Reduced cystathionine-γ-lyase (CSE) expression is involved in high glucose induced MMP14 expression in adipocytes and adipose tissues

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Abstract. In the present study, we investigate the effect of reduced cystathionine-γ-lyase (CSE) expression in high glucose induced metalloproteinases14 (MMP14) expression in adipocytes and visceral adipose tissues. Diabetic mice were prepared by injections of STZ and the expression of CSE, MMP14 in visceral adipose tissues were determined. Adipocytes were differentiated from 3T3-L1 cells and treated with high glucose (HG), H2S slow-releasing compound GYY4137 or transfected with CSE siRNA. Then the expression of CSE, MMP14 were determined by western blotting. CSE knockout mice were generated by crossing CSE +/- heterozygous mice and given intraperitoneally (i.p.) injections of GYY4137, and then the expression of CSE and MMP14 in visceral adipose tissues were determined by quantitative real-time PCR and western blotting. The following results were obtained from the study. In adipose tissues of diabetic mice, the mRNA and protein expression of MMP14 increased while the mRNA and protein expression of CSE decreased. In 3T3-L1 adipocytes, both HG DMEM and CSE siRNA transfection increased the mRNA and protein of MMP14. The addition of GYY4137 inhibited HG-induced upregulation of MMP14 expression. In CSE knockout mice, the mRNA and protein expression of MMP14 in adipose tissues increased, which could be inhibited by i.p. injections of GYY4137. In conclusion, high glucose increased the expression of MMP14 in adipocytes and visceral adipose tissues through inhibiting the expression of CSE.

Key words: High glucose, Adipocytes, Adipose tissues, Cystathionine-γ-lyase, Metalloproteinases14

MACROANGIOPATHIC AND MICROANGIOPATHIC COMPLICATIONS are major causes of mortality in diabetes. Endothelial dysfunction, characterized by shifting of the physiological properties of the vessel towards a vasoconstrictor, prothrombotic and proinflammatory state, plays a pivotal role in the development of functional and structural vascular alterations in diabetics with angiopathic complications [1, 2]. Metalloproteinases 14 (MMP14), a disintegrin and metalloproteinase10 (ADAM10), ADAM17 play important roles in endothelial dysfunction through cleaving extracellular domain of endoglin and fms-like tyrosine kinase-1, tumor necrosis factor-α (TNF-α). Soluble endoglin cleaved by MMP14, soluble fms-like tyrosine kinase-1 cleaved by ADAM10 and ADAM17 are two important markers for endothelial dysfunction, which induce endothelial dysfunction through blocking the endothelial protective effects mediated by transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) signaling [3-6]. In addition, ADAM17 also cleave extracellular domain of TNF-α and induce endothelial dysfunction through initiating inflammation in endothelial cells [7].

In recent years, adipose tissue has been regarded as an important endocrine organ which play important roles in endothelial dysfunction of cardiovascular diseases [8, 9]. Visceral adipose tissue has been confirmed to be more metabolically and biologically active than subcutaneous adipose tissue [10, 11]. In obesity and gestational diabetes mellitus (GDM), adipose tissue, rather than placental tissue, is thought to be the main source of elevated markers of endothelial dysfunction [12]. These results indicated that visceral adipose tissue might be the important source of endothelial dysfunction markers cleaved by MMP14, ADAM10, ADAM17 in high glucose environ-
ment. According to Zhou et al.’s study and our previous study, ADAM17 was expressed in adipocytes whereas ADAM10 was not expressed in adipocytes, and high glucose increased the activity and expression of ADAM17 in adipocytes [13, 14]. However, the effects of high glucose on the expression of MMP14 in adipocytes and adipose tissues remain to be elucidated.

Hydrogen sulfide (H$_2$S) has recently been suggested to be “the third endogenous gaseous signaling transmitter” in mammalian tissues. H$_2$S synthesis from L-cysteine in a range of mammalian tissues mainly through the activity of the enzymes including cystathionine-$\gamma$-lyase (CSE) and cystathionine-$\beta$-synthetase (CBS) [15]. Previous studies have shown that H$_2$S has several different properties in various tissues, including endothelial protection, anti-inflammatory, anti-oxidation, pro-angiogenesis [16-19]. In both of adipose tissues and adipocytes, CSE and CBS are identified. Pan’s study [20] and our previous study [14] both found that high glucose inhibits the expression of CSE and the production of H$_2$S in adipocytes. As previously described, sEng cleaved by MMP14 and sFlt-1 cleaved by ADAM17 are two important markers for endothelial dysfunction, and in diabetes, adipose tissues are considered to be the main source of sEng and sFlt-1 [12]. So we observed the role of H$_2$S in regulating MMP14, ADAM17 expression in adipocyte and adipose tissues. Our previous study has found that the reduced CES expression is involved in high glucose induced ADAM17 in adipocytes [14]. However, whether the reduced CES expression is involved in high glucose induced MMP14 expression in adipocytes and adipose tissues remain unknown. On this basis, we give the hypothesis that reduced CSE expression might be involved in modulation of high glucose induced MMP14 expression in adipocyte and adipose tissues. To test it, we firstly investigated the expression of MMP14 and CSE both in adipose tissues and adipocytes exposed to high glucose. Then whether downregulation of CSE was involved in high glucose induced aberrant expression of MMP14 was confirmed both in adipose tissues with CSE knockout (KO) and adipocytes with CSE knockdown. Furthermore, H$_2$S donor was used to reverse the effect of CSE KO or knockdown.

**Materials and Methods**

**Animal and tissue preparation**

6-weeks-old male C57BL/6 mice (Shanghai Laboratory Animal Co), weighing 17–19 g, were housed with regular light-dark cycles (lights on at 7:00 a.m., lights off at 7:00 p.m.) under controlled temperature (22 ± 2°C) and humidity (50 ± 10%), and were given standard diet and water *ad libitum*. They were allowed to acclimatize for 2 weeks before use. At 8 weeks of age, they were randomly assigned into 2 groups (n = 8 per group): diabetic and control groups. Mice in the diabetic group received one intraperitoneal injection with STZ (50 mg/kg body weight in 0.1 M citrate buffer pH 4.5, Sigma-Aldrich) to induce diabetes. Mice in the control group were injected intraperitoneal with vehicle (0.1 M citrate buffer pH 4.5) of equal volume. 7 and 14 days after injection, non-fasting blood glucose levels were determined by glucometer (Johnson & Johnson). The blood glucose cut-off was 16.7 mmol/L for including animals in diabetic group. At 14 days following the STZ injection, omental and mesenteric adipose tissue were collected and then stored at −80°C until assays.

The CSE$^{+/−}$ heterozygous mice (on a C57BL/6J background) were purchased from Shanghai Model Organisms Center, Inc. The CSE$^{−/−}$ males and females were bred to obtain wild type (WT, CSE$^{+/+}$), CSE$^{−/−}$ and CSE$^{+/−}$ littersmates. Then eight to twelve-week-old male CSE KO mice and age-matched male WT littersmates were randomly assigned to control and experimental groups (WT NC, CSE KO NC, WT GYY, and CSE KO GYY, n = 8 each). To evaluate the effect of exogenous H$_2$S on MMP14 in adipose tissues, the mice in WT GYY and CSE KO GYY groups were given intraperitoneally (i.p.) injections of 50 mg/kg GYY4137 (Cayman Chemical, United States) once daily for 2 weeks, while mice in WT NC and CSE KO NC groups were given i.p. injections of saline. The animals were sacrificed 2 weeks after GYY4137 administration for omental and mesenteric adipose tissues collection. Adipose tissues were stored at −80°C until assays.

All animal procedures were carried out in accordance with the guidelines for the use of laboratory animals published by the People’s Republic of China Ministry of Health (January 25, 1998), with the approval of the Ethical Committee of Experimental Animals of Second Military Medical University. Procedures were designed to minimize the number of animals used and their suffering.

**Adipocytes culture**

Adipocytes were cultured and differentiated as previously described [14]. Firstly, 3T3-L1 cells (Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were recovered and incubated in high-glucose (HG) DMEM (Gibco) containing 10% newborn calf serum (Gibco) at 37°C 5%CO2-95%air until confluency. Confluent 3T3L1 cells were then incubated in differentiation medium for 2 days after which the media were replaced by HG DMEM containing 10% FBS, supplemented with 100 milliunits/mL insulin every 2 days until >85% of the cells contained lipid droplets.
**Drug treatment**

LG DMEM contained 5.5 mmol/L glucose and HG DMEM contained 25.0 mmol/L glucose. GYY4137 (Sigma) was dissolved in PBS. After 7–10 days of differentiation, 3T3-L1 adipocytes were treated with serum free LG DMEM or serum free HG DMEM for 24 h. To determined the effect of H\(_2\)S, cells were treated with serum free HG DMEM containing GYY4137 (10 μM, 25 μM, 50 μM) or serum free HG DMEM without GYY4137 for 24 h.

**RNA interferences**

The Small interfering RNAs (siRNAs) for CSE were designed and synthesized by GenPharma Corporation (Shanghai, China). The siRNA used in the present study were illustrated in supplements (Supplementary Table 1). Control siRNA was scrambled sequence without any specific target. To knockdown the expression of CSE or MMP14, cultured 3T3-L1 adipocytes were transfected with CSE or MMP14 siRNA and negative control (NC) siRNA using Lipofectamine™ 2000 (Invitrogen) for 24 h.

**Total RNA extraction and quantitative real-time PCR**

Total RNA extraction and quantitative real-time PCR were carried out as described previously [21]. The comparative Ct (threshold cycle) method with arithmetic formulae (2\(^{-ΔΔCt}\)) was used to determine the relative quantitation of gene expression for both target and housekeeping genes β-actin [22].

**Western blot analysis**

Cultured adipocytes were scraped off the plate in the presence of cold Radio-Immunoprecipitation Assay (RIPA) lysis buffer containing protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Approximately 30–40 mg of adipose tissues were homogenized in cold RIPA lysis buffer containing protease inhibitor cocktail tablet. The lysates of adipocytes and adipose tissues were quickly centrifuged at 4°C for 15 minutes. The supernatant was collected and protein concentration was assayed using BCA Protein Assay Kit (Beyotime). 30 μg of protein samples were separated by 10% or 15% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After blockage for 2 hours, membranes were incubated with the antibody against CSE, MMP14, β-actin overnight at 4°C. Membranes were then washed and incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz). Immunoreactive proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (merck millipore) and Tanon 5200 Multi scanner. The ratio of band intensities to β-actin was obtained to quantify the relative protein expression level.

**Statistical analysis**

The data are presented as mean ± SEM. All data were tested for homogeneity of variance by Bartlett’s test before analyzing the significance. Individual comparisons were made by one-way ANOVA followed by Least Significant Difference (LSD)-t test for the data which were normally distributed. In all of the tests, \( p < 0.05 \) was considered to be significant.

**Results**

**Hyperglycemia upregulates the expression of MMP14 in adipose tissues**

To investigate the effects of hyperglycemia on the expression of MMP14 in adipose tissues, diabetic mice were prepared by injections of STZ. As shown in Supplementary Figs. 1 and 2, at 7 days and 14 days after STZ injection, fasting glucose levels were significantly higher in diabetic group mice than in control group. Then the expression of MMP14 in adipose, both of omental and mesenteric adipose tissue were determined by quantitative real-time PCR and western blotting. As shown in Fig. 1, the mRNA expression and protein expression of MMP14 in omental and mesenteric adipose tissues of diabetic mice were elevated.

**Hyperglycemia downregulates the expression of CSE in adipose tissues**

Our previous study has found that high glucose inhibits expression of H\(_2\)S synthetase CSE in adipocytes. In order to confirm the effect of hyperglycemia on the expression of H\(_2\)S synthetase in adipose tissues, the mRNA expression and protein expression of CSE in omental and mesenteric adipose tissue of diabetic mice were compared with control mice. As shown in Fig. 2, the expression of CSE in omental adipose tissues of diabetic group mice decreased.

**High glucose and CSE siRNA increases MMP14 expression in 3T3-L1 adipocytes**

To confirm the effect of hyperglycemia on the expression of MMP14 in adipose tissues, the effect of high glucose on the expression of MMP14 in adipocytes were determined. The expression of MMP14 in adipocytes treated with LG DMEM and HG DMEM were detected by western blotting. As shown in Fig. 3A and C, the expression of MMP14 significantly increased in 3T3-L1 adipocytes exposed to HG DMEM. Our previous study has found that high glucose inhibits the expression of H\(_2\)S enzyme CSE. In order to investigate whether the CSE decreasing is involved in the
MMP14 increasing in adipocytes, the expression of MMP14 in CSE-knockdown adipocytes were determined. As shown in Supplementary Fig. 3, transfection of CSE siRNA caused reduction of CSE expression. As shown in Fig. 3B and D, transfection of CSE siRNA resulted in increasing the expression of MMP14 in 3T3-L1 adipocytes exposed to LG DMEM.

Then we investigated the role of MMP14 in adipocytes through transfecting MMP14 siRNA. As shown in Supplementary Fig. 4, transfection of MMP14 siRNA significantly decreased the expression of MMP14. As shown in Supplementary Fig. 5, high glucose increased sEng production in adipocytes transfected with NC-siRNA and this effect was not occurred in 3T3-L1 adipocytes transfected with MMP14-siRNA.

H₂S donor inhibits high glucose-induced upregulation of MMP14 expression in 3T3-L1 adipocytes

In order to investigate the role of exogenous H₂S in high glucose-induced upregulation of MMP14 expression in adipocytes, cultured 3T3-L1 adipocytes were treated with high glucose DMEM containing H₂S slow-releasing compound GYY4137 for 24 h, and then the mRNA expression and protein expression of MMP14 were determined. As shown in Fig. 4, treatment of 3T3-L1 adipocytes with GYY4137 could resulted in a decrease in MMP14 expression in dose-dependent manner.

CSE knocking-out upregulates the expression of MMP14 in adipose tissues and H₂S donor decreases the expression of MMP14 in adipose tissues of CSE-knockout mice

To confirm the role of CSE in regulation of MMP14 expression in adipose tissues, the mRNA expression and protein expression of MMP14 in omental and mesenteric adipose tissues of CSE-knockout mice were determined by quantitative real-time PCR and western blotting. The expression of MMP14 was significantly higher in CSE-knockout omental and mesenteric adipose tissues than in wild type. To furtherly determine if exogenous H₂S can affect the expression of MMP14 in CSE-knockout mice,
the KO mice were given i.p. injections of GYY4137 or saline. As shown in Fig. 5, the upregulation effect of MMP14 in omental and mesenteric adipose tissues of CSE-knockout mice was rescued by GYY4137.

**Discussion**

The present study demonstrated that reduced expression of CSE induced by high glucose significantly stimulated MMP14 expression in adipocytes and adipose tissues. H2S synthase enzymes CSE was involved in high glucose induced MMP14 expression. Furthermore, exogenous H2S suppressed the expression of MMP14 induced by high glucose in adipocytes and adipose tissues.

In recent years, many studies found that visceral adipose tissue, which play important roles in endothelial dysfunction of cardiovascular diseases, is more metabolically and biologically active than subcutaneous adipose tissue [10, 11]. According to Lappas M’s study in obesity and GDM, visceral adipose tissue was major source of endothelial dysfunction markers, including sEng, sFlt-1 [12]. MMP14 is the main endoglin shedding protease, which participates in cleaving endoglin extracellular domain and releasing eEng. ADAM10 and ADAM17 are the main Flt-1 shedding proteases, which participates in cleaving Flt-1 extracellular domain and releasing sFlt-1. Our previous study has found that ADAM10 was not expressed in adipocytes, the expression of ADAM17 in adipocytes is upregulated by high glucose [14]. However, the expression of MMP14 in visceral adipose tissue of diabetics remains to be elucidated. In the present study, diabetic mice was obtained through STZ injection and the results showed that hyperglycemia significantly increased the expression of MMP14 in visceral adipose tissues. Consistent with the findings in diabetic mice, we found that high glucose significantly increased MMP14 expression in cultured adipocytes. These results in vivo and vitro suggest that hyperglycemia has upregulatory effect on the expression of MMP14 in visceral adipose tissues.

Until now, the mechanisms responsible for high glucose induced MMP14 expression in visceral adipose tissues and adipocytes remain unclear. sEng, which is shedded by MMP14, is responsible for the endothelial
dysfunction of diabetes. H₂S, which can be produced in wide spectrum of tissues through the activity of CBS and CSE, has protective effect in endothelial cells [15]. Our previous study has demonstrated that high glucose decreases the expression of CSE, which contributes to elevated ADAM17 expression in adipocytes, but CBS expression is not affected by high glucose [14]. Based on this, we investigate whether CSE is involved in high glucose induced MMP14 expression in visceral adipose tissues and adipocytes. Firstly, the effect of high glucose on the expression of CSE in visceral adipose tissues were confirmed in diabetic mice. The results showed that hyperglycemia significantly decreased the expression of CSE in visceral adipose tissues in diabetic mice, which was consistent with the change of CSE in adipocytes exposed to high glucose. Then the role of decreasing CSE on the expression of MMP14 was observed in CSE knocked-down adipocytes and CSE knocked-out visceral adipose tissues. We found the both knocking-down CSE and knocking-out CSE significantly increased MMP14 expression.

Above findings, including high glucose increased MMP14 expression and decreased CSE expression, knocking-down or knocking-out CSE increased MMP14 expression, suggested that decreased expression of CSE contribute to elevated MMP14 expression in adipocytes exposed to high glucose and visceral adipose tissues exposed to hyperglycemia. On the basis of these findings, we hypothesis that exogenous supply of H₂S may attenuate high glucose induced MMP14 expression. In order to test this hypothesis, endogenous H₂S was supplemented by GYY4137 in CSE decreased adipocytes and CSE knocked-out mice. GYY4137 is a watersoluble compound that releases low but consistent

Fig. 3 Effects of high glucose and CSE siRNA on the expression of MMP14 in 3T3-L1 adipocytes

3T3-L1 adipocytes were treated with HG DMEM or transfected with CSE siRNA for 24 h. The expression of MMP14 in 3T3-L1 adipocytes were determined by quantitative real-time PCR and western-blotting as described in materials and methods. (A) the effect of HG DMEM on the mRNA expression of MMP14 in 3T3-L1 adipocytes. (B) the effect of CSE siRNA transfection on the mRNA expression of MMP14 in 3T3-L1 adipocytes. (C) the effect of HG DMEM on the protein expression of MMP14 in 3T3-L1 adipocytes. (D) the effect of CSE siRNA transfection on the protein expression of MMP14 in 3T3-L1 adipocytes. Representative protein bands were presented on the top of corresponding histogram. Data were presented as mean ± SEM (n = 4 cultures). **p < 0.01 vs. indicated.
Effects of GYY4137 on high glucose induced MMP14 expression in 3T3-L1 adipocytes

3T3-L1 adipocytes were treated with HG DMEM containing increasing concentration of GYY4137 for 24 h. The expression of MMP14 in 3T3-L1 adipocytes were determined by quantitative real-time PCR and western-blotting. Representative protein bands were presented on the top of corresponding histogram. (A) the mRNA expression of MMP14 in adipocytes; (B) the protein expression of MMP14 in adipocytes. Data were presented as mean ± SEM (n = 4 cultures). *p < 0.05, **p < 0.01 vs. indicated.

Fig. 4

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Disclosure Statement

The authors have no conflicts of interest to declare.
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