Exogenous H₂S Preventing PDC-E1 Translocation to Nucleus and Inhibiting Vascular Smooth Muscle Cell Proliferation in Diabetic State

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Research

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Abstract

Background

The proliferation of vascular smooth muscle cells (VSMCs) is the main cause of diabetic vascular complications. Hydrogen sulfide (H_2S), a gaseous molecule, is involved in modulating multiple physiological functions. H_2S could inhibit VSMCs proliferation induced by hyperglycemia and hyperlipidemia, however, the mechanisms are unclear.

Results

Our results showed that H_2S level was lower and expression of proliferative protein for PCNA and CyclinD1 was higher in db/db mice aorta and VSMC treated by glucose and palmitate, whereas, exogenous H_2S decreased PCNA and CyclinD1 expression. We found that mitochondrial pyruvate dehydrogenase complex-E1 (PDC-E1) was significantly translocated to the nucleus of VSMCs with the treatment of high glucose and palmitate, and it increased the level of acetyl-CoA and histone acetylation (H3K9Ac). Exogenous H_2S inhibited PDC-E1 translocation from mitochondria to nucleus, due to PDC-E1 being modified via S-sulfhydration. In addition, PDC-E1 was mutated at Cys101. Overexpression of PDC-E1 mutated at Cys101 enhanced histone acetylation (H3K9Ac) and VSMCs proliferation.

Conclusions

These findings suggested that H_2S regulated PDC-E1 S-sulfhydration at Cys101 to prevent its translocation from mitochondria to nucleus and inhibit VSMCs proliferation in diabetic conditions.

Introduction

Vascular complications, such as atherosclerosis, in diabetes mellitus (DM), increase patient morbidity and mortality [1]. As a main component of the artery wall, the proliferation of vascular smooth muscle cells (VSMCs) plays a pivotal role in the initiation and development of diabetic vascular complications [2]. Recent studies have confirmed that chronic hyperglycemia / high glucose (HG) enhancing reactive oxygen species (ROS) accelerates the progress of VSMCs proliferation [3]. However, the mechanisms of VSMCs proliferation could not be fully explained. Due to the complexity of pathogenic mechanisms, a new insight is required to explore hyperglycemia/high glucose (HG)-induced VSMCs proliferation. Increasing evidence demonstrated that epigenetic regulation of gene expression is essential for cell proliferation and differentiation to explore modifications of core histones by methylation, phosphorylation, and acetylation [4]. In metazoan cells, the biosynthesis of acetyl-coenzyme A (CoA) in different subcellular compartments such as in mitochondria and nucleus, has been affirmed. ATP-citrate lyase (ACL) have been reported to be present in the nucleus. ACL, as the main enzyme for nuclear acetyl-CoA generation in mammalian cells, utilizes mitochondrial citrate as its substrate [5]. Recent studies have demonstrated that the pyruvate dehydrogenase complex (PDC) might be translocated from mitochondria to the nucleus in response to mitochondrial stress or the stimulus of growth factor in tumor cells. PDC

catalyzes pyruvate to produce acetyl-CoA in mitochondria and nucleus [4]. However, whether PDC regulates histone acetylation is still unknown.

Hydrogen sulfide (H_2S), as an important gasotransmitter in the cardiovascular system, is involved in vascular relaxation and anti-oxidation. Our previous study revealed that H_2S sustained mitochondrial ATP production through regulating the key enzymes of the mitochondrial tricarboxylic acid (TCA) cycle [6]. The novel studies have demonstrated that H_2S covalently modifies cysteine residues on target proteins, which is named as S-sulfhydration. This modification could modulate protein structure and activity [7,8]. Our group has also found that exogenous H_2S inhibited VSMCs proliferation in diabetic conditions [9]. However, its mechanism is not still clear. The aim of the present study was to explore whether H_2S could regulate PDC translocation from mitochondria to nucleus to effect on histone acetylation to inhibit VSMCs proliferation.

Materials And Methods

Animals.

Leptin receptor-deficient (db/db) mice (8–10 weeks old, n=60) and wild-type C57BL/6 mice (n=40) were purchased from the Animal Model Institute of Nanjing (Nanjing, China). The animals were housed under diurnal lighting conditions and fed standard mouse chow and water throughout the study period. Half of the db/db mice were put in the NaHS treatment group and treated with NaHS (80 μ mol/kg) by intraperitoneal injection every 2 days for 12 weeks. All animal experiments were performed by the Guide for the Care and Use of Laboratory Animals published by the China National Institute of Health and approved by the Animal Care Committees of Harbin Medical University, China.

Cell Culture and treatments

Vascular smooth muscle cells of mouse aorta (VCMCs) were purchased from the Chinese Academy of Sciences Cell Bank. The media for cell lines was supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. VSMCs were maintained at 37 °C in a humidified chamber containing 5% CO_2 incubator. Two days after seeding, the cultured VSMCs were randomly divided into the following groups and treatments: Control group (glucose, 25 mM), High Glucose (HG, 40 mM) + Palmitate (Pal, 500 μ M), HG+Pal+NaHS (100 μ M), HG+Pal+PPG (10 nM, an inhibitor of CSE), HG+Pal+MitoTempo (2 μ M), HG+Pal+NAC (100 μ M), and HG+Pal+NaHS+ACLI (50 μ M SB204990, an inhibitor of ATP citrate lyase), HG+Pal+NaHS+PDHI (100 μ M CPI-613, an inhibitor of PDC-E1). Drugs were added in the cultured medium for 24 h. VSMCs treated with high glucose and palmitate classically mimic the cells in hyperglycemia and hyperlipidemia.

Cell count

Make cell suspension from the treated cells. Slowly drip from the edge of the counting plate to fill the gap between the counting plate and the cover sheet. Wait a while, the counting plate under a low power lens

(10×10 times) to observe and count.

Functional nuclear Isolation

Isolating nucleus was performed with the nuclei isolation kit: nuclei PURE prep from Sigma Aldrich. Briefly, VSMCs were washed with PBS and scraped from the plate in the presence of lysis buffer. VSMCs were carefully placed on top of a 1.8 M sucrose gradient and the resulting suspension was centrifuged at 30,000 g for 45 min in a precooled swinging bucket ultracentrifuge. Nuclei were collected as a white pellet at the bottom of the centrifuge and washed with nuclei storage buffer (provided with the kit). Purity of nuclei was assessed by immunoblot. For functional experiments, isolated nuclei were used immediately.

Mitochondria isolation

VSMCs were washed twice with ice-cold PBS, resuspended in lysis buffer (mmol/L: 20 Hepes/KOH, pH 7.5, 10 KCl, 1.5 MgCl₂, 1.0 sodium EDTA, 1.0 sodium EGTA, 1.0 dithiothreitol, 0.1 PMSF, and 250 sucrose), and then homogenized by a homogenizer in ice/water. After removing the nuclei and cell debris by centrifugation at 1000 g for 10 min at 4 °C, the supernatants were further centrifuged at 10000 g for 10 min at 4 °C. The resulting mitochondrial pellets were resuspended in lysis buffer. The supernatants and mitochondrial fractions were stored at -80 °C.

Detection of H₂S in VSMCs using H₂S probe 7-Azido-4-Methylcoumarin

The fluorescence reaction of the sulfate diester in VSMCs was tested using 7-azido-4 methyl coumarin (C-7Az, Sigma), which has been shown to selectively respond to H₂S [10]. VSMCs were incubated with 50 μmol/LC-7Az PBS for 30 minutes and then the cells were washed with PBS. The fluorescence response of C-7Az in VSMCs was detected using a fluorescence microscope (Olympus, XSZ-D2, Tokyo, Japan) excited by a 720 nm laser. The results indicate that excitation fluorescence imaging can be used to detect H₂S by the triggering fluorescence reaction of C-7Az.

Flow cytometric analysis of cell cycle

Take cells in the logarithmic growth phase, inoculate 24 well plates or 2 mL in 6 well plates at 1×10⁶ cells/mL with 1mL, perform the required treatment (such as adding HG+Pal, NaHS), stop the culture after a specific time, and proceed to the next experiment. Centrifuge at 800 rpm for 5 min, collect the cell pellet, discard the supernatant, wash twice with pre-chilled PBS, add pre-chilled 70% ethanol, and fix at 4 °C for more than 4 h. Centrifuge at 1500 rpm for 5 min, discard the supernatant, wash once with 3 mL of PBS, add 400 μL of CCAA solution (PI stain solution, green), 100 μL RNase A (100 ugs/mL), and incubate at 4 °C in the dark for 30 min. Flow analysis: It is detected by flow cytometry with standard procedures and generally counts 20,000 to 30,000 cells, and the results are analyzed by cell cycle fitting software ModFit.

Immunofluorescence staining

VSMCs were fixed in 4% paraformaldehyde for 30 minutes and then permeabilized with 0.5% Triton X-100 for 30 min. The coverslips were blocked with 5% BSA for 1 h at 37 °C. Cells were incubated with anti-PDC-E1 antibodies overnight at 4 °C and incubated with anti-rabbit IgG for 1 h. Analysis and photomicrography were performed using a fluorescence microscope.

Immunoprecipitation

Briefly, isolated mitochondria and nuclues were resuspended in PBS and diluted to a concentration of 1 mg/mL. After three freeze-thaw cycles, a total amount of 500 µg of protein was used per sample for immunoprecipitation. Sepharose beads were conjugated with the anti-HSP70 antibody (10 µg antibody/500 µg protein) and incubated with samples overnight at 4 °C with gentle rotation. Following collecting of beads using centrifuge and three washing steps, precipitates were subjected to Western blotting analyses for detection of potential interacting proteins.

Mitochondrial and cellular ROS level analysis

Mitochondrial ROS and cellular ROS level analysis. Mitochondrial ROS production was measured using MitoSOX Red mitochondrial superoxide indicator (Invitrogen). VSMCs were treated with control, HG, NaHS and MitoTempo for 24 h. Cells were loaded with 5 µM MitoSOX Red at 37 °C for 30 min. Red fluorescence was measured at 583 nm following excitation at 488 nm using a fluorescence microscope. Intracellular ROS levels were examined using the DCFH-DA staining method based on the conversion of non-fluorescent DCFH-DA to the highly fluorescent DCF upon intracellular oxidation by ROS. VSMCs were seeded on coverslips and incubated (45 min, 37 °C, in the dark) in serum-free media containing DCFH-DA (10 µM) in the presence of control, HG, NaHS, NAC. After incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a spectrofluorometer with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DA in the absence of cells) was corrected by the inclusion of parallel blanks

Western blotting analysis

All cytoplasmic and nuclear were quantified by using the BCA Protein Assay kit (Beyotime, China). Samples were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose filter membranes. The antibodies used for western blot analysis included, anti-CSE (42 kDa, 1:1000), anti-CBS (61 kDa, 1:1000), anti-β-actin (42 kDa, 1:1000), anti-MMP2 (72 kDa, 1:1000), anti-MMP9 (67-92 kDa, 1:1000), anti-OPN (66 kDa, 1:1000), anti-α-smooth muscle actin (42 kDa, 1:1000), anti-CyclinD1 (36 kDa, 1:1000), anti-PCNA (36 kDa, 1:1000), anti-PDC-E1 (43 kDa, 1:1000), anti-H3 (17 kDa, 1:1000), anti-H3K9 (17 kDa, 1:1000), anti-H3K18 (17 kDa, 1:1000), anti-SOD (15 kDa, 1:1000), anti-CAT (55 kDa, 1:1000), anti-HSP70 (70 kDa, 1:1000), anti-COX IV (16 kDa, 1:1000), anti-Lamin B1 (66 kDa, 1:1000) antibody incubation, respectively leave at 4 °C overnight. All antibodies were from Proteintech Group, Inc, USA. Protein complexes were incubated for 1 hour at room temperature with an anti-mouse / anti-rabbit antibody. Densitometry was conducted with image processing and analysis program AlphaView SA and the data were expressed as relative units.

Point mutation of PDC-E1

Adenoviruses expressing GFP and PDC-E1-GFP were purchased from Cyagen Biosciences Inc. (Guangzhou, China). Mutation of the cysteine site at position **101** in PDC-E1 to alanine. The adenovirus was added directly to cells, and after 24 h for transfection, a new fresh medium was added. The cells were treated in different conditions 24 h after transfection, and the related proteins were detected by western blotting.

Pyruvate dehydrogenase activity assay

The PDH activity of the nuclear lysates was measured with a colorimetric PDH activity assay kit (GENMED, Shanghai, China) according to the manufacturer's instructions, and absorbance at 450 nm was measured kinetically for approximately 30 min at 37 °C after addition of PDH developer and PDH substrate. Sample blank values (without PDH substrate) were subtracted from the sample readings and PDH activity (nmole NADH/min) was normalized to mg of protein.

Acetyl-coenzyme A analysis

Acetyl-coenzyme A was measured according to the manufacturer's protocol (acetyl-coenzyme A assay kit, Sigma). All samples and standards should be run in duplicate. Add 10 µL of samples into duplicate wells of a 96 well plate. Bring samples to a final volume of 50 µL with Acetyl-CoA Assay Buffer. Include a blank sample for each sample by omitting the Conversion Enzyme in the Reaction Mix. To correct for background created by free Coenzyme A and succinyl-CoA, add 10 µL of Acetyl CoA Quencher to each sample, standard, and sample blank well. Incubate at room temperature for 5 min. Add 2 µL of Quench Remover, mix well, and incubate an additional 5 min. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 10 min at 37 °C. Protect the plate from light during the incubation. Measure fluorescence intensity ($\lambda_{ex} = 535 / \lambda_{em} = 587 \text{ nm}$) .

S-sulphydration Assay

S-sulphydration was performed as described previously [7]. VSMCs were homogenized in HEN buffer solution containing 250 mmol / L Hepes-NaOH, 1 mmol / L EDTA, 0.1 mmol / L neocuproine and 100 mmol / L deferoxamine to adjust the pH to 7.7. Cell lysate contained HEN buffer, 0.5% CHAP, 0.1% SDS, 20 mmol / L methyl methanethiosulfonate, 10 µg / mL leupetin, 5 µg / mL aprotinin and 1 mM protease inhibitor PMSF. Quantitative analysis was after the lysis of cells. VSMCs were added to the blocking buffer (HEN buffer with 2.5% SDS and 20 mM methyl methanethiosulfonate) at 50 °C for 60 min with frequent vortexing. Added 4 volumes of cold acetone to each 15 mL centrifuge tube and incubated at -20 °C for 1 hour. Then, centrifuged at 2000 g at 4 °C for 10 minutes. Removed the cold acetone. The cells were resuspended in 90 µL of HEN buffer (containing 1% SDS) and transferred to a new 1.5 mL EP tube. 4 mM N-[6-(biotinamido) hexyl]-3-(2-pyridyldithio) propionamide (biotin-HPDP stop solution) was added and incubated at 25 °C for 1 hour. After incubation for 3 h at 25 °C, biotinylated proteins were precipitated

by streptavidin-agarose beads, which were later washed with HEN buffer. The biotinylated proteins were eluted by SDS-PAGE and subjected to western blotting analysis using antibodies against PDC-E1.

Measurement of intracellular levels of polysulfide

Intracellular production of polysulfide was monitored using a newly developed fluorescent probe, SSP4, with slight modifications [11]. Briefly, VSMCs were loaded with $50 \mu\text{mol}\cdot\text{L}^{-1}$ SSP4 in a serum-free DMEM medium containing 0.003% Cremophor EL for 15 min at 37 °C in the dark. After being washed, SSP4 was detected using the fluorescence microscope (Olympus, XSZ-D2, Tokyo, Japan).

Statistical analysis

Statistical analysis of data was performed using Graphpad Prism. Two groups were compared using unpaired Student's *t*-tests and multiple groups using One-way ANOVA followed by multiple comparison tests. Statistical significance was detected at $P < 0.05$ level. Results were expressed as Mean $\pm$ SEM of multiple experiments.

Results

1. CSE expression and H₂S level in vascular smooth muscle cells in diabetic condition

In mammalian cells, the generation of H₂S is mainly dependent on cystathionine- γ - lyase (CSE) and cystathionine- β -synthase (CBS). The distribution of two enzymes for H₂S production has tissue specificity. CBS is the main enzyme for H₂S generation in nervous system. CSE is predominant enzyme for H₂S production in cardiovascular system. However, recent study has found that CBS also generates H₂S in cardiovascular system [12]. Our previous study has shown that the expression of CSE, an enzyme to generate H₂S, and H₂S level were decreased in the mesenteric artery in STZ-induced type 1 diabetes [13]. In this study, we chose db/db mice as the type 2 diabetes animal model. The expression of CSE in the thoracic artery was detected. The protein level of CSE was lower in db/db mice, compared to control mice. CSE protein level was recovered with the treatment of NaHS (db/db+NaHS) mice (Fig. 1A). The expression of CBS was similar among different groups (SFig. 1).

Vascular smooth muscle cells of mice aorta were cultured with high glucose (HG, 40 mM) and Palmitate (Pal, 500 μM) for 24 h to mimic hyperglycemia and hyperlipidemia of type 2 diabetes. Similarly, CSE expression was significantly decreased with the treatment of HG and Pal while exogenous H₂S recovered its expression. DL-propargylglycine (PPG, 10 nM) is an inhibitor of CSE. PPG treatment decreased the protein level of CSE (Fig. 1B). To explain the decrease of CSE protein level in HG+Pal group, we tested ubiquitylation level of CSE with immunoprecipitation. MG132 is an inhibitor of 26S proteasome. Our data showed that the ubiquitylation level of CSE was significantly increased in HG+Pal group, compared to control and NaHS-treated and MG132 groups (SFig. 2). H₂S fluorescence probe, 7-azido-4-methyl coumarin (C-7Az) was used to test H₂S content in VSMCs. The H₂S level in HG+Pal and PPG groups was

significantly lowered than that of control and exogenous H₂S groups (Fig. 1C). These results indicated that endogenous H₂S production in VSMCs decreased, due to CSE degradation, in hyperglycemia and hyperlipidemia conditions.

2. Effects of H₂S on proliferation and migration in VSMCs induced by high glucose and palmitate

We examined whether H₂S level regulated VSMCs proliferation. Proliferation assay showed that high glucose and palmitate could induce cell growth, whereas, exogenous H₂S blocked cell growth (Fig. 2A,B). H₂S significantly retarded cell progression at the G1 phase and the number of cells in the S phase decreased (Fig. 2C, Table1). Next, the protein level of CyclinD1, G1/S-checkpoint protein, and PCNA, which is responsible for governing the G1/S transition, was assessed. Our results showed that the protein level of CyclinD1 and PCNA was significantly increased in the thoracic arteries of db/db mice and VSMCs treated by high glucose and palmitate, compared to that with the treatment of exogenous H₂S (Fig. 2D,E). Additionally, we found that HG and Palmitate significantly enhanced VSMCs migration rates of two and three folds for 6, 12, and 24 h, respectively, compared to control groups, using the scratch assay. PPG also increased VSMCs migration rates. However, exogenous H₂S inhibited VSMCs migration rates (SFig. 3).

Hyper-proliferated SMCs induced a phenotypic switch of VSMCs. The biomarkers, α -SMA which represents the contractile type of SMCs, OPN and MMP2/9, which represent the synthetic phenotype, were examined. Our results showed that α -SMA expression in db/db mice and HG and Palmitate-stimulated VSMCs was significantly attenuated compared to that the treatment of exogenous H₂S. By contrast, the expression of OPN and MMP2/9 significantly increased in the thoracic arteries of db/db mice and VSMCs treated by high glucose and palmitate. Pre-treatment of PPG increased OPN, MMP2 and MMP9 expression (Fig. 2F,G). Taken together, these results validated that the decrease of H₂S promoted VSMCs proliferation in the diabetic state.

3. H₂S inhibiting PDC-E1 translocation from mitochondria to nucleus under high glucose and palmitate state.

PDC is composed of three catalytic enzymes: pyruvate dihydrolipoamide (E1), dihydrolipoamide transacetylase (E2), and dihydrolipoamide dehydrogenase (E3). PDC-E1 is responsible for catalyzing pyruvate to acetyl-CoA. Some study has demonstrated that PDC-E1 translocated from mitochondria to the nucleus under mitochondrial stress [14]. We first examined the nuclear presence of PDC-E1 in thoracic aorta and VSMCs under hyperglycemia and hyperlipidemia. We extracted the nucleus from VSMCs, Western blot analyzed that the protein level of PDC-E1 in db/db mice and VAMCs under high glucose and palmitate condition was higher than that in control and with the treatment of exogenous H₂S group (Fig. 3A,B). We also observed that PDC-E1 evidently localized within the nucleus (marked by DAPI) with the administration of high glucose and palmitate by immunofluorescence microscopy (Fig. 3C). To further confirm that PDC-E1 within the nucleus derived from mitochondria, we extracted the mitochondria and then measured its protein level. Our results found that the protein level of PDC-E1 in mitochondria of the

HG+Pal group was lower than that in control and with the treatment of NaHS groups (Fig. 3D). These results suggested that high glucose and palmitate promoted the translocation of PDC-E1 from mitochondria to the nucleus, however, exogenous H₂S could reduce PDC-E1 translocation.

To evaluate whether nuclear PDC-E1 was functional, we separated nuclei from VSMCs by high sucrose gradient centrifuged methods, avoiding of mitochondrial contamination. The activities of PDC-E1 in nuclei were tested, respectively. Our results found that the activity of PDC-E1 in HG+Pal group significantly increased, compared to control and with the treatment of NaHS (Fig. 3E). We then detected the content of acetyl-CoA in nuclei and found it in HG+Pal group was obviously higher than that in control and exogenous H₂S group (Fig. 3F). These results suggested that PDC-E1 translocation from mitochondria to nucleus with the treatment of high glucose and palmitate could be involved in acetyl-CoA production.

4. Nuclear PDC-E1 promoting Histone acetylation and cell proliferation

We have demonstrated that nuclear PDC-E1 generated acetyl-CoA, and then confirmed whether acetyl-CoA could modify histone. We first extracted the nuclei from thoracic aorta and VSMCs and examined the acetylation level. Our results showed that the acetylation level in db/db mice and administration of high glucose and palmitate was higher than that in control and treated as NaHS (Fig. 4A,B). Next, we detected the specific acetylation site of H3K9, which is involved in regulating VSMCs proliferative gene expression. Our results showed that after exposure to high glucose and palmitate, the acetylation level of H3K9 had increased compared to control and treated with NaHS groups (Fig. 4C,D). ATP-citrate lyase (ACL) is acetyl-CoA generating enzyme localized in cytosol and nuclei, which provides acetyl group for histone acetylation. We used ACLI (ACL inhibitor, SB204990, 50 μ M) and PDC-E1 inhibitor (PDHI, CPI-613, 100 μ M) to study the relative importance of these two enzymes in nuclear acetylation. Our data showed that PDHI (Fig. 4E) and ACLI (SFig. 4) obviously reduced the expression of Cyclin D1 and PCNA in VSMCs nucleus under diabetic condition. These results demonstrated that exogenous H₂S decreased the acetylation level of H3K9 via inhibiting PDC-E1 translocation from mitochondria to the nucleus.

5. mtHSP70 assisting PDC-E1 nuclear translocation from mitochondria

Evidence shows that mitochondria adapt to stress inducing by increased ROS level, which leads to the enhanced expression of heat shock proteins and mitochondrial transporters, promoting the communication with the nucleus [15]. We examined whether high glucose and palmitate can induce oxidative stress. We measured intracellular and mitochondrial reactive oxygen species (ROS) with fluorescent probe DCFH and MitoSOX. We found that quantification of DCFH and MitoSOX fluorescent intensity showed similar elevation with administration of high glucose and palmitate, while exogenous NaHS, NAC and MitoTempo significantly attenuated ROS levels in intracellular and mitochondria (Fig. 5A,B). MitoTempo is a suppressor of ROS production in mitochondria. The expression of mitochondrial catalase (Mito-CAT) and manganese-dependent superoxide dismutase (Mn-SOD) were tested to further examine the role of exogenous H₂S on ROS production in VSCMs treated with HG and palmitate. The

results showed that the expression of Mito-CAT and Mn-SOD, which was downregulated in HG+pal group, was upregulated by NaHS treatment (Fig. 5C). Our data reinforced the concept that exogenous H₂S attenuated oxidative stress in VSMCs.

Next, to investigate whether mitochondrial chaperone is involved in PDC-E1 translocation. We chose mtHSP70 (mitochondrial heat shock protein 70) based on previous studies that HSP70 took part in the nuclear transportation of several mitochondrial proteins [16,17]. We extracted mitochondria and Western blot assay found that the protein level of mtHSP70 was significantly higher in HG+pal group, compared to control, exogenous H₂S and MitoTempo groups (Fig. 5D). We also confirmed that expression of mtHSP70 in nuclear also obviously increased in HG+pal group (Fig. 5E). We performed immunoprecipitation to detect interaction between mtHSP70 and PDC-E1. Our results suggested that mtHSP70 may bind to PDC-E1 in nucleus and mitochondria (Fig. 5F,G). These results demonstrated that mtHSP70 assisted PDC-E1 translocation induced by mitochondria oxidative stress.

6. Exogenous H₂S regulating PDC-E1 S-sulfhydration to inhibit VSMCs proliferation PDC-E1.

In Fig. 3E, our results demonstrated that exogenous H₂S decreased the activity of PDC-E1. To further study how exogenous H₂S inhibiting PDC-E1 activity, we detected PDC-E1 S-sulfhydration. H₂S modifying specific cysteine residues of proteins, which is referred to S-sulfhydration, has been extensively studied. Protein S-sulfhydration as a kind of post-translational modification is involved in the alterations of protein structure and activity. SSP4, as a fluorescent probe, was used to detect the production of S-sulfhydration. The S-sulfhydration group, was obviously observed in the treatment of exogenous H₂S. Dithiothreitol (DTT, 1 mM), as an inhibitor of S-sulfhydration formation, reduced the effect of NaHS on S-sulfhydration formation (Fig. 6A). Furthermore, a biotin-switch assay was also used to measure protein modification of S-sulfhydration. Our results demonstrated that NaHS evidently enhanced PDC-E1 S-sulfhydration formation, whereas, DTT abolished the S-sulfhydration level of PDC-E1 (Fig. 6B). We used bioinformatics methods to analyze and predict the structure of the active center of PDC-E1. PDC-E1 consists of 390 amino acids and contains 11 cysteine residues. Based on bioinformatics analysis, the cysteine Cys101 of PDC-E1 in the active center was mutated to alanine (SFig. 5). Mutant of PDC-E1-Cys101 (PDC-E1m) overexpression plasmid was constructed. After PDC-E1m overexpression, H₂S could not reduce the expression of CyclinD1 and PCNA between high glucose and palmitate group and exogenous H₂S treatment group (Fig. 6C). Moreover, after PDC-E1m overexpression, the acetylation level of H3K9 had increased in HG+Pal and exogenous H₂S groups (Fig. 6D). Taken together, these results indicated that H₂S could inhibit VSMCs proliferation via elevating PDC-E1 S-sulfhydration modification and reducing PDC-E1 translocation .

Discussion

In our study, we demonstrated that PDC-E1 was present and functional in the nucleus in VSMCs under high glucose and palmitate condition. Nucleus PDC-E1 produced acetyl-CoA used for histone acetylation

to promote VSMCs proliferation. Exogenous H₂S inhibited PDC-E1 translocation from mitochondria to the nucleus via PDC-E1 S-sulphydration to decrease VSMCs proliferation.

Growing evidence has confirmed that VSMCs proliferation is characteristics in type 2 diabetes patients. When VSMCs proliferation occurs, VSMCs switch from contractile to synthetic phenotype. The cell cycle associated genes are overexpressed. In our study, the expression of PCNA and cyclin D1 was significantly increased under high glucose and palmitate condition. Some studies have revealed that when stress reaction happens, such as oxygen deficiency or high metabolic state, they induce the alteration of the post-translational modifications (PTMs) of histone [18,19]. PTMs have been considered as key contributors to control targeted gene expression in both physiological and pathophysiological conditions. For example, histone acetylation via histone acetyltransferase contributes to active transcription via rendering gene promoters more accessible to the transcription machinery [20]. Evidence demonstrates the emergence of metabolic enzymes as crucial modulators, such as ATP-citrate lyase and PDC-E1, are involved in cell proliferation. PDC-E1 is mitochondrial complex, as a gatekeeper to regulate pyruvate flux from the cytosol to mitochondria, which couples glycolysis to OXPHOS [21]. Relocalization of PDC-E1 has been observed in cancer cells that were serum-starved or stimulated by epidermal growth factor or a mitochondrial respiration inhibitor (rotenone) [22]. PDC-E1 converts mitochondrial pyruvate into acetyl-CoA. Thus, the concentration of acetyl-CoA in mitochondria can be 20–30 fold greater than that in the cytoplasm and nucleus because acetyl-CoA is membrane impermeable [23]. Therefore, PDC-E1 is necessary to translocate to the nuclei to generate acetyl-CoA from mitochondria. We confirmed that mitochondrial oxidative stress promoted PDC-E1 translocation to nuclei. In our study, we demonstrated that the activity of PDC-E1 and the acetyl-CoA content in the nucleus increased in the HG+Pal group. Exogenous H₂S reduced PDC-E1 activity and the acetyl-CoA content in the nucleus. H3K9 acetylation level was significantly increased were upregulated under hyperglycemia and hyperlipidemia condition, due to PDC-E1 translocation; however, exogenous H₂S reduced H3K9 acetylation level. These findings indicated that acetyl-CoA, as an important substrate for histone acetylation can be generated in the nucleus from pyruvate, which is dependant on PDC-E1 translocation in the nucleus.

Hydrogen Sulfide plays a crucial role in physiology and pathophysiology in cardiovascular systems. Recent studies showed that the persulfidation or S-sulphydration of reactive cysteines (i.e., Cys-SSH) contributes to cellular redox homeostasis. Persulfidation or S-sulphydration are generated by the transsulfuration pathway which catabolizes cysteine and cystathionine to generate hydrogen sulfide (H₂S) and H₂S-related sulfane sulfur compounds (referred to as H₂Sn) [24,25]. This pathway is of particular importance for the vascular system as CSE [26]. Emerging data suggest that hydropersulfide moiety (-SSH) in the active cysteine residues of target proteins can mediate cellular functions. In our study, we demonstrated that exogenous H₂S can modify PDC-E1 via S-sulphydration, which inhibited its activity and its translocation. Administration of DTT, a blocker of S-sulphydration, decreased the S-sulphydration level of PDC-E1. We overexpressed PDC-E1 in which Cys101 was mutated to Ala and demonstrated that treatment with exogenous H₂S decreased PDC-E1 S-sulphydration level and increased PDC-E1 translocation to the nuclei. This finding suggested that S-sulphydration of PDC-E1 at Cys101

might be one of the detailed mechanisms by which H₂S reduced PDC-E1 translocation to the nuclei and histone acetylation and inhibits VSMCs proliferation (Fig. 7).

In this study, it is noteworthy that PDC-E1, a key mitochondrial TCA cycle-related enzyme, translocated to the nuclei and that the relationship between this change in metabolic enzyme localization and waves of transcriptional activity has been confirmed. This study has provided evidence that H₂S increased PDC-E1 S-sulfhydration at Cys101 to regulate PDC-E1 translocation from mitochondria to the nucleus, thereby preventing VSMCs proliferation under hyperglycemia and hyperlipidemia condition. H₂S may be a useful therapeutic strategy in cardiovascular diseases in the future.

Abbreviation

Acetyl-CoA : [acetyl coenzymeA](#);

ACL : ATP citrate lyase;

C-7Az : 7-Azido-4-Methylcoumarin;

CBS : cystathionine β-synthase;

CSE : cystathionine γ-lyase;

DTT : dithiothreitol;

H₂S : hydrogen sulfide;

HG : high glucose;

NAC : N-acetyl-L-cysteine;

NaHS : Sodium hydrosulfide;

Pal : Palmitate;

PCNA : proliferating cell nuclear antigen;

PDC-E1 : pyruvate dehydrogenase complex E1;

PPG : poly propylene glycol;

ROS : reactive oxygen species;

VSMCs : vascular smooth muscle cells

Declarations

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Consent for publication

Not applicable

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All data generated during this study are included in this published article.

Competing interests

The authors declare that they have no conflict of interests.

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Figures

Figure 1

Exogenous H₂S affects CSE expression and H₂S content. (A) Quantification of CSE expression in tissues was carried out by western blotting. (n = 4) (B) VSMC infected with HG (40 mM) + palmitate (Pal, 400 μM), HG+Pal+NaHS (100 μM), HG+Pal+PPG (10 μM, an irreversible competitive CSE inhibitor) for 24 h. The CSE protein expression level was determined by western blotting. (n = 5) (C) 7-azido-4-methyl

coumarin probe staining was used to test H₂S level in VSMCs. (n = 4) Values are presented as the mean $\pm$ S.D. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2

Effects of exogenous H₂S on proliferation and phenotypic changes. (A) VSMC infected with HG (40 mM) + palmitate (Pal, 400 μ M), HG+Pal+NaHS (100 μ M), HG+Pal+PPG (10 μ M, an irreversible competitive CSE inhibitor). Cell counting method to detect the number of cells after 24 h treatment. (n = 4) (B) CCK8 detected the effects of different drug treatments on cell viability. (n = 6) (C) Flow cytometry method to detect the cell cycle of VSMCs. (n = 3) (D) PCNA and CyclinD1 expression levels in aorta from the control mice, db/db mice, and db/db mice treated with NaHS. (n = 4) (E) PCNA and CyclinD1 expression levels in VSMCs treated with HG (40 mM) + palmitate (Pal, 400 μ M), HG+Pal+NaHS (100 μ M), HG+Pal+PPG (10 μ M, an irreversible competitive CSE inhibitor). (n = 5) (F and G) The protein expression levels of α -SMA, MMP2, MMP9 and OPN were determined using western blotting analyses in tissues (F) and in VSMCs (G). (n = 4) Values are presented as the mean $\pm$ S.D. *P < 0.05, **P < 0.01.

Figure 3

H₂S inhibiting PDC-E1 translocation from mitochondria to nucleus under high glucose and palmitate state. (A and B) The nuclei in tissues (A) and cells (B) were extracted separately to detect the protein expression of PDC-E1 in the nuclei. (n = 4) (C) Immunofluorescence method to detect the nuclear localization of PDC-E1. (D) The levels of PDC-E1 in the mitochondria of VSMCs. (n = 5) (E) The activity of

PDC-E1 in the nuclei of VSMCs. (n = 4) (F) Detection of acetyl-CoA content in VSMCs. (n = 4) Values are presented as the mean $\pm$ S.D. *P < 0.05, **P < 0.01.

Figure 4

Nuclear PDC-E1 promoting Histone acetylation and cell proliferation (A and B) Relative acetylation levels in the nuclei of the thoracic aorta (A) and VSMCs (B). (C) The levels of H3 and H3K9ac in the nuclei of the thoracic aorta. (n = 4) (D) The levels of H3, H3K9ac, and H3K18ac in the nuclei of VSMCs. (n = 4) (E) PDC

inhibitor (PDHI, 100 μ M) inhibited the proliferation of VSMCs. (n = 3) Values are presented as the mean $\pm$ S.D. *P < 0.05.

Figure 5

Exogenous H2S inhibits VSMC oxidative stress. (A)The whole ROS level was measured by DCFH (green fluorescence), and (B) the mitochondrial ROS was measured by MitoSOX (red fluorescence). The mean fluorescence intensity was measured. (C) The mitochondrial Mito-CAT and Mn-SOD levels were examined by western blotting. (n = 4) (D) The mitochondria of VSMCs were extracted, and the protein expression of mtHSP70 between each group was detected by Western blotting. (n = 4) (E) VSMCs nuclear protein was extracted, and the protein expression of mtHSP70 between each group was detected by Western blotting. (n = 4) (F) The mitochondria of VSMCs were immunoprecipitated with anti-mtHSP70 antibody and then immunoblotted with antibodies specific for PDC-E1. (G) The nucleus of VSMCs were immunoprecipitated with anti-mtHSP70 antibody and then immunoblotted with antibodies specific for PDC-E1. Values are presented as the mean $\pm$ S.D. *P < 0.05.

Figure 6

Exogenous H₂S regulating PDC-E1 S-sulfhydration to promote PDC-E1 translocation. (A) The polysulfidation level was measured with a fluorescent probe, SSP4, in VSMCs. Scale bar = 100 μ m. DTT (1 mM, 30 min, an inhibitor of disulfide bond). (B) Quantification of polysulfidation in VSMCs was carried out by western blotting. (C) The mutation of cysteine 101 to alanine of PDC-E1 (PDC-E1m) and the vector control were transfected into VSMCs for 6 hours and then treated with HG (40 μ M), palmitate (Pal, 500 μ M) in the presence or absence of NaHS (100 μ M) for 24 hours. The expressions of PDC-E1, CyclinD1, PCNA were detected by western blotting. (n = 5) (D) The expressions of H3, H3K9ac were detected by western blotting after mutation. (n = 5) Values are presented as the mean $\pm$ S.D. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

Exogenous H₂S regulating PDC-E1 translocation from mitochondria to nucleus.

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