

Effects of hydrogen sulfide on myocardial fibrosis in diabetic rats: Changes in matrix metalloproteinases parameters

Ting Xiao, Ou Zeng, Jian Luo, Zhixiong Wu, Fang Li and Jun Yang*

Department of Cardiology, The First Affiliated Hospital of University of South China, Hengyang, 421001, China

Abstract. In order to explore the effects of hydrogen sulfide (H₂S) on myocardial fibrosis in diabetic rats and its underlying mechanisms, intraperitoneal injections of streptozotocin were used to establish the diabetes models and sodium hydrosulfide (NaHS) was used as an exogenous donor of H₂S. Eight weeks later, Van Gieson staining was used to observe pathological changes, and basic hydrolysis methods were adopted to measure hydroxyproline content, while Western Blot was used to determine the expression of MMP2, MMP7, MMP11, MMP13, MMP16, TIMP1 and TGFβ1. The results showed that significant myocardial fibrosis, decreased TIMP1 and MMP2 expression and increased MMP7, MMP11, MMP13, MMP16 expressions occurred in diabetic group, but all these changes were significantly reversed in diabetic rats after NaHS treatment. This suggests that H₂S could attenuate cardiac fibrosis induced by diabetes and its mechanisms may be related to its modulation of MMPs/TIMPs expression and regulation of TGFβ1.

Keywords: Hydrogen sulfide, diabetic myocardial fibrosis, matrix metalloproteinases, tissue inhibitors of metalloproteinases, transforming growth factor beta1

1. Introduction

During the past ten years, diabetes has grown into a common disease among people across the world, and has revealed its progression and symptoms to have similarities with those of cardiovascular diseases. Diabetic cardiomyopathy is one of the leading causes of increased morbidity and mortality among the diabetic population. According to some researches, high glucose levels are likely to lead to myocardial fibrosis [1]. The results of these research studies have made the prevention of diabetic myocardial fibrosis and its related mechanisms a relevant topic in the study of diabetic cardiomyopathy and cardiovascular diseases. Fibrosis is a key structural substrate that can cause cardiac failure, and subsequently sudden death [2]. This occurrence is a result of excess degradation and disruption of the extracellular matrix (ECM), or from the compilation of ECM proteins and formation of myocardial fibrosis.

* Address for correspondence: Jun Yang, Department of Cardiology, The First Affiliated Hospital of University of South China, Hengyang, 421001, China. Tel.: 0734-8578767; Fax: 0734-8279009; E-mail: yangjunincn@163.com.

Myocardial fibrosis is also a well-known cause of diastolic dysfunction and diastolic heart failure. Fibrosis is a crucial determinant of cardiac dysfunction caused by diabetes, which highlights the need for a better understanding of the mechanisms contributing to fibrosis caused by diabetes. The pathogenesis of diabetic myocardial fibrosis is relatively complex, and previous studies have suggested that dysregulation of matrix metalloproteinases (MMPs)/ tissue inhibitors of metalloproteinases (TIMPs) is involved in the progress of myocardial fibrosis [3]. MMPs are an endogenous family of enzymes responsible for ECM degradation. MMPs and their physiological inhibitors, TIMPs, play a key role in collagen remodeling in the heart. Most importantly, a time-and-space dependent window of TIMP/MMP balance may exist during the development of myocardial fibrosis and its progression to ventricular remodeling.

Hydrogen sulfide (H₂S) is a new type of gaseous signal molecule that has been discovered to offer multiple biological effects for cardiovascular diseases in some studies [4, 5], but it is still unclear whether endogenous hydrogen sulfide can participate in the occurrence and development of diabetic myocardial fibrosis, and if it is a possible regulatory mechanism. It is worth mentioning, however, that previous studies have reported exogenous H₂S can protect against diabetes-induced cardiac apoptosis, oxidative stress, dysfunction, hypertrophy and myocardial fibrosis [6, 7].

This paper intends to explore the role of endogenous H₂S in diabetic myocardial fibrosis and its possible mechanism by establishing a diabetic rat model induced by intraperitoneal injection of streptozotocin (STZ), and observing the impact of hydrogen sulfide on myocardial fibrosis, MMPs/TIMPs disorder and the transforming growth factor beta 1 (TGFβ1) expression in diabetic rats.

2. Materials and methods

2.1. Animals and reagents

Adult male Sprague-Dawley rats, weighing 288-300g, were obtained from the SJA Lab Animal Central of Changsha (Changsha, China). Sodium hydrosulfide (NaHS) was purchased from sigma Company (USA). STZ was purchased from MP Biomedicals Company (USA). Hydroxyproline detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibody for MMP2 (BA0569), MMP7 (BA2110), MMP11 (BA2203), MMP13 (BA2204), MMP16 (BA1280), TIMP1 (BA3727) and TGFβ1 (BA0290) were purchased from BOSTER (Wuhan, China). Anti-rabbit secondary antibody was purchased from KPL Technologies Company (USA).

2.2. Model establishment and experimental protocol

The experimental rats were divided into four groups: Control group (normal rats), DM group (diabetes rats), DM+NaHS group (diabetes rats treated with NaHS) and NaHS group (normal rats treated with NaHS). Diabetes was induced by a single intraperitoneal injection of STZ (40 mg/kg) dissolved quickly in 0.1 M sodium citrate buffer (pH = 4.4). 72 hours after STZ injection, only those rats with blood glucose levels ≥ 16.7 mM were considered successful diabetes model. The control group and DM group were intraperitoneally injected with physiological saline daily for 8 weeks, whereas, the DM+NaHS group and NaHS group were intraperitoneally administered with an equivalent volume of NaHS at a dose of 100 μ mol/kg daily for 8 weeks. At the end of the experiment, rats were sacrificed by intraperitoneal injection of chloral hydrate (350 mg/kg). Hearts were levaged with ice-cold normal saline and then removed.

2.3. Histopathological examination

Myocardium samples were fixed in 4% paraformaldehyde, paraffin embedded, sectioned and stained with Van Gieson staining, and at last observed at $\times 200$ magnification using a light microscope.

2.4. Hydroxyproline content assay

Myocardial hydroxyproline content was measured by basic hydrolysis method with a hydroxyproline detection kit. The left ventricular tissue of rats was cut into 10 mg pieces and added to 1 ml basic hydrolysates. Then, the samples were hydrolyzed at 100°C for 20 min. Absorbance was measured at 490 nm wavelengths, and the hydroxyproline content was calculated.

2.5. Western blot analysis

Total proteins were extracted and then were quantified. Protein was denatured, separated by SDS-PAGE electrophoretic and transferred to a PVDF membrane. The membranes were incubated with MMP2, MMP7, MMP11, MMP13, MMP16, TIMP1 and TGF β 1 antibody, respectively. Then primary antibodies were detected with a secondary antibody and finally the membranes were subjected to chemiluminescence detection assay.

2.6. Statistical analysis

SPSS18.0 was used for statistical analysis. Values are presented as mean \pm SD. Statistical differences between the groups were assessed by one-way ANOVA with SPSS 18.0 software. Difference were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Effects of hydrogen sulfide on pathological changes in diabetes rats

Figure 1 presents the pathological changes in the myocardium of rats from each group. Red staining reflects the intensity of fibrosis in the cardiac tissue. There was almost no obvious cardiac fibrosis in the control group and NaHS group. However, the content of collagen increased significantly in DM group. But compared with DM group, less collagen was observed in DM+NaHS group.

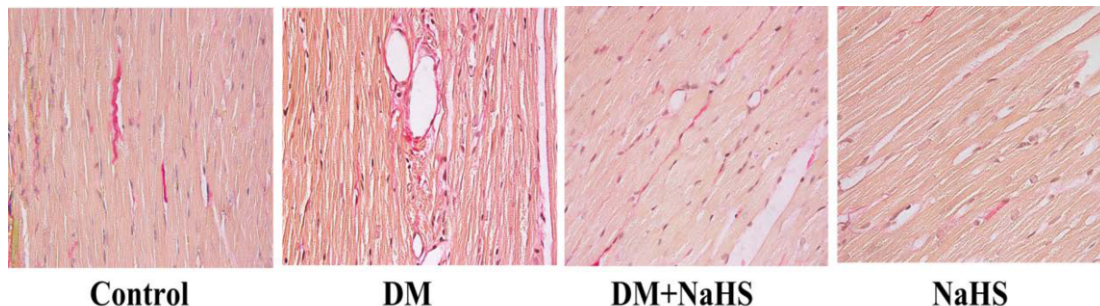


Fig. 1. Pathological changes of myocardium assessed by Van Gieson staining ($\times 200$).

3.2. Effects of hydrogen sulfide on hydroxyproline content in diabetes rats

Myocardial hydroxyproline content in each group is showed in Figure 2. Compared with control group, there was a significant higher level of hydroxyproline in DM group. However, compared with DM group, it decreased remarkably in DM+NaHS group.

3.3. Effects of hydrogen sulfide on MMP2, MMP7, MMP11, MMP13, MMP16 and TIMP1 expression in diabetes rats

As showed in Figures 3 and 4, compared with control group, there was an obvious higher expression of MMP7, MMP11, MMP13 and MMP16, but lower expression of MMP2 and TIMP1 in DM group. However, compared with DM group, the expression of MMP7, MMP11, MMP13 and MMP16 were significant decreased, while TIMP1 and MMP2 were significant increased in DM+NaHS group.

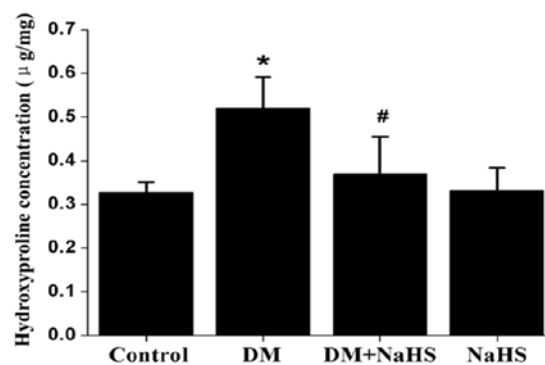


Fig. 2. Hydroxyproline contents in myocardial tissues from each group. * $P < 0.05$ vs control group; # $P < 0.05$ vs DM group.

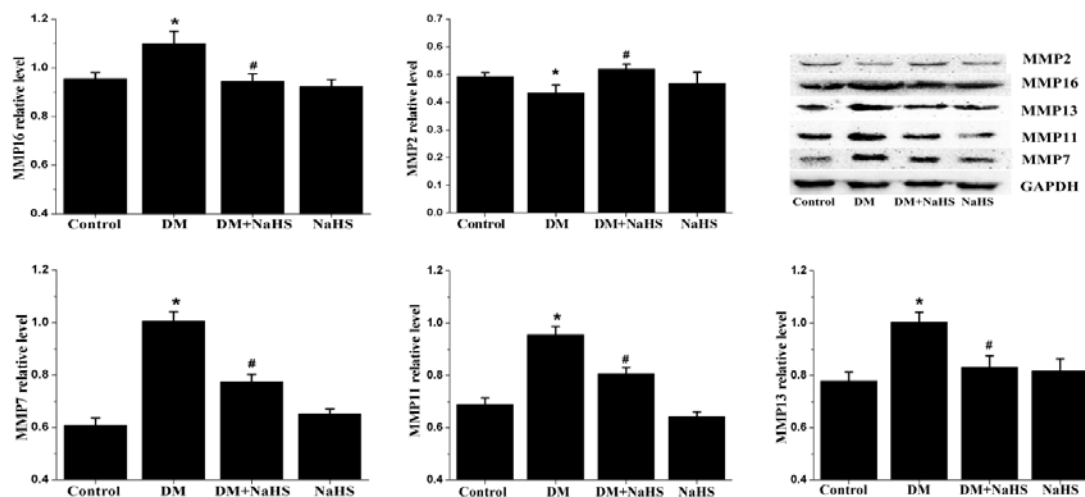


Fig. 3. Expression of MMPs in the myocardial tissues from each group (normalized by GAPDH). * $P < 0.05$ vs control group; # $P < 0.05$ vs DM group.

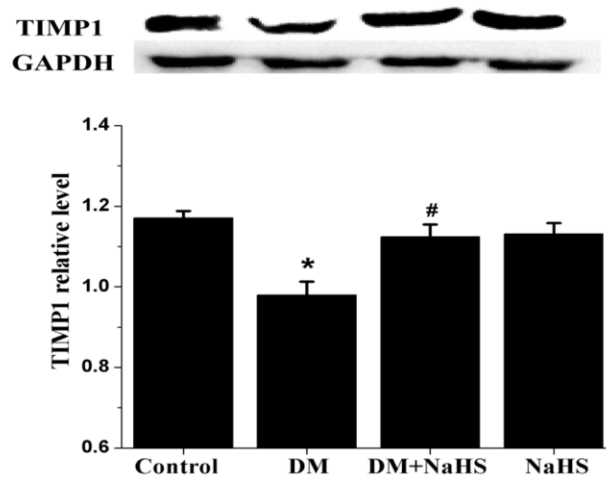


Fig. 4. Expression of TIMP1 in the myocardial tissues from each group (normalized by GAPDH). * $P < 0.05$ vs control group; # $P < 0.05$ vs DM group.

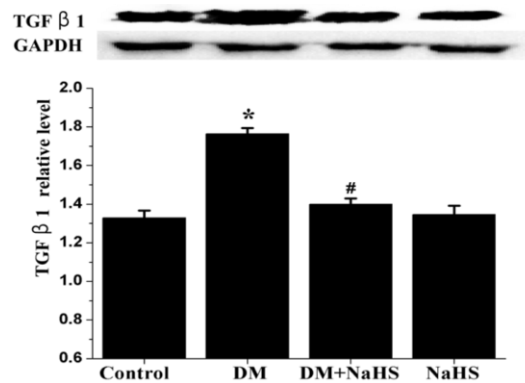


Fig. 5. Expression of TGF β 1 in the myocardial tissues from each group (normalized by GAPDH). * $P < 0.05$ vs control group; # $P < 0.05$ vs DM group.

3.4. Effects of hydrogen sulfide on TGF β 1 expression in diabetes rats

As showed in Figure 5, compared with control group, the expression of TGF β 1 was significant higher in DM group. However, compared with DM group, the expression of TGF β 1 was notably reduced in DM+NaHS group.

4. Discussion

Diabetic myocardial fibrosis, a main symbol of myocardial remodeling of diabetic cardiomyopathy, has complex pathogenesis and unclear mechanisms. Remodeling of the ECM is a key aspect of cardiac fibrosis. Altered levels of MMPs and their specific tissue inhibitors (TIMPs) have been involved in remodeling of the extracellular matrix during myocardial fibrosis [8]. Myocardial extracellular matrixes include collagen, adhesive glycoprotein and proteoglycan. The regulation disorder of MMPs

and TIMPs will cause a decrease in ECM degradation and an increase in accumulation, thus leading to myocardial fibrosis. When studying the structure of the heart, it becomes apparent that the myocardial ECM plays an integral role in maintaining the functionality of the heart's design. Diminished collagen cross-linking as a result of increased MMP activity has been associated with myocardial fibrosis and ventricular remodeling [9].

MMPs, a family of zinc-dependent endopeptidase, are quite homologous in structure and are able to effectively degrade the myocardial extracellular matrix. MMPs are known to degrade gelatin, as well as collagen type I and III, which are the main components of the myocardial extracellular matrix. TIMPs can specifically combine with the zinc ions in MMPs catalytic activity center via the cysteine residues located in N-terminal function areas, thus disbanding activated MMPs, or avoiding the activation of inactive MMPs by blocking the catalytic activity of MMPs. Therefore, the change of dynamic balance between MMPs and their inhibitors TIMPs will immediately lead to myocardial fibrosis. In the family of TIMPs, TIMP-1 has the strongest inhibitory effect on MMPs. MMP2 is a type of MMP more implicated in diabetic cardiac remodeling. Previous study reported that reduced MMP2 activity contributed to cardiac fibrosis under diabetic condition [10]. But some scholars also proved that myocardial fibrosis in human patients and animal models are accompanied by increased MMPs activity and decreased levels of TIMPs [11, 12]. TGF β 1 is widely accepted as a critical factor associated with fibrogenesis. It has been reported that TGF β 1 can stimulate fibroblast proliferation and production of ECM proteins, such as fibronectin and collagen [13]. In this study, myocardial fibrosis obviously exists in diabetic mice as shown in the result of Van Gieson Staining and increased level of hydroxyproline which is a hallmark of collagen production. Also, diabetes induced an increased expression of TGF β 1. Meanwhile, TIMP1 and MMP2 expression significantly decreases and MMP7, MMP11, MMP13, MMP16 expression obviously increases, which is similar to the observation discussed by other authors [14, 15].

Recently, hydrogen sulfide has been discovered to be an endogenous signaling gasotransmitter. Increasing evidence has shown that H₂S plays important roles in various systems, particularly in the cardiovascular system. To examine the role of H₂S in diabetic cardiomyopathy, we investigated cardiac fibrosis in an animal model of STZ-induced diabetes. To take the study even further, we analyzed changes in the cardiac extracellular matrix and the level of MMPs/TIMP and TGF β 1 in diabetic rats after treatment with NaHS. The results indicated that NaHS treatment normalized MMPs and TIMP expression, reduced cardiac TGF β 1 levels, and decreased cardiac fibrosis. The Nox/NADPH oxidase system is highly implicated in conditions of heart hypertrophy and fibrosis, and has been associated to the activation of MMPs [16]. Therefore, a plausible explanation for the protective effect of H₂S against MMPs dysregulation and cardiac fibrosis in the diabetic heart is the inhibition of cardiac Nox/NADPH oxidases expression reported by others [17].

During this study, we investigated the chronic effects of exogenous H₂S and proposed novel protective effects of H₂S on cardiac fibrosis. Our results demonstrated that H₂S has some beneficial effects on attenuating or suppressing the development of cardiac fibrosis induced by diabetes. The mechanisms may be at least partially related to its modulation of MMPs/TIMPs expression and the regulation of TGF β 1.

Acknowledgment

This study was supported by the National Natural Science Foundation of China (Grant No. 81202830).

References

- [1] K. Mizushige, T. Tsuji and T. Noma, Pioglitazone: Cardiovascular effects in prediabetic patients, *Cardiovascular Drug Reviews* **20** (2002), 329-340.
- [2] T. Tsoutsman, X. Wang, K. Garchow, B. Riser, S. Twigg and C. Semsarian, CCN2 plays a key role in extracellular matrix gene expression in severe hypertrophic cardiomyopathy and heart failure, *Journal of Molecular and Cellular Cardiology* **62** (2013), 164-178.
- [3] Y.Y. Li, C.F. McTiernan and A.M. Feldman, Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling, *Cardiovascular Research* **46** (2000), 214-224.
- [4] J.W. Calvert, W.A. Coetzee and D.J. Lefer, Novel insights into hydrogen sulfide-mediated cytoprotection, *Antioxidants Redox Sign* **12** (2012), 1203-1217.
- [5] H. Kimura, Production and physiological effects of hydrogen sulfide, *Antioxidants & Redox Signaling* **20** (2014), 783-793.
- [6] X. Zhang, L.H. Wang, Y.W. Wang, S.Y. Dong, X.N. Leng, J. Jia, Y.J. Zhao, H.L. Li, X.Y. Zhang, C.Q. Xu, G.D. Yang, L.Y. Wu, R. Wang, F.H. Lu and W.H. Zhang, Exogenous hydrogen sulfide attenuates diabetic myocardial injury through cardiac mitochondrial protection, *Molecular and Cellular Biochemistry* **371** (2012), 187-198.
- [7] X. Zhou, G.Y. An and X. Lu, Hydrogen sulfide attenuates the development of diabetic cardiomyopathy, *Clinical Science* **128** (2015), 325-335.
- [8] F.G. Spinale, Matrix metalloproteinases: Regulation and dysregulation in the failing heart, *Circulation Research* **90** (2002), 520-530.
- [9] E. Rizzi, C.S. Ceron, D.A. Guimaraes, C.M. Prado, M.A. Rossi, R.F. Gerlach and J.E. Tanus-Santos, Temporal changes in cardiac matrix metalloproteinase activity, oxidative stress, and TGF- β in renovascular hypertension-induced cardiac hypertrophy, *Experimental Molecular Pathology* **94** (2013), 1-9.
- [10] S.V. Linthout, U. Seeland, A. Riad, O. Eckhardt, M. Hohl, N. Dhayat, U. Richter, J.W. Fischer, M. Bohm, M. Pauschinger, H.P. Schultheiss and C. Tschope, Reduced MMP2 activity contributes to cardiac fibrosis in experimental diabetic cardiomyopathy, *Basic Research in Cardiology* **103** (2008), 319-327.
- [11] V. Polyakova, S. Hein, S. Kostin, T. Ziegelhoeffer and J. Schaper, Matrix metalloproteinases and their tissue inhibitors in pressure-overloaded human myocardium during heart failure progression, *Journal of the American College of Cardiology* **44** (2004), 1609-1618.
- [12] E. Vellaichamy, M.L. Khurana, J. Fink and K.N. Pandey, Involvement of the NF-kappa B/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor A, *Journal of Biological Chemistry* **280** (2005), 19230-19242.
- [13] M-S. Carlos, F-C. Isabel, G-C. Begona, E. Santos and M.L-N. Jose, Involvement of H- and N-Ras isoforms in transforming growth factor- β 1-induced proliferation and in collagen and fibronectin synthesis, *Experimental Cell Research* **312** (2006), 2093-2106.
- [14] J.M. Siddesha, A.J. Valente, S.S. Sakamuri, J.D. Gardner, P. Delafontaine, M. Noda and B. Chandrasekar, Acetylsalicylic acid inhibits IL-18-induced cardiac fibroblast migration through the induction of RECK, *Journal of Cellular Physiology* **229** (2014), 845-855.
- [15] T. Ashraf, M.A. Mekky and M.M.M. Omar, Tempol ameliorates cardiac fibrosis in streptozotocin-induced diabetic rats: role of oxidative stress in diabetic cardiomyopathy, *Naunyn-Schmiedeberg's Archives of Pharmacology* **386** (2013), 1071-1080.
- [16] M.Y. Wang, J. Zhang, S.J. Walker, R. Dworakowski, E.G. Lakatta and A.M. Shah, Involvement of NADPH oxidase in age-associated cardiac remodeling, *Journal of Molecular and Cellular Cardiology* **48** (2010) 765-772.
- [17] P.M. Snijder, R.A. de Boer, E.M. Bos, J.C. van den Born, W-P.T. Ruifrok, I. V-Baudoin, M.C.R.F. van Dijk, J-L. Hillebrands, H.G.D. Leuvenink and H. van Goor, Gaseous Hydrogen Sulfide Protects against Myocardial Ischemia-Reperfusion Injury in Mice Partially Independent from Hypometabolism, *PLoS One* **8** (2013), e63291.