SUPPLEMENTAL MATERIAL

Restoration of Defective Hydrogen Sulfide Production in Bone Marrow Cells from Diabetic Mice Rescues Their Impaired Tissue Reparative Function

Zhongjian Cheng, PhD[1]; Venkata Naga Srikanth Garikipati, PhD[1]; Emily Nickoloff, BS[1]; Chunlin Wang, BS[1]; David J Polhemus, BA[3]; Jibin Zhou, PhD[1]; Cynthia Benedict, PhD[1]; Mohsin Khan, PhD[1]; Suresh K Verma, PhD[1]; Joseph E. Rabinowitz, PhD[1,2]; David Lefer, PhD[3]; Raj Kishore, PhD[1,2#]

Author Affiliations: [1]From Center for Translational Medicine (ZJ.C, V.N.S.G, E.N, CL.W, JB.Z, C.B, M.K, S.K.V, J.R, R.K), [2]Department of Pharmacology (J.R, R.K), Lewis Katz School of Medicine, Temple University, 3500 Broad Street, Philadelphia, PA, 19140, U.S.A; [3]Department of Pharmacology and Experimental Therapeutics and Cardiovascular Center of Excellence (D.J.P, D.L), Louisiana State University Health Sciences Center, New Orleans, LA,70112 ,U.S.A

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[#]Address correspondence to:

Raj Kishore, PhD, Department of Pharmacology, Center for Translational Medicine, Lewis Katz School of Medicine, Temple University, 3500 Broad Street, Philadelphia, PA, 19140, U.S.A.

Phone: 215-707-2523

Fax: + 215-707-9890

E-mail: raj.kishore@temple.edu

Supplemental Materials and Methods

Mice

Twelve- to 14-week-old, male, db/db mice lacking the gene encoding for leptin receptor (Type II diabetic animal model) and their control nondiabetic db/+ mice were obtained from the Jackson Laboratories (Bar Harbor, ME). All animal experiments were approved by the Institutional Animal Care and Use Committee of Temple University.

Hind limb ischemia model

Induction of hind limb ischemia was performed in non-diabetic db/+ and diabetic db/db mice as described previously.[1] Briefly, mice were anesthetized by intraperitoneal injection of Avertin (i.p., 125 mg/kg) and the left femoral artery was ligated distal to the origin of the deep femoral artery and proximal to the popliteal artery. The left femoral artery was ligated proximal to the deep femoral artery and distal to saphenous artery, and the femoral artery was completely removed between the two ligatures avoiding injury of the femoral vein and nerve to preclude influence of inflammation and edema on arteriogenesis and angiogenesis. Postoperative analgesia was provided with buprenorphine (0.05 mg/kg).

Animal groups

Six animal groups were included: 1) db/+ mice with HLI (db/+ HLI); 2) db/db mice with HLI (db/db HLI); 3) db/db mice with HLI and local intramuscular injection of GFP-labeled diabetic BMCs (db/db HLI+BMC); 4) db/db mice with HLI and administration of DATS (2 mg/mouse/day) starting on the first day post-ligation for 3 weeks (db/db HLI+DATS); 5) db/db mice with HLI and local intramuscular injection of GFP-labeled diabetic BMCs plus administration of DATS (db/db HLI+BMC+DATS); 6) db/db mice with HLI and local

intramuscular injection of RFP-tagged CSE lentivirus-transfected diabetic BMC (db/db HLI+CSE-BMC).

Laser Doppler perfusion imaging of hind limb blood flow and sample collection

Blood flow measurements in each mouse's hind limbs were performed on 37^{o}C heated pads before and immediately after surgery, and on pre- (day 0) and post-ligation days 7, 14 and 21 using a laser Doppler perfusion imager (model LDI2-IR, Moor Instruments, Wilmington, DE) as previously described.[1] At each time point, an average of 4 measurements per animal was made on anesthetized (Avertin, i.p., 125 mg/kg) mice. To avoid the influence of light and temperature, the results were expressed as a ratio of perfusion in the left (ischemic) vs. right (non-ischemic) hind limb.[1] Perfusion was evaluated on the basis of colored histogram pixels and normalized for the limb surface analyzed. Laser Doppler blood flow data were expressed as the ratio of ischemic-limb to normal-limb blood flow.

At the end of each experiment (21 days post-ligation), mice were euthanized under anesthesia and gastrocnemius and medial thigh muscles were then harvested, embedded in paraffin for immunohistochemical analysis.

Isolation of BMCs

BMCs were isolated from bone marrow of 12- to 14-week-old, male db/+ or db/db mice as previously described.[2, 3] In brief, bone marrow-derived mononuclear cells were isolated from mice by density-gradient centrifugation with Histopaque-1083 (Sigma) and were macrophage-depleted by allowing attachment to uncoated plates for 1 hr. The unattached cells were removed and plated on culture dishes coated with 5 μg/ml human fibronectin (Sigma) and then cultured in endothelial cell basal medium-2 (EBM-2, CC-3156, LONZA) containing EBM-2MV bullet kit medium (CC-4176, LONZA) without hydrocortisone and 10% FBS. BMCs were cultured at 37^{o}C with 5% CO_2 in a humidified atmosphere. After 4 days in culture, non-adherent

cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7. Then the BMCs were used for further treatment and analysis.

GFP and RFP-tagged CSE transfection in BMCs

For labeling GFP and overexpression of RFP-labeled CSE in BMCs, the cells isolated from db/db mice were cultured in 6-well plates and grown to 80% confluence, then transduced with GFP-lentivirus (1 MOI, LV006, ABM) or RFP-labeled CSE lentivirus (1 MOI, LVP526776, ABM) for 48 hrs. To increase efficiency of lentivirus transfection, polybrene (8 μg/ml) was added together with the lentiviruses.

Hind limb intramuscular transplantation of BMCs

After femoral artery ligation, BMCs and CSE-BMCs were injected intramuscularly ($5x10^5$ cells in 100 μl PBS, at five injection sites, three sites in medial thigh and two sites in gastrocnemius muscles, respectively. 20 μl/per site) with a 27G needle as previously described.[4]

Immunohistochemistry and histology

Sections (5 μm thickness) were heated with citrate buffer at 5°C for 40 minutes for antigen retrieval. After blocking with 5% BSA in phosphate-buffered saline (PBS), sections were incubated overnight at 4°C with rabbit monoclonal antibody against CD31 (1:100, BD553370, BD), rat monoclonal antibody against CD68 (1:100, Ab53444, Abcam), rabbit monoclonal antibody against smooth muscle actin (SMA, 1:100, ab32575, Abcam). For every section, a negative control without primary antibody was processed simultaneously. After 15 minutes of washing in PBS, secondary antibodies were added for 1 hour at room temperature. Cell nuclei were counterstained with DAPI (Molecular Probes). CD31 and SMA were stained with fluorescent secondary antibody against rabbit. Staining was evaluated using a fluorescent or a

confocal microscope (Nikon TE3000 or Nikon TE3000 inverted). The capillary and arteriole density was then determined by counting the number of capillaries in each section of muscle. CD68 positive cells were stained with 3,3'-Diaminobenzidine (DAB, brown) and counterstained with hematoxylin.

Quantification of cell engraftment in ischemic hind limbs

Cell engraftment in the ischemic hind limb was quantified by histological analysis. Briefly, GFP-labeled BMCs or BMCs overexpressing RFP-labeled CSE (CSE-BMC) isolated from db/db mice ($5x10^5$ cells/mouse) were injected intramuscularly into ischemic hind limbs of db/db mice immediately after ligation of femoral artery. After 21 days, the ischemic hind limbs were harvested, and tissues were embedded and sectioned. Sections were incubated overnight at 4°C with Fluorescein griffonia (Bandeiraea) Simplicicolia Lectin I (1:100, Vector Lab, FL-1101), and chicken polyclonal antibody against GFP (1:100, A1026Z, Life Science) or rabbit polyclonal antibody against RFP (1:100, Rockland). For every section, a negative control without primary antibody was processed simultaneously. After 15 minutes of washing in PBS, secondary antibodies were added for 1 hr at room temperature. Then lectin, GFP and RFP were stained with fluorescent secondary antibody against rabbit and mouse, respectively. Cell nuclei were counterstained with DAPI (Molecular Probes). Five fields from four tissue sections were randomly selected, and the number of labeled cells was counted in each field as previously described.[5]

Tube formation assay

HCMVECs were treated with normal concentration of D-glucose (DG) (NG, 5 mM) or high concentration of DG (HG, 50 mM) for 48 hrs. To examine the role of deficiency of H_2S on HG-impaired tube formation, DATS (10 μM) was added in the culture medium with HG. To explore the role of CSE, CSE in HCMVECs was silenced with siRNA (sc-78973, Santa Cruz) or

overexpressed with transfection of CSE lentivirus (1 MOI, LVP526776, ABM). Then $4x10^4$ HCMVECs were plated on 120 µl matrigel (BD Falcon) in a 48-well plate. After incubation at 37°C in an atmosphere of 5% CO_2 for 16 hrs, the tubes were observed and photographed using a phase contrast microscope (x100 magnification, Nikon, TS100). The tube formation was counted by branch points in each image. The branch points for each tube structure were counted in each image.

To study the effect of diabetic BMCs on tube formation of endothelial cells (ECs) in diabetes, mouse microvascular endothelial cells (MMVECs) and BMCs were treated with NG or HG (50 mM) for 48 hrs. To examine the role of H_2S in diabetic BMC-mediated EC tube formation, the BMCs were treated with HG in the presence and absence of DATS (10 µM). Then the BMCs were trypsinized and labeled with PKH67 (green, MINI67-1KT, Sigma). Then a combination of $2x10^4$ HCMVECs and $2x10^4$ BMCs were plated on 120 µl matrigel (BD Falcon) in a 48-well plate. After incubation at 37°C in an atmosphere of 5% CO_2 for 16 hrs, the tubes were observed and photographed using a fluorescence microscope (x100 magnification, Nikon, TIE). The tube formation was counted by branch points in each image.

Scratch wound healing assay

Role of H_2S in HG-impaired HCMVEC migration was evaluated using a scratch wound assay as previously described.[6] Briefly, HCMVECs were cultured with EBM-2mv (CC-3156 and CC-4147, LONZA) and grown to 80% confluence in 6-well plates, then starved with serum free EBM-2mv for 6 hrs. Then the HCMVECs were treated with indicated concentrations of DG for 48 hrs. To examine the role of H_2S deficiency on HG-impaired cell migration, DATS (10 µM) was added together with 50 mM DG in the culture medium. To explore the role of CSE, the effect of CSE inhibitor PAG on wound healing (10 µM) for 48 hrs was examined. A clear area was then scraped in the monolayer with a 1000 µl pipette tip. After three washes with PBS, HCMVECs were cultured in EBM-2mv with same treatment. Wounded areas were

photographed with a phase contrast microscope (x100 magnification, Nikon, TS100) at 0 and 23 hrs, and to evaluate cells that migrated into the wound using ImageJ software as percentage of area covered by migrated cells divided by the area of initial wound/per field. Three different areas in each assay were randomly chosen.

RNA interference in HCMVECs

HCMVECs were transfected with CSE siRNA (60 nM, sc-78973, Santa Cruz) or negative control siRNA-NC (60 nM, siRNA-NC, sc-37007, Santa Cruz) accordance with the manufacturer's instructions. The transfection efficacy was determined by western blot using mouse monoclonal antibody against CSE (sc-365381, 1:1000, Santa Cruz) in HCMVECs treated with CSE siRNA for 72 hrs. Cells were subjected to experiments 24 hrs following transfection.

Intracellular H_2S production in a live cell by fluorescent probe

Intracellular free H_2S levels were determined in live BMCs and HCMVECs using a stable H_2S fluorescent probe as previously described[7] with modification. Briefly, cells were cultured on 25 cm round cover slips in 6-well plates with different treatments. At the end of experiment, the cells were washed with culture medium and incubated with 25 μM sulfidefluor 7AM (SF-7AM, Tocris, Cat. # 4943) in a cell culture incubator (37^{o}C; 5% CO_2; in the dark) for 30 minutes; washed with PBS three times; then received culture medium. The images were taken by inverted confocal microscope (Nikon TE3000).

Free H_2S in blood, tissues and cells by gas chromatograph

H_2S levels were measured in mouse BMCs, serum, or limb skeletal muscles by gas chromatography chemiluminescence (Agilent 7890 GC gas chromatography system and G660XA Series chemiluminescence detector) as previously described.[8] Briefly, free H_2S in fresh

plasma and tissue was liberated by incubating the samples in 1 M sodium citrate solution at 37 °C for 10 minutes. The resultant headspace gases were analyzed using the GC system.

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining for apoptosis was determined on BMCs or 5 µm-thick paraffin-embedded sections of the ischemic gastrocnemius muscle as per manufacturer's instructions (Cell death detection assay, Roche, Indianapolis, IN) according to the manufacturer's protocol as previously described.[9] TUNEL reaction mixture lacking terminal transferase (TdT) was used as a negative control. DAPI staining was used to count the total number of nuclei. TUNEL-stained tissue sections were also co-stained with rabbit monoclonal antibody against smooth muscle actin (SMA, 1:100, ab32575, Abcam). Counting the number of GFP^+/$TUNEL^+$ cells per high-power visual field (HVF) assessed cell death in ischemic gastrocnemius.

Migration assay

BMCs isolated from db/+ mice were treated with NG or HG (50 mM) in the presence or absence of DATS (10 µM) for 48 hrs. To study the role of CSE in HG-impaired BMCs migration, effect of CSE inhibitor DL-Propargyl Glycine (PAG, 100 µM, 48 hrs) was also examined.

Modified Boyden chamber cell migration assay was performed in chemotaxis chambers (Corning, Cat. # 3422). Briefly, BMCs were suspended in EC basal medium-2 (EBM-2, CC-3156, LONZA) in $2.5x10^5$/ml. We placed 100 µl EBM-2 in the upper chamber and 750 µl EBM-2 containing EBM-2MV bullet kit medium (CC-4176, LONZA) and 10% FBS without hydrocortisone in the lower chamber, respectively. Then we placed 200 µl cell suspension in the upper chamber. After 16 hrs incubation, the cells on the upper surface were removed and the cells on the underside were fixed with 4% formaldehyde and stained with Giemsa and DAPI,

respectively. The magnitude of BMC migration was evaluated by counting the migrated cells in 4 random high-power (×100) microscope fields.

Intracellular NO production in live HCMVECs

Intracellular NO production in live HCMVECs was determined as previously described.[10] Briefly, HCMVECs were treated with 10 μM 4-Amino-5-Methylamino-2',7'-Difluorofluorescein (DAF-FM, Sigma) in 37^{o}C and dark for 30 minutes. The images were taken by confocal microscope (Nikon TE3000 inverted). HCMVECs treated with L-NAME (100 μM) for 0.5 hr before DAF-FM staining served as negative controls.

Isolation of mouse lung microvascular ECs

Mouse lung microvascular endothelial cells (MMVECs) were isolated using collagenase as previously described,[11] and identified with staining of Von Willebrand factor (vWF, Santa Cruz SC-2780, 1:200) and CD31 (BD Biosciences; BD553370, 1:100).[10]

Real-time PCR

Gene expression levels of CSE, CBS, and MPST in BMCs or human CD34^{+} cells (mPB015F, Allcells) were determined by real-time PCR. RNA of the cells was extracted using RNeasy Mini RNA isolation kit (Gibco-Invitrogen). One μg of total RNA was reverse transcribed into cDNA using random hexamers (Gibco-Invitrogen). The gene expression was analyzed using the following primers:

CBS: forward: GATGCCGGAGAAGATGAGTATG; reverse: CAGGATCGACACCGATGATTT

CSE: forward: TTGCTAGAGGCAGCGATTAC; reverse: AGAGGGTAGCCCAGGATAAA

MPST: forward: CAGCTCGATCCCTCTTTCATC; reverse: GGTCCACCTTCTTCTCCTTAAAC

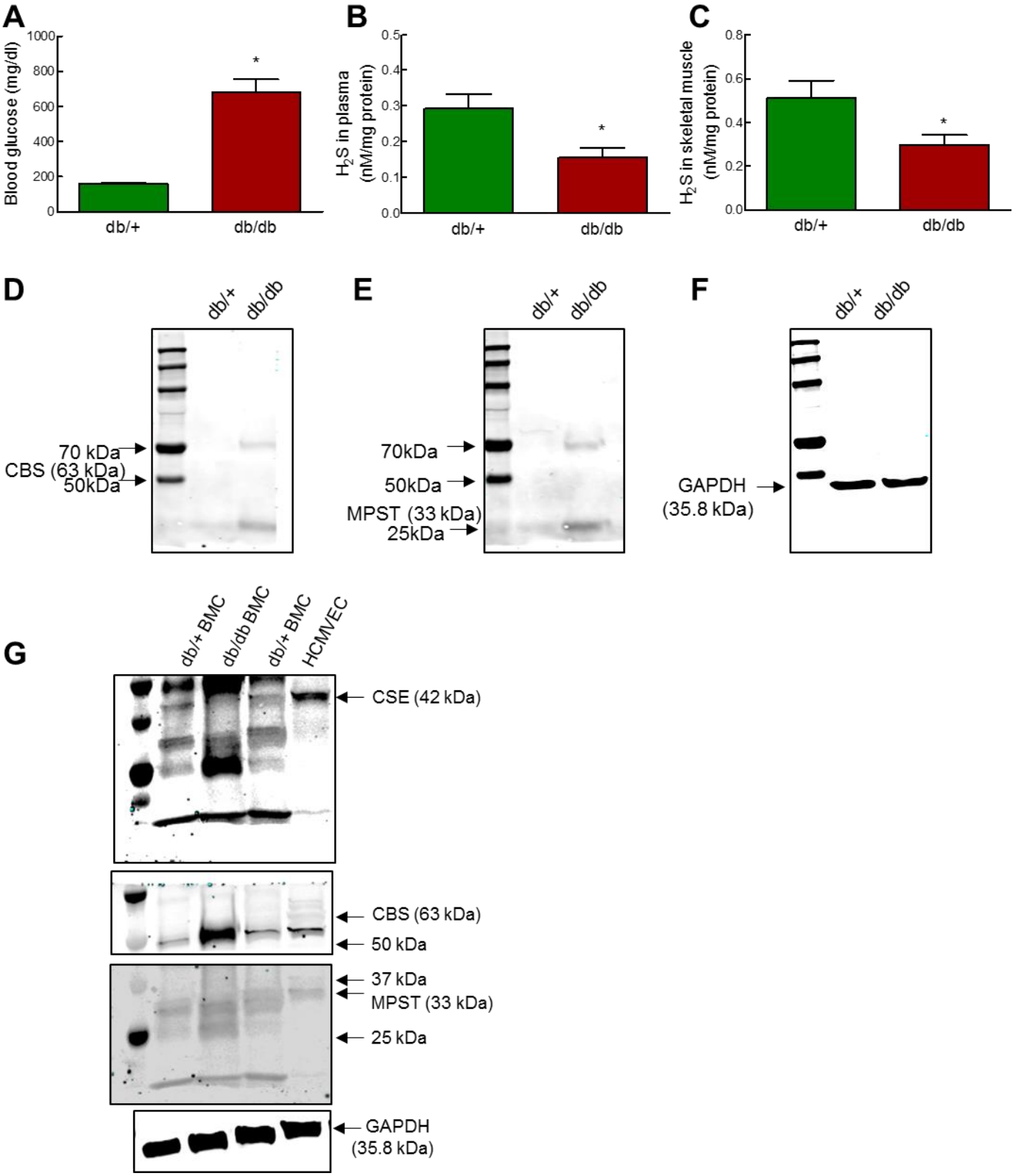
The resulting cDNA was quantified by real-time PCR using SYBR green PCR Master MIX (Roche, CA, USA) and using a Roche Light Cycler 4800 (Roche, www.roche.com). GAPDH was used as a housekeeping gene for normalization.

Statistics

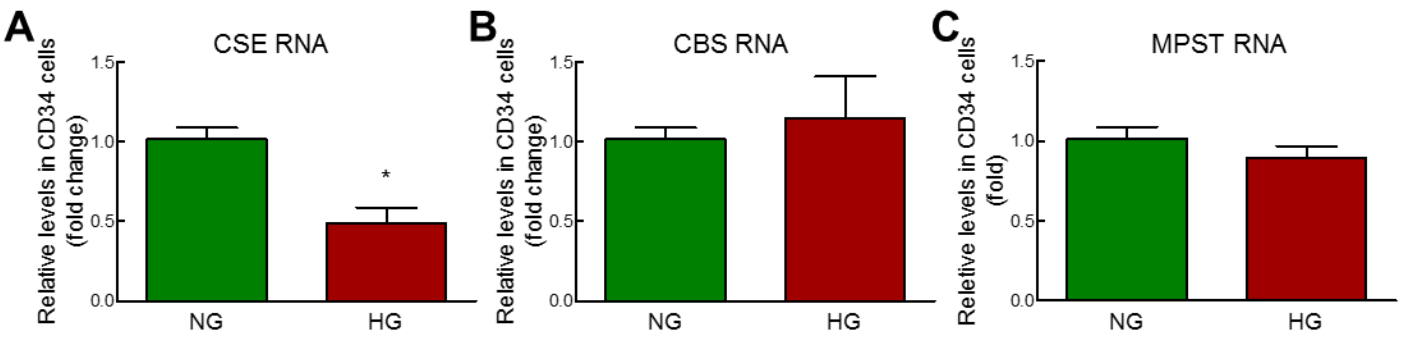
In vitro studies were repeated at least 3 times with triplicates/group/experiment. Results are expressed as the mean ± SEM. For statistical comparison of single parameters, independent *t* test was used for two groups and one way ANOVA with Bonferroni adjustment was performed for multiple groups. A probability value $p < 0.05$ was considered to be significant.

Supplemental Figures and Figure legends

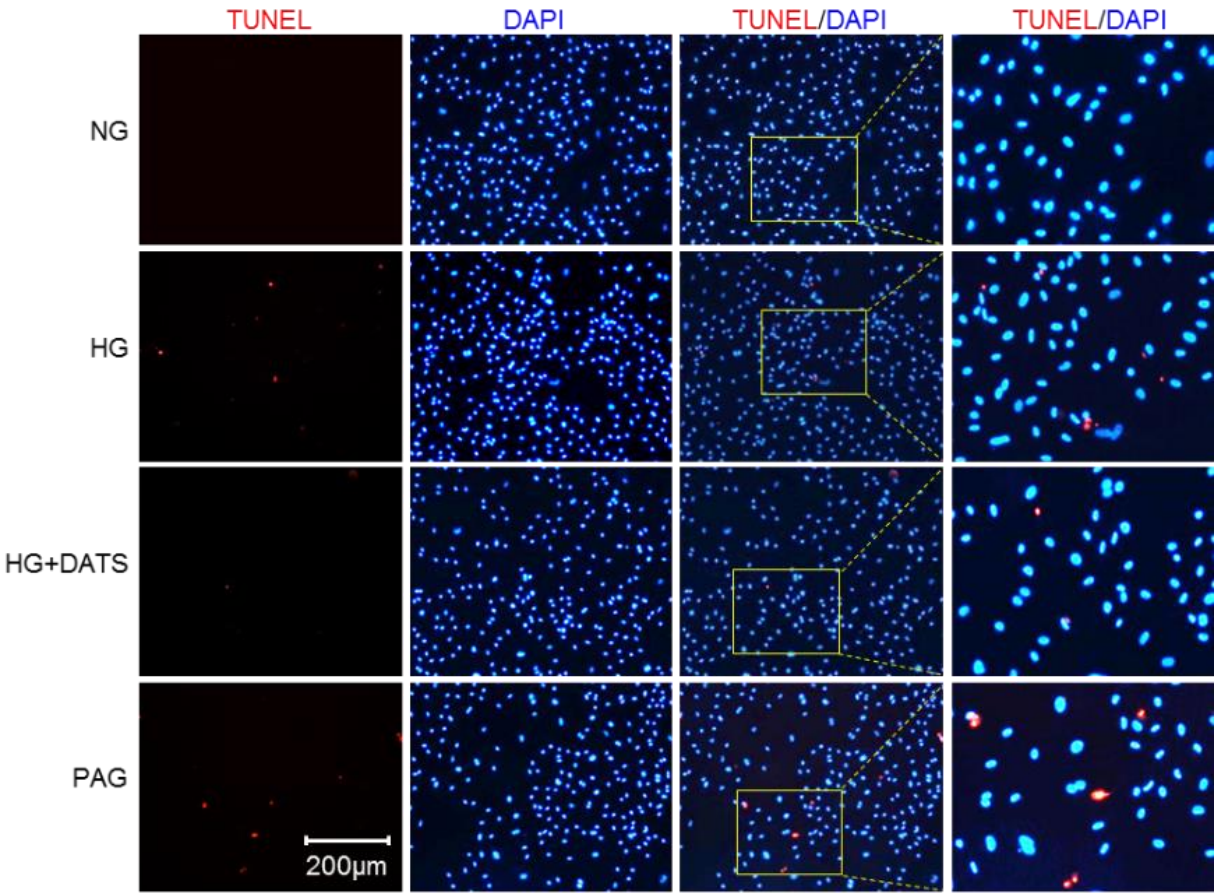
Supplementary Figure. 1



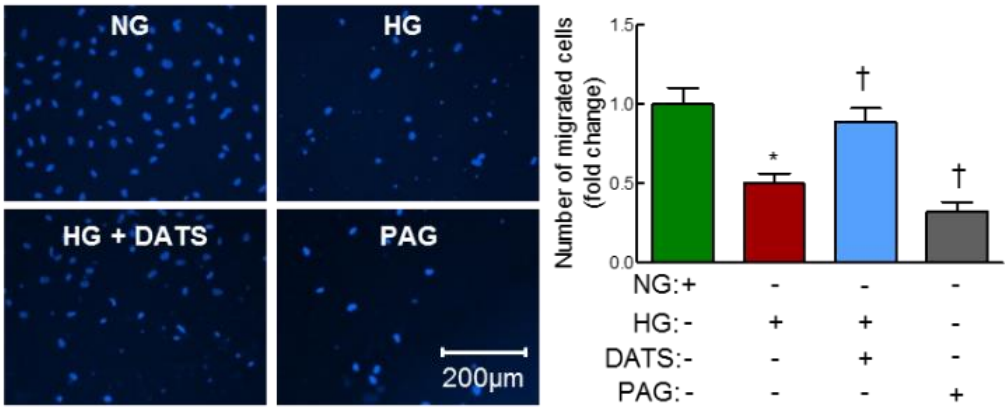
Supplementary Figure. 2



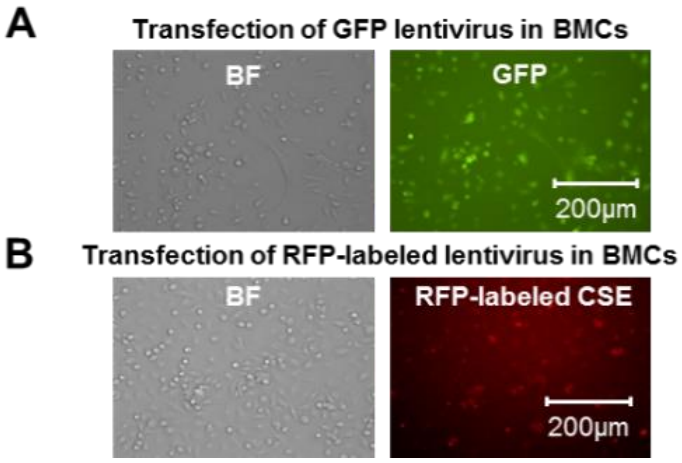
Supplementary Figure. 3



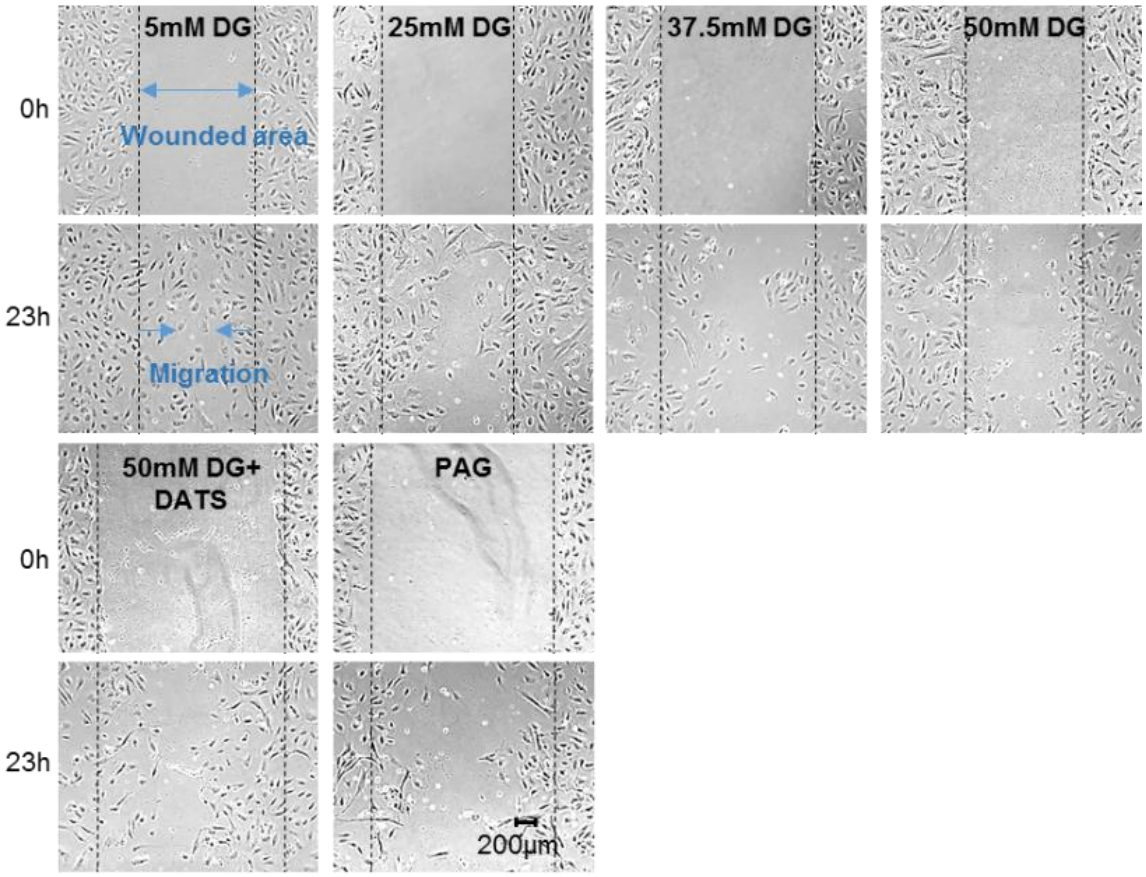
Supplementary Figure. 4



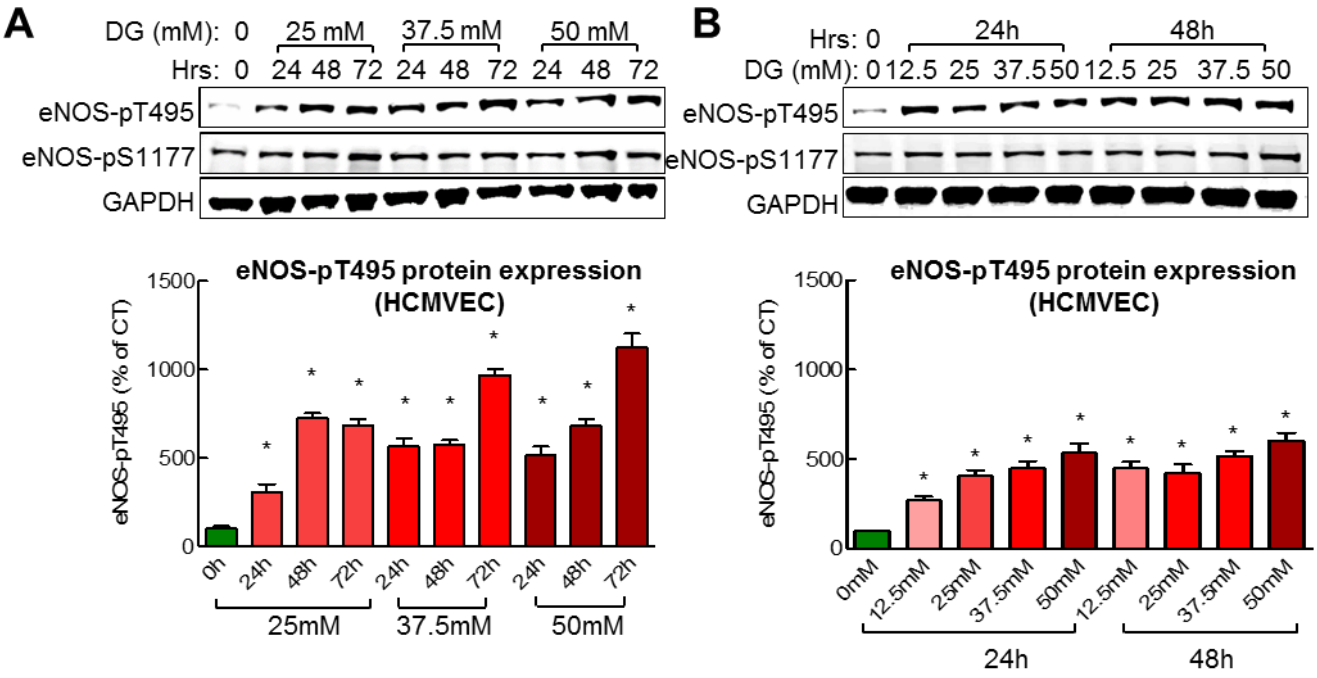
Supplementary Figure. 5



Supplementary Figure. 6



Supplementary Figure. 7



Supplementary Figure. 1. H_2S production was reduced in plasma and skeletal muscle from diabetic db/db mice. (A) Hyperglycemia was developed in male db/db mice. **(B)** and **(C)** Free H_2S levels detected by gas chromatography were decreased in plasma and skeletal muscle of diabetic db/db mice. **(D)** to **(G)** CBS and MPST protein levels were virtually undetected with 20 μg protein extract. Human cardiac microvascular endothelial cells (HCMVECs) lysate was used as a positive control. Blood, plasma, skeletal muscle of hind limbs, and BMCs were collected from 12-week-old, male db/+ and db/db mice. Free H_2S levels were measured by gas chromatography. n=5, *p<0.05 vs db/+ mice. BMCs, bone marrow cells; CBS, cystathionine-β-synthase; MPST, 3-mercaptopyruvate sulfurtransferase.

Supplementary Figure. 2. HG decreased CSE RNA levels in human $CD34^+$ cells. Average CSE **(A)**, CBS **(B)** and MPST **(C)** RNA levels. Human $CD34^+$ cells (mPB015F AllCell) were treated with normal D-glucose (NG, 5 mM) or HG (25 mM) for 24 hrs. CSE, CBS and MPST levels were examined by RT-PCR. n=3, *p<0.05 vs NG-treated $CD34^+$ cells. NG, normal glucose (5 mM DG).

Supplementary Figure. 3. DATS rescued HG-induced BMC death. Representative photomicrographs of TUNEL staining (red) in BMCs. Nuclei were stained with DAPI (blue). BMCs were isolated by gradient centrifugation from 12- to 14-week-old non-diabetic db/+ mice. BMCs were treated with HG (50 mM DG, 48 hrs) in the presence or absence of diallyl trisulfide (DATS, 10 μM) or CSE inhibitor DL-Propargyl Glycine (PAG, 100 μM) for 48 hrs. NG, normal glucose (5 mM DG)

Supplementary Figure. 4. DATS rescued HG-impaired BMC migration. Representative photographs of BMC migration using Boyden transwell chamber. BMCs were isolated by gradient centrifugation from 12- to 14-week-old db/+ mice. BMCs were treated with high HG (50 mM DG) or normal D-glucose (NG, 5 mM)) in the presence or absence of diallyl trisulfide (DATS, 10 μM) or CSE inhibitor DL-Propargyl Glycine (PAG, 100 μM) for 48 hrs. Nuclei were stained with DAPI (blue). n=3-5, *p<0.05 vs db/+ mice or NG-treated-BMCs; †p<0.05 vs HG-treated BMCs.

Supplementary Figure. 5. GFP or RFP-labeled CSE was transfected in BMCs by lentivirus. BMCs were transfected with GFP or RFP-labeled CSE lentivirus (MOI: 1) in the presence of polybrene (8 μg/ml) for 48 hrs. BMCs were isolated by gradient centrifugation from 12- to 14-week-old db/db mice. BF, bright field.

Supplementary Figure. 6. DATS rescued HG-impaired HCMVEC migration. Representative photomicrographs of HCMVEC migration by scratch healing assay. HCMVECs were treated with DG at indicated concentrations for 48 hrs. Quantification is shown in Figure 6F.

Supplementary Figure. 7. HG increased eNOS-pT495 in HCMVECs in a dose- and time-dependent manner. HCMVECs were treated with DG at indicated concentrations and times. Indicated doses of D-glucose were added to the cultured medium at hour 0. Samples were collected at the indicated times. n=5-7, *p<0.05 vs untreated HCMVECs. DG, D-glucose; eNOS, endothelial nitric oxide synthase; eNOS-pS1177, eNOS phosphorylation at serine 1177; eNOS-pT495, eNOS phosphorylation at threonine 495; HCMVECs, human cardiac microvascular endothelial cells.

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