Review Article

The Link Between Hyperhomocysteinemia and Hypomethylation: Implications for Cardiovascular Disease

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Abstract

Increased levels of homocysteine have been established as a risk factor for cardiovascular disease (CVD) by mechanisms still incompletely defined. S-Adenosylhomocysteine (SAH) is the metabolic precursor of homocysteine that accumulates in the setting of hyperhomocysteinemia and is a negative regulator of most cell methyltransferases. Several observations, summarized in the current review, support the concept that SAH, rather than homocysteine, may be the culprit in the CVD risk that has been associated with hyperhomocysteinemia. This review examines the biosynthesis and catabolism of homocysteine and how these pathways regulate accumulation of SAH. In addition, the epidemiological and experimental links between hyperhomocysteinemia and CVD are discussed, along with the evidence suggesting a role for SAH in the disease. Finally, the effects of SAH on the hypomethylation of DNA, RNA, and protein are examined, with an emphasis on how specific molecular targets may be mediators of homocysteine-associated vascular disease.

Keywords

homocysteine, S-Adenosylhomocysteine, endothelial dysfunction, epigenetics, epitranscriptomics, protein posttranslation modifications

Homocysteine Metabolism

Homocysteine is formed by the demethylation of the essential amino acid, methionine, via the formation of 2 intermediate compounds, S-Adenosylmethionine (SAM) and S-Adenosylhomocysteine (SAH; Figure 1).[1] Methionine is first converted to SAM through the catalytic action of methionine adenosyltransferase enzymes (MATs), forming SAM, a highly energetic compound. S-Adenosylmethionine is the methyl donor for the majority of cellular methylation reactions, which are catalyzed by specific methyltransferases that target important biomolecules, such as DNA, RNA, proteins, and lipids.[1] Nevertheless, in mammals, most methyl groups transferred from SAM are used in creatine formation, in phosphatidylcholine synthesis, and in the generation of sarcosine from glycine.[2] Following the transfer of a methyl group to an acceptor molecule, SAM is converted to SAH. Importantly, because SAH has an affinity for the catalytic site of most SAM-dependent methyltransferases that is equal or greater than that of SAM, SAH is a potent inhibitor of many methyltransferases and the SAM–SAH ratio is often used as an indicator for intracellular methylation capacity.[3]

S-Adenosylhomocysteine is further converted into homocysteine and adenosine by SAH hydrolase (SAHH), which is ubiquitously expressed in mammalian tissues. The formation of homocysteine from methionine is the only pathway of homocysteine biosynthesis in humans.[1]

Importantly, the SAHH reaction is reversible and presents a thermodynamic equilibrium that strongly favors SAH synthesis rather than its hydrolysis.[4] However, under normal physiological conditions, the reaction is directed toward SAH hydrolysis

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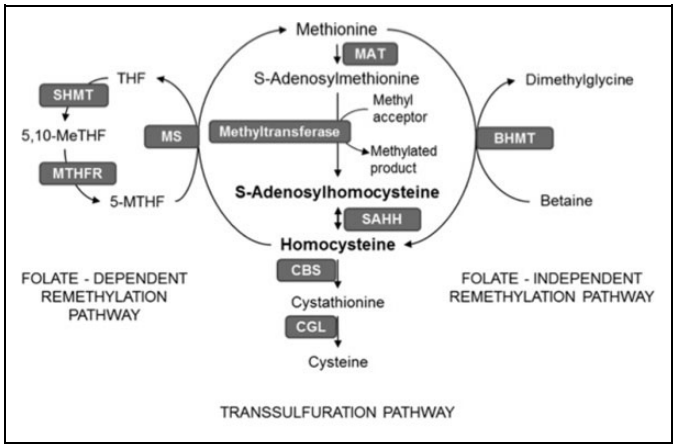

Figure 1. Schematic representation of homocysteine metabolism. The folate-dependent remethylation pathway and the transsulfuration pathway are dependent on the B vitamins—SHMT, CBS, and CGL are vitamin B_6 dependent; 5,10-methylenetetrahydrofolate reductase (MTHFR) utilizes vitamin B_2; methionine synthase (MS) is vitamin B_{12} dependent. BHMT indicates betaine–homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; MAT methionine adenosyltransferase; SAHH, S-Adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase.

due to the rapid metabolism or cellular export of homocysteine. Homocysteine is located at a metabolic branch point and can be either conserved by remethylation back to methionine or irreversibly degraded to cysteine via the transsulfuration pathway.[4]

Remethylation of homocysteine back to methionine occurs by 2 alternative metabolic pathways, the folate-dependent or folate-independent remethylation pathways (Figure 1). 5-Methyltetrahydrofolate (5-MTHF) is the active folate derivative and the main circulating form of folate in plasma. In the folate-dependent pathway, 5-MTHF supplies the methyl group used by the vitamin B_{12}-dependent methionine synthase (MS) to remethylate homocysteine and produce methionine and tetrahydrofolate (THF). The THF is then converted to 5,10-methylenetetrahydrofolate (5,10-MeTHF) in the presence of serine and vitamin B_6 by the enzyme serine hydroxymethyltransferase (SHMT). After reduction by 5,10-methylenetetrahydrofolate reductase (MTHFR), 5,10-MeTHF is converted into 5-MTHF, which will be available for the remethylation of a second molecule of homocysteine. The MTHFR uses flavin adenine dinucleotide (FAD; the active form of vitamin B_2) as a cofactor. The folate-dependent remethylation pathway is present in nearly all cells.[2,5] Additionally, in liver and kidney, remethylation also occurs by the folate-independent pathway in which methyl groups are donated by betaine (also known as trimethylglycine, a derivative of choline oxidation) in a reaction catalyzed by the enzyme betaine–homocysteine methyltransferase (BHMT).[2,5]

Homocysteine can be irreversibly metabolized through the transsulfuration pathway, which is mainly found in the liver, kidney, small intestine, and pancreas.[6] In the first step of this pathway, cystathionine β-synthase (CBS) catalyzes the condensation of homocysteine and serine to form cystathionine using pyridoxal phosphate (PLP or vitamin B_6) as a cofactor.[4] Cystathionine is further metabolized to produce cysteine by another PLP-requiring enzyme, cystathionine γ-lyase (CGL).[4,7] In addition to protein synthesis, cysteine is used in the synthesis of glutathione, an important cellular antioxidant.[4,8] The sulfur end product of cysteine metabolism is sulfate, which can be excreted by the kidneys.[9]

To maintain the optimal intracellular levels of homocysteine, cells may export homocysteine. The mechanisms that regulate homocysteine export into the extracellular compartment are not completely understood. Nevertheless, a mechanism involving the removal of the reduced form of homocysteine (with a free thiol group) to the extracellular compartment has been proposed. A separate mechanism appears to be responsible for the import of oxidized, disulfide forms of homocysteine into cells.[10] In plasma, the majority (98%-99%) of homocysteine is in disulfide form, as reduced homocysteine is rapidly oxidized, reacting with free thiol-containing molecules (including small thiol molecules, such as homocysteine or cysteine, and proteins with free cysteines, such as albumin) in plasma.[4,11] The remaining 1% to 2% of plasma homocysteine remains in its reduced form.[4,10,11] Total plasma homocysteine includes the sum of the circulating homocysteine molecules either in its reduced or in its oxidized forms.

The levels of plasma homocysteine are mainly regulated by the remethylation and transsulfuration pathways, as homocysteine is reabsorbed in the kidney and its urinary excretion is minimal.[12-14]

Hyperhomocysteinemia

Persistent elevation of homocysteine in the blood defines the condition called hyperhomocysteinemia. In healthy adults, fasting homocysteine concentrations in plasma are 5 to 15 μmol/L.[15-17] Hyperhomocysteinemia is classified, according to the levels of homocysteine accumulated, as mild (15-30 μmol/L), moderate (31-100 μmol/L), or severe (>100 μmol/L).[16,18,19]

Several factors can contribute to the circulating levels of homocysteine. In fact, plasma homocysteine levels are influenced by various nongenetic as well as genetic determinants.

Nongenetic determinants of plasma homocysteine include inadequate concentrations of B vitamins, which are substrates or cofactors for the major homocysteine regulating enzymes (Figure 1). As such, plasma concentrations of homocysteine are inversely related to plasma concentrations of folate, vitamin B_{12}, and vitamin B_6, as well as to the intake of these vitamins.[20] The most consistent association has been found with lower folate intake or with lower plasma concentrations of folate.[19] A modest inverse relationship has been reported between plasma concentrations of vitamin B_2 and homocysteine; however, this association is restricted to subjects carrying the *MTHFR* 677TT genotype (see subsequently). In addition, normal kidney function maintains optimal plasma levels of homocysteine, and impaired renal function is often

associated with mild to moderate hyperhomocysteinemia.[4,21] The majority (85%-100%) of patients with end-stage renal disease present with hyperhomocysteinemia, but the underlying mechanism is still not completely understood.[14,22] van Guldener et al reported that healthy kidneys do not remove homocysteine from the circulation, suggesting that the increased homocysteine levels observed in patients with renal disease are not due to impaired renal excretion of homocysteine.[12-14] Rather, it has been hypothesized that renal insufficiency causes the accumulation of uremic toxins that subsequently affect homocysteine metabolism, leading to hyperhomocysteinemia.[13,23] Plasma homocysteine concentrations increase with age. In fact, homocysteine levels approximately double from childhood to old age.[1,4] Gender also significantly affects the concentration of plasma homocysteine, with males having higher levels than females.[4,19] A rise in plasma homocysteine concentrations with menopause has also been described.[19] In addition, lifestyle factors can modulate the circulating levels of homocysteine. Tobacco smoking, high coffee consumption, alcoholism, and lack of exercise are examples of lifestyle factors associated with mild hyperhomocysteinemia.[24] Several pharmacological agents can also disturb homocysteine metabolism, such as certain anticarcinogenic agents (eg, methotrexate or sulfasalazine) and anticonvulsants (eg, carbamazepine or phenytoin).[25] Finally, pathologic conditions, such as hypothyroidism, psoriasis, lymphoblastic leukemia, and other malignancies, are also associated with hyperhomocysteinemia.[25]

Genetic determinants cause mild to severe hyperhomocysteinemia. Severe hyperhomocysteinemia is caused by rare genetic defects in either homocysteine transsulfuration or remethylation pathways. Cystathionine β-synthase deficiency or classical homocystinuria, an autosomal recessive disorder that affects the transsulfuration pathway, is the most common inborn error of homocysteine metabolism.[26] In addition to severe hyperhomocysteinemia and homocystinuria, CBS deficiency also results in hypocysteinemia, as well as hypermethioninemia caused by increased homocysteine remethylation.[26] Phenotypic consequences of CBS deficiency include thromboembolism and vascular occlusion, skeletal abnormalities, dislocation of the optic lenses, marfanoid features, and varying degrees of neurological impairment. In CBS-deficient patients, early homocysteine-lowering treatment significantly reduces the risk of life-threatening vascular events, despite imperfect biochemical control of homocysteine levels.[26]

Other genetic causes of hyperhomocysteinemia include rare mutations that result in MTHFR deficiency and mutations that cause cobalamin (vitamin B_{12}) deficiency.[27] Inborn errors of cobalamin metabolism can affect its absorption, transport, as well as its intracellular metabolism by inhibiting adenosylcobalamin synthesis and/or methionine synthase function.[28]

Several pieces of evidence led to the conclusion that the more common mild forms of hyperhomocysteinemia are, at least partially, genetically based.[25] For this reason, almost every gene involved in homocysteine metabolism has been analyzed for functional polymorphisms that could affect the circulating concentrations of homocysteine, and many genetic variants have been identified. The major known genetic determinant of plasma homocysteine levels in the general population is the 677C>T transition in the *MTHFR* gene.[29] MTHFR is an FAD-dependent enzyme involved in the folate-dependent remethylation of homocysteine. The common *MTHFR* C677 T polymorphism determines the synthesis of a thermolabile enzyme with reduced activity.[30] Homozygosity for the thermolabile MTHFR variant increases the plasma concentrations of homocysteine by approximately 25% in individuals with low folate status.[31]

Hyperhomocysteinemia and Cardiovascular Disease

Meta-analyses have shown that mild hyperhomocysteinemia is an independent risk factor for cardiovascular diseases (CVDs), with a prevalence of approximately 5% in the general population.[24,32,33]

The hypothesis that elevated homocysteine may contribute to vascular disease was first suggested by McCully in 1969 on the basis of postmortem observations of widespread arterial disease in young patients with markedly elevated homocysteine concentrations due to different genetic causes.[5,34,35] These observations formed the basis for the so-called *Homocysteine Theory* that suggests the importance of hyperhomocysteinemia in the development of atherosclerosis. McCully raised the important question of whether mild to moderate elevations in homocysteine, common in the general population, would increase the risk of vascular disease.[34,36]

In 1976, Wilcken and Wilcken provided the first evidence for an association between impaired homocysteine metabolism and CVD in the general population.[37] Since these observations, data from a large number of clinical and epidemiological studies have shown an important role for mild hyperhomocysteinemia as an independent risk factor for CVD and related mortality.[32] Large meta-analysis studies concluded that every increase of 5 μmol/L in plasma concentration of homocysteine increases the risk of CVD by approximately 20%, independent of traditional risk factors, such as diabetes, hypertension, smoking, and hypercholesterolemia.[38] As discussed further, in recent years, the concept that mild hyperhomocysteinemia is a causative risk factor for CVD has become controversial, as therapies that lower homocysteine have failed to improve outcomes in patients with established CVD.[39-43] Nonetheless, homocysteine has been shown to promote endothelial dysfunction, an early step in atherogenesis.

Homocysteine and Endothelial Dysfunction and Activation

The term "endothelial dysfunction" refers to the impairment of the normal homeostatic properties of the vascular endothelium, which include endothelium-dependent regulation of vascular tone, hemostasis, and inflammation. A decrease in nitric oxide (NO) bioavailability and an impairment of cell redox balance are major features of endothelial dysfunction. Endothelial

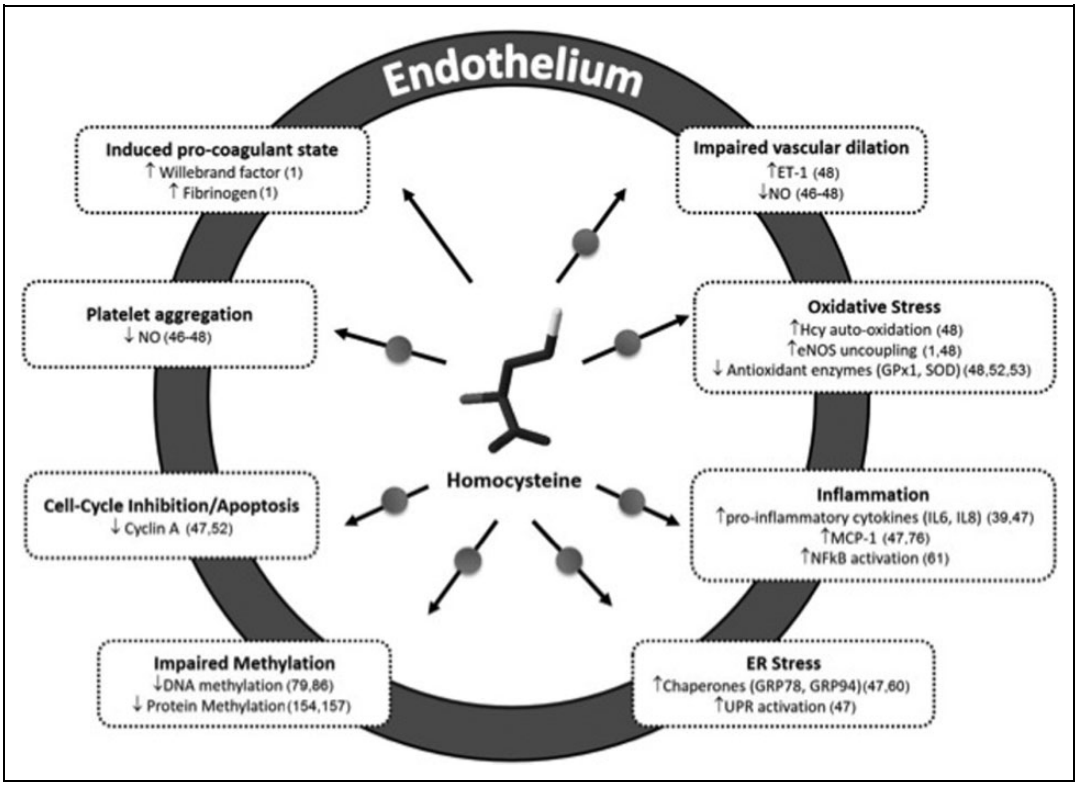

Figure 2. The harmful effects of homocysteine in the endothelium. Dots on the arrows indicate association of these effects with excess SAH and hypomethylation. eNOS indicates endothelial nitric oxide synthase; ET-1, endothelin 1; GPx1, glutathione peroxidase 1; GRP78, glucose-regulated protein 78; GRP94, glucose-regulated protein 94; Hcy, homocysteine; IL6, interleukin 6; IL8, interleukin 8; MCP-1,(monocyte chemoattractant protein 1; NF-κB, nuclear factor-kappa B; NO, nitric oxide; SAH, S-Adenosylhomocysteine; SOD, superoxide dismutase; UPR, unfolded protein response.

dysfunction often leads to a pro-inflammatory state (endothelial activation) that precedes the formation of atherosclerotic plaques.[44] The detrimental effect of homocysteine on the endothelium may explain the increased risk of CVD associated with hyperhomocysteinemia.[45]

Although many studies have shown that homocysteine disrupts endothelial homeostasis (summarized in Figure 2), the mechanisms by which homocysteine promotes endothelial dysfunction are not fully understood.

Nitric oxide is considered a major endogenous antiatherosclerotic molecule, as it is a potent vasodilator, and it inhibits monocyte and platelet adhesion, smooth muscle cell proliferation, and low-density lipoprotein (LDL) oxidation.[46,47] Increased plasma levels of homocysteine have been associated with impaired vascular tone, due to the decrease in NO bioavailability and increase in endothelin-1 (ET-1), a potent vasoconstrictor.[48] Similarly, vascular dysfunction was observed in an animal model of diet-induced mild hyperhomocysteinemia.[49] In addition, decreased levels of NO were observed in several vascular beds obtained from mice with mild hyperhomocysteinemia.[48] Moreover, oxidative stress was shown to contribute to the loss of bioavailable NO.[50,51]

There are several means by which homocysteine can promote the accumulation of damaging reactive oxygen species (ROS); these include homocysteine's auto-oxidation, endothelial nitric oxide synthase (eNOS) uncoupling, and the inhibition of the activity of important antioxidant enzymes, such as glutathione peroxidase 1 (GPx-1) or superoxide dismutase (SOD).[48,52,53] The reaction of superoxide with NO produces the strong oxidant peroxynitrite ($ONOO-$) and, simultaneously, decreases NO bioavailability.[45] Importantly, elevated homocysteine levels have also been associated with an increase in asymmetric dimethylarginine (ADMA) in endothelial cells.[54] Asymmetric dimethylarginine is a by-product of the hydrolysis of methylated proteins and an endogenous inhibitor of NO synthases.[54] Therefore, ADMA has been suggested as a mediator of reduced NO availability during hyperhomocysteinemia.[55]

More recently, it has been suggested that alterations in hydrogen sulfide (H_2S) production may also contribute to homocysteine-associated vascular disease. Hydrogen sulfide is a gasotransmitter produced in endothelial cells that participate in the regulation of endothelial integrity by exerting several beneficial physiological effects, including its anti-inflammatory and antioxidant actions.[23,56] Homocysteine is a precursor of H_2S, which is formed by alternative reactions catalyzed by the CBS and CGL enzymes of the transsulfuration pathway.[23,56] In endothelial cell cultures, CBS deficiency decreased H_2S production to inhibit cellular proliferation and migration. Other studies have shown that exogenous H_2S can

correct endothelial dysfunction *in vivo*.[57] Taken together, these findings suggest that CBS deficiency may decrease endothelial H_2S to impair vascular relaxation and contribute to endothelial dysfunction.[56,58] However, an absence of CBS can paradoxically augment H_2S production in liver due to CGL-dependent enzymatic pathways that utilize excess homocysteine, suggesting that the *in vivo* effects of CBS deficiency on vascular H_2S and endothelial function warrant further examination.[59]

Moreover, homocysteine induces endothelial inflammation and activation of the coagulation cascade, further contributing to the progression of atherosclerotic lesions. Homocysteine was shown to trigger the activation of nuclear factor κB (NF-κB), a transcription factor known to stimulate the production of cytokines, chemokines, leukocyte adhesion molecules, and hematopoietic growth factors in cell culture systems as well as in animal models.[60] In other studies using vascular endothelial cells, homocysteine increased the expression of adhesion molecules, such as E-selectin and vascular cell adhesion protein 1, that contribute to atherogenesis.[61,62] These adhesion molecules and cytokines enhance the binding of leukocytes to the endothelium and promote their transmigration to the vessel wall. More recently, it was shown that in humans, plasma levels of homocysteine correlate with those of interleukin 6 (IL-6) and IL-1 receptor antagonist.[47,63] Cell culture studies also showed that homocysteine favors a pro-coagulant state and platelet adhesion. Endothelial cells exposed to homocysteine had increased levels of tissue factor and enhanced activation of factor V to Va.[1] Furthermore, a correlation was found between homocysteine levels and fibrinogen, an acute-phase protein involved in vascular inflammation and a marker of endothelial dysfunction, in the plasma of patients with coronary artery disease, supporting the concept that excess homocysteine promotes inflammation and disrupts endothelial homeostasis in humans. Similarly, von Willebrand factor, another marker of endothelial damage, was also increased in plasma of patients with hyperhomocysteinemia having premature arterial disease.[1]

An alternative mechanism that may contribute to the homocysteine-induced inflammation and endothelial cell activation is protein homocysteinylation. Protein homocysteinylation is a posttranslational modification in which the homocysteine metabolite, Hcy-thiolactone, is incorporated into the polypeptide chain in place of methionine.[64,65] *In vivo* and *in vitro* studies have shown that protein homocysteinylation may impair or alter protein function.[65] Several plasma proteins have been shown to be targets for this modification by Hcy-thiolactone contributing to a atherogenic phenotype.[65,66] For example, homocysteinylated albumin induces inflammatory response and increases monocyte adhesion to human endothelial cells.[66] In addition, protein homocysteinylation may also contribute to apoptosis.[65]

Endoplasmic reticulum (ER) stress is another deleterious effect associated with high concentrations of homocysteine. Endoplasmic reticulum stress is characterized by an accumulation of misfolded proteins in the ER lumen, which triggers the unfolded protein response and increases the expression of stress response genes, such as *GRP78* and *GRP94*. Endoplasmic reticulum stress can induce inflammation and apoptosis, which can favor the progression of atherosclerotic lesions.[47]

Homocysteine causes a multitude of adverse effects that disrupt vascular cell homeostasis. For example, both ROS release and ER stress can activate apoptosis.[60] Similarly, homocysteine-induced peroxynitrite can induce apoptosis.[47] Oxidative stress can also contribute to LDL oxidation and the formation of foam cells to promote atherosclerotic plaque growth. Decreased NO levels lead to enhanced activation of coagulation pathways and increased platelet aggregation. Additionally, homocysteine can reduce endothelial expression of cyclin A, promoting cell cycle arrest and preventing replacement of damaged endothelial cells.[52] Thus, homocysteine can activate multiple pathways that together contribute to vascular disease progression.

S-Adenosylhomocysteine and CVD

As discussed earlier, several studies concluded that elevations of plasma homocysteine in the mild to moderate range were an independent risk factor for CVD in the general population.[32,38] Subsequently, several randomized control studies were conducted to examine whether homocysteine-lowering B-vitamin therapy would decrease the occurrence of adverse cardiovascular events. Surprisingly, despite a substantial, quick, and long-lasting effect on lowering the concentrations of plasma homocysteine, the majority of these vitamin therapies have shown no clear clinical benefit on vascular disease risk and mortality.[39-43] An alternative theory proposes that SAH, rather than homocysteine, may be a more accurate measure of CVD risk.[67-70] S-Adenosylhomocysteine is the homocysteine precursor that accumulates in the setting of hyperhomocysteinemia. Several studies report the positive correlation between increased homocysteine plasma levels and increased SAH intracellular and/or plasma levels.[70-73] Notably, growing evidence shows that B-vitamin treatments that reduce plasma homocysteine fail to lower plasma[74] and intracellular SAH.[75]

To date, there are few studies that have examined the association between SAH and CVD. Nevertheless, experiments in mice have established a link between excess SAH and CVD,[76] suggesting that SAH is a more sensitive biomarker for atherosclerosis than homocysteine.[69]

A prospective cohort study in coronary angiographic patients showed that increased plasma SAH levels are significantly correlated with an increase in CVD events.[70] Moreover, a cross-sectional cohort study of 402 individuals with low CVD risk found an association between SAH and subclinical atherosclerosis, implicating SAH as a marker of early atherosclerotic disease.[77] Taken together, these observations support the concept that elevated SAH, rather than homocysteine, may be the culprit in the CVD risk that has been previously associated with hyperhomocysteinemia.

S-Adenosylhomocysteine and Cell Hypomethylation

S-Adenosylhomocysteine accumulates in the setting of hyperhomocysteinemia due to the reversibility of SAHH reaction

that strongly favors SAH synthesis rather than its hydrolysis. However, under normal physiologic conditions, this reaction proceeds in the hydrolytic direction due to the efficient removal of homocysteine. Nonetheless, the seminal work by Yi et al showed that plasma levels of homocysteine were positively correlated with intracellular leukocyte SAH concentrations in a healthy population.[78] Additional studies further confirmed that SAH accumulates under hyperhomocysteinemia in humans and in animal models.[71,79,80]

S-Adenosylhomocysteine is, simultaneously, a by-product and a potent inhibitor of the activity of SAM-dependent methyltransferases. In fact, as mentioned earlier, the catalytic sites of most methyltransferases have a higher affinity for SAH compared to SAM. Hence, homocysteine-induced SAH accumulation may inhibit the activity of cell methyltransferases, thereby disrupting methylation homeostasis and promoting a hypomethylating environment. The SAM-dependent methyltransferases are responsible for the majority of cellular methylation reactions, including creatine synthesis, membrane phosphatidylcholine synthesis, and synthesis of neurotransmitters.[78] Additionally, DNA, RNA, and proteins are targets for methyltransferases, and the methylation state of these macromolecules can regulate gene expression patterns. Thus, we propose that excess SAH may contribute to the molecular basis of homocysteine-induced vascular toxicity by decreasing the methylation of these macromolecules.

Targets of SAH-Mediated Methylation Inhibition with Relevance for Gene Expression

DNA. Epigenetic markers, such as DNA methylation, regulate gene expression during development and adulthood, and they can determine cell- and tissue-specific gene expression. Methylation of the carbon 5 position of the cytosine ring is the most common modification of the double helix, and this cytosine methylation can modulate the transcriptional potential of genomic DNA.[81] In differentiated mammalian cells, cytosine methylation can occur in any nucleotide context; however, more than 98% occurs in cytosines that are followed by guanines, in a cytosine-phosphate-guanine (CpG) dinucleotide context.[82] CpG islands are sequences present in the genome that have a CG-rich base composition and high density of CpG dinucleotides.[83] The majority of the annotated gene promoters, including promoters of housekeeping genes, tissue-specific genes, and developmental regulator genes, contain CpG islands.[83] DNA methylation may alter gene expression: it can inhibit the binding of transcription factors and can recruit methyl-CpG binding domain proteins that promote the formation of a repressed chromatin state.[69] Transcriptional repression by DNA methylation has been demonstrated for promoters that contain CpG islands and also for promoters with low CpG density.[84]

Cytosine methylation in DNA is accomplished by the action of 3 DNA methyltransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B. DNMT1 is responsible for the maintenance of the DNA methylation patterns during replication, while the DNMT3A and DNMT3B function as *de novo* methyltransferases.[81] Importantly, SAH has been shown to inhibit the *in vitro* methyltransferase activity of each of the DNMT enzymes.[85] Other studies have also established that elevated plasma homocysteine levels are positively correlated with increased intracellular SAH and decreased DNA methylation in lymphocytes/leukocytes.[71,78] Accordingly, excess SAH levels have been strongly associated with altered gene expression in several studies.[86-88] Notably, SAH also induced tissue-specific changes in DNA methylation patterns that correlated with altered gene expression in hyperhomocysteinemic mice.[89]

S-Adenosylhomocysteine has been shown to disturb promoter methylation of many genes, including the *PDGF* (platelet-derived growth factor) gene,[86] the stress response-related *p66Shc* gene,[88,90] genes involved in the regulation of cholesterol biosynthesis, such as *SREBF1* (sterol regulatory element binding transcription factor 1) and *LDLR* (LDL receptor),[87] and those involved in cell stress and cell cycle regulation, such as the *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3) gene.[91] Additionally, inflammatory genes, such as *IL1B*, *IL6*, *IL8*, and *ICAM1* (intercellular adhesion molecule 1), that can be regulated by DNA methylation in some cancer cells,[92,93] may also be targets of SAH-mediated hypomethylation.[94]

Several lines of evidence support the concept that an elevation in homocysteine can lead to DNA hypomethylation, secondary to inhibition of DNMTs by SAH.[71,79,95,96] For instance, we and others observed that plasma homocysteine concentration has a positive correlation with SAH levels and a negative correlation with lymphocyte DNA methylation status in healthy individuals and in patients with CVD.[71,78] A genome-wide analysis study of DNA methylation, conducted in patients with chronic kidney disease presenting with high levels of both homocysteine and SAH, identified 52 differentially methylated pro-atherogenic genes.[95] These data provided evidence for both DNA hypomethylation and DNA hypermethylation at different gene *loci*, suggesting that mechanisms other than SAH-induced inhibition of DNMTs are also regulating epigenetic changes in this disease.

DNA hypomethylation has been linked to atherosclerotic disease in many other studies where the SAH status is unknown.[97,98] A significant reduction in the genomic methyl cytosine content was detected in advanced human atherosclerotic lesions[99] and in vascular lesions of mice lacking apolipoprotein E (ApoE), a well-established animal model of atherosclerosis.[100] The gene encoding the antioxidant extracellular SOD was also shown to be a target for regulation by DNA methylation. A study conducted in rabbits showed a marked reduction in the amount of methylated CpG in the *ec-sod* gene in atherosclerotic aortas when compared to healthy arteries.[101] Hiltunen et al reported that genomic hypomethylation occurs in atherosclerotic lesions in human, mouse, and rabbits and that it correlates with a higher transcriptional activity of some genes.[99] Namely, an association between the expression of PDGF-B and genomic hypomethylation was observed, suggesting that DNA hypomethylation could contribute to growth factor upregulation during atherosclerotic lesion

development.[99] Similarly, DNA hypomethylation was found n human atherosclerotic carotid plaques.[100] Although several studies report global hypomethylation in atherosclerotic plaques,[102,103] DNA global hypermethylation was also found in plaques.[104] The basis for these differences in methylation status is unclear but may relate to the stage of the plaque[105] or whether or not the plaque is symptomatic (ie, had a rupture).[96] Nonetheless, many studies have identified specific genes that are hypomethylated in atherosclerotic plaques and can be determinants for the disease.[98]

Folate levels are essential for the remethylation of homocysteine, and folate deficiency can lead to hyperhomocysteinemia. Many studies have focused on the effect of folate on DNA methylation, rather than the effects of homocysteine. Although most of these studies were conducted in cancer cells or patients, similar to the findings with elevated homocysteine, no consistent association was found between folate levels and global DNA methylation status across all studies.[106] Of importance, in a study of patients with uremic hyperhomocysteinemia, the negative correlation between homocysteine levels and DNA global methylation status was restored by folate therapy that increased DNA methylation and altered the patterns of gene expression.[107] This work by Ingrosso and colleagues represents the first human study that causally linked homocysteine and folate with altered gene expression via DNA hypomethylation.[107] However, to prove that SAH-induced hypomethylation contributes to disease mechanisms, additional information is needed about the relationship between DNA hypomethylation and the expression of disease-causing genes, as well as the role of SAH, homocysteine, and folate in these processes.

RNA. Although much less studied than other SAM-dependent methylation targets, RNA is highly methylated. To date, about 100 different methylation modifications in RNA have been described.[108] However, the function of most RNA modifications is unknown. Different RNA species, such as transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA), small nuclear RNA, and micro-RNA, can have different patterns of ribonucleotide methylation. Interestingly, some RNA methyltransferases target multiple RNA species, while others are specific for a single RNA species.[109]

The importance of RNA methylation is illustrated by the 5′ methylation cap in mRNA, which is crucial for mRNA stability and efficient gene expression.[110] In addition, several mechanisms have been proposed for tRNA and rRNA methylation-mediated regulation of translation and codon decoding.[109] RNA methylation has also been shown to affect RNA synthesis, maturation, transport, and splicing. The effect of methylation on RNA function depends on the RNA molecule that is being methylated, as well as the location of the methylated ribonucleotide within that molecule and the location of the methylation site within the ribonucleotide.

Although RNA methylation can occur on nitrogen, oxygen, and carbon, nitrogen is the most modified atom within the pyrimidine or purine base rings.[109] In addition to methylation sites on the ribonucleotide base, ribose can also be methylated. 2′-O-methylation is the most common type of ribose methylation.[111] Recently, the fat mass and obesity associated protein (FTO) and the alkylation repair homolog 5 (ALKBH5) were reported to mediate N6-methyladenosine demethylation.[110,112,113] The discovery that RNA methylation can be a dynamic modification raises the question of whether these modifications regulate gene expression. RNA methylation is per se an addition of information to the RNA sequence and its flexibility further increases RNA complexity and diversity, although the consequences of many of these modifications remain unclear.

The lack of information on the function of specific RNA methylations, as well as the fact that the activity and/or identity of many of the RNA methyltransferases remain unresolved, has hindered the study of these modifications in normal and disease conditions. Cell culture studies, as well as studies using purified enzymes, have shown that particular RNA methylation events are sensitive to SAH accumulation.[114-117] In addition, some RNA modifications may be altered under disease conditions. One study conducted in rabbits experiencing myocardial infarction reported altered patterns of liver tRNA methylation in these stressed rabbits compared to controls.[118] In particular, 1-methyladenosine content was decreased, whereas 7-methylguanosine and 1-methylguanosine contents were increased in the myocardial infarction group[118]; however, the specific function of each of these modifications remains unclear as is the molecular basis for these changes.[118] Several studies have also reported hypo- and hypermethylation of specific tRNA modifications in different types of cancer.[111,119-124]

Likewise, impaired rRNA methylation was found in leukemic blast cells, where rRNA maturation was compromised.[125,126] Additionally, genome association studies have also implicated gene variants of the mRNA demethylase *FTO* gene in cancer, neuronal development, and renal and CVDs, although the effect of these variants on RNA methylation was not analyzed in these studies.[113,127]

Transfer RNA is the most highly modified RNA species, and some of these modifications may have important roles in mediating cellular stress responses.[128] In 2010, the activity of ALKBH8, a mammalian methyltransferase that is able to methylate a number of different tRNA residues, was reported.[128] Its activity includes modifications that are essential for the correct translation of a subset of selenoproteins (proteins that contain selenocysteine in their polypeptide chain).[128,129] Selenocysteine (Sec) carrying tRNA (Figure 3) recognizes an UGA codon, which is a common signal for translation termination, as a target for Sec incorporation.[128,130,131] Thus, Sec-tRNA is essential for efficient translation of selenoproteins. There are 2 major Sec-tRNA isoforms, 5-methoxycarbonylmethyluridine (mcm5U) and 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm5Um), which differ by a single methyl group at the ribose of the U at position 34 (Figure 3).[128,130] ALKBH8 is responsible for the conversion of 5-carboxymethyluridine to mcm5U, a prerequisite step before the methylation of the 2′-O-ribose to form mcm5Um.[128,129] Several selenoproteins rely on the presence

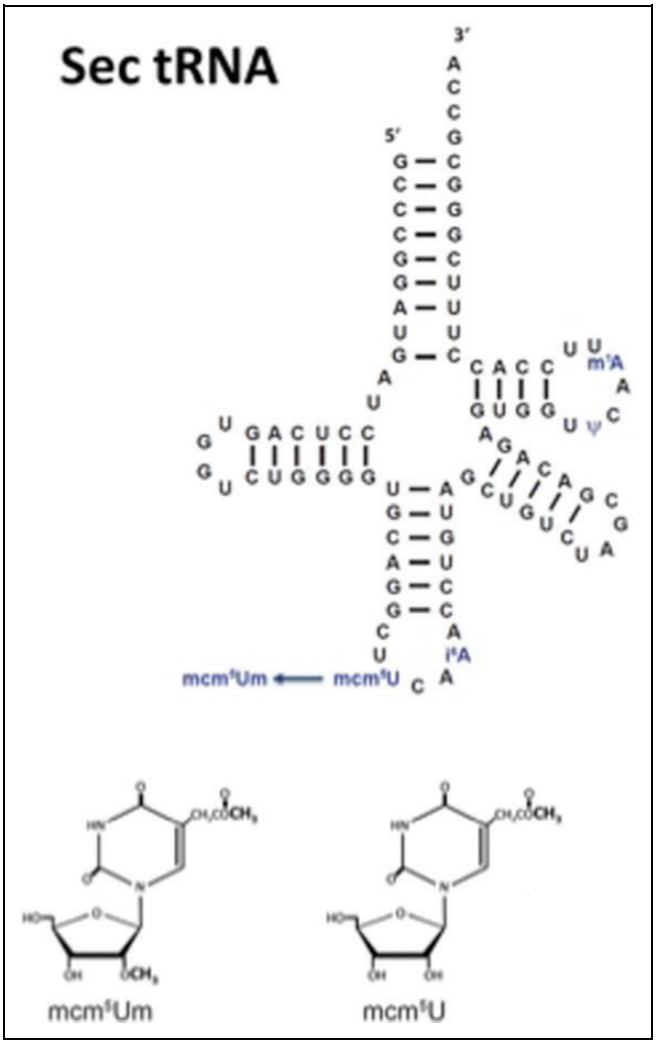

Figure 3. Human Sec-tRNA. The sequence and structure of the Sec-tRNA is shown at the top, and the modifications of the wobble uridine at position 34 of the anticodon (U34) are shown at the bottom. mcm5U indicates 5-methoxycarbonylmethyluridine; mcm5Um, 5-methoxycarbonylmethyl-2′-O-methyluridine; Sec, selenocysteine; tRNA, transfer RNA.

of mcm5Um in the Sec-tRNA to be efficiently translated, including GPx-1. Glutathione peroxidase 1 is a major antioxidant enzyme in human cells and its impaired translation is associated with elevated homocysteine levels.[53] Songe-Møller et al found that mice lacking ALKBH8 showed decreased mcm5U and mcm5Um content in tRNA with a consequent decrease in GPx-1 expression and increased susceptibility for oxidative stress.[128] Subsequent studies have also shown that a deficiency in ALKBH8 suppresses the expression of GPx-1 and other selenoproteins to cause oxidative stress in mouse embryonic fibroblasts.[132] Importantly, in addition to GPx-1, many other selenoproteins are involved in cell detoxification and redox regulation. Furthermore, selenium deficiency, which causes a decrease in select selenoprotein expression, has also been associated with oxidative stress.[130,133]

Studies in endothelial cells revealed that a hypomethylating environment, induced by excess SAH, impairs not only NO production but also the cellular redox state.[134,135] Previous studies reported a link between homocysteine-associated suppression of GPx-1 and endothelial dysfunction; however, our recent findings clarify the molecular mechanisms for decreased GPx-1 expression.[53,135] Our findings showed that SAH accumulation decreases Sec-tRNA mcm5Um levels, leading to the altered expression of a subset of selenoproteins, including GPx-1 in endothelial cells.[135] These results uncovered a specific mechanism by which SAH-mediated hypomethylation suppresses GPx-1 expression to promote oxidative stress and inflammatory activation of endothelial cells.

Protein. Protein methylation is a widespread posttranslational modification that modulates protein function and increases the structural diversity of the proteome. Furthermore, protein methylation can be reversible, similar to phosphorylation or acetylation, adding another means to regulate protein–protein interactions, protein stability, protein localization, and/or enzymatic activity.[136] Currently, 50 different protein lysine methyltransferases (PKMTs) and 11 protein arginine methyltransferases (PRMTs) are known.[137] The PKMTs can catalyze the transfer of 1, 2, or 3 methyl groups from SAM to lysine residues in a protein polypeptide chain, producing mono-, di-, or tri-methyl-lysine residues (Figure 4). Likewise, PRMTs can also mono-, di-, or tri-methylate arginine residues. Additionally, dimethylation by PRMTs can be symmetric, generating symmetric dimethylarginine residues, or asymmetric, producing ADMA residues[138,139] (Figure 4).

The methylation of histones is a well-known epigenetic mechanism that regulates gene expression by modulating chromatin structure.[103,140] Importantly, these methylation marks are highly dynamic and can be targeted by specific methylases and demethylases.[141] Furthermore, the effects of histone methylation on chromatin activation depend on the specific residues that are methylated.[140] Methylation of lysines 4, 36, and 79 in histone H3 are mainly associated with active transcription, while methylation at lysines 9 and 27 are associated with gene repression and heterochromatin formation. Furthermore, the lysine methylation status (mono-, di-, or trimethylation) can determine gene expression or repression. For example, trimethylated histone H3 lysine 4 is a mark of active promoters, while di- and trimethylated histone H3 lysine 9 (H3K9) residues are strongly associated with transcriptional repression.[140]

The establishment and maintenance of epigenetic gene silencing is fundamental to cell homeostasis.[142] The Polycomb group (PcG) of proteins are negative epigenetic regulators of transcription and represent evolutionarily conserved multiprotein complexes: the Polycomb repressive complexes (PRCs).[143] There are 2 main PRCs: PRC1 and PRC2. PRC2 catalyzes the dimethylation and trimethylation of histone H3 at lysine 27 (H3K27me2/3), which is a central feature of PcG-silenced chromatin, while PRC1 promotes lysine 119 monoubiquitylation of histone H2A (H2AUb1).[144] PRC2 complex is

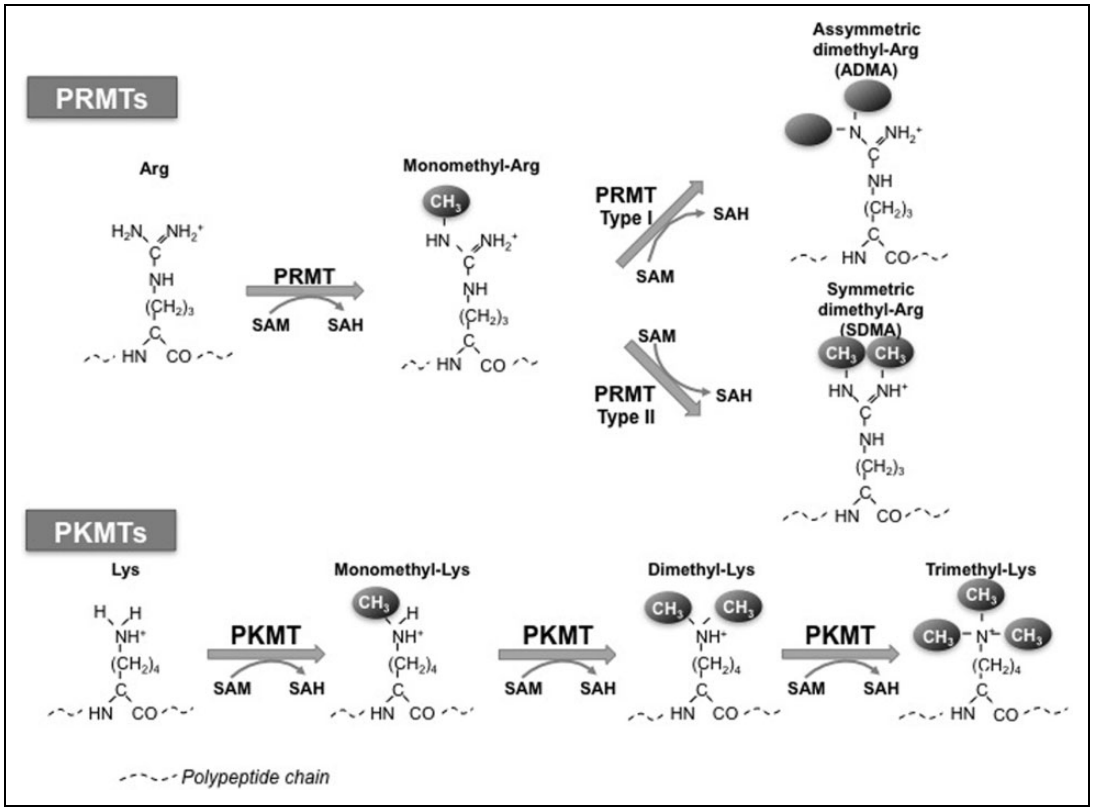

Figure 4. Protein methylation and SAH. Protein Arg and Lys residues are targets for the SAM-dependent PRMTs and PKMTs, respectively. Arg indicates arginine; Lys, lysine; PKMTs, protein lysine N-methyltransferases; PRMTs, protein arginine N-methyltransferases; SAH, S-Adenosylhomocysteine; SAM, S-Adenosylmethionine.

involved in the initiation of gene silencing, whereas PRC1 is responsible for stabilizing and maintaining gene repression. Enhancer of zeste homolog 2 (EZH2) is the SAM-dependent histone lysine methyltransferase (HKMT) that is the catalytic component of PCR2, and it epigenetically silences gene expression by mediating H3K27me3 methylation.[142,145] UTX (ubiquitously transcribed tetratricopeptide repeat on X chromosome demethylase) and JMJD3 (jumonji domain containing 3 demethylase) are H3K27-specific histone demethylases, which are capable of removing di- and trimethylation of H3K27.[139]

Similarly, G9a/GLP are 2 H3K9-specific HKMTs[146] that produce another repressive histone methylation. Several different demethylases were reported to remove H3K9 methylation, including multiple members of the Lysine demethylases (KDM) family.[139] Interestingly, similar to EZH2, G9a also relies on SAM as the methyl donor compound for its HKMT activity.[147,148]

Recent studies revealed that EZH2 epigenetically regulates cell proliferation, spreading, and angiogenesis in endothelial cells.[149] Additionally, EZH2 target genes that are suppressed by H3K27me3 include pro-inflammatory cytokines that can induce endothelial cell expression of adhesion molecules.[149] Excess SAH was found to cause a loss of the repressive histone marks, H3K27me3 and H3K9me3, in cancer cell lines,[150,151] and recent findings correlate a reduction in global histone H3K27me3 with the presence of advanced atherosclerotic plaques in human vessels.[152] Additionally, our recent studies provide mechanistic insight into how the regulation of EZH2 by SAH contributes to a pro-atherogenic environment. Thus, increased intracellular SAH leads to a decrease in EZH2 RNA, protein, and activity in endothelial cells.[94] Our studies further link the SAH-induced suppression of EZH2 and H3K27me3 to the activation of the pro inflammatory NF-κB pathway and the increased expression of adhesion molecules and inflammatory cytokines.[94] These results identified EZH2 as a new target of SAH regulation, demonstrating that EZH2 suppression and the subsequent NF-κB activation mediated by excess SAH accumulation may contribute to the adverse effects of SAH in the vasculature.

Other studies have shown an association of hypomethylation of nonhistone proteins with disease. Perna et al were the first to suggest the involvement of protein hypomethylation in hyperhomocysteinemia, reporting that SAH accumulation was associated with a decrease in methyl esterification of erythrocyte membrane proteins in patients with renal impairment.[153]

Specifically, the accumulation of intracellular SAH, and the concomitant decrease in the SAM–SAH ratio, resulted in a decrease in the methylation of ankyrin, a cytoskeletal protein that is involved in membrane stability and integrity.[153] Garcia and colleagues also observed that a decrease in the SAM–SAH ratio in rats resulted in impaired arginine methylation of proliferator-activated receptor-γ coactivator-1 (PGC-1α), a

transcriptional coactivator that regulates genes involved in energy metabolism.[154] Similarly, methylation of p21ras was also found to be suppressed by homocysteine under conditions that favored the accumulation of SAH.[155] In this study, the inhibition of vascular endothelial cell growth was correlated with an increase in SAH and a reduction in p21ras carboxyl methylation.[155] These observations suggest an additional mechanism by which SAH-induced hypomethylation could contribute to vascular dysfunction.

Our group reported that excess SAH altered global protein arginine methylation *in vitro* and *in vivo*.[138,155,156] In these studies, we observed that protein arginine methylation is a more susceptible target for SAH-mediated inhibition than DNA methylation.[138] Furthermore, we reported hypomethylation of proteins, including histones, in 2 independent animal models of hyperhomocysteinemia.[156,157] In support of these experimental findings, a recent study found hypomethylation of histones (and DNA) in human atherosclerotic carotid plaques compared to control arteries.[102] Taken together, these results provide evidence that protein methyltransferases are targets for SAH-mediated inhibition in the context of hyperhomocysteinemia and lay the groundwork for future investigation of specific protein methylation targets that may contribute to vascular disease.

Summary

The concept that homocysteine is a risk factor for cardiovascular disease has recently been questioned due to the lack of beneficial effects of treatments that lower circulating homocysteine. Growing evidence suggests that SAH, the homocysteine precursor, may be a better indicator of cardiovascular disease risk. In fact, recent studies show that treatments that augment SAH levels increase atherosclerotic lesion size in susceptible ApoE-deficient mice.[76]

Overall, several pieces of evidence implicate SAH as a key modulator of endothelial dysfunction by compromising RNA, DNA, and protein methylation. More importantly, many studies, including our own, support a role for excess SAH-induced hypomethylation in the pathogenesis of homocysteine-associated vascular disease.

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