Hyperhomocysteinemia and Hyperglycemia Induce and Potentiate Endothelial Dysfunction via \( \mu \)-Calpain Activation

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 \( \mu \)mol/L) and moderate (88 \( \mu \)mol/L) HHcy were induced in cystathionine \( \beta \)-synthase wild-type (\( \text{Cbs}^+/+ \)) and heterozygous-deficient (\( \text{Cbs}^{2/+} \)) 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 53 and 173 \( \mu \)mol/L in \( \text{Cbs}^+/+ \) and \( \text{Cbs}^{2/+} \) mice fed an 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 N\(^{\text{G}}\)-nitro-L-arginine methyl ester. HHcy potentiated HG-induced calpain activation in aortic endothelial cells isolated from Cbs mice. Calpain inhibitors rescued HHcy- and HG-induced ED in vivo and ex vivo. Moderate HHcy- and HG-induced \( \mu \)-calpain activation was potentiated by a combination of HHcy and HG in the mouse aorta. \( \mu \)-Calpain small interfering RNA (\( \mu \)-calpsiRNA) prevented HHcy/HG-induced ED in the mouse aorta and calpain activation in human aortic endothelial cells (HAECs) treated with DL-Hcy (500 \( \mu \)mol/L) and D-glucose (25 mmol) for 48 h. In addition, HHcy accelerated HG-induced superoxide production as determined by dihydroethidium and 3-nitrotyrosin 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 nitric oxide production and eNOS activity in HAECs, which were prevented by calpain inhibitors or \( \mu \)-calpsiRNA. HHcy aggravated HG-increased phosphorylation of eNOS at threonine 497/495 (eNOS-pThr497/495) in the mouse aorta and HAECs. HHcy/HG-induced eNOS-pThr497/495 was reversed by \( \mu \)-calpsiRNA and adenoviral transduced dominant negative protein kinase C (PKC)\( \beta \) in HAECs. HHcy and HG induced ED, which was potentiated by the combination of HHcy and HG via \( \mu \)-calpain/PKC\( \beta \) activation–induced eNOS-pThr497/495 and eNOS inactivation.

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 (HG), 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 macrovasculature via different signaling pathways in diabetes. However, it remains incompletely understood 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 ED.

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-eNOS-pThr495), 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 endothelial cells (ECs) (9). Activation of calpain mediates acute and chronic 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.

### RESEARCH DESIGN 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 body weight) 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 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 nitric oxide (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 small interfering RNA (calpsiRNA), and antioxidants in vivo or ex vivo. Thoracic aorta (5 mm) 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 ECs

Mouse aortic ECs (MAECs) were isolated using collagenase as previously described (17) and identified with staining of von Willebrand factor and CD31.

#### Cell Culture

Human aortic ECs (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 h in the presence or absence of selected pharmacological inhibitors, small interfering RNA (siRNA), or adenosin.

#### Calpain Activity

t-Butoxyxcarbonyl (t-BOC) assay was used for calpain activity measurement by treating ECs with t-BOC-leu- met-chloromethyl aminocoumarin (Molecular Probe) as previously described (19).

μ-Calpain and m-calpain are constitutively expressed in ECs (20). When calpains are activated, they undergo autoproteolysis, which removes the N-terminal ends (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 and aorta 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 \( \mu \)-calpain.

**In Situ Superoxide Production by Dihydroethidium Staining**

Cross-sections (30 \( \mu \)m) of fresh aorta segments were equilibrated for 30 min at 37°C in Krebs buffer and then incubated with dihydroethidium (3 \( \mu \)mol/L) for 30 min at 37°C in the dark.

**3-Nitrotyrosin Expression**

3-Nitrotyrosin in the mouse aorta (5-\( \mu \)m sections) was detected using an anti-3-nitrotyrosin monoclonal antibody (Santa Cruz) and costained with 3,3'-diaminobenzidine.

**NO Production and eNOS Activity**

NO production in HAECs was detected by staining with 4-amino-5-methylamino-2,7'-difluorescein (DAF-FM). To explore the role of calpain, especially \( \mu \)-calpain, DL-Hcy/D-Glu–treated HAECs were also treated with MDL-28170 (MDL; 20 \( \mu \)mol/L) and \( \mu \)-calpsiRNA, respectively. HAECs treated with an eNOS inhibitor \( \mathrm{N}^\mathrm{G} \)-nitro-L-arginine methyl ester (L-NAME; 100 \( \mu \)mol/L) for 1 h before DAF-FM staining were used as negative controls. eNOS activity in HAECs was determined by measuring the conversion of \(^3\text{H}\)-Arg to \(^3\text{H}\)-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, \( \text{O}_2^- \) produced by eNOS, a marker of eNOS uncoupling, was examined by dihydroethidium 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, U.K.) as described previously (25). Blood glucose levels were determined by a glucometer (HemoCue). Urinary 8-isoprostane and creatinine levels were determined by ELISA kit (Cayman).

**Statistical Analysis**

Results are expressed as the mean \( \pm \) 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 Cbs\(^{+/+}\) and Cbs\(^{-/-}\) mice, respectively (Fig. 1A). STZ injections resulted in HG in Cbs\(^{+/+}\) and Cbs\(^{-/-}\) mice (Fig. 1B).

**Figure 1** — Mice developed HHcy and/or HG by feeding with an HM diet and/or injection of STZ. A: t-Hcy levels. B: Blood glucose levels. C: Body weights. Values are mean \( \pm \) SEM; \( n = 5-10 \). *\( P < 0.05 \) vs. vehicle-treated corresponding mice on control diet; †\( P < 0.05 \) vs. vehicle-treated corresponding mice on HM diet; ‡\( P < 0.05 \) vs. STZ-treated corresponding mice on control diet. Cbs, cystathionine \( \beta \)-synthase. CT, control.

HM diet induced higher levels of plasma t-Hcy: moderate and severe HHcy in Cbs\(^{+/+}\) and Cbs\(^{-/-}\) mice with STZ injections, respectively (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 Cbs\(^{+/+}\)/HM mice had no effect on endothelial function (Fig. 2A, left panel), whereas moderate HHcy in Cbs\(^{-/-}\)/HM mice impaired vascular relaxation to ACh (\( P < 0.05 \)) (Fig. 2A, right panel; Supplementary Fig. 1A). HG impaired vascular relaxation to ACh in both Cbs\(^{+/+}\)/STZ and Cbs\(^{-/-}\)/STZ mice (\( P < 0.05 \)). A combination of moderate HHcy and HG in Cbs\(^{+/+}\)/HM/STZ mice potentiated ED to a greater level when compared with that from either Cbs\(^{-/-}\)/HM mice with moderate HHcy alone or Cbs\(^{+/+}\)/STZ
and Cbs−/−/STZ mice with HG alone (Fig. 2A). Notably, a combination of severe HHcy plus HG in Cbs−/−/HM/STZ mice potentiated ED to a greater level (P < 0.05) (Fig. 2A, right panel; Supplementary Fig. 1A). Vascular relaxation to ACh was completely abolished by eNOS inhibitor L-NAME in all groups (Fig. 2B, Supplementary Fig. 1B). Endothelium-independent vascular relaxation to SNP was not changed in all groups (Fig. 2C; Supplementary 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 (Supplementary Fig. 2) isolated from 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 administrated daily (2 mg/kg body weight, 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; Supplementary Fig. 3). Moreover, preincubation of aortic rings with calpain inhibitors MDL, calpeptin, and N-acetyl-Leu-Leu-Met-CHO (ALLM; 20 μmol/L, 1 h) markedly improved vascular relaxation to ACh in Cbs−/−/HM/STZ mice (P < 0.05) (Fig. 3B, right panel; Supplementary Fig. 3).

**Activation of μ-Calpain Mediates HHcy/HG-Induced ED**

μ-Calpain 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. 4G; Supplementary Fig. 4). Additionally, μ-calpsiRNA markedly reversed HHcy/HG-induced calpain activation in HAECs treated (P < 0.05) (Fig. 5H).

**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 O2− generation, and the combination of HHcy and HG further promoted O2− generation in the aortic endothelium of Cbs−/−/HM/STZ mice and in MAECs to a greater extent than either factor independently (Fig. 5A and B). Polyethylene glycol superoxide dismutase (PEG-SOD) significantly prevented the combination of HHcy and HG-induced O2−. Because peroxynitrite (ONOO−) is a strong and relatively stable oxidant species capable of causing nitrosylation of tyrosine residues on proteins, we also examined the content of 3-nitrotyrosin in the aorta of Cbs−/− mice. We found that moderate HHcy or HG alone significantly increased 3-nitrotyrosin 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-isoprostane, 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-isoprostane 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-isoprostane 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 preincubating the aortic rings with antioxidants PEG-SOD, Tempol, and apocynin for 1 h (P < 0.05) (Fig. 5E, Supplementary Fig. 5).

Oxidative Stress Mediates HHcy/HG-Induced \( \mu \)-Calpain Activation

To address the relevance to human pathogenesis, we examined the effect of HHcy and HG on \( \mathrm{O}_2^- \) production and 3-nitrotyrosin expression in HAECs. We found that DL-Hcy and D-Glu treatments for 48 h significantly increased \( \mathrm{O}_2^- \) production by 1.4- and 1.7-fold and 3-nitrotyrosin expression by 1.5- and 3.0-fold, respectively (P < 0.05) (Fig. 5F and G). The combination of DL-Hcy and D-Glu promoted \( \mathrm{O}_2^- \) production by 2.5-fold and

Activation of \( \mu \)-Calpain Mediates HHcy/HG-Induced NO Reduction and eNOS Inactivation

DL-Hcy and D-Glu for 48 h 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, \( \mu \)-calpsiRNA
Figure 4—m-Calpain activation regulated HHcy/HG-induced ED in mouse aorta. A: Structure and activation of μ/m-calpain large subunit. μ-Calpain and m-calpain activity was assessed by immunoblot analysis with a primary antibody that recognizes the NT domain 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-Hcy (500 μmol/L) and D-Glu (25 mmol/L) for 48 h with or without μ-calpsirNA in vitro for 72 h. Aortic rings were precontracted with phenylephrine (1 μmol/L) and examined for relaxation response to cumulative additions of ACh. G: Effect of μ-calpsirNA transfection in mouse aorta. Values are mean ± SEM; n = 3–5. *P < 0.05 vs. vehicle-treated corresponding mice on control diet (B) or control aorta (F); †P < 0.05 vs. vehicle-treated corresponding mice on HM diet (B); ‡P < 0.05 vs. STZ-treated corresponding mice on control diet (B); ††P < 0.05 vs. aorta treated with DL-Hcy/D-Glu (F). CT, control; m-calp, m-calpain; μ-calp, μ-calpain.
Figure 5—Oxidative stress–mediated HHcy/HG-induced ED and calpain activation. A: Images of in situ $\cdot O_2^-$ production in the aorta of Cbs-/+ mice (DHE staining).

B: Images and quantifications of in situ $\cdot O_2^-$ production in cultured 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 PEG-SOD (150 U/mL) for 48 h.

C: Images of 3-nitrotyrosin levels in the aorta.

D: Urinary 8-isoprostane levels (ELISA).

E: Endothelium-dependent relaxation in aorta of Cbs-/+ mice in the presence and absence of antioxidants PEG-SOD (150 U/mL), Tempol (1 mmol/L), or apocynin (10 μmol/L) for 1 h.

F: Images and quantifications of in situ $\cdot O_2^-$ production in HAECs.

G: Images and quantifications of 3-nitrotyrosin expression in HAECs.

H: Calpain activity in HAECs.

I: Effect of μ-calpsRNA 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 h or CtsiRNA and μ-calpsRNA for 72 h. Values are mean ± SEM; n = 3–5.

*P < 0.05 vs. vehicle-treated Cbs-/+ mice on control 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 HM diet (D) or DL-Hcy/D-Glu-treated MAECs/HAECs (B, F, G, H). CT, control.

DHE, dihydroethidium.
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 PKC$_b$2 Suppression**

Neither HHcy nor 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, nonselective PKC inhibitor GF109203X (GFX) and silencing of PKC$_b$2 gene by transfecting adenoviral transduced dominant negative PKC$_b$2 (Adv-dnPKC$_b$2) (Fig. 7D) rescued HHcy/HG-induced eNOS-pThr495 in HAECs (Fig. 7C). HHcy/HG-induced eNOS-pThr495 was also rescued by MDL and $\mu$-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 either DL-Hcy and D-Glu alone or the combination (Supplementary 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 (Supplementary Fig. 6B and 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 $\mu$-calpain/PKC$_b$2 activation-induced eNOS-pThr497/495 and eNOS inactivation (Fig. 8). We propose that $\mu$-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. $\mu$-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 to be related to NO reduction and enhanced reaction of NO with O$_2^-$, resulting in increased 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-nitrotyrosin 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 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 dimethylarginine dimethylaminohydrolase, which causes asymmetric dimethylarginine accumulation (32). We examined eNOS uncoupling under HHcy and/or HG conditions in HAECs and found that neither eNOS monomerization nor eNOS-produced O$_2^-$ was increased by HHcy and/or HG. These data suggest 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 calmodulin-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 current study, we found that the combination of HHcy and HG potentiated eNOS-pThr495, which was prevented by PKC inhibitor GFX. Within the PKC family, PKCβ has received much attention since it was first shown to be preferentially upregulated in

![Figure 6](image_url)

**Figure 6**—μ-calpain mediated HHcy/HG-induced NO reduction and eNOS inactivation in 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 h, CtRNA or μ-calpsiRNA for 72 h. L-NAME (100 μmol/L) was added in the last hour. Values are mean ± SEM; n = 3. *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. CT, control.
PKC was originally discovered as a kinase cleaved and activated by calpains (7), which tightly regulate their respective substrates through limited proteolytic cleavage.

Figure 7—Activation of μ-calpain and PKCβ2 mediated HHcy/HG-induced eNOS-pThr497/495. A: 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 h in the presence and absence of nonspecific PKC inhibitor GFX (2 μmol/L; 30 min), control adenovirus (Adv-CT; 200 MOI), dominant negative PKCβ2 adenovirus (Adv-dnPKCβ2; 200 MOI), or μ-calpsRNA for 72 h or MDL (20 mmol/L) for 48 h. D: Effect of Adv-dnPKCβ2 transfection in HAECs. Values are mean ± SEM; n = 3–5. *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. CT, control.

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.
cleavage. Activation of calpain has been implicated in diabetes-linked platelet aggregation (35), neurovascular dysfunction (36), and cardiomyocyte apoptosis (35). Recently, increased calpain activation was 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, calpain inhibitor MDL, ALLM, or calpeptin markedly 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 being 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 ECs (34). Nevertheless, here we show that both μ-calpain and PKCβ inhibition rescued HHcy/HG-induced eNOS-pThr497/495. These data suggest that activations of μ-calpain and PKCβ play critical roles in HHcy/HG-induced eNOS-pThr495 and eNOS inactivation. The molecular details for the interaction between PKCβ and μ-calpain activation under HHcy/HG conditions 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.

Reactive oxygen species induce calpain activation in retinal photoreceptor cells, cardiomyocytes, and pulmonary microvascular ECs (27,40,41). Inhibition of NADPH oxidase or reactive oxygen species 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 Ca2+ 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 O2− production and 3-nitrotyrosin 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–mediated calpain-independent 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 twofold 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 eNOS-pThr497/495 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 current study offers a novel insight into the proatherogenic 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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