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MMP-9- and NMDA receptor-mediated mechanism of diabetic renovascular remodeling and kidney dysfunction: hydrogen sulfide is a key modulator

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Abstract

Previously we reported that matrix metalloproteinase-9 (MMP-9) plays an important role in extracellular matrix (ECM) remodeling in diabetic kidney. Induction of NMDA-R and dysregulation of connexins (Cxs) were also observed. We concluded that this was due to decreased H₂S production by down regulation of CBS and CSE enzymes. However, the potential role of H₂S to mitigate ECM dysregulation and renal dysfunction was not clearly understood. The present study was undertaken to determine whether H₂S supplementation reduces MMP-9-induced ECM remodeling and dysfunction in diabetic kidney. Wild type (C57BL/6J), diabetic (Akita, C57BL/6J-*Ins²Akita*), MMP-9 knockout (MMP-9^{-/-}, M9KO) and double KO of Akita/MMP-9^{-/-} (DKO) mice were treated without or with 0.05 g/L of NaHS (as a source of H₂S) in drinking water for 30 days. Decreased tissue production and plasma content of H₂S in Akita mice were ameliorated with H₂S supplementation. Dysregulated expression of MMP-9, CBS, CSE, NMDA-R1 and Cxs-40, -43 were also normalized in Akita mice treated with H₂S. In addition, increased renovascular resistive index (RI), ECM deposition, plasma creatinine, and diminished renal vascular density and cortical blood flow in Akita mice were normalized with H₂S treatment. We conclude that diminished H₂S production in renal tissue and plasma levels in diabetes mediates adverse renal remodeling, and H₂S therapy improves renal function through MMP-9- and NMDA-R1-mediated pathway.

Keywords

Hydrogen sulfide; extracellular matrix; matrix metalloproteinase; connexins; collagen-elasticity; diabetes; renal microvasculature; nephropathy

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1. Introduction

Diabetic nephropathy is one of the leading causes of kidney disease characterized by progressive loss of renal function due to glomerular and tubular damage [1; 2]. Uncontrolled diabetes can lead to albuminuria, fluid buildup, increased blood pressure and ultimately renal failure. The early pathogenesis of diabetic nephropathy involves remodeling of extracellular matrix (ECM) within the glomerular and tubulointerstitial space [3; 4; 5]. The ECM comprises several protein components and among them collagen is the most abundant protein which gives structural support to the resident cells [6]. In contrast, another fibrillar protein elastin provides elasticity to tissues [7]. While a fine balance between collagen and elastin is important for normal kidney function, disruption of this balance due to modulation of ECM proteins and their regulatory peptidases in diabetes contributes to renal vascular impairment and failure [8; 9].

The matrix metalloproteinases (MMPs) are a group of Zn-dependent endopeptidases which controls ECM synthesis and degradation, including collagen and elastin [10; 11; 12; 13; 14]. An alteration of MMP activity over prolonged period of time causes adverse ECM remodeling which is often seen in fibrotic diseases such as diabetic nephropathy [8; 15]. Among MMPs, MMP-9 is a gelatinase and is highly expressed in the kidney [16] and serum in type-1 diabetic patients [17]. Previously, we reported that high levels of MMP-9 leads to diabetic renal remodeling which was associated with down regulation of hydrogen sulfide (H_2S) generating enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) [18]. Others have reported that low levels of H_2S contribute to vascular inflammation, endothelial dysfunction, and diabetic renal complication [19; 20]. Interestingly, supplementation of H_2S has been shown to improve renal function and reduces collagen expression in the kidney [21]. In a recent study, H_2S has also been shown to mitigate ECM remodeling in rat kidney by attenuation of oxidative stress, reduction of glomerular mesangial cell proliferation and inactivation of renin-angiotensin system (RAS) [22].

Physiological role of H_2S was initially reported through modulation of N-methyl D-aspartate receptor (NMDA-R) function in the mouse brain [23]. NMDA-R, which is a glutamate receptor, primarily regulates synaptic plasticity and memory function [24; 25]. However, it is also present in many other cell types and regulates a variety of functions [26; 27; 28]. In our previous study, we demonstrated reduced production and plasma levels of H_2S was associated with up regulation of NMDA-R1 and connexins (Cxs)-40 and -43 in the diabetic kidney [18]. However, whether H_2S modulates NMDA-R in the diabetic kidney and offers renal protection is not known.

In the present study, we aimed to determine whether H_2S can attenuate MMP-9 and NMDA-R1 expression in the diabetic kidney and mitigate adverse ECM remodeling. We also determined whether improvement of physiological parameters including renal resistive index, blood flow and renal function occurs following H_2S treatment. To achieve these goals we used Akita mice, a well-known model for type 1 diabetes mellitus, as an experimental model to mimic human disease. Hyperglycemia in these mice is due to spontaneous point mutation in the *Ins2* gene. This mutation leads to misfolding of insulin protein and thereby causes hypoinsulinemia and hyperglycemia. MMP-9 knock out (M9KO) and cross-breed of

Akita and M9KO (double knockout, DKO) mice were also used to determine the mechanisms of MMP-9-mediated renal remodeling and dysfunction. Our results indicated that H₂S generation and adverse remodeling in Akita kidney was MMP-9-dependent. Further, we provided with experimental evidence that supplementation of H₂S mitigated renovascular remodeling and improved renal function in diabetic mice through modulation of MMP-9, NMDA-R1 and Cxs pathways.

2. Materials and methods

2.1. Animals and protocol

Animal experiments were performed in accordance with the institutional animal care guidelines and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). C57BL/6J wild type (WT) mice, diabetic mice of C57BL/6J background (Akita, *Ins2^{Akita}*) and matrix metalloproteinase-9 (MMP-9) knockout (M9KO) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Akita and M9KO were crossbred to obtain double knockout (DKO) mice. All these animals were maintained in 12:12 h light–dark cycle with free access to rodent chow and water *ad libitum* in the animal care facility of the University of Louisville. Animals aged 14–16 weeks were used for the study. Treated groups received 0.05 g/L of NaHS, as a source of H₂S, in drinking water for 30 days.

In general, H₂S is 18% higher than the air. In the present study, we adopted our previously reported protocol where variability of H₂S treatment was minimal [29]. Furthermore, half-life of H₂S in water varies greatly with the presence of oxygen and certain metal ions. In our study, estimated half-life of H₂S in the drinking water was in between 30–36 hrs. To deliver appropriate amount, we replaced H₂S with freshly prepared solution every 24 hrs. In the end of experiments, animals were euthanized by using 2 × tri-bromo-ethanol (TBE), and blood samples and kidney were collected.

2.2. Antibodies and reagents

Rabbit polyclonal antibody to cystathionine β-synthase (sc-67154, CBS), mouse monoclonal antibody to cystathionine γ-lyase (sc-374249, CSE) and horseradish peroxidase-linked anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against MMP-9 (AB 19016; rabbit), NMDA-R1 (AB 9864, rabbit) and GAPDH (ABS 16, rabbit) were purchased from Millipore (Temecula, CA). Rabbit polyclonal antibody to connexin-40 (36-4900, Cx-40) and mouse monoclonal antibody to connexin-43 (35-5000, Cx-43) were purchased from Life Technologies (Grand Island, NY). Sodium hydrosulfide hydrate (NaHS; Cat # 161527) was from Sigma chemicals (St. Louis, MO). Polyvinylidene fluoride (PVDF) membrane was from Bio-Rad (Hercules, CA). All other chemicals used in this study were of analytical grade.

2.3. H₂S measurement

Plasma content and kidney tissue production of H₂S were measured. After euthanasia, blood was collected and isolated plasma was kept in an air-tight tube to prevent H₂S loss. Freshly isolated kidney tissues were homogenized with ice-cold PBS, collected in 1.5 ml Eppendorf

tube, and sealed immediately with parafilm. In this study, we used the modified protocol as described by Lu et al [30]. In brief, samples were centrifuged at a speed of $\times 11,000$ g for 10 minutes. 100 μ L aliquots from the supernatant or plasma were mixed separately with PBS (pH 7.4, 350 μ L) and $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ (1% W/V, 250 μ L) in a micro-centrifuge tube and sealed immediately. Next, N,N-dimethyl-p-phenylenediamine sulfate (20 mM, 133 μ L) in 7.2 M HCl, and FeCl_3 30 mM, 133 μ L) in 1.2 M HCl was added to the mixture, sealed and incubated at 37°C for 45 min. Trichloroacetic acid solution (10% w/v, 250 μ L) was added to the mixture to terminate the reaction. After centrifugation ($\times 2,700$ g for 5 mins), 200 μ L supernatant was transferred to a 96-well plate and the absorbance was measured at 670 nm in a spectrophotometer. Samples were assayed and H_2S was calculated against a calibration curve of known concentrations of NaHS (0.01 - 100 μ mol/L).

Here we account to report that we did not measure plasma H_2S levels at different time points following H_2S supplementation. To measure H_2S , each time we needed at least 100 μ L of plasma. Normally mice have approx. 58.5 ml of blood / kg body weight. Therefore, mice weighing about 25 g will ideally have total blood volume of 1.46 ml. If blood was drawn by using saphenous, tail, or sublingual vein or retro-orbital venous sinus, maximum 10% of total blood volume, i.e. 140 μ L of blood would have been available in a single occasion. It is difficult to get 100 μ L of plasma out of 140 μ L blood sample. Moreover, for repeated bleeds at shorter intervals suggested limit is $<1\%$, which is 14 μ L of blood in 24 hours. This amount of blood is insufficient for H_2S measurement using above mentioned method. Due to this technical issue we did not measure H_2S from mouse blood / plasma at different time points. However, we did measure baseline control values for H_2S and they were similar as sham controls.

2.4. Western blot analysis

Mouse kidney tissues from all groups were snap-frozen in liquid nitrogen for protein isolation. Kidneys were homogenized with RIPA lysis buffer (Boston BioProducts, Inc, Ashland, MA) containing PMSF and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) followed by protein estimation using Bradford method. Protein samples (200 μ g) were electrophoresed in SDS-PAGE and then transferred onto PVDF membranes. Membranes were blocked for 45 mins with 3% nonfat milk and incubated with appropriate primary antibody at 4°C overnight. Respective HRP conjugated secondary antibody incubation was for two hours at room temperature. After washing, membranes were developed using Luminata (Millipore, Temecula, CA). GAPDH was used as loading control and band intensity was quantified using ImageJ software (<http://imagej.nih.gov/ij/>).

2.5. Gelatin zymography for MMP-9 enzymatic activity measurement

Gelatin zymography was performed using 0.1% gelatin gel to measure MMP-9 activity. Briefly, 10% SDS-gel was made using gelatin as a substrate for MMP-9. Tissues were minced in cacodylic acid buffer and kept in cold room for overnight in a rotating shaker. The samples were then centrifuged and protein estimation was performed using Bradford method. Equal amount of protein was mixed with 2x loading dye and loaded in gelatin gel. After run, the gels were incubated in re-naturing buffer for an hour and transferred to developing buffer in a 37 °C shaker bath for overnight. Next day, the buffer was changed

and the gels were stained with Coomassie stain. MMP activity was detected as white bands against dark blue background and photograph of the gel was acquired using a digital camera.

2.6 Immunohistochemistry

To determine the localization of CBS and CSE enzymes, we used 5 μ m frozen kidney sections made using cryotome machine. A standard protocol (Abcam) was followed to perform immunohistochemistry. After blocking, primary antibodies (CBS or CSE) were applied overnight at 4 °C. Secondary antibodies labeled with Alexa fluor 488 and Alexa fluor 594 (Invitrogen) were applied for immunolocalization of these proteins. DAPI was used to counterstain nuclei. Slides, after staining, were analyzed for fluorescence intensity under a laser scanning confocal microscope (Olympus FluoView1000) using appropriate filter.

2.7. Renal ultrasonography for cortical blood flow and resistive index

Isoflurane anesthetized mice were placed supine on a heated table. After depilation, acoustic gel (Other-Sonic, Pharmaceutical Innovations, Newark, NJ) was applied on the skin and imaging was performed using Vevo 2100 system (Visual Sonics, Toronto, ON, Canada). The transducer, MS550 was held firmly by an integrated rail system during image capture. The kidney was scanned in the short axis. All measurements were done on the left side and included renal artery diameter, peak systolic and end diastolic blood flow velocity (mm/sec) in the renal artery and cortex by Pulsed-Wave Doppler mode. Cine loops were exported and analyzed to obtain resistive and pulsatility index.

2.8. Laser Doppler Flowmetry

Laser Doppler Flowmetry (LDF) is based on the reflection of laser beam light which changes in different wavelength (Doppler shift) when it is reflected by the moving RBCs in the microvasculature and a photodiode measures the emerged beam. The magnitude and frequency distribution of these wavelength changes are related to the number and velocity of moving RBCs. Laser Doppler cortical blood flow was measured according to the protocol described earlier [12]. Briefly, the animal, after anesthesia, was placed in right lateral position and the left kidney was exposed. Renal cortical blood flow was measured using Speckle Contrast Imager (Moor FLPI, Wilmington, DE). The contrast images were processed to produce a color-coded live flux image and a flux unit trace was also recorded for 2 min for all the mice.

2.9. Barium angiography for measuring renal vasculature

One ml Barium sulfate (50 mM) was infused at a constant pressure and flow with a syringe pump through a cannula as described previously [12]. Briefly, the mice were placed in the X-ray chamber and angiograms were captured with Kodak in vivo imaging system FX. Two min X-ray images were taken at 35 Kvp. Vessel enhancements were quantified using the software developed in University of Lubeck, Germany. The percentage of white pixels (vessels) was calculated against no. of pixels in the background by the program to calculate vascular percentage. ImageJ software was used to calculate the histograms and the values for vessel coverage were presented as mean value.

2.10. Histological collagen-elastin staining and quantification

Picrosirius red staining kit was used to measure collagen deposition following manufacturer's instructions (Polysciences Inc, Warrington, PA) with modification. 5 µm thick kidney sections were deparaffinized and hydrated prior to incubation, incubated with picrosirius red solution overnight, followed by 0.01N HCl treatment for 2 min. Slides were then dehydrated and mounted with permount (Fisher Scientific, NJ). Three to five high power field images were captured under light microscope. ImagePro Plus software (Media Cybernetics, Inc. Rockville, MD) was used to measure the intensity of red dye deposition in the renal cortex.

Elastin staining was performed on 5 µm thick sections after deparaffinization and hydration with graded alcohol. Sections were stained with Chromaview™ Elastin Stain Kit following manufacturer's instructions. Briefly, sections were kept in freshly prepared Verhoeff's Iron Haematoxylin for 15-20 minutes. Slides, after rinsing in water, were differentiated in working ferric chloride solution until sections appeared dark brown. They were then washed gently under running tap water for 2-3 minutes and re-stained with Van Gieson's stain solution for 1 minute. After thorough dehydration and clearing, slides were mounted using permount.

A computerized digital image analyzer, Image Pro plus software (version 7.0; Media Cybernetics Inc, Bethesda, MD) was used to analyze relative intensity of collagen deposition and elastin fibers. Briefly, digital images were captured using light microscope. After calibration, areas with red dye deposition were selected using the magic wand tool. This tool allows finding dark areas among light background and vice versa. Once selected, images were converted to RGB color channels. Following conversion, channels were extracted, semi-automatically measured the intensity of the channels (red channel selected for collagen and blue channel selected for elastin). After measurement, channels were merged with the original image. Collagen area fractions were calculated as follows: collagen area divided by total sampling area. Similar calculation was performed for elastin. All measurements were imported to Microsoft Excel, measured the relative intensity, analyzed and represented in bar diagram to demonstrate differences between groups.

2.11. Measurement of plasma creatinine level

The test kit Quantichrom™ Creatinine Assay Kit (DICT-500) was used for assessment of creatinine level in plasma samples. Briefly, according to the manufacturer's protocol, 2 mg/dL standards were made and 30 µl of diluted standards were transferred in to a 96-well clear bottom plate. Then 200 µl of working reagent was added to each well and tapped for mixing. The optical density was measured in SpectraMaxx (Molecular devices, CA, USA) just after the color development (~OD0) and then after 5 minutes (OD5) at 490-530 nm. The mean peak absorbance was considered at 510 nm. Creatinine concentration of the sample was calculated using the following formula $[(OD_{\text{sample 5}} - OD_{\text{sample 0}}) / (OD_{\text{standard 5}} - OD_{\text{standard 0}})] \times [STD]$ (mg/ dL).

2.12. Chemical-protein interactions: STITCH 4.0

Chemical-protein interaction data were retrieved from STITCH database (Ver. 4.0). STITCH is a resource to explore known and predicted interactions of chemicals and proteins. Chemicals are linked to other chemicals and/ or proteins by evidence derived from experiments, databases and the literature, containing interactions connecting over 300,000 chemicals and 2.6 million proteins from 1133 organisms.

The interaction data were curated as three interaction scores from several data sources of experiments, databases and text-mining representing the relevance of interactions. The experiment part consists of direct chemical-protein binding data with experimental evidence. The database part contains interaction data from pathway databases. The text-mining data was constructed by extracting information of interactions from literatures using text-mining techniques. In addition to the above three scores, STITCH database provides a combined score for a given chemical-protein interaction generated by combining three scores of corresponding evidence types. The score is an integer value ranging from 0 (no interaction) to 1 (high-confident interaction).

2.13. Statistical analysis

All values are presented as the mean $\pm$ standard error of the mean. Statistical analysis was performed using Primer of Biostatistics (Ver. 7; McGraw-Hill). Multiple comparisons were analyzed using one-way analysis of variance followed by Bonferroni's post hoc test. The threshold for significance was $p < 0.05$.

3. Results

3.1. Plasma and tissue H₂S production levels following NaHS treatment

Akita mice develop hyperglycemia at the age of 3-4 weeks and we previously reported that around 14-16 weeks of age these mice have ~550 mg/dl blood glucose levels [18]. In the present study, we measured plasma levels as well as tissue generation capability of H₂S and the summarized data are shown in fig. 1. Our results indicated that Akita mice had low levels of plasma H₂S and kidney tissue from these mice also produced decreased amount of H₂S ex vivo compared with their WT littermates (Fig.1A, B). The M9KO animals although exhibited decreased plasma H₂S levels and reduced ability to produce tissue H₂S, these difference were not significant compared with WT (Fig. 1A, B). These trends of tissue generation capacity and plasma levels of H₂S remained almost similar and non-significant in DKO mice compared with Akita mice (Fig.1A, B).

H₂S treatment in WT increased plasma H₂S levels (Fig.1A). Similar results were observed in tissue production levels (Fig. 1B), though the level of increase was non-significant compared with their respective controls. The increase of plasma H₂S levels and tissue production ability was slightly higher in M9KO mice, although these changes were not significant compared with their respective control groups (Fig. 1A, B). Interestingly, H₂S supplementation significantly increased plasma levels as well as tissue generation capability of H₂S, both in Akita and DKO mice, compared with their respective non-treated groups (Fig.1A, B).

3.2. Expression and activity of MMP-9 was mitigated in diabetic kidney by H₂S

Previously, we reported upregulation of MMP-9 in diabetic kidney [18; 31]. Similar to those findings, our present report also demonstrated upregulation of MMP-9 in Akita kidney (Fig. 2A), and the difference of expression levels was significant compared with WT littermates (Fig. 2A). As expected, null or minimal expression of MMP-9 was detected in M9KO and DKO mice. Interestingly, H₂S treatment attenuated increased expression of MMP-9 in Akita mice compared with non-treated animals (Fig. 2A).

We also performed in-gel gelatin zymography to measure enzymatic activity of MMP-9. Result reported in fig. 2B showed that MMP-9 activity was increased almost 4-folds in Akita animals compared with WT group. Negligible or no activity was observed in M9KO and DKO groups. When the animals were treated with H₂S, the activity level was reduced in WT as well as in Akita animals (Fig. 2B). No significant changes were observed in M9KO and DKO groups with H₂S treatment (Fig. 2B, C).

3.3. Effect of H₂S on CBS and CSE expression

In our earlier report we demonstrated down regulation of CBS and CSE enzymes in Akita kidney [18]. In the same study, we also demonstrated that knocking down of MMP-9 (M9KO) in Akita, i.e. crossbreed of M9KO and Akita, we termed as DKO, normalized CBS and CSE expression [18]. Similar to that finding, our present report also demonstrated that CBS and CSE was downregulated in Akita kidney (Fig. 3A), and the difference was significant compared with WT littermates (Fig. 3B). Corroborating with our previous finding, in the present study normalization of CBS and CSE in Akita mice was also measured (Fig. 3A, B). No apparent differences in CBS and CSE expression were observed in WT, M9KO and DKO mice without or with H₂S treatment (Fig. 3A, B).

We also performed immunostaining to demonstrate localization of these two enzymes in the kidney, and the data were presented in fig. 3C and D. In WT kidney, both these enzymes were localized in the glomerular region at basal level (Fig 3C). Expression of these enzymes was decreased in Akita animals compared with WT (Fig 3C). Treatment with H₂S increased localization of these enzymes in Akita animals compared with non-treated groups (Fig. 3C, D). No apparent change of expression levels were observed in WT, M9KO and DKO animals without or with H₂S treatment (Fig. 3C, D).

3.4. Expression of NMDA-R1 and gap junction proteins

Similar to our earlier observation [18], in the present study, we also found increased expression of NMDA-R1, Cx-40 and -43 in Akita animals compared with WT (Fig. 4A), and the differences were significant (Fig. 4B). In M9KO and DKO mice the expression levels of these molecules were attenuated compared with Akita mice (Fig. 4A). Interestingly, H₂S treatment also attenuated the expression of these three molecules in Akita mice (Fig. 4A). H₂S treatment however did not alter the expression levels of these proteins in M9KO and DKO mice, which remained at the basal levels similar to that of WT (Fig. 4A, B).

3.5. Renal resistive index and cortical blood flow

Renal resistive index (RI) reflects changes in intra-renal perfusion. Renal ultrasound was performed to detect changes in blood flow in the renal artery and cortical branches of the kidney. Baseline RI was considered 0.523 as found in WT animals (Fig. 5A). No significant change was observed in WT treated with H₂S (Fig. 5A). In Akita mice, RI of the main renal arteries and the interlobar arteries were significantly higher (0.664) compared with WT group (Fig. 5A). Following H₂S treatment, RI of Akita mice returned to baseline level (0.544) similar to that of WT mice (0.523). RI was relatively higher in DKO animals (0.616) before H₂S treatment but reduced significantly (0.522) following treatment (Fig. 5A). No significant changes were observed in RI level of M9KO animals without (0.553) or with H₂S treatment (0.528) (Fig. 5A).

We adopted Laser-Doppler Flowmetry (LDF) to quantify the blood flow in the renal cortex of the experimental and control animals. Results summarized in fig. 5B indicated a significant reduction in the renal cortical blood flow in Akita mice compared with WT mice. Interestingly, treatment with H₂S normalized renal blood perfusion in Akita mice as measured by LDF (Fig. 5B). However, blood flow did not alter in WT group treated with H₂S. Similarly, no changes were observed among M9KO mice without or with H₂S treatment (Fig. 5B). Significant reduction in blood flow was however observed in DKO mice which increased following H₂S treatment (Fig. 5B).

3.6. Renal microvascular density

Barium sulfate angiography was performed to evaluate renal vasculature. In figure 6A, representative Barium angiography image of WT control without H₂S treatment (left) and analysis of vessel coverage (right) using specialized software as described in section 2.9 was presented. Figure 6B indicated analyzed Barium perfused vasculature in dark background for all groups, and fig. 6C shown summarized % vessels coverage in the whole kidney. Results indicated that WT mice showed a well preserved renal architecture with no differences between control and H₂S treatment groups (Fig. 6B, C); whereas, Akita mice showed a significant decrease in total vascular coverage compared with WT mice (Fig. 6B, C). Following H₂S treatment, Akita kidney was increasingly perfused with more Barium as measured by % vessel coverage compared with non-treated group (Fig. 6B, C). M9KO animals showed no significant changes in renal vasculature without or with H₂S treatment (Fig. 6B, C). The renal vasculature in DKO animals was however reduced compared with WT (Fig. 6B, C). Interestingly, the vessel coverage was significantly increased in DKO mice following H₂S treatment (Fig. 6B, C).

Furthermore, to determine whether H₂S supplementation could sustain renal vascular perfusion in diabetic kidney, we performed a time course study. Our data suggested that diabetes deteriorated renal vascular perfusion over time (Fig. 6D). Interestingly, H₂S supplementation not only protected renal vascular perfusion in diabetes but also improved perfusion in Akita kidney in a time-dependent manner (Fig. 6D).

3.7. Collagen deposition in renal cortex

To measure the level of renovascular fibrosis, we performed picrosirius red staining and quantified collagen according to the method as described in section 2.10 in the renal tissue section. WT mice without H₂S treatment showed a thin layer of collagen around the periphery of the glomerulus and in the interstitium (Fig. 7A). These thin layers were even thinner in H₂S treated mice (Fig. 7A). A robust and significant increase of collagen deposition was observed in the peri-glomerular and tubulointerstitial space of Akita mice compared with WT mice (Fig. 7A, B). This increase in collagen deposition was prevented by H₂S treatment in Akita mice (Fig. 7A, B). M9KO mice showed similar pattern of collagen deposition without or with H₂S treatment and these were comparable with respective WT mice (Fig. 7A, B). In DKO mice, without or with H₂S treatment, collagen deposition remained unchanged and was comparable with respective WT littermates (Fig. 7A, B).

3.8. Elastin content in the renal perivascular region

Change of elastin content in the renal resistance arteries was measured by staining kidney sections with Verhoeff's Van Gieson stain. WT animals treated without or with H₂S showed a basal level of elastin content without statistical differences (Fig. 8A, B). In Akita mice elastin content in the perivascular region was significantly reduced compared with WT mice (Fig. 8A, B). The reduction was identified as thin internal elastic lamellae along with ruptured and dismantled fashion of media and disorganization in the media escorted by luminal narrowing. H₂S treatment significantly improved elastin content in Akita mice and it was almost to the basal level (Fig. 8A, B). There were no changes of elastin content was observed in M9KO animals without or with H₂S treatment (Fig. 8A, B). In contrast, DKO animals showed significant decrease in elastin content compared with WT, and this decrease was alleviated towards basal WT level following H₂S treatment (Fig. 8A, B).

3.9. Effect of H₂S on plasma creatinine level

Plasma creatinine and urea are established markers for determination of kidney function. However, plasma creatinine is a more sensitive index of kidney function compared with plasma urea level. Therefore, we measured plasma creatinine levels to estimate kidney function in our experiments. Results showed that basal level of creatinine in WT mice did not alter without or with H₂S treatment (Fig. 9). Interestingly, in Akita mice, the level was significantly higher compared with WT (Fig. 9). Following H₂S treatment the plasma creatinine content was significantly reduced in Akita animals (Fig. 9). The M9KO mice did not show significant increase in creatinine content compared with WT; neither had they showed differences without or with H₂S treatment (Fig. 9). However, in DKO mice, creatinine level was significantly higher compared with WT (Fig. 9). The DKO mice treated with H₂S showed towards basal level of creatinine (Fig. 9).

4. Discussion

Our present study demonstrated that MMP-9 was involved in diabetic renal remodeling through modulation of H₂S production in Akita mice. We also showed that decreased H₂S production and plasma availability in diabetes triggered a series of physiological cascades

involving NMDA-R1, Cx-40 and -43 proteins leading to collagen-elastin disruption, increase renal vascular resistive index, low renal perfusion and ultimately decline in renal function. The most interesting finding of the present study was that supplementation of H₂S was able to prevent or even reverse some of these above pathophysiological changes in diabetes, and therefore alleviated kidney dysfunction.

In streptozotocin-induced diabetic rat Zhou et al recently reported that H₂S treatment attenuated glomerular basement membrane thickening, mesangial matrix deposition, renal interstitial fibrosis and improved renal function [22]. Our present study is also in agreement with this previous study and further suggests that in genetic Akita diabetic mice, kidney exhibits reduced microvascular density, increased resistive index, and plasma creatinine levels in conjunction with collagen accumulation and gap junction protein alteration. Although we did not measure activation of renin-angiotensin system (RAS) and oxidative-redox balance, pathogenesis of renal dysfunction was shown to be mediated by activation of intrarenal RAS in streptozotocin-induced rat model, and H₂S alleviated development of diabetic nephropathy [32]. Furthermore, streptozotocin-induced spontaneously hypertensive rats (SHR) were shown to have lower levels of plasma and urinary H₂S [33]. In the same report renal dysfunction was documented by increased plasma creatinine and decreased renal cortical perfusion [33]. While all these studies were reported in diabetogen-induced animal model, the present study was performed in genetic diabetic model. In addition to further confirming the above previous finding that H₂S is a key regulator of diabetic nephropathy, our study demonstrated a distinct mechanism of diabetic renal remodeling and dysfunction involving MMP-9 and connexins.

Abundance of MMP-9 is known to cause ECM deposition and plays crucial role in severe cardiac and lung remodeling [34; 35]. Our present study demonstrated that MMP-9 expression was increased in Akita kidney. This resulted in decreased expression of H₂S metabolic enzymes, CBS and CSE, causing diminished plasma levels and tissue production of H₂S. Although the mechanism how increased MMP-9 diminished CBS and CSE enzymes was not known, it is reported that MMP-9 causes extensive modification of ECM and remodeling in the renal vasculature [36]. Since glucose induces oxidative stress [37], and oxidative radicals activate MMP-9 [18], it is possible that diabetes-mediated oxidative activation of MMP-9 in Akita mice modified ECM in the tissue, and thus mitigated activity of CBS and CSE in the vascular cells. This resulted in decreased H₂S production in the Akita kidney.

In vivo and in vitro experimental evidences suggest that H₂S regulates vascular remodeling through a wide array of mechanisms [38; 39; 40; 41]. In our study, we therefore treated Akita animals with H₂S to determine whether it modulates MMP-9, and thus remodeling process. Our results indicated that H₂S significantly reduced MMP-9 expression and activity in Akita mice. The current study also revealed that treatment with H₂S not only mitigated pathological remodeling, but also increased the level of CBS and CSE enzymes, and therefore contributed to possible restoration of endogenous H₂S generation. This was evident from the observation that tissue levels of H₂S production ability was significantly increased in diabetic kidney following H₂S treatment. The mechanism however is not known. Furthermore, although we observed increased levels of plasma H₂S and tissue

production in WT mice following H₂S supplementation compared with control animals, the differences were not significant (Fig. 1A, B). It is highly possible that blood gas pressure including partial H₂S pressure may already had at the upper limit; therefore, further supplementation of H₂S did not increase plasma H₂S levels and tissue production in WT. Whereas in Akita and DKO mice H₂S was in decreased levels compared with WT, and thus supplementation increased plasma levels of H₂S and tissue production towards basal levels in those animals. Exclusive and complete blood gas analysis will probably give the best answer why H₂S supplementation did not increase plasma H₂S levels and tissue production significantly in WT mice compared with non-treated animals. This needs future investigation.

A plethora of evidence suggest that H₂S regulates NMDA-R in many physiological and pathophysiological conditions [42; 43; 44; 45]. In the kidney, NMDA-R expresses abundantly in several tissue compartments including outer medulla, cortical brush boarder membrane and glomeruli [46]. Experimental evidences suggest that NMDA-R antagonist and co-agonist regulate glomerular as well as tubular function [46; 47]. We have previously shown increased expression of NMDA-R1, Cx-40 and -43 in Akita kidney and in hyperglycemic mouse glomerular endothelial cells (MAEC) in vitro [18]. We also found that H₂S treatment normalized these molecules in MAEC. However it was unknown whether similar results could be achieved in diabetic kidney. Our present report confirms that increased expression of NMDA-R1, Cx-40 and -43 were mitigated with H₂S treatment in vivo. Although MMP-9 knockout in diabetic mice (DKO) attenuated expression of these gap junction proteins, H₂S treatment completely normalized their expression in Akita and DKO mice, suggesting that the mechanism of gap junction molecules regulation by H₂S in diabetes is MMP-9-independent.

Reports from several laboratories indicated that an increased renal resistive index (RI) reflects changes in intra-renal perfusion. It is also related to systemic hemodynamics, thus providing useful prognostic information about type-1 diabetes [48; 49]. Type-2 diabetes patient has also been reported to have increased RI with decreased creatinine clearance [50]. Not only had that in chronic kidney disease patient showed increased RI associated with interstitial fibrosis, atherosclerosis and reduced renal function [51]. Therefore, RI is an important tool to assess renal health and possible illness. We performed renal ultrasound in our study to detect changes of blood flow in the renal artery and cortical branches within the kidney. Data revealed that Akita mice developed increased RI which was indicative of impaired renal perfusion and possible dysfunction. H₂S treatment increased renal perfusion, and thus RI was normalized in Akita mice. We also used laser Doppler flowmetry (LDF) to measure the flow across renal cortex to explore whether diabetes altered regional blood flow in the kidney. This technique is very advanced and gives real time changes (2D measurement) in tissue perfusion [12]. Using LDF we measured basal level of blood flow (in flux units) in WT animals. Although blood flow did not change in WT mice treated with H₂S vs non-treated mice, the flow was significantly improved in Akita mice suggesting that microvascular relaxation mediated by H₂S decreased renal RI and allowed more blood to flow through the renal microvessels. To our knowledge this is the first report in which we demonstrate amelioration of increased renal RI and decreased blood flow by H₂S treatment in diabetes.

Barium contrast X-ray angiography is a useful tool for vascular imaging. Our group has previously been exploited this technique to elucidate vascular heterogeneity in Akita and other experimental murine models [52]. Using the same sophisticated method for assessing vasculature we also reported that reduction in arcuate and interlobular renal arteries occurred in *Timp-2^{-/-}* animals [12]. In the present study, we also exploited this technique for estimation of total kidney vasculature in our experimental animals that could possibly give some clue why RI and renal blood flow was reduced in Akita kidney. Summarized data presented in fig 6 revealed that percent vessel coverage was significantly reduced in Akita mice vs WT. The mechanism is probably due to the increased resistance in the pre-terminal branches causing vessel collapse resulting in blood flow reduction to the distal part. This reduction was almost normalized in Akita mice treated with H₂S suggesting that the opening of micro vessels, probably terminal arcuate and interlobular arteries permitted more Barium to penetrate distal part of the vessels, and thus increased calculated total vessels coverage. Non-significant changes were observed among M9KO and WT animals treated without or with H₂S. However, reduced vessel density in DKO was restored with H₂S treatment. Interestingly, in DKO mice, although the vessel density was reduced significantly it was not as robust as Akita compared with WT. Thus, the results suggested that MMP-9 was involved in renal microvascular narrowing in diabetes, and by knocking out MMP-9 in Akita mice (DKO) we were able to partially improve Barium perfusion through microvessels. Nonetheless, H₂S treatment further improved Barium perfusion and thus total renal microvascular coverage in DKO mice was increased. This suggests further beneficial effects of H₂S on the top of MMP-9 knockout in diabetes, and perhaps a contributing factor in improving renal RI and blood flow.

Accumulation of ECM which includes collagen deposition and elastin degradation is an important event in renovascular remodeling in diabetic nephropathy. It is reported that urinary collagen levels are high in patients with T2D [53]. Increased amount of collagen was found in patients with diabetic nephropathy and the expression level was robust in patients with superior glomerular lesions [54]. Elastin degradation is a well-known feature in vascular pathology. Recent report suggests that high glucose is responsible for elastin degradation in vascular smooth muscle cells [55]. Using Sprague Dawley rat abdominal aorta injury model, Basalyga et al showed that vascular remodeling occurred via calcium chloride induced chronic degeneration of elastic fibers mediated by MMPs [56]. In the present study, we measured amount of collagen deposition and results suggested that collagen deposition was high in interstitium and glomerular basement membrane in diabetic Akita mice compared with WT control. Interestingly, H₂S treatment attenuated accumulation of total collagen in the peri-glomerular as well as in tubulointerstitial space in Akita mice. This is in agreement with a recent report in which H₂S has been shown to reduce collagen fiber accumulation in unilateral ureteral obstruction (UVO) mice renal interstitium [57]. On the contrary to collagen accumulation, elastin measurement indicated increased degeneration of elastic fibers in the perivascular region of Akita mice resulting vessel collapse. Previously, it has been reported that external H₂S modulated elastin degradation in pulmonary artery wall of male Wistar rats with hypoxia [58]. Our study is in agreement with this previous report and suggests that H₂S also alleviates renal elastin degradation in Akita mice.

The use of plasma creatinine measurement is a very popular tool for assessing renal function in mouse models. High levels of plasma creatinine were measured in Akita and DKO animals in our present study. When these animals were treated with H₂S, plasma creatinine levels were reduced to basal level indicating normalization of renal function and further supported our findings that increased RI and reduced blood flow in diabetes mitigated renal function, and H₂S treatment through improved perfusion restored normal function in diabetes. This result is in accordance with the recent finding that exogenous H₂S prevents progression of diabetic nephropathy in SHR rats [33].

Protein-chemical connections between H₂S machinery, ECM components and GAP junction proteins as found by STITCH, EMBL (<http://stitch.embl.de/>) analysis was depicted in figure 10. This is a database of protein-chemical interactions, which integrates different sources of experimental and manually curated scientific reports with text-mining information and predicted interaction [59]. Protein-chemical interactions are essential for living system. These interactions are essential for cellular metabolism, signal transduction and most pharmaceutical interventions. Here, we have used this interactive software to detect and study a variety of cellular functions and the impact of drug treatment on the system. Figure 10 showed the possible interactions between H₂S producing machinery (CBS, CTH, and MPST); ECM components (MMP-9, TIMP-1, Elastin) and GAP junction proteins (Gja5 or Connexin-40, Gja1 or Connexin-43) in mice. Protein-chemical interaction analysis indicated that MMP-9 was strongly related to CBS and CSE via calcium and pyridoxal-5'-phosphate. On the other hand, GAP junctions proteins (Cx-40 and -43) were also strongly connected with MMP-9 via cadherin pathway. Additionally, interaction between Cx-40/-43 and CBS/CTH was also stronger via protein-kinase C (prkcc) pathway. Thus, the analysis demonstrated a stronger connection and interaction between H₂S machinery, gap junction proteins and ECM components as suggested by STITCH (Fig. 10). The association also determines that ECM components act with GAP junction protein via cadherin which is another scope for future research. Finally, GAP junction proteins are connected to H₂S system via protein kinase C and pyridoxal 5'-phosphate pathway which also needs to be explored in future.

Our study has few limitations. 1) Supplementation of H₂S through drinking water was not the perfect method to treat animals since the same procedure could not be used to treat patients. 2) Our measurement of H₂S in biological samples was not the ideal as some may have been lost during processing and Zn-trapping. In addition, this method might have been identified some non-H₂S sulfur metabolites. 3) We measured vascular density at 0, 15 and 30 days without or with H₂S supplementation only in Akita mice (Fig. 6D). In other groups, we measured at the end of 30 days following H₂S supplementation (Fig. 6B). Similar to Akita, a baseline angiography at 0 day and a time course study would have been given comprehensive insights into the progression of renal microvascular recovery in all the groups. This deserves a separate study.

In conclusion, our present report demonstrates that in diabetes, intense renovascular remodeling occurs due to increased expression of MMP-9 and low levels of H₂S. Although MMP-9 deletion partially mitigated remodeling in Akita, the normal kidney function was maintained through H₂S supplementation in Akita and DKO mice. Thus, supplementation of

H₂S promises a potential therapeutic approach to prevent diabetic nephropathy by preserving renal microvascular architecture and function. A number of H₂S-realising compounds have been synthesized, such as GYY4137 and AP39, to deliver therapeutic H₂S. Inspiringly, compound GYY4137 has already been tested in animal models, and has shown to improve vasculopathy [60; 61]. Subsequent clinical trials are ongoing to test the safety and efficacy of H₂S donating compounds and these results are yet to publish. As we wait for the outcomes, studies targeting diabetic subjects are needed to confirm the improvement of renopathy by these H₂S-donating compounds for full therapeutic potential.

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Highlights

In diabetes:

- Decreased H₂S and increased MMP-9 activity result in renovascular remodeling
- Reduced vascular density and increased resistance decreases renal cortical blood flow
- Vascular collagen deposition is decreased and elastin expression is increased with H₂S
- H₂S therapy normalizes the expression of MMP-9, NMDA-R1, and connexins-40 and -43
- H₂S alleviates renovascular remodeling, improves renal blood flow and function

Fig. 1. NaHS treatment increased tissue production and plasma levels of H₂S in diabetic animals Animals were treated without or NaHS (0.05 g/ L) for 30 days as mentioned in Materials and Methods. **(A)** Fifty milligram (50 mg) of kidney tissue from each animal group was homogenized and assayed for H₂S measurement as described in Materials and Methods. **(B)** Hundred microliter of plasma from each animal was used for H₂S content measurement. H₂S measurement for both tissue and plasma was performed within 36 h of sample collection. Data represents mean $\pm$ SEM, (n = 8).

Fig. 2. Increased expression and activity of MMP-9 in diabetes was by H₂S

Kidneys were homogenized in appropriate buffer, protein extracted and loaded onto SDS gel as described in Materials and Methods. (A) MMP-9 protein expression. GAPDH was used as loading control. (B) Gelatin zymography image showing MMP-9 activity. (C) Fold changes of respective proteins. (D) Bar diagram represents fold change of the protein and enzyme activity. Data represents mean $\pm$ SEM from seven independent experiments (n = 8).

Fig. 3. Decreased expression and localization of CBS & CSE were normalized by H₂S in diabetic mice

Kidneys were homogenized in RIPA buffer and loaded onto SDS gel as described in Materials and Methods. **(A)** Protein expression of CBS and CSE. GAPDH was used as loading control. **(B)** Bar diagram represents fold changes of CBS and CSE expression. Data represents mean $\pm$ SEM from five independent experiments ($n = 8$). **(C and D)** Immunostaining images of CBS (green; pointed with red arrow) and CSE (red; pointed with yellow arrow) enzymes in kidney tissues.

Fig. 4. Up regulated NMDA-R1, Cx-40 and -43 in diabetic animals were normalized with H₂S treatment

200 µg of kidney tissue protein were loaded onto SDS gel and immunoblotted as described in Materials and Methods. **(A)** Expression of NMDA-R1, Cx-40 and -43 proteins without or with H₂S treatment. GAPDH was used as control. **(B)** Fold changes of respective proteins. Bar graphs represent mean $\pm$ SEM from five independent experiments (n = 8).

Fig. 5. Renal resistive index and cortical blood flow measurement. (A) Renal resistive index was reduced in diabetic animals following H₂S treatment

Kidney ultrasound was performed to measure renal resistive index (RI) using the Visual sonics Vevo Ultra imaging system as described in Materials and Methods. Values are represented as RI. Resistance of renal arterial flow to the kidney was measured by RI and calculated as (peak systolic velocity – end-diastolic velocity)/peak systolic velocity. Median values are shown in the “box-and-whisker plot”. Box plots represent mean RI $\pm$ SEM (n=5). (B) Renal cortical blood flow was increased significantly following H₂S treatment in diabetic mice. Renal cortical blood flow was measured in the left kidney as described in Materials and Methods. Box plots represent mean flux units in the renal cortex $\pm$ SEM (n=5).

Fig. 6. H₂S treatment increased renal vascular perfusion in diabetic mice

To visualize renal vascular architecture, 50 mM of barium sulfate was infused via infra-renal aorta at constant pressure and time. **(A)** Representative Barium x-ray kidney angiogram of WT mice without H₂S treatment is shown here as an example (left); FOV, field of view. Analysis of its vessel coverage using VesSeg tool as described in Materials and Methods is shown on the right. **(B)** Vessel segment analysis of angiogram images from WT, AKITA, M9KO and DKO groups without or with H₂S treatment. Total vessel area was calculated using ImageJ software. **(C)** Bar diagram represents the mean percent change in renal vessel coverage against the background $\pm$ SEM ($n = 8$ / group). **(D)** Representative time course angiography of Akita kidney without or with H₂S treatment.

Fig. 7. H_2S reduced increased deposition of collagen in diabetic kidney

(A) Representative photomicrographs of collagen staining with picrosirius red. Blue arrows indicated peri-glomerular and green arrows indicated tubulointerstitial collagen staining (original image $\times 25$ magnifications). (B) Bar diagram showed total collagen area against the background presented as mean relative intensity in arbitrary unit (A.U.). Data represents mean $\pm$ SEM (n=5/group).

Fig. 8. H₂S treatment increased elastin expression in diabetic animals

(A) Kidney sections were stained with Van Gieson's stain and dark brown in the photomicrographs, indicated by blue arrows, depicted elastin in the renal perivascular region (original image $\times 25$ magnifications). (B) Bar diagram represents the total elastin area against the background presented as mean relative intensity $\pm$ SEM (n= 5/ group).

Fig. 9. Increased plasma levels of creatinine were reduced significantly after H₂S treatment in diabetic animals

End-point plasma creatinine levels in WT, Akita, M9KO and DKO mice were measured before and after H₂S treatment. Each experiment was performed in triplicate and values presented as mean $\pm$ SEM (n=5/group).

Fig. 10. STITCH network connecting H₂S machinery, ECM and GAP junction protein

For eight different compounds, investigated in this study, interacting proteins and chemicals are shown. Stronger associations are represented by thicker lines. Protein-protein interactions are shown in **blue**, chemical-protein interactions are shown in **green**. CBS: cystathionine β-synthase; CTH: cystathionine γ-lyase; MPST: 3-mercaptopyruvate sulfurtransferase; MMP-9: Matrix metalloproteinase 9; TIMP1: Tissue inhibitor of metalloproteinase 1; Eln: Elastin; Gja1: Connexin-43; Gja5: Connexin-40; Cdh2: Cadherin 2; Prkcc: protein kinase C γ.