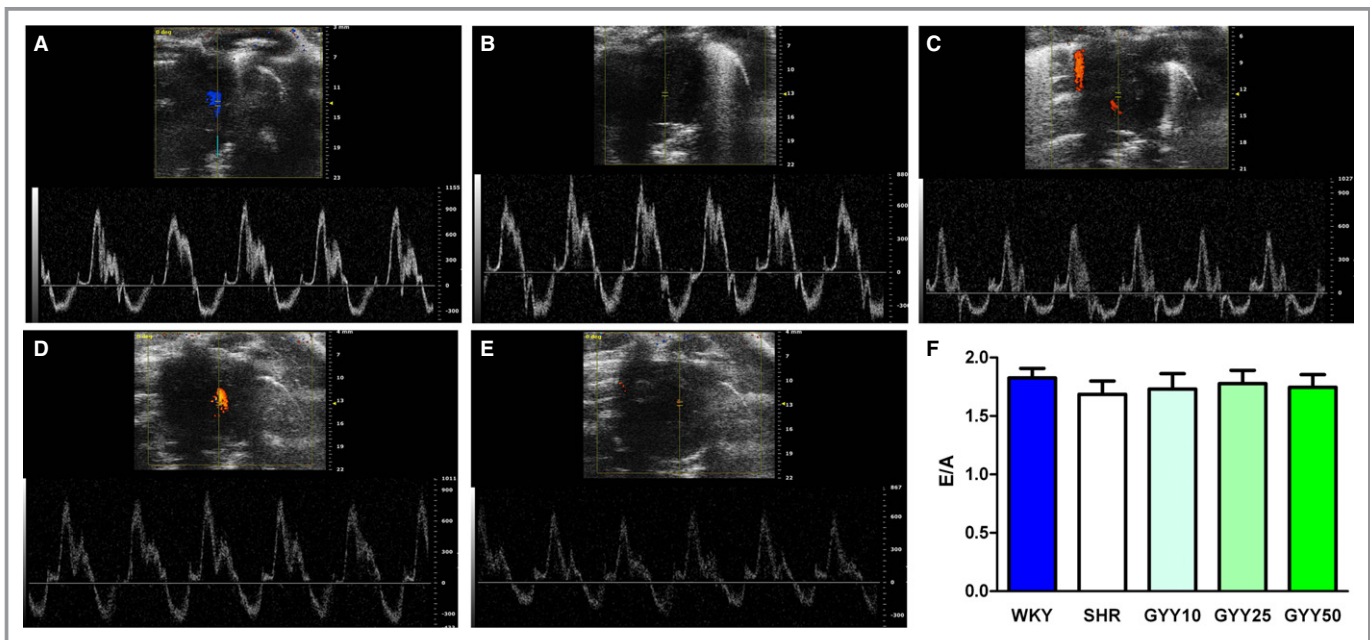


Figure 4. Effect of GYY4137 on cardiac diastolic function in SHRs. Male SHRs and WKY rats aged 12 weeks were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg per day (GYY10), 25 mg/kg per day (GYY25), or 50 mg/kg per day (GYY50) for 4 weeks. Cardiac diastolic function was assessed by the ratio of the peak of initial flow velocity (E wave) and the atrial contraction (A wave) of the transmitral flow (E/A). A, WKY rats. B, SHRs. C, GYY10. D, GYY25. E, GYY50. F, Quantitation of E/A ratio after GYY4137 treatment for 4 weeks. n=8. SHR indicates spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat.

Table 2. Effect of GYY4137 Treatment for 4 Weeks on Hemodynamic Parameters in SHR (n=10)

Parameter	WKY	SHR	GYY10	GYY25	GYY50
LVESP, mm Hg	104±9	185±8**	180±8	161±9 ^{††}	148±10 ^{††}
LVEDP, mm Hg	3.32±1.64	5.21±2.31	5.77±2.34	5.02±2.22	4.29±1.51
dp/dt _{max} , mm Hg/s	7301±801	8012±916	7674±1039	7235±990	7077±927
−dp/dt _{max} , mm Hg/s	6734±835	7102±872	7062±890	6422±553	6282±843
τ, ms	8.58±1.37	12.02±1.40**	11.03±1.56	10.71±1.55	10.35±1.48

−dp/dt_{max} indicates maximal left ventricular pressure decay; dp/dt_{max}, maximal positive left ventricular pressure development; GYY10, GYY4137 10 mg/kg/day; GYY25, GYY4137 25 mg/kg/day; GYY50, GYY4137 50 mg/kg/day; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat.

***P*<0.01 vs WKY, ^{††}*P*<0.01 vs SHR analyzed with 1-way analysis of variance followed by Bonferroni post hoc test.

H₂S Improves Myocardial Structure and Cardiac Function

Invasive arterial blood pressure measurement showed that SHRs aged 12 weeks treated with GYY4137 at 25 or 50 mg/kg per day for 4 weeks (but not 10 mg/kg per day) displayed decreased systolic blood pressure, diastolic blood pressure, and mean arterial pressure (Figure 2). M-mode echocardiography demonstrated that both interventricular septum and LV posterior wall thickness in SHRs aged 16 weeks were greater than those of age-matched normotensive Wistar-Kyoto controls and were attenuated by 4-week treatment with the 3 doses of GYY4137 (Figure 3A and 3B). LV end-diastolic diameter was increased after GYY4137 administration with no discernible effects on LV end-systolic diameter, LV ejection fraction, fractional shortening (Figure 3C and 3D), or E/A ratio (Figure 4). There were no significant differences in hemodynamic

parameters between groups except for LV end-systolic pressure (Table 2), suggesting that H₂S does not affect cardiac systolic or diastolic function in SHRs aged 16 weeks.

H₂S Attenuates Myocardial Hypertrophy in SHR and Ang II–Induced Neonatal Rat Cardiomyocyte Hypertrophy

Hematoxylin and eosin staining revealed cardiomyocytes in SHRs to be enlarged, and this was prevented by GYY4137 treatment for 4 weeks (Figure 3E and 3F). Compared with the Wistar-Kyoto group, heart weight, heart mass index, LV mass index, and LV weight:tibia length ratio (all indices of hypertrophy) were increased in the untreated SHR group, and these changes were prevented by H₂S (Figure 3G through 3I). ANP was expressed to a greater degree at both the mRNA and

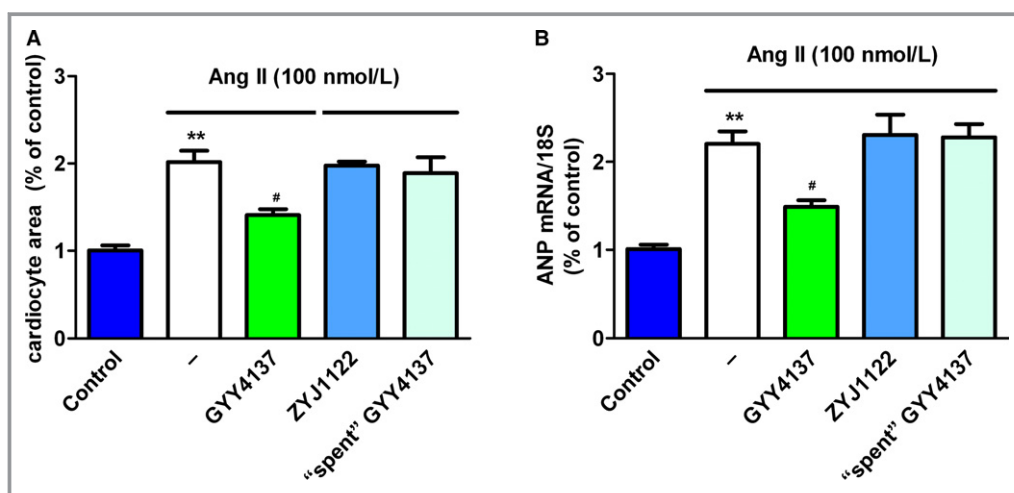


Figure 5. Effect of ZYJ1122 and "spent" GYY4137 on Ang II-stimulated neonatal rat cardiomyocytes hypertrophy. A, Neonatal rat cardiomyocytes were pre-treated with GYY4137 (50 μmol/L), ZYJ1122 (50 μmol/L) or "spent" GYY4137 (50 μmol/L of GYY4137 dissolved in cell culture medium for 72 hours) for 4 hours followed by Ang II (100 nmol/L) for a further 24 hours. Cells were digested and cell surface area was calculated. B, Quantification of ANP mRNA by real-time PCR. Untreated cells served as control (con). n=4. ***P*<0.01 vs con; #*P*<0.05, vs cells treated with Ang II alone. Ang II indicates angiotensin II; ANP, atrial natriuretic peptide.

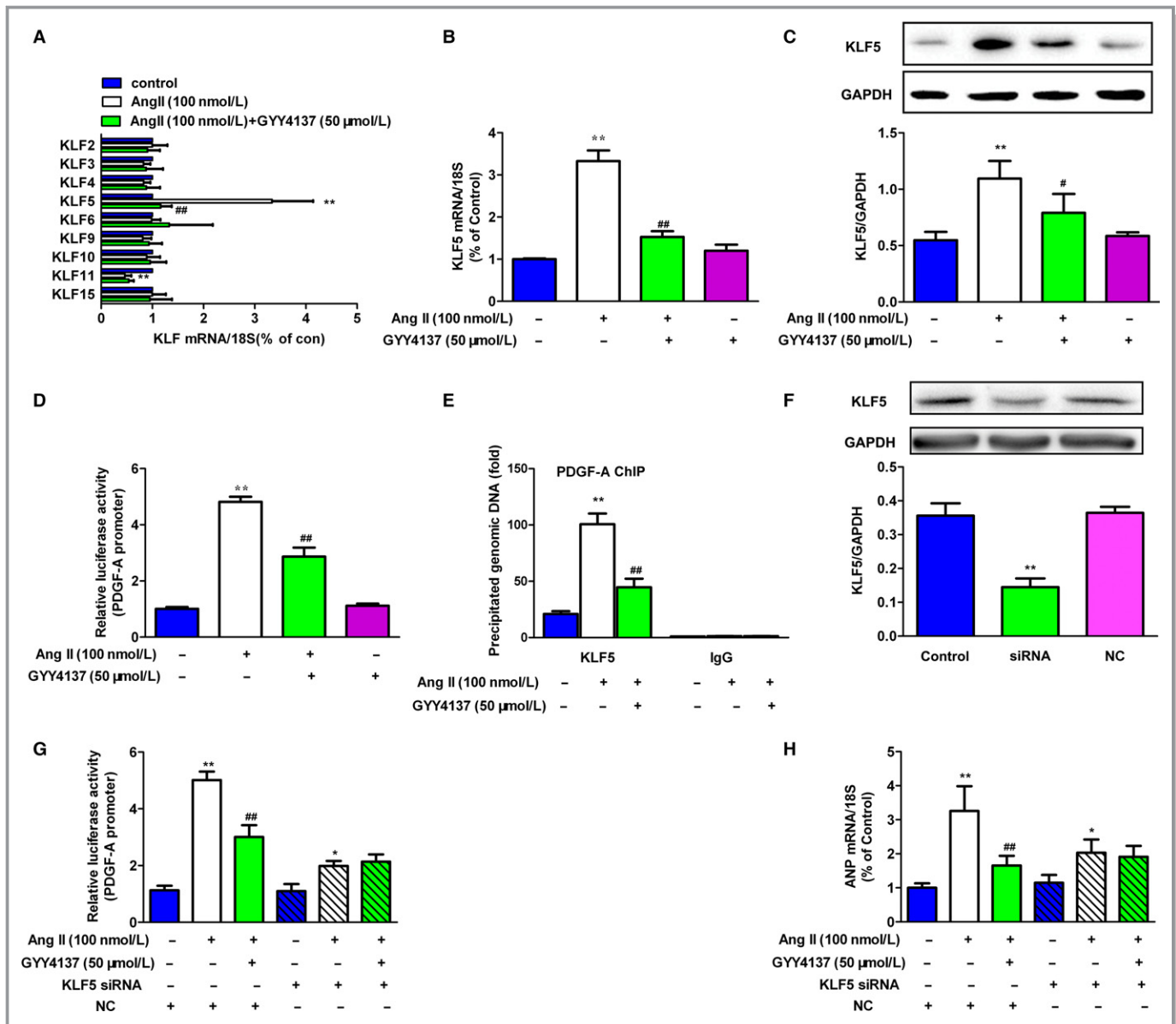


Figure 6. Effect of GYY4137 on KLF5 expression in Ang II–stimulated neonatal rat cardiomyocytes. A, Neonatal rat cardiomyocytes were pretreated with GYY4137 (50 μmol/L) for 4 hours before incubation with Ang II (100 nmol/L) for a further 24 hours. Quantification of KLF mRNA by real-time PCR. B, Quantification of KLF5 mRNA expression by real-time PCR. C, Quantification of KLF5 protein expression by Western blotting. D, PDGF-A promoter luciferase activity was measured. E, Chromatin fragments for ChIP assays were immunoprecipitated with anti-KLF5 antibody. Precipitated DNA was amplified by real-time PCR with primers spanning the PDGF-A promoter region. A–E, n=4. ***P*<0.01 vs cells not undergoing any drug treatment; #*P*<0.05, ##*P*<0.01 vs cells treated with Ang II alone. F, After rat KLF5-specific siRNA (KLF5 siRNA) or NC siRNA transfection for 48 hours, KLF5 protein expression was measured with Western blotting (n=4). ***P*<0.01 vs NC. G, After siRNA transfection for 24 hours, cells were pretreated with GYY4137 (50 μmol/L) for 4 hours followed by Ang II (100 nmol/L) stimulation for 24 hours. PDGF-A promoter luciferase activity was measured. Four independent experiments were performed. H, Quantification of ANP mRNA expression by real-time PCR. G and H, n=4. **P*<0.05, ***P*<0.01 vs cells not undergoing any drug treatment (with corresponding siRNA transfection), ##*P*<0.01 vs cells treated with Ang II alone (with corresponding siRNA transfection). Ang II indicates angiotensin II; ANP, atrial natriuretic peptide; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; KLF, Krüppel-like factor; NC, nonspecific control; PCR, polymerase chain reaction; PDGF-A, platelet-derived growth factor A.

protein levels in myocardium from SHRs, and this increase in expression was attenuated by GYY4137 treatment for 4 weeks (Figure 3J and 3K).

Cultured neonatal rat cardiomyocytes were induced into hypertrophy with Ang II, as shown histologically by increased cardiomyocyte surface area and enhanced ANP

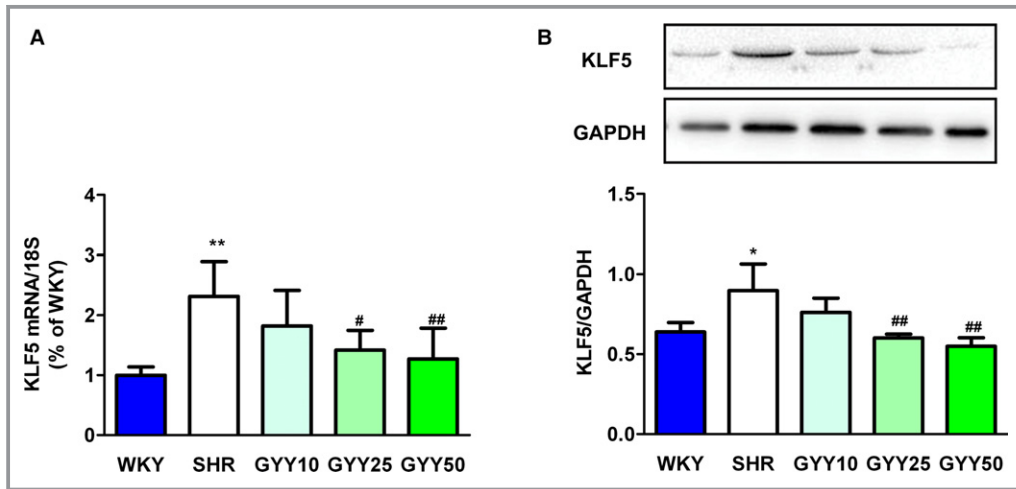


Figure 7. Effect of GYY4137 on KLF5 expression in SHR. A, Male SHRs and WKY rats aged 12 weeks were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg per day (GYY10), 25 mg/kg per day (GYY25) or 50 mg/kg per day (GYY50) for 4 weeks. Quantification of KLF5 mRNA in myocardium by real-time polymerase chain reaction. B, Quantification of KLF5 protein in myocardium by Western blotting. n=4 to 8. * P <0.05, ** P <0.01 vs WKY; # P <0.05, ## P <0.01 vs SHR. KLF5 indicates Krüppel-like factor 5; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat.

mRNA and protein expression; these changes were all inhibited by pretreatment with GYY4137 (12.5–50 μ mol/L) (Figure 3L–3N). Because 50 μ mol/L GYY4137 produced the most profound effects in these parameters, this concentration was used in the following experiments. To exclude the

influence of structure of GYY4137 on the attenuated responses to hypertrophy, ZYJ1122 (a structural analogue of GYY4137 lacking sulfur) and “spent” GYY4137 (GYY4137 dissolved for 72 hours with no definitive H₂S release) were used in the experiment. In addition, we found that only

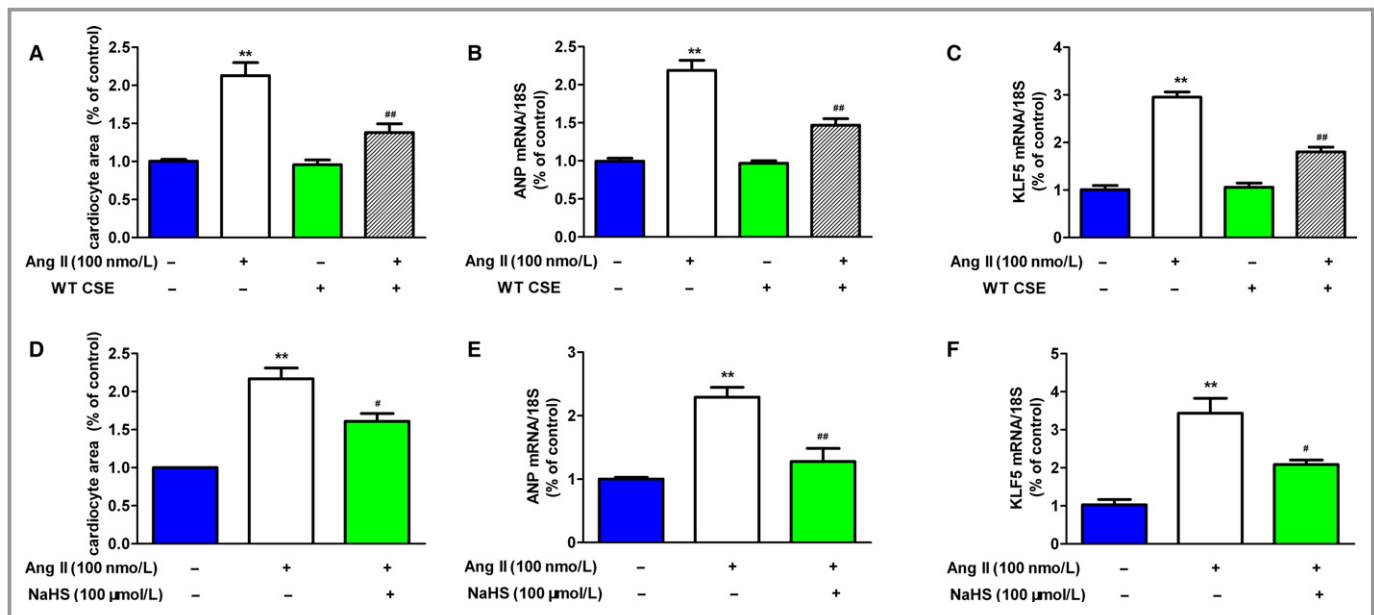


Figure 8. Effect of CSE overexpression and NaHS on Ang II-stimulated neonatal rat cardiomyocytes hypertrophy and KLF5 expression. A, After WT CSE was transfected for 24 hours, neonatal rat cardiomyocytes were stimulated with Ang II (100 nmol/L) for 24 hours. Cells were digested, and cell surface area was calculated. B and C, Quantification of ANP and KLF5 mRNA by real-time PCR. D, Neonatal rat cardiomyocytes were pretreated with NaHS (100 μ mol/L) for 4 hours followed by Ang II (100 nmol/L) for a further 24 hours. Cells were digested, and cell surface area was calculated. E and F, Quantification of ANP and KLF5 mRNA by real-time PCR. n=4. Untreated cells served as control. ** P <0.01 vs control; ## P <0.01 vs cells treated with Ang II alone. Ang II indicates angiotensin II; ANP, atrial natriuretic peptide; CSE, cystathionine γ -lyase; KLF5, Krüppel-like factor 5; PCR, polymerase chain reaction; WT, wild type.

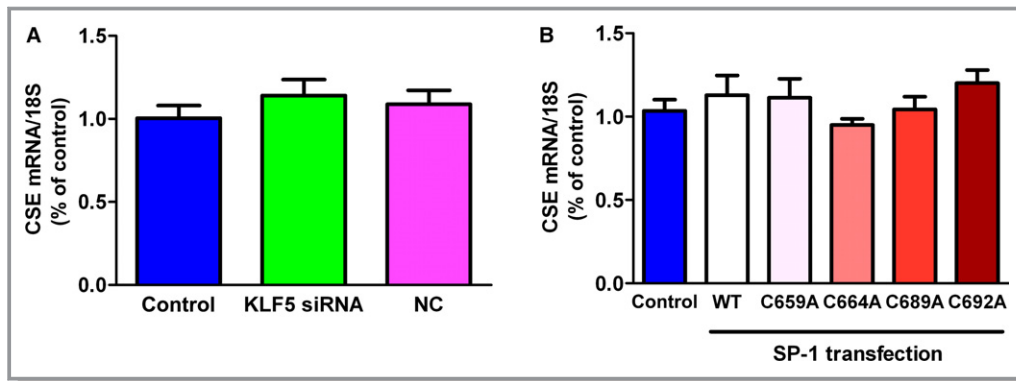


Figure 9. Effect of KLF5 siRNA or mutated SP-1 on CSE expression. A, After rat KLF5-specific siRNA or NC siRNA transfection for 48 hours, CSE mRNA was measured with real-time PCR. B, After plasmid transfection of WT SP-1 or mutated SP-1 at Cys659, Cys664, Cys689, or Cys692 for 48 hours, CSE mRNA was measured with real-time PCR (n=4). CSE indicates cystathionine γ -lyase; KLF5, Krüppel-like factor 5; NC, nonspecific control; PCR, polymerase chain reaction; SP-1, specificity protein 1; WT, wild type.

GGY4137—not ZYJ1122 or “spent” GYY4137—was able to decrease cardiocyte area and ANP mRNA expression in Ang II-stimulated neonatal rat cardiomyocytes (Figure 5).

H₂S Diminishes KLF5 Expression Both In Vitro and In Vivo

Quantitative analysis for KLF mRNA expression indicated that mRNA expression of KLF5 was increased and that of KLF11 was decreased in neonatal rat cardiomyocytes after Ang II stimulation. The increased KLF5 expression, but not the decreased KLF11 expression, was reversed by GYY4137 pretreatment (Figure 6A). Moreover, H₂S diminished KLF5 mRNA and protein expression in Ang II-stimulated cardiomyocytes (Figure 6B and 6C), whereas no alteration in KLF5 expression was detected if cardiomyocytes were treated with GYY4137 alone. GYY4137 administered for 4 weeks exerted a similar attenuating effect on KLF5 expression in myocardium of SHR (Figure 7A and 7B). It is noted that both CSE overexpression (endogenously increasing H₂S) and NaHS (another traditional H₂S donor) also decreased cardiocyte area and reduced mRNA expression of ANP and KLF5 in Ang II-stimulated cardiomyocytes (Figure 8A through 8F).

H₂S Suppresses Ang II-Stimulated Neonatal Rat Cardiomyocyte Hypertrophy in a KLF5-Dependent Manner

Ang II increased PDGF-A promoter activity and enhanced the recruitment of KLF5 to the PDGF-A promoter, which was decreased after GYY4137 coincubation (Figure 6D and 6E). KLF5 siRNA attenuated the effect of GYY4137 on decreasing PDGF-A promoter activity and ANP mRNA expression (Figure 6F through 6H). Moreover, there was no significant

difference on CSE mRNA expression if KLF5 was knocked down (Figure 9A). These data suggest that H₂S suppresses Ang II-stimulated cardiomyocyte hypertrophy in a KLF5-dependent manner.

H₂S Attenuates KLF5 Transcriptional Activation in Ang II-Stimulated Neonatal Rat Cardiomyocytes

Luciferase (Luc) reporter assays revealed higher KLF5 promoter (−2300/+236) activity with Ang II stimulation, and GYY4137 reversed this effect (Figure 10A). In a series of deletion constructs at −2300/+236, −363/+236, and −67/+236, the attenuating effects of GYY4137 on KLF5 promoter activity were observed in −2300 Luc, −363 Luc, and −67 Luc, respectively; however, GYY4137-induced inhibition of KLF5 promoter activity was abolished in −32 Luc (Figure 10B). It appears that an important site between 67 and 32 bp upstream of the KLF5 promoter sequence is responsible for the attenuating effect of H₂S on KLF5 transcription. Interestingly, the putative SP-1 binding site (5′-GGCGGG-3′) is present in this region of the KLF5 promoter. Electrophoresis mobility shift assay suggested that GYY4137 decreased binding activity between SP-1 and DNA (Figure 10C and 10D), and chromatin immunoprecipitation assay confirmed that GYY4137 decreased SP-1 binding activity to the KLF5 promoter, which was enhanced after Ang II stimulation (Figure 10E). Moreover, the attenuating effect of GYY4137 on KLF5 promoter activity and KLF5 mRNA expression was weakened after SP-1 protein knockdown (Figure 10F through 10H). Taken together, our results suggest that H₂S decreases SP-1 binding to the KLF5 promoter and attenuates KLF5 transcriptional activity in Ang II-stimulated cardiomyocytes. As a transcription factor, KLF5 exerts its potential biological activity only once it enters the nucleus to bind with target

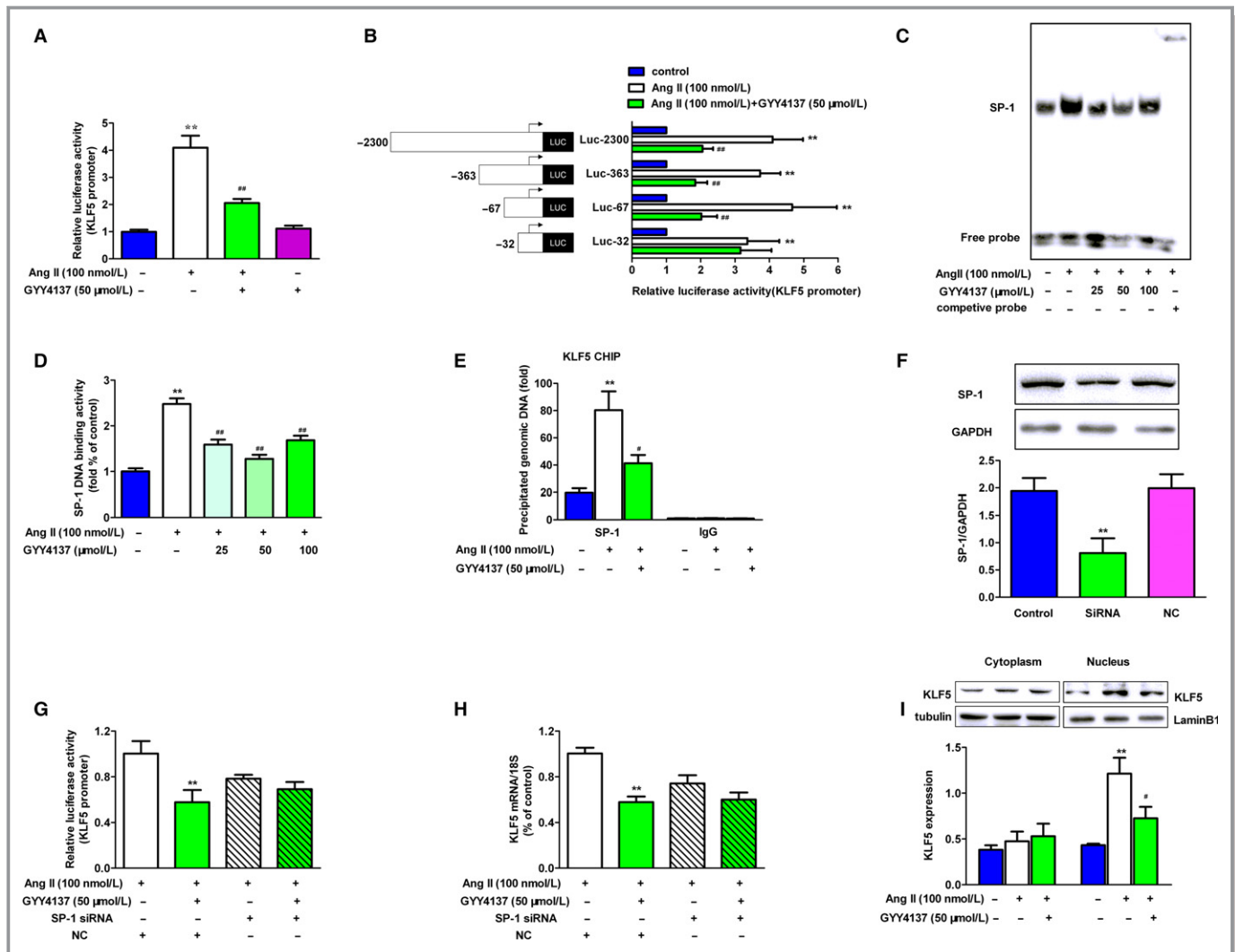


Figure 10. Effect of GYY4137 on KLF5 transcriptional activity in neonatal rat cardiomyocytes. A and B, Neonatal rat cardiomyocytes were pretreated with GYY4137 (50 μmol/L) for 4 hours before incubation with Ang II (100 nmol/L) for a further 24 hours. KLF5 promoter activity was determined using a dual-Luc reporter assay system. C and D, Electrophoresis mobility shift assay was performed with a DNA probe harboring the SP-1 site. E, Chromatin fragments for ChIP assays were immunoprecipitated with anti-SP-1 antibody. Precipitated DNA was amplified by real-time polymerase chain reaction with primers spanning the KLF5 promoter region. F, After rat SP-1-specific siRNA or NC siRNA transfection for 48 hours, SP-1 protein expression was measured. $**P < 0.01$ vs NC. G and H, After siRNA transfection for 24 hours, cells were pretreated with GYY4137 (50 μmol/L) for 4 hours followed by Ang II (100 nmol/L) stimulation for 24 hours. KLF5 promoter luciferase activity (G) and KLF5 mRNA expression (H) were measured. I, KLF5 expression in cytoplasm and nucleus from cardiomyocytes was measured. Untreated cells served as control ($n=4$). A–E and G–I, $**P < 0.01$ vs control; $\#P < 0.05$, $\#\#P < 0.01$ vs cells treated with Ang II alone. Ang II indicates angiotensin II; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; KLF5, Krüppel-like factor 5; Luc, luciferase; NC, nonspecific control; SP-1, specificity protein 1.

genes. After Ang II stimulation for 24 hours, we found that KLF5 protein expression in the nuclei of cardiomyocytes was increased, and GYY4137 attenuated this increase, with no discernible change in cytoplasm (Figure 10I).

H₂S Sulfhydrated SP-1 at Cys664 to Regulate KLF5 Transcription Activity and Prevent Myocardial Hypertrophy

We found stronger S-sulfhydration on SP-1 after GYY4137 administration in neonatal rat cardiomyocytes (Figure 11A)

and myocardium of SHR (Figure 11B). According to the structure and bioinformatics analysis of SP-1, there were 11 cysteine residues on SP-1, 4 of which (at 659, 664, 689, and 692) located into the domain to bind with KLF5 promoter. Next, SP-1 mutated at Cys659, Cys664, Cys689, or Cys692 to Ala (C659A, C664A, C689A or C692A), or wild type was transfected into neonatal rat cardiomyocytes. GYY4137 still enhanced S-sulfhydration on SP-1 after overexpression of wild-type SP-1 or mutated SP-1 at Cys659, Cys689 or Cys692 but not at Cys664 overexpression (Figure 11C). GYY4137 was able to attenuate KLF5

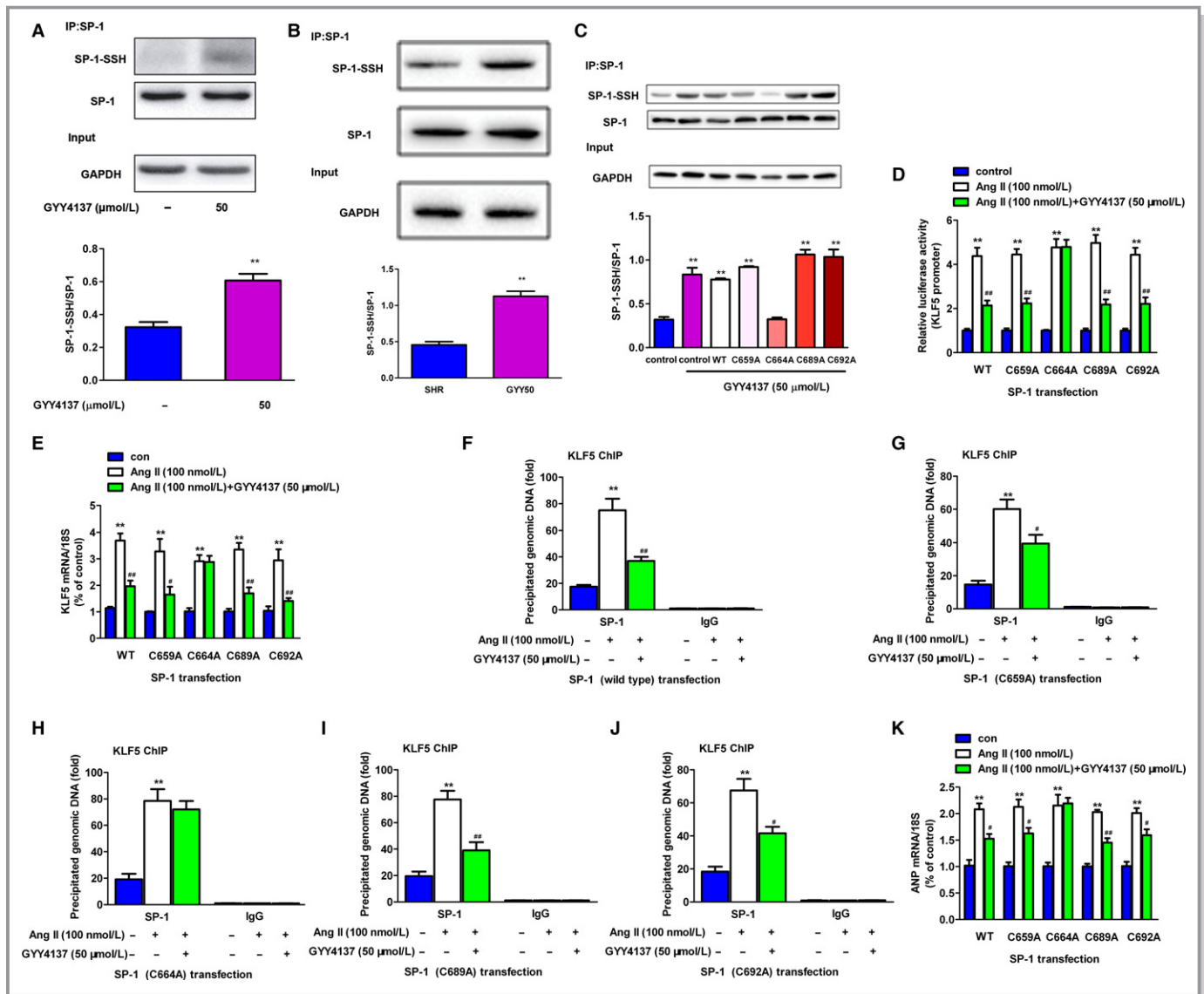


Figure 11. GYY4137 sulfhydrated SP-1 at Cys664 to regulate KLF5 transcription activity and to prevent myocardial hypertrophy. A, Neonatal rat cardiomyocytes were incubated with GYY4137 (50 μmol/L) for 24 hours. SP-1-SSH was detected with the “Tag-Switch” method. B, Male SHRs aged 12 weeks were given GYY4137 by intraperitoneal injection at doses of 50 mg/kg per day (GYY50) for 4 weeks. SP-1-SSH in myocardium was detected with the Tag-Switch method. C, After plasmid transfection of WT SP-1 or mutated SP-1 at Cys659, Cys664, Cys689 or Cys692 for 48 hours, SP-1-SSH was detected with the Tag-Switch method. D and E, After plasmid transfection of WT SP-1 or mutated SP-1 for 24 hours, cells were pretreated with GYY4137 (50 μmol/L) for 4 hours followed by Ang II (100 nmol/L) stimulation for 24 hours. KLF5 promoter luciferase activity (D) and KLF5 mRNA expression (E) were measured. F–J, Chromatin fragments for ChIP assays were immunoprecipitated with anti-SP-1 antibody. Precipitated DNA was amplified by real-time polymerase chain reaction with primers spanning the KLF5 promoter region. K, ANP mRNA expression was measured. Untreated cells served as control (n=4). ***P*<0.01 vs control; #*P*<0.05, ##*P*<0.01 vs cells treated with Ang II alone. Ang II indicates angiotensin II; ANP, atrial natriuretic peptide; ChIP, chromatin immunoprecipitation; IgG, immunoglobulin G; IP, immunoprecipitation; KLF5, Krüppel-like factor 5; SHR, spontaneously hypertensive rat; SP-1, specificity protein 1; SSH, S-sulfhydration; WT, wild type.

promoter activity and mRNA expression in Ang II-stimulated cardiomyocytes after SP-1-wild type, SP-1-C659A, SP-1-C689A, or SP-1-C692A but not SP-1-C664A overexpression (Figure 11D and 11E). Chromatin immunoprecipitation assay confirmed that GYY4137 could decrease SP-1 binding activity to the KLF5 promoter, which was enhanced after

Ang II stimulation but not in mutated SP-1 at Cys664A overexpression (Figure 11F through 11J). Moreover, GYY4137 inhibited hypertrophy, represented as reduced ANP mRNA expression, after SP-1 mutated at Cys659, Cys689, and Cys692 but not at Cys664 (Figure 11K). No significant difference was noted on CSE mRNA expression if

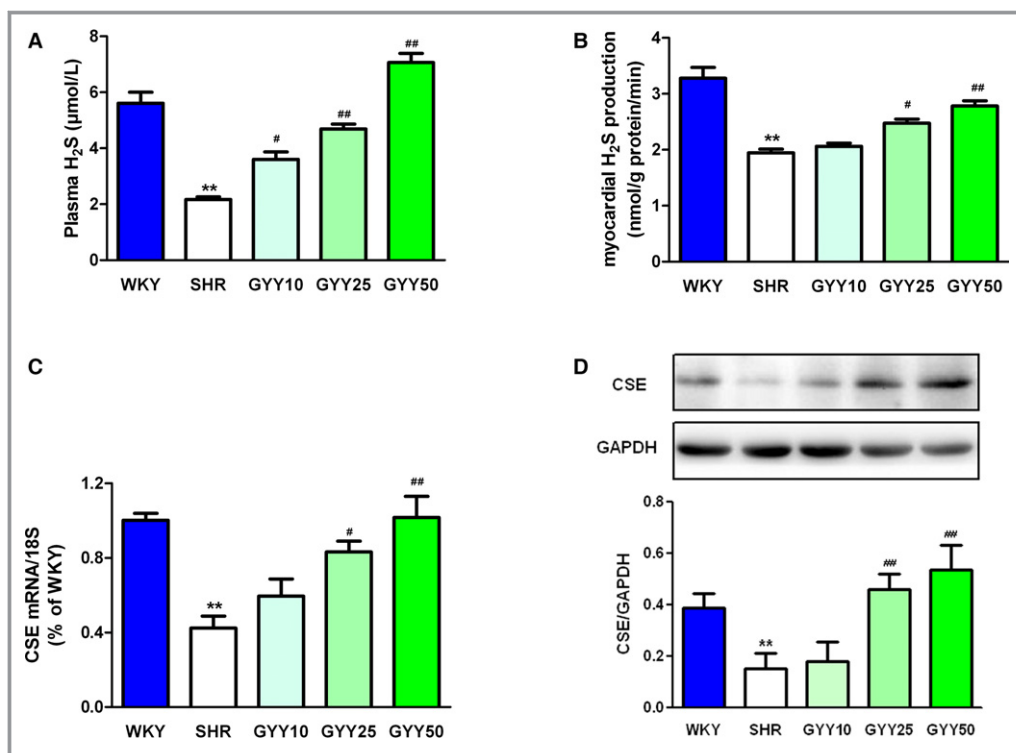


Figure 12. Effect of GYY4137 on the CSE/H₂S system in SHR rats. Male SHR and WKY rats aged 12 weeks were given GYY4137 by intraperitoneal injection at doses of 10 mg/kg per day (GYY10), 25 mg/kg per day (GYY25), or 50 mg/kg per day (GYY50) for 4 weeks. A, Plasma H₂S concentration. B, H₂S in myocardium. C and D, CSE expression as quantified by real-time polymerase chain reaction and Western blotting (n=6 to 8). ***P*<0.01 vs WKY rats; #*P*<0.05, ##*P*<0.01 vs SHRs. CSE indicates cystathionine γ -lyase; H₂S, hydrogen sulfide; SHR, spontaneously hypertensive rat; WKY rat, Wistar-Kyoto rat.

wild-type or mutated SP-1 was transfected into cardiomyocytes (Figure 9B).

Discussion

In the present study, we found that H₂S levels in both plasma and myocardium and CSE mRNA and protein expression levels in myocardium were decreased in SHRs (Figure 12) and in patients with myocardial hypertrophy, regardless of Ang II level. These data suggested that the impaired H₂S/CSE system contributes to hypertrophy rather than Ang II. Moreover, we found greater KLF5 mRNA and protein expression in hypertrophic myocardium from patients. These data tie in well with our findings in rodents and raise the possibility that H₂S supplementation might be a clinically useful therapeutic strategy in the future for attenuating cardiac hypertrophy.

The role, if any, of H₂S on myocardial hypertrophy has previously been entirely unclear. NaHS has been reported to suppress cardiac hypertrophy, possibly by modifying expression of Cx43 in rats with abdominal aortic coarctation.¹⁵ In rats with isoproterenol-induced heart failure, NaHS protects against myocardial hypertrophy by suppressing local renin

production.⁶; however, NaHS treatment for 3 months was not found to reduce myocardial hypertrophy in a previous study on SHRs.¹⁷ Divergent effects of H₂S on myocardial hypertrophy may be explained by different types of H₂S donors used in such studies, different treatment durations, different animal models, different ages, or a combination of these factors. GYY4137, an H₂S slow-releasing compound, was used in our present study. When incubated in aqueous buffer (pH 7.4, 37°C), GYY4137 released H₂S, climbed for 15 minutes, and then plateaued at 75 minutes. After administration (intravenous or intraperitoneal) of GYY4137 to anesthetized rats, plasma H₂S concentration was increased at 30 minutes and remained elevated over the 180-minute time course of the experiment.⁷ We found that GYY4137 at doses of 10, 25, and 50 mg/kg per day attenuated myocardial hypertrophy in SHRs; however, high blood pressure is a predominant factor for the development of myocardial hypertrophy, and blood pressure also decreases in SHRs treated with H₂S.^{7,18} Moreover, H₂S is an endothelium-derived hyperpolarizing factor, activating potassium channels to relax vascular smooth muscle.¹⁹ Nevertheless, we found that, at low dose, GYY4137 also attenuated myocardial hypertrophy without any discernible antihypertensive effect in our study, suggesting

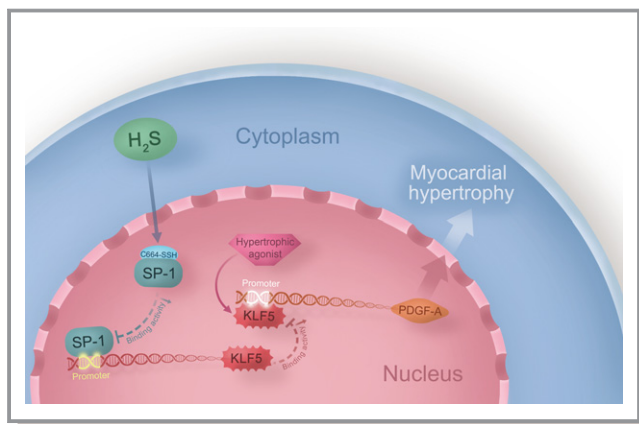


Figure 13. Schematic depicting the proposed signal mechanism by which H₂S attenuates myocardial hypertrophy. H₂S sulfhydrates SP-1 at Cys664 to attenuate SP-1 binding activity with KLF5 promoter, decreases KLF5 expression, and then reduces KLF5 binding activity with PDGF-A promoter to inhibit myocardial hypertrophy. H₂S, hydrogen sulfide; KLF5, Krüppel-like factor 5; PDGF-A, platelet-derived growth factor A; SP-1, specificity protein 1.

that the inhibitory effect of H₂S on myocardial hypertrophy is created, at least in part, through a blood pressure-independent mechanism.

The KLF family of gene regulatory proteins is composed of transcription factors that are implicated in many biological processes, including proliferation, apoptosis, differentiation, development, survival, and responses to external stress.² The primary KLF knockout phenotype includes cardiovascular development abnormalities and higher susceptibility to injury or cardiac failure, which suggests that KLF plays an important regulatory role in the cardiovascular system.^{20,21} We hypothesized that KLF might be responsible for the attenuation of myocardial hypertrophy in response to H₂S. We first used real-time PCR to determine whether H₂S alters expression of KLF family members. In our study, KLF11 expression was impaired after Ang II stimulation in cardiomyocytes. KLF11 plays important roles in transforming growth factor β -mediated cell growth and differentiation.²² Knockdown of KLF11 potentiates basal and transforming growth factor β -stimulated endothelin-1 expression in human endothelial cells.²² In our study, however, the reduction in KLF11 could not be restored by H₂S, suggesting that KLF11 is not involved in its protective effect on cardiomyocyte hypertrophy. Moreover, GYY4137 pretreatment only attenuated KLF5 mRNA levels in the presence of Ang II stimulation. Consequently, we focused on KLF5 in subsequent experiments.

KLF5 is a key regulator of cardiovascular remodeling.²³ Heterozygous KLF5 knockout mice (KLF5^{+/-}) show diminished levels of arterial wall thickening, angiogenesis, cardiac hypertrophy, and interstitial fibrosis in response to stress.³ In cultured cardiac fibroblasts, Ang II increases expression of

KLF5 and PDGF-A, which controls tissue remodeling.³ Haploinsufficiency of KLF5 suppresses cardiac fibrosis and hypertrophy elicited by moderate-intensity pressure overload.²⁴ It has also been reported previously that KLF5 can interact with many other transcription factors (eg, c-Jun, retinoic acid receptor α , CREB binding protein, PPAR- δ) and can activate various gene promoters such as a non-muscle-type myosin heavy chain gene SMemb, plasminogen activator inhibitor 1, inducible nitric oxide synthase, PDGF-A, early growth response 1, and vascular endothelial growth factor receptors, at least in vitro, and thereby regulate the expression of many genes.²⁵ In our study, the attenuating effect of H₂S on PDGF-A promoter activity and ANP mRNA expression was weakened after KLF5 knockdown, suggesting that H₂S might act on a transcriptional element and suppress PDGF-A promoter activity and cardiomyocyte hypertrophy in a KLF5-dependent manner.

KLF5 can also be regulated by a variety of transcription factors and nuclear receptors, such as retinoic acid receptor α , nuclear factor κ B, PPAR- γ , and p300.^{26,27} In our study, we found that H₂S can decrease SP-1 binding to the KLF5 promoter and thus suppress KLF5 expression. There are abundant exposed cysteine groups in the structure of SP-1, and it is possible that H₂S may target SP-1 by S-sulfhydration. Several reports found that S-sulfhydration is a newly defined posttranslational modification and plays an important role in regulating (mostly inhibiting) protein activity and physiological effects.^{14,28–30} We found that there was stronger S-sulfhydration on SP-1 after GYY4137 incubation. In addition, GYY4137 regulated KLF5 transcriptional activity and inhibited hypertrophy after SP-1 mutated at Cys659, Cys689, and Cys692 but not Cys664 overexpression. This suggests that sulfhydration of SP-1 at Cys664 by H₂S might be one of the detailed mechanisms to suppress KLF5 transcription activity and thus inhibit progression of myocardial hypertrophy (Figure 13).

In conclusion, this study provides evidence that H₂S increases SP-1 sulfhydration at Cys664 to regulate KLF5 transcription activity to prevent the development of myocardial hypertrophy. These data highlight a novel role for KLF5 in the protective effect of H₂S on myocardial hypertrophy. An H₂S-related compound may be a useful therapeutic strategy in clinical conditions associated with excessive hypertension and myocardial hypertrophy in the future.

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Disclosures

None.

References

- Georgiopoulou VV, Kalogeropoulos AP, Raggi P, Butler J. Prevention, diagnosis, and treatment of hypertensive heart disease. *Cardiol Clin*. 2010;28:675–691.
- McConnell BB, Yang VW. Mammalian Kruppel-like factors in health and diseases. *Physiol Rev*. 2010;90:1337–1381.
- Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R. Kruppel-like zinc-finger transcription factor KLF5/BTE2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med*. 2002;8:856–863.
- Wang R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol Rev*. 2012;92:791–896.
- Liu YH, Lu M, Hu LF, Wong PT, Webb GD, Bian JS. Hydrogen sulfide in the mammalian cardiovascular system. *Antioxid Redox Signal*. 2012;17:141–185.
- Liu Z, Han Y, Li L, Lu H, Meng G, Li X, Shirhan M, Peh MT, Xie L, Zhou S, Wang X, Chen Q, Dai W, Tan CH, Pan S, Moore PK, Ji Y. The hydrogen sulfide donor, GYY4137, exhibits anti-atherosclerotic activity in high fat fed apolipoprotein E (-/-) mice. *Br J Pharmacol*. 2013;169:1795–1809.
- Li L, Whiteman M, Guan YY, Neo KL, Cheng Y, Lee SW, Zhao Y, Baskar R, Tan CH, Moore PK. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. *Circulation*. 2008;117:2351–2360.
- Ahmad FU, Sattar MA, Rathore HA, Abdullah MH, Tan S, Abdullah NA, Johns EJ. Exogenous hydrogen sulfide (H₂S) reduces blood pressure and prevents the progression of diabetic nephropathy in spontaneously hypertensive rats. *Ren Fail*. 2012;34:203–210.
- Du J, Huang Y, Yan H, Zhang Q, Zhao M, Zhu M, Liu J, Chen SX, Bu D, Tang C, Jin H. Hydrogen sulfide suppresses oxidized low-density lipoprotein (ox-LDL)-stimulated monocyte chemoattractant protein 1 generation from macrophages via the nuclear factor kappaB (NF-kappaB) pathway. *J Biol Chem*. 2014;289:9741–9753.
- Flannigan KL, Agbor TA, Motta JP, Ferraz JG, Wang R, Buret AG, Wallace JL. Proresolution effects of hydrogen sulfide during colitis are mediated through hypoxia-inducible factor-1alpha. *FASEB J*. 2015;29:1591–1602.
- Kan J, Guo W, Huang C, Bao G, Zhu Y, Zhu YZ. S-propargyl-cysteine, a novel water-soluble modulator of endogenous hydrogen sulfide, promotes angiogenesis through activation of signal transducer and activator of transcription 3. *Antioxid Redox Signal*. 2014;20:2303–2316.
- Xie L, Feng H, Li S, Meng G, Liu S, Tang X, Ma Y, Han Y, Xiao Y, Gu Y, Shao Y, Park CM, Xian M, Huang Y, Ferro A, Wang R, Moore PK, Wang H, Ji Y. SIRT3 mediates the anti-oxidant effect of hydrogen sulfide in endothelial cells. *Antioxid Redox Signal*. 2016;24:329–343.
- Lee ZW, Zhou J, Chen CS, Zhao Y, Tan CH, Li L, Moore PK, Deng LW. The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One*. 2011;6:e21077.
- Zhang D, Macinkovic I, Devarie-Baez NO, Pan J, Park CM, Carroll KS, Filipovic MR, Xian M. Detection of protein S-sulfhydration by a tag-switch technique. *Angew Chem Int Ed Engl*. 2014;53:575–581.
- Huang J, Wang D, Zheng J, Huang X, Jin H. Hydrogen sulfide attenuates cardiac hypertrophy and fibrosis induced by abdominal aortic coarctation in rats. *Mol Med Rep*. 2012;5:923–928.
- Liu YH, Lu M, Xie ZZ, Hua F, Xie L, Gao JH, Koh YH, Bian JS. Hydrogen sulfide prevents heart failure development via inhibition of renin release from mast cells in isoproterenol-treated rats. *Antioxid Redox Signal*. 2014;20:759–769.
- Shi YX, Chen Y, Zhu YZ, Huang GY, Moore PK, Huang SH, Yao T, Zhu YC. Chronic sodium hydrosulfide treatment decreases medial thickening of intramyocardial coronary arterioles, interstitial fibrosis, and ROS production in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2007;293:H2093–H2100.
- Lu M, Liu YH, Goh HS, Wang JJ, Yong QC, Wang R, Bian JS. Hydrogen sulfide inhibits plasma renin activity. *J Am Soc Nephrol*. 2010;21:993–1002.
- Mustafa AK, Sikka G, Gazi SK, Steppan J, Jung SM, Bhunia AK, Barodka VM, Gazi FK, Barrow RK, Wang R, Amzel LM, Berkowitz DE, Snyder SH. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ Res*. 2011;109:1259–1268.
- Nemer M, Horb ME. The KLF family of transcriptional regulators in cardiomyocyte proliferation and differentiation. *Cell Cycle*. 2007;6:117–121.
- Pearson R, Fleetwood J, Eaton S, Crossley M, Bao S. Kruppel-like transcription factors: a functional family. *Int J Biochem Cell Biol*. 2008;40:1996–2001.
- Spittau B, Kriegstein K. Klf10 and Klf11 as mediators of TGF-beta superfamily signaling. *Cell Tissue Res*. 2012;347:65–72.
- Nagai R, Suzuki T, Aizawa K, Shindo T, Manabe I. Significance of the transcription factor KLF5 in cardiovascular remodeling. *J Thromb Haemost*. 2005;3:1569–1576.
- Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, Shindo T, Sano M, Otsu K, Snider P, Conway SJ, Nagai R. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. *J Clin Invest*. 2010;120:254–265.
- Nagai R, Shindo T, Manabe I, Suzuki T, Kurabayashi M. KLF5/BTE2, a Kruppel-like zinc-finger type transcription factor, mediates both smooth muscle cell activation and cardiac hypertrophy. *Adv Exp Med Biol*. 2003;538:57–65; discussion 66.
- Drosatos K, Pollak NM, Pol CJ, Ntziachristos P, Willecke F, Valenti MC, Trent CM, Hu Y, Guo S, Aifantis I, Goldberg IJ. Cardiac myocyte KLF5 regulates Ppara expression and cardiac function. *Circ Res*. 2016;118:241–253.
- He M, Han M, Zheng B, Shu YN, Wen JK. Angiotensin II stimulates KLF5 phosphorylation and its interaction with c-Jun leading to suppression of p21 expression in vascular smooth muscle cells. *J Biochem*. 2009;146:683–691.
- Lu C, Kavalier A, Lukyanov E, Gross SS. S-sulfhydration/desulfhydration and S-nitrosylation/denitrosylation: a common paradigm for gasotransmitter signaling by H₂S and NO. *Methods*. 2013;62:177–181.
- Paul BD, Snyder SH. H₂S: a novel gasotransmitter that signals by sulfhydration. *Trends Biochem Sci*. 2015;40:687–700.
- Paul BD, Snyder SH. H₂S signalling through protein sulfhydration and beyond. *Nat Rev Mol Cell Biol*. 2012;13:499–507.