Hydrogen Sulfide Attenuates Cardiac Dysfunction After Heart Failure Via Induction of Angiogenesis

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Background—Hydrogen sulfide (H$_2$S) has been shown to induce angiogenesis in in vitro models and to promote vessel growth in the setting of hindlimb ischemia. The goal of the present study was to determine the therapeutic potential of a stable, long-acting H$_2$S donor, diallyl trisulfide, in a model of pressure-overload heart failure and to assess the effects of chronic H$_2$S therapy on myocardial vascular density and angiogenesis.

Methods and Results—Transverse aortic constriction was performed in mice (C57BL/6J; 8–10 weeks of age). Mice received either vehicle or diallyl trisulfide (200 µg/kg) starting 24 hours after transverse aortic constriction and were followed up for 12 weeks using echocardiography. H$_2$S therapy with diallyl trisulfide improved left ventricular remodeling and preserved left ventricular function in the setting of transverse aortic constriction. H$_2$S therapy increased the expression of the proangiogenic factor, vascular endothelial cell growth factor, and decreased the angiogenesis inhibitor, angiostatin. Further studies revealed that H$_2$S therapy increased the expression of the proliferation marker, Ki67, as well as increased the phosphorylation of endothelial NO synthase and the bioavailability of NO. Importantly, these changes were associated with an increase in vascular density within the H$_2$S-treated hearts.

Conclusions—These results suggest that H$_2$S therapy attenuates left ventricular remodeling and dysfunction in the setting of heart failure by creating a proangiogenic environment for the growth of new vessels. (Circ Heart Fail. 2013;6:1077-1086.)

Key Words: angiogenesis ■ diallyl trisulfide ■ endothelial nitric oxide synthase ■ H$_2$S donor ■ nitric oxide

Heart failure is a heterogeneous syndrome that can result from a number of common disease stimuli, including, but not limited to long-standing hypertension, myocardial infarction, or ischemia associated with coronary artery disease. The prevalence of heart failure has increased dramatically because modern therapies have reduced the in-hospital mortality of acute myocardial infarction. In the United States, it has become the most common discharge diagnosis in patients aged ≥65 years and the primary cause of readmission within 60 days of discharge. Current treatments for heart failure are woefully inadequate, and the availability of hearts for transplantation is severely limited. Therefore, adjunct pharmacotherapies designed to coincide with the standard means of care are needed to decrease the extent of injury leading to the development of heart failure.

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For centuries, the consumption of garlic has been recognized for its health benefits. For instance, garlic has been associated with reducing cardiovascular risk and diabetes mellitus, stimulating the immune system, protecting against infection, and inducing anticancer effects. The organosulfur compounds, such as diallyl trisulfide (DATS), found in garlic are considered to be responsible for its pharmacological activity. Recent evidence indicates that DATS mediates the vasoactive properties of garlic via the sustained release of hydrogen sulfide (H$_2$S). H$_2$S is a gaseous signaling molecule with a diverse physiological profile. In the heart, treatment with exogenous H$_2$S or modulation of the endogenous production of H$_2$S through the cardiatic-specific overexpression of the H$_2$S-generating enzyme, cystathionine γ-lyase, protects against acute myocardial ischemia-reperfusion injury and heart failure by attenuating oxidative stress, inhibiting apoptosis, and reducing inflammation. In contrast, pharmacological inhibition or genetic deficiency of cystathionine γ-lyase results in vascular dysfunction and an exacerbation of myocardial injury. Furthermore, DATS administration was recently shown to reduce infarct size and improve contractile function after acute myocardial ischemia-reperfusion injury by restoring cardiac H$_2$S levels.
Angiogenesis is a complex biological process characterized by extracellular matrix remodeling and changes in endothelial cell behavior that leads to increased growth, migration, and assembly into capillary structures.\textsuperscript{12} It remains an attractive therapeutic option for the treatment of heart failure. Recently, several in vitro studies indicate that $\text{H}_2\text{~S}$ induces angiogenesis,\textsuperscript{13,14} and there is evidence that $\text{H}_2\text{~S}$ promotes vessel growth in a wound-healing manner\textsuperscript{15} and in the setting of hindlimb ischemia.\textsuperscript{16} However, there are not any data about the ability of $\text{H}_2\text{~S}$ to induce vessel formation in the setting of heart failure. The goal of the present study was to determine the therapeutic potential of a stable, long-acting $\text{H}_2\text{~S}$ donor, DATS, in a model of pressure-overload heart failure and to assess the effects of chronic $\text{H}_2\text{~S}$ therapy on myocardial vascular density and angiogenesis.

**Methods**

An expanded version of the Methods can be found in the online-only Data Supplement.

**Mice**

Male C57BL/6J mice 8 to 10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). All experimental protocol were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

**DATS Preparation and Administration**

DATS (LKT Labs, St. Paul, MN) was maintained in sealed amber glass ampules and kept at $-20^\circ\text{C}$ until use. On the day of experimentation, a fresh ampule of DATS was opened; 5 µL of DATS was diluted in 500 µL of 100% dimethyl sulfoxide (DMSO) followed by further dilution in sterile saline to obtain the correct dosage to be delivered in a volume of 50 µL. The resulting concentration of DMSO in this dosage was 1%. Vehicle consisted of a solution of 1% DMSO in sterile saline. DATS (200 µg/kg) or Vehicle (1% DMSO) groups were injected intraperitoneally once per day for 12 weeks after transverse aortic constriction (TAC). This dose of DATS was selected on the basis of our previous experience investigating DATS in murine models of cardiac ischemia/reperfusion injury.\textsuperscript{11}

**TAC Protocol and Echocardiography**

To create pressure overload, TAC procedure was performed in mice by placing a 7-0 silk suture around the aortic arch between the brachiocephalic trunk and the left carotid artery. The suture was ligated around a 27G blunt needle. The needle was immediately removed after ligation. At 2 days before TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) ultrasound scanner. At 2 days before TAC procedure, baseline transthoracic echocardiogram was performed in C57/BL6 mice and evaluated cardiac structure and function using 2-dimensional echocardiography (Figure 1A). For these experiments, Vehicle (1% DMSO) or DATS (200 µg/kg) was administered intraperitoneally daily starting at 24 hours after TAC surgery. Analysis at 1 week after TAC revealed that DATS increased both circulating and cardiac free $\text{H}_2\text{~S}$ and sulfane sulfur (bound sulfide) levels (Figure 1B–1G). After TAC procedure, echocardiography was also performed in the same manner for up to 12 weeks. To determine cardiac structure and function, intraventricular septal end-diastolic thickness, left ventricular (LV) end-diastolic dimension, LV end-systolic dimension, and LV ejection fraction were analyzed from M-mode images.

**Histology**

Hearts were collected at the indicated times, fixed in 10% buffered formalin, embedded in paraffin and stained with Masson’s trichrome and Picrosirius Red.

**Vascular Density and Cellular Proliferation Measurements**

Angiogenic index measurements from frozen tissue sections were performed as previously described.\textsuperscript{18} Briefly, hearts from the experimental groups were collected, dissected, and embedded in OCT (optimal cutting temperature compound) freezing medium. Frozen tissue blocks were cut into 5-µm sections and slides fixed for staining. Slides were stained with anti-CD31 antibody to calculate an angiogenesis index or with anti-Ki67 antibody to determine cellular proliferation index. We also performed formalin fixed tissue immunochemistry using anti–von Willebrand factor antibody staining to quantify the number of capillaries per unit area.

**Western Blot Analysis**

Myocardial tissue samples were taken homogenized, and lysates were used for Western blot analysis as previously described.\textsuperscript{17}

**Measurement of Nitrite Levels**

Nitrite analysis was performed as previously described.\textsuperscript{19}

**Measurement of $\text{H}_2\text{~S}$ and Sulfane Sulfur**

$\text{H}_2\text{~S}$ and sulfane sulfur levels were measured in heart and blood according to previously described methods.\textsuperscript{15}

**Statistical Analysis**

All data are expressed as means±SEM. Statistical significance was evaluated using unpaired Student $t$ test for comparison between 2 means and a 1-way ANOVA with Tukey test as the post hoc analysis for comparison among 3 means by use of Prism 5 (GraphPad Software Inc). For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the post hoc analysis was used. The following comparisons were made separately: (1) baseline versus postbaseline measurements at each time point for the DATS and vehicle groups and (2) DATS versus vehicle measurements at each time point. The $P$ value for these evaluations was adjusted by applying the Bonferroni correction for multiple comparisons. A value of $P<0.05$ denoted statistical significance, and $P$ values were 2-sided.

**Results**

**DATS Therapy Attenuates Cardiac Dysfunction After TAC**

To investigate the effects of DATS on pressure overload–induced cardiac hypertrophy and dysfunction, we performed TAC procedure in C57/BL6 mice and evaluated cardiac structure and function using 2-dimensional echocardiography (Figure 1A). For these experiments, Vehicle (1% DMSO) or DATS (200 µg/kg) was administered intraperitoneally daily starting at 24 hours after TAC surgery. Analysis at 1 week after TAC revealed that DATS increased both circulating and cardiac free $\text{H}_2\text{~S}$ and sulfane sulfur (bound sulfide) levels (Figure 1B–1G). At 6 weeks after TAC, both Vehicle and DATS-treated animals displayed a similar degree of intraventricular septal end-diastolic wall thickness (Figure 2A). However, administration of DATS significantly reduced LV cavity diameters, LV end-diastolic dimension, and LV end-systolic dimension, in treated mice at 6 weeks up to 12 weeks after TAC when compared with Vehicle-treated mice (Figure 2B and 2C). In addition, DATS treatment improved cardiac function beginning at 3 weeks after TAC (Figure 2D) as evidenced by improved LV ejection fraction. In addition, DATS-treated mice displayed significantly less of an increase in heart weight/tibia length ratios and less pulmonary edema when compared with Vehicle-treated mice at 12 weeks after TAC (Figure 2E and 2F).

**DATS Attenuates Myocardial Fibrosis After TAC**

We investigated the extent of LV fibrosis at 6 weeks after TAC (Figure 3). Histological analysis of Masson’s Trichrome and...
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Picrosirius Red stained sections revealed extensive areas of intermuscular and perivascular fibrosis in hearts from Vehicle-treated mice (Figure 3B and 3C; P<0.001 for Picrosirius Red and P<0.05 for Masson’s Trichrome versus Sham). Although fibrosis was evident in the sections taken from DATS-treated hearts, it was significantly less when compared with the Vehicle-treated hearts (P<0.01 for Picrosirius Red and P<0.05 for Masson’s Trichrome).

DATS Augments Myocardial Angiogenic Factors and Vascular Density After TAC

DATS significantly (P<0.001) increased myocardial CD31 protein levels compared with both Sham and Vehicle-treated mice (Figure 4A). At 6 weeks after DATS, treated mice exhibited significantly greater cardiac vascular endothelial growth factor (VEGF)-A protein expression compared with Sham mice (Figure 4A; P<0.05). Moreover, DATS also significantly increased basic fibroblast growth factor protein levels in the myocardium as compared with Sham mice (Figure 4B; P<0.05). Furthermore, DATS significantly reduced angiostatin protein, a known endogenous inhibitor of angiogenesis (Figure 4C; P<0.001 versus Sham). We also measured the capillary density and angiogenic index of the heart after TAC. Figure 5 shows that both the von Willebrand factor capillary density (Figure 5A and 5C) and the CD31:DAPI angiogenic index (Figure 5B and 5D) were increased by DATS therapy compared with Sham or Vehicle-treated groups. This increase in vascular density corresponds to an increase in cellular proliferation (Ki67:DAPI ratio) of the DATS treatment group (Figure 5B and 5E). Together, these results clearly demonstrate that DATS therapy significantly increases vascular density and cell proliferation in heart after TAC.

DATS Augments eNOS-NO Signaling After TAC

We next investigated whether DATS treatment modulated Akt phosphorylation in the heart after TAC (Figure 6). Total Akt expression was not different among all groups. However, a significant (P<0.05) increase in the phosphorylation of Akt at serine residue 473 (Akt-PSer473) was observed in hearts treated with DATS at 6 weeks after TAC compared with Sham.

Figure 1. Diallyl trisulfide (DATS) increases sulfide levels after transverse aortic constriction (TAC). A, Mice (C57 BL6/J) were subjected to TAC surgery and were studied for a period of 12 weeks after TAC. The H2S donor, DATS, was initiated at a dose of 200 µg/kg (daily IP injection) at 24 hours after TAC surgery. Vehicle-treated mice were administered 1% dimethyl sulfoxide (DMSO). Baseline echocardiography was performed 1 week before TAC and thereafter at 1, 3, 6, 9, and 12 weeks after TAC. Additional mice were euthanized at 6 weeks after TAC. Heart and lung tissue were collected for assessment of cardiac and lung weights, cardiac histology, and myocardial molecular determinations. In addition, blood samples were also collected for measurement of NO intermediates. Representative gas chromatograph peaks and summary data for (B–D) serum and (E–G) myocardial levels of free H2S and sulfane sulfur after 1 week of TAC in groups of mice injected daily with vehicle or DATS. Results are expressed as mean±SEM. Numbers in bars represent sample size.
mice. NO generated from endothelial NO synthase (eNOS) is known to modulate vascular angiogenesis and promote vascular and myocardial cell cytoprotection during ischemic conditions. Activation of AMP-activated protein kinase (AMPK) increases the phosphorylation and activity of eNOS. There were no differences in the phosphorylation of AMPK at threonine residue 172 (AMPK-PThr172) and total AMPK expression in the heart among all groups (Figure 6B). However, we observed a significant increase in the phosphorylation of eNOS at serine residue 1177 (eNOS-PSer1177; activation site) and a significant decrease in the phosphorylation of eNOS at threonine residue 495 (eNOS-PThr495; inhibition site) after treatment with DATS (Figure 6C) when compared with either Sham (P<0.05) or Vehicle-treated mice (P<0.01). There were
no differences in total eNOS expression in the heart among all groups. Furthermore, DATS treatment increased cardiac nitrite levels (Figure 6D) after TAC compared with Vehicle mice (P<0.01). Nitrite is an established biomarker for NO, suggesting that eNOS activation resulted in increased NO bioavailability after DATS treatment.

DATS Increases Glutathione Peroxidase 1 and Heme Oxygenase 1 Expression After TAC

We next investigated the effects of the administration of DATS after TAC on various antioxidants. We observed no significant difference in the protein levels of the glutathione subunit, glutamate-cysteine ligase, or heme oxygenase 2 (HO-2) among all groups (Figure 7A). However, DATS significantly increased the expression of glutathione peroxidase 1 (GPx-1) compared with Sham (P<0.05). We also observed a significant increase in HO-1 expression after TAC compared with Sham (P<0.01) and Vehicle-treated mice (P<0.05; Figure 7B).

Discussion

In response to myocardial injury, the LV undergoes morphological changes, resulting in ventricular remodeling that are initially considered adaptive. However, in response to sustained pathological stimuli, such as increased pressure, LV remodeling becomes maladaptive, leading to the development of heart failure. Moreover, the morphological and functional changes that accompany LV remodeling serve as predictors of morbidity and mortality. The underlying mechanisms of LV remodeling include many biological reactions, such as cell death, inflammation, oxidative stress, and development of fibrosis. These reactive processes stimulate each other and advance from acute cellular reactions to chronic anatomic and functional changes. In the current study, chronic administration of DATS, an organosulfur compound that augments H\(_2\)S levels, provides protection against the adverse remodeling associated with TAC by increasing circulating and cardiac sulfide levels. Specifically, we found that DATS therapy attenuated LV dilatation and dysfunction, attenuated the development of perivascular and intermuscular fibrosis, and attenuated the development of cardiac hypertrophy. These findings are in concert with previous studies, which demonstrated that both exogenous and endogenously derived H\(_2\)S exhibit potent cytoprotective effects in different models of heart failure. Together, these findings support recent experimental data demonstrating that H\(_2\)S is an important mediator of both cell survival and remodeling in the heart after the induction of heart failure.

Recent studies indicate that the heart progresses rapidly from a compensatory hypertrophic state to a state of decompensated failure when vascular growth cannot keep pace with pathological myocyte growth. VEGF, a potent angiogenic cytokine, plays a central role in coronary vascular network growth under these conditions, as evidenced by the finding that a reduction in VEGF signaling contributes to the rapid progression from compensatory cardiac hypertrophy to failure.
Recent evidence indicates that H$_2$S is a strong promoter of angiogenesis. For instance, exposing cultured endothelial cells to H$_2$S stimulates cell proliferation, migration, and tube formation. Additionally, H$_2$S promotes angiogenesis in vivo, as evidenced by an increase in the neovascularization of implanted matrigel plugs in mice after treatment with sodium hydrosulfide. Finally, the proangiogenic effects of H$_2$S are evident in models of chronic vascular disease, such as hindlimb ischemia. In the current study, H$_2$S therapy increased the protein expression of VEGF after TAC. This was associated with increased vascular density in the treated hearts, as evidenced by the observed increase in the expression of von Willebrand factor and CD31 and an increase in the proliferation marker, Ki67. H$_2$S therapy also decreased the expression of angiostatin, an inhibitor of angiogenesis, and increased the expression of basic fibroblast growth factor, a factor that facilitates the formation of new blood vessels. NO has also been reported to play a role in mediating VEGF-induced angiogenesis. Although NO and H$_2$S signaling have traditionally been considered to operate via distinct pathways, there is evidence of cross-talk between the 2 pathways. For instance, H$_2$S therapy improves survival after cardiac arrest and cardiopulmonary resuscitation in an eNOS-dependent manner and provides cardioprotection against acute myocardial ischemia-reperfusion injury by activating eNOS/NO.

Additionally, Coletta et al demonstrated an unexpected level of cooperation, as well as a mutual reliance between these signaling pathways to promote angiogenesis. Specifically, blocking either eNOS/NO or H$_2$S markedly reduces the angiogenic effects of the other. In agreement with previous reports, we observed a significant increase in the phosphorylation of eNOS (activation site) after H$_2$S therapy. We also found that H$_2$S increased cardiac nitrite levels, which indicates an encouragement of NO production after exogenous H$_2$S administration. Taken together, this evidence suggests that H$_2$S therapy does not simply prevent vessel dropout after TAC, but rather creates a proangiogenic environment for the growth of new vessels. Given that this correlates with the observed improvements in LV remodeling and function, the induction of angiogenesis via VEGF/NO signaling seems to play a major role in the cardioprotection afforded by H$_2$S.

VEGF, in part, regulates multiple angiogenic cellular responses, including survival, migration, and differentiation through the activation of Akt signaling. Although we did find that DATS therapy significantly increased Akt phosphorylation when compared with Sham mice, we only observed a nonsignificant trend between the DATS and Vehicle-treated mice. Based on these findings, there is not enough evidence to suggest a role for Akt in mediating the H$_2$S-induced angiogenesis or cardioprotection. VEGF also regulates eNOS/NO
signaling via protein kinase C,

and further studies are needed to determine the exact mechanism by which \( \text{H}_2\text{S} \) induces angiogenesis and cardioprotection. There is considerable evidence to suggest that oxidative stress plays a prominent role in the development of LV remodeling during heart failure.

The increased oxidative stress of the failing heart is a result of an increased production of reactive oxygen species and an overwhelmed antioxidant defense system. Therefore, the capacity of cardiac myocytes to maintain homeostasis during periods of oxidative stress resides in the ability to activate or induce protective enzymes. \( \text{H}_2\text{S} \) has previously been shown to act either as a direct antioxidant and upregulate antioxidant defenses. Previously, we have reported that \( \text{H}_2\text{S} \) attenuates oxidative stress in the heart by activating nuclear factor E2-related factor (Nrf2), a member of the nuclear factor E2 family of nuclear basic leucine zipper transcription factors. \( \text{H}_2\text{S} \) has previously been shown to act either as a direct antioxidant and upregulate antioxidant defenses. 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In the current study, we expand on these previous findings and demonstrate that \( \text{H}_2\text{S} \) therapy increases HO-1 in a model of pressure-induced heart failure. The increase in HO-1 is especially notable, given that it is associated with the production of CO, the third member of the
gasotransmitter family. Although the levels of CO were not evaluated in the current study, we did find that H$_2$S therapy increased NO levels. Therefore, it is intriguing to speculate that H$_2$S therapy has the ability to increase the levels of the other 2 gasotransmitters. This suggests that the activation of one of the endogenously produced gases can lead to the activation of the other 2. The specific role of H$_2$S because it influences both NO and CO is worthy of further exploration because the administration of 1 gasotransmitter that can have control over the others would likely prove to be clinically powerful, advantageous, and efficient.

Accumulating evidence indicates that H$_2$S signals modify cysteine residues in proteins via a process termed sulfhydration. Sulfhydration is similar to nitrosylation, which is the process by which NO modifies proteins. However, unlike nitrosylation, which often results in the inhibition of target protein activity, sulfhydration seems to enhance activity. For instance, the antiapoptotic actions of nuclear factor κB are dependent on H$_2$S sulfhydrating its p65 subunit. In addition, H$_2$S induces endothelial cell and smooth muscle cell hyperpolarization and vasorelaxation by sulfhydrating ATP-sensitive potassium channels. Finally, H$_2$S has recently been shown to sulfhydrate Keap1, which results in the release and translocation of Nrf2 to the nucleus. Although the degree of protein sulfhydration was not evaluated in the current study, it can be speculated that the observed improvements in LV remodeling and function are a result of this post-translational modification. Therefore, future studies are needed to evaluate specific protein targets to determine the full mechanism(s) by which H$_2$S provides protection in the setting of heart failure.

The attractiveness of using H$_2$S as a therapeutic strategy to treat heart failure stems from the ability of H$_2$S to activate multiple protective pathways at the same time. In the current study, we provide evidence that H$_2$S protects via a VEGF-eNOS-NO proangiogenic pathway and a GPx-1-HO-1 antioxidant pathway. In addition to our current findings, treatment with H$_2$S could also limit the development of LV remodeling and dysfunction associated with heart failure through its ability to inhibit inflammation, modulate mitochondrial function, and inhibit apoptosis. Although these protective pathways can be separated into distinct signaling cascades, there can be overlap between the signaling molecules. For example, H$_2$S can provide antioxidant effects via an Nrf2-HO-1 pathway. Moreover, there is evidence that NO possesses antioxidant effects and can activate Nrf2 and HO-1. Moreover, activation of either of these antioxidant pathways could not only lead

**Figure 7.** Diallyl trisulfide (DATS) upregulates GPx1 and HO-1 after transverse aortic constriction (TAC). Representative immunoblots and densitometric analysis of (A) glutamate-cysteine ligase (GCLC) and Glutathione Peroxidase 1 (GPx1) and (B) heme oxygenase 1 (HO-1) and HO-2 in hearts from Sham, Vehicle-, and DATS-treated mice at 6 weeks of TAC. Results are expressed as mean±SEM. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.
to a reduction of oxidative stress, but could also contribute to a reduction in cell death and improvement in mitochondrial function. Together this would provide a more suitable environment for the VEGF-eNOS-NO pathway to induce the growth of new vessels. Given the diverse signaling profile of H₂S, it is likely that all of these pathways work together and in parallel to prevent the adverse remodeling of heart failure.

In summary, this study provides novel evidence that chronic H₂S therapy attenuates LV remodeling and preserves cardiac function in the setting of pressure-induced heart failure. Our data suggest that H₂S mediates these protective effects by inducing angiogenesis via an increase in VEGF and eNOS/NO. Additionally, the current study indicates that H₂S upregulates the endogenous antioxidants, GPx1 and HO-1. Together, these findings continue to support the emerging concept that treatment strategies aimed at increasing the levels of H₂S may be of clinical importance in the treatment of heart failure.

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Disclosures

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CLINICAL PERSPECTIVE

Adjunct pharmacotherapies designed to coincide with the standard means of care are needed to decrease the extent of injury leading to the development of heart failure. In this regard, therapeutic strategies aimed at increasing the levels of the gaseous signaling molecule, hydrogen sulfide, have come to be a focus of interest given their ability to exert cytoprotective effects in various models of cardiac injury. In the current study, chronic administration of diallyl trisulfide, an organosulfur compound that augments hydrogen sulfide levels, provides protection against the adverse remodeling associated with transverse aortic constriction by increasing circulating and cardiac sulfide levels. Specifically, we found that diallyl trisulfide therapy attenuated LV dilation and dysfunction, attenuated the development of perivascular and intermuscular fibrosis, and attenuated the development of cardiac hypertrophy. Our data indicated that diallyl trisulfide mediated these protective effects by inducing angiogenesis and alleviating oxidative stress. Together, these findings continue to support the emerging concept that treatment strategies aimed at increasing the levels of hydrogen sulfide may be of clinical importance in the treatment of heart failure.
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Supplemental Material

Hydrogen Sulfide Attenuates Cardiac Dysfunction Following Heart Failure via Induction of Angiogenesis

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Expanded Methods

Materials and Methods

Mice

Male C57BL/6J mice 8-10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). All experimental protocol were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

DATS Preparation and administration

DATS (LKT Labs, St. Paul, MN) was maintained in sealed amber glass ampules and kept at -20 °C until use. On the day of experimentation a fresh ampule of DATS was opened. 5 μl of DATS was diluted in 500 μl of 100% DMSO. For in vivo experiments, the DATS in 100% DMSO solution was further diluted in sterile saline to obtain the correct dosage to be delivered in a volume of 50 μl. The resulting concentration of DMSO in this dosage was 1%. Vehicle consisted of a solution of 1% DMSO in sterile saline. DATS (200 μg/kg) or Vehicle (1% DMSO) groups were injected intraperitoneally once per day for 12 weeks following TAC.

Transverse Aortic Constriction (TAC) Protocol

To create pressure overload, TAC procedure was performed in mice. Mice were
anesthetized with Ketamine (100 mg/kg) and Xylazine (8 mg/kg) and the core body temperature was maintained in normal range (36-37°C). Then mice were orally intubated and placed on a rodent ventilator to maintain respiration during the surgical procedures. The second intercostal muscle was incised to visualize the aortic arch. Following identification and dissection of the aortic arch, 7-0 silk suture was placed around the aortic arch between the brachiocephalic trunk and the left carotid artery and ligated around a 27G blunt needle. The needle was immediately removed after ligation. The chest was surgically closed and mice were put in a recovery with 100 % oxygen along with a surgical warming pad to maintain core body temperature within normal limits. At the end of the experimental protocol (i.e. 6 or 12 weeks following TAC surgery) mice were euthanized and the hearts, lungs and blood samples were collected, and hearts and lungs were weighed.

Echocardiography

At 2 days prior to TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) under anesthesia with isoflurane (0.25 to 0.50%) supplemented with 100% O2. Following TAC procedure, echocardiography was also performed in same manner for up to 12 weeks. To determine cardiac structure and function, intraventricular septal end diastolic dimension (IVSd), LV end diastolic dimension (LVEDD), LV end systolic dimension (LVESD), and LV ejection fraction (LVEF) were analyzed from M-mode images.
**Histology**

Hearts were collected at the indicated times, fixed in 10% buffered formalin, embedded in paraffin stained with Masson’s trichrome and Picrosirius Red (to detect fibrosis). Digital images were analyzed using ImageJ.

**Vascular density measurements**

Angiogenic index measurements from frozen tissue sections were performed as previously described \(^1,2\). Briefly hearts from the experimental groups were collected, dissected, and embedded in OCT freezing medium. Frozen tissue blocks were cut into 5 µm sections and slides fixed for staining. After FBS blockade, primary anti-CD31 antibody was added to each section at a 1:200 dilution and incubated at 37°C for 1 hour. Slides were then washed with PBS with 1% FBS and subsequently incubated with secondary Cy3 conjugated anti-rat antibody at a 1:250 dilution incubated at room temperature (RT) for 1 hour. Slides were washed with PBS with 1% FBS and mounted with cover slips using Vectashield DAPI. Images were captured using a Hamamatsu digital camera in conjunction with a Nikon TE-2000 epifluorescence microscope (Nikon Corporation, USA) at 200X magnification. Simple PCI software version 6.0 (Compix Inc., Sewickly, PA, USA) was used to measure the area of CD31 and DAPI positive staining to calculate an angiogenesis index expressed as the ratio between CD31 and DAPI positive regions.
We also performed formalin fixed tissue immunohistochemistry using anti-vWF antibody staining to quantify the number of capillaries per unit area. NovaRED staining of vWF was performed using the VECTOR NovaRED peroxidase substrate kit. Briefly, formalin fixed tissue sections were treated with primary and secondary antibodies and NovaRED peroxidase substrate then added for 10 minutes at room temperature in a humidified chamber. After PBS wash, tissue sections were counterstained with hematoxylin. 8 random photomicrograph fields on 2 different tissue sections per mouse were obtained using an Olympus BX53 microscope (Olympus, USA). Capillary density as measured by the number of vWF positive vessels per mm$^2$ was counted in a blinded manner.

**Cellular proliferation measurements**

Immunofluorescent staining of the cell proliferation was performed as previously reported $^{1,2}$. Frozen tissue sections of heart were incubated with primary anti-Ki67 antibody (1:350) at 37$^\circ$C for 1. Slides were washed with PBS with 1% FBS, and secondary DTAF anti-rabbit (1:150) conjugated antibody was added and incubated at room temperature for another 1 hour. Slides were finally washed and mounted with cover slips using Vectashield DAPI. Images were acquired as described above. Cellular proliferation (proliferation index) was determined as the ratio between regions positive for Ki67 and DAPI positive areas.
**Western Blot Analysis**

Myocardial tissue samples were taken homogenized and lysates were used for Western blot analysis. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: CD 31 (1:2,000 Abcam) VEGF-A (1:25,000 Cell Signaling); b-FGF (1:2,000 Cell Signaling); Angiostatin (1:3,000 Abcam); Phosphorylated Akt (Ser 473) (1:5,000, Cell Signaling Technology); AMPK and Phosphorylated AMPK (Thr 172) (1:5,000, Cell Signaling Technology); eNOS (1:5,000, BD Transduction Laboratories); Phosphorylated eNOS (Ser 1177) (1:1,000 Cell Signaling Technology); Phosphorylated eNOS (Thr 495) (1:1,000 Cell Signaling Technology); GCLC (1:3,000 Abcam); HO1 (1:10,000 Abcam) Immunoblots were next processed with the appropriate secondary antibodies (Cell Signaling) for 1 hr at room temperature. Immunoblots were then probed with a SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) to visualize signal, followed by exposure to X-ray film.

**Measurement of Nitrite Levels**

Nitrite analysis was performed as previously described.³
Measurement of Hydrogen Sulfide and Sulfane Sulfur

Hydrogen sulfide and sulfane sulfur levels were measured in heart and blood according to previously described methods\textsuperscript{4}. For heart tissue, the amount of H\textsubscript{2}S is reported as nmol/mg wet weight. For the blood, the amount of H\textsubscript{2}S is reported as µM.

Statistical analysis.

All data are expressed as mean ± SEM. Statistical significance was evaluated using unpaired Student t-test for comparison between 2 means and a 1-way ANOVA with a Tukey test as the posthoc analysis for comparison among 3 or more means by use of Prism 5 (GraphPad Software Inc). For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. The following comparisons were made separately: (1) baseline vs. post-baseline measurements at each time point for the DATS and vehicle groups and (2) DATS vs. vehicle measurements at each time point. The p-value for these evaluations was adjusted by applying the Bonferroni correction for multiple comparisons. A value of p<0.05 denoted statistical significance and p-values were two-sided.
References


