

Vascular Dysfunction in Hyperhomocyst(e)inemia. Implications for Atherothrombotic Disease

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Elevated plasma homocyst(e)ine is currently accepted as a major, independent risk factor for atherosclerosis and venous thrombosis. Even moderate hyperhomocyst(e)inemia is prospectively associated with increased risk of mortality in patients with cardiovascular disease. However, the underlying mechanisms resulting in vascular damage are not clearly defined.

The endothelium exerts fundamental control on the vascular tone, coagulation and fibrinolysis. Injury to the endothelium followed by dysfunction is an early key event preceding manifestation of vessel pathology. Acute and chronic exposure of endothelium to homocyst(e)ine induces impairment of endothelial function associated with altered homeostasis and morphologic changes of the vessel wall.

Investigations of the role of homocyst(e)ine in the endothelium-dependent function in healthy subjects and cardiovascular patients have recently added important clinical insight with implications for the treatment of cardiovascular disease. Importantly, the damaging effects of hyperhomocyst(e)inemia on endothelial function are, at least in part, reversible in patients with established vascular disease, supporting further the hypothesis that homocyst(e)ine-lowering through vitamin supplementation may have vasoprotective effects.

Key words: Homocysteine; Endothelium; Coagulation; Free radicals.

Abbreviations: AT III, antithrombin III; BAEC, bovine aortic endothelial cells; CAD, coronary artery disease; CBS, cystathionine- β -synthase; CVD, cerebrovascular disease; EDRF, endothelium-derived relaxing factor; ET-1, endothelin-1; FMD, flow-mediated dilatation; GPx, glutathionine peroxidase; Hcy, homocysteine; H₂O₂, hydrogen peroxide; HUVEC, human umbilical vein endothelial cells; MS, methionine synthase; MDA, malondialdehyde; NO, nitric oxide; O₂⁻, superoxide anion; OH[·], hydroxyl radical; oMLT, oral methionine loading test; PAI-1, plasminogen activator inhibitor; PAOD, peripheral arterial occlusive disease; PGI₂, prostaglandin I₂; ROS, reactive oxygen species; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine; TAS, total antioxidant status; TBARS, thiobarbi-

turic acid reactive substances; TF, tissue factor; tHcy, total plasma homocysteine; TM, thrombomodulin; t-PA, tissue-type plasminogen activator; vWF, von Willebrand factor.

Introduction

Elevated plasma levels of total homocysteine (homocyst(e)ine, tHcy) are currently considered a major, independent risk factor for venous thrombosis (1) and cardiovascular disease (2). It was in the early sixties that a new disorder in methionine metabolism – then termed homocystinuria due to the large amounts of homocystine found in the patients' urine – was first suggested to cause mental and physical retardation (3). Documentation of other typical pathological findings in homocystinuria including morphologic vascular changes and thrombosis (4) was soon followed by identification of the underlying enzymatic defect, cystathionine- β -synthase (CBS) deficiency (5).

However, it was only in 1969 that among other metabolic changes occurring in various enzymatic defects of methionine metabolism, tHcy was concluded to be the most likely cause of the wide-spread vascular pathologies found in infant autopsies (6, 7). Until then, the role of homocysteine (Hcy) as a potential cause for atherosclerosis and thrombosis was limited to the very rare congenital errors of methionine metabolism associated with marked elevation of plasma tHcy concentrations up to >300 μ mol/l. First indication that much lower plasma tHcy concentrations may also contribute to the etiology of vascular pathology came from a paper published in 1976, showing significantly higher Hcy (actually cysteine-homocysteine mixed disulfides) levels in patients with angiographically confirmed coronary artery disease (CAD) compared to controls (8).

Comparable tHcy levels (approx. 15–30 μ mol/l, termed mild hyperhomocyst(e)inemia) can be found in approx. 20–30% of patients with coronary, cerebrovascular, and peripheral vascular disease (9) and it is for this reason that Hcy has stirred an enormous interest in the search for a potential new risk factor with implications for the greater part of the general population.

Since then, numerous case-control studies have confirmed the original observation and have established beyond doubt an association between elevated plasma tHcy levels and atherothrombotic disease (10). Several of these studies found a dose-response relationship, further indicating potential causality between the disease and plasma tHcy levels. Thus in meta-analyses, the odds ratio for a 5 μ mol/l increment in tHcy was calculated as 1.7 for CAD, and 1.5 for cerebrovascular dis-

ease (CVD) and peripheral arterial occlusive disease (PAOD) (11). It appeared that a total of 10% of the population's CAD risk is attributable to tHcy. It is noteworthy that the difference in plasma tHcy concentrations between patients and controls (age- and sex-matched) is usually only a mere 2–3 $\mu\text{mol/l}$ (12) and that the associated risk appears to be without a threshold (11).

It must be considered, however, that retrospective studies may suggest an association but lack the power to prove causality, and therefore prospective studies have generally more significance in determining a causal role. Prospective studies published to date have yielded heterogeneous outcomes, and generally the association appears to be somewhat weaker. However, a strong predictive value for elevated plasma tHcy concentrations, again in a dose-response fashion, was found for mortality from cardiovascular disease (13) and for all-cause mortality (14).

Currently, the causal role of tHcy in the etiology of atherothrombotic disease is not clearly established. However, important evidence for potential causality comes from animal and *in vitro* studies, and especially *in vivo* studies investigating the function of vascular endothelium, the major mediating site of pathology in atherosclerosis. Several important observations have been made recently, suggesting that even mild hyperhomocyst(e)inemia can severely impair vascular function and thus can significantly contribute to atherothrombotic disease in man.

Metabolism of Methionine/Homocyst(e)ine

Hcy is found exclusively as an intermediate product of methionine metabolism and is present in every cell of the human body. There is no genetic coding for this amino acid. Although methionine is an essential amino acid and is derived from dietary (animal) protein, it is very unlikely that dietary uptake alone has a major impact on plasma and tissue Hcy concentrations, as most cells have two metabolic pathways available to metabolise Hcy. In cells with both pathways functional, Hcy is distributed equally under normal circumstances (15). Plasma tHcy concentration is generally determined by genetic, nutritional, physiologic and environmental factors (16). The B-complex vitamins (folic acid, cobalamin, pyridoxal phosphate) are especially important in maintaining Hcy homeostasis, with folic acid being the most important determinant of plasma levels (2).

In an initial step, dietary methionine is converted to S-adenosyl-methionine (SAM) by the enzyme methionine adenosyltransferase (EC 2.5.1.6). SAM is the most important methyl group donor and is involved in at least 100 known methylation reactions that include the synthesis of numerous neurotransmitters, hormones and nucleic acids, with fundamental importance for proliferation, differentiation and function of cells (17). Lack of methylation will therefore have severe effects on gene expression, protein translation, chemotaxis and signal transduction (17).

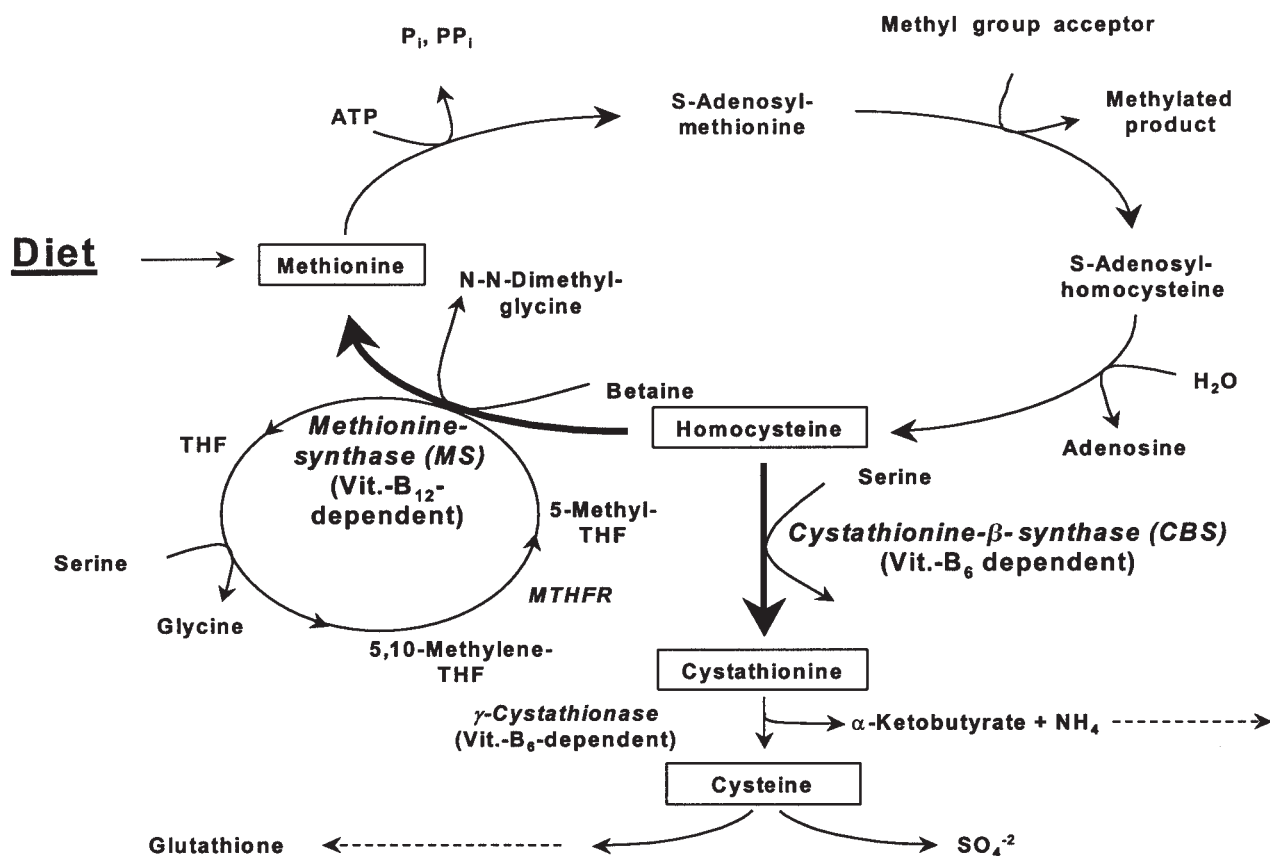


Fig. 1 Metabolism of methionine/homocysteine. MTHFR= methylenetetrahydrofolate reductase, ATP=adenosine 5' triphosphate, P_i =inorganic phosphate, PP_i =pyrophosphate, THF=tetrahydrofolate.

After donation of methyl groups, S-adenosyl-homocysteine (SAH) is formed, which is then hydrolyzed, in a reversible reaction, to Hcy and adenosine by the enzyme SAH-hydrolase (EC 3.3.1.1). It must be noted, that up this point, all reactions were vitamin-independent.

Hcy is potentially cytotoxic. Therefore, intracellular accumulation of Hcy necessitates either its conversion to non-cytotoxic metabolites or its export to the circulation (18). Thus, elevated plasma levels generally reflect a disturbance of Hcy homeostasis

Intracellularly, Hcy can be remethylated to form methionine completing the methionine cycle, or enter the irreversible transsulfuration pathway. Both remethylation and transsulfuration pathways are regulated by SAM, which acts as a positive allosteric effector for the transsulfuration pathway and as a negative allosteric effector for the remethylation pathway (19). With abundant methionine supply, concomitant elevated SAM will direct Hcy towards transsulfuration (simultaneously inhibiting remethylation), and *vice versa*.

The vitamin B₁₂-dependent enzyme methionine synthase (MS) (EC 2.1.1.13) converts Hcy to methionine, using 5-methyltetrahydrofolate as the methyl group donor (remethylation). Transfer of the methyl group to Hcy will yield tetrahydrofolate, the biologically active form of folic acid that is essentially involved in one-carbon metabolism and thus in the synthesis of purines and pyrimidines for the DNA synthesis. Thus, deficiencies of folic acid and vitamin B₁₂ may both impair remethylation, potentially leading to hyperhomocyst(e)inemia. However, Hcy is also important for the generation of active folic acid and thus one-carbon metabolism. This reaction is therefore fundamental to the organism, and all nucleated cells express MS.

Hcy may also undergo irreversible degradation *via* the transsulfuration pathway. This reaction is initiated by the vitamin B₆-dependent CBS (EC 4.2.1.22). Hcy condenses with serin, generating cystathionine, and is further converted to cysteine by the enzyme cystathionase (EC 4.4.1.1) yielding α -ketobutyrate and ammonia. The transsulfuration is completed here and cysteine may then undergo catabolism to inorganic sulfate, taurine, glutathione or pyruvate.

Terminology

Hcy itself is a thiol (sulfhydryl-)containing 4-carbon α -amino acid (20), but several related forms may be present in human plasma and tissues. Rapidly increasing research has made it important to distinguish between homocysteine forms to avoid confusion.

Of the total plasma concentration, about 98% exists in the oxidized form as disulfides. Oxidation occurs within minutes to hours after free Hcy is released into circulation (21). Approx. 85% is bound to protein (mainly to albumin with a single free cysteine residue at position 34); the remainder occurs in non-protein bound forms including mainly homocystine (homocysteine that had underwent auto-oxidation) or homocysteine-cysteine mixed disulfide (22). The disulfide link-

age represents the common feature of all oxidized forms of Hcy. Only approx. 2% occurs as the (free) thiol (23). Under pathological conditions, the relative distribution of the fractions may differ. For instance, the concentration of the free reduced form can account for up to 20% of total plasma Hcy in patients with homocystinuria (24). It was recently suggested, that this form is mainly responsible for effects on vascular endothelium, but is not distinguished when measuring total plasma Hcy (25). Homocysteine-thiolactone is a physiological, stable 5-membered ring condensation product of Hcy. It is produced by methionyl-tRNA aminoacyl synthetase (26), and will be hydrolyzed to Hcy by soluble and vessel-bound esterases (27), preventing accumulation in the circulation. However, elevation of intracellular homocysteine-thiolactone, especially in the presence of B-complex vitamin deficiency, can reach 20% with damaging effects on cell homeostasis (28).

When determined in plasma or serum, homocysteine is commonly termed homocyst(e)ine, because it includes a reduction step yielding the thiol. Most currently used laboratory methods include this reducing treatment. Therefore all forms, free and protein-bound, are measured and the moiety is referred to as the sum of reduced (-SH) and oxidized (-S-S-) forms of homocysteine, termed "homocyst(e)ine" (or total homocysteine: tHcy). We therefore frequently use this term in this paper, because for clinical and experimental purposes, most authors have used the determination of "homocyst(e)ine", and that is most likely to continue in the future.

Vascular Endothelium and Homocyst(e)ine in Coagulation and Fibrinolysis

Normal endothelium exerts fundamental control on coagulation and fibrinolysis to inhibit intravascular coagulation and yet close a vessel after trauma through generation of compounds important to the maintenance of vessel patency and blood fluidity. Impairment of vascular endothelial function may alter homeostasis, and induce vascular dysfunction, and eventually morphologic changes, of the vessel wall.

Vascular endothelial cells express thrombomodulin (TM), the molecular mooring site required by thrombin for activation of circulating anticoagulant protein C (29). Activation of protein C simultaneously promotes its anticoagulant activity through inactivation of factors Va and VIIIa (30) and also increases fibrinolytic activity by causing a decrease in the activity of plasminogen activator-inhibitor 1 (PAI-1) (31) and an increase in the activity of tissue-type plasminogen activator (t-PA) (32), leading to enhanced hydrolysis of (inactive) plasminogen to (functional) plasmin with consecutive breakup of fibrin.

Both plasminogen activators and plasminogen activator inhibitors are secreted by vascular endothelial cells and express receptors for t-PA that further localize activation of plasminogen to the endothelial cell sur-

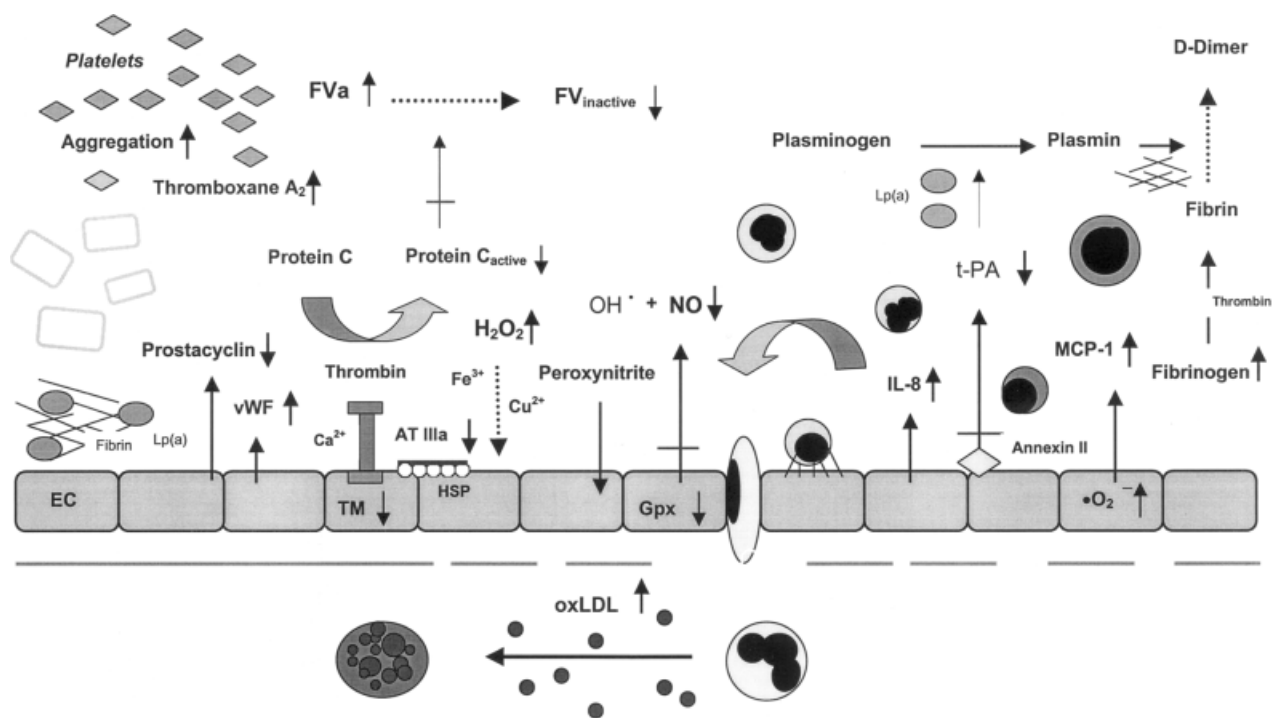


Fig. 2 Homocysteine-mediated effects on the endothelium. Arrows and bars indicate potential selected actions. Gpx = glutathione peroxidase, TM = thrombomodulin, vWF = von Willebrand factor, IL-8 = interleukin 8, Fe = iron, Cu = copper, HSP = heparan sulfate proteoglycan, AT IIIa = activated an-

tithrombin III, MCP-1 = monocyte chemoattractant protein 1, FV = factor V, Lp(a) = Lipoprotein (a), t-PA = tissue-type plasminogen activator, oxLDL = oxidized low-density lipoprotein, EC = endothelial cell.

face (33). Hcy inhibits t-PA receptor function as preincubation of human endothelial cells with Hcy results in a selective 65% reduction of the cellular binding sites for, and activity of t-PA, in a time- and dose-dependent fashion (no effect on plasminogen binding is observed) (34). This blockade of t-PA binding to endothelial cells could be mediated through selective blocking of annexin II by Hcy, a phospholipid-binding protein expressed on endothelial cells that serves as docking site for t-PA (35). Interestingly, Hcy does not inhibit the secretion of PAI-1 (36).

Protein C activation was markedly reduced in bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) when exposed to Hcy in a dose-dependent manner (37).

Exposure of cultured endothelial cells to Hcy leads to increased conversion of endogenous factor V to activated factor V (FVa) (38). Both findings may be explained by a decreased capacity of endothelial cells to activate protein C as a consequence of diminished activity and surface expression of TM. Indeed, Lentz *et al.* found increased TM mRNA and protein synthesis in HUVEC cells (36); however, TM was not expressed on the cell surface, suggesting interference of homocysteine with the secretory process. Furthermore, irreversible inactivation of protein C and TM, but not thrombin, was reported by the authors. This was supported by the finding that, despite a two- to three-fold increase in TM mRNA, Hcy inactivated the cofactor activity of TM (39). While these *in vitro* experiments used Hcy concentrations between 0.5 mM and 10 mM, a sig-

nificant reduction of aortic TM anticoagulant activity was demonstrated in a diet-induced hyperhomocysteinemic monkey model with moderate increase of plasma Hcy levels (40), although the role of Hcy in the observed changes could not be clarified.

Von Willebrand factor (vWF) is a multimeric glycoprotein synthesized and secreted by the endothelial cells that is active in the control of thrombosis. vWF promotes thrombosis through adhesion and shear-stress-induced aggregation of platelets on the exposed subendothelial connective tissue of injured vessels (41). Hcy (1.0 mM) inhibited endoplasmic reticulum-dependent processing and secretion of vWF in HUVEC cells (42). Incubation with 2 mmol/l Hcy resulted in complete loss of vWF multimers, consistent with impaired exit from the endoplasmic reticulum. It is therefore suggested, that Hcy does not necessarily prevent vWF assembly and dimer formation but may alter intracellular transport mechanisms and thus lead to retention of proteins. Endoplasmic reticulum retention may be caused by altering the intracellular redox potential through the Hcy's free thiol group.

Selective inhibition of intracellular transport of vWF and TM could predispose to thrombosis by altering the balance between procoagulant and anticoagulant proteins.

Heparan sulfate proteoglycans are synthesized and expressed on the surface of endothelial cells, where they are tightly bound to the cell surface and exhibit anticoagulant activity through binding and activating antithrombin III (AT III) (43). Activated AT III may then irre-

versibly bind to, and neutralize, thrombin and other serine proteases of the clotting cascade (factors XIIIa, XIa, IXa and Xa).

In vitro, Hcy concentrations as low as 0.1 mM inhibit AT III binding activity by suppressing the expression of anticoagulant heparan sulfate proteoglycans and thus altering its binding to cultured porcine aortic endothelial cells (44). The amount of AT III bound to the cell surface is reduced in a dose- and time-dependent fashion.

Tissue factor (TF) is another small transmembrane glycoprotein which initiates coagulation by binding to factor VIIa. This binary complex proteolytically activates factor IX and X, triggering the downstream coagulation pathway (45). Incubation of endothelial cells in culture with Hcy (0.1–0.6 mM) increases TF expression and activity in a time- and dose-dependent manner (46).

Hcy may thus affect several functions of the endothelium with the alteration of antithrombotic mechanisms, consequently favoring thrombus formation.

Homocysteine and Vascular Tone

Normal vascular endothelium keeps the vessel in a state of relaxation, mainly through basal synthesis and release of vasodilators. The most important vasodilators are endothelium-derived relaxing factor (EDRF; =nitric oxide, NO) and prostacyclin (or prostaglandin I₂, PGI₂). It was first shown in 1980, that intact endothelium was required for the acetylcholin-induced vasodilation, mediated through a vasodilator, then termed EDRF (47). This substance has since been identified as the soluble gas NO (48) that needs to be permanently synthesized and released for continuous vascular relaxation. NO is synthesized from L-arginine by nitric oxide oxidases (49), yielding citrulline. Furthermore, NO has antiaggregation properties towards platelets and inhibits aggregation of monocytes on the endothelium.

NO is supported in its platelet anti-aggregatory effect by PGI₂, another vasodilator synthesized by endothelial cells. PGI₂ is formed from arachidonic acid in a series of reactions initiated by the enzyme cyclooxygenase (50). These substances counteract the vasoconstrictive effects of *e.g.* angiotensin II or endothelin-1 (ET-1), and this causes long-lasting vasoconstriction (51). ET-1 production is inhibited by NO and PGI₂ (52).

Hcy was found to stimulate secretion of NO in cultured BAEC cells after brief exposure (15 min), forming the adduct S-nitroso-homocysteine, itself a potent vasodilator with anti-aggregatory effect on platelets and an inhibitor of H₂O₂ formation (53). Stimulated endothelial cells exposed to increasing concentrations of Hcy may produce more NO than control cells (54). This response may be seen as a physiologic defense mechanism of vascular endothelium against Hcy-mediated cytotoxicity. S-nitrosation of homocysteine may thus represent an endogenous cytoprotective, antithrombotic cellular regulatory mechanism. However, a marked impairment of NO response upon prolonged

exposure to Hcy (> 3 hours) was also observed (54), suggesting an inability to sustain S-nitrosation owing to an imbalance between the production of NO by progressively dysfunctional endothelial cells and the level of Hcy.

Homocyst(e)ine and Oxidative Stress

Oxidant stress plays a key role in endothelial dysfunction (55). The endothelial Hcy-mediated cytotoxicity (56) is, in part, attributable to the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radical (HO•), mediated through the SH groups (57) during the (auto-) oxidation of Hcy to homocystine or other mixed disulfides (58, 59), enhanced by the presence of transition metals such as copper (Cu²⁺) and iron (Fe³⁺) (60). Intracellularly, multiple mechanisms serve to attenuate the toxicity of Hcy-induced oxidative stress, including glutathione, glutathione peroxidase (GPx) and catalase (61). Additionally, S-nitrosation of Hcy effectively inhibits sulfhydryl-dependent H₂O₂ generation (53). It is therefore suggested that stimulation of endothelial cells to increase NO production might help prevent the generation of H₂O₂ and thereby serve to detoxify this atherothrombotic thiol.

However, chronic exposure to Hcy impairs the basal NO production and thus bioavailability of NO, independently of eNOS activity, by generating H₂O₂, and by decreasing intracellular GPx in unstimulated BAEC (60).

Through enhanced NO release, the endothelium can modify the toxicity of Hcy for a limited time. However, deficiency of bioactive NO, as GPx expression, decreases and oxidative by-products of tHcy begin to accumulate leading to endothelial dysfunction.

Thiols, such as Hcy, may further oxidize low-density lipoprotein (LDL) *via* reactions including ROS-mediated mechanisms (61).

The role of Hcy-mediated oxidative stress and lipid peroxidation in the etiology of atherosclerosis is supported by findings in the animal model. Feeding rabbits a methionine-rich diet for 6–9 months resulted in a significant increase in thiobarbituric acid-reactive substances (TBARS) in plasma and aorta, and antioxidant enzyme activity changes associated with typical atherosclerotic changes in the aorta (62). Hyperhomocyst(e)inemia, induced by intermittent exposure to nitrous oxide for 4 weeks, induced significantly higher levels of malondialdehyde (MDA, a sensitive marker of lipid peroxidation) in cardiac tissue, suggesting increased *in vivo* lipid peroxidation in hyperhomocysteinemia (63).

In a subset (100 males) of participants in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study, plasma tHcy levels were significantly associated with F₂-isoprostane levels (another sensitive marker of *in vivo* lipid peroxidation), increasing linearly across quintiles of tHcy (64). Following an oral methionine load, subjects with a pathological loading test reached significantly higher values of TBARS after 4

and 6 hours as compared to subjects with normal loading test results (65).

Homocyst(e)ine in Clinical Studies of Endothelial Function

Endothelial injury leads to vascular dysfunction with an impairment of endothelial vasoregulatory mechanisms. Endothelial dysfunction is considered the initial step in atherosclerosis and is thought to precede overt manifestations of vascular disease by many years (66).

Measuring various markers of endothelium-derived regulatory proteins (67) can be useful in demonstrating beneficial effects of specific treatments on the endothelium (68). Clinical and functional estimates of endothelial dysfunction come from measuring endothelium-dependent vasodilatation invasively (for instance direct infusion of acetylcholine) (69) or non-invasively (flow-mediated vasodilatation, FMD), using arterial diameter response to hyperemic flow detected with high-resolution ultrasound (vessel tracking system) (70). Additional information on the reactivity of peripheral resistance vessels can be obtained through venous occlusion plethysmography (71). Commonly, the brachial artery is used to investigate vascular endothelial function. FMD in the brachial artery correlates closely with functional (72) and morphologic (73) changes of the coronary arteries, and is thus considered a useful investigation in healthy subjects and patients with known vascular pathologies.

Investigating healthy subjects, fasting tHcy repeatedly emerged as the strongest predictor for impaired FMD, independent of age, sex, body mass index, blood pressure, vitamin status and cholesterol (74, 75). The response to endothelium-independent, nitroglycerin-induced dilatation remained unchanged.

Acute hyperhomocyst(e)inemia using an oral methionine loading test (oMLT) induces substantial linear impairment of FMD in healthy subjects (76) and leads to a significant increase in vWF in patients with vascular disease, reflecting endothelial dysfunction (77). Even in healthy first-degree relatives of patients with mild hyperhomocyst(e)inemia, the increase in tHcy after a methionine load is an independent predictor of endothelial dysfunction (78). In these studies, only endothelium-dependent FMD (which is largely mediated by nitric oxide) was associated with hyperhomocyst(e)inemia, therefore suggesting impaired endothelial NO activity as the underlying mechanism. However, intake of 2 g vitamin C (79) or pretreatment with oral vitamin C (1 g/day/for 1 week) prevented oMLT-induced endothelial dysfunction in healthy subjects, indicating that the adverse effects of Hcy on endothelium are, in part, mediated through oxidative stress (80). oMLT-induced impairment of FMD was also reported by Chao *et al.* (81), however, they were unable to demonstrate changes in oxidative status (measured as P-selectin levels and phosphatidylcholine hydroperoxide, a major product of lipid peroxidation). They suggested an age-related effect thus indirectly supporting the hypothesis of NO impairment.

Importantly, Hcy-induced endothelial dysfunction does not even require intake of a full (standardized) oMLT (100 mg/kg), but is measured after small physiological increments in plasma tHcy (82), corroborating the potential implication of Hcy as risk factor for vascular disease.

Lowering Plasma Homocyst(e)ine Concentrations and Endothelial Function

Folic acid intake in doses between 0.3 and 15 mg has been reported to effectively lower tHcy levels (83) but only very few clinical studies investigated the effect of vitamin supplementation and lowering tHcy on vascular endothelial function.

Oral folic acid supplementation improves the arterial endothelium-dependent vascular function (FMD) of the brachial artery in healthy subjects with mild hyperhomocyst(e)inemia (84), with no effect on endothelium-independent responses (85). It must be noted that folic acid has been shown to possess some independent antioxidative capacity *in vitro* (86) and the beneficial effect on the endothelial function could thus have been a side effect of the folate intake rather than tHcy lowering.

Folic acid administration is capable of preventing the impairment of the endothelial function and the increase in oxidative stress (increase of MDA excretion in the urine) induced by an acute oral fat load (87), further suggesting a protective effect on (lipid-induced) urinary-damage end products. In a placebo-controlled study including CAD-patients, folic acid supplementation significantly improved endothelial dysfunction (88) suggesting that patients with established vascular pathology may also benefit from folate intake and/or tHcy lowering.

Our own group investigated the effect of tHcy lowering through vitamin supplementation on resistance vessel reactivity in patients with angiographically documented CAD (unpublished). Patients served as their own controls in a blinded on-off therapy trial. Plasma folate increased by 347% in all individuals after 6 weeks (5 mg folic acid/day) lowering tHcy by 21.1%. However, significant improvement of resistance vessel reactivity was limited to subjects in whom tHcy was lowered ≥ 2 $\mu\text{mol/l}$, whereas total antioxidant status (TAS) remained unchanged. TAS in non-responders (tHcy lowering < 2 $\mu\text{mol/l}$) increased but endothelial function remained unchanged. We conclude that endothelial dysfunction in patients with established vascular disease is, at least in part, reversible by oral treatment with folic acid. However, the effect is subject to lowering tHcy by 2 $\mu\text{mol/l}$ or more, and is not a consequence of folate increase itself.

Conclusions

Injury to the endothelium followed by dysfunction is a key event preceding manifestation of vessel pathology. Fundamental insight into the pathomechanisms asso-

ciated with Hcy is provided by *in vitro* and *in vivo* studies mentioned above

The damaging effects of Hcy on vascular endothelium are biologically plausible and highlight the potential role of elevated tHcy concentrations in the etiology of atherosclerosis and venous thrombosis.

It is possible that hyperhomocyst(e)inemia simply reflects an association with another underlying cause and tHcy must be seen as a sensitive marker for *e.g.* intracellular vitamin deficiency ultimately leading to vascular disease. This is supported by the overall predictive value of prospective studies.

However, clinical studies clearly showing impaired vascular endothelial function associated with hyperhomocyst(e)inemia, and its reversibility by Hcy-lowering in healthy subjects and patients with established vascular disease, argue strongly in favor of causality.

Atherothrombosis is multifactorial and there is reason to believe that other coexisting risk factors are required for, and considerably augment the Hcy-induced damage.

Apart from clarifying the potential of Hcy-lowering cardiovascular prevention in prospective trials, it is essential to identify subjects at risk and those who may benefit most from lowering plasma homocyst(e)ine concentrations.

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