Hyperhomocysteinemia and its role in the development of atherosclerosis

A.B. Lawrence de Koning, Geoff H. Werstuck, Ji Zhou, Richard C. Austin*

Department of Pathology and Molecular Medicine, McMaster University and the Henderson Research Centre, Hamilton, Ontario, Canada

Received 31 December 2002; received in revised form 25 April 2003; accepted 25 April 2003

Abstract

Numerous epidemiological studies have demonstrated that hyperhomocysteinemia (HHcy) is a strong and independent risk factor for cardiovascular disease. HHcy can result from a deficiency in the enzymes or vitamin cofactors required for homocysteine metabolism. Several hypotheses have been proposed to explain the cellular mechanisms by which HHcy promotes cardiovascular disease, including oxidative stress, endoplasmic reticulum (ER) stress and the activation of pro-inflammatory factors. Studies using genetic- and diet-induced animal models of HHcy have now demonstrated a direct causal relationship between HHcy, endothelial dysfunction and accelerated atherosclerosis. These recently established animal models of HHcy provide investigators with important in vivo tools to (i) further understand the cellular mechanisms by which HHcy contributes to endothelial dysfunction and atherosclerosis, and (ii) develop therapeutic agents useful in the treatment of cardiovascular disease. © 2003 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Hyperhomocysteinemia; Atherosclerosis; Endothelial dysfunction; Endoplasmic reticulum stress

1. Introduction

Atherosclerosis, the principal cause of cardiovascular disease and stroke, is a complex, chronic process that is initiated at sites of endothelial cell injury and culminates in the formation of stratified lesions of the arterial wall [1–5]. Subendothelial infiltration of monocytc cells, proliferation and migration of smooth muscle cells, cholesterol deposition, and elaboration of extracellular matrix are hallmark features of atherosclerotic lesions. Cholesterol-laden smooth muscle cells and macrophages, morphologically recognized as foam cells, are observed at all stages of lesion development [6,7]. The importance of cholesterol and its oxidized derivatives in the pathogenesis of atherosclerosis is supported by studies demonstrating the presence of cholesterol within the lesions from humans and animals [8–12]. Traditionally, cholesterol and its oxidized derivatives are thought to accumulate in atherosclerotic lesions, thereby contributing to the formation of advanced, multilayered atheromas. Although advanced atherosclerotic lesions cause progressive narrowing of the vessel lumen that can lead to ischemic symptoms, acute coronary syndromes usually result from lesion rupture and thrombosis [1–4].

Conventional risk factors for cardiovascular disease, including hypercholesterolemia, hypertension, smoking and diabetes, account for approximately 50% of all cases [1,2,12]. Evidence now indicates that HHcy, which occurs in approximately 5 to 7% of the general population, is an important, independent risk factor for atherosclerosis and thrombotic disease [13–20]. Furthermore, up to 40% of patients diagnosed with premature coronary artery disease, peripheral vascular disease or recurrent venous thrombosis have HHcy [13–18].

In this review article, we will summarize the genetic and nutritional factors that induce HHcy and further examine the clinical evidence implicating HHcy as an independent risk factor for cardiovascular disease. In addition, potential mechanisms by which homocysteine accelerates atherosclerosis will be discussed in light of the important findings recently reported for both genetic- and diet-induced animal models of HHcy.

2. Genetic and nutritional factors causing HHcy

Homocysteine is a thiol-containing amino acid that is formed during the metabolic conversion of methionine to
Fig. 1. Metabolism of homocysteine. Dietary methionine is converted to the methyl donor S-adenosylmethionine (SAM) and is demethylated to S-adenosylhomocysteine (SAH), which is subsequently cleaved into adenosine and homocysteine. Through the transsulfuration pathway, homocysteine is converted to cystathionine and cysteine by the enzyme cystathionine β-synthase (CBS) and the cofactor vitamin B6 (methylcobalamin).

Homocysteine can also be remethylated through the folate cycle. This pathway depends on the enzyme methionine synthase (MS) and vitamin B12 as well as the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) and folate, which enters the cycle as tetrahydrofolate (THF). In liver and kidney, homocysteine is also remethylated by the enzyme betaine homocysteine methyltransferase (BHMT), which transfers a methyl group to homocysteine via demethylation of betaine to dimethylglycine (DMG).

cysteine (Figure 1). Once synthesized, homocysteine may either be metabolized to cysteine by the transsulfuration pathway or converted back to methionine by the remethylation pathway [16,19,20]. Mutations in genes responsible for homocysteine metabolism can result in homocystinuria, a severe form of HHcy [20]. The most common genetic cause of homocystinuria, homozygous cystathionine β-synthase (CBS) deficiency, results in plasma homocysteine concentrations of up to 400 μmol/L, compared to normal plasma levels of ~10 μmol/L [16,19,20]. Homozygous CBS deficiency is inherited as an autosomal recessive disorder with pleiotropic clinical manifestations, including mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities and hepatic steatosis [16,20]. Furthermore, patients are at higher risk for premature atherosclerosis and thrombotic disease, which is the major cause of death [16,19,21–23]. While homozygous CBS deficiency is rare, heterozygous CBS deficiency occurs in approximately 1% of the general population and is associated with premature atherosclerosis and thrombotic disease in phenotypically normal individuals [13,15,16,21–23].

Deficiency of 5,10-methylenetetrahydrofolate reductase (MTHFR), the enzyme involved in folate-dependent remethylation of homocysteine to methionine, also causes severe HHcy and can lead to premature atherosclerosis and thrombotic disease [24–26]. Additionally, nutritional deficiencies in other vitamin cofactors required for homocysteine metabolism, namely folate, vitamin B6 (pyridoxal phosphate), B12 (methylcobalamin), and B2 (riboflavin) can promote HHcy [27–31].

It has been estimated that inadequate intake of B-vitamins and folate may account for approximately two thirds of all cases of HHcy [28]. The decision of both American and Canadian governments to enrich cereal grain flour with folate in the late 1990s to prevent neural tube defects (NTDs) was thus seen as having great potential in preventing HHcy in the general public. Cohort studies have since showed that folate fortification of cereal flour products has been effective in reducing total plasma homocysteine levels in the general population [32–34]. It is not yet known, however, if lowering of total plasma homocysteine via folate supplementation is effective in reducing the risk of developing atherosclerosis and thrombosis [32]. Prospective studies are currently underway to examine this association. Regardless of the underlying condition leading to HHcy, the relationship between elevated plasma homocysteine levels and vascular disease persists.

3. Homocysteine and vascular disease

Patients suffering from homocystinuria develop extensive arterial intimal thickening and fibrous plaques rich in smooth muscle cells and collagen [20,23,35]. These fibrous lesions greatly outnumber fatty atherosclerotic lesions in the major arteries of homocystinuric patients and, combined with abnormally accelerated thrombosis, lead to tissue infarction and death at an early age [35]. Venous thrombosis and, to a lesser extent, arterial thrombosis are common in patients with homocystinuria [36]. Homocysteine-induced thrombosis seems to result from disturbances in the thrombotic potential of endothelial cells rather than changes in platelet physiology. This leads to the development of a prothrombotic phenotype consistent with vascular injury (reviewed in [19,20]).

It is estimated that 5 to 7% of the general population has mild to moderate HHcy [13–16] and are at an increased risk of developing coronary atherosclerosis and vascular thrombosis in later life. Individuals with mild HHcy also have an increased prevalence of carotid artery stenosis, premature peripheral and cerebral vascular atherosclerosis [19–21,23]. Epidemiological data has generally showed a strong association between plasma homocysteine and the premature development of atherosclerotic cardiovascular disease. Many case-control and cross-sectional studies have identified an association between total plasma homocysteine levels and the development of carotid, coronary, peripheral and aortic atherosclerotic disease [37]. The multicentre European Concerted Action Project (750 patients, 800 controls), for example, revealed that HHcy confers an independent risk similar to smoking and hypercholesterolemia for developing occlusive arterial disease [38]. Additionally, this study suggested that mild HHcy may have a synergistic effect when combined with other risk factors, such as diabetes and hypertension. Control of mild HHcy may therefore be critically important in attenuating lesion formation.
and preventing atherosclerosis when other established risk factors are present. Unlike case-control studies, however, prospective cohort studies have not shown a robust or consistent association between HHcy and atherosclerosis [39]. This seems to be especially true for prospective studies of healthy individuals with no previous history of cardiovascular disease. Contrasting findings from prospective studies have been suggested to be a result of differing experimental designs and endpoints or perhaps even genetic or nutritional heterogeneity of test populations, resulting in different cardiovascular risk profiles in the populations studied [40]. Despite these conflicting prospective studies, mild HHcy is generally regarded as a causal, independent risk factor for occlusive vascular disease [18,41,42].

4. Potential cellular mechanisms by which homocysteine promotes atherosclerosis

4.1. Inflammatory response

In general, the development and progression of atherosclerosis is considered to be a form of chronic inflammation [1–3,11]. In support of these findings, in vitro studies have demonstrated that homocysteine enhances the production of several pro-inflammatory cytokines. Expression of monocyte chemoattractant protein 1 (MCP-1) is increased in cultured human vascular endothelial cells, smooth muscle cells and monocytes treated with homocysteine [43–45]. MCP-1 is known to enhance monocyte endothelial binding and recruitment to the subendothelial cell space, a critical early step in the formation of fatty streaks [11]. Homocysteine has also been shown to increase expression of IL-8 [43], a T-lymphocyte and neutrophil chemoattractant, in cultured endothelial cells. Homocysteine-induced expression of MCP-1 and IL-8 in monocytes and endothelial cells has been shown to occur through activation of NF-κB, a transcription factor involved in mediating downstream inflammatory processes [46]. Active NF-κB, which is found in atherosclerotic lesions, stimulates production of cytokines, chemokines, interferons, leukocyte adhesion molecules, hemopoietic growth factors and major histocompatibility (MHC) class I molecules—all of which are thought to influence vascular inflammation and, ultimately, atherogenesis [46]. Recent data substantiating the in vivo activation of NF-κB and downstream inflammatory marker expression in atherosclerotic lesions in hyperhomocysteinemic apolipoprotein E (apoE)-deficient mice further supports the concept that homocysteine contributes to the development of atherosclerosis by causing vascular inflammation [47].

4.2. Oxidative stress

Homocysteine is injurious to vascular endothelial cells and impairs normal cellular function. One of the most recognizable physiological changes associated with endothelial damage by HHcy is the impairment of vasodilation [48–52]. Abnormal vasomotor response is believed to be an early step in the formation of atherosclerotic lesions [53]. Mild to moderate HHcy in both human patients and animal models has been shown to impair normal endothelium-dependent vasodilation [49,51,54]. B vitamin supplementation, which has been shown to lower plasma homocysteine levels, is effective in restoring normal endothelial function [55]. The additional observation that antioxidants prevent impairment in endothelium-dependent relaxation suggests the involvement of reactive oxygen species (ROS) [47–54].

The thiol group of homocysteine readily undergoes auto-oxidation in plasma to generate ROS, and it has been suggested that homocysteine induces cell injury/dysfunction via a mechanism involving oxidative stress [50,56]. However, this hypothesis fails to explain why cysteine, which is present in plasma at 20 to 30 fold higher concentrations than homocysteine and is more readily auto-oxidized, does not cause endothelial cell injury and is not considered a risk factor for cardiovascular disease [57–59]. Recent studies have also demonstrated that homocysteine does not significantly increase the production of ROS via auto-oxidation and is largely involved in antioxidant and reductive cellular biochemistry [60]. Based on these findings, a re-examination of the role of oxidative stress due to the auto-oxidation of homocysteine is warranted.

Homocysteine-induced oxidative stress may occur through other mechanisms. Various ex vivo studies using vascular tissues have implicated HHcy in causing abnormal vascular relaxation responses by enhancing the intracellular production of superoxide anion (O$_2^-$) [53,61,62]. O$_2^-$ is believed to react with and decrease the availability of endothelial nitric oxide (NO) to yield peroxynitrite, thereby limiting normal vasodilation responses [63,64]. O$_2^-$ and peroxynitrite are also known to contribute to the oxidative modification of tissues, resulting in the formation of lipid peroxides and nitrosated end products such as 3-nitrotyrosine. The observations that homocysteine decreases the expression of a wide range of antioxidant enzymes [65,66] and impairs endothelial NO bioavailability by inhibiting glutathione peroxidase activity [52,67] raises the possibility that homocysteine sensitizes cells to the cytotoxic effects of agents or conditions known to generate ROS. Decreased NO bioavailability has also been shown ex vivo to increase the expression of MCP-1, which may enhance intravascular monocyte recruitment and lead to accelerated lesion formation [68].

4.3. Endoplasmic reticulum stress

In eukaryotic cells, the endoplasmic reticulum (ER) is the cellular organelle where secretory proteins or proteins destined for the plasma membrane undergo a variety of modifications, including disulphide bond formation, glycosylation, folding and oligomeric assembly (see reviews [69–71]). To assist in the correct folding of newly synthe-
Fig. 2. Proximal sensors IRE-1, ATF-6 and PERK co-ordinately regulate UPR signaling in eukaryotes. ER transmembrane protein IRE-1 dimerizes and autophosphorylates upon release of GRP78 to bind unfolded proteins. Endoribonuclease activity of IRE-1 cleaves mammalian XBP-1 mRNA to remove a 26-base pair intron. Translation of alternatively spliced XBP-1 mRNA yields XBP-1 protein containing a novel C-terminus that binds to ER stress response element (ERSE) promoters, increasing transcription of ER chaperone genes. ATF-6 migrates to the Golgi following GRP78 release and is cleaved by site 1 and site 2 (S1/S2) proteases. The released 50 to 60 kDa ATF-6 transactivation domain migrates to the nucleus and binds to ERSE, increasing transcription of ER chaperones. PERK is activated via dimerization and autophosphorylation following GRP78 release. PERK catalyzes the phosphorylation of eukaryotic initiation factor-2α (eIF-2α), which prevents eIF-2α from initiating translation, thereby limiting the unfolded protein load on the ER. Activation of the PERK pathway has also been shown to mediate the transcriptional activation of ER stress response genes.

sized proteins and to prevent aggregation of folding intermediates, the ER contains a high concentration of molecular chaperones including the 78-kDa glucose-regulated protein (GRP78), GRP94, calnexin, calreticulin, protein disulphide isomerase and ERp72. These chaperones act as a quality control system by ensuring that only correctly folded proteins are allowed to enter the Golgi for further processing and secretion. The observation that these chaperones are induced in response to a variety of pathological agents and/or conditions that cause ER stress [70] suggests that they play a critical role in quality processes during protein folding and processing.

ER stress results in the activation of the unfolded protein response (UPR), an intracellular signaling pathway essential for survival of cells undergoing ER stress (Figure 2). In mammalian cells, the UPR is mediated via three ER-responsive sensors: a type-I ER transmembrane protein kinase (IRE-1), the activating transcription factor 6 (ATF-6) and the PKR like ER kinase (PERK). Activation of these three pathways is mediated by GRP78, which is bound to each sensor in the absence of ER stress. During the accumulation of unfolded proteins in the ER, GRP78 dissociates from its respective sensor to interact with unfolded proteins, thereby facilitating the activation of IRE-1, ATF-6 and PERK [72–74]. Activation of both IRE-1 and ATF-6 increase the expression of ER-resident chaperones. IRE-1 is a stress-activated transmembrane protein kinase having endoribonuclease activity. Following ER stress, IRE-1 dimerizes and is autophosphorylated, thereby allowing IRE-1 to act as an endoribonuclease in the alternative splicing of XBP-1 mRNA. The removal of a 26 base pair intron results in a translation frameshift that permits XBP-1 to act as a transcriptional activator of genes containing upstream ER stress response elements (ERSE). ATF-6 is a transcription factor that is localized to the ER membrane in unstressed cells. Upon ER stress, ATF-6 is transported to the Golgi where the cytosolic transactivation domain of ATF-6 is cleaved from the membrane by specific proteases (S1P and S2P) that also recognize and cleave sterol regulatory element-binding proteins (SREBPs) under conditions of sterol starvation. Following release, the transactivation domain of ATF-6 localizes to the nucleus where it binds to ERSE, thereby activating transcription of numerous UPR-responsive genes, including GRP78, GADD153 and Erp72. Concomitant with an increase in the transcriptional activation of UPR-responsive genes, ER stress also leads to a rapid, general decrease in protein synthesis, a cellular process mediated by the transmembrane protein kinase, PERK. Activation of PERK causes phosphorylation of eukaryotic initiation factor-2α (eIF-2α), which effectively blocks mRNA translation initiation to help relieve the unfolded protein burden on the ER. Recent studies have also demonstrated that PERK-dependent eIF-2α phosphorylation is required for transcriptional activation of a wide range of UPR-responsive genes [75,76]. As a result, the UPR co-ordinately enhances cell survival by ensuring that the adverse effects of ER stress are dealt with in a timely and efficient manner. Failure to elicit a functional UPR following ER stress can result in programmed cell death via specific ER-dependent pathways and contribute to the pathogenesis of a number of human diseases, including diabetes, Alzheimer’s disease, Parkinson’s disease and cancer [71].

A recently proposed mechanism of vascular injury involves the ability of homocysteine to cause ER stress by disrupting disulphide bond formation and causing misfolding of proteins traversing the ER [65,66,77–79]. We as well as others have previously reported that elevated levels of intracellular homocysteine increase the expression of several ER stress response genes, including GRP78, GRP94, Herp and RTP [65,66,77,78,80]. In addition, homocysteine induces the expression of GADD153 [65,66,80], a basic region leucine zipper transcription factor involved in ER stress-induced cell death [81]. These effects in gene expression directly involve the UPR because homocysteine treatment has been shown to induce the activation of both PERK [82,83] and IRE-1 [84,85]. Transient and chronic ER stress elicited by homocysteine has been shown to adversely affect several cellular functions involved in the development and progression of ath-
erosclerosis, including lipid dysregulation, programmed cell death and inflammation. We have recently demonstrated that homocysteine-induced ER stress causes dysregulation of lipid biosynthesis by activating the SREBPs [80]. ER-resident transcription factors responsible for the induction of genes in the cholesterol/triglyceride biosynthesis and uptake pathways [86,87]. The observation that stable over-expression of GRP78, which protects cells from agents and/or conditions known to cause ER stress, inhibits homocysteine-induced cholesterol gene expression in cultured human cells further supports an association between ER stress and lipid metabolism [80]. Additional studies have demonstrated that activation of the UPR increases lipid biosynthesis in yeast [88,89] and dolichol biosynthesis [90], which is a component of the cholesterol biosynthesis pathway in cultured mammalian cells. Our findings suggest that activation of the UPR by homocysteine promotes the overproduction of ER lipid components, including cholesterol and triglycerides, resulting in the accumulation of lipids in affected cell types, including hepatocytes, smooth muscle cells and macrophages. This dysregulation in lipid biosynthesis and uptake may explain the paradox as to why lipid-rich atherosclerotic lesions develop in hyperhomocysteinemic patients with normal serum lipid profiles.

Recent studies have now shown that homocysteine induces programmed cell death in cultured human vascular endothelial cells and that this effect involves activation of the UPR [85]. Homocysteine-induced cell death was mimicked by other ER stress agents and was dependent on IRE-1 signaling. Activation of IRE-1 by homocysteine leads to a rapid and sustained activation of JNK protein kinases [91], a result consistent with the finding that activation of JNK by ER stress involves binding of IRE-1 to TRAF2 [92]. Because persistent activation of JNK correlates with cell death [93], these studies provide further support for a mechanism involving homocysteine-induced programmed cell death. In addition, caspase-3 or a caspase-like protease was shown to be essential for homocysteine-induced programmed cell death, a result consistent with the ability of homocysteine thiolactone, a cyclic thioester derivative of homocysteine synthesized by specific aminocyl-tRNA synthetases [94], to induce programmed cell death in HL-60 cells [95]. Although caspase-7 mediated caspase-12 activation has been implicated in the coupling of ER stress to programmed cell death [96], there are presently no reported studies examining the effect of homocysteine on the activation of these caspases. Given that programmed cell death has been widely documented to occur in animal and human atherosclerotic lesions, and that programmed cell death and ER stress are increased in atherosclerotic lesions from mice fed hyperhomocysteinemic diets (Zhou and Austin, unpublished results), it is tempting to suggest that homocysteine could adversely affect the stability and/or thrombogenicity of atherosclerotic lesions through its ability to elicit ER stress. Further studies are, however, required to identify the pro-apoptotic factors involved in this process as well as their in vivo relevance to the development and progression of atherosclerosis.

Previous work by Pahl and colleagues [97–100] has demonstrated that the in vitro activation of NF-κB occurs in cells undergoing ER stress. This suggests that homocysteine-induced ER stress could contribute to the development of atherosclerosis by directly enhancing an inflammatory response via NF-κB activation. Recent studies have now demonstrated that HHcy enhances activation of NF-κB in atherosclerotic lesions and in selective tissues from apoE-deficient mice having diet-induced HHcy [47]. Although the mechanism responsible for this proinflammatory effect of HHcy is unknown, homocysteine-induced ER stress could enhance the phosphorylation and activation of JNK, thereby leading to further inflammation and programmed cell death [92]. Based on these recent findings, homocysteine-induced ER stress may prove to be an important mechanism that not only contributes to the development of atherosclerosis but also influences other specific diseases, including diabetes and hypertension [71].

4.4. Additional mechanisms

In addition to the established mechanisms described above, other studies have suggested that elevated levels of homocysteine can elicit other effects that contribute to atherogenesis and thrombotic disease. It has been reported that homocysteine stimulates the proliferation of cultured vascular smooth muscle cells [101–103] and upregulates smooth muscle cell collagen [104], findings consistent with the observation that patients with severe HHcy have widespread premature atherosclerosis with intimal thickening and collagen-rich, fibrous lesions [15]. Several potential mechanisms have also been proposed to explain endothelial cell dysfunction during HHcy, including direct cell injury [50,56], growth arrest [65] and altered cellular methylation status [105]. In addition to causing endothelial cell dysfunction, elevated levels of homocysteine have been shown to increase the procoagulant activity of cultured endothelial cells by enhancing tissue factor [106–108] and factor V [109] activities, inhibiting cell surface thrombomodulin [110] and protein C [111], and impairing tissue plasminogen activator binding to its endothelial cell receptor, annexin II [112].

Homocysteine thiolactone, the cyclic thioester of homocysteine, has recently received attention for its potential role in atherosclerosis and thrombotic disease [94,113]. In addition to the transsulphuration and remethylation pathways, intracellular homocysteine can be converted by methionyl-tRNA synthase into a homocysteine-AMP complex. This complex is subsequently catabolised to homocysteine thiolactone, thereby preventing the incorporation of homocysteine into nascent polypeptide chains. However, homocysteine thiolactone has unique reactive properties that can lead to the homocysteinylination of lysine residues and free amine groups on numerous cellular proteins, thereby resulting in
decreased biological activity and premature degradation [113]. In addition, homocysteine thiolactone secreted into the circulation may induce widespread modification of plasma proteins that could potentially contribute to the development of atherosclerosis and thrombotic disease. Recent studies have demonstrated that homocysteine thiolactone decreases paraoxonase activity associated with HDL, thereby rendering HDL less protective against oxidative damage and against toxicity of homocysteine thiolactone [114]. Additional studies using physiologically relevant in vivo models of HHcy are necessary to further understand the atherogenic role of homocysteine thiolactone.

5. Animal models of hyperhomocysteinemia

Recent findings from genetic- and diet-induced animal models of HHcy have significantly enhanced the status of homocysteine as a risk factor for atherosclerosis. They have also supported and extended many of the proposed in vitro mechanisms and have provided a more physiological perspective on the role of homocysteine in the induction of cardiovascular disease.

Manipulation of plasma homocysteine can be accomplished by dietary and/or genetic approaches. The addition of methionine and/or depletion of B vitamins and folate in the diet can be used to induce mild to severe HHcy. Genetically engineered mice deficient in CBS or MTHFR have also been used as animal models of HHcy. Homozygous CBS-deficient mice have total plasma homocysteine levels 40 times greater than normal and suffer from severe growth retardation, hepatic steatosis and ectopia lentis – phenotypic changes also observed in patients with homozygous CBS deficiency [115]. Heterozygous CBS-deficient mice, however, do not suffer from the same developmental defects as homozygotes. These animals have twice the normal concentration of plasma homocysteine, making them ideal models to study mild HHcy. Recently, the chronic, mild HHcy present in heterozygous CBS-deficient mice has been shown to cause endothelial dysfunction by decreasing vascular NO bioavailability, thereby leading to impaired vasorelaxation [48,52]. This association is not unique to CBS-deficient mice, as endothelial dysfunction has also been shown in rabbits and cynomolgus monkeys (Macaca fascicularis) having diet-induced HHcy [53,116–118]. Although CBS-deficient mice and nonmurine models of HHcy exhibit endothelial dysfunction, it is important to note that they do not develop atherosclerosis [115–118].

MTHFR-deficient mice have been recently developed to examine the effects of HHcy resulting from genetic deficiencies in the remethylation pathway [119]. Although MTHFR-deficient mice share basic phenotypic similarities with CBS-deficient mice, they are unique in ways that may predispose them to developing atherosclerosis. Mature heterozygous and homozygous MTHFR-deficient mice accumulate lipid in their proximal aorta which is reminiscent of early atherosclerotic lesions [119]. This is significant, given that plasma lipid levels are normal in these animals. Although the arterial lipid inclusions are not as elaborate as lesions observed in murine models of atherosclerosis, such as apoE-deficient or LDL receptor-deficient mice, they are the first observations showing that mild HHcy is involved in the formation of vascular lesions.

Studies in our laboratory examined the molecular and cellular consequences of diet-induced HHcy in wild-type and heterozygous CBS-deficient mice [80]. Both mild and severe HHcy were shown to induce ER stress and abnormal hepatic accumulation of lipid in each strain of mice. ER stress was shown to promote SREBP activation, thereby resulting in increased expression of enzymes encoding genes for cholesterol and triglyceride biosynthesis, as well as the LDL receptor. SREBP dysregulation resulted in hepatic steatosis, a characteristic feature observed in human homocystinuric patients. Interestingly, plasma lipid levels remained unchanged despite increases in hepatic VLDL-triglyceride secretion rates. These observations suggest that hepatic steatosis in HHcy does not result from impaired lipid export, but instead is due to increased lipid biosynthesis coupled with enhanced uptake of LDL by the liver. Should this effect of homocysteine on lipid metabolism and uptake be confirmed in vascular cells involved in atherogenesis, this could potentially explain the lipid rich arterial lesions associated with mild HHcy in patients with normal lipid profiles.

One of the earliest detectable cellular responses in the development of atherosclerotic lesions is the binding of monocyctic cells to the vascular endothelium. Recent studies have now demonstrated that the number of monocytes present on the surface of aortic endothelium is significantly elevated in rats with diet-induced HHcy, although typical atherosclerotic lesions were not observed [120]. A significant increase in the expression of MCP-1, VCAM-1 and E-selectin was also observed in the aortic endothelium of hyperhomocysteinemic rats, thereby providing a potential cellular mechanism responsible for the increase in monocyte binding and recruitment. The additional observation that folate supplementation prevented an increase in plasma homocysteine levels, decreased monocyte binding to the endothelium and inhibited the expression of MCP-1, VCAM-1 and E-selectin, emphasizes a potential antiathero- genic effect of lowering plasma homocysteine.

Since the development and progression of atherosclerosis is limited in some animal models of HHcy, we and other researchers have generated dietary HHcy in genetically altered mice prone to developing atherosclerosis. An established model of spontaneous atherosclerosis is the apoE-deficient mouse [121]. These mice develop hypercholesterolemia because of impaired reverse cholesterol transport to the liver, thereby leading to the accumulation of cholesterol-rich remnants in plasma. This persistent and severe hypercholesterolemia (up to 5 times normal) leads to the premature development of complex atheroscle-
The ability of homocysteine to activate and increase numbers of lesion-resident macrophages. As discussed previously, the ability of homocysteine to activate and increase numbers of lesion-resident macrophages. As discussed previously, the ability of homocysteine to activate and increase numbers of lesion-resident macrophages.

Two separate studies published in the last two years demonstrate that mild HHcy accelerates atherogenesis in apoE-deficient mice. Zhou et al. [122] showed that apoE-deficient mice fed methionine- or homocysteine-enriched diets developed larger, more complex and more numerous arterial lesions, compared to mice fed control diets (Figure 3). An important feature of these pathological changes was that they occurred without a significant elevation in the concentration of plasma lipids. Many of these lesions were rich in collagen, probably owing to the ability of homocysteine to stimulate SMC proliferation and extracellular matrix deposition [104,123]. Zhou et al. further demonstrated that the differences in lesion size and frequency were seen only between groups following 3 months of dietary treatment compared to 12 months, suggesting that HHcy mediates the early stages of atherogenesis [122]. In a separate study using apoE-deficient mice fed diets supplemented with methionine to induce HHcy, Hofmann et al. [47] found significant increases in atherosclerotic lesion area, compared to mice fed control diets. Furthermore, diet-induced HHcy promoted NF-κB activation in aortic tissue, leading to the increased expression of the pro-inflammatory adhesion molecule VCAM-1 as well as the pro-inflammatory mediators RAGE and EN-RAGE. Increased expression of pro-inflammatory genes in the aorta of hyperhomocysteinemic animals is also consistent with the finding of increased plasma concentrations of TNF-α and increased numbers of lesion-resident macrophages. As discussed previously, the ability of homocysteine to activate NF-κB and enhance vascular inflammatory processes may in part be related to its ability to cause ER stress [97–99].

Our finding that markers of ER stress are increased in the livers [80] and atherosclerotic lesions (Zhou and Austin, unpublished results) of mice with diet-induced HHcy provides support for this concept. Lesions with increased expression of the collagen degrading matrix metalloproteinase-9 were also observed, which could potentially promote lesion instability and rupture. The increased collagen in the lesions observed by Zhou et al. [122] is in contrast to the prediction of plaque instability by Hofmann et al. [47]. Both studies, however, are in agreement that diet induced HHcy in the presence of a hyperlipidemic state accelerates the development of atherosclerosis. A recent study by Wang et al. [124], using apoE and CBS double knockout mice, showed that atherosclerotic lesion area and lesion lipid content increased with plasma homocysteine concentrations, independent of diet. Overall, these data strongly suggest that HHcy amplifies proatherogenic cellular processes when combined with hyperlipidemia. As previously mentioned, case-control epidemiological studies seem to implicate HHcy in compounding the overall risk of cardiovascular disease when in the presence of additional risk factors such as hypertension and diabetes [35,37,38]. Supporting this hypothesis, Matthias et al. [125] showed that spontaneously hypertensive rats fed high doses of methionine developed more complex arterial lesions compared to rats fed normal diets.

Collectively, these studies have helped elucidate the causal role of homocysteine in the development of endothelial dysfunction and atherosclerosis. Furthermore, these animal models are valuable in vivo tools to further examine potential therapeutic approaches in lowering plasma homocysteine while decreasing the prevalence of cardiovascular disease.

6. Conclusions and questions

HHcy is an independent risk factor for cardiovascular disease. Recent studies have identified three major cellular mechanisms by which HHcy may contribute to the development of endothelial dysfunction and atherosclerosis. They include the induction of pro-inflammatory factors, oxidative stress, and ER stress. Genetic- and diet-induced animal models of HHcy have now demonstrated a causal relationship between HHcy and accelerated atherosclerosis. They have also provided important insights into the role of HHcy in endothelial dysfunction and the development of atherosclerosis. These studies, however, raise some interesting and relevant questions. Although HHcy seems to be very important in early lesion development, what role does it play in plaque stability and/or thrombogenicity? Can HHcy, in the absence of hypercholesterolemia or other significant risk factors, enhance lesion development in humans? Does dietary enrichment of vitamins essential for the
metabolism of homocysteine protect against cardiovascular disease? Answering these and other important questions will certainly enhance our understanding of the role of HHcy in the development and progression of atherosclerosis.

Acknowledgments

The author’s work is supported by Research Grants from the Heart and Stroke Foundation of Ontario (T-4005) and the Canadian Institutes of Health Research (MOP-49417). R.C. Austin is a Career Investigator of the Heart and Stroke Foundation of Ontario. We apologize to many of our colleagues whose work could not be directly acknowledged due to space limitations.

Note added to proof

Recent studies by Ji and Kaplowitz [126] have demonstrated increased HHcy, ER stress and liver injury in an alcohol-fed mouse model of HHcy.

References


Matthias D, Becker CH, Riezler R, Kindling PH. Homocysteine induced arteriosclerosis-like alterations of the aorta in normotensive and hypertensive rats following application of high doses of methionine. Atherosclerosis 1996;122:201-16.