Hyperhomocysteinemia and hyperglycemia induce and potentiate endothelial dysfunction via μ-calpain activation

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Abstract

Plasma homocysteine (Hcy) levels are positively correlated with cardiovascular mortality in diabetes. However, the joint effect of hyperhomocysteinemia (HHcy) and hyperglycemia (HG) on endothelial dysfunction (ED) and the underlying mechanisms have not been studied.

Mild (22 µmol/L) and moderate HHcy (88 µmol/L) were induced in cystathionine β-synthase wild type (Cbs+/+) and heterozygous deficient (Cbs+/−) mice by a high methionine (HM) diet. HG was induced by consecutive injection of streptozotocin. We found that HG worsened HHcy and elevated Hcy levels to 55 µmol/L and 173 µmol/L in Cbs+/+ and Cbs+/− mice fed a HM diet, respectively. Both mild and moderate HHcy aggravated HG-impaired endothelium-dependent vascular relaxation to acetylcholine, which was completely abolished by endothelial nitric oxide synthase (eNOS) inhibitor L-NAME. HHcy potentiated HG-induced calpain activation in aortic endothelial cells isolated from Cbs mice. Calpain inhibitors rescued HHcy- and HHcy/HG-induced ED in vivo and ex vivo. Moderate HHcy and HG-induced µ-calpain activation was potentiated by a combination of HHcy and HG in the mouse aorta. µ-calpain siRNA (µ-calpsiRNA) prevented HHcy/HG-induced ED in the mouse aorta and calpain activation in human aortic endothelial cells (HAECs) treated with DL-homocysteine (500 µmol/L) and D-glucose (25 mmol) for 48 hrs. In addition, HHcy accelerated HG-induced superoxide production as determined by DHE and 3-NT staining and urinary 8-isoprostane/creatinine assay. Antioxidants rescued HHcy/HG-induced ED in mouse aortas and calpain activation in cultured HAECs. Finally, HHcy potentiated HG-suppressed NO production and eNOS activity in HAECs, which were prevented by calpain inhibitors or µ-calpain siRNA. HHcy aggravated HG-increased phosphorylation of eNOS at threonine
497/495 in the mouse aorta and HAECS. HHcy/HG induced eNOSp-Thr497/495 was reversed by μ-calpsiRNA and adenoviral transduced dominant negative PKCβ2 in HAECS.

HHcy and HG induced ED, which was potentiated by the combination of HHcy and HG via μ-calpain/PKCβ2 activation-induced eNOSp-Thr497/495 and eNOS inactivation.

Key words: hyperhomocysteinemia, hyperglycemia, diabetes, endothelial dysfunction, calpain.
Introduction

Cardiovascular disease (CVD) is one of the most prevalent complications and a major cause of premature mortality in patients with diabetes. Numerous factors have been suggested related to CVD in diabetes, such as hyperinsulinemia, hyperlipidemia, hyperglycemia, obesity, and smoking. Recently, accumulative evidence indicated that hyperhomocysteinemia (HHcy), referring to elevated concentrations of plasma homocysteine (Hcy), is also linked to CVD in diabetes.

HHcy has been established as an independent and significant risk factor for CVD [1]. Recent studies shown a high prevalence of HHcy in patients with diabetes, and that plasma concentration of Hcy is positively correlated with macrovascular diseases [2], cardiovascular morbidity and mortality [3] in diabetes. Endothelial dysfunction (ED) is an early event in the development of CVD which is defined by reduced endothelium-dependent vascular relaxation to acetylcholine (ACh) [4]. It has been suggested that increased plasma Hcy and blood glucose (HG) levels may be responsible for ED in micro- and macro-vasculature via different signaling pathways in diabetes. However, whether and how a combination of HHcy and HG, which is commonly seen in human and associated largely with increased cardiovascular mortality has a joint effect on the development of CVD, is unknown.

We and others reported that HHcy impairs endothelial function in mouse aortas [5], cremaster microvasculatures [5], and small mesenteric arteries [6]. We also demonstrated that HHcy induces ED, protein kinase C (PKC)-mediated phosphorylation of endothelial nitric oxide synthase (eNOS) at threonine 495 (PKC-eNOSp-Thr495), and inactivation of eNOS in mouse thoracic aortas [5].
PKC is an important signaling molecule associated with ED in diabetes, and a substrate of calpain, a family of calcium-dependent cysteine-proteases [7, 8]. Calpain cleaves and constitutively activates PKC, which leads to induction of a variety of signal transduction processes [7, 8]. In the calpain family, μ- and m-calpain are well-characterized and abundantly expressed in ECs [9]. Activation of calpain mediates acute and chronic hyperglycemia (HG)-induced leukocytes-endothelium integration in rat mesenteric arteries [10, 11]. Calpain activation was also found in primary hepatocytes of Cbs^+/+ mice and Hcy-treated cultured rat heart microvascular ECs [12, 13]. The role of calpain and its molecular targets in HHcy- and HHcy/HG-related ED, however, has not been studied.

In this study, we examined the joint effects and underlying mechanisms of HHcy and HG on endothelial function in our newly developed HHcy/HG mouse model using cystathionine β-synthase heterozygous (Cbs^+/+) and wild type (Cbs^+/+) mice. We provide strong evidence showing that μ-calpain activation plays a critical role in HHcy/HG-induced macrovascular ED.
Material and Methods

**Experimental animals and sample preparations** — HHcy was induced in male $Cbs^{-/-}$ or $Cbs^{+/-}$ mice (Jackson Laboratory) by feeding 8-week old mice with our newly designed high methionine (HM, 2%, 07794, Harlan Teklad) diet in which folic acid and B vitamins are reduced to the sufficient basal levels [14] for 8 weeks. HG was induced by injection of streptozotocin (STZ) (i.p., 40 mg/kg BW) for 5 consecutive days at the age of 8 weeks. All animals received humane care in compliance with institutional guidelines 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.

One week after the last STZ injection, mice with blood glucose levels above 16.7 mmol/L were used for HG studies. Endothelial function was assessed by endothelium-dependent vascular relaxation to acetylcholine (ACh) and –independent relaxation to sodium nitroprusside (SNP) in the mouse thoracic aorta using a multiple-wire myograph (DMT 610) [6, 15, 16]. Roles of NO, calpain, and μ-calpain activations, and oxidative stress in HHcy- and/or HG-induced ED were examined in the presence of eNOS inhibitor, calpain inhibitors, calpain siRNA (calpsiRNA), and antioxidants in vivo or ex vivo. 5 mm thoracic aorta were fixed in 4% buffered paraformaldehyde and cut with the microtome (HM310) for immunostaining. The rest of aorta was used for Western blot.

**Isolation of mouse aortic endothelial cells** — Mouse aortic endothelial cells (MAECs) were isolated using collagenase as previously described [17], and identified with staining of VWF and CD31.
**Cell culture** — Human aortic endothelial cells (HAECs) were cultured as previously described [18].

MAECs or HAECs were treated with DL-Hcy (200 or 500 µmol/L) and/or D-glucose (D-Glu, 25 mmol/L) for 24 or 48 hrs in the presence or absence of selected pharmacological inhibitors, siRNA or adenovirus.

**Calpain activity** — “t-BOC” assay was used for calpain activity measurement by treating ECs with t-butoxycarbonyl-leu-met-chloromethyl aminocoumarin (Boc-LM-CMAC, Molecular Probe) as previously described [19].

μ- and m-calpain are constitutively expressed in ECs [20]. When calpains are activated, they undergo autoproteolysis which removes the N-terminal (NT, 27 and 19 amino acids from the large subunit (catalytic subunit) of μ- and m-calpain, respectively, **Fig. 4A** [20]. Therefore, an antibody directed against calpain’s N-terminus can be used to determine calpain’s proteolytic activity; lower N-terminus levels indicate higher proteolytic activity [9].

We examined μ- and m-calpain activity by Western blot analysis using polyclonal antibodies against NH₂ terminus domain of μ- and m-calpain (Abcam), respectively, as previously described [10, 21, 22]. Total μ- and m-calpain expression was also detected using polyclonal antibodies against domain IV (Abcam).

**RNA interference in ECs and aorta** — HAECs were transfected with μ-calpsiRNA or control siRNA (CTsiRNA) in accordance with the manufacturer's instructions (Santa Cruz). The transfection efficacy was determined by Western blot using rabbit polyclonal antibody against μ-calpain.

**In situ superoxide production by DHE staining** — 30 µm cross-section of fresh aorta
segments were equilibrated for 30 minutes at 37°C in Krebs buffer, and then incubated with dihydroethidium (DHE, 3 µmol/L) for 30 minutes at 37°C in the dark.

**3-nitrotyrosin (3-NT) expression** — 3-NT in the mouse aorta (5 µm sections) was detected using an anti-3-NT monoclonal antibody (Santa Cruz) and co-stained with 3, 3′-diaminobenzidine (DAB).

**NO production and eNOS activity** — NO production in HAECs was detected by staining with 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein (DAF-FM). To explore the role of calpain, especially µ-calpain, DL-Hcy/D-Glu-treated HAECs were also treated with MDL28170 (MDL, 20 µmol/L) and µ-calpsiRNA, respectively. HAECs treated with an eNOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME,100 µmol/L) for 1 hr before DAF-FM staining were used as negative controls. eNOS activity in HAECs was determined by measuring the conversion of ^3H-Arg to ^3H-citrulline as previously described [5].

**eNOS uncoupling** — eNOS monomer, an indicator of eNOS uncoupling was examined by Western blot using low-temperature SDS-PAGE as previously described [23]. Moreover, O_2^- produced by eNOS, a maker of eNOS uncoupling, was examined by DHE staining in the presence and absence of L-NAME [24].

**Biochemical determinations** — Total plasma Hcy (t-Hcy) levels were measured using a Biochrom 30 amino analyzer (Cambridge, UK) as described previously [25]. Blood glucose levels were determined by a glucometer (HemoCue). Urinary 8-isoprostan and creatinine levels were determined by ELISA kit (Cayman).

**Statistical analysis** — Results are expressed as the mean ± SEM. For statistical comparison of single parameters, unpaired-t test was used for two groups from the same
mouse strain or from different mouse strains. One-way ANOVA with Tukey adjustment was performed for multiple groups in each mouse strain. $p < 0.05$ was considered to be significant.

**Reagents** — All reagents were purchased from Sigma, unless otherwise stated.
Results

Plasma t-Hcy and blood glucose levels, and body weights — Mild and moderate HHcy were induced by HM diet in \( \text{Cbs}^{+/+} \) and \( \text{Cbs}^{-/-} \) mice, respectively (Fig. 1A). STZ injections resulted in HG in \( \text{Cbs}^{+/+} \) and \( \text{Cbs}^{-/-} \) mice (Fig. 1B). HM diet induced higher levels of plasma t-Hcy; moderate and severe HHcy in \( \text{Cbs}^{+/+} \) and \( \text{Cbs}^{-/-} \) mice with STZ injections, respectively (p<0.05). (Fig. 1A). Body weight was significantly reduced in mice with either HHcy or HG alone (Fig. 1C), and to a greater extent in mice with HHcy/HG (p<0.05).

Suppressed NO bioavailability mediates HHcy/HG-induced ED — Mild HHcy in \( \text{Cbs}^{+/+} \)/HM mice had no effect on endothelial function (Fig. 2A. left panel), whereas moderate HHcy in \( \text{Cbs}^{+/+} \)/HM mice impaired vascular relaxation to ACh (p<0.05, Fig. 2A. right panel, Online Fig. 1A). HG impaired vascular relaxation to ACh in both \( \text{Cbs}^{+/+} \)/STZ and \( \text{Cbs}^{-/-} \)/STZ mice (p<0.05). A combination of moderate HHcy and HG in \( \text{Cbs}^{+/+} \)/HM/STZ mice potentiated ED to a greater level when compared to that from either \( \text{Cbs}^{+/+} \)/HM mice with moderate HHcy alone or \( \text{Cbs}^{+/+} \)/STZ and \( \text{Cbs}^{-/-} \)/STZ mice with HG alone (Fig. 2A). Notably, a combination of severe HHcy plus HG in \( \text{Cbs}^{+/+} \)/HM/STZ mice potentiated ED to a greater level (p<0.05, Fig. 2A right panel, Online Fig. 1A). Vascular relaxation to ACh was completely abolished by eNOS inhibitor L-NAME in all groups (Fig. 2B, Online Fig. 1B). Endothelium-independent vascular relaxation to SNP was not changed in all groups (Fig. 2C, Online Fig. 1C).

Activation of calpain mediates HHcy/HG-induced ED — To study the role of calpain in HHcy- and HHcy/HG-induced ED, we first examined calpain activity in MAECs (Online Fig. 2) isolated from \( \text{Cbs}^{+/+} \) mice using t-BOC assay. Calpain activity was increased by 1.44-
and 1.75-fold in MAECs from mice with moderate HHcy and HG alone, respectively (p<0.05, Fig. 3A). Notably, calpain activity was potentiated by 3.26-fold (p<0.05) in mice with combined severe HHcy and HG. We next tested whether HHcy and/or HG-mediated calpain activation regulates ED by examining the effects of calpain inhibitors. Calpain inhibitor MDL-28170 (MDL) administrated daily (2 mg/kg BW, i.p.) for 2 weeks [26] largely improved endothelial function in Cbs++/HM and Cbs++/HM/STZ mice (p<0.05, Fig. 3B left panel, online Fig. 3). Moreover, preincubation of aortic rings with calpain inhibitors MDL, calpeptin, and ALLM (20 µmol/L, 1 hr) markedly improved vascular relaxation to ACh in Cbs++/HM/STZ mice (p<0.05, Fig. 3B right panel, online Fig. 3).

Activation of µ-calpain mediates HHcy/HG-induced ED — µ- and m-calpain are constitutively expressed in ECs [20]. We found that moderate HHcy in Cbs+++/HM mice, but not mild HHcy in Cbs+++/HM mice, significantly increased µ-calpain activity as µ-calpain NT levels were reduced to 17% (p<0.05, Fig. 4B). HG alone increased µ-calpain activity as µ-calpain NT levels were reduced to 32% and 13% in Cbs+++/STZ and Cbs+++/STZ mice, respectively (p<0.05). The combination of HHcy and HG activated µ-calpain in both mouse strains to a greater extent (9% and 2% of µ-calpain NT levels, respectively, p<0.05, Fig. 4B). m-calpain activity was not changed (Fig. 4C). HHcy and/or HG had no effect on total µ- and m-calpain protein levels (Fig. 4D and E). Moreover, silencing the µ-calpain gene by µ-calpsiRNA (Fig. 4G) significantly rescued HHcy/HG-induced ED in mouse aorta treated with Hcy and glucose (p<0.05, Fig. 4F and Online Fig. 4). Additionally, µ-calpsiRNA markedly reversed HHcy/HG-induced calpain activation in HAECs treated (p<0.05, Fig. 5E).

HHcy and HG induce oxidative stress to a greater extent than either factor.
independently — One of the most important potential mechanisms leading to calpain activation is oxidative stress [27]. We found that moderate HHcy or HG alone markedly increased $O_2^-$ generation, and the combination of HHcy and HG further promoted $O_2^-$ generation in the aortic endothelium of $Cbs^{-/-}$/HM/STZ mice and in MAECs to a greater extent than either factor independently (Fig. 5A, B). PEG-SOD significantly prevented the combination of HHcy and HG-induced $O_2^-$. Because peroxynitrite (ONOO\textsuperscript{-}) is a strong and relatively stable oxidant species capable of causing nitrosylation of tyrosine residues (3-NT) on proteins, we also examined the content of 3-NT in the aorta of $Cbs^{-/-}$ mice. We found that moderate HHcy or HG alone significantly increased 3-NT levels in the aortic endothelium, which was dramatically elevated in the combination of HHcy and HG (Fig. 5C). Moreover, HHcy alone increased the levels of urinary 8-isoprostan, a prostaglandin-like compound formed from the free radical-catalyzed peroxidation of arachidonic acid, by 1.8- and 2.2-fold in $Cbs^{+/+}$/HM and $Cbs^{+/+}$/HM mice, respectively (p<0.05, Fig. 5D). HG alone increased urinary 8-isoprostan levels by 3.4 and 4.6-fold in $Cbs^{+/+}$/STZ and $Cbs^{+/+}$/STZ mice, respectively. A combination of moderate or severe HHcy and HG markedly enhanced urinary 8-isoprostan levels by 9.4- and 9.7-fold in $Cbs^{+/+}$ HM/STZ and $Cbs^{+/+}$ HM/STZ mice, respectively.

**Oxidative stress mediates HHcy/HG-induced ED —** The vascular relaxation to ACh was significantly improved in the aorta of $Cbs^{+/+}$ HM/STZ mice by pre-incubating the aortic rings with antioxidants PEG-SOD, Tempol, and apocynin for 1hr (p<0.05, Fig. 5E, Online Fig. 5).

**Oxidative stress mediates HHcy/HG induced μ-calpain activation —** To address the
relevance to human pathogenesis, we examined the effect of HHcy and HG on O$_2^-$ production and 3-NT expression in HAECs. We found that DL-Hcy and D-Glu treatment for 48 hrs significantly increased O$_2^-$ production by 1.4- and 1.7-fold, and 3-NT expression by 1.5- and 3.0-fold, respectively (p<0.05, Fig 5 F, G). The combination of DL-Hcy and D-Glu promoted O$_2^-$ production by 2.5-fold and 3-NT expression by 5-fold, respectively. PEG-SOD significantly prevented O$_2^-$ production and 3-NT expression in HAECs. Next we examined the effects of oxidative stress on calpain, especially µ-calpain activity. We found calpain activity was increased by 1.8- and 2.3-fold in HAECs treated with DL-Hcy and D-Glu for 48 hrs, respectively (Fig. 5H). The combination of DL-Hcy and D-Glu increased calpain activity by 4.9-fold (Fig. 5F, p<0.05). PEG-SOD significantly decreased HHcy/HG-induced calpain activation by 49% (p<0.05). We also examined the role of µ-calpain in HHcy/HG-induced calpain activation by knocking down µ-calpain gene in HAECs with µ-calpsiRNA (72 hrs) (Fig. 5G). We found that µ-calpsiRNA rescued HHcy/HG-induced calpain activation (Fig. 5F), suggesting µ-calpain plays a major role.

Activation of µ-calpain mediates HHcy/HG-induced NO reduction and eNOS inactivation — DL-Hcy and D-Glu for 48 hrs significantly reduced NO production to 78% and 36% and eNOS activity to 69% and 52% (p<0.05, Fig. 6) in HAECs, respectively. The combination of DL-Hcy plus D-Glu further reduced NO production and eNOS activity to 26% and 35%, respectively (p<0.05). Calpain inhibitor MDL largely improved NO production and eNOS activity from 26% to 86% and 35% to 74%, respectively (p<0.05, Fig. 6). Moreover, µ-calpsiRNA markedly improved NO production from 26% to 94% and eNOS activity from 26% to 86% in Hcy/glucose treated HAECs (p<0.05, Fig. 6). eNOS inhibitor L-NAME
diminished NO generation and eNOS activity to 5.7% and 10%, respectively.

**HHcy/HG-induced eNOS-pThr495 is rescued by PKCβ2 suppression** — Neither HHcy or HG changed eNOS protein levels in the mouse aorta (p<0.05, Fig. 7A, left panel). In HAECs, DL-Hcy decreased eNOS protein levels by 59% from 100%, whereas, D-Glu had no effect on eNOS protein levels both in the presence or absence of DL-Hcy (Fig. 7A, right panel).

Phosphorylation of eNOS at threonine 497 (rodent) or 495 (human) (eNOS-pThr497/495) by activation of PKC—a substrate of calpain [7, 8], is a negative regulator for eNOS activation. We found that HHcy and HG increased eNOS-pThr497/495 (mouse/human) levels by 1.7- and 3.6-fold in the Cbs+/+ mouse aorta (p<0.05, Fig. 7B left panel), and 1.6- and 1.5-fold in HAECs (p<0.05, Fig. 7B right panel), respectively. The combination of HHcy and HG potentiated eNOS-pThr497/495 levels by 5.3- and 2.2-fold in the mouse aorta and HAECs, respectively (p<0.05). Moreover, non-selective PKC inhibitor GFX and silencing of PKCβ2 gene by transfecting adenoviral transduced dominant negative PKCβ2 (Adv-dnPKCβ2) rescued HHcy/HG-induced eNOS-pThr497/495 in HAECs (Fig. 7D). HHcy/HG-induced eNOS-pThr495 was also rescued by MDL and µ-calpsiRNA.

Another possible mechanism for eNOS inactivation is eNOS uncoupling; eNOS is converted from an NO-producing enzyme (dimer) to the monomer form that generates O$_2^-$ [28]. We found that eNOS monomer, a maker for eNOS uncoupling, was not increased by neither DL-Hcy and D-Glu alone nor the combination (Online Fig. 6A). We also examined the content of O$_2^-$ produced by eNOS, another indicator of eNOS uncoupling, in the HAECs treated with the combination of DL-Hcy and D-Glu. We found that HHcy/HG-induced O$_2^-$
was not changed by eNOS inhibitor L-NAME, suggesting eNOS uncoupling is not involved (Online Fig. 6 B, C).
Discussion

We investigated the individual and combined effects of HHcy and HG on endothelial function in the thoracic aortas of $Cbs^{+/+}$ and $Cbs^{-/-}$ mice. We reported two major novel findings. Firstly, HG worsens HHcy, but not vice versa, in mice. Secondly, HHcy and HG induce ED and potentiate each other’s effect, and Hcy dose-dependently aggravated HG-induced ED via μ-calpain/PKCβ2 activation-induced eNOSp-Thr497/495 and eNOS inactivation (Fig. 8). We propose that μ-calpain activation causes ED and contributes to HHcy-heightened cardiovascular risk seen in diabetic patients. The interaction between HHcy and HG metabolism is not known. μ-calpain-induced eNOS inactivation has not been addressed in HHcy and the combination of HHcy plus HG. Our study provides novel mechanistic insights for vascular diseases in these metabolic disorders.

NO is synthesized within ECs by eNOS. Loss of NO bioavailability and eNOS activity has been implicated in several disease states such as coronary artery disease, hypertension, heart failure, HHcy, and diabetes. Here we found that both HHcy and HG impaired vascular relaxation to ACh. Notably, Hcy dose-dependently aggravated HG-induced ED. Moreover, eNOS inhibitor L-NAME completely blocked vascular relaxation to ACh in the aortas from mice with HHcy and/or HG, suggesting that decrease of NO bioavailability plays a major role.

Decreased NO bioavailability has been suggested be related to NO reduction and enhanced reaction of NO with $O_2^-$ resulting in increased peroxynitrite (ONOO⁻) formation. Here we found that the combination of HHcy and HG potentiated HHcy- or HG -induced NO reduction and eNOS inactivation in HAECs. In addition, HHcy and HG also potentiated
3-NT expression, a maker for ONOO’, in mouse aorta and HAECs. Thus we demonstrated that HHcy/HG decreases NO bioavailability via inactivation of eNOS and enhanced reaction of NO with \( \text{O}_2^- \).

Under pathological stimuli, eNOS uncoupling is suggested to be one of the important principles for eNOS inactivation [29]. It was suggested that HHcy induces eNOS-uncoupling by decreasing L-arginine uptake [30], intracellular BH\(_4\) bioavailability [31], and catabolic degradation activity of DDAH which causes ADMA accumulation [32]. We examined eNOS uncoupling under HHcy and/or HG condition in HAECs and found that neither eNOS monomerization nor eNOS-produced \( \text{O}_2^- \) was increased by HHcy and/or HG. This data suggests that eNOS uncoupling may not be the mechanism mediating HHcy, HG, or their combined effects in eNOS inactivation. This is consistent with our previous findings that HHcy inactivates eNOS mostly via PKC/eNOS-pThr495, but not eNOS uncoupling, as BH\(_4\), sepiapterin, and L-arginine did not rescue HHcy-induced eNOS inactivation in MAECs [5].

Thr497 (rodent)/495 (human) in the CaM-binding domain is one of key negative regulatory sites for eNOS activity. eNOS Thr497/495 can be phosphorylated by AMP-activated kinase and PKC, resulting in reduced eNOS catalytic activity. We previously reported that PKC activation mediates HHcy-induced eNOS-pThr495 and eNOS inactivation in HAECs, and proposed that PKC-induced eNOS-pThr495/497 may play a major role in HHcy-induced ED in microvasculature [5]. In the present study, we found that the combination of HHcy and HG potentiated eNOS-pThr495 which was prevented by PKC inhibitor GF109203X (GFX). Within the PKC family, PKC\( \beta \) has received much attention since it was first shown to be preferentially upregulated in diabetic vascular tissue [33].
PKCβ2 activation mediates HG-induced ED [34] and cardiomyocyte apoptosis [35]. Here, for the first time, we demonstrated that PKCβ2 activation contributes to HHcy/HG-induced eNOS inactivation because PKC inhibitor GFX and Adv-dnPKCβ2 transduction rescued HHcy/HG-induced eNOS-pThr495 in HAECs.

PKC was originally discovered as a kinase cleaved and activated by calpains [7] which tightly regulate their respective substrates through limited proteolytic cleavage. Activation of calpain has been implicated in diabetes-linked platelet aggregation [35], neurovascular dysfunction [36], and cardiomyocyte apoptosis [35]. Recently, increased calpain activation is linked to HG-induced microvascular inflammatory responses, eNOS inactivation, and NO reduction [10, 11]. Here we provided strong evidence that the combination of HHcy and HG potentiates ED via activation of calpain since calpain inhibitor MDL, ALLM, or calpeptin markedly improved vascular relaxation to ACh. Moreover, μ- but not m-calpain activation plays important role in HHcy/HG-induced calpain activation as μ-calpsiRNA markedly inhibited calpain activation and improved endothelial function. Taken together, we demonstrated, for the first time, that μ-calpain activation regulates HHcy/HG-induced ED in mouse aorta via decrease of NO bioavailability.

The interaction between PKC and calpain has been debated. Recent studies suggested that besides as the upstream regulator of PKC activation, calpain can also serve as a downstream target of PKC signaling. PKC induces calpain phosphorylation in cultured cancer cells [37]. PKC inhibitor BIM-1 decreases calpain activity in mouse microvascular endothelial cells [34]. Nevertheless, here we show that both μ-calpain and PKCβ inhibition rescued HHcy/HG-induced eNOS-pThr495. These data suggest that activations of μ-calpain
and PKCβ2 play critical roles in HHcy/HG-induced eNOS-pThr495 and eNOS inactivation. The molecular details for the interaction between PKCβ2 and μ-calpain activation under HHcy/HG condition are warranted.

Activation of calpain leads to disruption of eNOS [38, 39], thus decreasing NO bioavailability. In good accordance with our previous finding [5], we provided evidence that HHcy decreased eNOS expression. However, the combination of HHcy and HG did not further decrease eNOS expression both in HAECs and mouse aortas, suggesting that the degradation of eNOS by calpain does not play a major role in HHcy/HG-induced NO reduction and ED.

ROS induces calpain activation in retinal photoreceptor cells, cardiomyocytes, and pulmonary microvascular ECs [27, 40, 41]. Inhibition of NADPH oxidase or ROS production significantly prevents calpain activation [41]. Oxidative stress-induced calpain activation is suggested to be related to cysteine oxidation of plasma membrane calcium ATPase [42] and free radical-activated L-type voltage-sensitive calcium channels-induced Ca^{2+} overloading [43]. Moreover, ONOO· is responsible for the cleavage of the N-terminus of latent μ-calpain [35]. We show here that the combination of HHcy and HG potentiated HHcy- or HG-induced O_2· production and 3-NT expression in MAECs and HAECs. Antioxidants PEG-SOD, Tempol, and apocynin improved HHcy/HG-induced ED. Moreover, HHcy/HG-induced calpain activity was decreased by 49% by PEG-SOD (Fig. 5H), suggesting that oxidative stress-induced calpain signaling pathways play an important role in HHcy/HG-induced ED. Further studies to address oxidative stress-mediated calpain-independent signaling pathways in HHcy/HG-induced NO reduction and ED are
warranted.

In diabetes patients, many factors may influence plasma Hcy levels, including age, renal function [44], CBS activity, and circulating insulin concentrations [45]. We found that HG increased plasma Hcy levels by 2-fold in mice, and augmented glomerular sclerosis and albuminuria levels (data not shown). We conclude that HHcy and HG interfere with each other, and speculate that higher levels of plasma Hcy in HG-mice may be due to renal damage. Studies are underway to determine the underlying mechanisms of the worsened Hcy and glucose metabolism in the combined metabolic disorder of HHcy and HG.

Conclusions

HG worsens HHcy. HHcy and HG induce ED, which is potentiated by a combination of HHcy and HG via µ-calpain/PKCβ2 activation-induced eNOSp-Thr497/495 and eNOS inactivation. µ-calpain activation-caused ED may contribute to heightened cardiovascular risk seen in both Type 1 and Type 2 diabetes patients with HHcy. The present study offers a novel insight into the pro-atherogenic role of µ-calpain under the combination of HHcy and HG, and proposes µ-calpain as a critical therapeutic target for HHcy-aggravated cardiovascular complications in diabetes.

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ZJC. designed and conducted the experiments, analyzed and integrated the data, and
wrote the manuscript. XHJ contributed to discussion and technical assistance. MP and PF helped with the eNOS activity assay, animal care and tissue collections. JM contributed to discussion and English editing of the manuscript. KM and RKG took confocal images. SE, RS, MM, and XFY contributed to the discussion. HW supervised this study and participated in designing the experiments, analyzing the data, and manuscript writing. HW is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the accuracy of the data analysis, and edited the manuscript.

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Disclosures

None.
References


Figure legends

**Fig. 1.** Mice developed hyperhomocysteinemia and/or hyperglycemia by feeding with a high methionine (HM) diet and/or injection of streptozotocin (STZ). 

A. Total plasma homocysteine (tHcy) levels. 

B. Blood glucose levels. 

C. Body weights. n=5-10, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on control (CT) diet; †p<0.05 vs vehicle-treated corresponding mice on HM diet; ‡p<0.05 vs STZ-treated corresponding mice on CT diet. 

*Cbs*, cystathionine β-synthase.

**Fig. 2.** Hyperhomocysteinemia aggravated hyperglycemia-induced endothelial dysfunction in mouse thoracic aorta mainly via nitric oxide. 

A. Endothelium-dependent vascular relaxation to acetylcholine (ACh). 

B. Endothelium-dependent vascular relaxation to ACh in the presence of NOS inhibitor N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L, 30 min). 

C. Endothelium-independent vascular relaxation to sodium nitroprusside (SNP). Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to cumulative additions of ACh or SNP. n=5-10, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on CT diet; †p<0.05 vs vehicle-treated corresponding mice on HM diet; ‡p<0.05 vs STZ-treated corresponding mice on CT diet.

**Fig. 3.** Activation of calpain mediated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction in mouse aorta. 

A. Images and quantifications of calpain activity in cultured mouse aortic endothelial cells (MAECs, P\(_0\)) isolated from Cbs\(^{+/+}\) mice. MAECs from STZ-treated Cbs\(^{+/+}\) mice on HM diet treated with MDL28170 (MDL, 20 µmol/L, 1hr) served as a negative control. 

B. Endothelium-dependent vascular relaxation to ACh in the aorta of Cbs\(^{+/+}\)
and STZ-treated $Cbs^{+/-}$ mice on HM diet pretreated with calpain inhibitors *in vivo* (**left panel**) or *ex vivo* (**right panel**). Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to accumulative concentrations of ACh. n=3-10, values are mean ± SEM. *p<0.05 vs vehicle-treated $Cbs^{+/-}$ mice on CT diet; †p<0.05 vs vehicle-treated $Cbs^{+/-}$ mice on HM diet; ‡p<0.05 vs STZ-treated $Cbs^{+/-}$ mice on CT diet; #p<0.05 vs STZ-treated $Cbs^{+/-}$ mice on HM diet.

**Fig. 4.** µ-calpain activation regulated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction in mouse aorta. **A.** Structure and activation of µ/m-calpain large subunit. µ- and m-calpain activity was assessed by immunoblot analysis with a primary antibody that recognizes the N-terminal domain (NT) of µ- or m-calpain large subunit (catalytic subunit), which is autolyzed in active calpains. Total µ- or m-calpain levels were also assessed by a primary antibody that recognizes µ- or m-calpain domain IV. **B.** NT domain containing µ-calpain. **C.** NT domain containing m-calpain. NT domain containing µ/m-calpain served as an inverse indicator of µ/m-calpain activity. **D.** IV domain containing µ-calpain. **E.** IV domain containing m-calpain. **F.** Endothelium-dependent vascular relaxation to ACh in control mouse aorta treated with or without DL-homocysteine (DL-Hcy, 500 µmol/L) and D-glucose (D-Glu, 25 mmol/L) for 48 hrs with or without µ-calpain siRNA (µ-calpsiRNA) *in vitro* for 72 hrs. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to cumulative additions of ACh. **G.** Effect of µ-calpsiRNA transfection in mouse aorta. n=3-5, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on CT diet (**B**) or control aorta (**F**); †p<0.05 vs vehicle-treated corresponding mice on high methionine (HM) diet (**B**); ‡p<0.05 vs STZ-treated corresponding mice on CT diet (**B**);
#p<0.05 vs aorta treated with DL-Hcy/D-Glu (F). CTsiRNA, control siRNA; m-calp, m-calpain; µ-calp, µ-calpain.

**Fig. 5. Oxidative stress mediated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction and calpain activation.**

**A.** Images of *in situ* ·O$_2^-$ production in the aorta. **B.** Images and quantifications of *in situ* ·O$_2^-$ production in cultured mouse aortic endothelial cells (MAECs, p0) isolated from control mice. MAECs were treated with DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol/L) with or without polyethylene glycol superoxide dismutase (PEG-SOD, 150 U/mL) for 48 hrs. **C.** Images of 3-nitrotyrosine (3-NT) levels in the aorta. **D.** Urinary 8-isoprostane levels. **E.** Endothelium-dependent vascular relaxation in aorta of STZ-treated *Cbs*$^{−/−}$ mouse on HM diet in the presence and absence of antioxidants PEG-SOD (150 U/mL), Tempol (1 mmol/L), or apocynin (10 µmol/L) for 1 hr. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation to cumulative additions of ACh. **F.** Images and quantifications of *in situ* ·O$_2^-$ production in HAECs. **G.** Images and quantifications of 3-nitrotyrosin (3-NT) expression in HAECs. **H.** Calpain activity in human aortic endothelial cells (HAECs). **I.** Effect of µ-calpain siRNA (µ-calpsiRNA) transfection in HAECs. HAECs (p8 to 9) were treated with or without DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol/L) in the presence or absence of PEG-SOD (150 U/mL) or MDL (20 µmol/L) for 48 hrs, or control siRNA (CTsiRNA) and µ-calpsiRNA for 72 hrs. n=3-5, values are mean ± SEM. *p<0.05 vs vehicle-treated *Cbs*$^{−/−}$ mice on CT diet (D) or control MAECs/HAECs (B, F, G, H); †p<0.05 vs vehicle-treated *Cbs*$^{−/−}$ mice on HM diet (D) or DL-Hcy-treated MAECs (B, F, G, H); ‡p<0.05 vs STZ-treated *Cbs*$^{−/−}$ mice on CT diet (D) or DL-Hcy-treated MAECs (B, F, G, H).
D-Glu-treated MAECs/HAECs (B, F, G, H); #p<0.05 vs STZ-treated Cbs<sup>+/−</sup> mice on HM diet (E) or DL-Hcy/D-Glu-treated MAECs/HAECs (B, F, G, H). DHE, dihydroethidium.

**Fig. 6.** µ-calpain mediated hyperhomocysteinemia/hyperglycemia-induced nitric oxide (NO) reduction and endothelial NO synthase (eNOS) inactivation in human aortic endothelial cells (HAECs). A. DAF-FM staining of NO production. B. Quantifications of NO production. C. eNOS activity. HAECs were treated with and without DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol) in the presence and absence of MDL for 48 hrs, control siRNA (CTsiRNA) or µ-calpain siRNA (µ-calpsiRNA) for 72 hrs. L-NAME (100 µmol/L) was added in the last hour. n=3, values are mean ± SEM. *p<0.05 vs control; †p<0.05 vs DL-Hcy-treated HAECs; ‡p<0.05 vs D-Glu-treated HAECs; #p<0.05 vs DL-Hcy/D-Glu-treated HAECs. DAF-FM, 4-Amino-5-methylamino-2′,7′-difluorescein.

**Fig. 7.** Activation of µ-calpain and PKCβ<sub>2</sub> mediated hyperhomocysteinemia/hyperglycemia-induced phosphorylation of eNOS at threonine 497 (mouse)/495 (human) (eNOSpThr497/495). A. endothelial nitric oxide synthase (eNOS) protein levels in mouse aorta of Cbs<sup>+/−</sup> mice (left panel) and HAECs (right panel). B. eNOS-pThr497/495 protein levels in mouse aorta of Cbs<sup>+/−</sup> mice (left panel) and in HAECs (right panel). C. eNOS-pThr495 protein levels in HAECs. HAECs were treated with DL-Hcy (500 µmol/L) and D-Glu (25 mmol/L) for 48 hrs in the presence and absence of non-specific PKC inhibitor GFX (2 µmol/L, 30 min.), control adenovirus (Adv-CT, 200 MOI), dominant negative PKCβ<sub>2</sub> adenovirus (Adv-dnPKCβ<sub>2</sub>, 200 MOI) or µ-calpain siRNA (µ-calpsiRNA) for 72 hrs, or MDL (20 mmol/L) for 48 hrs. D. Effect of Adv-dnPKCβ<sub>2</sub> transfection in HAECs.
n=3-5, values are mean ± SEM. *p<0.05 vs control; †p<0.05 vs DL-Hcy-treated HAECs; ‡p<0.05 vs D-Glu-treated HAECs; #p<0.05 vs DL-Hcy/D-Glu-treated HAECs.

Fig. 8. Mechanisms of hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction. Adv-dnPKCβ2, dominant negative PKCβ2 adenovirus; ALLM, N-Acetyl-Leu-Leu-Met-CHO; ED, endothelial dysfunction; eNOS, endothelial nitric oxide synthase; eNOS-pThr495/497, phosphorylation of eNOS at threonine 495/497; GFX, GF109203X; PEG-SOD, polyethylene glycol superoxide dismutase; NO, nitric oxide; O₂⁻, superoxide; OONO⁻, peroxynitrite.
Fig. 1. Mice developed hyperhomocysteinemia and/or hyperglycemia by feeding with a high methionine (HM) diet and/or injection of streptozotocin (STZ). A. Total plasma homocysteine (tHcy) levels. B. Blood glucose levels. C. Body weights. n=5-10, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on control (CT) diet; †p<0.05 vs vehicle-treated corresponding mice on HM diet; ‡p<0.05 vs STZ-treated corresponding mice on CT diet. Cbs, cystathionine β-synthase.
Fig 2

Fig. 2. Hyperhomocysteinemia aggravated hyperglycemia-induced endothelial dysfunction in mouse thoracic aorta mainly via nitric oxide. A. Endothelium-dependent vascular relaxation to acetylcholine (ACh). B. Endothelium-dependent vascular relaxation to ACh in the presence of NOS inhibitor N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L, 30 min). C. Endothelium-independent vascular relaxation to sodium nitroprusside (SNP). Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to cumulative additions of ACh or SNP. n=5-10, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on CT diet; †p<0.05 vs vehicle-treated corresponding mice on HM diet; ‡p<0.05 vs STZ-treated corresponding mice on CT diet.
**Fig 3. Activation of calpain mediated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction in mouse aorta.**

A. Images and quantifications of calpain activity in cultured mouse aortic endothelial cells (MAECs, P₀) isolated from Cbs⁺/⁻ mice. MAECs from STZ-treated Cbs⁺/⁻ mice on HM diet treated with MDL28170 (MDL, 20 µmol/L, 1hr) served as a negative control. B. Endothelium-dependent vascular relaxation to ACh in the aorta of Cbs⁺/⁻ mice, with calpain inhibitors in vivo (left panel) or ex vivo (right panel). Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to accumulative concentrations of ACh. n=3-10, values are mean ± SEM. *p<0.05 vs vehicle-treated Cbs⁺/⁻ mice on CT diet; †p<0.05 vs vehicle-treated Cbs⁺/⁻ mice on HM diet; ‡p<0.05 vs STZ-treated Cbs⁺/⁻ mice on CT diet; #p<0.05 vs STZ-treated Cbs⁺/⁻ mice on HM diet.
**A** Structure and activation of μ/m-calpain big subunit

**B** NT domain containing μ-calpain

**C** NT domain containing m-calpain

**D** IV domain containing μ-calpain

**E** IV domain containing m-calpain

**F** Endothelium-dependent relaxation (preincubated with μ-calpsiRNA)

**G** μ-calpain knockdown in mouse aorta by siRNA
Fig. 4. µ-calpain activation regulated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction in mouse aorta. A. Structure and activation of µ/m-calpain large subunit. µ- and m-calpain activity was assessed by immunoblot analysis with a primary antibody that recognizes the N-terminal domain (NT) of µ- or m-calpain large subunit (catalytic subunit), which is autolyzed in active calpains. Total µ- or m-calpain levels were also assessed by a primary antibody that recognizes µ- or m-calpain domain IV. B. NT domain containing µ-calpain. C. NT domain containing m-calpain. NT domain containing µ/m-calpain served as an inverse indicator of µ/m-calpain activity. D. IV domain containing µ-calpain. E. IV domain containing m-calpain. F. Endothelium-dependent vascular relaxation to ACh in control mouse aorta treated with or without DL-homocysteine (DL-Hcy, 500 µmol/L) and D-glucose (D-Glu, 25 mmol/L) for 48 hrs with or without µ-calpain siRNA (µ-calpsiRNA) in vitro for 72 hrs. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to cumulative additions of ACh. G. Effect of µ-calpsiRNA transfection in mouse aorta. n=3-5, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on CT diet (B) or control aorta (F); †p<0.05 vs vehicle-treated corresponding mice on high methionine (HM) diet (B); ‡p<0.05 vs STZ-treated corresponding mice on CT diet (B); #p<0.05 vs aorta treated with DL-Hcy/D-Glu (F). CTsiRNA, control siRNA; m-calp, m-calpain; µ-cal, µ-calpain.
Diabetes

**A**  
*In situ* $\cdot$$\cdot$$\cdot$O$_2$ production in aorta of Cbs$^{-/-}$ mice (DHE staining)

**B**  
*In situ* $\cdot$$\cdot$$\cdot$O$_2$ production in MAECs (DHE staining)

**C**  
3-NT expression in aorta of Cbs$^{-/-}$ mice (DAB staining)

**D**  
Urinary 8-isoprostane/creatinine levels (ELISA)

**E**  
Endothelium-dependent relaxation (preincubated with antioxidants)

**F**  
*In situ* $\cdot$$\cdot$$\cdot$O$_2$ production in HAECs (DHE staining)
Fig. 5. Oxidative stress mediated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction and calpain activation. A. Images of in situ \( \cdot \text{O}_2 \) production in the aorta. B. Images and quantifications of in situ \( \cdot \text{O}_2 \) production in cultured mouse aortic endothelial cells (MAECs, p0) isolated from control mice. MAECs were treated with DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol/L) with or without polyethylene glycol superoxide dismutase (PEG-SOD, 150 U/mL) for 48 hrs. C. Images of 3-nitrotyrosine (3-NT) levels in the aorta. D. Urinary 8-isoprostane levels. E. Endothelium-dependent vascular relaxation in aorta of STZ-treated Cbs\(^{-/-}\) mouse on HM diet in the presence and absence of antioxidants PEG-SOD (150 U/mL), Tempol (1 mmol/L), or apocynin (10 µmol/L) for 1hr. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation to cumulative additions of ACh. F. Images and quantifications of in situ \( \cdot \text{O}_2 \) production in HAECs. G. Images and quantifications of 3-nitrotyrosin (3-NT) expression in HAECs. H. Calpain activity in human aortic endothelial cells (HAECs). I. Effect of \( \mu \)-calpsiRNA transfection in HAECs. HAECs (p8 to 9) were treated with or without DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol/L) in the presence or absence of PEG-SOD (150 U/mL) or MDL (20 µmol/L) for 48 hrs, or CTsiRNA and \( \mu \)-calpsiRNA for 72 hrs. n=3-5, values are mean ± SEM. *p<0.05 vs vehicle-treated Cbs\(^{-/-}\) mice on CT diet (D) or control MAECs/HAECs (B, F, G, H); †p<0.05 vs vehicle-treated Cbs\(^{-/-}\) mice on HM diet (D) or DL-Hcy-treated MAECs (B, F, G, H); ‡p<0.05 vs STZ-treated Cbs\(^{-/-}\) mice on CT diet (D) or D-Glu-treated MAECs/HAECs (B, F, G, H); #p<0.05 vs STZ-treated Cbs\(^{-/-}\) mice on HM diet (E) or DL-Hcy/D-Glu-treated MAECs/HAECs (B, F, G, H). DHE, dihydroethidium.
Fig. 6. µ-calpain mediated hyperhomocysteinemia/hyperglycemia-induced nitric oxide (NO) reduction and endothelial NO synthase (eNOS) inactivation in human aortic endothelial cells (HAECs). A. DAF-FM staining of NO production. B. Quantifications of NO production. C. eNOS activity. HAECs were treated with and without DL-Hcy (500 µmol/L) and/or D-Glu (25 mmol) in the presence and absence of MDL for 48 hrs, CTsiRNA or µ-calpsiRNA for 72 hrs. L-NAME (100 µmol/L) was added in the last hour. n=3, values are mean ± SEM. *p<0.05 vs control; †p<0.05 vs DL-Hcy-treated HAECs; ‡p<0.05 vs D-Glu-treated HAECs; #p<0.05 vs DL-Hcy/D-Glu-treated HAECs. DAF-FM, 4-Amino-5-methylamino-2',7'-difluorescein.
Fig. 7. Activation of μ-calpain and PKCβ2 mediated hyperhomocysteinemia/hyperglycemia-induced phosphorylation of eNOS at threonine 497 (mouse)/495 (human) (eNOSpThr497/495). A. endothelial nitric oxide synthase (eNOS) protein levels in mouse aorta of Cbs+/− mice (left panel) and HAECS (right panel). B. eNOS-pThr497/495 protein levels in mouse aorta of Cbs+/− mice (left panel) and in HAECS (right panel).  C. eNOS-pThr495 protein levels in HAECS. HAECS were treated with DL-Hcy (500 μmol/L) and D-Glu (25 mmol/L) for 48 hrs in the presence and absence of non-specific PKC inhibitor GFX (2 μmol/L, 30 min.), control adenovirus (Adv-CT, 200 MOI), dominant negative PKCβ2 adenovirus (Adv-dnPCKβ2, 200 MOI) or μ-calpsiRNA for 72 hrs, or MDL (20 mmol/L) for 48 hrs. D. Effect of Adv-dnPCKβ2 transfection in HAECS. n=3-5, values are mean ± SEM. *p<0.05 vs control; †p<0.05 vs DL-Hcy-treated HAECS; ‡p<0.05 vs D-Glu-treated HAECS; #p<0.05 vs DL-Hcy/D-Glu-treated HAECS.
Fig. 8. Mechanisms of hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction. Adv-dnPKCβ2, dominant negative PKCβ2 adenovirus; ALLM, N-Acetyl-Leu-Leu-Met-CHO; ED, endothelial dysfunction; eNOS, endothelial nitric oxide synthase; eNOS-pThr495/497, phosphorylation of eNOS at threonine 495/497; GFX, GF109203X; PEG-SOD, polyethylene glycol superoxide dismutase; NO, nitric oxide; O₂⁻, superoxide; OONO⁻, peroxynitrite; µ-calpsiRNA, µ-calpainsiRNA.
Online Fig. 1. Hyperhomocysteinemia aggravated hyperglycemia-induced endothelial dysfunction in mouse thoracic aorta mainly via nitric oxide. 

A. Endothelium-dependent vascular relaxation to ACh. 

B. Endothelium-dependent vascular relaxation to ACh in the presence of L-NAME (100 µmol/L, 30 min). 

C. Endothelium-independent vascular relaxation to SNP. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to cumulative additions of ACh or SNP, and represented as the area under the curve (AUC). n=5-10, values are mean ± SEM. *p<0.05 vs vehicle-treated corresponding mice on CT diet; †p<0.05 vs vehicle-treated corresponding mice on HM diet; ‡p<0.05 vs STZ-treated corresponding mice on CT diet.
Online Fig. 2. Identification of mouse aortic endothelial cells (MAECs). Cultured MAECs isolated from control Cbs<sup>++</sup> mice (p0) were identified by endothelial makers VWF (red, Von Willebrand factor, vWF, Santa Cruz, SC-2780, 1:200) and CD31 (green, CD31, BD Biosciences, BD553370, 1:100). Endothelial cells were stained with DAPI (blue, 4',6-Diamidino-2-Phenylindole).
Online Fig 3. Activation of calpain mediated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction in mouse aorta. Endothelium-dependent vascular relaxation to ACh in the aorta of Cbs<sup>+/−</sup> and STZ-treated Cbs<sup>+/−</sup> mice on HM diet pretreated with calpain inhibitors in vivo (left panel) or ex vivo (right panel). In in vivo, calpain inhibitor MDL was administrated by i.p. injection (2 mg/kg/day, 2 weeks) whereas in ex vivo, aortic rings were incubated with calpain inhibitors MDL, calpeptin and ALLM (20 µmol/L for all) for 1 hr. Aortic rings were pre-contracted with phenylephrine (1 µmol/L) and examined for relaxation response to accumulative concentrations of ACh, and represented as the AUC. n=3-10, values are mean ± SEM. *p<0.05 vs vehicle-treated Cbs<sup>+/−</sup> mice on CT diet; †p<0.05 vs vehicle-treated Cbs<sup>+/−</sup> mice on HM diet; ‡p<0.05 vs STZ-treated Cbs<sup>+/−</sup> mice on CT diet; #p<0.05 vs STZ-treated Cbs<sup>+/−</sup> mice on HM diet.
Online Fig 4

**Endothelium-dependent relaxation (preincubated with μ-calpsiRNA)**

Online Fig. 4. *μ-calpain* siRNA rescued hyperhomocysteinemia/hyperglycemia-induced ED. Endothelium-dependent vascular relaxation to ACh in control mouse aorta treated with or without DL-Hcy (500 μmol/L) and D-Glu (25 mmol/L) for 48 hrs in the presence and absence of μ-calpsiRNA *in vitro*. Aortic rings were pre-contracted with phenylephrine (1 μmol/L) and examined for relaxation response to cumulative additions of ACh and represented as the AUC. n=3-5, values are mean ± SEM. *p<0.05 vs control aorta; #p<0.05 vs aorta treated with DL-Hcy/D-Glu and DL-Hcy/D-Glu + CTsiRNA. CT siRNA, control siRNA.
Online Fig. 5. Oxidative stress regulated hyperhomocysteinemia/hyperglycemia-induced endothelial dysfunction. Endothelium-dependent vascular relaxation in aorta of STZ-treated Cbs<sup>-/-</sup> mice on HM diet in the presence and absence of antioxidants PEG-SOD (150 U/mL), Tempol (1 mmol/L), or apocynin (10 μmol/L) for 1hr. Aortic rings were pre-contracted with phenylephrine (1 μmol/L) and examined for relaxation to cumulative additions of ACh and represented as the AUC. n=3-5, values are mean ± SEM. #p<0.05 vs STZ-treated Cbs<sup>-/-</sup> mice on HM diet.
Online Fig 6. Endothelial nitric oxide synthase (eNOS) uncoupling was not regulated by hyperhomocysteinemia/hyperglycemia. A. eNOS dimer and monomer expression in HAECs. HAECs were treated with and without DL-Hcy (500 or 1000 µmol/L) and/or D-glucose (D-Glu, 25 mmol/L) for 24 or 48 hrs. B. Images of in situ $\cdot$O$_2^-$ production in HAECs. C. Quantifications of O$_2^-$ production. HAECs were treated with or without DL-Hcy (500 µmol/L) and D-Glu (25 mmol/L) for 24 hrs. n=3-5, values are mean ± SEM. *p<0.05 vs control; †p<0.05 vs DL-Hcy-treated HAECs in corresponding time point; ‡p<0.05 vs D-Glu-treated HAECs in corresponding time point. DHE, dihydroethidium.