

## Review

# Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease

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## Abstract

Hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular disease, including ischemic heart disease, stroke, and peripheral vascular disease. Mutations in the enzymes responsible for homocysteine metabolism, particularly cystathionine  $\beta$ -synthase (CBS) or 5,10-methylenetetrahydrofolate reductase (MTHFR), result in severe forms of HHcy. Additionally, nutritional deficiencies in B vitamin cofactors required for homocysteine metabolism, including folic acid, vitamin B6 (pyridoxal phosphate), and/or B12 (methylcobalamin), can induce HHcy. Studies using animal models of genetic- and diet-induced HHcy have recently demonstrated a causal relationship between HHcy, endothelial dysfunction, and accelerated atherosclerosis. Dietary enrichment in B vitamins attenuates these adverse effects of HHcy. Although oxidative stress and activation of proinflammatory factors have been proposed to explain the atherogenic effects of HHcy, recent *in vitro* and *in vivo* studies demonstrate that HHcy induces endoplasmic reticulum (ER) stress, leading to activation of the unfolded protein response (UPR). This review summarizes the current role of HHcy in endothelial dysfunction and explores the cellular mechanisms, including ER stress, that contribute to atherothrombosis.

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**Keywords:** hyperhomocysteinemia; atherosclerosis; endothelial dysfunction; endoplasmic reticulum stress; apoptosis

**Abbreviations:** apoE, apolipoprotein E; CBS, cystathionine  $\beta$ -synthase; GADD153, growth arrest- and DNA damage-inducible gene 153; HHcy, hyperhomocysteinemia; eIF2 $\alpha$ , eukaryotic initiation factor-2 $\alpha$ ; ER, endoplasmic reticulum; GRP, glucose-

regulated protein; MCP-1, monocyte chemoattractant protein 1; MTHFR, 5,10-methylene tetrahydrofolate reductase; PERK, PKR-like ER kinase; ROS, reactive oxygen species; TDAG51, T-cell death-associated gene 51; UPR, unfolded protein response; XBP-1, X-box binding protein-1

## Introduction

Clinical manifestations of atherothrombotic disease, cardiac arrhythmias, myocardial infarction and stroke account for the majority of deaths in North America.<sup>1–4</sup> Atherothrombosis is a complex, chronic process that is initiated at sites of endothelial cell injury and culminates in atherosclerotic lesion disruption with superimposed thrombus formation.<sup>1–5</sup> Cholesterol deposition, infiltration of monocytic cells, proliferation and migration of smooth muscle cells, and elaboration of extracellular matrix are characteristic features of atherosclerotic lesions. Cholesterol-enriched macrophages, morphologically recognized as foam cells, are also observed during lesion development.<sup>6,7</sup> It is well established that the acute clinical manifestations of atherothrombotic disease result from lesion rupture, triggering thrombus formation and vessel occlusion.<sup>2,8</sup>

Apoptotic cell death is a hallmark feature of both animal and human atherosclerotic lesions.<sup>9–11</sup> Apoptotic cells can be observed throughout advanced atherosclerotic lesions, but are most prominent in and around the lipid-rich core. It has been suggested that apoptosis increases the risk of lesion rupture by decreasing the number of viable smooth muscle cells necessary for collagen production and stabilization of the fibrous cap. Furthermore, apoptosis enhances thrombogenicity by increasing the number of tissue factor-rich apoptotic cells within the atherosclerotic lesion.<sup>12,13</sup> However, the cellular pathways responsible for this effect and their relevance to atherothrombotic disease are incompletely understood.

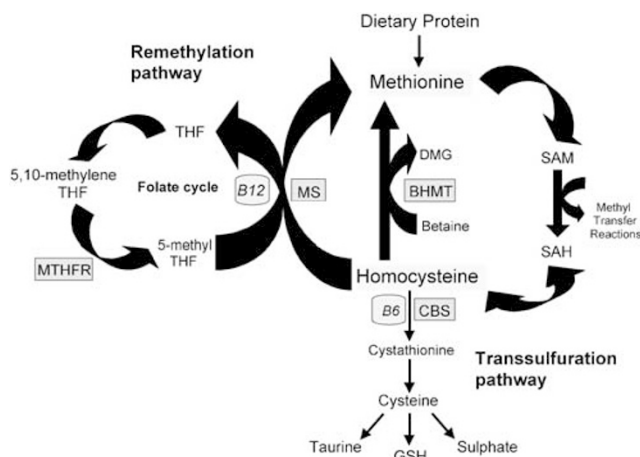
Hyperhomocysteinemia (HHcy) is a pathological condition characterized by an increase in plasma concentration of total homocysteine.<sup>14–21</sup> Numerous clinical and epidemiological studies have indicated that HHcy is an independent risk factor for atherothrombotic disease. Up to 40% of patients diagnosed with premature coronary artery disease, peripheral vascular disease, or recurrent venous thrombosis present with HHcy.<sup>14–19</sup> Recent studies have now demonstrated that homocysteine causes endothelial cell dysfunction and induces apoptotic cell death in cell types relevant to atherothrombotic disease, including endothelial cells<sup>22,23</sup> and smooth muscle cells.<sup>24</sup> Furthermore, a direct causal relationship between induction of HHcy and accelerated atherosclerosis has been reported in apolipoprotein E (apoE)-deficient mice with diet- and/or genetic-induced HHcy.<sup>25–28</sup> Given that oral administration of folic acid or a

combination of B vitamins can decrease HHcy and attenuate atherogenesis in these animal models,<sup>26,28</sup> there is growing interest for treatment of HHcy as a strategy for prevention of atherothrombotic disease.

In this review article, we will summarize the genetic and dietary factors that induce HHcy. In addition, the cellular mechanisms that may explain how HHcy causes endothelial cell dysfunction and accelerates atherosclerotic lesion development will be discussed in light of recent findings in animal models of HHcy.

## Genetic and Nutritional Factors that Induce HHcy

Homocysteine is a thiol amino-acid synthesized during the metabolic conversion of methionine to cysteine. Once generated, homocysteine is either metabolized to cysteine via the transsulfuration pathway or remethylated to methionine via the remethylation pathway (Figure 1).<sup>17,20,21</sup> Mutations in genes responsible for the metabolism of homocysteine, including cystathionine  $\beta$ -synthase (CBS), methionine synthase (MS), 5,10-methylenetetrahydrofolate reductase (MTHFR), or betaine homocysteine methyltransferase (BHMT), can result in severe forms of HHcy, termed homocystinuria. The most common genetic cause of homocystinuria, homozygous CBS deficiency, results in plasma total homocysteine concentrations of up to 400  $\mu\text{mol/l}$ , compared to normal levels of 10–12  $\mu\text{mol/l}$ .<sup>17,21</sup> CBS deficiency is associated with a wide range of clinical manifestations, including mental retardation, ectopia lentis, osteoporosis, skeletal abnormalities and hepatic steatosis.<sup>17</sup>



**Figure 1** Homocysteine metabolic pathways. Dietary methionine is converted to the methyl donor S-adenosylmethionine (SAM) and is demethylated to S-adenosylhomocysteine (SAH) and homocysteine. In the transsulfuration pathway, homocysteine is converted to cystathionine by the enzyme cystathionine  $\beta$ -synthase (CBS) and the cofactor vitamin B6 (pyridoxyl phosphate). Once formed from cystathionine, cysteine can be utilized in a number of cellular functions, including protein synthesis and glutathione (GSH) production. Homocysteine can also be remethylated through the folate cycle. This pathway requires the enzyme methionine synthase (MS) and vitamin B12 as well as the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) and folic acid, 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).

Furthermore, patients are at high risk for cardiovascular disease, which is the major cause of death.<sup>29–31</sup> Although homozygous CBS deficiency is rare, the incidence of heterozygous CBS deficiency may be found in up to 1% of the general population and is associated with premature cardiovascular disease in phenotypically normal individuals.<sup>29–31</sup> Deficiency of MTHFR causes severe HHcy and can lead to premature atherosclerosis and thrombotic disease.<sup>32–34</sup> Renal insufficiency or nutritional deficiencies of B vitamins required for homocysteine metabolism, namely folic acid, vitamin B6 (pyridoxal phosphate), and/or vitamin B12 (methylcobalamin), also cause HHcy.<sup>35,36</sup> It is estimated that inadequate intake of vitamins accounts for two-thirds of all cases of HHcy.<sup>36</sup> Although vitamin supplementation has been shown to be effective in lowering plasma homocysteine levels, it remains to be determined if the risk of cardiovascular disease is decreased.

## HHcy and Endothelial Cell Dysfunction

The term ‘endothelial dysfunction’ refers to impairment of the normal homeostatic properties of vascular endothelium, which include endothelium-dependent regulation of vascular tone, hemostasis, and inflammation.<sup>37</sup> Endothelial dysfunction is commonly detected as impairment of endothelium-dependent relaxation of blood vessels, and is predictive of adverse cardiovascular outcomes.<sup>38</sup> Many studies using animal models and human subjects have demonstrated that HHcy induces endothelial dysfunction.<sup>39,40</sup> The degree of impairment of endothelium-dependent relaxation during HHcy is similar to that of other risk factors such as hypercholesterolemia and hypertension.<sup>39</sup>

The mechanisms by which HHcy induces endothelial dysfunction are incompletely defined. However, one consistent finding from studies of experimental HHcy is impairment of vasodilation mediated by endothelium-derived nitric oxide.<sup>39</sup> Nitric oxide is a potent vasodilator that is produced by the endothelial isoform of nitric oxide synthase (eNOS) in response to physiological stimuli, and is a major mediator of endothelium-dependent relaxation.<sup>37</sup> Expression of eNOS does not appear to be decreased during HHcy, and vasodilator responses to nitroprusside or nitroglycerin are preserved, suggesting that sensitivity of vascular muscle to nitric oxide is relatively normal.<sup>41</sup> It is likely, therefore, that HHcy decreases nitric oxide bioavailability through alternative mechanisms, such as accelerated oxidative inactivation of nitric oxide.<sup>40</sup>

Homocysteine-induced oxidative inactivation of nitric oxide has been observed *in vitro* in cultured endothelial cells,<sup>42</sup> and evidence for increased oxidative inactivation of nitric oxide during HHcy has been obtained in animals using both pharmacological<sup>41,43–46</sup> and genetic<sup>47,48</sup> approaches. Several types of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and peroxynitrite, may contribute to the oxidative inactivation of endothelium-derived nitric oxide in HHcy.<sup>40</sup>

Another potential mechanism for endothelial dysfunction during HHcy is inhibition of nitric oxide production caused by asymmetric dimethylarginine (ADMA), an endogenous eNOS

inhibitor. Elevated plasma levels of ADMA have been demonstrated in a non-human primate model of HHcy.<sup>49</sup> In human subjects, plasma levels of ADMA increase rapidly after acute methionine loading and elevation of plasma ADMA correlates with impairment of endothelium-dependent relaxation.<sup>50</sup> Elevation of ADMA in HHcy may be caused by decreased catabolism of ADMA by dimethylarginine dimethylaminohydrolases (DDAH), which hydrolyzes ADMA to citrulline and methylamines.<sup>51</sup>

Although recent experimental work on homocysteine-induced endothelial dysfunction has focused on the role of nitric oxide and oxidative stress, it should be recognized that homocysteine has several other properties that may adversely affect the endothelium. Homocysteine induces ER stress,<sup>52–56</sup> stimulates proinflammatory responses,<sup>57</sup> and alters the methylation of regulatory proteins such as Ras;<sup>58</sup> each of these effects may result in activation of endothelial cell death pathways. Delineating the role of these potential mechanisms in producing endothelial dysfunction should be a priority for future studies.

## HHcy and Atherothrombosis: Potential Cellular Mechanisms

### Inflammation

Atherothrombotic disease is considered to be a form of chronic inflammation.<sup>1–3</sup> Cell culture studies have demonstrated that homocysteine induces the production of several proinflammatory cytokines. Treatment of cultured human vascular endothelial cells, smooth muscle cells, and monocytes with homocysteine induces the expression of monocyte chemoattractant protein 1 (MCP-1).<sup>59–61</sup> MCP-1 enhances the binding of monocytes to the endothelium and their recruitment to the subendothelial cell space, a critical step in atherosclerotic lesion development. Homocysteine has also been shown to increase expression of IL-8,<sup>59</sup> a T lymphocyte and neutrophil chemoattractant, in cultured endothelial cells. Homocysteine-induced expression of MCP-1 and IL-8 has been shown to occur through activation of NF- $\kappa$ B, a transcription factor known to stimulate the production of cytokines, chemokines, leukocyte adhesion molecules, and hemopoietic growth factors – all of which are thought to contribute to vascular inflammation and atherogenesis.<sup>1–3</sup> The recent observations that NF- $\kappa$ B activation and downstream proinflammatory mediators and cytokines are increased in atherosclerotic lesions from hyperhomocysteinemic apolipoprotein E-deficient mice<sup>28,62</sup> further supports the concept that HHcy accelerates atherogenesis by causing vascular inflammation.

### Oxidative stress

The highly reactive thiol group of homocysteine is readily oxidized to form ROS,<sup>42</sup> suggesting that homocysteine induces cell injury/dysfunction through a mechanism involving auto-oxidation and oxidative damage. However, this hypothesis fails to explain why cysteine, which is present in plasma at much higher concentrations than homocysteine and is readily auto-oxidized, does not cause endothelial cell injury and is not

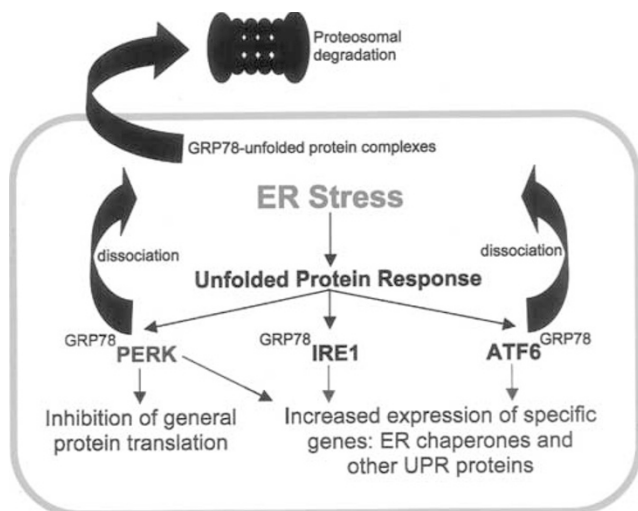
considered a risk factor for cardiovascular disease.<sup>63</sup> Recent studies have also demonstrated that homocysteine is largely involved in antioxidant and reductive cellular biochemistry.<sup>64</sup> In fact, the homocysteine-dependent transsulfuration pathway is critical in the maintenance of the intracellular glutathione pools, and the regulation of this pathway is sensitive to oxidative stress conditions.<sup>65,66</sup>

Homocysteine-induced oxidative stress may impact atherogenesis by mechanisms unrelated to auto-oxidation. Various *ex vivo* studies using vascular tissues have implicated HHcy in causing abnormal vascular relaxation responses by inducing the intracellular production of superoxide.<sup>67–69</sup> Superoxide is believed to react with endothelial nitric oxide to yield peroxynitrite, thereby limiting the normal vasodilation response.<sup>70,71</sup> Both superoxide and peroxynitrite contribute to the modification of tissues, resulting in the generation of lipid peroxides and in the case of peroxynitrite, the modification of proteins by tyrosine nitration and the formation of 3-nitrotyrosine. The recent findings that heme oxygenase-1 (HO-1) and glutathione peroxidase (GPx) expression and activity are impaired in cultured vascular endothelial cells treated with homocysteine suggest that HHcy can inhibit the antioxidant potential of cells.<sup>48,52,72</sup> This is particularly relevant to atherothrombosis given that (i) HHcy increases vascular dysfunction in GPx-deficient mice,<sup>48</sup> (ii) overexpression of GPx attenuates homocysteine-induced endothelial dysfunction,<sup>47</sup> and (iii) overexpression of HO-1 inhibits the development of atherosclerosis in apoE<sup>−/−</sup> mice.<sup>73</sup>

### Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR)

In eukaryotic cells, the ER is the principal site for folding and maturation of transmembrane, secretory, and ER-resident proteins.<sup>74–78</sup> To assist in the correct folding of newly synthesized proteins and to prevent aggregation of folding intermediates, the ER contains numerous molecular chaperones such as GRP78, GRP94, calnexin, calreticulin, and protein disulfide isomerase. These chaperones act as a quality control system by ensuring that only correctly folded proteins are processed prior to entering the Golgi for further processing and secretion. Pathological conditions and/or agents that interfere with proper folding and maturation of proteins activate the UPR, an integrated intracellular signaling pathway that responds to ER stress by increasing the expression of UPR responsive genes (including the ER-resident molecular chaperones), attenuating global protein translation and degrading unfolded proteins (Figure 2).

The UPR is mediated via the ER-resident sensors IRE-1, ATF-6, and PERK-like ER kinase (PERK), and activation of these sensors depends on their dissociation from GRP78 following ER stress.<sup>74–78</sup> As a result, the UPR coordinately 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 as well as prolonged or severe ER stress can result in apoptotic cell death and contribute to the pathogenesis of a number of human diseases, including Alzheimer's disease, Parkinson's disease, and diabetes.<sup>76,77</sup>



**Figure 2** ER stress and the unfolded protein response (UPR). The UPR is regulated in eukaryotes by the proximal sensors IRE-1, ATF-6, and PERK. Activation of these sensors occurs following their dissociation from GRP78 in response to ER stress. Once activated, the UPR functions as an integrated, intracellular signaling pathway to attenuate protein translation, increase ER chaperone expression, and enhance degradation of unfolded proteins via the proteasome

One proposed mechanism of vascular injury by homocysteine involves ER stress and activation of the UPR.<sup>52–56,78</sup> We as well as others have reported that elevated levels of intracellular homocysteine elicit ER stress, thereby leading to activation of the UPR and increased expression of ER stress response genes, including GRP78, GRP94, Herp, and RTP.<sup>52–56</sup> In addition, homocysteine induces the expression of growth arrest- and DNA damage-inducible gene 153 (GADD153)<sup>79</sup> and T-cell death-associated gene 51 (TDAG51),<sup>22</sup> proapoptotic factors induced by ER stress. These effects in gene expression directly involve the UPR because homocysteine treatment has been shown to induce the activation of both PERK<sup>80,81</sup> and IRE-1.<sup>23</sup>

Severe or prolonged ER stress elicited by homocysteine has been shown to activate several cellular functions involved in the development and progression of atherosclerosis, including dysregulation of lipid metabolism, apoptotic cell death, and inflammation. We as well as others have demonstrated recently that homocysteine-induced ER stress causes dysregulation of lipid biosynthesis in hepatocytes by activating the SREBPs,<sup>56,82</sup> which are ER-resident transcription factors responsible for the induction of genes in the cholesterol/triglyceride biosynthesis and uptake pathways.<sup>83</sup> Stable overexpression 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, providing further support for an association between ER stress and lipid metabolism.<sup>56</sup> Consistent with these findings, ER stress, increased expression of cholesterol biosynthetic genes, and hepatic steatosis were observed in mice with diet-induced HHcy. Further studies, however, are required to determine if this mechanism contributes to the atherogenic effect of HHcy.

## HHcy, ER Stress and Apoptotic Cell Death

Recent studies have demonstrated that homocysteine induces apoptotic cell death in cultured human vascular endothelial cells through activation of the UPR.<sup>23</sup> Apoptotic cell death was dependent on IRE-1 signaling and was also induced by other ER stress agents, including tunicamycin and thapsigargin. Homocysteine-induced IRE-1 activation causes a rapid and sustained activation of JNK protein kinases,<sup>84</sup> a result consistent with the finding that activation of JNK by ER stress involves binding of IRE-1 to TRAF2.<sup>85</sup> Since persistent JNK activation is associated with apoptotic cell death,<sup>86</sup> these studies provide further support for a homocysteine-induced mechanism involving UPR-dependent apoptotic cell death. Furthermore, caspase-3 activation is essential for homocysteine-induced apoptotic cell death, a result consistent with the ability of homocysteine thiolactone, a cyclic thioester derivative of homocysteine,<sup>87</sup> to induce apoptotic cell death in HL-60 cells.<sup>88</sup> Although caspase-7 and/or caspase-12 activation have been implicated in the coupling of ER stress to apoptotic cell death,<sup>89–91</sup> there are presently no reported studies examining the effect of homocysteine on the activation of these caspases. Given that apoptosis has been widely documented to occur in animal and human atherosclerotic lesions, and that apoptotic cell death and ER stress are increased in atherosclerotic lesions from mice fed hyperhomocysteinemic diets (Zhou and Austin, submitted), it is possible that homocysteine-induced ER stress could adversely affect the stability and/or thrombogenicity of atherosclerotic lesions.

## HHcy, TDAG51 and Detachment-Mediated Apoptotic Cell Death

It has been reported that homocysteine induces endothelial cell detachment<sup>92</sup> and causes apoptotic cell death via a detachment-mediated process.<sup>93,94</sup> Treatment of primary trophoblast cells with pathophysiological levels of homocysteine resulted in cellular flattening and enlargement, extension of pseudopodia, and cellular vacuolization.<sup>93</sup> Furthermore, homocysteine, but not cysteine, induced trophoblast apoptosis as measured by loss of adhesion, increased mitochondrial cytochrome *c* release, and DNA cleavage. Similar results were also observed in a human histiocytic lymphoma cell line U937 treated with homocysteine.<sup>94</sup>

We have recently demonstrated that TDAG51 is induced by homocysteine, causes apoptotic cell death by impairing cell adhesion, and contributes to the development of atherosclerosis in apoE-deficient mice with diet-induced HHcy.<sup>22</sup> TDAG51 is a member of a novel pleckstrin homology-related gene family that consists of Ipl/Tssc and Tih.<sup>95</sup> Sequence analysis has revealed that TDAG51 contains a central motif resembling a pleckstrin homology domain and several distinctive C-terminal proline–glutamine (PQ) and proline–histidine (PH) repeats. Given that proteins with pleckstrin homology domains are important in cytoskeletal organization<sup>96</sup> and that PQ/PH repeats can regulate transcription and/or apoptotic cell death,<sup>97–99</sup> it has been suggested that TDAG51 functions to regulate cytoskeletal integrity and/or to

mediate apoptosis.<sup>100,101</sup> Recent studies have demonstrated that decreased expression of TDAG51 in metastatic melanoma confers resistance to apoptosis.<sup>102</sup> In contrast, constitutive overexpression of TDAG51 increases apoptotic sensitivity and impairs melanoma cell proliferation. However, the precise mechanism by which TDAG51 mediates apoptotic cell death is not known. Although TDAG51 has been shown *in vitro* to play critical roles in the upregulation of Fas gene expression and activation-induced apoptosis of T cells,<sup>100</sup> TDAG51-deficient mice express normal levels of Fas with no impairment in T-cell apoptosis.<sup>103</sup> Furthermore, TDAG51 fails to increase Fas expression and does not promote Fas-dependent apoptotic cell death in rat H19-7 neuronal cells.<sup>101</sup> Based on these reports, there is no clear evidence implicating TDAG51 in the Fas-dependent cell death pathway. However, our findings that overexpression of TDAG51 leads to dramatic alterations in cell shape and increases detachment of cells from their appropriate matrix<sup>22</sup> support a mechanism involving detachment-induced apoptosis or anoikis. Furthermore, the observation that TDAG51-induced shape changes are independent of caspase activation and occur prior to activation of the cell death program is consistent with this mechanism.<sup>22</sup> Given that TDAG51 colocalizes with focal adhesion kinase and disrupts the actin cytoskeleton,<sup>22</sup> it is conceivable that TDAG51 acts to impair focal adhesion complex assembly and attenuate survival signaling from the extracellular matrix prior to initiation of apoptosis.

In addition to its ability to decrease cell growth and promote apoptotic cell death, TDAG51 has been reported to inhibit protein synthesis, possibly by interacting with cellular factors involved in the regulation of protein translation.<sup>104</sup> It is well established that activation of the UPR by ER stress leads to decreased rates of protein synthesis,<sup>105</sup> a process mediated by the ER-resident kinase, PERK.<sup>106–108</sup> Once activated, PERK phosphorylates Ser51 of eukaryotic initiation factor-2 $\alpha$  (eIF2 $\alpha$ ), thereby inhibiting cellular mRNA translation and inducing transcriptional activation of a wide range of ER stress-inducible genes, including GRP78, GADD153, and TDAG51.<sup>106</sup> Given that homocysteine causes ER stress, the ability of homocysteine to increase TDAG51 expression, activate PERK,<sup>80,81</sup> and induce eIF2 $\alpha$  phosphorylation<sup>80</sup> is not surprising. Although these studies provide evidence that TDAG51 plays a role in protein translation, cell growth, cytoskeletal organization, and apoptotic cell death, its physiological function and role in atherothrombotic disease remain to be determined.

## Animal Models of HHcy-induced Atherogenesis

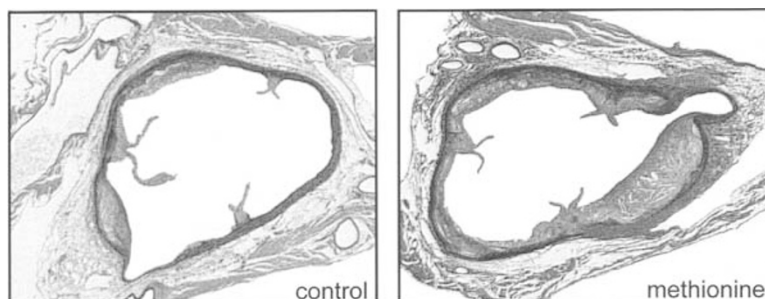
Supplementation of methionine, homocysteine, and/or depletion of B vitamins and folic acid in the diet can induce mild to severe HHcy.<sup>25–28</sup> Transgenic mice deficient in CBS or MTHFR have also been used as animal models of HHcy. Homozygous CBS-deficient mice have total plasma homocysteine levels 40-fold greater than normal and suffer from severe growth retardation, hepatic steatosis, and dislocation of the lens<sup>109</sup> – phenotypic changes also observed in human patients with homocystinuria.<sup>16–21</sup> Heterozygous CBS-defi-

cient mice have twice the normal concentration of total plasma homocysteine, but do not present with the same developmental defects observed in homozygotes, thereby making them ideal models to study mild HHcy. Heterozygous CBS-deficient mice develop endothelial dysfunction by decreasing vascular nitric oxide bioavailability, thereby leading to impaired vasorelaxation.<sup>41,46</sup> This association is not unique to CBS-deficient mice, as endothelial dysfunction has also been observed in other animal models of HHcy, including rabbits and monkeys.<sup>67,110–113</sup> Although CBS-deficient mice or non-murine models of HHcy exhibit endothelial dysfunction, there is no evidence of atherosclerotic lesion development.<sup>111–113</sup>

MTHFR-deficient mice share a similar phenotype with CBS-deficient mice but may be more prone to developing atherosclerosis.<sup>114</sup> Adult heterozygous and homozygous MTHFR-deficient mice show aortic lipid accumulation that is reminiscent of early atherosclerotic lesions. Interestingly, plasma lipid levels are normal in these animals. Although the lipid accumulation is not as advanced as that observed in other murine models of atherosclerosis, including apoE- or LDL receptor-deficient mice, these observations are the first to show that mild HHcy contributes to atherosclerotic lesion development.

Binding of monocyte cells to the vascular endothelium is one of the earliest detectable events in atherosclerotic lesion development. Recent studies have now demonstrated that the binding of monocytes to the endothelium is significantly elevated in rats with diet-induced HHcy, although typical atherosclerotic lesions were not observed.<sup>115</sup> The increased expression of MCP-1, VCAM-1, and E-selectin in the aortic endothelium of hyperhomocysteinemic rats suggests a potential cellular mechanism responsible for the enhanced monocyte binding and recruitment. The additional observation that supplementation of the diets with folic acid prevented an increase in total plasma homocysteine levels, decreased monocyte binding to the endothelium, and inhibited the expression MCP-1, VCAM-1, and E-selectin further supports an antiatherogenic effect of lowering plasma homocysteine.

Since atherosclerotic lesion development is limited in most animal models of HHcy, we and other researchers have generated dietary HHcy in genetically altered mice prone to developing atherosclerosis. Several recent studies have demonstrated that mild HHcy accelerates atherogenesis in apoE-deficient mice.<sup>25–28</sup> Zhou *et al*<sup>25,26</sup> demonstrated that apoE-deficient mice fed chow diets supplemented with either methionine or homocysteine developed larger, more complex and more numerous arterial lesions, compared to mice fed control diets (Figure 3). Lesions were rich in smooth muscle cells and collagen, a result consistent with the ability of homocysteine to stimulate SMC proliferation and extracellular matrix deposition.<sup>116</sup> The effects of the hyperhomocysteinemic diets on lesion size and frequency were seen following 3 months, but not 12 months, of dietary treatment, suggesting that HHcy mainly influences the early stages of atherogenesis. Hoffman *et al*<sup>28</sup> also found significant increases in atherosclerotic lesion area in apoE-deficient mice fed a high methionine diet, compared to mice fed control diet. Furthermore, HHcy was associated with an increase in NF- $\kappa$ B activation in aortic tissue, leading to the increased expression



**Figure 3** Atherosclerotic lesions in the aortic root of apoE-deficient mice fed a control diet or a high methionine diet to induce HHcy. Lesion sizes in mice fed high methionine diet were significantly larger, compared to mice fed control diet. Sections were stained with Orcein to reveal the elastic lamina (kindly provided by Ji Zhou and Erling Falk)

of the proinflammatory adhesion molecule VCAM-1 and the proinflammatory mediators RAGE and EN-RAGE. As discussed previously, the ability of homocysteine to activate NF- $\kappa$ B and enhance vascular inflammatory processes may in part be related to its ability to cause ER stress. Our finding that ER stress and NF- $\kappa$ B activation are increased in the atherosclerotic lesions of mice with diet-induced HHcy provides support for this concept.<sup>62</sup> A recent study by Wang *et al*<sup>67</sup> has also shown that genetic hyperhomocysteinemia in a hyperlipidemic background accelerates atherosclerosis. Using compound apoE and CBS double knockout mice, atherosclerotic lesion area and lipid content increased with total plasma homocysteine concentrations, independent of diet.

In addition to its effects on atherogenesis, diet-induced hyperhomocysteinemia in apoE-deficient mice increases the expression of tissue factor (TF),<sup>28</sup> an integral membrane glycoprotein essential for the initiation of blood coagulation.<sup>117</sup> Consistent with these findings, *in vitro* studies have shown that homocysteine can induce TF procoagulant activity.<sup>118</sup> Evidence now indicates that an increase in TF expression and/or activity can enhance thrombin generation, thereby increasing lesion thrombogenicity and the risk of thrombotic complications.<sup>1,119,120</sup> Thus, an increase in TF expression and/or activity has the potential to enhance thrombosis. Our recent findings that overexpression of GRP78 inhibits TF procoagulant activity<sup>121</sup> suggest that inhibition of ER stress has the potential to suppress the prothrombotic potential of cells.

Collectively, these animal models of HHcy have supported and extended many of the proposed *in vitro* mechanisms and provide a physiological perspective on the role of HHcy in atherosclerotic lesion development. Although these studies demonstrate that HHcy accelerates atherogenesis when combined with hyperlipidemia, additional studies will be necessary to determine if HHcy accelerates atherogenesis in the presence of other cardiovascular risk factors, including diabetes and hypertension.

## Conclusions and Questions

HHcy is an independent risk factor for cardiovascular disease. Several cellular mechanisms have been proposed to explain the effects of HHcy on endothelial dysfunction and atherosclerosis, including induction of proinflammatory factors, oxidative stress, and ER stress. Animal models of HHcy have

demonstrated a causal relationship between HHcy, endothelial cell dysfunction, and accelerated atherosclerosis. These studies raise some interesting and relevant questions. Although HHcy accelerates early lesion development, what role does it play in plaque stability and/or thrombogenicity? Does HHcy, in the absence of hypercholesterolemia or other cardiovascular risk factors, enhance lesion development? Does dietary enrichment in B vitamins essential for the metabolism of homocysteine protect against cardiovascular disease? Do other risk factors such as hypercholesterolemia, diabetes, and/or hypertension contribute to atherothrombotic disease through a mechanism involving ER stress? This is particularly intriguing given that the accumulation of free cholesterol in the ER of cultured mouse peritoneal macrophages depletes ER calcium stores and leads to ER stress, UPR activation, and apoptotic cell death.<sup>122</sup> Furthermore, an association between free cholesterol accumulation, apoptotic cell death, and markers of ER stress were reported in advanced atherosclerotic lesions from apoE-deficient mice. Answers to these and other important questions will undoubtedly enhance our understanding of the cellular mechanisms that mediate atherothrombotic disease and its clinical outcomes.

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