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Research paper

Hyperhomocysteinemia potentiates diabetes-impaired EDHF-induced vascular relaxation: Role of insufficient hydrogen sulfide

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ABSTRACT

Insufficient hydrogen sulfide (H_2S) has been implicated in Type 2 diabetic mellitus (T2DM) and hyperhomocysteinemia (HHcy)-related cardiovascular complications. We investigated the role of H_2S in T2DM and HHcy-induced endothelial dysfunction in small mesenteric artery (SMA) of db/db mice fed a high methionine (HM) diet. HM diet (8 weeks) induced HHcy in both T2DM db/db mice and non-diabetic db/+ mice (total plasma Hcy: 48.4 and 31.3 μM, respectively), and aggravated the impaired endothelium-derived hyperpolarization factor (EDHF)-induced endothelium-dependent relaxation to acetylcholine (ACh), determined by the presence of eNOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) and prostacyclin (PGI_2) inhibitor indomethacin (INDO), in SMA from db/db mice but not that from db/+ mice. A non-selective Ca^{2+}-active potassium channel (K_{Ca}) opener NS309 rescued T2DM/HHcy-impaired EDHF-mediated vascular relaxation to ACh. EDHF-induced relaxation to ACh was inhibited by a non-selective K_{Ca} blocker TEA and intermediate-conductance K_{Ca} blocker (IK_{Ca}) Tram-34, but not by small-conductance K_{Ca} (SK_{Ca}) blocker Apamin. HHcy potentiated the reduction of free sulfide, H_2S and cystathionine γ-lyase protein, which converts L-cysteine to H_2S, in SMA of db/db mice. Importantly, a stable H_2S donor DATS diminished the enhanced O_2^- production in SMAs and lung endothelial cells of T2DM/HHcy mice. Antioxidant PEG-SOD and DATS improved T2DM/HHcy impaired relaxation to ACh. Moreover, HHcy increased hyperglycemia-induced IK_{Ca} tyrosine nitration in human micro-vascular endothelial cells. EDHF-induced vascular relaxation to L-cysteine was not altered, whereas such relaxation to NaHS was potentiated by HHcy in SMA of db/db mice which was abolished by ATP-sensitive potassium channel blocker Glycolamide but not by K_{Ca} blockers.

Conclusions: Intermediate HHcy potentiated H_2S reduction via CSE-downregulation in microvasculature of T2DM mice. H_2S is justified as an EDHF. Insufficient H_2S impaired EDHF-induced vascular relaxation via oxidative stress and IK_{Ca} inactivation in T2DM/HHcy mice. H_2S therapy may be beneficial for prevention and treatment of micro-vascular complications in patients with T2DM and HHcy.

1. Introduction

Diabetes is the most prevalent metabolic disorders and is estimated to affect 400 million or 4.4% of population worldwide in the next 20 years [1,2]. Type 2 diabetic mellitus (T2DM) is the most common form of diabetes. In adults, about 90–95% of all diagnosed cases of diabetes are T2DM. In T2DM, the micro-vascular dysfunction encompasses long-term complications, such as retinopathy, nephropathy and neuropathy which impose a major public health burden.

Endothelium plays a key role in the control of vascular homeostasis by releasing vasodilator substances, including nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor

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(EDHF), and vasoconstrictor substances, such as angiotensin II, endothelin-1, thromboxane A_2, and prostaglandin H_2, in response to pathophysiological stimulation [3,4]. It is generally accepted that NO predominantly controls relaxation of macro-vasculature, whereas EDHF primarily controls relaxation of micro-vasculature and becomes more important when vessel diameter decreases [5–7].

EDHF is proposed to be a substance and/or electrical signal that is synthesized or generated in and released from endothelium under pathophysiological stimuli. EDHF action is to hyperpolarize vascular smooth muscle cells (VSMCs), resulting in vascular relaxation [8,9]. Vascular relaxation to acetylcholine (ACh) in the presence of a combination of eNOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) and PGI_2 inhibitor indomethacin (INDO) are used to determine EDHF-induced endothelium-dependent vascular relaxation. Although extensive studied, the nature of EDHF remains unclear. Many factors have been suggested to be EDHF which induces endothelium-dependent vascular hyperpolarization and vascular relaxation in the presence of L-NAME + INDO, such as epoxyeicosatrienoic acids (EETs), H_2O_2, gap junctions [10]. Very recently, study demonstrated that hydrogen sulfide (H_2S) may be one of major EDHF regulating endothelial function in micro-vasculature [11]. Numerous studies supported the concept that EDHF-mediated vascular relaxation is elicited by the opening of Ca^{2+}-activated potassium channels (K_{Ca}) in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) [12]. The K_{Ca} family consists of small conductance K_{Ca} (SK_{Ca}, including $SK_{Ca}1$, $SK_{Ca}2$, $SK_{Ca}3$), intermediate conductance K_{Ca} (IK_{Ca}) and large conductance K_{Ca} (BK_{Ca}) subtypes. SK_{Ca} and IK_{Ca} predominantly expressed in ECs, whereas BK_{Ca} is preferentially located in VSMCs [13,14].

Endothelial dysfunction (ED) is an early event in the development of vascular abnormalities prior to any visible morphological changes and is characterized by the impairment of endothelium-dependent vasodilatation. Many factors are related to ED, including diabetes, hypertension, smoke, obese and elevated plasma homocysteine level called hyperhomocysteinemia (HHcy). We have reported that HHcy impairs endothelium-dependent vascular relaxation to ACh in the presence of L-NAME + INDO in mouse micro-vasculature (small mesenteric artery, SMA) via oxidation/nitration of SK_{Ca}/IK_{Ca} [13]. Recently, we also observed that HHcy potentiates hyperglycemia-induced ED in mouse aorta via activation of μ-calpain [15]. HHcy appeared to be a stronger risk factor for cardiovascular disease (CVD) in patients with T2DM [16]. However, whether HHcy impairs endothelial function in micro-vasculature in T2DM and the underlying mechanisms have not been studied.

H_2S is an endogenously produced gasotransmitter that is critical for the regulation of cardiovascular homeostasis.[17–19]. In mammalian species, H_2S is produced enzymatically by cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST), of which CSE is primarily expressed in the cardiovascular system, including cardiomyocytes, ECs and VSMCs [20]. It was reported that H_2S production was reduced in mice with HHcy [21] and T2DM [22,23]. A role of H_2S in the pathogenesis of T2DM has been suggested, as circulating levels of H_2S were found to be inversely proportional to cardiovascular complications in T2DM animals [22,24] The negative association between T2DM and H_2S is also reinforced by decreased plasma H_2S levels in patients with T2DM [23,25]. Recently, we provided strong evidence that H_2S reduction is related to T2DM-induced bone marrow cell (BMC) dysfunction and impaired ischemic tissue repair [26]. Restoration of H_2S production in T2DM mice improves reparative property of BMCs [26]. In this study, we examined the effects and underlying mechanisms of HHcy on T2DM-impaired endothelial function in SMA of db/db mice.

2. Materials and methods

2.1. Experimental animals and sample collections

HHcy was induced in male T2DM db/db mice (Jackson Laboratory) by feeding 8-week-old mice with our newly designed high-methionine (HM) diet (methionine, 2%, 07794, Harlan Teklad), in which folic acid and B vitamins are reduced to the sufficient basal levels,[15] for 8 weeks. Diet contains equal amount of folic acid and B vitamins but less methionine ((CT, 0.37%), 07793, Harlan Teklad) was used as control diet (CT). Non-T2DM db/+ mice were served as controls. All animals received humane care in compliance with institutional guideline and the "Guide for the Care and Use of Laboratory Animals" prepared by the "Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council". At the end of experiments, 24 h metabolic parameters including water intake and urine excretion were collected in metabolic cages; body weight, heart weight and kidneys weight were recorded. Blood was collected by cardiac puncture. Lungs were perfused with PBS and digested with collagenase II for mouse lung endothelial cells (MLECs) isolation. Second order of SMAs were dissected for vascular reactivity assessment.

2.2. Plasma total homocysteine (t-Hcy) concentrations

t-Hcy levels were analyzed using a Biochrom 30 amino acid analyzer (Cambridge, UK) as we previously described [27,28].

2.3. Oral glucose tolerance test (OGTT) and blood glucose

OGTT and blood glucose were analyzed as previous describe [15].

2.4. Free sulfide levels in small mesenteric arteries (SMAs)

Free sulfide in SMAs was measured by RP-HPLC after derivatization with excess monobromobimane (MBB) as stable products sulfide-dibimane (SDB) as previously described [29].

2.5. H_2S production in SMAs by fluorescent probe

H_2S levels were determined in fresh SMAs using a stable H_2S fluorescent probe as previously described [26].

2.6. SMAs preparation for vessel reactive assessment

Mice were sacrificed under anesthesia. Mesenteric bed was removed and prepared for vascular reactive assessment as we previously described [13].

2.6.1. Vascular contractile responses

After an equilibration period, SMA rings were exposed to potassium chloride (KCl, 120 mM). Following a second round of washing and equilibration with Krebs, vascular contractile responses to cumulative additions of phenylephrine (PE, 10 nM to 33 μM) were determined.

2.6.2. Endothelium-dependent and -independent vascular relaxation responses

The presence of intact endothelium in the vascular preparations was confirmed as described previously [30]. Endothelium-dependent relaxation responses to cumulative concentrations of ACh (10 nM to 33 μM) and endothelium-independent relaxation responses to sodium nitroprusside (SNP, 1 nM to 10 μM) in rings pre-contracted with PE (1 μM) were determined.

2.6.3. EDHF-induced vascular relaxation to ACh

EDHF-induced vascular relaxation responses to ACh were determined in the presence of N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM, a NOS inhibitor that blocks NO production) plus indomethacin (INDO, 10 μM, a non-selective cyclooxygenase (COX) inhibitor that blocks the formation of PGI_2) for 30 min. To dissect the mechanisms underlying EDHF-induced relaxation, SMAs were incubated with either a non-selective K_{Ca} inhibitor TEA (1 mM), or a

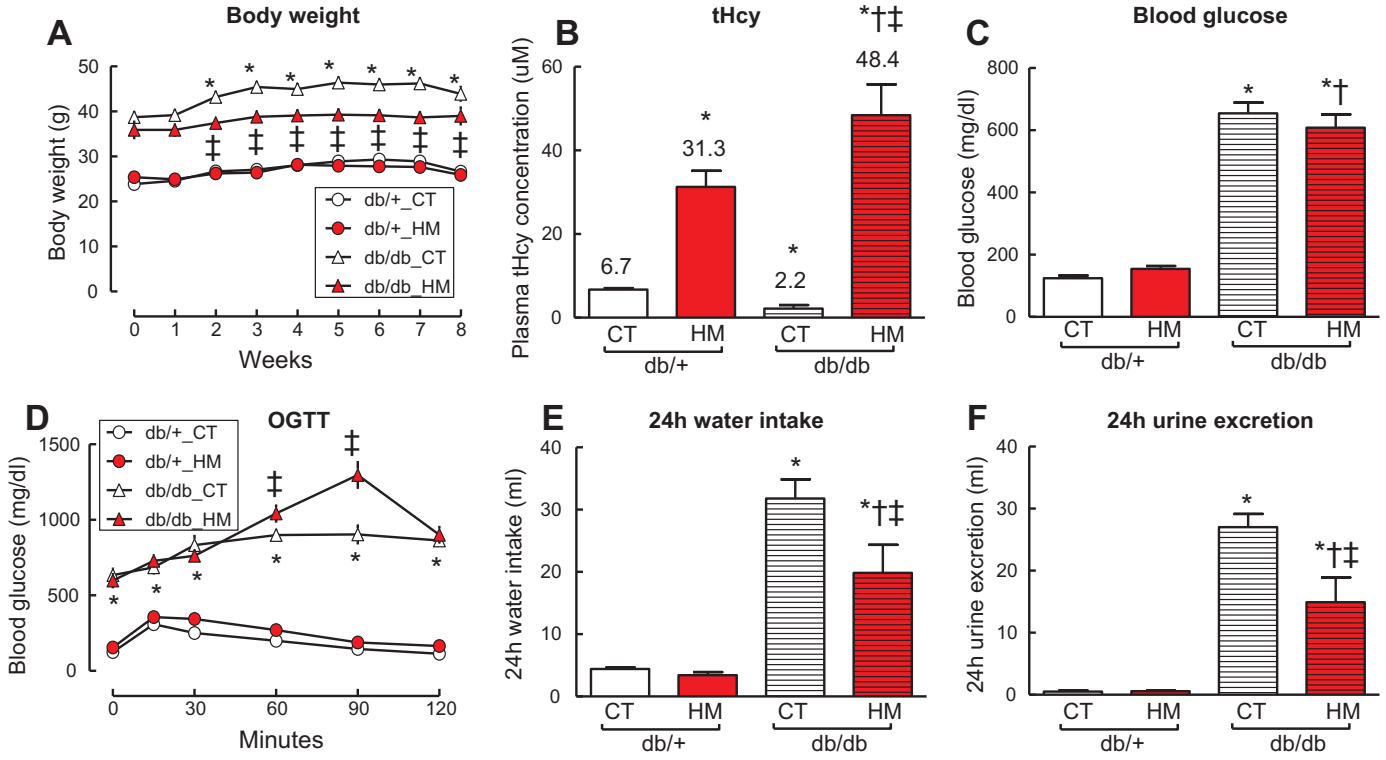

Fig. 1. HHcy aggravated glucose intolerance and reduced body weight of db/db mice. A. Body weight. **B.** Total plasma homocysteine (tHcy) levels. **C.** Blood glucose levels. **D**. Oral glucose tolerance test (OGTT). **E**. 24 h water intake. **F**. 24 h urine excretion. At the age of 8-week, db/+ and db/db mice were fed with a high methionine diet (HM, methionine: 2% w/w) for 8 weeks. db/+ and db/db mice fed with a control diet (CT, methionine: 0.37% w/w) served as controls. n = 5–10, * $p < 0.05$ vs db/+ mice on CT diet (db/+_CT); † $p < 0.05$ vs db/+ mice on HM diet; ‡ $p < 0.05$ vs db/db mice on CT diet (db/db_CT). HHcy, hyperhomocysteinemia.

selective SK_{Ca} inhibitor Apamin (1 μM) and/or a selective IK_{Ca} inhibitor Tram34 (1 μM) for 30 min in the presence of INDO and L-NAME, respectively. Further, NS309 (1 μM, 30 min), IK_{Ca}/SK_{Ca} opener, was used to determine the role of IK_{Ca} and SK_{Ca} in T2DM (db/db mice)- and T2DM/HHcy (db/db mice fed with HM diet)-impaired EDHF-induced endothelium-dependent vascular relaxation in the presence of L-NAME + INDO as we described previously [31].

The role of oxidative stress in T2DM/HHcy-impaired EDHF-induced relaxation was examined by pre-incubating the SMAs with antioxidants polyethylene glycol (PEG)-superoxide dismutase (PEG-SOD, 150 U/ml) for 1 h before testing vascular relaxation responses to ACh in the presence of L-NAME + INDO. To examine the role of insufficient H_2S production on T2DM/HHcy-impaired EDHF-induced relaxation to ACh, SMAs were treated with a stable H_2S donor DATS (5 μM) for 30 min before testing vascular relaxation responses to ACh in the presence of L-NAME + INDO.

2.6.4. EDHF-induced vascular relaxation by H_2S or L-cysteine

EDHF-induced vascular relaxation in response to NaHS (10–60 μM) or L-cysteine (10 μM) were determined in the presence of L-NAME (100 μM) and INDO (10 μM) for 30 min. To dissect the role of K_{Ca}, the SMAs were incubated with either TEA (1 mM), Apamin (1 μM) or Tram34 (1 μM) for 30 min in the presence of INDO and L-NAME, respectively.

Moreover, to assess the role of ATP-activated potassium channel (K_{ATP}) in T2DM- and T2DM/HHcy-impaired ED, vascular relaxation response to H_2S was examined in the presence of K_{ATP} inhibitor glybenclamide (GLB, 10 μM, 30 min), L-NAME and INDO.

2.7. Isolation of mouse lung endothelial cells (MLECs)

MLECs were isolated and identified as described previously [15,32].

2.8. Superoxide production in SMAs and MLECs

Superoxide generation in fresh SMA segments and MLECs were measured by staining with dihydroethidium (DHE) as described previously [13,15].

2.9. IK_{Ca} tyrosine nitration in SMAs by immunohistochemistry

Tyrosine nitration is a marker of oxidative stress. To explore whether IK_{Ca} is oxidized under T2DM/HHcy condition in SMAs, we examined the co-localization of IK_{Ca} and 3-nitrotyrosine (3-NT) as described previously [13].

2.10. Cell culture

Human cardiac micro-vascular endothelial cells (HCMVECs, CC7030, Lonza Inc, Allendale, USA) were cultured in EGM® − 2 MV medium [13,26]. HCMVECs were treated with D-glucose (25 mM) with or without DL-homocysteine (DL-Hcy, 500 mM) for 48 h [15,26].

2.11. Western blot and immunoprecipitation

IK_{Ca} oxidation was determined in HCMVECs treated with D-glucose with or without DL-Hcy for 48 h by immunoprecipitation of 200 μg protein with a mouse monoclonal antibody against 3-NT (SC32757, Santa Cruz) followed by blotting with antibodies against IK_{Ca} (H-120, SC32949, Santa Cruz).

2.12. Chemicals

All chemicals, if not specified above, were purchased from Sigma-Aldrich (St. Louis, MO).

2.13. 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 (unpaired) and one way ANOVA (Tukey) with adjustment was performed for multiple groups. A probability value $p < 0.05$ was considered to be significant.

3. Results

3.1. Body, plasma total Hcy and blood glucose levels, glucose tolerance

Body weight was reduced in db/db mice with HHcy (Fig. 1**A**, $p < 0.05$). Plasma total Hcy (t-Hcy) level was decreased in diabetic db/db mice compared with that in non-diabetic db/+ (2.15 ± 0.88 and 6.74 ± 0.36 μM, respectively, Fig. 1**B**, $p < 0.05$). The HM diet elevated t-Hcy level to 31.3 ± 3.8 μM in db/+ mice and 48.4 ± 3.4 μM in db/db mice (Fig. 1**B,** $p < 0.05$). Blood glucose level was significant higher in db/db mice (654 ± 35.1 mg/dl) compared with that in non-diabetic db/+ mice (124 ± 8.5 mg/dl) (Fig. 1**C**). HHcy did not change blood glucose levels in both db/+ mice and db/db mice, but aggravated glucose intolerance in db/db mice (Fig. 1**C** and **D**, $p < 0.05$).

3.2. Heart and kidneys weights, and 24 h metabolic parameters

Heart weights were not changed by either T2DM, HHcy, or the combination (Supplementary Fig. A to C). HM diet-induced intermediate HHcy, defined as plasma tHcy level between 30 and 100 μM [33,34], reduced 24 h water intake and urine excretion, but increased kidneys weights in db/db mice (Fig. 1**E** to **F** and Supplementary Fig. D to F, $p < 0.05$). Whether HHcy aggravated renal damage in db/db mice needs further investigation.

3.3. HHcy had no significant effect on SMA contractile response to KCl

HHcy did not change vascular contractile response to the single dose of KCl in SMA from either db/db or db/+ mice (Fig. 2**A**). The db/db mice had a trend of reduced contraction to KCl compared to such response in the db/+ mice when fed a control diet. Intermediate HHcy had no significant effect on vascular contraction to KCl compared to their parallel control group on control diet. Contractile response to PE was not changed by either T2DM and/or HHcy (Fig. 2**B**).

3.4. HHcy potentiated T2DM-impaired endothelium-dependent vascular relaxation to ACh

Endothelium-dependent vascular relaxation responses to accumulative concentrations of ACh were impaired in SMAs of db/db mice compared with that of db/+ mice (Fig. 2**C**, maximal relaxation: 80.8 ± 5.0 and 94.3 ± 2.7, respectively, $p < 0.05$**)**. HM diet-induced intermediate HHcy had a trend of impairing vascular relaxation to ACh in db/+ mice and significantly aggravated T2DM-impaired relaxation to ACh in db/db mice (Fig. 2**C**, maximal relaxation: 62.7 ± 6.1, $p < 0.05$). Neither T2DM nor HHcy alone or a combination of T2DM and HHcy affected endothelium-independent vascular relaxation to SNP in SMAs (Fig. 2**D**).

3.5. HHcy aggravated T2DM-impaired vascular relaxation to ACh in the presence of eNOS and COX inhibitors

It is generally accepted that EDHF predominantly controls vascular relaxation in micro-vasculature [5–7]. Because that HM diet-induced intermediate HHcy had no effect either on endothelium-dependent or endothelium-independent relaxation in SMAs of db/+ mice, we examined the effect of HHcy on T2DM/HHcy-impaired EDHF-induced relaxation to ACh in the SMAs of db/db mice. We found that EDHF-induced relaxation to ACh was impaired in db/db mice compared to that in db/+ mice (maximal relaxation: 60.4 ± 2.1 and 69.6 ± 3.9, respectively, Fig. 3**A,** $p < 0.05$), and that HM diet-induced HHcy further aggravated T2DM-impaired EDHF-induced relaxation to ACh in db/db mice (maximal relaxation: 48.3 ± 7.7% ($p < 0.05$)).

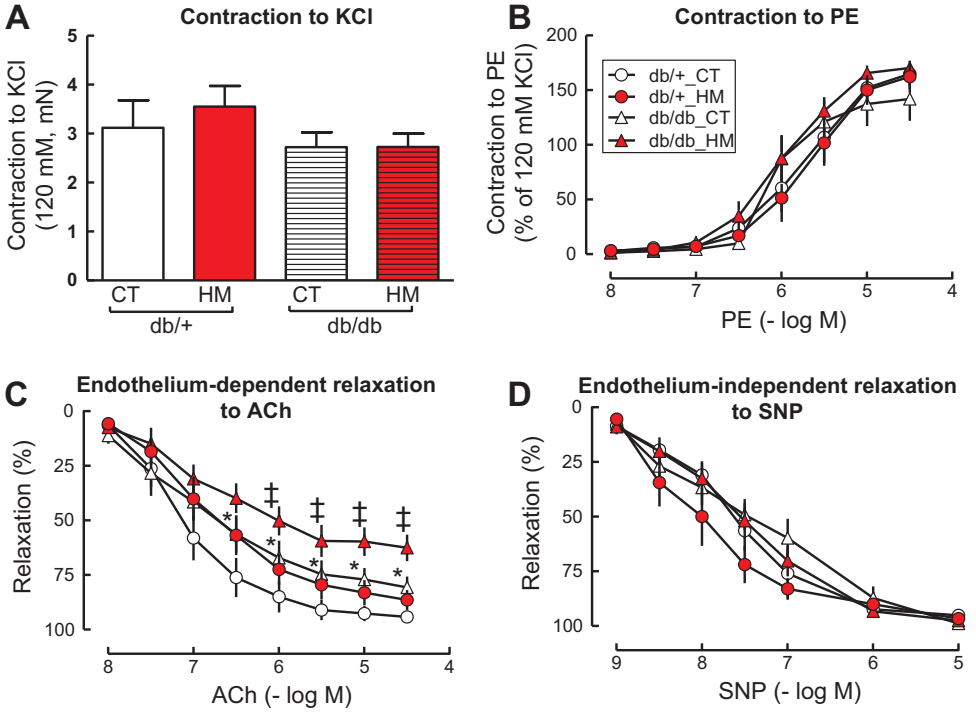

Fig. 2. HHcy aggravated T2DM-impaired endothelium-dependent vascular relaxation to ACh in SMA of db/db mice. A and **B.** Vascular contractile response to potassium chloride (KCl, **A**) and phenylephrine (PE, **B**). **C.** Endothelium-dependent vascular relaxation to acetylcholine (ACh). **D.** Endothelium-independent vascular relaxation to sodium nitroprusside (SNP). Small mesenteric arterial rings were pre-contracted with phenylephrine (1 μM) and examined for relaxation response to cumulative additions of ACh or SNP. n = 5–10, *$p < 0.05$ vs db/+ mice on CT diet (db/+ _CT); ‡$p < 0.05$ vs db/db mice on CT diet (db/db_CT). SMAs, small mesenteric arteries.

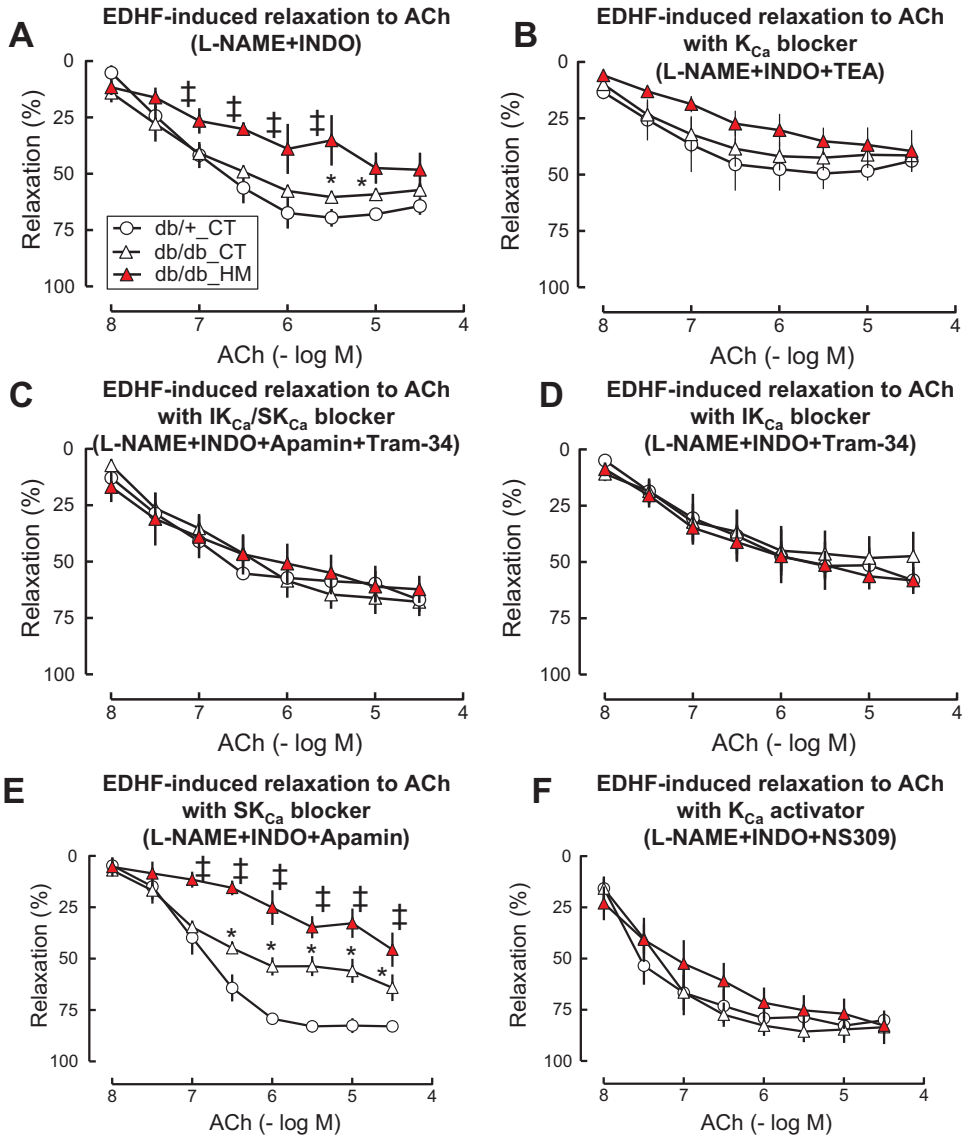

Fig. 3. HHcy aggravated impaired EDHF-induced relaxation to ACh in SMA of db/db mice via IK_{Ca} inhibition. EDHF-induced relaxation was determined by pretreated the SMA with NOS inhibitor L-NAME (100 μM) and COX inhibitor (INDO, 10 μM) and indicated inhibitors, precontracted with phenylephrine (1 μM), and examined for relaxation to acetylcholine (ACh). **A**. EDHF-induced relaxation to ACh. **B**. EDHF-induced relaxation in the presence of SK_{Ca}/Ik_{Ca} blocker TEA (1 mM). **C**. EDHF-induced relaxation in the presence of SK_{Ca} blocker Apamin (1 μM) and IK_c blocker Tram-34 (1 μM). **D**. EDHF-induced relaxation in the presence of Ik_{Ca} blocker Tram-34. **E**. EDHF-induced relaxation in the presence of SK_{ca} blocker Apamin. **F**. EDHF-induced relaxation in the presence of SK_{Ca}/IK_{Ca} activator NS309 (10 μM). n = 5–10. *p < 0.05 vs db/+ mice on CT diet (db/+_CT); ‡p < 0.05 vs db/db mice on CT diet (db/db_CT). COX, cyclooxygenase; HHcy, hyperhomocysteinemia; INDO, indomethacin; L-NAME, N^G-Nitro-L-arginine methyl ester; NOS, NO synthase.

3.6. HHcy worsen T2DM-impaired EDHF-induced vascular relaxation to ACh via IK_{Ca} inactivation

Because EDHF induces endothelium-dependent vascular relaxation to ACh is mainly via K_{Ca} opening in EC [12], we tested the effect of different K_{Ca} blockers on EDHF-induced vascular relaxation to ACh. We found a non-selective K_{Ca} blocker TEA completely diminished either T2DM- or T2DM pluc HHcy-impaired EDHF-induced vascular relaxation to ACh (Fig. 3**B**). IK_{Ca} blocker Tram-34 or the combination of IK_{Ca} blocker Tram-34 and SK_{Ca} blocker Apamine but not Apamine alone also diminished T2DM- and T2DM/HHcy-impaired EDHF-induced relaxation to ACh mice (Fig. 3**C** to **E)** in SMAs. Moreover, we found that NS309, a non-selective activator of K_{Ca}, completely restored EDHF-induced relaxation responses to ACh in db/db and db/db/HHcy mice (Fig. 3**F**, $p < 0.05$), suggesting that inactivation of IK_{Ca}, but not SK_{Ca}, plays a major role in HHcy-potentiated diabetes-impaired EDHF-induced vascular relaxation in db/db and db/db/HHcy mice.

3.7. HHcy aggravated T2DM-induced H_2S deficiency and CSE protein reduction in SMAs of db/db mice

We examined H_2S production in SMA with two different methodologies. Using a florescent probe, we found that H_2S production was decreased to 65% in db/db mice compared with that in db/+ mice ($p < 0.05$) and further reduced to 33.7% by the addition of HHcy (Fig. 4**A** and **B**, $p < 0.05$). Using RP-HPLC detection, free sulfide levels were reduced to 63.7% in SMAs of db/db mice (45.9 pM/mg) which were further reduced to 48.4% by the addition of HHcy (34.9 pM/mg) compared to that in db/+ mice (72.1 pM/mg), respectively (Fig. 4**C,** $p < 0.05$). We and others recently reported that cystathionine β-lyase (CSE) is a dominant H_2S-synthesizing enzyme in cardiovascular system [19,35,36]. Thus we examined the effect of T2DM alone and a combination of T2DM and HHcy on CSE level in SMA. We found that CSE protein levels were decreased to 58% ($p < 0.05$) in SMAs of db/db mice which were further reduced to 25.4% ($p < 0.05$) by HHcy (Fig. 4**D,** $p < 0.05$).

3.8. H_2S donor DATS rescued T2DM/HHcy-induced oxidative stress in SMAs and mouse lung endothelial cells (MLECs)

Insufficient H_2S has been suggested to be one of the potential sources of oxidative stress in diseases conditions [37]. Here, we examined in situ superoxide (O_2^-) generation in SMAs, and found that O_2^- was increased in db/db SMAs by 365%, further elevated by HHcy to 455% (Fig. 5**A**, $p < 0.05$). To explore the role of insufficient H_2S on T2DM/HHcy-induced oxidative stress, we applied DATS, a stable H_2S

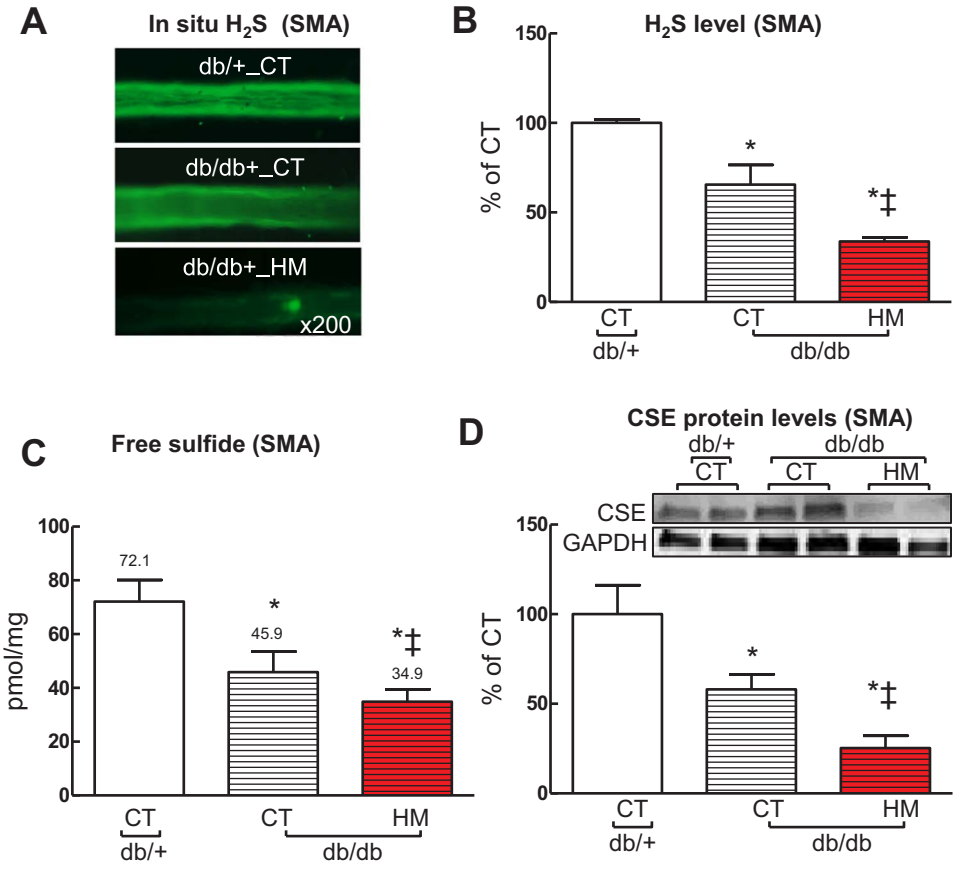

Fig. 4. HHcy potentiated H_2S deficiency and CSE downregulation in SMA of db/db mice. A. Reprehensive images of intracellular H_2S production in SMAs stained with fluorescent probe sulfidefluor 7AM (SF-7AM, 25 μM, for 30 min). **B.** Quantification of Intracellular H_2S production in SMAs. **C.** Free sulfide levels in SMAs measured by reversed phase HPLC. **D.** CSE protein levels in SMAs. n = 3–5 *p < 0.05 vs db/+ mice on CT diet (db/+_CT); ‡p < 0.05 vs db/db mice on CT diet (db/db_CT). CSE, cystathionine γ-lyase.

donor, and found that DATS effectively reduced O_2^- levels to 245% in SMAs from db/db/HHcy mice (Fig. 5**A**, p < 0.05). O_2^- production was increased to 248% in MLECs from db/db and further elevated by HHcy to 314% (Fig. 5**C,** p < 0.05) which was completely reversed by DATS to 113% (Fig. 5**C**, p < 0.05). Finally, we found that antioxidant PEG-SOD and DATS improved EDHF-induced relaxation to ACh in SMAs of db/db HHcy mice (maximal relaxation: 43.1–70.1% and 50.7–73.8%, respectively, Fig. 5**D** and **E**, p < 0.05). These data suggest that H_2S deficiency is, at least in part, a source of oxidative stress in micro-vasculature and responsible for T2DM/HHcy-impaired EDHF-induced relaxation.

3.9. HHcy potentiated IK_{Ca} oxidation/tyrosine nitration in micro-vascular endothelial cells

One of the major potential mechanisms leading to inactivation of K_{Ca} is oxidative stress [13,38]. We used HCMVEC as a relevant cellular model for studying the underlying mechanisms of T2DM and T2DM/HHcy-mediated IK_{Ca} inactivation in EC. By immunoprecipitation with antibody of 3-NT, a marker of oxidative stress, followed with IK_{Ca} Western blot, we found that IK_{Ca} oxidation was increased by high glucose (25 mM D-glucose) by 1.37-fold (Fig. 6**A**, p < 0.05), and by the combination of D-glucose plus DL-Hcy by 3.51-fold (Fig. 6**A**, p < 0.05) in HCMVECs. Finally, we confirmed such IK_{Ca} oxidation induction in the endothelium by co-immunostaining of 3-NT and IK_{Ca} in the cross sections of mouse SMA (Fig. 6**B**). These data suggest that HHcy aggravated T2DM-induced IK_{Ca} oxidation in micro-vascular ECs.

3.10. NaHS caused EDHF-induced vascular relaxation, which was enhanced in T2DM SMAs and potentiated by HHcy via ATP-activated potassium channel (K_{ATP}) activation

H_2S has been suggested to be one of EDHF causing EC and SMC hyperpolarization via K_{Ca} and K_{ATP} [39]. We hypothesized that insufficient H_2S is responsible for T2DM/HHcy-impaired EDHF-induced vascular relaxation in micro-vasculature of mice. To validate this hypothesis, we firstly tested whether reduced H_2S in T2DM/HHcy is responsible for impaired EDHF-induced relaxation by examining EDHF-induced relaxation to L-cysteine, a precursor of endogenous H_2S for 30 min. Surprisingly, we found that L-cysteine can cause EDHF-induced vascular relaxation, which was not altered in db/db and db/db/HHcy mice (Fig. 7**A**), suggesting that L-cysteine display EDHF function but not directly contribute to H_2S deficiency related EDHF dysfunction in T2DM and T2DM/HHcy mice. Next we examined EDHF-induced vascular relaxation to exogenous H_2S. We found that vascular relaxation to NaHS in the presence of L-NAME plus INDO was enhanced in db/db mice compared with that in db/+ mice (maximal relaxation: 77.4 ± 4.4 vs 50.6 ± 5.0, Fig. 7**B**, p < 0.05). HHcy further enhanced EDHF-induced relaxation to NaHS in db/db mice to a greater extend (maximal relaxation: 85.7 ± 1.2, p < 0.05). Increased sensitivity to NaHS in T2DM/HHcy SMA was abolished by K_{ATP} blocker GLB, but not changed in the presence of three K_{Ca} blockers (Fig. 7**C** to **F**). Our findings suggest that the increased sensitivity of SMAs to H_2S in SMAs of T2DM and T2MD/HHcy mice is largely due to K_{ATP} activation, and may play a compensatory effect for insufficient endogenous H_2S production.

4. Discussion

We investigated the single and combined effects of HHcy and T2DM on endothelial function of mouse micro-vasculature SMA and reported four major novel findings. Firstly, T2DM worsens HHcy induced by HM diet. Secondly, HHcy aggravated T2DM-impaired EDHF-induced vascular relaxation to ACh in SMA via IK_{Ca} inactivation. Thirdly, H_2S deficiency, due to downregulation of CSE, contributes to IK_{Ca} oxidation. Fourthly, H_2S and L-cysteine display EDHF function and could be EDHF.

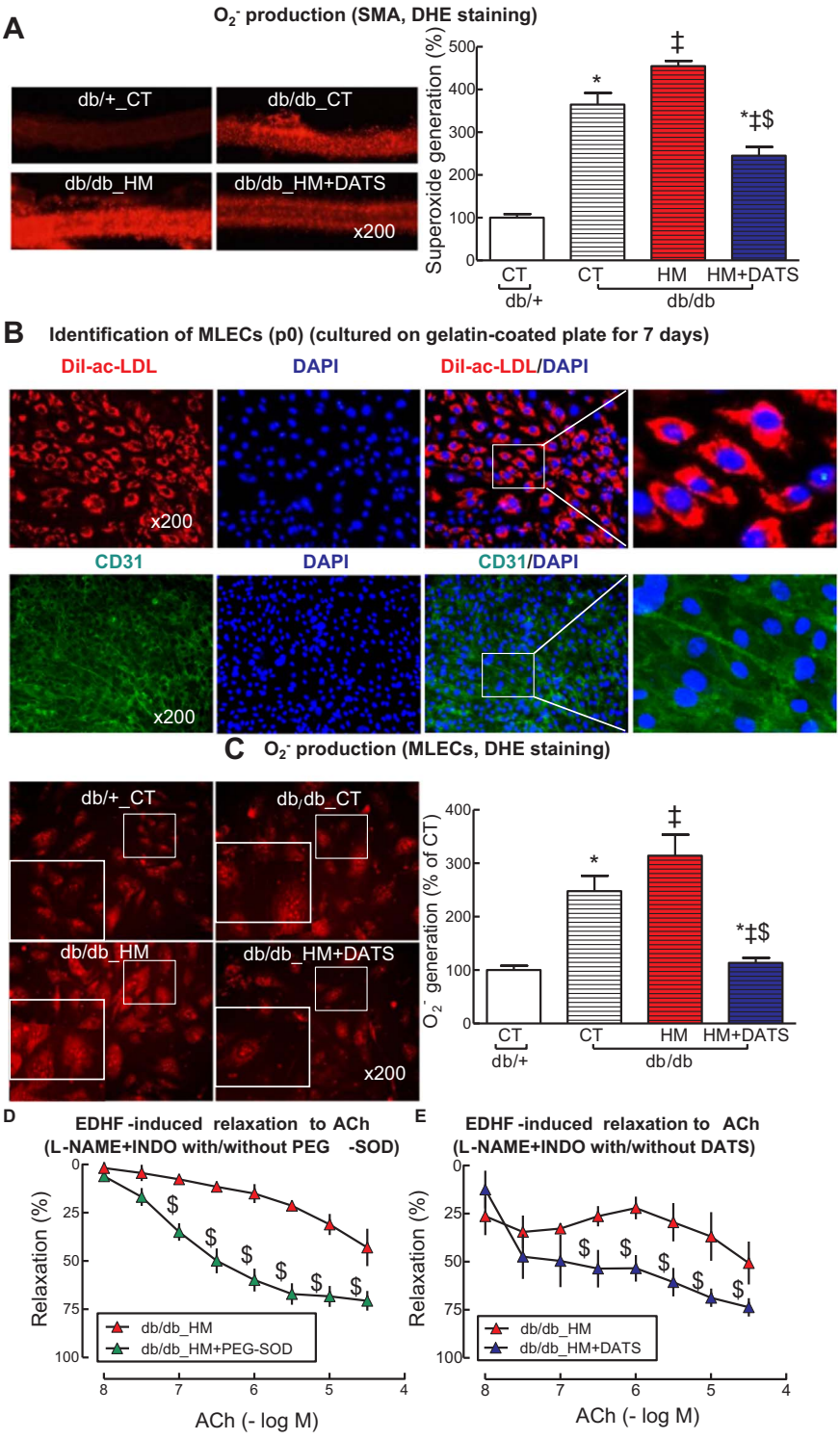

Fig. 5. H_2S donor DATS rescued HHcy-induced oxidative stress and -impaired EDHF-induced relaxation to ACh in SMA of db/db mice with HHcy. A. Representative images (Left panel) and quantifications (Right panel) of in situ O_2^- production in SMAs stained with DHE. The SMAs from db/db mice fed with HM diet were treated with or without DATS (5 μM) for 30 min before DHE staining. B. Identification of primary mouse lung endothelial cells (MLECs) isolated from lung of db/+ and db/db by Dil-ac-LDL uptake and CD31 staining. C. Representative images (Left panel) and quantifications of O_2^- production (Right panel) in mouse MLECs (DHE staining). The MLECs from db/db mice fed with HM diet were treated with or without DATS (5 μM) for 30 min. D. EDHF-induced relaxation to ACh in SMAs of db/db mice fed with HM diet in the presence of L-NAME + INDO (30 min) with or without PEG-SOD (150 U/ml) for 1 h. E EDHF-induced relaxation to ACh in SMAs of db/db mice fed with HM diet in the presence or L-NAME + INDO with or without DATS (5 μM) for 30 min. Then the SMAs were contracted with phenylephrine (1 μM), and examined for relaxation to ACh. n = 3–5. *p < 0.05 vs db/+ mice on CT diet (db/+_CT); ‡p < 0.05 vs db/db mice on CT diet (db/db_CT); $p < 0.05 vs db/db mice on HM diet (db/db_HM). DATS, diallyl trisulfide; DHE, dihydroethidium. PEG-SOD, polyethylene glycol superoxide dismutase.

We propose that administration of H_2S may open a novel avenue for prevention and therapeutics of CVD in patients with T2DM and HHcy.

We used db/+ mice as the non-diabetic control and found that db/+ mice on 8 week HM diet developed intermediate HHcy (tHcy = 31.3 μM), which neither impaired endothelium-dependent relaxation to ACh, nor affected vascular contraction to KCl, PE and the endothelium-independent relaxation to SNP (Fig. 1). This is in good accordance with our previous findings showing that diet-induced mild HHcy (22.0 μM) did not impair endothelial function in non-diabetic mice, but intermediate HHcy (tHcy = 52.6 μM) aggravated hyperglycemia-impaired endothelial function in the aorta of STZ-treated mice [15]. We also reported that severe HHcy (tHcy = 169.5 μM) impaired endothelium-dependent vascular relaxation to Ach, as well as EDHF-induced relaxation, in SMAs of non-diabetic mice [13]. In the present study, we induced intermediate HHcy in db/db mice by using our own designed HM diet fed and examined its effect on vascular reactivity with a focus on EDHD-induced vascular relaxation in the micro-vasculature.

We discovered that HHcy aggravated impaired EDHF-induced relaxation to Ach in SMA of db/db mice, determined by measuring

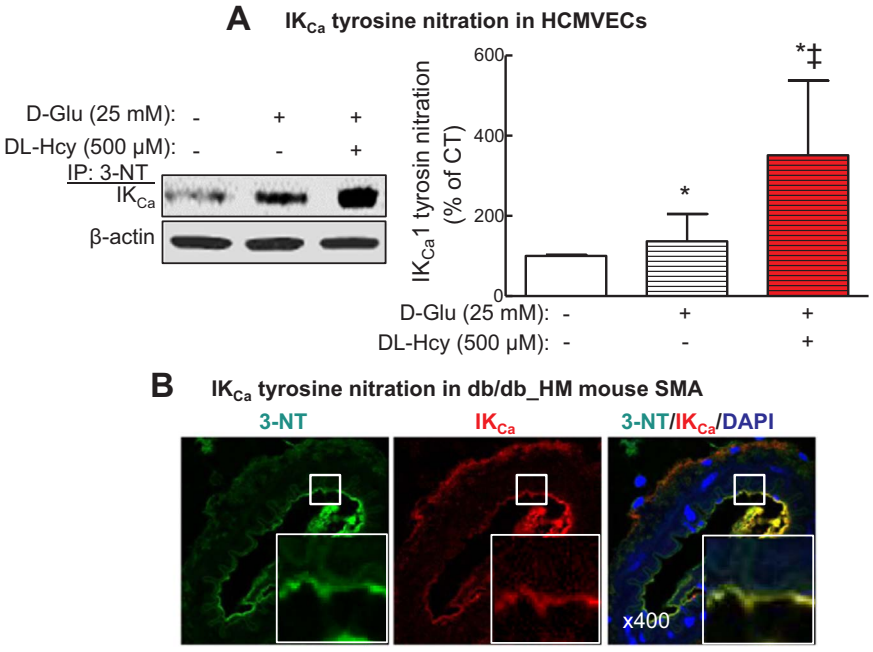

Fig. 6. HHcy potentiated hyperglycemia-induced tyrosine nitration of IK_{Ca}. **A**. IK_{Ca} levels in HCMVECs after immunoprecipitation with 3-NT antibody. HCMVECs were treated with D-glucose (D-Glu, 25 mM) or D-Glu plus DL-homocysteine (DL-Hcy, 500 μM) for 48 h. *p < 0.05 vs HCMVECs treated with vehicle; ‡p < 0.05 vs HCMVECs treated with D-Glu). **B**. Representative images of co-staining of 3-nitrotyrosine (3-NT, green) and IK_{Ca} (red) and DAPI (blue) in SMAs of db/db mice fed with HM diet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

endothelium-dependent vascular relaxation to ACh in the presence of L-NAME and INDO, a standard approach for EDHF-induced relaxation study [6,8,13]. EDHF is defined as substance or electrical signal generated in and released from endothelium thereby hyperpolarizes VSMC resulting in vascular relaxation [9]. It is generally agreed that EDHF is the major contributor for micro-vasculature relaxation.

Although the nature of EDHF remains unclear, the endothelium-dependent vascular relaxation to ACh in the presence of L-NAME + INDO, are suggested to be initiated by the activation of endothelial IK_{Ca} and SK_{Ca} which result in endothelial cell (EC) hyperpolarizationf [45]. Hyperpolarization of endothelium is then transferred to VSMC by synthesizing or generating signals capable of diffusing through BK_{Ca} and K_{ATP} in VSMC or myoendothelial gap junctions resulting in vascular relaxation. Although both IK_{Ca} and SK_{Ca} regulate EDHF-induced vascular relaxation, plenty of evidence showed that IK_{Ca} plays a major role in the regulation of microcardiovascular homeostasis in mice and humans [46]. Previously, inactivation of IK_{Ca}-related ED was reported in SMA of db/db mice [8]. We also demonstrated that HHcy impairs EDHF-induced vascular relaxation to ACh in SMA by inhibiting SK_{Ca}/IK_{Ca} activities via oxidation and tyrosine nitration related mechanisms [13]. In this study, we observed that EDHF-induced relaxation to ACh was inhibited by a non-selective K_{Ca} blocker TEA and intermediate-conductance K_{Ca} blocker (IK_{Ca}) Tram-34, but not by small-conductance K_{Ca} (SK_{Ca}) blocker Apamin. Our findings suggest that HHcy potentiates T2DM-impaired EDHF function in SMAs via IK_{Ca} inactivation.

Our study confirmed that H_2S is an effective EDHF, because that L-cysteine and NaHS, precursor and donor of H_2S, induced vascular relaxation to ACh in the presence of L-NAME plus INDO (Fig. 7). However, the detail molecular mechanisms of H_2S-induced vascular relaxation in the presence of L-NAME + INDO need further investigation. H_2S has been suggested as a new type of EDHF [40]. H_2S induced cell hyperpolarization measured by patch clamp [41]. H_2S-induced vascular relaxation is endothelium-dependent. Removal of endothelium completely abolished the NaHS-induced relaxation in human and rat mesenteric arteries [42,43]. Vascular relaxation to ACh in SMAs was virtually abolished in the presence of L-NAME + INDO in CSE-deleted mice [11,39]. We demonstrated that the levels of H_2S, free sulfide and CSE protein were all reduced and further potentiated by intermediate HHCy in T2DM mice SMA, similar and associated with impaired EDHD-induced SMA relaxation. These data lead us to hypothesize that reduced CSE, the key enzyme for H_2S generation, may be the cause of H_2S reduction, which is responsible for T2DM-induced and HHcy-potentiated impairment of EDHF-induced relaxation. Our results are in good accordance with recent findings that vascular relaxation response to NaHS was enhanced in the aorta of non-obese diabetic mice [22], and in polyamory arteries of STZ-treated diabetic mice [44].

Previously, we and others reported that oxidative stress is one of the major sources for IK_{Ca} inactivation [13]. Here we show that H_2S is a powerful antioxidant. We employed two methods to measure H_2S levels, in situ staining with a H_2S probe and analytical quantitation of free sulfide using HPLC. We found that HHcy potentiated H_2S and free sulfide reduction in the SMAs of diabetic db/db mice, and that H_2S donor DATS rescued T2DM/HHcy-induced superoxide production in SMAs and MLECs (Fig. 5). In summary, our H_2S studies demonstrate that: 1) HHcy aggravated H_2S reduction, CSE downregulation and oxidative stress in SMAs of T2DM mice; 2) HHcy aggravated T2DM-impaired EDHF function, at least in part, via oxidation/inactivation of IK_{Ca}; 3) DATS, a stable H_2S donor, rescued oxidative stress in SMAs and MLECs of T2DM mice; 4) Antioxidant PEG-SOD and DATS improved EDHF function in SMAs of T2DM/HHcy mice. These findings allow us to propose a working model of T2DM/HHcy-induced ED in micro-vasculature. We conclude that HHcy aggravated H_2S reduction via CSE suppression in diabetic micro-vascular ECs, leading to IK_{Ca} oxidation/inactivation and ED (Fig. 8).

The underlying mechanisms of H_2S as an antioxidant remain unclear. It may be related with: 1, quenching free radicals as a chemical reductant; 2, scavenging free radical via nonenzymatic antioxidants; 3, activating antioxidant enzymes; 4, inhibiting mitochondrial free radicals production [37], We previously reported that HHcy induced oxidative stress in SMA [13] and enhanced hyperglycemia-induced superoxide generation in aortic arteries [15,47]. The current studies provide additional evidence suggesting that insufficient H_2S is likely, at least one of mediators of oxidative stress in T2DM/HHcy which induces ED in micro-vasculature.

Together with the effect of D-glucose and D-glucose/DL-Hcy on induction of IK_{Ca} oxidation in HCMVECs (Fig. 6), we believe that deficient H_2S production/oxidative stress and tyrosine nitration of IK_{Ca} play important role in impaired EDHF function in T2DM/HHcy mice. Restoration of H_2S bioavailability may open a novel avenue for prevention and treatment of micro-vascular complications in patients with T2DM/HHcy. Details studies to explore underlying mechanisms and

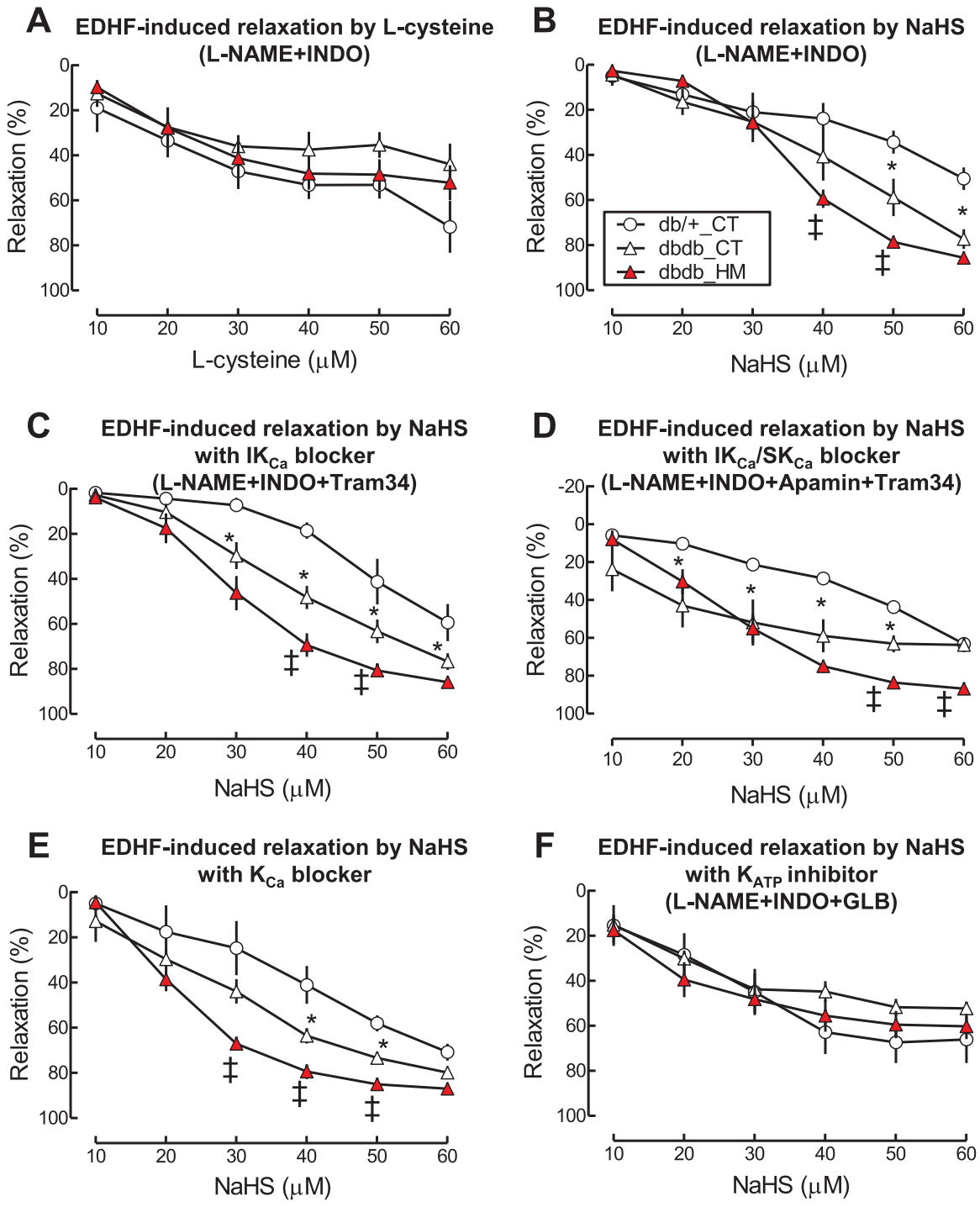

Fig. 7. HHcy sensitized H_2S donor NaHS-induced vascular relaxation in SMA of db/db mice via activation of K_{ATP}. EDHF-**induced** relaxation in SMA was determined by pretreated the SMA with NOS inhibitor L-NAME (100 μM) and COX inhibitor (INDO, 10 μM) and indicated inhibitors, precontracted with phenylephrine (1 μM), and examined for relaxation to L-cysteine or NaHS. **A**. EDHF-induced relaxation by L-cysteine (10 μM). **B**. EDHF-induced relaxation by NaHS. **C**. EDHF-induced relaxation to NaHS in the presence of IK_{Ca} inhibitor Tram-34 (1 μM). **D**. EDHF-induced relaxation to NaHS in the presence of IK_{Ca} inhibitor Tram-34 and SK_{Ca} inhibitor Apamin (1 μM). **E**. EDHF-induced relaxation to NaHS in the presence of K_{Ca} inhibitor TEA (1 mM). **F**. EDHF-induced relaxation to NaHS in the presence of K_{ATP} inhibitor glibenclamide (GLB, 3 μM). n = 5–8 *p < 0.05 vs db/+ mice on CT diet (db/+_CT); ‡p < 0.05 vs db/db mice on CT diet (db/db_CT).

effects of deficient H_2S on IK_{Ca} oxidation and inactivation may lead to the identification of novel therapeutic targets.

Previous studies showed that H_2S induces EC and VSMC hyperpolarization via opening IK_{Ca}/SK_{Ca} in ECs and BK_{Ca}/K_{ATP} in VSMCs, respectively [39,48,49]. Selective IK_{Ca} and SK_{Ca} channel blockers, Charybdotoxin and Apamin inhibited H_2S-induced vascular relaxation in SMA of CSE knockout mice [39] and aorta of Sprague-Dawley rats [49], but failed to influence the concentration-dependent relaxation induced by NaHS in human mesenteric arteries [42]. Moreover, Non-specific K_{Ca} blocker TEA completely inhibited vascular relaxation to NaHS in the presence of L-NAME + INDO in rat cerebral arteries.[50]. Interestingly, we discovered an enhanced EDHF relaxation to NaHS in T2DM/HHcy mice, which was not inhibited by any of K_{Ca} channel blockers (Fig. 7 B-E). Because that K_{ATP} may also mediate H_2S-induced relaxation [39], we also tested the effect of K_{ATP} blocker glibenclamide (GLC) and found that GLC rescued enhanced relaxation to NaHS in T2DM/HHcy mice.

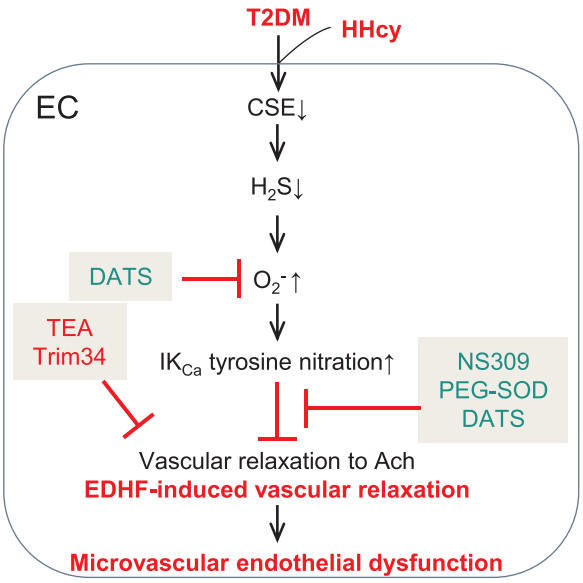

Fig. 8. Mechanism of HHcy-potentiated diabetes-impaired EDHF-induced vascular relaxation. Working model of T2DM/HHcy-induced endothelial dysfunction in microvasculature.

We then conclude that K_{ATP} channel activation is responsible for enhanced EDHF relaxation to NaHS in SMA in TD2M and TD2M/HHcy mice. This difference, compared to previous findings, may be related with the size and type of vessels, and species. Moreover, because enhanced relaxation to exogenous H_2S (NaHS) in the presence of L-NAME plus INDO in T2DM and T2DM/HHcy mice was inhibited by K_{ATP} blocker GLC, we suggest that K_{ATP} is the major target of H_2S in VSMCs which was sensitized by T2DM and HHcy. Our findings support the previous concept that H_2S targets K_{ATP} in VSMCs thus hyperpolarizes VSMCs leading to vasorelaxation [49]. Our findings are also in line with recent studies that H_2S induced vascular relaxation in K_{ATP}-dependent manner in rat aortic arteries and SMAs [8,43]. K_{ATP} blocker GLB reduced vascular relaxation response to H_2S in rat SMAs [43] and K_{ATP} opener pinacidil induced vascular relaxation in rat SMAs whereas K_{ATP} blocker GLB inhibited H_2S induced vascular relaxation [49]. The enhanced vascular relaxation to H_2S in SMAs of T2DM and T2DM/HHcy mice in the presence of L-NAME plus INDO may be a complementary effect to overcome insufficient H_2S production and ED in micro-vasculature under metabolic disorder. It would be ideal to examine whether KCl can completely block vascular relaxation response to H_2S in the presence of L-NAME + INDO thus confirm H_2S as an EDHF regulating endothelial function under T2DM/HHcy condition. It is an ongoing effort in our laboratory to continually explore mechanisms underlying H_2S-induced endothelium-dependent vascular relaxation.

In addition, we observed some resistant relaxations to ACh in the presence of K_{Ca} blockers and NO/PGI_2 inhibitors (Fig. 3**B**). Several potential factors may contribute to these resistant relaxation, including: 1) NO, PGI_2 and K_{Ca} channels may not be completely inhibited, because of the dosage limitation; 2) ACh may stimulate other relaxation in the presence of NO/PGI_2 inhibitors independent from $K_{Ca;}$ 3) Other residual relaxant factors independent from NO, PGI_2 and K_{Ca}. Future studies should explore other EDHF relaxation factors and identify novel therapeutic targets.

In good accordance with our previous study that HM induced higher plasma total Hcy (t-Hcy) levels in mice with hyperglycemia [15], we also observed that the plasma t-Hcy level is higher in db/db mice fed with a HM diet compared that in db/+ mice (Fig. 1**B**). The interaction between HHcy and hyperglycemia/T2DM is not known and need to be addressed in the future studies.

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No potential conflicts of interest relevant to this article were reported.

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Author contribution

Conceived and designed the experiments: ZJC, RK and HW. Performed the experiments: ZJC, XQS, XHJ, HMS, PF and MC. Analyzed the data: ZJC and HW. Contributed reagents/materials/analysis tools: ZJC, YJ, RK, CK, XFY, RK and HW. Wrote the paper: ZJC and HW. ZJC and HW are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2018.02.006.

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