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Mechanisms of homocysteine-induced damage to the endothelial, medial and adventitial layers of the arterial wall

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Abstract

Homocysteine (Hcy) is a non-protein forming amino acid which is the direct metabolic precursor of methionine. Increased concentration of serum Hcy is considered a risk factor for cardiovascular disease and is specifically linked to various diseases of the vasculature. Serum Hcy is associated with atherosclerosis, hypertension and aneurysms of the aorta in humans, though the precise mechanisms by which Hcy contributes to these conditions remain elusive. Results from clinical trials that successfully lowered serum Hcy without reducing features of vascular disease in cardiovascular patients has cast doubt on whether or not Hcy directly impacts the vasculature. However, studies in animals and in cell culture suggest that Hcy has a vast array of toxic effects on the vasculature, with demonstrated roles in endothelial dysfunction, medial remodeling and adventitial inflammation. It is hypothesized that rather than serum Hcy, tissue-bound Hcy and the incorporation of Hcy into proteins could underlie the toxic effects of Hcy on the vasculature. In this review, we present evidence for Hcy-associated vascular disease in humans, and we critically examine the possible mechanisms by which Hcy specifically impacts the endothelial, medial and adventitial layers of the arterial wall. Deciphering the mechanisms by which Hcy interacts with proteins in the arterial wall will allow for a better understanding of the pathomechanisms of hyperhomocysteinemia and will help to define a better prevention at the appropriate window of life.

Keywords:

Homocysteine, Vascular Disease, Folate, Atherosclerosis, Vitamin B12

Abbreviations:

Hcy: Homocysteine, *HHcy*: Hyperhomocysteinemia, *MMP*: Matrix metalloproteinase, *ECs*: Endothelial cells, *NO*: Nitric oxide, *HUVECs*: Human umbilical vein endothelial cells, *NFκB*: Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, *ApoE^{-/-}*: Apolipoprotein E-deficient, *RAGE*: Receptor for advanced glycation end-products, *Ero1α*: Endoplasmic reticulum oxidoreductin 1-Alpha, *PERK*: PKR-like ER kinase, *IRE1*: Inositol-requiring enzyme 1, *N-Hcy*: N-Homocysteine, *SMCs*: Smooth Muscle Cells.

1. Introduction

Diseases of the cardiovascular system, including those which impact the blood vessels, are a prominent cause of mortality¹. Therefore, great efforts have been made to understand the risk factors and underlying mechanisms of cardiovascular disease development and progression. A common feature of various forms of vascular disease is a marked rise in serum Homocysteine (Hcy)². Hcy is a non-protein forming amino acid produced as an intermediate byproduct of methionine metabolism (**Figure 1**). It can be further metabolized to cystathionine via cystathionine β -synthetase, a vitamin B6-dependent enzyme. Alternatively, Hcy can be remethylated back to methionine either by betaine Hcy methyltransferase or by methionine synthase, a vitamin B12-dependent enzyme. Folate is also required for the remethylation of Hcy by providing 5-methyltetrahydrofolate (**Figure 1**). The complexity of Hcy metabolism presents challenges to human health, as errors in any one of the crucial steps in this process can lead to alterations in Hcy concentrations. For example, mutations in cystathionine β -synthetase, methylenetetrahydrofolate reductase, methionine synthase, methionine synthase reductase and mutations in the genes involved in B12 metabolism produce inherited disorders that are known to cause homocystinuria. These disorders and others relating to Hcy metabolism put the patient at risk for various pathologies, including vascular disease.

A link between Hcy and vascular disease was first established in 1969 when Kilmer McCully reported that two children with homocystinuria presented with vascular pathologies that were normally reserved for elderly adults³. He noted that a 2-month-old boy had advanced stage arteriosclerosis suggestive of advanced cardiovascular disease, which was accompanied by reduced methylenetetrahydrofolate reductase activity in the liver and kidney. As well, he reported that an 8-year-old child with cystathionine β -synthetase deficiency died from what appeared to be stroke-related arteriosclerosis in the carotid artery. These findings provoked extensive research, which showed an association between Hcy and hypertension⁴⁻⁶ and prompted the study of Hcy in various vascular complications afflicting small and large vessels, including endothelial dysfunction, atherosclerosis, and aneurysms. There is compelling evidence to suggest that Hcy induces

endothelial damage, medial proliferation⁷ and adventitial activation⁸. In this review, we discuss important implications for Hcy related vascular pathologies, and how Hcy particularly impacts each layer of the arterial wall (**Figure 2**).

2. Evidence for Homocysteine-induced vasculopathy in humans

It is well known that Hcy is a predictor of cardiovascular disease in humans. In fact, an increase in plasma Hcy levels by 5 μ M from normal levels is reported to augment the risk of cardiovascular disease by 20%^{9, 10}. Furthermore, there is a strong correlation between elevated levels of plasma Hcy and vascular disease¹¹⁻¹³, though whether Hcy causes vascular disease or is simply a bystander is not completely understood. There are a number of conditions that produce a rise in serum Hcy, or hyperhomocysteinemia (HHcy), in humans, many of which also have features of vascular disease.

Homocystinuria due to cystathionine β -synthetase deficiency accounts among the most common inherited disorder of methionine metabolism and results in the accumulation of Hcy and methionine in the blood and urine¹⁴. The clinical spectrum of this disease is vast, and vascular complications are a prominent feature^{15, 16}. Although most often associated with venous thrombosis^{17, 18}, there is evidence for large arterial pathology associated with cystathionine β -synthetase deficiency. Similar to findings in patients with Marfan syndrome, a subset of patients with homocystinuria had a dilated aortic root¹⁹. Interestingly, emerging evidence from a phase 1/phase 2 clinical trial suggests that taurine may be a promising treatment option for vascular complications associated with homocystinuria, as it improved otherwise diminished endothelial function in cystathionine β -synthetase deficient patients²⁰. Further research should be employed to determine if this treatment is effective in other forms of Hcy-related vascular disease.

HHcy can also be induced by deficiencies in vitamin B12²¹, vitamin B6²² and folate²³. In a study of vitamin B12 deficiency in pediatric patients, the concentration of serum Hcy was increased and was markedly correlated with the intimal-medial thickness of the carotid artery, reminiscent of subclinical atherosclerosis²⁴. Similarly, in a study of mild HHcy in adults, low folate serum concentrations were observed in patients with

atherosclerosis²⁵. Moreover, population studies found significant inverse relationships between vitamin B12 and both plasma Hcy and the maximum diameter of aneurysmal abdominal aortas in elderly adults^{26, 27}. These findings suggest that HHcy induced by nutritional deficiencies impacting Hcy metabolism may play a role in various vascular pathologies, including the development of atherosclerotic plaques and aortic aneurysms. Interestingly, renal disease is also associated with HHcy, through reduced filtration and other mechanisms incompletely understood. End stage renal disease impact the transulfuration capacity of Hcy, ultimately leading to increased Hcy plasma concentrations²⁸. In a large meta-analysis of observational and interventional trials, the authors concluded that total blood levels of Hcy were a risk factor for both cardiovascular disease and death in patients with end stage renal disease²⁹, suggesting a role for HHcy in exacerbating negative outcomes for patients with various forms of kidney disease.

Although a relationship between HHcy and abdominal aortic aneurysms has been well established³⁰, the expression of Hcy in aortic tissue from these patients was only recently investigated. In a study of aortic tissue from 30 patients with abdominal aortic aneurysms, the authors found a marked increase in Hcy expression in the aorta, and a significant increase in Hcy synthesized from isolated aortic SMCs compared to controls³¹. Furthermore, when incubated with Hcy, abdominal aortic aneurysms tissue samples resulted in increased matrix metalloproteinase (MMP)2 activity, which could further weaken the vessel wall and lead to aneurysm rupture and dissection³². Less is known about the relationship between Hcy and thoracic aortic aneurysms, though plasma Hcy levels are more elevated in Marfan syndrome patients with severe cardiovascular manifestations, particularly those related to thoracic aortic dilatation³³.

Although there is a clear relationship between Hcy and vascular disease, the underlying mechanisms are not fully understood. It has been suggested that irreversible protein homocysteinylation could play a role in vascular injury, as N-Homocysteinylated proteins were identified in the aorta from patients undergoing cardiac surgery³⁴. Furthermore, N-homocysteinylation appears to be proatherosclerotic, although these processes have not been mechanistically elucidated³⁵. Further research into the

impact of N-homocysteinylated proteins on human vascular disease is needed. As Hcy is associated with large and small vessel diseases, it is suspected that Hcy elicits negative effects on the vasculature by impacting various layers of the arterial wall. In the next sections of this review, we will discuss evidence for Hcy-induced vascular damage in animal and cell culture models at each layer of the arterial wall, particularly for larger-caliber blood vessels such as the aorta.

3. Experimental evidence for homocysteine-induced vascular pathology

3.1 Homocysteine and the endothelium

The endothelium plays a critical role in maintaining vascular wall homeostasis by regulating vascular tone, permeability, inflammation and cell growth. Therefore, damage to the endothelium or perturbed function of endothelial cells (ECs) has major adverse consequences for vascular stability and function. This perturbed endothelial process, termed endothelial dysfunction, can also trigger inflammatory events which underlie the cascade of events involved in the development of atherosclerotic lesions³⁶.

Findings in culture studies of ECs and in animal models of hyperhomocysteinemia suggest that Hcy is associated with endothelial dysfunction^{37, 38}. In particular, evidence suggests that Hcy causes a reduction in the ability of ECs to regulate vascular tone. Endothelial cells maintain vascular tone by releasing specialized signaling molecules, including nitric oxide (NO) and prostacyclin, potent vasoactive mediators³⁹. It is thought that Hcy impairs the ability of ECs to maintain vascular tone by reducing their ability to produce NO (**Figure 2**). This was confirmed in a study of human umbilical vein endothelial cells (HUVECs), where treatment with Hcy caused a reduction in NO production by suppressing nitric oxide synthase activity⁴⁰. In studies of patients undergoing cardiac surgery, the concentration of vascular Hcy⁴¹, but not plasma Hcy⁴², was associated with vascular superoxide production, suggesting a role for tissue-bound Hcy in endothelial dysfunction. Moreover, vasomotor regulation in large and small vessels is critically inhibited in various murine models of hyperhomocysteinemia, with impaired bioavailability of endothelium derived NO as the proposed mechanism⁴³⁻⁴⁷.

Furthermore, Hcy may interfere with the hydrogen sulfide signaling pathway, a pathway that is closely interconnected with the NO pathway in maintaining vascular homeostasis⁴⁸. In line with this hypothesis, exogenous hydrogen sulfide protected cultured ECs from Hcy-induced damage⁴⁹ and protected against Hcy-induced endothelial dysfunction⁵⁰.

There is also compelling evidence to suggest that Hcy contributes to endothelial dysfunction by inducing the stimulation of inflammatory cascades in ECs (**Figure 2**). Several cell culture studies have shown that Hcy induces vascular inflammation through inducing the release of inflammatory cytokines by ECs, including interleukin-6, interleukin-8 and tumor necrosis factor- α ⁵¹⁻⁵³, which may be due, at least in part, to the activation of NF κ B⁵⁴. Animal models of HHcy have yielded similar results. For instance, in aortic tissue from apolipoprotein E-deficient (ApoE^{-/-}) mice, induction of HHcy was associated with a 10-fold increase in RAGE, a multiligand signal transduction receptor linked to the proinflammatory response, and increased nuclear translocation of NF κ B⁵⁵. Another mechanism by which Hcy induces vascular inflammation could be through the induction of endoplasmic reticulum stress, as an increase in Ero1 α expression was observed in the thoracic aorta of HHcy mice in association with endoplasmic reticulum stress and endothelial inflammation. Furthermore, knockdown of Ero1 α in HUVECs from these mice ameliorated Hcy-induced endoplasmic reticulum stress and the activation of PERK and IRE1 inflammatory pathways³⁸.

Both endothelial dysfunction and inflammation are hallmarks of atherosclerosis. Hence, it is no surprise that HHcy is strongly associated with the development of atherosclerotic lesions⁵⁵⁻⁵⁸. Although the mechanisms are still being explored, it is thought that Hcy contributes to early events of atherosclerosis, particularly through its actions on ECs. In addition to inducing inflammation, culture studies have revealed that Hcy induces endothelial cell apoptosis (**Figure 2**), which is another feature of atherosclerotic lesions^{37,59}. Supporting the notion that Hcy contributes to early processes in the formation of atherosclerotic lesions, a time-course study showed that HHcy promotes early atherosclerosis, but does not necessarily cause early rupture or weakening

of the atherosclerotic plaque⁵⁷. Moreover, HHcy was observed in association with increased oxidative stress and accelerated atherosclerotic changes in a mouse model of atherosclerosis^{60, 61}. Further support for the contribution of Hcy to atherosclerosis comes from studies of betaine supplementation in atherosclerotic animals. Betaine treatment has been reported to decrease Hcy levels⁶², and supplementation with Betaine has been reported to decrease the risk of atherosclerosis in HHcy mice⁶³, and to decrease the size of atherosclerotic lesions in ApoE^{-/-} mice⁶⁴.

One mechanism by which Hcy may impact the endothelium is through irreversible protein homocysteinylated. Through an error-editing function of methionyl-tRNA synthetase (MARS), Hcy can be converted to Hcy thiolactone, a chemically active metabolite that acylates free amino groups⁶⁵. This reaction yields homocysteinylated proteins which are unable to retain their biological activity⁶⁶. In cultured HUVEC cells, high concentrations of extracellular Hcy yielded high Hcy thiolactone expression and the incorporation of Hcy into proteins⁶⁷ (**Figure 2**). Furthermore, in a recent report that extensively studied the impact of different Hcy metabolites on HUVECs, it was suggested that Hcy thiolactone and N-homocysteine (N-Hcy) both have the potential to disturb vascular homeostasis by altering the expression of genes involved in blood clotting⁶⁸. Interestingly, in ApoE^{-/-} mice, N-Hcy proteins were accumulated in atherosclerotic lesions, suggesting a potential role for homocysteinylated proteins in atherosclerosis.

Taken together, these data show that Hcy can provoke and/or exacerbate vascular disease by inducing endothelial dysfunction and inflammation. There is evidence that Hcy elicits its effects on endothelial cells through its incorporation into proteins, rendering them biologically inactive. However, further research is needed to outline the precise mechanisms of Hcy-induced endothelial damage and how this contributes to atherosclerosis and other vascular diseases.

3.2 Homocysteine and the medial layer of large arteries

The media of large arteries is comprised of circumferential layers of interconnected smooth muscle cells (SMCs), which are separated by continuous elastin filaments, collagen and proteoglycans in an intricately structured manner. To a great extent, the medial layer is responsible for the structural and elastic properties of the artery. Therefore, processes that elicit damage to any component of the medial layer resulting in medial remodeling could impact the structural and functional integrity of the artery, resulting in detrimental vascular disease processes.

As with ECs, it is thought that Hcy can influence changes in SMCs that are conducive to the development of atherosclerosis. Under normal circumstances, vascular SMCs are non-proliferative and contractile, residing in the medial layer where they provide stability and contractile forces to the vessel. However, in response to short-term exposure of Hcy, human and rat aortic SMCs are induced to undergo widespread proliferation⁶⁹⁻⁷¹ (**Figure 2**). This is reminiscent of SMCs in atherosclerosis, where SMC proliferation is a hallmark feature⁷². Furthermore, in guinea pigs fed a high cholesterol diet, the induction of HHcy through high methionine intake induced SMC proliferation in the aorta, alongside other features of atherosclerosis, including increased vessel thickness, and the presence of fatty streaks⁷³. The reduction in this response after Hcy-lowering administration of betaine provided further proof that this effect was due to Hcy⁷³.

Another feature of non-contractile SMCs is their ability to synthesize extracellular matrix constituents, including collagen and elastin. This synthetic phenotype of SMCs is normally reserved for vasculogenesis. However, during vascular disease, SMCs can be summoned to perform non-contractile functions, including elaboration and degradation of extracellular matrix components. In addition to inducing cellular proliferation, Hcy provoked significant collagen synthesis and accumulation in cultured SMCs¹¹ (**Figure 2**). Moreover, the accumulation of insoluble collagen was found in aortas of rats fed a diet supplemented with Hcy for 6 weeks⁷⁴. Collagen accumulation in the aorta and other vessels produces vessel stiffening, increased resistance and hypertension⁷⁵. Further evidence for Hcy-induced collagen synthesis and vascular remodeling came from studies

in mice that showed HHcy association with numerous features of aortic remodeling^{76, 77}. For instance, HHcy achieved by cystathionine β -synthetase heterozygous knockout induced increased collagen deposition and elastin fragmentation, resulting in increased aortic wall thickness and increased aortic resistance in mice⁷⁶. Moreover, another model of HHcy induced by a high methionine diet in mice showed a similar increased arterial resistance⁷⁷. Synthetic SMCs also produce growth factors including connective tissue growth factor, which is up-regulated in atherosclerotic plaques. In cultured SMCs, Hcy induced the expression of connective tissue growth factor⁷⁸ (**Figure 2**), suggesting another mechanism by which Hcy contributes to medial remodeling and atherosclerosis.

The above results imply that Hcy induces vascular remodeling and vessel stiffening by provoking medial SMCs to synthesize and deposit excessive collagen in the vessel wall. Likewise, there is evidence suggesting that Hcy may also contribute to vascular remodeling by inducing the production of elastin degrading enzymes in the aortic media. In the aforementioned study by Ovechkin et al., increased activity of MMP-2 and -9 was detected in the aorta in association with elastin degradation in HHcy mice⁷⁶ (**Figure 2**). Moreover, elastin degradation was detected in the aorta of ApoE^{-/-} mice following induction of HHcy, and this was associated with increased expression and activity of MMP9⁵⁵.

A detrimental consequence of aortic medial remodeling is the formation of aneurysms, which can progressively expand and dissect, causing sudden death. Aneurysms of the abdominal aorta are one of the leading causes of death in aging individuals, and there are currently no proven drug strategies to reverse their progression. Interestingly, experimental evidence suggests that Hcy could contribute to the incidence and progression of abdominal aortic aneurysms by inducing the up-regulation of various inflammatory pathways^{8, 79, 80}. On the other hand, aneurysms of the ascending aorta are usually non-inflammatory⁸¹ and are rather caused by extracellular matrix degradation⁸², suggesting a different role for Hcy in ascending aortic aneurysm formation. We recently showed that Hcy is accumulated in the ascending aorta of aged rats in response to fetal programming, and that it is associated with marked elastin degradation. Furthermore, we

found evidence for homocysteinylated extracellular matrix proteins in the aortic media, including elastin and actin-binding proteins (Balint B, submitted; **Figure 2**). These results suggest a role for protein homocysteinylolation in ascending aortic remodeling, providing insight into another mechanism by which Hcy contributes to vascular disease.

3.3 Homocysteine and the Adventitia

The adventitia is the outmost layer of the blood vessel. Although vascular inflammation is classically thought to start in the endothelium, there is evidence suggesting that inflammation of the vascular wall can also be initiated in the adventitial layer and progress through the media towards the endothelium⁸³. As mentioned above, Hcy induces an inflammatory response in ECs. However, the role of Hcy on adventitial cells is less understood. One study of aortic aneurysm in ApoE^{-/-} mice found that HHcy exaggerated inflammatory cell recruitment in the adventitia and increased the activity of MMPs, particularly in the adventitial layer⁸ (**Figure 2**). Another study found that HHcy caused increased deposition of adventitial collagen (**Figure 2**) and enhanced adventitial hyperplasia in response to balloon injury in carotid arteries of the rat⁸⁴. These findings indicate a role for Hcy-induced adventitial pathology but further research is needed to better understand how Hcy aggravates the adventitia.

4. Implications for Hcy-targeted therapy of vascular disease

The notion that Hcy is associated with vascular disease has prompted the initiation of randomized trials to evaluate the impact of decreasing serum Hcy among patients with cardiovascular disease. However, despite reducing serum Hcy levels, interventions with B-vitamin supplements have failed to elicit any promising cardioprotective effects⁸⁵⁻⁸⁷. These disconcerting results have sparked controversy in the field, challenging the idea that Hcy has a causal role in vascular pathogenesis. In favor of the Hcy-mediated vascular disease hypothesis, it has been suggested that the Hcy-lowering effects of supplemental B-vitamins is offset by a toxic response to the vitamins themselves that further exacerbates vascular lesions⁸⁸. However, although B-vitamin supplementation successfully decreases serum Hcy concentrations, it is unknown whether it has the ability to decrease tissue-bound or intracellular Hcy. Current evidence suggests that Hcy elicits

its negative effects directly in the vessel wall by inducing modifications to vascular cell function, and by irreversibly incorporating into cellular and extracellular matrix proteins (**Figure 2**). These pathological changes induced by Hcy would likely be unaffected by serum Hcy-lowering treatments. Therefore, understanding the specific effects of Hcy on each layer of the arterial wall and on each specific component, including ECs, SMCs and extracellular matrix constituents will allow for the development of targeted prevention against Hcy-associated vascular disease in the appropriate period of life.

5. Conclusions

There is compelling evidence from studies in humans, animals and cell culture to suggest that Hcy plays a causative role in the formation and progression of vascular disease. Furthermore, it is clear that Hcy has a diverse set of pathological functions as it elicits toxic effects on all layers of the arterial wall (**Figure 2**). The impact of Hcy on the greater vasculature and the mechanisms by which it elicits toxic effects on all layers of the vessel wall remains an imperative and exciting field of research. Although Hcy-lowering vitamin B supplementations have failed to relieve vascular complications associated with Hcy, further research is needed to determine if reducing intracellular and tissue-bound Hcy in the appropriate period of life could subsequently alleviate vascular disease.

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Author Contributions:

BB and JLG wrote the manuscript. BB, JLG and RMGR designed and coordinated the article template and concept. All authors discussed and commented on the manuscript and participated in the preparation and revision of the manuscript.

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