Increased endogenous $H_2S$ generation by CBS, CSE and 3MST gene therapy improves ex vivo renovascular relaxation in hyperhomocysteinemia

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Abstract

Hydrogen sulfide (H₂S) has recently been identified as a regulator of various physiological events, including vasodilation, angiogenesis, anti-apoptotic and cellular signaling. Endogenously, H₂S is produced as a metabolite of homocysteine (Hcy) by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST). Although Hcy is recognized as a vascular risk factor at an elevated level (HHcy) and contributes to vascular injury leading to renovascular dysfunction, the exact mechanism is unclear. The goal of the current study was to investigate whether conversion of Hcy to H₂S improves renovascular function. Ex vivo renal artery culture with CBS, CSE and 3MST triple genes therapy generated more H₂S in the presence of Hcy and these arteries were more responsive to endothelial-dependent vasodilation compared to non-transfected arteries treated with high Hcy. Cross section of triple genes delivered renal arteries immunostaining suggested increased expression of CD31, VEGF and diminished expression of anti-angiogenic factor, endostatin. In vitro endothelial cell culture demonstrated increased mitophagy during high levels of Hcy and was mitigated by triple genes delivery. Also, dephosphorylated Akt and phosphorylated FoxO3 in HHcy were reversed by H₂S or triple genes delivery. Upregulated MMP-13 and downregulated TIMP-1 in HHcy were normalized by overexpression of triple genes. Together, these results suggest that H₂S play a key role in renovasculopathy during HHcy and is mediated through Akt/FoxO3 pathways. We conclude that conversion of Hcy to H₂S by CBS, CSE or 3MST triple genes therapy improves renovascular function in HHcy.
Introduction

Homocysteine (Hcy) is an established vascular risk factor and promotes vascular diseases at elevated level, known as hyperhomocysteinemia (HHcy), including endothelial dysfunction, smooth muscle proliferation, and matrix remodeling. While the exact mechanism of these pathophysiological conditions are not clear and may be multifaceted, many laboratories have identified oxidative stress (57), epigenetic modification of proteins (47), cellular apoptosis and autophagy (36, 55) are involved in HHcy-associated vascular disorders. Recently, a growing body of evidences suggested that depletion of hydrogen sulfide (H2S) in the body during HHcy is one of the possible mechanisms of vasculopathies (2, 50).

Hydrogen sulfide (H2S) is a gaseous molecule of immense physiological importance that extends from antioxidant properties (28), vasorelaxation (32), neurotransmitter (33), angiogenic agent (48) and many more, few to name. Physiologically, Hcy metabolizes by three enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) to produce H2S. These three H2S producing enzymes has wide range of tissue distributions in common, such as brain, liver and kidney (22, 27, 44, 45), in addition to their organ specificity. One of the important physiological roles of these three transsulfuration enzymes is to generate H2S in the body using either Hcy or cysteine as a substrate. While main source of H2S is cysteine under normal physiological conditions, Hcy becomes preferred source of H2S production in HHcy (9). Thus, it is expected that HHcy will result in higher level of H2S in the body. Paradoxically, during pathological level, HHcy causes down regulation of H2S leading to vascular disease, such as impairment of endothelium (14). The mechanism(s) of such paradoxical effect of HHcy in H2S regulation and vascular disorders are not clear.

In the recent years several mechanisms are proposed to define depletion of H2S during HHcy to explain Hcy-associated vasculopathies. For example, we and others have previously reported attenuation of CSE in HHcy as a regulatory mechanism of H2S depletion (5, 39). The exact mechanism, however, is far from clear. Additionally, in an Hcy-independent study we have recently reported the regulatory role of H2S in vascular endothelial growth factor (VEGF) and their inhibitors during heart failure (19). However, the role of H2S in Hcy-induced modulation of these factors in vascular bed and combined effects of CBS, CSE and 3MST enzymes in modulation of Hcy-associated renal vasculopathy has never been tested.

Another signature of vascular disease underlies on extracellular matrix (ECM) components in HHcy (59, 60). Proteinases and their tissue inhibitors play major roles in the formation and degradation of ECM under physiological and pathological conditions (53, 56). Matrix metalloproteinases (MMPs) maintain tissue homeostasis in the matrix, and therefore,
contribute to ECM modulation. Among MMPs, we have previously reported that MMP-2 and -9 are involved in renal matrix remodeling, in particular collagen IV modulation, associated with HHcy (39). On the other hand MMP-13 is an interstitial collagengase that degrades collagen I & II (3), and has significant renal expression related to ECM remodeling during progressive renal diseases (1, 30). Although MMPs are directly involved in matrix degradation, endogenously their activities are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs), of which TIMP-1 inactivates most MMPs (43, 52, 59). It is reported that reduction of Hcy level is directly associated with mitigation of MMP activity (40) and matrix accumulation (59); however, the precise role of H2S in regulation of MMP-13 and TIMP-1 in HHcy related renovascular remodeling is not clearly defined.

While MMP/TIMP balance maintains ECM homeostasis, autophagic degradation of cellular components, such as mitochondria also play role in vascular remodeling (54). Autophagic degradation of mitochondria denotes mitophagy. This is a normal physiological process to remove debris or recycle cellular components. Although the existence of mitophagy is well known, the phenomena whether the mitochondria are randomly or selectively targeted are unclear. Mitochondria are the site of oxidative phosphorylation, and respiration produces reactive oxygen species (ROS) (11, 26). As a major source of ROS production, mitochondria are especially prone to oxidative damages. Hcy is also known to cause oxidative damage of cellular organelles (34). However, to our knowledge, the effect of HHcy on mitophagy, particularly in the vascular mitochondria, and possible modulatory role of H2S on renal vascular dysfunction is not elicted.

Reports are also available that oxidative stress induces cell death through dephosphorylation of Akt signaling pathways (18). Hcy induces oxidative stress and HHcy reduces H2S production, which is an antioxidant molecule (36). Akt in the downstream pathway activates transcription factor FoxO. Importantly, FoxO play roles in cell survival and is negatively regulated by Akt-activation during oxidative stress (25). It is well reported that H2S minimizes oxidative stress; however, the role of H2S in Akt activation and FoxO regulation in the downstream pathway during HHcy are not defined.

Taking into account of Hcy pathobiology and beneficial role of H2S in health and disease, the current study was undertaken to address the mechanism of renovascular dysfunction in HHcy and ameliorating role of H2S, if any. Since the renal artery plays an important role to regulate renal function in normal and pathophysiological conditions, we chose to delineate functional status of renal artery in HHcy and defined the implications of triple genes therapy
(CBS, CSE and 3MST) to modulate this function through H$_2$S generation. Additionally, the involvement of Akt/FoxO signaling pathway in this mechanism was explored in cellular level.

**Materials and Methods:**

**Animal model**

C57BL/6J (wild type, WT) mice of ages 12-16 weeks were used for this study. Mice were obtained from Jackson Laboratories Inc, Maine, USA, and housed in the animal care facility of the University of Louisville. The mice were anesthetized with Tribromoethanol (TBE, 240 mg/Kg body weight) and sacrificed to collect renal artery. All animal procedures were in accordance with the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

**Antibodies and reagents**

Cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) antibodies were purchased from Novus Biologicals, Littleton, CO. Anti-CD31, anti-VEGF and anti-endostatin antibodies were purchased from Abcam (San Francisco, CA). Antibodies against LC3AI/II, mTOR, BNIP3 and Beclin 1 were from Cell Signaling. Anti-phospho-Akt, anti-phospho FoxO3a, anti-MMP-13, anti-TIMP-1, anti-β-actin, anti-GAPDH and HRP conjugated secondary antibodies were from Millipore. Reactive oxygen species (ROS), particularly hydrogen peroxide (H$_2$O$_2$), detection reagent DCFDA {5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; CM-H2DCFDA} was from Invitrogen. All other reagents were used from commercially available highest grade.

**Cell culture**

Mouse aortic endothelial cells (MAECs) were from Cellbiologics (Chicago, IL). Cells were cultured and maintained in DMEM/F-12 (50/50) medium containing 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (Mediatech, Herndon, VA). T-25 flasks were grown in a tissue culture incubator, trypsinized (0.25% trypsin, 0.1% EDTA in HBSS without Ca$^{2+}$, Mg$^{2+}$, and sodium bicarbonate; Mediatech), and plated onto 12-well TPP (Techno Plastic Products, Trasadingen, Switzerland) cell culture plate. For the transfection study cells were allowed to grow about 50% confluence and then transfected with either CBS, CSE, 3MST or triple genes. Transfection efficacy was measured by GFP vector in separate experiments. After 48 h of transfection, cells either lysed to measured CBS, CSE, and 3MST protein expression and / or to detect H$_2$S generation capability, or treated with or without homocysteine (Hcy, 75 µM) to collect
mitochondria. Cells were also cultured for 48 h in the presence of H$_2$S (30 µM) and Hcy (75 µM), and immunostained with LC3AI/II antibody secondarily conjugated with FITC. For flow cytometry analysis mitochondria isolated from the cells, immunostained with LC3AI/II and analyzed by flow cytometry. ROS production were measured in the isolated mitochondria treated with Hcy (75 µM) for 48h using CM-H2DCFDA as a substrate. For phosphorylation study, cells pre-treated with or without H$_2$S (30 µM) were treated with Hcy for 30 min, and proteins were analyzed. Empty plasmid was used as a control for all transfection study.

Renal artery culture, overexpression of genes and myobath study

The renal artery was used for ex vivo gene transfection, culture and to measure functional status. The artery was isolated from anesthetized mice and placed in ice cold physiological salt solution (PSS) containing (in mM), NaCl 118, KCl 4.7, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 12.5 and glucose 11.1, pH 7.4. Fat and connective tissues were cleaned off, and the vessels were cut into 2 mm pieces. Four to five arterial rings were placed in each well of a 12-well cell culture plate (TPP, Switzerland) containing 1 ml complete cell culture medium (DMEM/F-12 50/50, 10% FCS, 1% L-glutamine and 1% Penicilin-Streptomycin solution (Cellgro, Mediatech, Inc). Arterial explants were transfected with plasmid having CBS, CSE and 3MST genes (0.4 µg DNA/cm$^2$ of growth area) or empty plasmid using jetPRIME transfection reagent (Polyplus transfection Inc. NY) following manufacturer’s instructions. After 6 h, arterial rings were gently mounted on the bottom of Matrigel well (BD Biosciences). For effective mounting, a very thin layer of matrigel (BD Biosciences) was applied to the outer surface of vessel to be attached to the well, and immediately placed on the well. One ml complete cell culture medium was added to the wells, and plates were kept in 37°C cell culture incubator for 48 h. The transfection efficiency was 30-40% as determined by the percentage of GFP-positive cells through flow cytometry 24 h after transfection. After 48 h, arterial rings were taken off the matrigel, and any excess matrigel was removed. Rings were mounted with two tungsten wires (Scientific Instruments Services, Ringoes, NJ) of same diameter (0.002 ) attached to the myobath, and were placed in 25 ml organ bath filled with PSS at 37°C. The PSS in the myobath was constantly aerated with O$_2$:CO$_2$, 95%:5%, respectively. Rings were stretched gradually to obtain 0.5 g optimal resting tensions and were equilibrated for an hour. After equilibration, phenylephrine (Phe) of 10$^{-6}$ to 10$^{-2}$ M was added in the organ bath to make a final concentration of 10$^{-9}$ to 10$^{-5}$ M, respectively. Acetylcholine (Ach) was added to the organ bath in similar manner as described for Phe to detect endothelial-dependent vasorelaxation. The tissue
responses were recorded graphically using mp100 software for 10 minutes of each for each drug concentration.

**Detection of tissue capability to generate H$_2$S**

The capability of renal arterial tissue to generate H$_2$S was determined according to the previously adopted method (41).

**Tissue sectioning**

At the end of experiment, cultured renal arterial tissue were placed in tissue freezing media (Triangle Biomedical Sciences, Inc., Durham, NC) and were frozen in liquid nitrogen. Frozen blocks with the molds were placed in a $-70^\circ$C freezer until serial sections were made. Cryosections (Leica CM1850) of 3 $\mu$m thicknesses were put on glass slides and immunostained with anti-CD31, anti-VEGF, anti-endostatin and anti-CSE antibodies with appropriate secondary fluorescence antibodies to measure expression of these molecules under laser scanning confocal microscopy (Olympus FluoView 1000).

**Immunostaining**

Cryosections on the slide or MAECs grown in chamber slides (Lab-Tek II, Thermo Fisher Scientific) were washed with PBS (pH 7.4), fixed with 3.7% paraformaldehyde containing 0.25% l-$\alpha$-lysophosphatidylcholine for 30 min followed by three washes with PBS 5 min each. Tissues then blocked with 1% BSA for 15 min, washed with PBS (3X, 5 min each) and appropriate primary antibody (1:100 dilutions in 1% BSA) was added, and incubated for overnight at 4°C with gentle agitation. Excess antibody washed by PBS (3X, 5 min each) wash and secondary fluorescence conjugated antibody (1:500 dilutions in 1% BSA) was added, and incubated for 2 h at room temperature. Unbound secondary antibodies removed by PBS wash (3X, 5 min each), tissues stained with nuclear stain DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) where ever mentioned in the figure, and fluorescence was visualized in a laser scanning confocal microscope (Olympus Fluoview 1000) with appropriate filter.

**Immunoblotting**

Protein was isolated from cells using RIPA lysis buffer (Thermo Scientific Inc., Rockford, IL), containing protease inhibitors and PMSF (phenylmethylsulfonyl fluoride). Protein content in the samples was estimated by BCA assay, and equal amount of total protein was loaded in each well of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels. Protein
separated by electrophoresis, transferred to PVDF (Polyvinylidene fluoride) membrane and incubated with primary antibody followed by secondary HRP conjugated antibody. An ECL plus Western blotting reagent (GE Health Care, Little Chalfont, Buckinghamshire) was used to detect the protein of interests. To normalize expressed protein in the Western blot, membranes were stripped with membrane-stripping buffer (Boston BioProducts, Worcester, MA) and reprobed with either β-actin or GAPDH antibody. Intensity of bands was detected by Gel –Doc software, and was normalized with their corresponding β-actin/GAPDH control.

Measurement of ROS
Reactive oxygen species, in particular hydrogen peroxide (H₂O₂), hydroxyl (HO•-) and peroxyl (ROO•-) radicals in the isolated mitochondria was detected by CM-H₂DCFDA {5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester} reagent following manufacturer’s instructions. This dye is non-fluorescent in reduced form, but after cellular oxidation becomes fluorescent. Briefly, mitochondria were isolated from experimental cells, re-suspended in PBS containing 10 µM CM-H₂DCFDA and maintained at 37°C in the dark for 30 minutes. Cells washed to remove excess dye and analyzed by fluorescence spectrophotometer.

Flow cytometry
At the end of experiments, mitochondria were isolated and immunolabeled with LC3AI/II antibody. Mitochondria were then washed and labeled with FITC conjugated secondary antibody. Cells washed and 10,000 events were analyzed by flow cytometry (Accuri 6). Isotype control was used for this study.

Statistical analysis
Values were taken as means ± standard error (SE) of measurement. The number of experiment was carried out for each of the experiment is mentioned in the figure legends. The difference between mean values of multiple experiments was analyzed by one-way ANOVA (unless otherwise mentioned) followed by Scheffe’s post hoc analysis. Paired t-Test was used to determine significance difference between groups and p < 0.05 was considered significant.
Results

Over expression of genes, tissue generation of $\text{H}_2\text{S}$ and vascular reactivity of renal artery in ex vivo condition

Arterial explants overexpressed with CBS, CSE, 3MST or triple genes were used for this study. Transfection efficacy of gene was determined by GFP assay (Figure 1A). Briefly, a similar group of tissue that were overexpressed with CBS, CSE, 3MST or triple genes was transfected with pcDNA3.1/GFP plasmid vector and after 48 h of transfection tissue section were observed under confocal microscope. Results indicated that the tissues expressed GFP (Figure 1A). Since CSE mainly localizes in the vascular smooth muscle cells (VSMCs), we confirmed its overexpression in VSMCs by immunostaining (Figure 1B). Furthermore, tissue protein was analyzed by immunoblotting to confirm overexpression of CBS, CSE and 3MST (Figures 1C-D).

Next, we measured tissue ability to generate $\text{H}_2\text{S}$. Results indicated that the tissues which overexpressed CBS, CSE or 3MST had greater ability to generate $\text{H}_2\text{S}$ compared to non-transfected tissue (Figure 2A). Interestingly, triple genes delivered tissue generated almost double amount of $\text{H}_2\text{S}$ compared to control, and about 70-80% more than individual gene therapy (Figure 2A).

Since Hcy is known to cause impaired endothelial-dependent vascular relaxation and $\text{H}_2\text{S}$ causes vasodilation, we measured vascular endothelial reactivity (Acetylcholine-induced endothelial-dependent) of phenylephrine (Phe, $10^{-5}$ M) pre-contracted vessels of Hcy, $\text{H}_2\text{S}$ and $\text{H}_2\text{S}$ generating genes transfected arterial rings. In myobath, Phe pre-contracted WT renal arterial rings which received either triple genes or $\text{H}_2\text{S}$, and incubated with homocysteine (Hcy) over 48 h, were challenged with cumulative dose-dependent Ach (Acetylcholine, $10^{-9}$-$10^{-5}$ M) relaxation. Results indicated that arterial rings incubated with Hcy (WT + Hcy) had impaired endothelial-dependent vasorelaxation compared to control groups (WT) (Figure 2B). Interestingly, endothelial-dependent vasorelaxation was improved in the arterial rings incubated with Hcy that were overexpressed triple genes (WT + triple genes + Hcy) (Figure 2C). Similar result was obtained from the group of WT + $\text{H}_2\text{S}$ + Hcy (Figure 2D). No significant changes in vascular reactivity were recorded in the control (WT) vs WT + triple genes delivered groups (Figure 2C) or WT vs WT+$\text{H}_2\text{S}$ supplemented groups (Figure 2D) without Hcy treatment.

We suspected inhibition of eNOS (endothelial nitric oxide synthase) by Hcy led to diminished endothelial-dependent vascular relaxation and improvement of relaxation was due to either increase of eNOS by triple genes or through protective mechanism by $\text{H}_2\text{S}$ in HHcy. To examine this, we pre-incubated (and incubation continued until end of the experiments) vessels, as shown in figures (2B, C & D), with L-NAME ($N^G$-nitro-L-arginine methyl ester, 10 µM), a
specific inhibitor of eNOS. These vessels were pre-contracted with Phe (Phenylephrine, $10^{-5}$ M) and dose-dependent responses to Ach were measured. Results as shown in figures 2B, C & D indicated that L-NAME incubated vessels were unresponsive to Ach-induced relaxation.

After vascular reactivity study, we analyzed tissues to detect eNOS expression through immunoblotting. Results indicated that Hcy diminished eNOS expression, whereas triple genes ameliorated eNOS expression in HHcy (Figure 2E).

To determine whether endothelial-independent relaxation was impaired in HHcy, and gene therapy had any role to ameliorate vasorelaxation, we performed endothelial-independent vasorelaxation by SNP (Sodium nitroprusside). Results indicated that there were virtually no differences of endothelial-independent relaxation, as detected by cumulative SNP ($10^{-9}$-$10^{-5}$ M) challenge (Fig. 2F).

**Triple genes delivery improved CD31, VEGF and diminished endostatin expressions in hyperhomocysteinemia**

Immunostaining of arterial explants indicated that HHcy attenuated CD31 and VEGF, whereas endostatin expression was abrogated (Figure 3). Overexpressed genes of CBS, CSE and 3MST reversed the effect of Hcy on the expression of these angiogenic and anti-angiogenic factors (Figure 3).

**Overexpression of triple genes increased H$_2$S generation in HHcy**

Mouse aortic endothelial cells (MAECs) were used for in vitro transfection study. Transfection efficacy of plasmid vector was determined by GFP expression (Figure 4A). Over expression of CBS, CSE and 3MST was measured by Western blot and results indicated that transfected cells were overexpressing these protein compared to control (Figure 4B). Since 3MST is reported to localize in the mitochondria, expression of 3MST in the isolated mitochondria after transfection was detected by immunoblotting. Results indicated that 3MST was overexpressed in the mitochondria (Figure 4C). Not only had that, isolated mitochondria of 3MST transfected cells produced increased amount of H$_2$S compared to non-transfected cells (Figure 4C, bar diagram). More importantly, while Hcy significantly attenuated H$_2$S generation in the control cells (non-transfected cells); the overall ability to generate H$_2$S, in the presence of Hcy, by the cells overexpressing CBS, CSE and 3MST genes was significantly increased compared to control (Figure 4D). Generation of H$_2$S was maximally measured from the triple genes delivered cells (Figure 4D).
Triple genes delivery prevented mitophagy in HHcy

To determine whether H$_2$S mitigates Hcy-induced mitophagy, we incubated MAECs with or without Hcy pretreated with H$_2$S (30 µM). Expression of LC3I/II, a marker of autophagy/mitophagy was dramatically increased in cells incubated with Hcy (75 µM) for 48 h (Figure 5A). Supplementation of H$_2$S (30 µM) along with Hcy diminished expression of LC3I/II (Figure 5A). Immunoblotting results of isolated mitochondria extracted protein indicated and further confirmed expression of LC3I/II in the mitochondria in HHcy (Figures 5B & C). Triple genes delivery abolished Hcy-induced expression of LC3I/II in mitochondria (Figures 5B & C). Additionally, markers of mitophagic molecules, such as BNIP3 and Beclin 1 were upregulated in mitochondria; whereas mTOR was attenuated by Hcy treatment (Figures 5B & D). These effects were normalized in the mitochondria of MAECs, which overexpressed CBS, CSE and 3MST triple genes and received Hcy treatment for 48 h (Figures 5B & D).

Triple genes delivery mitigated Hcy-induced mitophagy and ROS production

Immunostaining as well as immunoblotting of mitophagic markers as shown in figure 5 indicated mitophagy in HHcy and preventive role of H$_2$S from Hcy-induced mitophagy in MAECs. To further verify we isolated mitochondria from triple genes delivered MAECs treated with or without Hcy. As shown in the figure 6A, flow cytometry data indicated that a significant number of mitochondria (13%, Figures 6A & B) expressed LC3I/II treated with Hcy. Interestingly, mitochondria of triple genes delivered cells were not expressing this marker.

To determine whether Hcy induced ROS in mitochondria that may have played a role in mitophagy, we isolated mitochondria and measured ROS by DCFDA substrate. Figure 6C indicated that Hcy increased ROS production, particularly H$_2$O$_2$, about 50% more compared to the mitochondria isolated from control cells. Individual gene of CBS, CSE or 3MST delivery to the cells mitigated ROS production; however, the production of ROS was almost normalized in the mitochondria of triple genes delivered cells (Figure 6C).

H$_2$S regulated Akt and Foxo3 activation, and MMP/TIMP expressions in HHcy

We determined the phosphorylation of survival kinase Akt and its downstream regulator FoxO3a in HHcy, since they are important regulators of mitophagy. Also, any modulatory role of H$_2$S in this pathway was determined. Results indicated that Hcy mitigated activation of Akt and induced phosphorylation FoxO3a (Figures 7A & B); whereas, H$_2$S reversed these effects of Hcy. Similarly, triple genes therapy normalized Hcy regulated mitigation of Akt activation (Figures 7C
& D). Also, increased expression of MMP-13 and attenuated expression of TIMP-1 in Hcy treated cell were normalized by triple genes therapy (Figures 7C & D).

Discussion

It is well documented that hyperhomocysteinemia (HHcy) impairs endothelial function and remodels vascular bed, especially extracellular matrix (ECM), and diminishes vascular function in pathophysiological conditions (4, 13, 15, 16, 24, 39). Here, we demonstrated with evidence that overexpression of CBS, CSE and 3MST (cystathionine β-synthase, cystathionine-γ-lyase and 3-mercaptopyruvate sulfurtransferase, respectively) genes mitigated HHcy effect on vascular bed by converting Hcy to H2S. The benefit of gene delivery was achieved by two ways: first, by metabolizing Hcy that minimized stress, and second, by converting Hcy to H2S, which protected tissue from oxidative damage due to HHcy. In addition, involvement of mitophagy, MMP/TIMP axis and Akt/FoxO3a pathway has been demonstrated in endothelial pathobiology during HHcy. Furthermore, the regulatory mechanism of H2S to modulate vascular illness in HHcy was delineated with experimental evidences.

Remodeling of vessel is a dynamic and physiological process, and many factors are involved in this process including angiogenic and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) is an angiogenic factor that promotes endothelial cell proliferation, migration and tube formation (17). These events are essential for the development of new blood vessels from pre-existing ones. This molecule specifically target endothelial cells and promotes their proliferation, survival, migration, and sprouting (61). On the other hand, anti-angiogenic factor, such as endostatin, inhibits vascular growth by down regulating VEGF (20). Hcy has been reported to inhibit endothelial cell proliferation, migration and tube formation suggesting that Hcy inhibit vascular growth (6, 7, 31). Contrary to anti-angiogenic effects of Hcy on vasculature, recent report suggested that H2S, a metabolic product of Hcy, is an endogenous factor of vascular growth and management through VEGF-dependent pathway (35). In a clinical study Tepper et al reported that human endothelial progenitor cells from diabetic subjects exhibited impaired proliferation, adhesion and subsequent incorporation into vascular structures (51). This suggested a clear correlation between impaired endothelial proliferation and structural anomaly in a clinical setting. Although this study did not link impaired endothelial proliferation and function, through an in vitro study Tanaka et al reported a distinct link between improved proliferation and function in endothelial cells using honeycomb-patterned polymer film (49). Our present report in agreement with this previous study suggested improved endothelial function by triple genes therapy in HHcy through eNOS-dependent pathway (Figure 2C), and also perhaps
through endothelial proliferation mechanism (Figures 2E & 3). This was most likely achieved through two different mechanisms: first, Hcy toxicity to vascular cells was diminished by accelerated metabolism of Hcy by triple genes; and second, accelerated Hcy metabolism produced angiogenic H$_2$S, which promoted endothelial proliferation as evidenced by CD31 upregulation by triggering VEGF and its inhibitor, endostatin (Figure 3).

Results from our study (Figure 4D) and others suggested that there was reduced production of H$_2$S in the presence of high Hcy. It’s no surprise to ask a question why the production of H$_2$S reduces during HHcy, when it is a substrate of the transsulfuration enzymes. Previously we reported possible mechanisms of this paradoxical effect (38). Briefly, although cysteine is the main substrate of CSE, and CSE expresses mainly in vascular bed, during HHcy, Hcy competes for binding to CSE with cysteine. Therefore, increased Hcy level decreases H$_2$S production from cysteine through substrate inhibition (5, 46). In addition, protein homocysteinylated is a major reaction in the presence of thiolactone and homocysteinylated led to protein damage (23). It is possible that, though there is no direct evidence, the activity of CSE may be severely impaired during HHcy resulting in attenuated H$_2$S generation. This is still an active interest in our laboratory; however, presently we do not have supporting evidence to establish this hypothesis. Future rigorous studies are needed to verify this plausibility.

Another mechanism of vessel remodeling is through cellular autophagy and mitophagy. Mitophagy denotes degradation of mitochondria through autophagy, and autophagy is a process whereby cellular components are degraded by engulfment into autophagosomes (8). In this regard several molecules, such as LC3 (microtubule-associated protein light chain 3), mTOR (mammalian target of rapamycin), BNIP3 (BCL2 adenovirus E1B 19 kDa protein-interacting protein 3) and Beclin 1 play key roles in mitophagic events. While LC3, BNIP3 and Beclin 1 are autophagic markers (58), mTOR regulates cell growth, cell proliferation, cell motility and cell survival (21). Although mitophagy is finely tuned to reutilize cellular energy during stress, several lines of investigations has reported cellular oxidative stress-mediated mitophagy in the disease pathogenesis (29). Hcy is known to cause oxidative damage of cellular organelles (34). However, the effect of HHcy on mitophagy, particularly in the vascular mitochondria, and its consequences on vascular dysfunction is not well established. Our present report demonstrated that Hcy initiated mitophagy by inhibiting mTOR and inducing LC3AI/II, BNIP3 as well as Beklin 1 (Figures 5 & 6A & B). Supplementation of H$_2$S as well as triple genes overexpression mitigated mitophagy (Figures 5 & 6A & B). These results suggested two possible different mechanisms of H$_2$S effect on cellular mitophagy in HHcy. In the first mechanism supplemented H$_2$S protected mitochondria from being subjected to mitophagy (Figure 5A). In the second
mechanism triple genes overexpression of transsulfuration enzymes utilized Hcy as a substrate to produce H$_2$S. This second mechanism therefore minimized Hcy effect on mitophagy by reducing Hcy stress (Figures 5B & 6A), as well as by enhancing generation of antioxidant H$_2$S (Figures 4C & D). It is reported that autophagy resulted in necrotic cell death in human umbilical vein endothelial cells (10), and this mechanism may contribute to the deterioration of vascular endothelial function (10). It is possible in our study that diminished endothelial function in HHcy treated vessels (Figure 2B) may, in part, due to autophagic / mitophagic death of functional endothelial and / or vascular smooth muscle cells. Although we have demonstrated here with evidence that Hcy-induced mitophagy in in vitro endothelial cells (Figures 5, 6A & B), which may have contributed to impaired endothelial-dependent vascular function in arterial explants (Figure 2B), further studies are needed to dissect whether only endothelial cells are involved in vascular dysfunction or it is a combined effect of vascular smooth muscle and endothelial cells. Additionally, in our experiments, gene therapy improved but did not normalize endothelial function. This could be due to partial transfection, as we have reported in the method that about 30-40% cells was GFP positive.

The signaling molecule of Akt is well recognized for its anti-apoptotic activity and known as a survival kinase (12). FoxO proteins are a group of Forkhead family of transcription factors and studies have indicated that decreased Akt signaling activates autophagy transcription-dependent mechanism involving Foxo3 (42). However, the involvement of Akt/FoxO signaling cascades in Hcy-induced mitophagy and the regulatory role of H$_2$S, if any, are not defined in vascular diseases. Our present report demonstrated that Hcy dephosphorylated Akt and downstream pathway upregulated FoxO (Figure 7). H$_2$S as well as triple genes overexpression ameliorated Akt activation and mitigated FoxO. This result suggested that Akt/FoxO pathway may have a role in the induction of mitophagy/autophagy in HHcy. Contrary to H$_2$S supplementation, which modulated Akt/FoxO pathways, triple genes delivery modulated Hcy effect by metabolizing Hcy to H$_2$S rather than direct interfering of these signaling cascades. This result demonstrated differential role of endogenous vs exogenous H$_2$S in Hcy signaling.

Chronic HHcy alters extracellular matrix (ECM) components (59, 60) and matrix metalloproteinases (MMPs) maintain ECM homeostasis in the matrix. Among MMPs, MMP-13 have significant renal expression related to ECM remodeling during progressive renal diseases (1, 30). MMPs activities are endogenously regulated by inhibitors of metalloproteinases (TIMPs), and TIMP-1 inactivates most MMPs (43, 52, 59). Although, reduction of Hcy level is directly associated with amelioration of MMP activity (40) and matrix accumulation (59), the mechanism of MMP/ TIMP-1 regulation in HHcy, and possible role of H$_2$S
is not well known. We have previously reported mtMMP-9 (mitochondrial MMP-9)-mediated
autophagy/mitophagy in cardiomyocytes during HHcy through N-methyl-d-aspartate receptor 1
(NMDA-R1)-dependent pathway (55). Here we demonstrated that Hcy induced MMP-13 and
mitigated TIMP-1 (Figure 7C). Interestingly, triple genes delivery normalized the expression of
MMP-13 suggesting a triggering mechanism of this metalloproteinase by H₂S in HHcy.

In our study we chose to transfect renal arteries with CBS, CSE and 3MST genes of
transsulfuration enzymes ex vivo rather than in vivo injection for the following reasons: 1) to
avoid immune response; 2) to increase efficacy of transfection with minimum amount of DNA;
and 3) to avoid potential health problems to the animal, such as toxicity and inflammatory
responses with large amount of DNA. Furthermore, in our experiment normal renal arteries, i.e.
arteries from animal that are non-hyperhomocysteinemic were used. In ex vivo condition these
arteries were treated with high Hcy (75 µM) to measure endothelial dysfunction, and triple genes
were delivered to measure any protective role of gene therapy from Hcy threat. It is fact that in
our study 3MST did not increase H₂S generation significantly in the renal arterial tissue
compared to control. However, 3MST transfection significantly increased H₂S generation in
cultured MAECs mitochondria (Figure 4C). It is possible that the renal arterial tissue was
insufficient to measure 3MST-mediated H₂S generation in our experiment. Nonetheless, triple
genes therapy significantly increased H₂S generation vs control and improved endothelial-
dependent vasorelaxation in presence of Hcy vs Hcy alone (Figures 2A & C, respectively).
Improved endothelial-dependent vasorelaxation in triple genes delivered vessels were, at least
in part, by increased eNOS activity as evidenced by eNOS inhibition (Figure 2C). Taking
together we believe this functional improvement was achieved as a result of combined effects of
three transsulfuration enzymes. The study could have been considerably strengthened and
more clinically relevant if renal arteries from HHcy animals, such as CBS+/-, would have been
considered. However, arteries of CBS+/- animals were already exposed to higher levels of Hcy
in their circulation as well as in vasculature, due to impaired metabolism, and may have
damaged endothelial integrity. The idea of the present study was to demonstrate the protective
role of gene therapy in preserving endothelial function by Hcy metabolism and efficient
production of H₂S. The study, however, was not designed to regenerate damaged endothelial
cells from HHcy animals, such as CBS+/-, ex vivo. Therefore, we used WT arteries instead.
Nevertheless, the study that aims to repair and regenerate endothelial cells is off in urgent need
to demonstrate effectiveness of genes therapy to restore endothelial function in HHcy.

In conclusion, the important aspects of our study is that overexpression of Hcy
metabolizing genes CBS, CSE and 3MST improved endothelial function in HHcy by triggering
angio- and anti-angiogenic factors through H$_2$S generation. In addition, mitophagy and MMP/TIMP were also regulated by gene therapy possibly through H$_2$S regulated Akt/FoxO pathway. This is an interesting finding, where gene delivery could not only be exploited to minimize Hcy toxicity, but also to protect cell by generating anti-oxidative agent, H$_2$S.

**Limitations of the study:**

This study has following limitations: 1) the renal arteries were cultured for 48 h, which is longer than standard incubation time. This raises a concern regarding the viability of the vessels such a long time after harvesting. In addition, as shown in figure 2B, the level of relaxation of treated renal vessels to Ach reaches 20% by most, which is lower than usually observed in normal vessel. This again raises a concern of whether this related to loss of vasoreactivity due to long incubation time. 2) Ex vivo study, instead of in vivo gene treatment was performed to avoid immune response and potential health problems to animals. Since the safety and toxicity of the treatment in our present study is yet unproved in vivo, we consider this is a potential limitation of our study. 3) The idea tested in this study was to observe any protective effect of gene therapy in preserving endothelial function, rather than endothelial regeneration. In the discussion, although we have discussed the reason for examining normal arteries, the fact that the treatment works in normal artery does not guarantee it would succeed under disease condition. An experimental model close to renal vascular disease would be helpful to explore the effectiveness of the therapy in this area. Lastly, but not least, 4) in our experiment reduced ROS was found after the gene therapy. In this case, although we did not measure, an endothelium-independent increase in NO availability could be another mechanism, which warrants future investigation.

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References


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Figure legends

Figure 1:

(A) Transfection efficacy in arterial explants. Renal arterial explants from wild type (WT, C57BL/6J) were transfected with pcDNA3.1/GFP, pcDNA3.1/CBS, pME18S-CSE-HA, pME18S-3MST or triple genes of CBS, CSE and 3MST as described in the materials and methods, and earlier report (38). After 48 h, GFP transfected artery was cryosectioned, and transfection efficacy was determined by examining pcDNA/GFP fluorescence in fluorescent microscope (Scale bar, 50 µm).

(B) CSE overexpression in VSMC. Since CSE is mainly localized in the vascular smooth muscle cell (VSMC), enhanced expression of CSE in the arterial section after gene delivery was confirmed by immunostaining. Red fluorescence indicated expression of CSE, and blue fluorescence indicated nuclear stain with DAPI (Scale bar, 100 µm).

(C) & (D) Immunoblotting and protein expression levels. Enhanced expression of CBS, CSE and 3MST protein in the renal arterial explant was further confirmed by immunoblotting following gene transfection (Data mean ± SE, n = 3; *indicates p<0.05 vs control).

Figure 2:

(A) Gene delivery increased generation of H$_2$S in arterial explants. WT arterial explants after 48 h of gene transfection were homogenized and tissue capability to generate H$_2$S was determined by our previously adopted method (41) using Hcy as a substrate. Data represents mean ± SE, n = 4-5 independent experiments; *indicates p<0.05 vs control, †p<0.01 vs individual gene transfection.

(B) Hcy impaired endothelial-dependent vasorelaxation. Renal arterial explants of WT mice were cut into 2 mm pieces, and cultured with or without Hcy (75 µM) for 48 h following protocol as described in the methods. Arterial rings were mounted in a myobath containing PSS as described in methods. A group of rings, which were incubated with Hcy mounted in myobath containing L-NAME (N$^\text{G}$-nitro-L-arginine methyl ester, 10 µM), an eNOS inhibitor. Rings were pre-contracted with Phe (10$^{-5}$M) and later challenged with dose-dependent acetylcholine (Ach) as indicated in the figure. Vessels which received L-NAME were virtually unresponsive to Ach, indicating blocked eNOS activity. Data analyzed by two-way ANOVA and represented as mean ± SE, n = 4-5; *indicates p<0.05 vs WT at same dose of Ach.

(C) Triple gene therapy improved endothelial-dependent vasorelaxation in HHcy. Renal arterial explants of WT mice were cut into 2 mm pieces, transfected with triple genes and
cultured with or without Hcy (75 µM) as indicated for 48 h. Arterial rings of WT and WT+Triples genes served as controls for WT + Hcy and WT + Triples genes + Hcy, respectively. Rings were mounted between two tungsten wires, hanged in myobath as described earlier. In another set of experiment, WT arterial rings received L-NAME (10 µM) in the myobath, in addition to triple genes and Hcy treatment. Endothelial-dependent vasorelaxation was measured in cumulative Ach doses. Data analyzed by two-way ANOVA and represented as mean ± SE, n = 4-5 independent experiments; *indicates p<0.05 WT+Triple genes + Hcy vs WT +Hcy.

(D) H2S improved endothelial-dependent vasorelaxation in HHcy. Arterial rings were incubated with Hcy and with or without H2S (30 µM, in the form of NaHS). Endothelial-dependent vasorelaxation was measured as described earlier. Rings which received L-NAME in myobath were unresponsive to Ach, indicating blocked eNOS activity. Data analyzed by two-way ANOVA and represented as mean ± SE, n = 4-5 independent experiments; *indicates p<0.05 WT+H2S + Hcy vs WT +Hcy.

(E) Expression of eNOS. After myobath study, arterial rings were homogenized in RIPA lysis buffer and eNOS expression was measured by immunoblotting. Data mean ± SE, n = 4-5; *indicates p<0.01 vs WT and †p<0.05 vs WT + Hcy.

(F) No changes in endothelial-independent vascular relaxation were observed among the groups. Phe (10^{-5} M) pre-contracted vessels were challenged with dose-dependent (10^{-9}-10^{-5} M) SNP (Sodium nitroprusside), a direct NO donor. No differences were recorded between WT, WT+Hcy and WT+Triples genes vessels, indicating unchanged vascular smooth muscles reactivity among the groups. Data analyzed by two-way ANOVA and represented as mean ± SE, n = 4.

Figure 3: Gene therapy induced CD31 and VEGF, and diminished endostatin in renal artery explants in HHcy. CBS, CSE and 3MST genes were transfected in WT arterial explants, and explants were cultured in matrigel for 48 h in the presence of Hcy (75 µM). Explants were cryosectioned and immunostained with appropriate antibodies secondarily conjugated with FITC. Fluorescence images were taken under confocal microscope (Red arrows indicated endothelial lining; Scale bar, 50 µm; A.U., arbitrary unit). Bar diagram: data represents mean ± SE, n = 7; *p<0.01 vs control and †p<0.01 vs triple genes + Hcy.

Figure 4: Triple genes expression and H2S generation in endothelial cells.
(A) Mouse aortic endothelial cells (MAECs) were transfected with either CBS, CSE, 3MST or triple genes. Expression of GFP vector indicating successful transfection.

(B) After 48 h of transfection cell were lysed and expression of CBS, CSE, and 3MST protein were measured by Western blot.

(C) In isolated mitochondria, localized expression of 3MST was confirmed by immunoblotting. Immunoblotting of mitochondria extracted protein with anti-GAPDH antibody (glyceraldehyde 3-phosphate dehydrogenase) indicated isolation of pure mitochondria, which is free from cytosolic fraction of conserved GAPDH protein. Immunoblot reprobed with COX IV (cytochrome c oxidase) antibody and used as a mitochondrial loading control. The capability of H₂S generation by isolated mitochondria from 3MST transfected cells were measured following protocol as described in methods. Bar diagram showed 3MST transfected mitochondria generated increased amount of H₂S vs control in presence of Hcy (Data mean ± SE, n=4; *p<0.05 vs control).

(D) Cells capability to generate H₂S in presence of Hcy was measured as indicated in the method and our previously adopted protocol (37). Representative data from n = 5 independent experiments. Data mean ± SE, n=4; †p<0.05 vs control and *p<0.05 vs Hcy.

Figure 5:

Hcy-induced mitophagy was mitigated by H₂S and gene therapy.

(A) Immunostaining: MAECs were cultured for 48 h in the presence or absence of H₂S (30 µM, in the form of NaHS) and Hcy (75 µM) as shown in the figure. Cells immunostained with LC3AII antibody secondarily conjugated with FITC (green). Nucleus stained with DAPI (blue).

(B) Hcy-induced expression of mitophagy markers, mTOR, Beclin 1, BNIP3 and ratio of LC3AII were mitigated by CBS, CSE and 3MST genes transfection. MAECs were transfected with genes as indicated in the figure and treated with or without Hcy (75 µM) for 48 h. Mitochondria isolated, and lysed in RIPA lysis buffer. Equal amount of protein were analyzed for mitophagy markers as indicated. GAPDH immunoblotting of mitochondria extracted protein indicated isolation of pure mitochondria, which is free from cytosolic GAPDH. Immunoblot reprobed with COX IV antibody and used as a mitochondrial loading control.

(C) Ratio of LC3AII/I, and (D) Densitometric analyses of mTOR, BNIP3 and Beclin 1. Data represents mean ± SE, n=4; *p<0.01 vs control and †p<0.01 vs Hcy.

Figure 6:

Triple gene delivery mitigated mitophagy and mitochondrial ROS production.
(A) Flow cytometry: Mitochondria isolated from the treated MAECs as shown, immunostained with LC3AI/II and analyzed by flow cytometry. Appropriate controls were taken.

(B) Bar diagram showed % of mitochondria expressing LC3AI/II marker as an indication of mitophagy. Data mean ± SE, n = 4/group; *p<0.05 vs control and †<0.01 vs Hcy.

(C) MAECs were transfected either with single gene or triple genes as shown, and cultured for 48 h in the presence of Hcy (75 µM). Mitochondria were isolated and ROS production was detected using DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) as a substrate. Data presented as mean ± SE, n = 5 experiments; *indicates p<0.05 vs control, †indicates p<0.05 vs Hcy.

Figure 7:

(A)-(B) Hcy-induced dephosphorylation of Akt and phosphorylation of FoxO3a mitigated by H₂S.

(A) MAECs pre-treated with or without H₂S (30 µM, in the form of NaHS)) were treated with Hcy (75 µM) for 30 min as indicated in the figure. A group without any treatment served as control and a group pretreated with H₂S served as control for Hcy+H₂S. At the end of experiment cells were lysed and proteins were analyzed by Western blot.

B) Bar diagram showed densitometric analyses of phospho-protein expression. Data mean ± SE, n = 4; *p<0.05 vs control and †<0.05 vs Hcy treatment.

(C) & (D) Gene therapy normalized Akt activation and MMP-13/TIMP-1 imbalance. In a separate experiment (C) cells were transfected with CBS, CSE and 3MST genes, and treated with Hcy (75 µM). Expression of phospho-Akt after 30 min of Hcy treatment, and MMP-13 and TIMP-1 after 48 h of Hcy treatment were measured by Western blot.

(D) Bar diagram showed densitometric analyses of protein expression. Data mean ± SE, n = 4; *p<0.01 vs control and †<0.05 vs Hcy treatment.
Figure 2

E

F

SNP (M)

Response (% relaxation)

WT

WT+Hcy

WT+Triple

WT+Triple+Hcy

Fold changes x WT

eNOS

β-actin

*   †   †
Figure 3

CD31

VEGF

Endostatin

- + Hcy + Triple genes + Hcy

CD31 expression (A.U.)

VEGF expression (A.U.)

Endostatin expression (A.U.)

Figure 3
Figure 7

A

p-Akt
p-FoxO3a
β-actin

B

Protein/β-actin (Fold change)

Control
Hcy
Hcy+H₂S
H₂S

C

p-Akt
β-actin
MMP-13
TIMP-1

D

pAkt
MMP-13
TIMP-1

Control
Hcy
Triple
Triple+Hcy