Original Paper

High Glucose Induces Mouse Mesangial Cell Overproliferation via Inhibition of Hydrogen Sulfide Synthesis in a TLR-4-Dependent Manner

Tao Ding Wei Chen Juan Li Jiarong Ding Haiyan Hu Xiaobin Mei

Department of Nephrology, Shanghai Changhai Hospital Affiliated to Second Military Medical University, Shanghai, China

Key Words

Hydrogen sulfide • High glucose • Mesangial cells • TLR4

Abstract

Background/Aims: Overproliferation of mesangial cells was believed to play an important role in the progress of diabetic nephropathy, one of the primary complications of diabetes. Hydrogen sulfide (H_2S), a well-known and pungent gas with the distinctive smell of rotten eggs, was discovered to play a protective role in diabetic nephropathy. **Methods:** MTT assay was used to examine the viability of mesangial cells. Small interfering RNA was used to knock down the expression of TLR4 while specific inhibitor LY294002 to suppress the function of PI3K. H_2S generation rate was determined by a H_2S micro-respiration sensor. **Results:** Glucose of 25mM induced significant mesangial cells proliferation, which was accomplished by significantly inhibited endogenous H_2S synthesis. And exogenous H_2S treatment by NaHS markedly mitigated the overproliferation of mouse mesangial cells. Furthermore, it was found that H_2S deficiency could result in TLR4 activation. And H_2S supplementation remarkably inhibited TLR4 expression and curbed the mesangial cell overproliferation. Besides, PI3K/Akt pathway inhibition also significantly ameliorated the cell overproliferation. **Conclusion:** High glucose (HG) induces mouse mesangial cell overproliferation via inhibition of hydrogen sulfide synthesis in a TLR-4-dependent manner. And PI3K/Akt pathway might also play a vital part in the HG-induced mesangial cell overproliferation.

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Introduction

Diabetes mellitus, a common chronic metabolic disease, is characteristic of a group of disorders in hemorheology, microcirculation and cellular metabolism, and could lead to ischemia, hypoxia, tissue hydropsia and more severely end-organ irreversible damage. Nephropathy is one of the leading causes of mortality in diabetes complication, which is featured by overproliferation of mesangial cells. The current paper focused on the potential

Xiaobin Mei

Department of Nephrology, Shanghai Changhai Hospital, Second Military Medical University, Shanghai 200433, (China)
Tel. +8621-31161407, E-Mail xiaobinmei2016@qq.com

mechanism underlying the overproliferation of mesangial cells, and aimed to provide a new potential target for the prevention and treatment of diabetic nephropathy.

Previous studies have shown that high plasma glucose in diabetes could result in reversible changes in some related genes and phenotypes. Mesangiolysis and exuberant mesangial repair then develop, ultimately resulting in marked increase in mesangial matrix [1, 2]. However, the involved mechanisms and pathways are not yet fully elucidated.

Hydrogen sulfide (H_2S) is a well-known and pungent gas with the distinctive smell of rotten eggs discovered to be synthesized enzymatically in mammalian and human tissues. The prior studying about hydrogen sulfide to its discovery was concentrated on the resulted air/water pollution and the toxicity/inhibition in central nervous system and respiration system. Since its discover in mammalian in 1980s, hydrogen sulfide was shown to be take effect in various physiology and pathways involved in hippocampus, digestion and vascular smooth muscles [2, 3].

In mammalian and human tissues, the bulk of endogenous H_2S synthesis appears to be from the PLP (pyridoxal-5'-phosphate)-dependent enzymes cystathionine- γ -lyase (CSE) and cystathionine- β -synthase (CBS) via the amino acids cysteine, homocysteine and cystathionine [4]. It been widely suggested that the expression of CSE and CBS in rodents and humans showed a marked degree of tissue specificity. The current literature consensus was that CBS was the predominant source of H_2S in the brain and nervous tissue, and that in the vasculature (e.g. smooth muscle and endothelium) CSE was the major source of H_2S [2]. It also shares other similarities with carbon monoxide (CO) and nitric oxide (NO) which are both well accepted gasotransmitters, in that they exert biological effects at low concentrations, toxic effects at high concentrations [2-4]. Due to their wide biological applications, the gasotransmitters were not yet clearly elucidated in the receptors and functioning mechanism [5].

Hydrogen sulfide was demonstrated to be regulating cellular proliferation. H_2S is the first identified endogenous gaseous opener of ATP-sensitive K^+ channels in vascular smooth muscle cells. Through the activation of ATP-sensitive K^+ channels, H_2S lowers blood pressure, protects the heart from ischemia and reperfusion injury, inhibits insulin secretion in pancreatic β cells, and exerts anti-inflammatory, anti-nociceptive and anti-apoptotic effects. Yang et al. also showed that genetic deletion of CSE by which H_2S is physiologically generated in mice markedly reduces H_2S levels in the serum, heart, aorta, and other tissues [6-8]. Interestingly, H_2S was reported by many to play an important role in diabetic nephropathy [9-12]. Hence it was implied that exogenous H_2S might attenuate the progression of diabetic nephropathy by inhibiting the mesangial cell proliferation.

Toll-like receptor (TLR) family was a well-accepted group of pathogen-associated molecular patterns (PAMPs) receptors [13]. Moreover, it has been proved that TLRs could trigger aseptic inflammation by recognizing various endogenous cellular components, such as heat shock protein 90 and hyaluronic acid [14, 15]. More importantly, TLR4 seemed to be mediating the pathophysiology in diabetes: type 2 diabetic subjects had significantly increased TLR4 mRNA, and related ligands in monocytes compared with control subjects [16, 17]; *in vitro*, high glucose induces toll-like receptor expression in human monocytes [18]. To investigate the involvement of TLR4 in the diabetic mesangial cell overproliferation, we adopted TLR4-defecient model using siRNA technique *in vitro* and gene knock-out *in vivo*. It was found that high glucose induced the mRNA expression of TLR4 in a concentration-dependent way, which was attenuated by siRNA knock-down of TLR4 *in vitro* and abated by TLR4 knock-out in C3H/HeouJ mice. More interestingly, expression of CBS/CSE was augmented after TLR4 inhibition *in vitro* and *in vivo*.

Therefore, we hypothesized that TLR4 overexpression led to a lacking of endogenous hydrogen sulfide, resulting in the mesangial cell overproliferation in diabetic nephropathy. Hence certain techniques would be adopted to demonstrate our hypothesis like siRNA transfection (CSE), genetic co-immunoprecipitation etc.

Materials and Methods

Cell culture and treatment

Mouse mesangial cells (purchased from the Shanghai Chinese Academy of Sciences) were culture in low-glucose DMEM medium supplemented with 10% FBS at 37°C, CO₂ incubator according to the manufacturer's instructions. Cells at a density of 1×10^5 /mL were seeded in 6-well plates to reach 80-90% confluence. 0.25% trypsin with 0.03% EDTA was used to digest the cells. The normal glucose group was treated with 5.5mM glucose while the high glucose 25 mM, 30 mM, 35 mM and 40 mM. After overnight fasting, cells were incubated with normal or high glucose for 24 hours. NaHS (Sigma) was added to the mesangial cells at the meantime of glucose, at different concentrations of 5 μ M, 10 μ M, 30 μ M, 50 μ M and 100 μ M. The proliferation of mesangial cells was examined by MTT (3-[4, 5-dimethylthiazol -2-yl] - 2, 5-diphenyl tetrazolium bromide, Sigma Aldrich) assay as previously described [19, 20].

RNA interference and PI3K inhibition

The small interfering RNA for TLR4 was obtained from Santa Cruz (sc-40261). Transfection of siRNA to murine mesangial cells was performed using siPORT NeoFx transfection agent (Ambion) according to the manufacturer's instruction. LY294002, (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was used as a specific inhibitor of phosphatidylinositol 3-kinase [21].

Real-time H₂S production determination

H₂S production was determined real-time as previously described [20, 22]. Briefly, cells were collected in PBS containing protease inhibitor (2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 mg/ml aprotinin) and homogenized for 15 s on ice. Then the suspension was centrifuged at 5000 rpm for 5 min, after which the supernatants were placed into a temperature-controlled micro-respiration chamber (Unisense) to measure H₂S production using a miniaturized H₂S micro-respiration sensor (Model H₂S-MRCh; Unisense) coupled with a Unisense PA2000 amplifier. The H₂S production was standardized by total protein concentration determined by modified Bradford assay.

Western blot analysis

Western blot analysis was conducted as previous described. Mouse mesangial cells were homogenized and lysated with cold RIPA lysis buffer (P0013C, Beyotime). Protein of 30 μ g per lane was loaded in 10% SDS-PAGE followed by transfer to nitrocellulose membranes. Blocked with 5% skim milk powder dissolved in PBS, the bolts were incubated with antibodies against overnight at 4°C at a dilution of 1:1000. The primary antibody used in the experiment were TLR4 (25) (Santa Cruz, sc-293072), CSE (CTH P-15) (Santa Cruz, sc-131905), phosph-Akt (Ser473, Santa Cruz, sc-293125), Akt (Santa Cruz, sc-5298), phosphor-PI3K (Santa Cruz, sc-130211), and PI 3-kinase C2 α Antibody (H-300, Santa Cruz, sc-67306). Secondary horseradish peroxidase-conjugated antibody was applied to the membrane for 1h at room temperature. The enhanced chemiluminescence western blotting detection system (Santa Cruz) was used to visualize immunoreactive proteins. A GeneGnome HR scanner was adopted to quantify the bolts of the membranes by GeneTools Software. Actin expression was obtained to quantify the relative protein expression level.

Statistical analysis

Data were expressed as means $\pm$ SEM. Two-tailed Student's t test was adopted for the compare of statistical significance between only two groups, while one-way analysis of variance (ANOVA) for among more than two groups followed by post hoc the Student-Newman-Keuls test. SPSS 16.0 (SPSS, Inc.) was used and less-than-0.05 P value was considered significant [23].

Results

High glucose inhibited the synthesis of endogenous H₂S and induced the overproliferation in mouse mesangial cells

First, we investigated the effect of glucose on cell viability on mouse mesangial cells incubated in 5mM (control), 20 mM, 25 mM and 30 mM glucose. It was found that high glucose incubation resulted in

significant overproliferation of the renal mesangial cells in mice, in a concentration-independent manner (Fig. 1A). Glucose of 25 mM induced the high increment in the cell viability. Then the endogenous H_2S synthesis was assessed in mouse mesangial cells cultured in high glucose. Western blot analysis detected significantly decreased expression of CSE in 20 mM, 25 mM and 30 mM glucose groups compared to control groups (Fig. 1C). Besides, the H_2S generation rate was significantly reduced in cells incubated in 25 mM and 30 mM glucose compared to control (Fig. 1B). At last, H_2S supplementation was conducted via NaHS administration. It was found that NaHS treatment significantly curbed the mesangial cell overproliferation.

TLR4 activation was found to be involved in the high glucose-induced mesangial cell overproliferation

Western blot showed that high glucose (25 mM) incubation significantly activated the expression of TLR4 (Fig. 1D). Downregulation of TLR4 by siRNA could reverse the overproliferation of mesangial cells in mice (Fig. 2C). Interestingly, silencing of CSE also significantly increased the TLR4 expression in mouse mesangial cells (Fig. 2B); NaHS treatment could significantly inhibit the TLR4 activation and curbed the cell overproliferation (Fig. 2B). Besides, TLR4 downregulation did not significantly alter the inhibition of the CSE expression induced by high glucose (Fig. 2A). However, TLR4 silencing could markedly abolish the cell overproliferation resulted from CSE downregulation. These results implied that TLR4 might be in the downstream of CSE pathway in the hyperglycemia-induced mesangial cell overproliferation.

Downregulation of TLR4 inhibited PI3K-Akt pathway and curbed the overproliferation of mouse mesangial cells

We then investigate the effect of TLR4 on PI3K-Akt pathway in mesangial cells. Western blot analysis showed that the phosphorylation of PI3K and Akt was significantly enhanced by hyperglycemia and reduced to normal level after TLR4 knock-down, although the total expression of PI3K and Akt remained unchanged (Fig. 3A and B). Inhibition of PI3K by LY294002 also significantly mitigated high-glucose-induced cell proliferation as was demonstrated in Fig. 3C.

Fig. 1. High glucose (HG) induced significant overproliferation of mouse mesangial cells, which could be partly attributed to endogenous H_2S synthesis deficiency or TLR4 activation. (A) HG induced significant overproliferation in mouse mesangial cells. Mannitol treatment of 25 mM did not significantly alter the viability in mouse mesangial cells. (Values were mean $\pm$ SEM; * P < 0.05, ** P < 0.01 vs. 5 mM glu; ### P < 0.01 vs. 25 mM glu; n = 4). (B) H_2S generation rate was significantly reduced by HG treatment of 25 mM and 30 mM, compared to 5 mM glu. (Values were mean $\pm$ SEM; * P < 0.05, ** P < 0.01 vs. 5 mM glu; n = 4). (C) CSE expression was significantly reduced by HG treatment in a concentration-independent manner. (Values were mean $\pm$ SEM; * P < 0.05, ** P < 0.01 vs. 5 mM glu; n = 4). (D) TLR4 expression was significantly reduced by HG treatment of 25 mM and 30 mM. (Values were mean $\pm$ SEM; * P < 0.05, ** P < 0.01 vs. 5 mM glu; n = 4).

Fig. 2. CSE inhibition-induced TLR4 activation was possibly involved in the mesangial cell overproliferation resulted by HG. (A) TLR4 suppression did not alter the CSE expression in mouse mesangial cells treated by high glucose. (Values were mean $\pm$ SEM; $**P < 0.01$ vs. 5 mM glu + con siR; $n = 4$). (B) Inhibition of CSE resulted in activation of TLR4, which was reversed by NaHS treatment. (Values were mean $\pm$ SEM; $*P < 0.05$ vs. con siR; $#P < 0.05$ vs. CSE siR; $n = 4$). (C) Silencing of TLR4 significantly reversed the cell overproliferation induced by HG. (Values were mean $\pm$ SEM; $*P < 0.05$, $**P < 0.01$ vs. 5 mM glu + con siR; $##P < 0.01$ vs. 25 mM glu + con siR; $n = 4$). (D) TLR4 suppression significantly curbed the mesangial cell overproliferation induced by CSE silencing. (Values were mean $\pm$ SEM; $**P < 0.01$ vs. 5 mM glu + con siR, $##P < 0.01$ vs. 25 mM glu + con siR; $n = 4$).

Fig. 3. Inhibition of PI3K pathway significantly suppressed HG-induced mesangial cell proliferation in a TLR4-dependent way. (A, B) HG led to significant increase in the expression of PI3K/Akt in mouse mesangial cells, which was down-modulated by TLR4 silencing. (Values were mean $\pm$ SEM; $**P < 0.01$ vs. 5 mM glu + con siR, $##P < 0.01$ vs. 25 mM glu + con siR; $n = 4$). (C) Inhibition of PI3K significantly curbed the mesangial cell overproliferation induced by HG treatment. (Values were mean $\pm$ SEM; $**P < 0.01$ vs. 5 mM glu; $##P < 0.01$ vs. 25 mM glu; $n = 4$).

Discussion

The main finding of the current paper was that diabetic renal lesions could be induced by the downregulation of hydrogen sulfide which was dependent of augmented TLR4 expression and reduced H_2S synthesis. In addition, TLR4-mediated PI3K-Akt pathway activation partially contributed to the pathophysiology of diabetic nephropathy.

For several centuries, hydrogen sulfide (H_2S) had been known to be highly toxic. However, more recently H_2S has been recognized as mediating a wide range of physiological effects. Nitric oxide (NO) is a gas with biological and regulatory properties and a very short half-life (few seconds), whose characters are shared by H_2S . Like NO, H_2S synthesis and bioavailability were vital to the physiological and pathophysiological processes. H_2S is a highly lipophilic molecule able to freely penetrate the membranes of cells of all types by diffusion and without the requirement for specialized membrane transporters [24]. Through its presence and enzymatic production, H_2S has gained attention for its signaling capabilities as important gasotransmitters like nitric oxide (NO) and carbon monoxide (CO) [4].

Accumulating evidence has shown that upregulation of H_2S exerts protective effect on diabetes-induced damages such as cardiac fibrosis, glomerular podocyte injury, wound healing impairment etc. [25-35]. Besides, a reduction in hydrogen sulfide (H_2S) has been suggested to play a vital role in diabetic vascular complications including diabetic nephropathy [29]. It was demonstrated that lower concentrations of H_2S were responsible for the development and progression of diabetic nephropathy [36]. H_2S generation in Type 2 diabetes with nephropathy is inversely associated with the CSE/CBS mRNA expression. A disturbed H_2S metabolism is present in diabetes associated with nephropathy [37]. Furthermore, H_2S was reported to have a neuroprotective effect against the cognitive diseases [38-40]. The proposed mechanisms underlying the protective effect of H_2S involved oxidative stress [25] and autophagy [41].

Diabetic nephropathy was a leading cause of end-stage kidney disease worldwide. Marked mesangial matrix expansion was characteristic as renal lesions in diabetic patients [42]. Not only mesangial cells, upregulated hydrogen sulfide could also attenuate high glucose-induced mouse podocyte (MPC) injury [30]. However, the exact mechanisms and pathways by which H_2S exerts its multitude of effects are not yet fully understood. Our results have demonstrated that overproliferation of mouse mesangial cells could be inhibited by H_2S upregulation through addition of an exogenous donor (NaHS) or overexpression of CSE/CBS.

TLRs, one family of pathogen-associated molecular patterns (PAMPs) receptors, are integral membrane glycoproteins with cytoplasmic tails, and play a role in the early recognition and host defense against pathogenic microorganisms [43, 44]. TLR-4 also recognizes other. TLR-4, like other TLRs, is able to recognize endotoxin (LPS) and microbial molecules (glycolipids, respiratory syncytial virus fusion protein and heat shock proteins) and endogenous damage-induced tissue molecules producing intracellular signals responsively [45, 46]. To note, hyperglycemia was shown to induce TLR4 expression. Pahwa et al. found that TLR4 mediate hyperglycemia induced macrovascular aortic endothelial cell inflammation and perturbation of the endothelial glycocalyx [47]. And high glucose could also induce and activate TLR4 in endothelial cells of diabetic retinopathy [48, 49]. The present study demonstrated that TLR4 knock-down curbed the abnormal proliferation of mesangial cells induced by hyperglycemia, which was in accordance with the others' reports.

Interesting, TLR4 was reported to be associated with H_2S synthesis and bioavailability. Tamizhselvi et al. [50] reported that H_2S may up-regulate the TLR4 pathway via substance P. In accordance, our *in-vitro* experiment showed that the downregulation of H_2S could markedly activate the TLR4 expression in the mouse mesangial cells. Vice versa, TLR4 was reported to upregulate CBS expression through NF- κ B activation in rat [51]. Zheng et al. found that compared with their wide-type counterparts, TLR4 knockout mice showed elevated expression of H_2S -synthesizing enzyme CSE, therefore abolishing the inflammation effect of lipopolysaccharide [52].

The phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt) signaling pathway was suppressed in diabetes [53, 54]. Our results showed that TLR4 interfering suppressed the PI3K/Akt pathway, mitigating the abnormal mesangial cell proliferation. The present study demonstrated that the effect of H_2S against hyperglycemia-induced mesangial cell proliferation might be associated with the down-regulation of the PI3K/Akt signaling pathway. More evidences had shown that PI3K/Akt signaling pathway participated the physiological effect of H_2S [55-57].

In summary, we found that HG inhibited the synthesis of endogenous H₂S and induced the overproliferation in mouse mesangial cells; TLR4 activation was involved in the HG-induced mesangial cell overproliferation; downregulation of TLR4 inhibited PI3K-Akt pathway and curbed the overproliferation of mouse mesangial cells. It was concluded that high glucose could induce mouse mesangial cell overproliferation via inhibition of hydrogen sulfide synthesis in a TLR-4-dependent manner.

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Disclosure Statement

None.

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