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Hyperhomocysteinemia and Endothelial Dysfunction

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Abstract

Hyperhomocysteinemia (HHcy) is a significant and independent risk factor for cardiovascular diseases. Endothelial dysfunction (ED) is the earliest indicator of atherosclerosis and vascular diseases. We and others have shown that HHcy induced ED in human and in animal models of HHcy induced by either high-methionine load or genetic deficiency. Six mechanisms have been suggested explaining HHcy-induced ED. These include 1) nitric oxide inhibition, 2) prostanoids regulation, 3) endothelium-derived hyperpolarizing factors suppression, 4) angiotensin II receptor-1 activation, 5) endothelin-1 induction, and 6) oxidative stress. The goal of this review is to elaborate these mechanisms and to discuss biological and molecular events related to HHcy-induced ED.

Keywords

Hyperhomocysteinemia; endothelial dysfunction; vascular relaxation/vascular contractile responses

Hyperhomocysteinemia and Cardiovascular Diseases

In healthy individuals, plasma homocysteine (Hcy) level is between 5-10 μmol/L. Elevation of plasma Hcy levels creates a condition called hyperhomocysteinemia (HHcy), which is characterized into three ranges; moderate (16-30 μmol/L), intermediate (31 - 100 μmol/L), and severe (>100 μmol/L) HHcy [1]. In the general population, modulate elevations in plasma Hcy and may be due to inherited enzyme variants and/or a relative deficiency of folate, vitamin B_6, which are required for the normal metabolism of Hcy. Two strongest determinants of plasma Hcy concentration are folate status and renal function.

McCully suggested that HHcy are causally related to cardiovascular diseases (CVD) in 1969 [2]. However, this theory was neglected for a long time, probably due to the low prevalence of inborn errors of metabolism that were accompanied by severe HHcy. In 1976, HHcy gained a world-wide interest, when Wilcken and Wilcken observed an abnormal rise in plasma Hcy after oral methionine (Met) loading in patients with coronary artery disease [3]. Since then, abundant retrospective and prospective observational studies have shown a relation between plasma total homocysteine (tHcy) level and coronary artery disease, peripheral artery disease, stroke, or venous thrombosis [4,5]. In a metaanalysis by Boushey *et al.*, an increase of 5 μmol/L in plasma Hcy enhanced the risk of CVD by 1.6-to 1.8-fold [4]. Another recent meta-analysis by den Heijer *et al.*, also shown that an 25% elevation (about 3 μmol/L) tHcy was associated with about a 10% higher risk of cardiovascular events and a 20% higher risk of stroke after adjustment for other known risk factors [5].

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Homocysteine Metabolism

The single source of Hcy in humans is the demethylation of essential amino acid, Met, *via* two intermediate compounds, S-adenosylmethionine (SAM), a major donor of cellular methylation, and S-adenosylhomocysteine (SAH), a potent inhibitor of biological transmethylation (Fig. 1). SAM is the methyl donor in over 115 different cellular methyltransferase reactions, including those of DNA, RNA, proteins and lipids [6]. With the transfer of the methyl group, SAM is converted into SAH. Hcy can utilize adenosine, a normal constituent of all body fluids to forma SAH, a potent inhibitor of cellular methylation, thereby causing cellular hypomethylation. The intracellular SAM/SAH ratio has been used as a predictor of cellular methylation capacity. SHA is further converted into Hcy, a nonproteinogenic amino acid containing a free sulfhydryl group, and adenosine by the SAH hydrolase, which is widely distributed in mammalian tissues. Hcy can be further metabolized *via* two alternative pathways: it may be irreversibly degraded through the transsulfuration pathway or remethylated to Met *via* remethylation pathway. Hcy is transsulfurated to cystathionine and cystaine cystathionine β-synthase (CBS), vitamin B_6 dependently in the liver and kidneys. Hcy can be also remethylated to Met by folate dependent and non-dependent pathways. When the cellular capacity to metabolize Hcy is exceed, this amino acid will be exported to the extracellular compartment to achieve equilibrium concentration in the circulation.

Hcy is present in different forms. About 70-80% is protein-bound. Non-protein-bound Hcy is found in “mixed disulfide” (i.e. the dimmer of Hcy and cysteine), homocystine (the oxidized disulfide of Hcy) and reduced Hcy. Reduced Hcy forms only about 1% of the tHcy content. Laboratories usually measure tHcy concentration, which is the sum of all Hcy fractions [7].

Endothelial Injury as the Primary Mechanism for Hcy-Related CVD

During the last two decades, biomedical research has been actively exploring the role of Hcy in atherosclerosis and the underlying mechanisms [8-10]. Among the proposed mechanisms, we believe that endothelial injury is the primary mechanism for Hcy-related CVD. Our laboratory were the first to report: 1) Hcy, at clinically relevant concentrations, arrests cell growth and suppresses cyclin A transcription in Endothelial cells (ECs) *via* hypomethylation-related mechanism [8,11]. These findings, which cannot be mimicked by cysteine, led us to propose that hypomethylation is the major biochemical mechanism in Hcy vascular disease. 2) HHcy accelerates atherosclerosis in mice [12] and inhibits high density lipoprotein biosynthesis in human and mice [13]. 3) Severe HHcy impairs reendothelialization and increases neointimal formation in mice [14]. 4) Hcy demethylates cyclin A promoter and inhibits DNA methyltransferases 1 activity in ECs [15]. 5) Hcy transport is differentially regulated in vascular cells and is predominantly *via* a lysosomal dependent alanine-serine-cysteine transport system [16]. 6) Hcy inhibits cyclin A transcription and cell growth by inhibiting DNA methylation through suppression of DNA methyltransferase 1 in ECs [17]. It is possible that HHcy induces endothelial injury and ED leading to the development of atherosclerosis.

Homocysteine and Endothelial Dysfunction

The endothelium is a single layer of cells lining all blood vessels. It plays an important role in many physiological functions, including the control of blood cell trafficking, vasomotor tone, vessel permeability, and hemostatic balance. ECs produce a wide variety of substances in response to various physical and chemical stimuli, including vasodilator substances (i.e. nitric oxide, NO; prostacyclin, PGI_2; and endothelium-derived hyperpolarizing factor, EDHF), and vasoconstrictor substances (i.e. endothelin-1, ET-1; angiotensin II, Ang II; thromboxane A_2, TXA_2 or free radicals) [18].

ED is defined as an impairment of endothelium-dependent relaxation of blood vessels. It is the earliest indicator of the development of CVD and precedes the appearance of atherosclerotic plaque and the frank symptoms of peripheral vascular disease. Chronic exposure to physical and chemical stimuli can lead to ED. Impaired endothelial vasomotor function was observed in human and experimental models of HHcy in minipigs, monkey, rats and mice either with high-Met diet or the deletion of the CBS gene [19,20]. Further, endothelium-dependent vascular relaxation response to acetylcholine (ACh) and bradykinin was also impaired in the pig common carotid arteries pretreated with Hcy (50 and 100 μmol/L) for 24 h, rabbit thoracic aorta rings incubated with Hcy (0.1, 1, and 10 mmol/L) for 3 h [21]. Superfusion of Hcy (1 mmol/L) on the cerebral cortex impaired cerebrovascular vasodilatory responses induced by ACh in rabbits [22]. Six mechanisms have been suggested explaining HHcy-induced ED. These include 1) NO inhibition, 2) prostanoids regulation, 3) EDHF inhibition, 4) angiotensin II receptor 1 (AT_1) activation, 5) endothelin-1 (ET-1) induction, and 6) oxidative stress. We summarized these mechanisms in Fig. 2 and discussed the biological and molecular events related to HHcy-induced ED below.

1) NO Inhibition

Multiple studies suggested that HHcy induces ED *via* NO inhibition. NO is a potent vasodilator that activates soluble guanylyl cyclase in vascular muscle, resulting in accumulation of cyclic guanosine monophosphate (cGMP) and relaxation. It was reported that bioavailability of endothelium-derived nitric oxide was reduced in the aorta, mesenteric arterioles, cerebral arterioles and arterials from gracilis muscle of mice with mild HHcy [20]. We found that endothelium-dependent vascular relaxation response to ACh was impaired in the aortic and cremaster microvascular arterials from $CBS^{-/-}$ mice. Preinucabtion with N^G-nitro-L-arginine methyl ester (L-NAME), an endothelial nitric oxide synthase (eNOS) inhibitor, abolished vascular relaxation response to ACh, indicating that decreased NO bioavailability was involved in HHcy-induced ED (Fig. 3) [19]. A suppressive effect of Hcy on endogenous bioavailable NO is also supported by the previous finding that incubation of arterial strips with Hcy significantly attenuated the endothelium-dependent vascular relaxation response to calcium ionophore A23187 [23]. NO bioavailability could be diminished by the reaction of NO with superoxide anion ($O_2^{-\cdot}$) or by the reaction of thiol groups in the Hcy molecule with NO. Exposure of ECs to Hcy led to the formation of S-nitrosohomocysteine, decreasing the bioactivity of NO [24].

Effect of HHcy on eNOS expression on vasculature was controversy by either upregulate [25] or downregulate [19]. We have found that HHcy inhibited eNOS activity in human aortic ECs *via* PKC and threonine 495 phosphorylation pathways [19]. Recent evidence suggests that asymmetric dimethylarginine (ADMA), an endogenous inhibitor eNOS, may mediate HHcy-induced ED [20]. The supporting evidence for the ADMA hypothesis is that Hcy reduced the activity of dimethylaminohyrolase (DDAH), the enzyme that degrades ADMA, in cultured endothelial cell line ECV304 [26]. However, we did not find an elevated plasma ADMA levels in $CBS^{-/-}$ mice, a genetic model of severe HHcy, by using HPLC analysis [19]. In addition, tetrahydrobiopterin (BH_4), a cofactor of eNOS, was recently reported to attenuate Hcy-mediated impairment of endothelium-dependent relaxation in rats aortic rings [27]. ED in the mesenteric artery from pregnant $CBS^{+/-}$ mice was restored by preincubation with sepiaptern, a precursor of BH_4 [28]. Very recently, It was reported that 5-MTHF has beneficial effects on endothelial function and vascular superoxide production in human atherosclerosis, by preventing peroxynitrite-mediated BH_4 oxidation and improving eNOS coupling [29]. Interestingly, we have found that neither argine (1 mmol/L) nor BH_4 (10 μmol/L) or sepiapterin (10 μmol/L) could prevent the inhibitory effect of Hcy on eNOS activity in cultured human and mouse aortic ECs [19].

2) Prostanoids Regulation

Prostanoids including prostaglandins and thromboxanes are generated from aracidonic acid by cyclooxygenase (COX). Prostacyclin (PGI_2) is the major vasodilatory prostanoid produced in ECs and is released in response to shear stress, hypoxia, or to substances that stimulate NO formation. PGI_2 relaxes vascular smooth muscle by activation of adenylate cyclase (AC), and thus leads to increased production of cyclic adenosine monophosphate (cAMP). In addition to the vasodilatory prostanoids, ECs also produce vasoconstrictive prostanoids such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin H_2 (PGH_2), or thromboxane A_2 (TXA_2). Contractile prostaglandins induced vascular contraction by inhibiting activity of AC, and thus lead to decreased generation of cAMP Previous studies found that a high Hcy concentration increased TXA_2 and 8-iso-$PGF_{2\alpha}$ (a metabolite of $PGF_{2\alpha}$) biosynthesis [30-32]. Preincubation with a non-specific COX inhibitor indomethacin or PGH_2/TXA_2 receptor antagonist SQ 29,548 restored ED in the skeletal muscle arterioles from Wistar rats fed with high-Met (1 g/kg) diet for 4 weeks [31]. In the arterioles of HHcy rats, increased flow-induced constriction was abolished by indomethacin or SQ 29,548 or the TXA_2 synthase inhibitor CGS 13,080 [32]. PGI_2 generation seems not significantly affected by HHcy [33]. Whether contractile prostanoids induction in HHcy is the result of COX activation and/or due to increased release of arachidonic acid remains unclear. In fact, recent evidence that Hcy induced arachidonic acid release and formation of its end product TXB_2 in platelet may provide partial explanation [34].

Taken together, HHcy induces ED, probably partially, *via* aracidonic acid-prostanoids pathway. The induction of TXA_2, PGH_2 and $PGF_{2\alpha}$, but not PGI_2, seems contributing to HHcy-induced ED. Further studies exploring the role and mechanisms of HHcy-induced imbalance of vasodilatory and vasocontractive prostanoids should provide important insights.

3) Endothelium-Derived Hyperpolarizing Factors (EDHF) Inhibition

Accumulative evidence is mounting that EDHF is a major determinant of vascular tone, especially in small resistance vessels [18]. The nature of EDHF is still not entirely elucidated. The endothelium-mediated relaxation that remains resistant to NOS and COX inhibition is thought to be mediated by EDHF. Current evidence suggests that EDHF-mediated responses are initiated by activation of endothelial K^+ channels with resultant hyperpolarization of ECs. This endothelial hyperpolarization spreads to the underlying smooth muscle layer through myoendothelial gap junctions, or the efflux K^+ from the ECs elicit hyperpolarization of the adjacent smooth muscle cells. Epoxyeicosatrienoic acid likely has a regulatory role in this pathway [35]. EDHF may reflect a purely electrical coupling process between cells and involve myoendothelial gap junctions that are constituted, potentially, from the five connexins (Cx 37, 40, 43, 44 and 45) found in vascular tissues [36-38]. Several risk factors for atherothrombotic CVD have been reported to affect the EDHF pathway, including hypertension [39], diabetes [40], hypercholesterolemia [39]. Role of Hcy on EDHF still remains unclear. EDHF-mediated renal vasodilatory response was impaired in the Wistar rats during acute and chronic HHcy [41]. Furthermore, HHcy-induced impairment of EDHF was suggested to be related to redistribution of Cx43 [42], and SAH related down-regulation of Cx44 in ECs [38]. In addition, oxidative stress, implicated as a pathological mechanism in HHcy, has also been shown to disrupt gap junctional communication [43]. Understanding the mechanisms of HHcy-induced EDHF impairment could be beneficial for the prevention and/or therapy of microangiopathy in HHcy.

Endothelium-independent dilation induced by sodium nitroprusside (SNP), a nitric oxide donor, however, has been reported to be reduced in HHcy by some authors [44] or not reduced by others [45]. The differences on endothelium-independent relaxation response to SNP may

be due to difference on tHcy levels, duration of HHcy, species, and type of vessels, and synergetic effects of other factors such as hyperglycemia and dyslipidemia.

4) Angiotensin II Receptor-1 Activation

Angiotensin II (Ang II), an octapeptide hormone, is the active component of the renin-angiotensin system. Ang II is a multifunctional peptide that has numerous actions on vascular smooth muscle, such as the modulation of vasomotor tonus. Accordingly, Ang II plays a fundamental role in controlling the functional and structural integrity of the arterial wall and may be important in physiological processes, regulating blood pressure and pathological mechanisms underlying vascular disease. Therefore, understanding whether Ang II is involved in HHcy-induced ED may provide a fundamental insight in the treatment of HHcy-related vascular diseases. However little is known about the effect of this elevation upon the vascular reactivity to Ang II. Recent study found that AT_1 activated in mouse aortic ECs treated with Hcy (100 μmol/L) for 48 h [46]. Furthermore, HHcy-induced activation of AT_1 receptor involves matrix metalloproteinase-9 and collagen type-1 modulation using extracellular signal-regulated kinase-1/2 and signal transducer and activator of transcription 3 signaling cascades [46]. Valsartan, a AT_1 receptor blocker, attenuated pathological ventricular hypertrophy induced by HHcy in rats [47]. Vasoconstraction to Ang II was increased in the carotid artery from Wistar rats fed with Met (0.1, 1 and 2g/kg body weight) in the drinking water for 8 and 16 weeks [48]. Preincubation with indomethacin, normalized the contractile response to Ang II. This study suggested that HHcy-induced vasocontractile response to Ang II appears to be related to the release of vasoconstrictor prostanoids [48].

5) Endothelin-1 Induction

Endothlin-1 (ET-1) is a 21-residue peptide synthesized and secreted by EC. It is classically defined as a potent vasoconstrictor and mitogen for VSMCs. ET-1 often acts as a paracrine hormone, binding to transmembrane ET_A receptor expressed on VSMC. However, ET-1 also elicits a vasodilatory response mediated by transmembrane ET_B receptors on vascular ECs. Because regulation of vascular tone is one of the most specific endothelial functions so far described, ET-1 is used as a powerful marker to study endothelial function.

Several studies showed that HHcy activate or induce ET-1. Preincubation with Hcy (500 μmol/L) for 24 h increased the secretion and mRNA levels of ET-1 in human umbilical vein ECs [49]. It is suggested that Hcy induces ERK phosphorylation *via* reactive oxygen species (ROS) accumulation, and activates transcriptional factor AP-1, which in turn elicits additional expression and secretion of ET-1 [49,50]. Clinically, plasma Hcy level showed significantly positive correlation with plasma ET-1 level in the patients with disturbed glucose metabolism [51]. Hcy-induced ET-1 release is dependent on hyperglycaemia and reactive oxygen species (ROS) production in bovine aortic ECs [52]. Recently, Duan *et al.*, reported that phytoestrogen alpha-zearalanol ablated Hcy-elicited ET-1 secretion/mRNA induction and ROS accumulation [49]. In contrast, other studies shown that pathophysiological concentrations of Hcy reduced both ET-1 production and preproET-1 mRNA levels in cultured EC potentially through oxidative products [53,54]. Because of the contradictory observations stated, the role of ET-1 in HHcy-induced ED is uncertain and need to be further evaluated.

6) Role of Free Radical

Oxidation has been suggested as a primary biochemical mechanism responsible for HHcy-related pathogenesis. Experiments using animal models with genetically or diet-induced HHcy [55] or Hcy treated cultured ECs [56] showed an increased accumulation of ROS. Five mechanisms have been proposed for Hcy-induced oxidative stress. These include: 1) inhibition of the activity of cellular antioxidant enzymes such as cellular glutathione peroxidase or heme oxygenase -1, 2) Hcy autooxidation, 3) nitric oxide synthase (NOS)-dependent generation of

superoxide anion (O^{-}_{2}) *via* uncoupling of eNOS, 4) disruption of extracellular superoxide dismutase from endothelial surfaces, and 5) activation of NADPH oxidases.

ROS and oxidant stress promote the formation of nitrotyrosine, an indictor of the NO and superoxide radical reaction, resulting in the formation of the strong oxidant peroxynitrite. Peroxynitrite, besides other effects, leads to tyrosine nitration. The letter event may alter protein function, and therefore, induce cellular dysfunction. Pretreatment with catechin, a flavonoid antioxidant that reduces ROS levels, decreased Hcy-dependent formation of nitrotyrosine [57]. Peroxynitrite can also directly damage the electron-transport chain in mitochondria and thereby induce mitochondrial and consequently ED [58]. Apocynin, a NADPH oxidase inhibitor, not only attenuated aortic superoxide and peroxynitrite to control levels but also restored endothelium-dependent relaxation in the aortas of HHcy rats [59]. Tempol, a cell permeable mimetic of SOD, improved endothelial function in the rat femoral arteries preincubation with D,L-Hcy (100 μM) for 4 h [60]. Tempol also improved ED in the absence of Hcy after the initial incubation period [60]. Decreased NO bioavailability by reaction of NO and superoxide is likely a major mechanism on oxidative stress-induced ED. Superoxide also plays an important role in impairment of EDHF by oxidation of myoendothelial gap junction proteins [43].

7) Vascular Contractile Response to Phenylephrine

Phenylephrine (PE) induces smooth muscles cell contraction by stimulating α_1-adrenoreceptors, which causes a rapid calcium release from scarcoplasmic reticulum and prevents calcium reuptake. PE also depolarizes the arterial smooth muscle cells and consequently activates voltage-operated Ca^{2+} channels in the plasma membrane of the smooth muscle, leading to an increased influx of Ca^{2+}. The sustained contractions result from calcium influx through the receptor-operated calcium channels. Vascular contractile response to PE is either increased [61,62] or decreased in the presence of HHcy [63]. Preincubation with Hcy (10 mmol/L) for 3 h, increased vascular contractile response to PE in pulmonary arteries of guinea-pig [62]. In addition, long-term high Met load (1g/kg body weight) for 4 weeks increased vasoreactivity to PE in the rat skeletal muscle arteriolars [64]. Furthermore, increased contractile response to PE by HHcy was significantly inhibited by the concomitant incubation with either SOD plus catalase, Tiron or PJ34 (an inhibitor of polyADP-ribose polymerase) [62,64], suggesting the involvement of oxidative stress. Increased contractile response to PE can be also inhibited by preincubation with L-NAME, suggesting HHcy reduced activity of NO in arterioles may contribute to the microvascular impairment described in HHcy [64]. Very recently, Andrade *et al.*, demonstrated that the enhanced PE-induced vascular contraction in carotid artery due to HHcy is endothelium-dependent and involves a loss of the inhibitory effect of relaxant α_{1D}-adrenoceptors by reducing NO biodisponibility [61].

Enhanced vasoreactivity to PE in HHcy may be *via* endothelium-dependent pathway, and may be related to oxidative stress, decreased NO bioavailability, loss of the inhibitory effect of relaxant α_{1D}- adrenoceptors by reducing NO biodisponibility.

Hcy-Lowering Therapy and ED

Several studies have described an improvement in endothelial function following administration of folic acid (5 or 10 mg/day), but not with low dose of folic acid (400 μg/day) in human [65,66]. More recently, It was reported that folic acid supplementation at high dose (0.071 μg/g/day, equivalent to a 5 mg/70 kg/day human dose) but not low dose (0.0057 μg/g/day, equivalent to a 400 μg/70kg/day human dose) for two weeks restored ED in the mesenteric artery from CBS (+/-) mice [67]. This suggests that the dosage of folic acid may be critical for improvement of ED in HHcy. Improvement of endothelial function does not consistently correlate with total Hcy lowering [66]. However, some studies found a significant positive

correlation between tHcy reduction and improvement in endothelial function [68]. The efficacy of folic acid supplementation on improving ED and prevent Hcy-related cardiovascular events remains an open questions and requires further evaluation.

Conclusion

In summary, extensive experimental evidence, both *in vitro* and *in vivo*, indicates that HHcy impairs endothelial function. As summarized in Fig 2, HHcy changes vascular tone by regulating endothelium-dependent vasodilator and constrictor substances, including decreasing NO bioavailability, increasing contractile prostanoids as well as interfering myoendothelial communication. Although oxidative stress and eNOS uncoupling have been suggested to play a critical role in HHcy-induced ED, in our study, preincubation with antioxidant SOD plus catalase did not restored ED in the aortic arterial from $CBS^{-/-}$ mice (Fig. 3) [19]. Further, adenoviral transduced SOD or GPX-1 overexpression did not restore eNOS activity suppressed by Hcy in cultured mouse ECs [19]. Similarly, results of randomized controlled trials of antioxidant vitamin supplements in large numbers of participants has been ambiguous or contradictory [69]. This discrepancy remains to be resolved in future experimental and clinical studies. Studies on the mechanisms of HHcy-induced impairment of microvasculature myoendothelial gap junction, such as Cx43 and Cx44, should be an interesting area for future research. Further, the effect of HHcy on ET-1 secretion/generation may be relevant for CVD in diabetes. Finally, the beneficial effects on the endothelial function by supplementation of high dose folic acid (5-10 mg/day) may provide potential therapeutic advantage for HHcy-related cardiovascular diseases.

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Abbreviation

AC	Adenylate cyclase

ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
Ang II	Angiotensin II
AT_1	Angiotensin II receptor-1
BH_4	Tetrahydrobiopterin
cAMP	Cyclic adenosine monophosphate
CBS	Cystathionine β-systhase
COX	Cyclooxygenase
CVD	Cardiovascular disease
Cx	Connexin
DDAH	Dimethylaminohyrolase
ECs	Endothelial cells
ED	Endothelial dysfunction
EDHF	Endothelium-derived hyperpolarizing factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A	Endothelin-1 receptor A
Hcy	Homocysteine
HHcy	Homocystinemia
Met	Methionine
MS	Methionine synthase
MTHER	5,10-methylenetetrahydrofolate reducatase
NO	Nitric oxide
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
SNP	Sodium nitruprusside
SOD	Superoxide dismutase
ROS	Reactive oxygen species
tHcy	Plasma total homocysteine
TXA_2	Thromboxane A_2

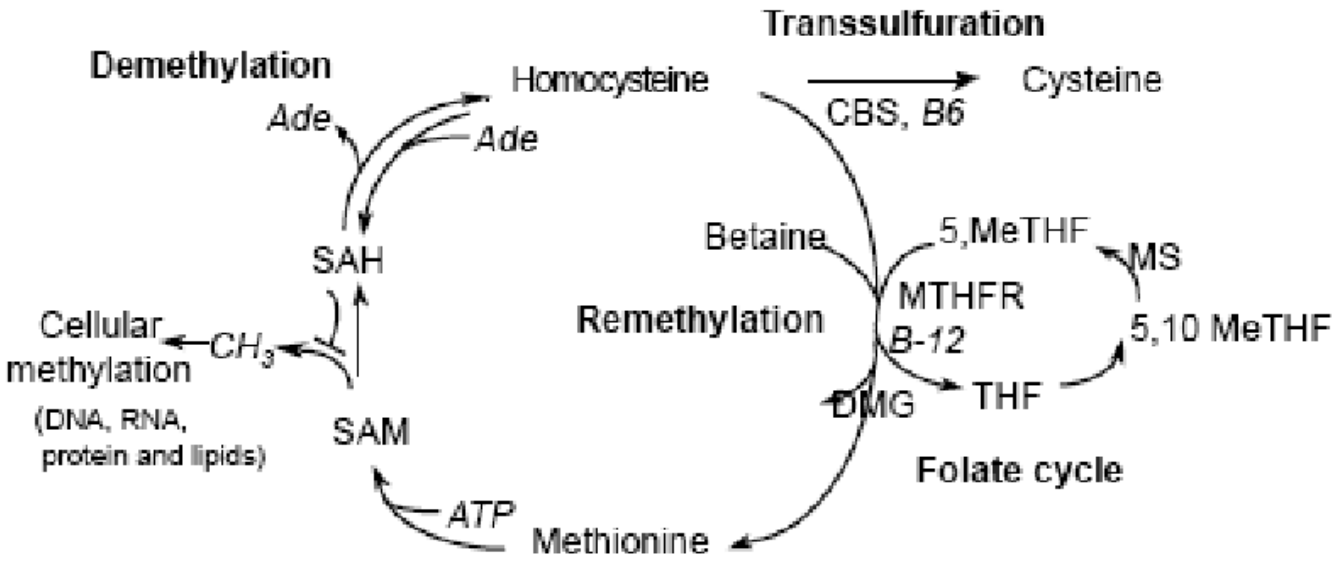

Fig. (1). The pathways of homocysteine metabolism

Ade, Adenosine; CBS, cystathionine-b-synthase; DMG, dimethyglycine; MeTHF, methylenetetrahydrofolate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH,S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

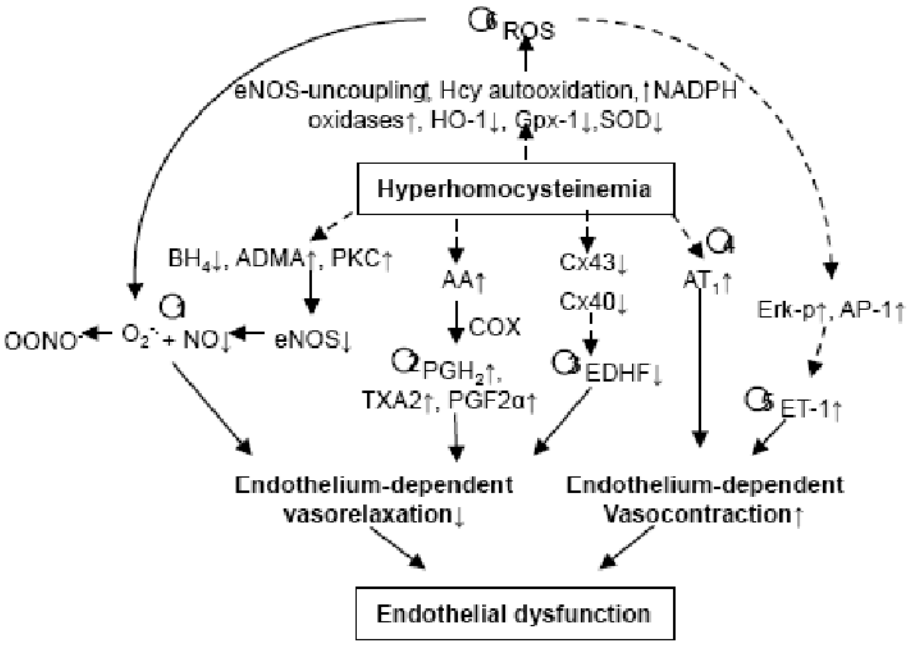

Fig. (2). Potential mechanisms of HHcy-induced endothelial dysfunction

Six mechanisms have been suggested explaining HHcy-induced ED; 1) nitric oxide inhibition, 2) prostacyclin regulation, 3) endothelium-derived hyperpolarizing factors suppression, 4) angiotensin II receptor-1 activation, 5) endothelin-1 induction, and 6) oxidative stress. AA, aracidonic acid; AP-1, activator protein-1; AT1, angiotensin II receptor 1; eNOS, endothelial NO synthase; ADMA, asymmetric dimethylarginie, an eNOS inhibitor; BH4, tetrahydrobiopterin; COX, cyclooxygenase; Cx, connexins; EDHF, endothelium-derived hyperpolarizing factor; Erk-p, phosphorylated extracellular signal-regulated protein kinase, ET-1, endothelin-1; Gpx-1, glutathione peroxidase, an antioxidant enzyme; HO-1, heme oxygenase-1; O2-•, superoxide anion; OONO-, peroxynitrite anion; BH4, tetrahydrobiopterin, a cofactor of eNOS; NADPH, a cofactor of eNOS; NO, nitric oxide; PKC, protein kinase C; PGH2, protaglandin H2; PGF2α, prostaglandin F2α; ROS, reactive oxygen species; SOD, superoxide dismutase; TXA2, thromboxane A2,

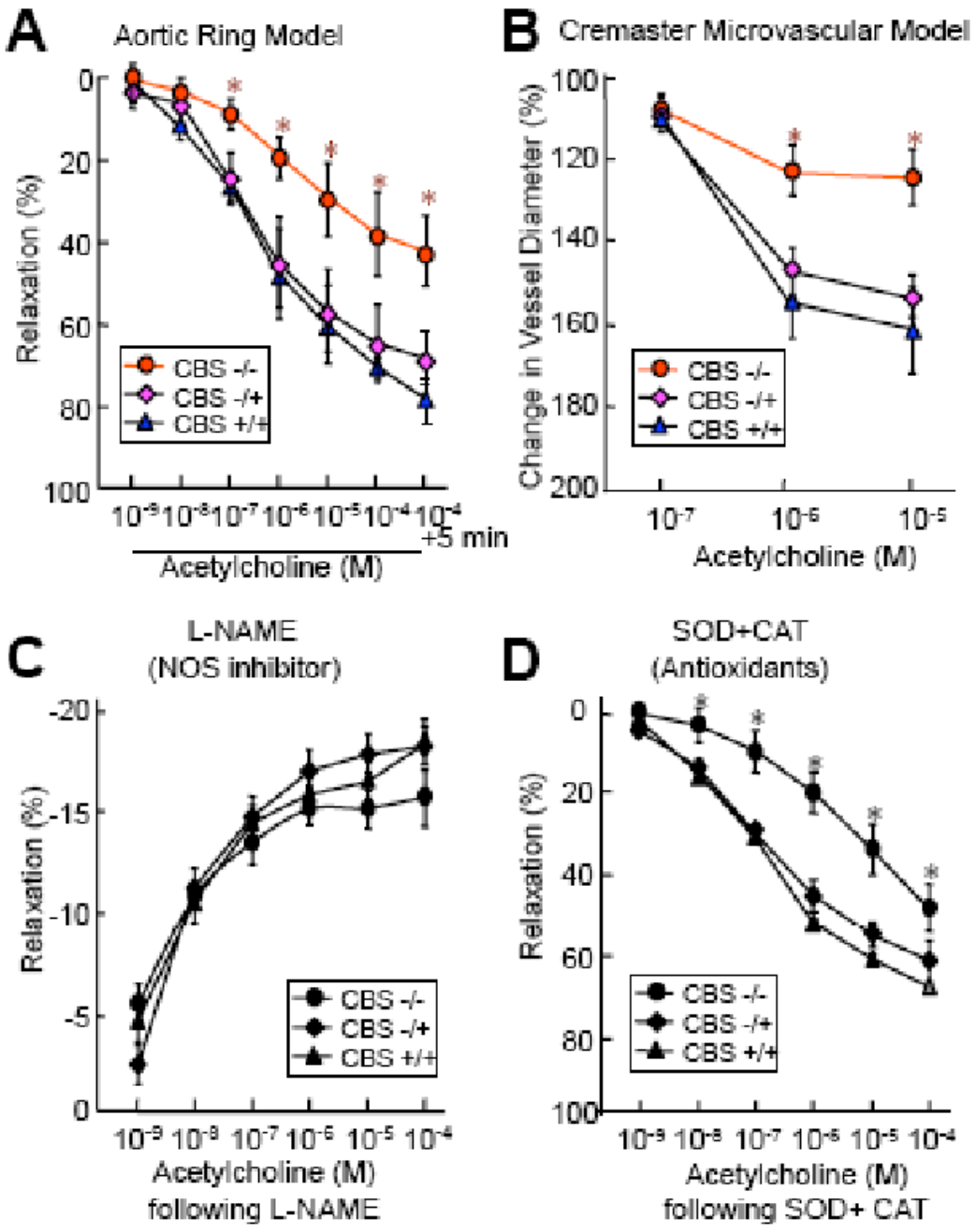

Fig. (3). Severe HHcy Impaired endothelial dependent arterial relaxation *via* eNOS inhibition

Mouse vascular reactivity was examined using two ex-vivo functional models, aortic ring and intravital videomicroscopy of the cremaster. Mouse thoracic aortas were harvested from CBS KO mice at the age of 10 weeks. **A. Ex vivo aortic vasomotor response.** Vessel rings were precontracted and then contracted with 10-6 M phenylephrine. Dose-responsive relaxation was measured in response to cumulative addition of acetylcholine (10-9-10-4M) in 1 min interval. **B. Cremaster microvascular vasomotor response.** Mouse cremaster micrevasculature was pinned radially, visualized through a upright microscope and continually superfused with Krebs buffer. The inner lumen diameter of the arteriole was measured before and after superfusion with acetylcholine (10-7-10-5M) in a 3 min interval. C & D. Vasorelaxation to

acetylcholine following NG-nitro-L-arginine methyl ester (L-NAME) and superoxide dismutase (SOD) + catalase (CAT). Aortic rings were precontracted with KCl, treated with 30 mM L-NAME for 20 min or 200 U/ml SOD plus 100 U/ml CAT for 2 min, and contracted with phenylephrine. Dose-response relaxation was measured in response to cumulative addition of acetylcholine at 1 min intervals. n=9, * $P \leq 0.01$ verses CBS-/+ or CBS+/+ mice with identical treatment.