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ORIGINAL ARTICLE

Hydrogen sulfide protects against vascular remodeling from endothelial damage

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Abstract Remodeling by its very nature implied synthesis and degradation of extracellular matrix (ECM) proteins. Although oxidative stress, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) have been implicated in vascular remodeling, the differential role of MMPs versus TIMPs and oxidative stress in vascular remodeling was unclear. TIMP-3 induced vascular cell apoptosis, therefore, we hypothesized that during vascular injury TIMP-3, MMP-9 and -12 (elastin-degrading MMP) were increased, whereas MMP-2 (constitutive MMP) and TIMP-4 (cardioprotective TIMP) decreased. Because of the potent anti-oxidant, vasorelaxing, anti-hypertensive agent, hydrogen sulfide (H_2S) was used to mitigate the vascular remodeling due to the differential expression of MMP and TIMP. Carotid artery injury was created by inserting a PE-10 catheter and rotating several times before pulling out. The insertion hole was sealed. Mice were grouped: wild type (WT), wild-type damaged artery (WTD), WT + NaHS (sodium hydrogen sulfide, precursor of H_2S) treatment (30 μmol/L in drinking water/6 weeks) and WTD + NaHS treatment. Carotid arteries were analyzed for oxidative stress and remodeling, by measuring super oxide dismutase-1 (SOD1), p47 (NADPH oxidase subunit), nitrotyrosine, MMPs and TIMPs by in situ immunolabeling and by Western blot analyses. The results suggested robust increase in p47, nitrotyrosine, MMP-9, MMP-12, TIMP-3 and decrease in SOD1 and MMP-2 levels in the injured arteries. The treatment with H_2S ameliorated these effects. We concluded that p47, TIMP-3, MMP-9 and -12 were increased where as SOD-1, MMP-2 and TIMP-4 were decreased in the injured arteries. The treatment with H_2S mitigated the vascular remodeling by normalizing the levels of redox stress, MMPs and TIMPs.

Keywords SOD · P47 · NaHS · Nitrotyrosine · MMP-12 · ROS · RNS · RTS

Abbreviations

AVF	Aorta–venacava fistula.
BW	Body weight
H_2S	Hydrogen sulfide
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenosine dihydrogenphosphate
NaHS	Sodium hydrogen sulfide
p47	Protein of 47 kDa
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RTS	Reactive thiol species
SOD	Superoxide dismutase
STS	Sodium thiosulfide
TIMP	Tissue inhibitor of metalloproteinase
WT	Wild type
WTD	Wild-type damaged artery

Introduction

Damage to the endothelial layer of the cardio vasculature is of special interest since the vasculature is constantly exposed to hemodynamic shearing forces as well as rapid changes in pressure. Moreover, there is a physical

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disturbance of vasculature when inserting such mechanical interventional devices as cardiac stents to restore blood flow from occluded regions. Furthermore, H_2S has been shown to act as a reliever of cellular oxidative stress as well as a vasodilator that relieves physiological stress accompanied with hypertension. We aimed to investigate the expression as well as localization of several different proteins involved in oxidative stress and remodeling to determine whether hydrogen sulfide could alleviate physiological and interventional stress.

Superoxide dismutase (SOD) is a protein whose function is to destroy free radicals in the body (Kartha et al. 2008). The role of tissue inhibitors of metalloproteinases (TIMPs) is to inhibit their respective matrix metalloproteinases (MMPS) that to involve in constitutive and pathological remodeling process (Luo 2005). Moreover, a balance has been noted between MMPs and TIMPs such that if MMPs are higher, then the inhibitors are lower (Malemud 2006). Upon exposure, stress cells express proteins that are involved in remodeling (Luo 2005). For example, oxidative stress results in increased expression of MMP-2 and MMP-9 (Sen et al. 2007a). Moreover, MMPs are known to be part of the proliferation process of vascular smooth muscle cells after balloon injury (Holven et al. 2003). Our laboratory has demonstrated that arteriovenous fistula (AVF) heart failure produced an increase in MMP-2 and MMP-9 while this increase was reduced to control levels with addition of sodium thiosulfate (STS) with the restoration of H_2S levels. This suggested that STS modulated contractility partially via H_2S production. There was an increase in MMP-2 and MMP-9 expression in kidney of hyperhomocysteinemic mice, a condition that increases oxidative stress (Sen et al. 2009); moreover, hydrogen sulfide treatment reduced expression of MMP-2 and MMP-9 significantly (Sen et al. 2009).

One mechanism by which hydrogen sulfide acts is via activation of potassium-dependent ATP channels that causes vasodilation in cardiovascular protection (Zhang et al. 2007c). The concentration of H_2S plays important role in the protection versus prosecution. For instance, low H_2S concentration could result in vasoconstriction due to blocked vasoactive effect of nitric oxide (NO) and formation of nitrosothiol (Ali et al. 2006; Webb et al. 2008; Whiteman et al. 2006). Under certain concentrations, H_2S can act as an anti-oxidant by acting as a peroxynitrate scavenger (Whiteman et al. 2004). There are conflicting data on the signal transduction of H_2S in mitogen-activated protein kinase and phosphatidy inositol-3-kinase/Akt pathway (Hu et al. 2007). Moreover, p47 is involved in oxidative stress via the NF-κB pathway that induces MMP/TIMP axis leading to cardiac dilation and failure (Henderson and Tyagi 2006).

Based on the roles of these several proteins, we hypothesized that damaging conditions would result in an increase in ROS and hydrogen sulfide would relieve oxidative stress. Moreover, we hypothesized that damaging conditions would increase pathological remodeling instigated by MMP-2 and that H_2S would restore normal remodeling.

Methods

Animal

Wild-type (WT, C57BL/6J) male mice aged 8 weeks (weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were grouped: (wild type WT), WT + NaHS, WTD (WT-damaged artery), WTD + NaHS and housed in the animal care facility at University of Louisville. Mice were treated with 30 μm/L NaHS for 8 weeks in drinking water. Every alternate day drinking waters was supplemented with NaHS. To anesthetize the mouse, mouse was injected with avertin (2.5% solution), intraperitoneal injection of 10 mg/g bodyweight. The right common carotid artery was identified and was cannulated with a polyethylene catheter PE-10. The catheter was slowly advanced as far as possible towards the brain. Endothelial cell layer damage was done by moving catheter back and forth several times. Then, catheter was removed. The incision was closed using 6.0 self-absorbing sutures. The sham surgery was used in the same way except the cannulation. After 8 weeks of treatment, animals were killed and carotid arteries were removed and stored at 80°C until further analysis.

Chemicals

Horseradish peroxidase (HRP)-conjugated antibodies, MMP-12, TIMP-3, TIMP-4 and nitrotyrosine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); sodium chloride, protease inhibitor cocktail, sodium hydrosulfide hydrate and antibody for β-actin were purchased from Sigma (St. Louis, MO). RIPA buffer was from Boston Bioproducts (Worcester, MA). Secondary antibodies, conjugated with Alexa 555 dye or with Alexa 488 were purchased from Invitrogen (Carlsbad, CA). Antibody against MMP-2 was purchased from Novus (Littleton, CO). Antibodies against p47 and SOD-1 were purchased from Millipore (Billerica, MA). Antibody against MMP-9 was purchased from ABCAM (Cambridge, MA).

Preparation of samples: western blot analysis and immunodetection

After treatment, carotid arteries were frozen with liquid nitrogen and lyses buffer (in mM: 50 Tris–Cl, pH 7.4, 150

NaCl, 1% Triton X-100 and 1 EGTA) along with freshly prepared inhibitors (1 mM PMSF, 1 μg/mL leupeptin, 200 μM sodium orthovandate and 1 μg/mL aprotinin). The frozen samples were pulverized with mortar and pestle and the pulverized frozen shards were placed in a microfuge tubes. Afterwards, samples were sonicated at low setting for three times and 6 s each. Samples were placed on ice between sonication events. Afterward, samples were centrifuged at 10,000*g* for 10 min to collect the cellular debris. Protein estimation was conducted via Bradford method and analyzed on a spectrophotometer. Equal amounts of protein were loaded on to SDS-PAGE of 10% polyacrylamide gels. Protein was then blotted onto a polyvinylidenedifluoride membrane. After being transferred, blots were washed with Tris-buffered saline (TBS) for 5 min at room temperature and incubated in blocking buffer for 1 h at room temperature. The blots were then incubated with the indicated primary antibodies, appropriate dilutions in 3% fat-free milk solution of TBST (0.1% Tween 20 + TBS) overnight at 4°C using gentle agitation. The blots were washed four times (5-min wash each time) with TBST and incubated with HRP-conjugated secondary antibody (1:3,000 dilutions in 3% milk-TBST). After being washed with TBST four times (10 min wash each time), the proteins of interest were detected using an ECL plus kit (Amersham Biosciences, Piscataway, NJ). The membranes were then stripped using 0.2 M NaOH solution for 30 min at room temperature and reprobed with β-actin as standard.

Confocal microscopy

The tissue slices were taken from carotid vessel and fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature. Tissue was washed and permeabilized with 0.1% Triton X-100 for 20 min. After being washed, the slices were incubated with primary that could be either anti-SOD1, anti-p47, anti-TIMP-3 and anti-TIMP-4 antibody, or anti-MMP-9 (Sigma) (1:500 dilution, prepared in 0.02% Tween 20/PBS) overnight at 4°C. Cells were washed, and goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) (1:600 dilutions, prepared in PBS) was applied for 3 h at room temperature. Tissue was washed and incubated with 50 ng/mL Mitotracker Red in the dark for 25 min. After additional washes with PBS to remove unbound Mitotracker, the cells were mounted onto the glass slides. The images were acquired using a laser confocal microscope (FluoView 1000).To enable the comparison of changes in fluorescence intensity and punctate staining pattern; the images were acquired under the identical set of conditions. FITC fluorescence was imaged using a band pass filter set at 488 nm excitation and 510–540 nm emission. Mitotracker Red was imaged using a He–Ne laser (excitation 579 nm and emission 599 nm) and also green was used.

Data analysis and statistics

Values are mean ± SE from at least three different experiments. The data were analyzed by Student's *t* test for comparison of the results between various treatment groups. $P < 0.05$ was considered to indicate statistical significance.

Results

To determine the expression of p47 (green) and SOD1 (red) (Fig. 1a), the carotid artery sections were analyzed by confocal microscope. A graph form was depicted using selected region of the intensity that was representative of the injured carotid artery (Fig. 1b). There were equal lowest basal intensity levels for WT and WT + NaHS groups. There was a significantly increase in the intensity of p47 in WTD group relative to control groups. Moreover, the intensity of WTD + NaHS was decreased significantly from WTD groups, but still remained above control levels. The SOD-1 expression was highest in control groups WT, WT + NaHS, but was significantly decreased in WTD groups. WTD + NaHS showed an intermediate level.

Because oxidative stress caused activation of latent MMPs, and to determine the extent of oxidative stress the levels of nitrotyrosine were measured in vascular tissue homogenates. The data in Fig. 2 showed nitrotyrosine expression for the following four groups: WT, WTD, WT + NaHS, WTD + NaHS. There was significant elevation of nitrotyrosine radical in WTD with an elevation of 5.0 relative to control ($P < 0.05$, $n = 3$). Interestingly, there was an amelioration in nitrotyrosine generation relative to control ($P < 0.05$, $n = 3$) in WTD + NaHS group. There were no significant differences between WT and WT + NaHS groups.

Because MMP-12 specifically degraded elastin, we measured MMP-12 in arterial tissue homogenates. The data in Fig. 3 showed MMP-12 for the following four groups: WT, WTD, WT + NAHS, WTD + NaHS. The results suggested a significant 3.0 increase ($P < 0.05$, $n = 3$) in MMP-12 expression in WTD group as compared to WT group. This increase was mitigated by NaHS treatment.

The data in Fig. 4 showed the expression profile of MMP-2 and MMP-9 using confocal microscopy. There was an increase in MMP-9 expression in WTD group and a decrease in expression to basal levels in WTD + NaHS group. The two controls had a baseline expression that was

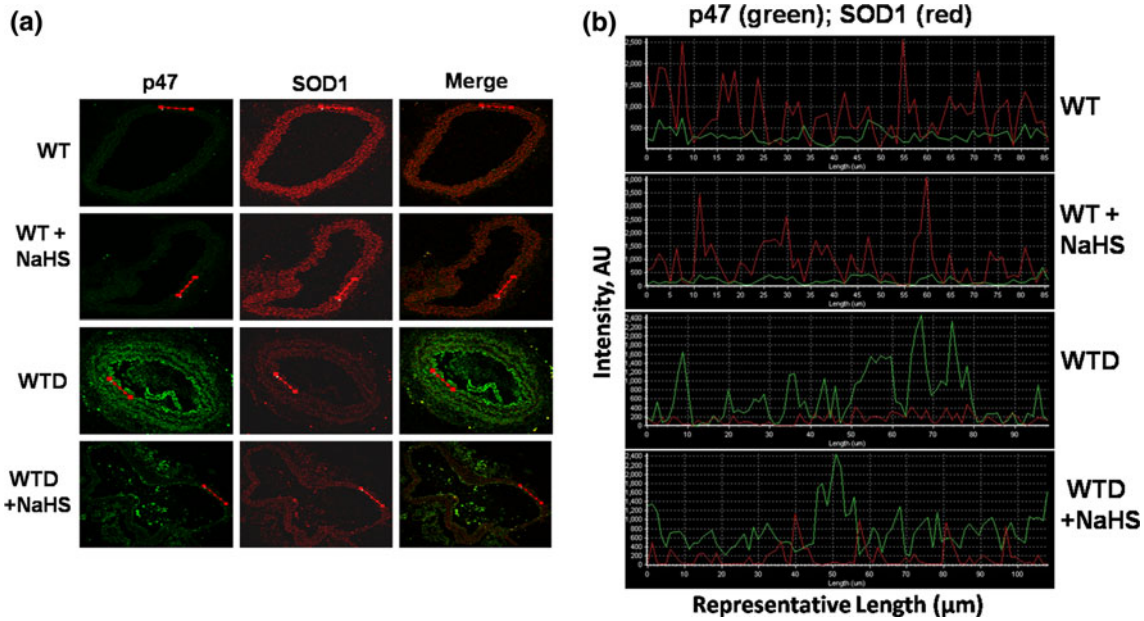

Fig. 1 **a** Carotid artery cross sections from WT-sham (WT), WT-damaged-injured (WTD), WT + NaHS and WTD + NaHS treated with NaHS for 6 weeks were stained with p47 and SOD1 antibodies. The secondary antibodies were labeled with *green* for p47 and *red* for SOD1. The serial images were merged. The *lines* represent the injury regions. **b** The injury regions of the vessels were scanned for intensity for the respective antibody labeling. The *lines* were drawn and matched with p47 (*green*) and SOD1 (*red*) labels. Higher the intensity, higher was the expression

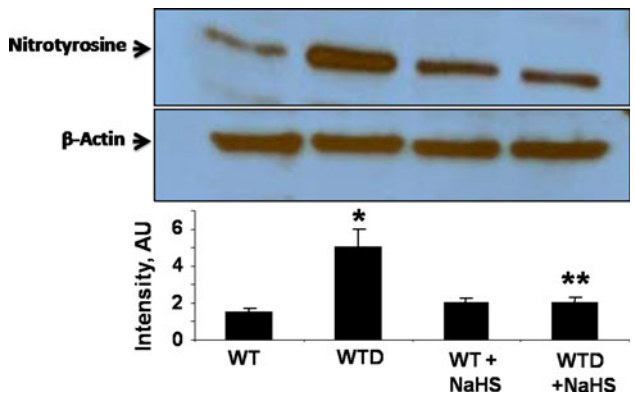

Fig. 2 Carotid arteries tissue homogenates were prepared and analyzed by western blot analysis for nitrotyrosine: the homogenates from WT-sham (WT), WT-damaged- injured (WTD), WT + NaHS and WTD + NaHS treated with NaHS for 6 weeks were analyzed using anti-nitrotyrosine antibody. The gel panel was the representative western blot with β-actin control. The *bar graph* represented the bands scanned intensity in arbitrary unit (AU). Each *bar* represented mean + SEM from $n = 6$ in each group. *$P < 0.05$ as compared to WT; **compared to WTD

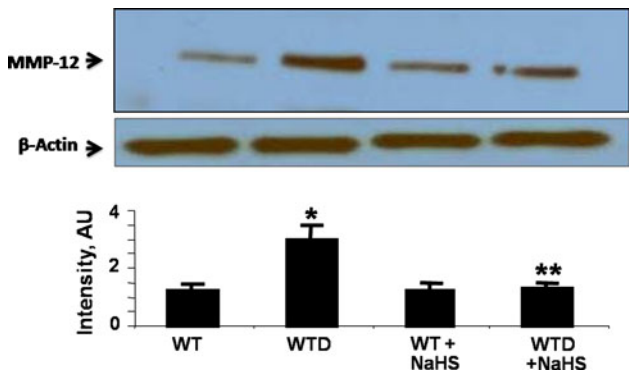

Fig. 3 Carotid arteries tissue homogenates were prepared and analyzed by western blot analysis for MMP-12: the homogenates from WT-sham (WT), WT-damaged-injured (WTD), WT + NaHS and WTD + NaHS treated with NaHS for 6 weeks were analyzed using anti-MMP-12 antibody. The gel panel was the representative western blot with β-actin control. The *bar graph* represented the bands scanned intensity in arbitrary unit (AU). Each *bar* represented mean + SEM from $n = 6$ in each group. *$P < 0.05$ as compared to WT; **compared to WTD

lower than WTD and WTD + H_2S. WTD had the greatest expression of MMP-9 while WTD + H_2S had a reduced expression as compared to WTD. WTD + NaHS group had intensity only slightly greater than control values. The levels of MMP-2 did not demonstrate significant difference in between the groups.

Figure 5 showed confocal analyses of carotid artery sections, to determine expression levels of TIMP-3 (green) and TIMP-4 (red). The lowest intensities for TIMP-3 were found in control groups, WT, WT + NaHS. WTD showed a significant increase in intensity relative to controls. WTD + NaHS group showed an intensity that was decreased from WTD, and almost that of control values. TIMP-4 expression

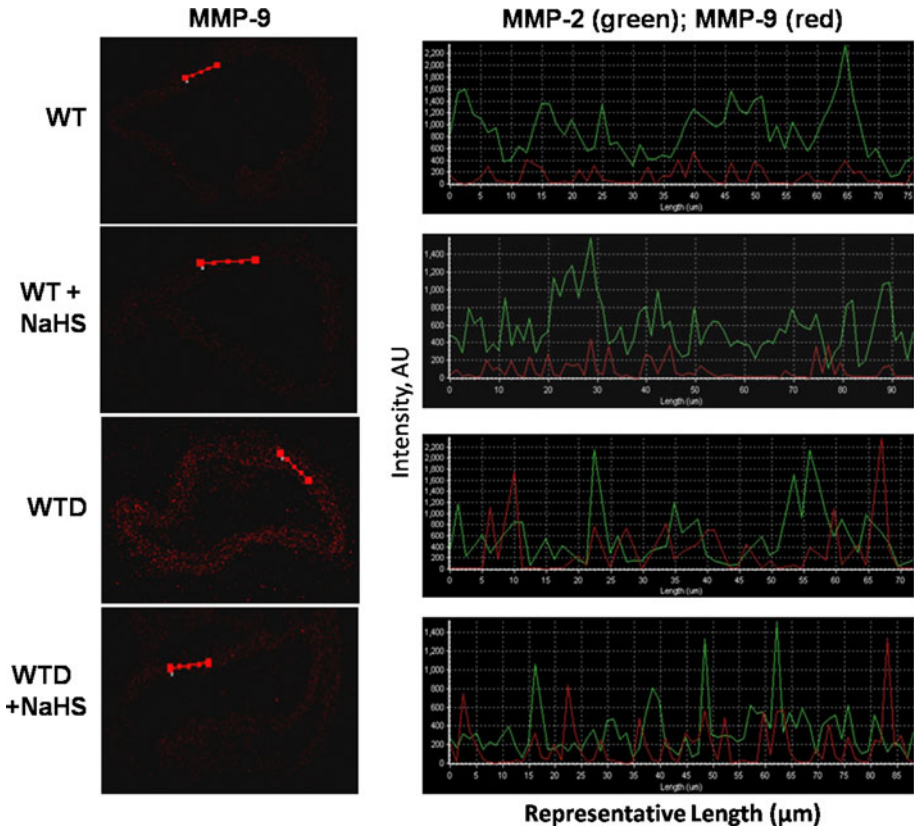

Fig. 4 Carotid artery cross sections from WT-sham (WT), WT-damaged- injured (WTD), WT + NaHS and WTD + NaHS treated with NaHS for 6 weeks were stained with MMP-9 antibody (*left column panels*). The *line* panels represent the injury regions of the vessels, scanned for intensity for the respective antibody labeling. The *lines* were drawn and matched with MMP-2 (*green*) and MMP-9 (*red*) labels. Higher the intensity, higher was the expression. The secondary antibodies were labeled with *green* for MMP-2 and *red* for MMP-9

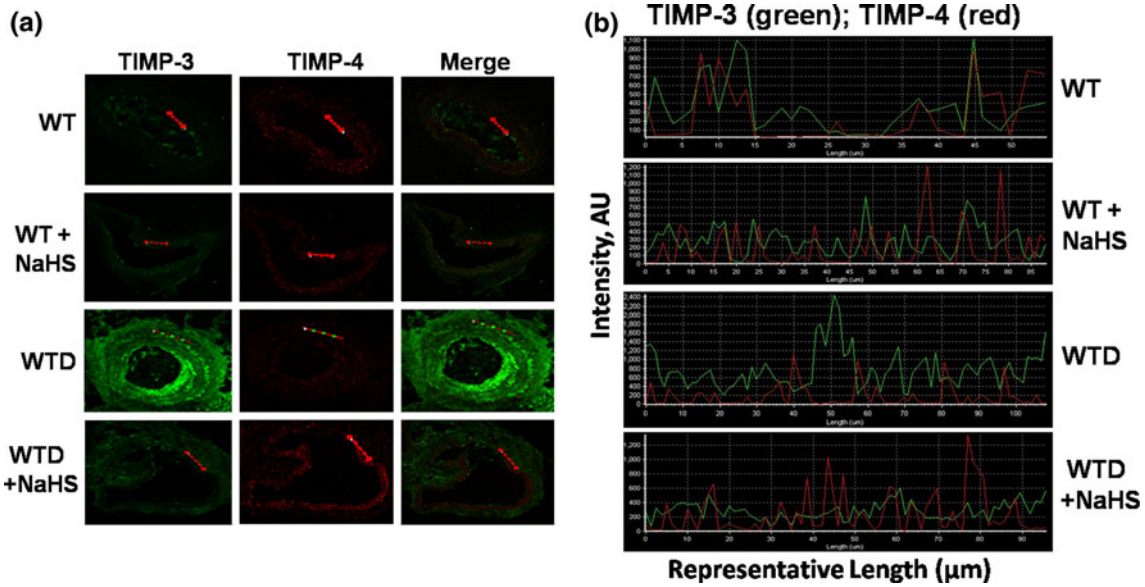

Fig. 5 **a** Carotid artery cross sections from WT-sham (WT), WT-damaged-injured (WTD), WT + NaHS and WTD + NaHS treated with NaHS for 6 weeks were stained with TIMP-3 and TIMP-4 antibodies. The secondary antibodies were labeled with *green* for TIMP-3 and *red* for TIMP-4. The serial images were merged. The *lines* represent the injury regions. **b** The injury regions of the vessels were scanned for intensity for the respective antibody labeling. The *lines* were drawn and matched with TIMP-3 (*green*) and TIMP-4 (*red*) labels. Higher the intensity, higher was the expression

appeared to have increased under WTD + NaHS conditions relative to all other conditions. Figure 5a and b showed an increase in TIMP-3 expression for WTD group, but a reduced expression for WTD + NaHS group to levels near control WT and WT + NaHS groups. Moreover, there was no change in expression of TIMP-4 in any of the groups.

Discussion

The utility of hydrogen sulfide as a therapeutic agent has been gaining momentum; however, several studies have indicated conflicting results on its beneficial or detrimental effect. The differences in the results of these studies seem to be contingent on a garden variety of variables: dosage use, length of exposure, method of administration and tissue sample. Hydrogen sulfide could act as a vasodilator and potent anti-oxidant under some conditions, or could act as a vasoconstrictor and, therefore, contribute to ROS generation under other experimental conditions. For instance, H_2S can activate potassium-dependent ATP channel that can cause vasodilatation and elicit cardiovascular protection (Zhang et al. 2007c). Interaction between H_2S and NO is very complex. Low H_2S concentration could result in vasoconstriction due to blocked vasoprotective effect of NO and formation of nitrosothiol (Ali et al. 2006; Webb et al. 2008; Whiteman et al. 2006). Moreover, local oxygen concentration has also effect on whether H_2S acts as a vasoconstrictor or dilator (Koenitzer et al. 2007). Under certain concentrations, H_2S can act as anti-oxidant by acting as a peroxynitrate scavenger (Whiteman et al. 2004). Moreover, there are conflicting data regarding the signal transduction pathways of mitogen-activated protein kinase and phosphatidyl inositol-3-kinase/Akt, both act as inhibitory and activating factors depending on the cell line (Hu et al. 2007). These contradictory findings may best be explained by the concentration of H_2S (Wagner et al. 2009).

It is still under debate the role of H_2S as a metabolic mediator or toxic gas (Tisherman and Drabek 2008). H_2S has been found to have both proinflammatory (Collin et al. 2005; Li et al. 2005; Zhang et al. 2006, 2007a, b) and anti-inflammatory effects (Whiteman et al. 2004; Elrod et al. 2007; Hu et al. 2007; Sivarajah et al. 2009). Moreover, the route of administration is also something to be considered. One study reported that the lower levels of H_2S defend organs from oxidative stress and other pathology (Zhang et al. 2008). Moreover, other authors have reported that H_2S induces ROS and RNS formation at higher levels, while decreasing hydrogen peroxide, peroxinitrite and superoxide anion generation at lower levels. One experiment reported that H_2S increased SOD and glutathionine-peroxidase (GSH-Px) (Liu et al. 2009). We similarly found that under damaging conditions, nitrotyrosine expression was increased significantly relative to WT, indicating that endothelial damage resulted in increased RNS. Moreover, hydrogen sulfide treatment decreased this expression to within the range of control levels such that WTD + NAHS were not significantly elevated above controls.

High-micromolar concentrations have been accompanied by cytotoxic effects that result from free radical generation: glutathionine deletion, intracellular iron release, pro-apoptotic pathway (Yang et al. 2007). However, low micromolar concentration has been shown to promote cytoprotection that includes anti-necrosis and anti-apoptosis (Rose et al. 2005), or proapoptosis (Adhikari and Bhatia 2008; Baskar et al. 2007; Cao et al. 2006) that depends on the experimental conditions. Perhaps, our experimental condition could have been further modified to create a greater decrease in free radical production upon addition of NaHS. For instance, it is important to note that control conditions had an elevated trend of increasing nitrotyrosine content, but did not reach significance relative to control values.

STS is known as an anti-oxidant and calcium solubilizer (Hayden et al. 2005; Meissner et al. 2006), and has been shown to modulate H_2S production (Sen et al. 2008). For instance, our laboratory conducted an experiment of heart failure using AVF. Under these conditions, hydrogen sulfide production was decreased in left ventricular tissue. This was accompanied by ventricular contractile dysfunction as determined by M-mode echocardiograms. However, the addition of sodium thiosulfide increased hydrogen sulfide production and also returned contractile function to basal control levels (Sen et al. 2008). Moreover, AVF heart failure produced an increase in MMP-2 and MMP-9 while this increase was reduced to control levels with the addition of sodium thiosulfide and restoration of H_2S level. This suggested that sodium thiosulfide modulated contractility partially via H_2S production. Another study from our laboratory determined the role of hydrogen sulfide in HHcy renal damage (Sen et al. 2009). There was an increase in MMP-2 and MMP-9 expression in kidney of HHcy; moreover, hydrogen sulfide treatment reduced the expression of MMP-2 and MMP-9 significantly (Sen et al. 2009). We found results consistent with these results for MMP-9 in carotid arteries.

The protein, p47, is involved in oxidative stress via the NF-κB pathway that induces MMP/TIMP axis leading to cardiac dilation and failure (Henderson and Tyagi 2006). It was found that p47 expression was the highest in intensity under WTD group, whereas controls, WT and WT + NaHS, had the least expression of p47. WTD + NaHS had an intensity that was lesser than WTD alone, thereby indicating the relief of p47-induced stress. This indicates a positive effect of H_2S in relieving oxidative stress by decreasing a protein involved in a pathway that mediates these events. Because p47 is involved in the pathway of NF-κB, changes in the localization of NF-κB are important.

Superoxide dismutase is responsible for destroying free superoxide radicals in the body; this occurs be converting superoxide radicals to molecular oxygen and hydrogen peroxide within cytoplasm and mitochondria. Moreover, mutations in SOD are associated with amyotrophic lateral

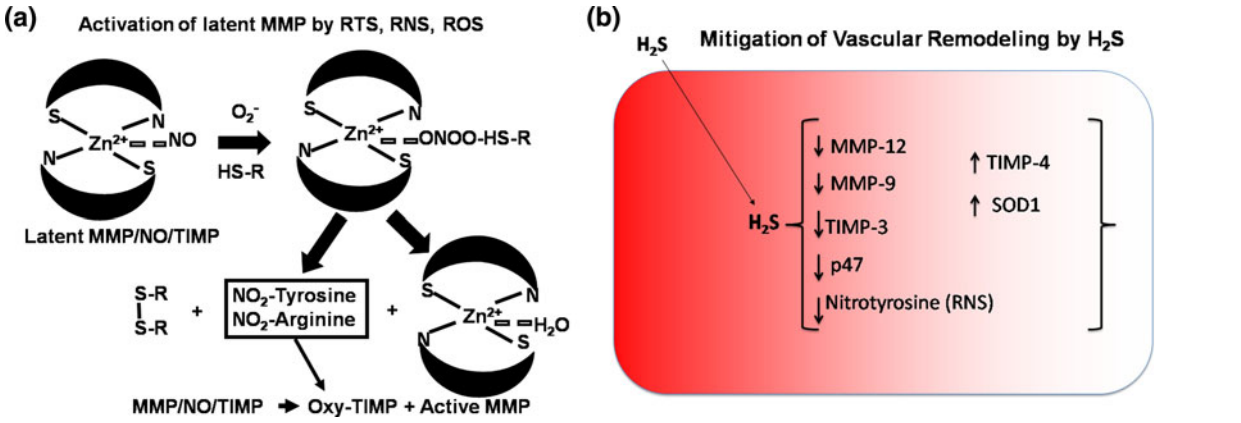

Fig. 6 **a** Schematic presentation of MMP/NO/TIMP in latent ternary complex in the basement membrane of the endothelial cells. During increase in oxidative stress, reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive thiol species (RTS, R-SH) are increased that modified the latent MMP complex, leading thiol-peroxinitrite intermediate. The intermediate lead to oxidized TIMP by nitration and in the process generates active MMP, oxidized thiols (R-S-S-R). **b** The treatment with H_2S mitigated the oxidative stress and the remodeling

sclerosis (Al-Chalabi and Leigh 2000; Conwit 2006). An environment that increases oxidative stress would also show a decrease in SOD (Kartha et al. 2008). Consistent with this notion, we found the following: WT and NaHS had basal expressions of SOD1 that were higher than the damaging groups, WTD and WTD + NaHS. Moreover, there was a significant increase in intensities of SOD1 under WTD + NaHS conditions versus WTD alone.

TIMPs play an important role in inhibiting certain MMPs that are involved in remodeling of the extracellular matrix (ECM) (Luo 2005). MMPs contain a large difference in both constitutive versus pathological remodeling (Shastry et al. 2005; Tyagi et al. 2005a, b). Studies have also shown that MMPs are expressed in a kind of balance with TIMPs such that if one is increased, the other is decreased (Malemud 2006). Several experiments from our laboratory have shown that when TIMP-3 increased, TIMP-4 decreased (Moshal et al. 2005, 2006; Shastry et al. 2006). In HHcy, increased expression of MMP-2 and MMP-9 has been reported in cardiovascular and neurovascular diseases (Lominadze et al. 2006; Sen et al. 2007a, b). Hcy has been shown to increase oxidative stress that increases the expressions of MMP-2 and MMP-9 and proceed to degrade ECM and change collagen/elastin ratio (Herzlich et al. 1996). MMPs can degrade proteins within the arterial wall that consist of the following: collagen, laminin, elastin, fibronectin (Raymond et al. 2004; Wald et al, 2002). MMPs are known to be involved in proliferation of vascular smooth muscle cells after balloon injury by degrading ECM (Holven et al. 2003). TIMP-3 was shown to be upregulated after vascular injury and also associated with collagen accumulation (Moshal et al. 2008). Baker et al. (1998) has also shown that TIMP-3 induces apoptosis in vascular smooth muscle cells, as well as regressing neo-intimal growth. We found that TIMP-3 is increased significantly in WTD group relative to control groups, WT, and WT + NaHS. Moreover, this increase in TIMP-3 expression is relieved to nearly control values upon addition of NaHS (WTD + NaHS).This suggests a reduction in apoptosis upon addition of NaHS.

We concluded based on these results that further investigation is necessary to optimize the conditions that permit the greatest relief of oxidative stress and pathological remodeling and activate the latent MMPs (Fig. 6a). We provide a model for our results of hydrogen sulfide under damaging conditions: increased SOD1, decrease TIMP-3, decreased p47 and decreased MMP-9. MMP-2 showed a decreasing trend and nitrotyrosine levels were within control levels such that there was no significant increase relative to control values after H_2S treatment (Fig. 6b).

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