

Brief Review

Hyperhomocysteinemia and atherosclerosis

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Abstract: Arteriosclerosis and its complications, such as heart attack and stroke, are the major causes of death in developed countries. It was believed that age, hyperlipidemia, hypertension, diabetes and smoking are common risk factors for cardiovascular disease. In addition, overwhelming clinical and epidemiological studies have identified homocysteine (Hcy) as a significant and independent risk factor for cardiovascular disease. In healthy individuals, plasma Hcy is between 5 and 10 $\mu\text{mol/L}$. One cause of severe hyperhomocysteinemia (HHcy) is the deficiency of cystathionine β -synthase (CBS), which converts Hcy to cystathionine. CBS homozygous deficiency results in severe HHcy with Hcy levels up to 100 to 500 $\mu\text{mol/L}$. Patients with severe HHcy usually present with neurological abnormalities, premature arteriosclerosis. It has been reported that lowering plasma Hcy improved endothelial dysfunction and reduced incidence of major adverse events after percutaneous coronary intervention. The mechanisms by which Hcy induces atherosclerosis are largely unknown. Several biological mechanisms have been proposed to explain cardiovascular pathological changes associated with HHcy. These include: (1) endothelial cell damage and impaired endothelial function; (2) dysregulation of cholesterol and triglyceride biosynthesis; (3) stimulation of vascular smooth muscle cell proliferation; (4) thrombosis activation and (5) activation of monocytes. Four major biochemical mechanisms have been proposed to explain the vascular pathology of Hcy. These include: (1) autooxidation through the production of reactive oxygen species; (2) hypomethylation by forming SAH, a potent inhibitor of biological transmethylation; (3) nitrosylation by binding to nitric oxide or (4) protein homocysteinylation by incorporating into protein. In summary, our studies, as well as data from other laboratories support the concept that Hcy is causally linked to atherosclerosis, and is not merely associated with the disease. Although folic acid, vitamin B12 and B6 can lower plasma Hcy levels, the long-term effects on cardiovascular disease risk are still unknown and judgments about therapeutic benefits await the findings of ongoing clinical trials.

Key words: homocysteine; atherosclerosis

血浆同型半胱氨酸水平升高与动脉粥样硬化

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摘要: 心血管疾病已成为当今全球性致残与致死的最重要原因之一。目前确定的冠心病的危险因素主要包括高龄、血脂异常、高血压、糖尿病、吸烟、肥胖症。大量的临床试验及流行病学研究已经证实血浆同型半胱氨酸水平升高是心血管疾病的一个独立的危险因素。健康人的血浆同型半胱氨酸水平为 5~10 $\mu\text{mol/L}$ 。血浆同型半胱氨酸水平严重升高的主要原因是胱硫醚- β -合成酶(cystathionine- β -synthase, CBS)基因的缺陷。CBS 基因缺陷的纯合体可导致血浆同型半胱氨酸水平升高至 100~500 $\mu\text{mol/L}$ 。血浆同型半胱氨酸水平严重升高的病人通常伴随神经系统异常、早发性的动脉粥样硬化。叶酸、维生素 B6 和 B12 治疗能降低血浆同型半胱氨酸的水平并改善血管内皮功能、减少经皮冠状动脉腔内成形术(percutaneous transluminal coronary angioplasty, PTCA)术后并发症。迄今为止, 血浆同型半胱氨酸水平升高引起心血管疾病的发病机制并未完全明了, 目前认为主要与以下几个方面有关: (1) 内皮细胞损伤及功能障碍。我们实验室在 CBS 基因敲除的小鼠模型上证实血浆同型半胱氨酸水平升高能抑制 eNOS 的活性, 导致主动脉内皮功能的障碍。我们还在细胞模型上证实了同型半胱氨酸能显著抑制内皮细胞的增殖。(2) 胆固醇和甘油三酯生物合成代谢异常。我们实验室在 apoE、CBS 双基因敲除的小鼠模型上证实血浆同型半胱氨酸水平升高能改变肝脏的脂肪代谢, 增加巨噬细胞对修饰 LDL 的摄取, 从而导致胆固醇脂和甘油三酯在血管壁的堆积, 促

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进动脉粥样斑块的形成。(3)刺激血管平滑肌细胞增殖。此外还发现同型半胱氨酸能激活蛋白激酶C信号途径,促进胶原蛋白的合成,抑制弹性蛋白和胶原蛋白的交联。(4)激活血栓形成。(5)激活单核细胞。目前认为同型半胱氨酸主要通过以下几个化学机制致病:(1)自氧化产生活性氧。同型半胱氨酸在自氧化的过程中能产生大量的活性氧,从而引起血液中脂蛋白和细胞膜脂质的过氧化损伤,并进一步引起内皮功能的障碍。(2)在腺苷的参与下形成SAH,一种甲基转移抑制剂,导致细胞内的低甲基化。(3)与一氧化氮结合形成亚硝酰物。(4)参与蛋白质的合成。总之,我们和其他实验室的研究结果均表明同型半胱氨酸不仅与动脉粥样硬化相关,而且具有致病效应。尽管补充叶酸、维生素B6和B12等治疗能降低血浆同型半胱氨酸的水平,但是否能降低心血管疾病的风险仍有待于大量的动物研究及临床试验。

关键词: 同型半胱氨酸; 动脉粥样硬化

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Abbreviation: ACAT, acyl CoA:cholesterol acyltransferase; Ade, adenosine; apoE, apolipoprotein E; CBS, cystathionine β -synthase; CE, cholesterol ester; CEH, cholesterol ester hydrolase; DMG, dimethylglycine; FC, free cholesterol; HDL, high density lipoprotein; HHcy, hyperhomocysteinemia; HHcyc, hyperhomocysteinemic; LDL, low density lipoprotein; MeTHF, methylenetetrahydrofolate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; NEFA, non-esterified fatty acid; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SR, scavenger receptors; THF, tetrahydrofolate; TG, triglyceride; TC, total cholesterol; TGS, triglyceride synthase; TLC, thin layer chromatography;

Despite considerable advances in our understanding of the etiology of arteriosclerosis, the established risk factors do not fully explain its occurrence: about 30% cardiovascular disease cannot be explained by conventional risk factors^[1,2]. Although overwhelming clinical and epidemiological studies have identified homocysteine (Hcy) as a significant and independent risk factor for cardiovascular disease^[3,4], the mechanisms by which Hcy induces atherosclerosis are largely unknown. We have previously suggested that Hcy promotes atherosclerosis by stimulating vascular smooth muscle cell proliferation and inhibiting endothelial cell growth^[5-7], and have recently reported that hyperhomocysteinemia (HHcy) accelerates atherosclerosis in a mouse hyperhomocysteinemic (HHcyc) model with and without dietary manipulation^[8]. Our studies, together with studies discussed in this review, support the hypothesis that Hcy is a causative factor for atherosclerosis.

Hcy metabolism and HHcy

Hcy is a highly reactive sulfhydryl-containing amino acid and an intermediate in methionine metabolism (Fig.1). Methionine forms S-adenosylmethionine (SAM), a major donor of cellular methylation. SAM, which converts to S-adenosylhomocysteine (SAH) by donating a methyl group for cellular methylation, is then converted to Hcy. Further metabolism of Hcy occurs through two pathways to reduce total Hcy concentrations in the cells and blood; trans-sulfuration to cysteine, catalyzed by vitamin B6 dependent cystathionine β -synthase (CBS), and remethylation to methionine by receiving a methyl group from betaine or methylenetetrahydrofolate (MeTHF), catalyzed by methion-

ine synthase (MS) or methylenetetrahydrofolate reductase (MTHFR). Vitamin B12 and folic acid serve as important co-enzymes for remethylation. Multiple factors interacting with Hcy metabolism determine its plasma concentration. These include genetic abnormalities, nutritional defects, renal impairment, age and gender, all of which play a role in accelerated cardiovascular disease. Genetic defects in the enzymes (CBS, MS or MTHFR) and dietary defect of the cofactors (folic acid, vitamins B6 and B12) involved in trans-sulfuration and remethylation pathways would impair Hcy clearance and increase plasma Hcy levels.

HHcy and cardiovascular disease

In healthy individuals, plasma Hcy is between 5 and 10 $\mu\text{mol/L}$. The earliest case of severe HHcy was reported 40 years ago^[9]. One cause of severe HHcy is a deficiency of CBS, which converts Hcy to cystathionine (Fig.1)^[10]. CBS homozygous deficiency results in severe HHcy with Hcy levels up to 100 to 500 $\mu\text{mol/L}$. These are rare disorders with a frequency of 1 out of 75 000 in the general population. Patients with severe HHcy who usually present with neurological abnormalities, premature arteriosclerosis, develop cerebral thrombosis or myocardial infarction around 30 years old. In 1969, Dr McCully first suggested that elevated Hcy levels are responsible for widespread vascular lesions in HHcyc infants, and that moderate HHcy may be a potential cause for cardiovascular disease^[11]. This hypothesis was overlooked for many years, until the later recognition of Hcy as an independent risk factor for myocardial infarction and stroke in general population by reports of prospective studies^[3,4]. It is now recognized that HHcy is a common risk factor for cardiovascular disease,

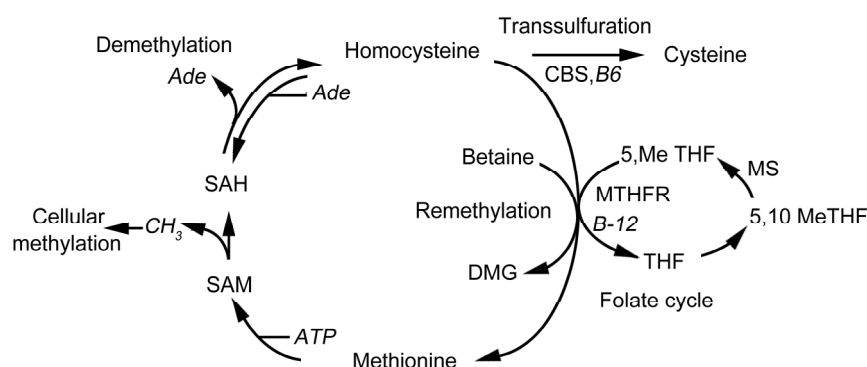


Fig. 1. Pathways of Hcy metabolism (SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Ade, adenosine; THF, tetrahydrofolate; MeTHF, methylenetetrahydrofolate; CBS, cystathionine- β -synthase; DMG, dimethylglycine; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase).

similar to those for smoking and hyperlipidemia^[12], despite controversy about whether moderate HHcy is a causative agent or only a marker of cardiovascular disease.

Hcy lowering and cardiovascular disease

Severe and moderate HHcy can be treated with vitamins supplements of folate, B6, and B12^[13,14]. Folate supplementations, in daily doses of at least 0.4 mg, have been shown to reduce plasma Hcy levels even in healthy individuals^[15,16]. The addition of vitamins B12 and/or B6, to folic acid supplementation may provide a small further reduction in Hcy levels in certain groups of patients. Renal impairment is an important cause of HHcy, and individuals with HHcy secondary to renal disease commonly require significantly higher doses of folic acid (5–40 mg) to achieve maximal therapeutic effect^[17]. Importantly, it has been reported that lowering plasma Hcy improved endothelial dysfunction, a marker of atherothrombotic risk^[18,19]. Recently, short-term Hcy-lowering therapy (12 months) has suggested some clinical benefit of reduced incidence of major adverse events after percutaneous coronary intervention^[20]. However, the long-term effects of reduced plasma Hcy levels on cardiovascular disease risk are still unknown and judgments about therapeutic benefits await the findings of ongoing clinical trials.

Biological mechanisms of Hcy pathology

A large body of *in vitro* studies has demonstrated significant biological effects caused by Hcy. Several biological mechanisms have been proposed to explain cardiovascular pathological changes associated with HHcy. These include endothelial cell damage^[7], impaired endothelial function^[21,22], dysregulation of cholesterol and triglyceride (TG) biosynthesis^[23], thrombosis activation^[24], stimulation of vascular smooth muscle cell (VSMC) proliferation^[5,25], and ac-

tivation of monocytes^[26]. Because VSMC proliferation is one of the hallmarks of atherosclerosis, early studies of Hcy vascular biology focused on VSMC. We were the first to report a significant growth-promoting effect of Hcy on human and rat aortic smooth muscle cells^[5,25]. We have also shown that Hcy increased cyclin A gene expression and activated cyclin A promote through the ATF/cyclic AMP-responsive element-binding site. Several studies from other laboratories supported these findings and confirmed the mitogenic effects of Hcy on VSMC^[27–29]. In addition, Hcy activates the protein kinase C pathway, increases c-myc and c-myb expression^[30], increases collagen synthesis^[28] and inhibits lysyl oxidase, a key enzyme in elastin and collagen crosslinking^[31] in VSMC. Thus, it is generally agreed that Hcy, at high concentrations, promotes atherosclerosis, at least, in part, by stimulate VSMC proliferation.

Proposed biochemical mechanisms

Although multiple mechanistic studies have demonstrated significant biological effects and important molecular events caused by Hcy, the biochemical mechanisms by which HHcy promotes arteriosclerosis remain largely unknown. Four major biochemical mechanisms have been proposed to explain the vascular pathology of Hcy. These include (1) autooxidation through the production of reactive oxygen species^[32], (2) hypomethylation by forming SAH, a potent inhibitor of biological transmethylation (Fig.1)^[33], (3) nitrosylation by binding to nitric oxide, or (4) protein homocysteinylation by incorporating into protein^[34].

Hcy autooxidation mechanisms

Oxidation has been proposed as a primary biochemical mechanism responsible for Hcy pathogenesis^[24]. Hcy contains a free sulfhydryl group (SH), which dominates the redox property. Hcy can be autooxidized with another Hcy

molecule, and generate the disulfide and reactive oxygen species (Fig.2). In a biological system, Hcy can form a mixed disulfide with other free sulfhydryl amino acid or sulfhydryl containing proteins via similar oxidation mechanism, thereby generating reactive oxygen species. During Hcy oxidation, the liberated reactive oxygen species could initiate lipid peroxidation in circulating lipoproteins and in the cell membranes, a process that could lead to impaired endothelial function. In addition, Hcy-mediated oxidation of low-density lipoprotein (LDL) could form lipid derivatives that trigger platelet activation, releasing growth factors that cause smooth muscle cell proliferation and vascular hypertrophy.

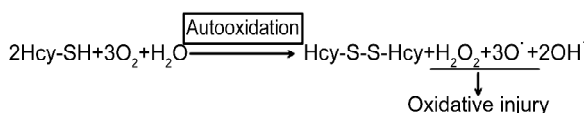


Fig. 2. Homocysteine autooxidation. Hcy-SH, free Hcy; Hcy-S-S-Hcy, homocystine (disulfide); O₂, oxygen; H₂O, water; H₂O₂, hydrogen peroxide; O[•], singlet oxygen; OH[•], hydroxyl radical.

Hcy hypomethylation mechanisms

Paradoxically, most of the reported biological effects of Hcy in vascular cells were observed at Hcy concentrations about 100-fold higher than those found in human HHcy. The putative pro-oxidative effects of autooxidation of its free sulfhydryl group can be mimicked by cysteine, a non-pathogenic biothiol. We have proposed that hypomethylation is a specific biochemical mechanism by which Hcy induces vascular injury that leads to cardiovascular disease [33]. Through the adenosylation pathway, Hcy can utilize adenosine, a normal constituent of all body fluids, to form

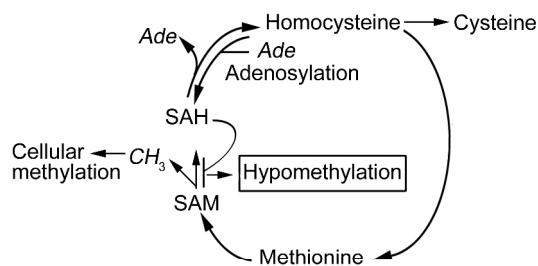


Fig. 3. Hcy hypomethylation (SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Ade, adenosine).

SAH, a potent inhibitor of cellular methylation, thereby causing cellular hypomethylation (Fig.3). Notably, this pathway is not shared by cysteine. The formation of SAH depends on Hcy availability and is not affected by adenosine accumulation^[35].

We tested this hypothesis on cultured vascular cells, and found that clinically relevant concentrations (20–50 μmol/L) of Hcy and homocystine (the disulfide), in the presence of adenosine, dramatically inhibited [³H]thymidine incorporation, an indicator of DNA synthesis, and cell proliferation in a dose-dependent manner in endothelial cells from different species (Fig.4). This inhibitory effect appears to be specific to endothelial cells; Hcy did not inhibit DNA synthesis in aortic smooth muscle cells or fibroblasts. Interestingly, this is a free sulfhydryl group-independent effect, because homocystine, which lacks the free sulfhydryl group, had a similar inhibitory effect on endothelial cell growth. Importantly, this is not mimicked by cysteine or cysteine (the disulfide)^[7]. Therefore, we have identified a Hcy unique, cell type specific, growth inhibitory effect at clinically relevant concentrations in endothelial cells. Because damage to endothelial cells is a key feature of

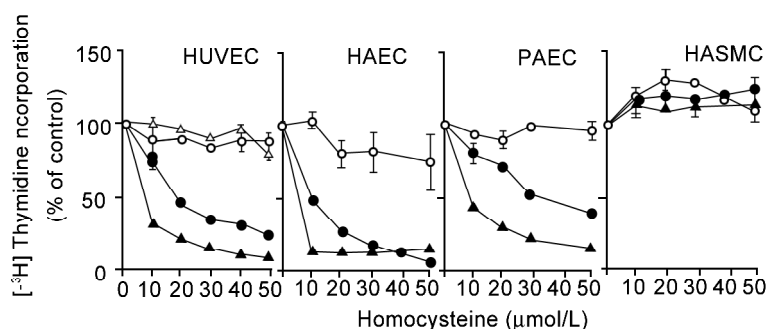


Fig. 4. Effect of Hcy on DNA synthesis in endothelial cells. Human umbilical vein endothelial cell (HUVEC), human aortic endothelial cells (HAEC), porcine aortic endothelial cells (PAEC) and human aortic smooth muscle cells (HASMC) were incubated with control medium containing 50 μmol/L adenosine and 10 μmol/L EHNA (an adenosine deaminase inhibitor, added to stabilize adenosine) in the absence (control, open circles) or presence (open triangles) of L-Cys, DL-Hcy (filled circles), L-homocystine (filled triangles), at the indicated concentrations. Cells at (70–80)% confluence were treated with described chemicals for 24 h and metabolically labeled with 37 kBq/ml [³H]thymidine for the last 3 h. [³H]-thymidine incorporation was measured in a liquid scintillation counter. Values represent mean ± SD from three independent experiments from 3 wells (n = 9). These results were published in J Biol Chem 1997; 272: 25380-25385.

arteriosclerosis, the growth inhibition of endothelial cells may represent an important mechanism for Hcy-induced arteriosclerosis.

Because the ratio of SAH to SAM represents a measure of cellular methylation status, we determined the SAH/SAM ratio in vascular cells by metabolically labeling with [14 C] adenosine and analyzing its metabolites by two-dimensional thin layer chromatography (2D-TLC) (Fig. 5). Hcy, but not cysteine markedly increased the level of SAH in human

aortic endothelial cells and increased the SAH/SAM ratio by 6- to 8-fold. It had little effect on the ratio in human aortic smooth muscle cells. These results suggest that Hcy-induced increases in the SAH/SAM ratio (and subsequent cellular hypomethylation) may mediate inhibition of endothelial cell growth by Hcy. Also, the differential effect of Hcy, at physiologically relevant concentrations on endothelial cell and VSMC growth may be explained by its ability to dramatically increase the SAH/SAM ratio in endothe-

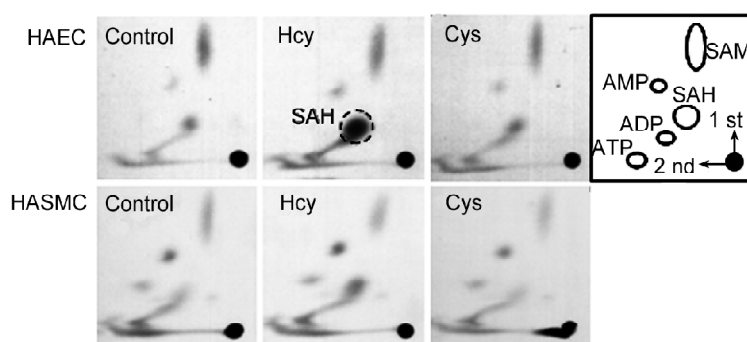


Fig. 5. Increase in SAH content in endothelial cells by Hcy. Human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC) were cultured as in Fig. 4 and exposed to 50 μ mol/L *DL*-Hcy or *L*-Cys in six-well plates and metabolically labeled with [14 C] adenosine for 5 h. Adenosine metabolites were analyzed by two-dimensional chromatography. Standard compounds (inset, SAH, SAM, ADP, AMP, and ATP) were applied on the plate and localized under UV light. These results were published in J Biol chem 272: 1997; 25380-25385.

lial cells but not in VSMC.

In addition, we have also found that Hcy decreases carboxyl methylation of Ras, and arrests endothelial cell growth at the G₁/S transition of the cell cycle^[7]. It is likely that the accumulation of SAH inhibits cellular methylation, leading to endothelial cell growth inhibition. Recently, we found that Hcy dramatically decreases cyclin A transcription and that adenovirus-transduced cyclin A expression rescued endothelial cell growth from Hcy treatment^[6].

This work is corroborated by reports that elevated Hcy levels in patients are linked to increase SAH and impaired erythrocyte membrane protein methylation^[36]. In addition, CBS knock out mice have increased SAH levels and decreased DNA methylation^[37, 38]. Collectively, these studies suggest that Hcy-induced inhibition of endothelial cell growth may play an important role in the pathogenesis of arteriosclerosis and thrombosis by preventing repair of aged or injured cells, and that impaired methylation due to increased levels of SAH might be a key mechanism in Hcy-dependent atherosclerosis.

Dietary-induced HHcy animal models

Given that most of the Hcy studies have focused on the effects of Hcy on cellular and vascular function, it is of great importance to evaluate the direct role of Hcy in car-

diovascular pathogenesis in animal models. Several dietary animal models of HHcy have been used to study Hcy-mediated vascular pathogenesis. Diet-induced HHcy is associated with vascular dysfunction in the monkey probably due to the inhibition of nitric oxide synthesis^[39], linked with vascular elastic structural damage in the minipig^[40], correlated with increased post-injury intimal hyperplasia^[41] and leukocyte-endothelium interaction^[42] in the rat, and vascular dysfunction^[43] and increased intimal hyperplasia^[44] in mice.

CBS-deficient mice

A genetic HHcy model with the gene deletion of CBS, which catalyzes Hcy conversion to cystathionine, has been used recently in studies of Hcy pathology (Fig. 1). The CBS-deficient mice resemble human HHcy patients; those that are homozygous for CBS deficiency have plasma Hcy levels of about fifty-times normal; while heterozygotes have plasma Hcy levels approximately two-times normal^[45]. Homozygous CBS-deficient mice (CBS^{-/-}) have a short life span and develop hepatic steatosis. Heterozygous CBS-deficient mice (CBS^{+/-}) have endothelial dysfunction probably due to nitric oxide inactivation resulting from increased production of reactive oxygen species following the inhibition of glutathione peroxidase, an antioxidant enzyme^[46].

This effect has been associated with SAM dependent methylation inhibition^[38,47]. CBS^{-/-} mice have increased hepatic cholesterol and TG levels through increasing hepatic expression of genes involved in cholesterol and TG synthesis by activating sterol regulatory element-binding proteins if fed a HHcyc diet^[23]. However, atherosclerotic lesions have not been observed in CBS knockout mice. Therefore, it is necessary to test the Hcy theory of atherosclerosis in animals that develop spontaneous atherosclerosis to determine whether HHcy is harmful under atherogenic conditions.

ApoE deficient mice

Several genetic models of atherosclerosis in mouse have been established and characterized^[48]. Mice with a targeted disruption in the gene of apolipoprotein E (apoE), which mediates the removal of plasma lipoproteins through its interaction with LDL receptors and other receptors, are severely hypercholesterolemic and develop spontaneous aortic atherosclerotic lesions^[49, 50]. The homozygous (apoE^{-/-}) mouse is the most frequently used genetic atherosclerosis animal model. These mice develop advanced lesions at a young age if fed an atherogenic diet. Recently, diet-induced HHcy in apoE^{-/-} has revealed accelerated atherosclerosis^[51] and enhanced vascular inflammation^[44]. However, dietary manipulations elicit broad physiological changes that can confound data interpretation. Therefore, an animal model of HHcy, which is isolated from broad dietary effects, would be useful in better defining mechanistic relationship between HHcy and atherogenesis.

CBS/apoE double knockout mice

Recently, we have created double knockout mice with targeted deletions of the apoE and CBS genes by breeding CBS^{-/-} females with apoE^{-/-} males. The general health and body weight of CBS^{+/-}/apoE^{-/-} and CBS^{-/-}/apoE^{-/-} were not different compared to normal mice. CBS^{-/-}/apoE^{-/-} had a high incidence of death during the first 3 postnatal weeks, which is similar as the CBS^{-/-}. About 5% of CBS^{-/-}/apoE^{-/-} survived to 15 weeks of age, about 2% to 6 months. This animal serves as a faithful model of human hypercholesterolemia and HHcy, and is susceptible to atherosclerosis. We used this model to determine the effect of Hcy on atherosclerosis and lipid metabolism in animals fed a regular diet, an atherogenic high-fat diet, or an atherogenic high-fat plus HHcyc high-methionine diet.

CBS/apoE double knockout mice are HHcyc and hypercholesterolemic

CBS gene deficiency, on an apoE knockout background,

produced a two-fold increase in plasma Hcy levels in CBS heterozygote mice (CBS^{+/-}/apoE^{-/-}) compared to CBS wild type animals (CBS^{+/+}/apoE^{-/-}). Severe HHcy was found in CBS homozygotes (CBS^{-/-}/apoE^{-/-}) (Table 1). The ratios of plasma Hcy levels were 1:2: 54 (CBS^{+/+}/apoE^{-/-} : CBS^{+/-}/apoE^{-/-} : CBS^{-/-}/apoE^{-/-}), which is greater than those observed in CBS single knockout mice (1:2:40)^[45]. The double knockout mice had significantly increased plasma total cholesterol (TC) levels, which were similar to those in apoE single knockout mice^[50]. HHcy caused by CBS gene deletion did not change the levels of plasma non-esterified fatty acid (NEFA), but was associated with significantly increased plasma TC levels and decreased plasma TG levels in the absence of dietary manipulation.

Notably, a high fat diet not only elevated NEFA and TC concentrations, but also doubled Hcy levels in both CBS^{+/+} and CBS^{+/-} mice. However, this may be related, in part, to the higher content of methionine and lower content of choline in the high fat diet. A prior study using a high methionine plus low folate diet in CBS^{-/-} mice found a 15-fold increase (from 6.2 to 92.8 mmol/L) in Hcy levels^[52], whereas we observed a 21-fold increase (from 7.4 to 154.9 μ mol/L) in Hcy levels in CBS^{-/-}/apoE^{-/-} mice fed a high methionine plus high fat diet compared to mice on a control diet, and an 11-fold increase (from 14.2 to 154.9 μ mol/L) compared to mice on only a high fat diet (Table 1). These data suggest that the high intake of dietary cholesterol and fat, might contribute to the increase of Hcy levels as well. Thus, the combination of HHcy and hyperlipidemia may increase the occurrence of atherosclerosis. The combination of high fat plus high methionine diet in CBS^{-/-} mice is an easily produced model of severe HHcy, which will permit large-scale *in vivo* functional assessments that were not possible with the CBS^{-/-} mice. Our finding is partially consistent with the report of Austin^[23] showing that Hcy increases cholesterol and TG content of HepG2 cells and that diet-induced HHcy increases the accumulation and synthesis of hepatic cholesterol and TG in mice.

HHcy accelerates aortic lesion in CBS/apoE double knockout mice

At 15 weeks, atherosclerotic lesions were apparent in apoE^{-/-} mice at the branch points of the aortic arch and at all the ostia of the intercostal arteries (Fig.6A). HHcyc apoE^{-/-} mice had slightly larger lesion areas in the aortic arch but this was not statistically significant at this time point (Fig. 6A). At 6 months of age, lesions were enhanced in the aortic arch and significantly increased with CBS gene deletion in a dose-dependent manner. At one year of age, advanced lesions were observed in all apoE^{-/-} mice in the aortic arch;

Table 1. Plasma levels of Hcy, NEFA, TC and TG

Genotype	n	Hcy ($\mu\text{mol/L}$)	NEFA (mEq/l)	TC (mg/dl)	TG (mg/dl)
Regular diet					
CBS ^{+/+} /ApoE ^{-/-}	14	3.8 $\pm$ 0.9	0.74 $\pm$ 0.42	387 $\pm$ 130	128 $\pm$ 64
CBS ^{-/-} /ApoE ^{-/-}	15	7.4 $\pm$ 2.9	0.73 $\pm$ 0.12	442 $\pm$ 111	145 $\pm$ 56
CBS ^{-/-} /ApoE ^{-/-}	12	210.4 $\pm$ 80.1 ^{*†p<0.001}	0.83 $\pm$ 0.03	559 $\pm$ 84 ^{*P<0.001†p=0.006}	72 $\pm$ 21 ^{*p=0.009†p<0.001}
HF diet					
CBS ^{+/+} /ApoE ^{-/-}	12	5.9 $\pm$ 2.0 ^{*p=0.039}	1.37 $\pm$ 0.35 ^{*p=0.099}	1271 $\pm$ 233 ^{*p<0.001}	166 $\pm$ 40 ^{*p=0.095}
CBS ^{-/-} /ApoE ^{-/-}	12	14.2 $\pm$ 7.8 ^{†p=0.011}	1.61 $\pm$ 0.54 ^{†p=0.029}	1366 $\pm$ 303 ^{†p<0.001}	259 $\pm$ 166 ^{†p=0.02}
HF + HM diet					
CBS ^{-/-} /ApoE ^{-/-}	14	154.9 $\pm$ 90.7 ^{‡p<0.001}	1.10 $\pm$ 0.27	1545 $\pm$ 264	162 $\pm$ 89

Mice were grown under regular diet, or fed a high fat or a high fat+ high methionine diet at 8 weeks of age for 3 months. Values are mean $\pm$ SD, *P* values from *t*-test. *, comparison *versus* CBS^{+/+}/ApoE^{-/-}; †, comparison *versus* CBS^{-/-}/ApoE^{-/-}; ‡, comparison *versus* CBS^{+/+}/ApoE^{-/-} on high fat diet. Hcy, homocysteine; NEFA, non-esterified fatty acid; TC, total cholesterol; TG, triglyceride; HF, high fat; HM, high methionine. These results were published in Blood 2003; 101: 3901-3907.

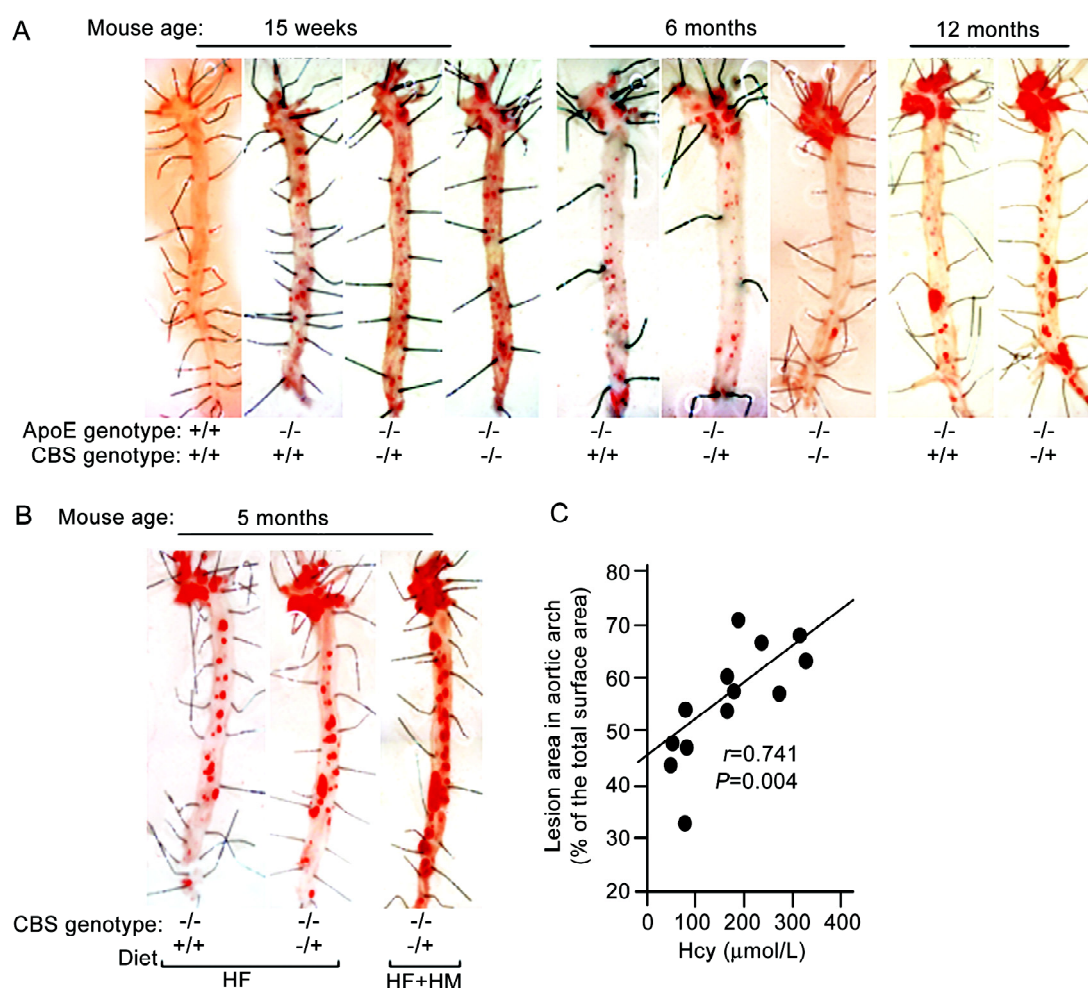


Fig. 6. HHcy increased atherosclerosis lesions. *A*, *B*: Sudan IV staining of atherosclerotic lesions in mouse aortas. Mice were killed at the ages of indicated. Lesions were examined by Sudan IV staining. Lesions shown up as red plaques. *C*: Regression analysis of the correlation of lesion area with plasma concentrations of Hcy in mice. Each spot represents one mouse. HF, high fat; HF+HM, high fat plus high methionine. These results were published in Blood 2003; 101: 3901-3907.

however, lesions were significantly increased by the coexistence of HHcy. Advanced lesions in the aortic arch were also observed at 5 months of age in CBS^{+/+}/apoE^{-/-} mice that had been fed with HF or HF+HM diet for 3 months (Fig. 6B). These lesions were comparable to those of CBS^{+/+}/apoE^{-/-} at one-year-old of age on regular diet (Fig. 6A). In HHcyc mice, the increases of plasma Hcy levels were significantly correlated with increases in atherosclerotic lesion area in the aortic arch (Fig. 6C). These results indicate that genetically induced mild HHcy enhances atherogenesis in old mice, whereas both genetic and dietary severe HHcy significantly increases lesion formation in young mice.

The increased lesion formation in CBS^{-/-}/apoE^{-/-} animals is associated with elevated TC which is known to be strong risk factors for cardiovascular disease, suggesting that HHcy affects hepatic lipid metabolism thereby further increasing cardiovascular disease risk. Plasma TG probably does not correlate with increased lesion formation because this remains within the normal range of TG concentrations in the double knockout mice. In addition, the role of plasma TG as an independent cardiovascular disease risk factor is controversial and unresolved. A high fat plus high methionine diet consistently resulted in severe atherosclerosis and HHcy in CBS^{+/+}/apoE^{-/-}, thereby confirming the atherogenicity of Hcy and validating the model for severe HHcy.

Hcy increases the uptake of acetylated-LDL in peritoneal macrophages

We have demonstrated that both genetic and dietary HHcy increased aortic lesion formation and neutral lipid (CE and TG) content in the lesions of apoE^{-/-} mice in the vessel wall [8]. Models for the mechanism of atherogenic lipid accumulation in vascular lesions emphasize increased LDL uptake by macrophages into the vessel wall. *In vitro* studies have established that LDL can be modified by oxidation, acetylation, glycation, methylation and other conditions [53, 54]. During Hcy autooxidation, liberated reactive oxygen species could initiate lipid peroxidation and lead to impaired endothelial function and the formation of atherogenic LDL [55]. Although Hcy and other thiols induce LDL peroxidation *in vitro* [56, 57], no difference in the extent of oxidation of LDL has been found in patients with moderate and severe HHcy in case-control studies [58, 59]. We speculated that Hcy increases lipid accumulates in the lesions of the double knockout mice via enhanced LDL uptake. To test this possibility, we incubated mouse macrophages with [¹²⁵I]acetylated-LDL or [¹²⁵I]native-LDL and then measured protein-associated [¹²⁵I] radioactivity. As shown in Fig. 7, native-LDL uptake was somewhat decreased in macroph-

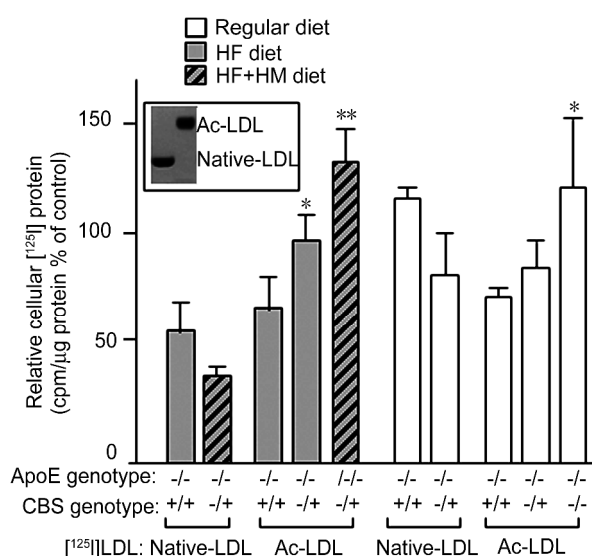


Fig. 7. Effect of hyperhomocysteinemia on LDL uptake. Mouse peritoneal macrophages were incubated with 20 μ g/ml of [¹²⁵I]Ac-LDL or [¹²⁵I]native-LDL for 2 h. Protein-associated [¹²⁵I]radioactivity were measured and normalized as [¹²⁵I] counts per mg protein. The uptake activity of [¹²⁵I]Ac-LDL in macrophages from CBS^{+/+}/ApoE^{-/-} mice under HF diet was set as the control. LDL uptake activity is expressed as relative cellular [¹²⁵I]protein (% of control mean values). Inset is the image of LDLs on 0.75% agarose gel showing the electrophoretic mobility change by acetylation. Values represent mean $\pm$ SD from 3 independent assays ($n=9$). * $P<0.01$ versus CBS^{+/+}/ApoE^{-/-}, * $P<0.01$ versus CBS^{+/+}/ApoE^{-/-} in the same group. HF, high fat; HF+HM, high fat plus high methionine; Ac-LDL, acetylated-LDL. These results were published in Blood 2003; 101: 3901-3907.

ages from HHcyc mice. In sharp contrast, HHcy resulting from CBS gene deletion significantly increased acetylated-LDL uptake. A similar pattern was observed in macrophages from dietary-HHcyc mice. These data indicate that HHcy increases the uptake of acetyl-LDL by macrophages.

Potential mechanisms of Hcy enhanced acetylated-LDL uptake

At current stage, it is not clear yet whether the enhanced uptake of modified-LDL by macrophages contribute to the increased atherosclerosis lesion in HHcy. We do not know how Hcy enhances modified-LDL uptake by macrophages. We propose that hypomethylation is a specific biochemical mechanism by which Hcy induces vascular injury [33]. Hcy can utilize adenosine to form SAH, a potent inhibitor of cellular methylation (Fig. 3). Elevated Hcy levels in patients are linked to increased SAH and impaired erythrocyte membrane protein methylation [60]. CBS-deficient mice have in-

creased SAH levels and decreased DNA methylation^[47]. Hcy arrests endothelial cell growth and increases cellular SAH in a cell type specific way^[7]. It is relevant that methylation of LDL abolished its recognition by LDL receptors^[61], retarded the degradation of aggregated LDL by macrophages^[62], and decreased CE formation in macrophages^[63]. It is possible that HHcy may inhibit lipid or protein methylation in LDL, which may result in increased endocytosis of LDL-derived CE in the lesions.

In addition, we considered that enhanced uptake of modified-LDL may account for the increase in lesion lipid content. Modified-LDL stimulates the secretion of cytokines and growth factors from vascular cells, and in contrast to native-LDL, is avidly taken up by macrophages in a process that is mediated by interaction with a family of scavenger receptors (SR)^[64]. Modified-LDL binds to SR (class A and B). SR-A and SR-B are detected in macrophage-rich areas within atherosclerotic lesions of apoE knockout mice^[65], and in human atherosclerotic lesions^[66]. SR-A is pro-atherogenic under hyperlipidemic conditions and both apoE and LDL receptor-deficient mice have reduced atherosclerosis in the absence of SR-A.

The interaction of SR-A with ligands induces cellular signaling leading to gene transcription and cytokine release. SR-B1 binds to high density lipoprotein (HDL) with high affinity^[67]. The expression of SR-B family members (SR-B1 & CD-36) is inducible. Unlike LDL receptors (LDLR), macrophage SR is not regulated by the cellular cholesterol content; hence, macrophage uptake of modified-LDL can contribute to the cellular accumulation of CE and eventually to increased atherosclerosis. Whereas uptake of native LDL by macrophages from HHcy mice was decreased, uptake of acetylated-LDL was higher in the HHcy mice than in control mice. Thus, the enhanced uptake of a modified LDL by macrophages from the HHcy mice could account for the observed increase in lesion severity in these mice.

Summary

Our studies, as well as data from other laboratories support the concept that Hcy is causally linked to atherosclerosis, and is not merely associated with the disease. Our recent findings on HHcy atherosclerosis mice support a model in which HHcy promotes atherosclerosis by altering hepatic lipid metabolism and increasing the uptake of modified-LDL in macrophages leading to the accumulation of CE and TG in the vessel wall. Results from lipid analyses and the LDL uptake assay suggest that HHcy increases plasma cholesterol and decreases HDL-

cholesterol. It is important to closely examine cholesterol and HDL metabolism, and LDL uptake regulation by HHcy in the HHcy mice. In addition, mechanistic assessments should examine the involvement of Hcy-related oxidation and hypomethylation in altered lipid metabolism in HHcy. Furthermore, therapeutic approaches using folic acid, vitamin B12 and B6, or other reagents on HHcy and hypercholesterolemic CBS/apoE double knockout should generate important quantitative data to evaluate therapeutic benefit. Considering no clinical data supporting the hypothesis that lowering plasma Hcy levels is cardio protective, these studies should yield insights into the mechanistic link between HHcy and atherosclerosis, and provide ultimate proof of causality of HHcy in vascular disease. Our studies suggest that Hcy alters hepatic lipid metabolism, and that Hcy-lowering therapy may improve hyperlipidemia and therefore result in cardio protection. Vitamin supplements can be recommended to HHcy patients who are at the risk of cardiovascular disease. Future trials with lipoprotein assessment and morbidity/mortality endpoints are needed to prove the hepatic lipid metabolism cross talk and the causality of HHcy.

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