

Review

Interactions of Homocysteine, Nitric Oxide, Folate and Radicals in the Progressively Damaged Endothelium

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The endothelium exerts fundamental control over vascular tone, and injury to the endothelium followed by dysfunction is an early key event preceding manifestation of vessel pathology. Both elevated plasma homocysteine and low folate status have been identified as major and independent risk factors for atherosclerosis and have stirred an enormous and still increasing interest. The damaging effects of hyperhomocysteinemia on endothelial function are, at least in part, reversible through folate supplementation. Because of the inverse relationship between plasma folate and homocysteine levels, however, it is difficult to discriminate between their respective effects. Endothelial dysfunction refers mainly to reduced bioavailability of nitric oxide (NO), which is involved in homocysteine-mediated vascular damage. Accumulating evidence further suggests that radical oxygen species are fundamentally involved in hyperhomocysteinemia. NO production is determined by cofactors such as tetrahydrobiopterin, which is oxidized and depleted in conditions of oxidant stress by peroxynitrite. Deficiency of tetrahydrofolate contributes to uncoupling, turning the NO synthase into a superoxide radical-producing enzyme. It appears that progression of vascular disease is likely to determine the multiple interactions between homocysteine, NO, oxygen radicals and folate. Folate has only recently been found to exert direct anti-oxidative effects and contribute to restoration of impaired NO metabolism. Understanding of the complex interactions between homocysteine, radicals, NO and folate offers promising perspectives in the individual treatment of vascular disease. Thus, preventive and therapeutic strategies may require a more distinct approach and better discrimination of target groups for greatest possible efficacy. Clin Chem Lab Med 2003; 41(11):1444–1454

Key words: Homocysteine; Endothelial dysfunction; Nitric oxide; Radicals; Folate.

Abbreviations: ADMA, asymmetrical dimethylarginine; BH₄, tetrahydrobiopterin; DDAH, dimethylarginine di-

methylaminohydrolase; EC, endothelial cell; ED, endothelial dysfunction; eNOS, endothelial NO synthase; GPx, glutathione peroxidase; GTP, guanosine triphosphate; Hcy, homocysteine; H₂O₂, hydrogen peroxide; HO•, hydroxyl radical; IL, interleukin; LDL, low density lipoprotein; L-NMMA, N^G-monomethyl-L-arginine; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; O₂⁻, superoxide anion; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SAM, S-adenosyl-methionine; SOD, superoxide dismutase; SREBP, sterol regulatory element binding protein; TNF-α, tumor necrosis factor-α; TXA₂, thromboxane A₂.

Introduction

Mild elevations of homocysteine (Hcy) in plasma (~12–30 μmol/l) are found in ~20–30% of patients with coronary, cerebrovascular and peripheral vascular disease (1–5). Simple supplementation with B-vitamins effectively lowers Hcy, and it is for this reason that Hcy has stirred an enormous and still increasing interest in the search for a new potential and modifiable risk factor with implications for the greater part of the general population. Folate deficiency has also been identified as an independent risk factor for atherosclerosis (6–11). With growing insight into molecular mechanisms it has become increasingly clear that moderate folate deficiencies are implicated in the process of chronic diseases including atherothrombotic vessel pathologies. Because of the inverse relationship between plasma folate and Hcy levels, however, it is difficult to discriminate between their respective effects, although numerous intrinsic mechanisms have been reviewed to explain cellular dysfunction through either low folate or hyperhomocysteinemia (4, 10).

Hcy induces a large number of cellular dysfunctions that all fit with biological plausibility into the generally accepted response-to-injury hypothesis of the etiology of atherothrombotic disease (12). Within this hypothesis, obligatory injury to the vessel leads to functional impairment of endothelial cells (EC), termed endothelial dysfunction (ED). ED is considered to be the initial step in the progression of atherosclerosis and is thought to precede the overt manifestation of vascular disease by many years (12). ED may principally relate to all EC dysfunctions but is more commonly used to describe the impairment of vasodilator response (relaxation) of the endothelium to mechanical or pharma-

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cological stimuli. Thus ED refers mainly to reduced bioavailability of nitric oxide (NO), resulting in impaired endothelium-dependent vasodilatation (13, 14). This situation is associated with profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome (15). Consequently, ED has been increasingly recognized as a surrogate end point for cardiovascular risk (16). Commonly, the brachial artery is used to investigate vascular endothelial function. Flow-mediated vasodilatation (FMD) in the brachial artery correlates closely with functional (17) and morphological (18) changes in the coronary arteries, and is thus considered a useful investigation in healthy subjects and patients with known vascular pathologies. Much of current knowledge of Hcy-mediated vascular injury is derived from studies on ED.

Accumulating evidence suggests that oxidant stress alters many functions of the endothelium, including modulation of vascular tone, thus inducing ED (19). Indeed, radical oxygen species (ROS), and hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) in particular, play a fundamental role in hyperhomocysteinemia (20). Hcy impairs endothelium-dependent vasodilatation (21), even in physiological increments (22). Previous studies have suggested that hyperhomocysteinemia induces ED by decreasing bioavailability of NO (23, 24), an effect that can be reversed by administration of antioxidants and O_2^- scavengers in particular, suggesting a key function for ROS (3, 23, 25–28).

Folic acid supplementation was found to reverse Hcy-mediated impairment of NO metabolism (29), with improvement of ED in atherosclerotic (30, 31) and healthy (hyperhomocysteinemic) subjects (32, 33). In patients with known vascular disease, the beneficial effect of folic acid on endothelial function has been attributed to reduction of intracellular O_2^- (34, 35), independent of changes in plasma Hcy levels (34–36). Others, however, suggested that the beneficial effects on ED are mediated by reduced concentrations of free moieties of Hcy in plasma (37) or exclusively through the decrease in Hcy, largely independent of folate (30, 34, 37). However, this was questioned by findings of improvement in ED after acute administration of high-dose folic acid, an effect presumably independent of Hcy-lowering (35, 36, 38).

It appears that progression of vascular disease is likely to determine a much more complex set of interactions, as is illustrated by the seemingly conflicting outcomes of investigations. Better understanding of the molecular mechanisms, however, would allow more precise targeting of high-risk groups.

This paper aims to review the current knowledge on the molecular interplay of NO, Hcy, folates and ROS in health and disease. Better insight may help to explain heterogeneous findings from clinical studies on the human endothelium investigating the therapeutic potential of folate supplementation and/or Hcy-lowering.

Homocysteine Metabolism and Its Particularity in Endothelium

Hcy is a thiol-containing amino acid that is found exclusively as an intermediate product metabolized from methionine and derived from dietary protein. Metabolism takes place in every cell of the human body, and plasma concentrations of Hcy are generally determined by genetic, nutritional, physiological and environmental factors (5). In an initial step, dietary methionine is first converted to S-adenosyl-methionine (SAM) by the enzyme methionine adenosyltransferase (EC 2.5.1.6.). SAM is the most important methyl group donor with fundamental importance for cell proliferation, differentiation and function (39). Lack of methylation will therefore have severe effects on gene expression, protein translation, chemotaxis and signal transduction (39).

Most cells have two metabolic pathways available to metabolize Hcy; that is, vitamin B₆-dependent trans-sulfuration to form cysteine irreversibly, and resynthesis to methionine in a folate- and vitamin B₁₂-dependent remethylation. In cells with both pathways functionally available, Hcy is distributed equally under normal circumstances (40). The vitamin B₁₂-dependent enzyme methionine synthase (MS) (EC 2.1.1.13) converts Hcy to methionine, using 5-methyltetrahydrofolate (5-MTHF) as the methyl group donor. 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 DNA synthesis. Deficiencies in folic acid and vitamin B₁₂ may both individually impair remethylation. Intracellular accumulation of Hcy necessitates either the conversion to non-cytotoxic metabolites or export of Hcy to the circulation, potentially leading to hyperhomocysteinemia (41). Elevated plasma levels thus generally reflect a disturbance of Hcy homeostasis.

Most importantly for the vascular endothelium, the enzyme responsible for the trans-sulfuration pathway has not yet been found to be present in EC; thus, catabolism of potentially cytotoxic Hcy in vascular cells is limited to the remethylation pathway catalyzed by MS, and thus depends entirely on the sufficient distribution of folate and vitamin B₁₂. Deficiency of these vitamins in conjunction with the restricted metabolic capacity for Hcy may therefore exert particularly deleterious effects on the cardiovascular system,

The Healthy Endothelium. Vascular Tone and Detoxification of Homocysteine

The normal vascular endothelium keeps the vessel in a state of relaxation (vascular tone), mainly through permanent synthesis and continuous release of the vasodilatory substance, first named endothelium-derived relaxing factor (EDRF) and subsequently identified as the soluble gas nitric oxide (NO) (42). NO is basally synthesized from 5-electron oxidation of L-arginine by the

catalytic action of calcium-dependent nitric oxide oxidases (NOS) at concentrations of about 2 to 20 nmol/l, yielding citrulline (43). Three NOS enzymes (EC 1.14.13.39) have been characterized, of which type III is restricted to endothelial cells and consequently called endothelial NO synthase (eNOS). The complex process of NOS activity involves the transfer of electrons from NAD(P)H and the reduction of molecular oxygen (44). The reaction consumes 1.5 mol of NAD(P)H, and 2 mol of oxygen per mol of citrulline formed. NOS reduces molecular oxygen to O_2^- at the flavin-binding sites in the absence of sufficient L-arginine (45). NOS requires calmodulin and the pteridin tetrahydrobiopterin (BH_4) as cofactors. The enzyme functions as a dimer consisting of two identical monomers, which can be divided into two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain (46). The former contains binding sites for one molecule each of NAD(P)H, and the flavins flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), whereas the latter binds heme and BH_4 , as well as the substrate L-arginine. The reductase and the oxygenase domains of NOS are distinct catalytic units, which together provide the complete machinery required for NO production. BH_4 is an increasingly recognized key feature of NOS, and is synthesized from guanosine triphosphate (GTP) in at least four enzymatic steps. BH_4 increases the affinity of the enzyme for L-arginine and shares structural similarities with the active form of folic acid, 5-MTHF (47). BH_4 stabilizes NOS dimers once formed, and the precise location at the domain-domain interface suggests a possible direct role in electron transfer (48–50). Despite these clues the full role of BH_4 in NOS catalysis remains to be elucidated. NO is highly reactive, unstable and soluble in lipids, and thus readily diffuses across cell membranes. NO undergoes rapid conversion and has a half-life in the range of only 5 to 20 seconds in blood (51–53) and can become inactivated by its interaction with superoxide radical (O_2^-), a fundamental reaction involved in decrease of NO-bioavailability. NO diffuses to the subjacent vascular smooth muscle, where it regulates cell proliferation and remodelling (14, 54), and relaxation of smooth muscle and vascular tone through increase of cyclic guanosine monophosphate (cGMP) (13). The endothelially derived NO also diffuses to the luminal surface of the endothelium, where it exerts a number of important physiological effects, including scavenging of O_2 (55), inhibition of platelet adherence and aggregation (56), regulation of basal myocardial function (57), modulation of endothelial layer permeability (58) and attenuation of leukocyte-endothelial interaction *via* expression of adhesion molecules such as P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (14, 59, 60). Down-regulation by NO involves inhibition of protein kinase C (PKC) activation (61) and of nuclear factor- κ B (NF- κ B). Another potentially vasoprotective function of NO in the healthy endothelium is detoxification of Hcy through reaction to S-nitroso-Hcy (62). NO can be inactivated by O_2^- and stabilized by superoxide dismutase (SOD).

SOD is a secretory glycoprotein with an affinity for heparin-like substances. Direct reaction of NO with a thiol can also result in generation of O_2^- ; however, the production rate under physiological conditions is low and O_2^- will be readily scavenged, if not by thiols themselves, then by ascorbate and SOD (63). Under physiological conditions, endogenous antioxidant defences minimize this interaction and maintain what seems to be a tenuous balance between O_2^- and NO. Furthermore, under healthy conditions NO detoxifies Hcy through reaction-forming S-nitroso-Hcy, itself acting as a vasodilator, preventing platelet aggregation and providing anti-proliferative effects (64).

Endogenous inhibitors of NOS that have recently received much attention are N^G -monomethyl-L-arginine (L-NMMA) and asymmetrical dimethylarginine (ADMA) (65). An elevated level of ADMA is a risk factor for ED. L-NMMA competes with L-arginine as a substrate, but itself cannot be oxidized to NO. ADMA is synthesized by methylation of the side-chain guanidino nitrogen atoms of L-arginine residues within proteins, due to the action of methyltransferases that utilize SAM as the methyl group donor (66). The pathways for ADMA clearance are normally through renal excretion and through hydrolyzation by dimethylarginine dimethylaminohydrolase (DDAH).

The Diseased Endothelium. Reduced Bioavailability and NO due to Oxidative Inactivation and Uncoupling of NOS

Aging and diseased endothelium is especially prone to cellular dysfunctions. Activated transcription factor NF- κ B has been identified in human atherosclerotic plaques but is absent, or nearly so, in vessels devoid of atherosclerosis. ED is a hallmark event in the diseased vessel, due to decreased NO bioavailability as a potential consequence of inactivation through increased oxidative stress and/or uncoupling of NOS. Furthermore, oxidative-modified low density lipoprotein (LDL) was demonstrated to impair DDAH activity and increase the concentration of the endogenous NO inhibitor ADMA (67). O_2^- is increasingly generated not only by activated neutrophils, but also by the vascular endothelium *via* xanthine oxidase, cyclo-oxygenase, NAD(P)H oxidase activity, the mitochondrial respiratory chain (68, 69) and uncoupled endothelial NOS (70, 71). NOS is capable of producing not only NO, but also O_2^- , H_2O_2 and peroxynitrite. This unusual property is a consequence of the dimeric nature of the enzyme, in which the two subunits are able to function independently (50). Saturating L-arginine concentrations and the presence of sub-saturating levels of BH_4 lead to the simultaneous production of NO and O_2^- by the two subunits, respectively. The actual outcome will depend on the relative rates of the two reactions, and BH_4 takes a central role in this switch (72). Due to the large difference in binding affinity between BH_4 -binding sites, only at very high BH_4 concentrations will NOS function purely as an NO-producing synthase (73). The synthesis of BH_4 , a

cofactor critical for the production of NO by NOS, is increased by inflammatory stimuli, owing to an increase in the expression of the rate-limiting enzyme in BH₄ synthesis, GTP cyclo-hydrolase I (EC 3.5.4.16) (74). BH₄ augments NO formation, whereas its deficiency decreases NO bioactivity and increases O₂⁻ generation by NOS. Depending on substrate and cofactor availability, NOS can then primarily catalyze O₂⁻ production instead of NO, a harmful condition termed "uncoupling". This reaction is substantially accelerated under inflammatory-like conditions when both substrates are present in high concentrations. Under basal conditions, NO undergoes a rapid bi-radical reaction with O₂⁻ in direct equimolar concentrations to form the potent oxidant peroxynitrite (ONOO⁻). The rate of this reaction is determined by the ratio of NO and O₂⁻ and the activity of SOD in the cellular environment. The presence of O₂⁻ is counteracted by the antioxidant enzyme SOD, essential to catalyze its dismutation, and has been shown to protect cells from oxygen-free radicals (75). Evidence indicates that SOD is an important mediator in modulating vascular tone and inhibiting atherosclerosis. Thus, decreased secretion of SOD could weaken the defence against oxidative stress in the vascular wall. Peroxynitrite is formed from the reaction of NO and O₂⁻ at the rapid rate of $5-6.7 \times 10^9 \text{ mol/l}^{-1}\text{s}^{-1}$ (76). The reaction is fast enough to out-compete endogenous SOD, which neutralizes O₂⁻ at a rate of only $2 \times 10^9 \text{ mol/l}^{-1}\text{s}^{-1}$ (77). Thus, the formation kinetics favor peroxynitrate formation, especially in diseased vessels. Importantly, a considerable proportion of BH₄ undergoes oxidation under conditions associated with heightened oxidative stress such as peroxynitrite, contributing to BH₄ depletion (78). Indeed, recent evidence indicates that oxidation of BH₄ by peroxynitrite substantially contributes to uncoupling of NOS *in vivo* (79). Thus, in the presence of low concentrations or absence of L-arginine or BH₄, NOS catalyzes the uncoupled reaction of oxygen, leading to the production of O₂⁻ and H₂O₂ (72).

Although ROS, and O₂⁻ in particular, can themselves trigger a variety of signal transduction processes leading to pathogenic cellular conditions (80), the detrimental effects of O₂⁻ on endothelial function and progression of atherosclerosis are likely to be mediated by the scavenging of NO. Support comes from investigations of intra-arterial infusion of BH₄ that have demonstrated improvement of endothelium (NO)-dependent vasodilatation in chronic smokers, suggesting that depletion of BH₄ may have a fundamental impact on the transformation of NOS into an O₂⁻-generating enzyme in humans (81). O₂⁻ production and formation of peroxynitrite are fundamentally involved in the development of nitrate tolerance following prolonged exposure to organic nitrates such as nitroglycerine (82).

The formation of peroxynitrite from NO and O₂⁻ has been implicated in the pathology of a large number of conditions involving oxidative stress such as atherosclerosis and myocardial dysfunction (83). Peroxynitrite is capable of inducing cellular injury by lipid peroxidation, DNA fragmentation, damage to proteins and lipids, depletion of important plasma antioxidants such

as glutathione and cysteine and nitration of proteins leading to cellular dysfunction (76, 83). The effect of augmented oxidation and deficiency of BH₄ by peroxynitrite causes uncoupling of NOS, leading to serve as additional sources of O₂⁻ production (72).

Hyperhomocysteinemia Enhances Endothelium Dysfunction

Homocysteine has been shown to activate EC through up-regulation of components of the inflammatory cascade, including activation of transcription factors such as NF- κ B and PKC (84–86). NF- κ B activates a variety of target genes relevant to the pathophysiology of the vessel wall, including cytokines, chemokines and leukocyte adhesion molecules, as well as genes that regulate cell proliferation and mediate cell survival. Hcy most likely activates NF- κ B through creation of oxidative stress by altering the redox thiol status of the cell (87). Other transcription factors have also been found to be stimulated or altered by Hcy; these include sterol regulatory element binding protein (SREBP) (88) and peroxisome proliferator-activated receptors (PPAR) (85, 89). Hcy-mediated activation of SREBP up-regulates expression of HMG-CoA reductase with increased biosynthesis and cellular uptake of cholesterol, the depletion of BH₄ being a secondary effect (88). Statins inhibit HMG-CoA reductase and augment gene expression with up-regulation of GTP cyclo-hydrolase I, thereby increasing BH₄ levels in EC (90). PPARs directly modulate vessel wall functions. They were shown to inhibit the activation of inflammatory response genes, such as interleukin (IL) IL-2, IL-6, IL-8, tumor necrosis factor- α (TNF- α) and metalloproteinases (MMPs) by negatively interfering with NF- κ B and other signalling pathways in cells of the vascular wall. Thus, Hcy may enhance vascular constrictive remodelling by inactivating PPAR- α and - γ in EC and vascular smooth muscle cell (VSMC) (89). As a consequence, there is increased production of pro-inflammatory cytokines, and expression of adhesion molecules and chemotactic factors in hyperhomocysteinemia (71) (Figure 1).

Hcy was found to stimulate secretion of NO in cultured EC after brief exposure (15 min), forming the adduct S-nitroso-Hcy, itself a potent vasodilator with an anti-aggregatory effect on platelets and an inhibitor of H₂O₂ formation (64). Thus, through enhanced NO release, the endothelium can modify the toxicity of Hcy for a limited time and this response may be seen as a physiological defence mechanism of vascular endothelium against Hcy-mediated cytotoxicity. However, chronic exposure of Hcy impairs the basal NO production and thus bioavailability of NO, by generating ROS and also by decreasing intracellular glutathione peroxidase (GPx) (64, 91). The adverse vascular properties of Hcy may, in part, relate to a progressive imbalance between the concentrations of Hcy and the production of NO in progressively dysfunctional EC as expected in progressively damaged vessels. Thus, Hcy potentially contributes to the formation of O₂⁻ and peroxynitrite,

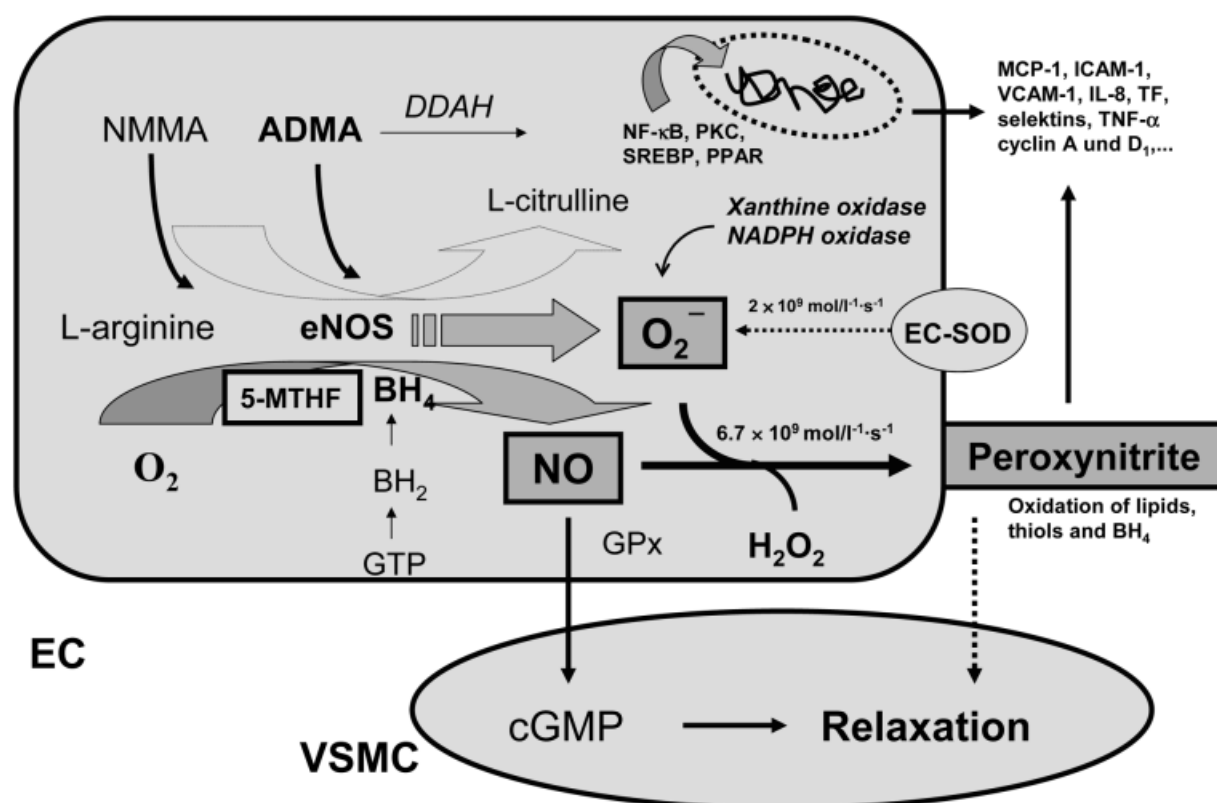


Figure 1 NO, oxygen radicals and the dysfunctional endothelial cell. ADMA, asymmetrical dimethylarginine; BH₂, dihydropterin; BH₄, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; DDAH, dimethylarginine dimethylaminohydrolase; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; GPx, glutathione peroxidase; GTP, guanosine triphosphate; H₂O₂, hydrogen peroxide; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin-8; L-NMMA, N^G-mono-

methyl-L-arginine; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; 5-MTHF, 5-methyltetrahydrofolate; O₂⁻, superoxide anion; PKC, protein kinase C; PPAR, peroxisome proliferator activated receptor; SOD, superoxide dismutase; SREBP, sterol regulatory element binding protein; TF, tissue factor; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cell.

oxidation of BH₄ and ultimately NOS uncoupling (26, 92). Hcy increases leukocyte rolling, adherence and transmigration, thus provoking leukocyte-endothelium interaction by suppression of NO (93).

ADMA, the endogenous inhibitor of NOS, is elevated in atherosclerosis and mediates ED during (acute) hyperhomocysteinemia (94). Methionine loading increases SAM and thus substrate availability for methyltransferases, thereby facilitating the synthesis of ADMA. Another explanation for elevated ADMA concentrations in hyperhomocysteinemia is decreased activity of DDAH, the enzyme required for metabolism of ADMA to citrulline and dimethylamine. In fact, inhibition of DDAH by Hcy has been demonstrated recently (95).

The activity and expression of NAD(P)H oxidases are regulated by pro-inflammatory cytokines such as TNF-α (96). Hcy stimulates gene expression of a large number of pro-inflammatory cytokines including TNF-α, which in turn enhances up-regulation of NAD(P)H oxidase and NOS, augmenting ROS (O₂⁻) production (71). It was further demonstrated that clinically relevant concentrations of Hcy (10 μmol/l) significantly alter human monocyte function by up-regulating MCP-1 and IL-8 expression and secretion *via* enhanced formation of in-

tracellular ROS originated from an NAD(P)H oxidase source *via* calmodulin or PKC signalling pathways (86) Hcy-induced ROS subsequently activate mitogen-activated protein kinase (p38 and extracellular signal-regulated kinase 1/2) and NF-κB in a PPAR-γ activator-sensitive manner (86). In addition to NO inactivation, synthesis of the vasoconstrictor prostaglandin thromboxane A₂ (TXA₂) is promoted in hyperhomocysteinemia, when xanthine oxidase-derived O₂⁻ scavenges NO-forming peroxynitrite, promoting release of TXA₂ and resulting in vasoconstriction (69). This was demonstrated in changes in diameter of isolated arterioles from methionine diet-induced rats measured by video microscopy. This observation was confirmed by *in vitro* studies using microvascular EC cultures showing inactivation of NO by O₂⁻ and generation of peroxynitrite induced by high concentrations of Hcy (97). Collectively it seems that in hyperhomocysteinemia, O₂⁻ eliminates NO by forming peroxynitrite, which then promotes formation of TXA₂.

The endothelial Hcy-mediated cytotoxicity is, in part, attributable to the generation of ROS, such as H₂O₂, O₂⁻ and hydroxyl radical (HO•) by way of the SH group during the (auto-) oxidation of Hcy to homocyst(e)line or other mixed disulfides (91, 98), enhanced by the pres-

ence of transition metals such as copper (Cu^{2+}) and iron (Fe^{3+}) (99). Hcy may oxidize LDL *via* reactions including ROS-mediated mechanisms (100), itself a very strong stimulus for ED. Hcy further increases oxidative stress by inhibiting expression of function of key antioxidant enzymes such as GPx (91) and SOD. Hcy induces endoplasmic reticulum stress in vascular EC and reduces the secretion and expression of extracellular SOD (101). Furthermore, Hcy decreases the binding of SOD to vascular endothelial surfaces by degradation of endothelial heparan sulfate proteoglycan, which results in the loss of ability to protect endothelial cell surfaces from oxidative stress (102).

Potential Mechanisms Explaining a Beneficial Effect of Folic Acid in Dysfunctional Endothelium

Lowering homocysteine concentrations

Hcy is recognized as an independent risk factor for atherosclerosis in the coronary, cerebral and peripheral vasculature. Nearly all atherogenic effects of Hcy have been demonstrated in a dose-response relationship. Importantly, Hcy-mediated ED is induced by small physiological increments in plasma tHcy (27), corroborating the potential implication of Hcy as a risk factor for vascular disease (4). Thus, by lowering Hcy levels through folate administration, all of the described Hcy-mediated effects as outlined above may potentially be reversed or attenuated (1, 4, 5, 10, 20, 103).

Effect on NOS uncoupling

NOS activity of intact cells is largely dependent on intracellular concentrations of BH_4 (104). Under certain pathological conditions, NOS can switch from mainly NO synthesis to production of O_2^- (uncoupling) (105). However, only fully reduced tetrahydroanalogues are active and support NO generation, whereas the dihydrobiopterins are inhibitory. As 5-MTHF stimulates the reduction of quinonoid dihydropterin (q- BH_2) to BH_4 by dihydropterin reductase (EC 1.6.99.7), folate acts indirectly on restoration of NOS activity through increase of cofactor BH_4 . Furthermore, BH_4 shares structural similarities with 5-MTHF, and both agents have been shown to improve endothelium-dependent vasodilatation (47). Supplementation with BH_4 or sepiapterin, an oxidized BH_4 analogue that generates BH_4 on two sequential enzymatic reductions by sepiapterin reductase and dihydrofolate reductase, has also been demonstrated to improve ED, notably by stimulating NOS activity (78). The polypeptide located in the N-terminal pteridine-binding domain of NOS shares structural similarities with the folate binding site of dihydrofolate reductase (106–108). In fact, 5-MTHF appears capable of fitting into the active site and can interact in an almost identical manner as the natural cofactor BH_4 . It was further shown that 5-MTHF binds to this active pterin site of NOS and mimics the orientation (stereospecificity) of BH_4 . Apparently 5-MTHF interacts with NOS in an analogous fashion, yet independent of BH_4 ,

to improve ED. Alternatively, 5-MTHF may also enhance the binding of BH_4 to NOS in a fashion of BH_4 stabilization, an effect reported for ascorbic acid (109). Through direct interaction with NOS, particularly in BH_4 -deficient vessels, 5-MTHF attenuates O_2^- production (110) and thus supports conversion of NOS uncoupling from a peroxynitrite synthase and O_2^- producing enzyme back to NOS, improving ED particularly in the diseased vessel.

Direct and indirect antioxidant properties of folic acid

Hcy increases oxidative stress by inhibiting the expression of antioxidant enzymes. Attenuation of this inhibitory effect on GPx and SOD through Hcy-lowering by folate treatment may thus indirectly lead to stimulation of enzyme activities and limitation of ROS. Reversal of uncoupled NOS and reduction of ADMA and L-arginine all increase NO bioavailability, and this may be seen as an indirect anti-oxidative side effect of treatment with folic acid (111). Folate protects against oxidative modifications of LDL-cholesterol, independent of lowering of Hcy (112). In contrast, folate deficiency was found to result in increased lipid peroxidation in rats (113) and decrease in cellular antioxidant defence (108). Indeed, 5-MTHF possesses direct anti-oxidative capacity (110) and (using lucigenin-enhanced chemiluminescence) it was observed that 5-MTHF could reduce O_2^- generation by two ROS-generating systems: xanthine oxidase and NOS (36).

These findings are supported by observations in humans that folic acid abolished the Hcy-induced increases in endothelial O_2^- (34), and administration of folic acid added significantly to total anti-oxidative status in serum of coronary artery disease (CAD) patients (30). In healthy volunteers, pre-treatment with folic acid prevented the postprandial lipid-induced ED and increase of urinary radical damage end products (115), corroborating the (radical scavenging) function of folic acid. Although its direct anti-oxidative effectiveness is several times lower than ascorbate, 5-MTHF can directly scavenge O_2^- . Application of high doses of folic acid may consequently exert direct anti-oxidative effects of clinical relevance, but may make it difficult to discriminate those from Hcy-mediated effects on the endothelium.

Conclusions

Current evidence suggests that endothelial function is not determined solely by the individual risk factor burden, but rather may be regarded as an integrated index of all atherogenic and athero-protective factors present in an individual at a given point of time. Injury to the endothelium followed by dysfunction is an early key event preceding manifestation of vessel pathology. In line with this hypothesis, ED was shown to constitute an independent predictor of cardiovascular events, providing valuable prognostic information additional to that derived from conventional risk factor assess-

ment. Treatment and modification of risk factors may improve endothelial function and in turn prognosis. Hence, given its reversibility and granted the availability of a diagnostic tool to identify patients at risk and to control the efficacy of therapy in clinical practice, ED is an attractive primary target in the effort to optimize individual therapeutic strategies to reduce cardiovascular morbidity and mortality.

It remains possible that hyperhomocyst(e)inemia simply reflects an association with another underlying cause and Hcy may just serve as a sensitive marker for, e.g., intracellular vitamin deficiency, ultimately leading to vascular disease. However, clinical investigations clearly showing impaired vascular endothelial function associated with hyperhomocyst(e)inemia and reversibility through Hcy-lowering in healthy subjects and patients with established vascular disease argue strongly in favor of causality. 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. Atherothrombosis is multifactorial and there is reason to believe that coexistence of additional risk factors is required and considerably augments the likelihood of Hcy-induced damage. It must be noted that many of the identified atherogenic effects demonstrated for Hcy are unspecific. However, the presence of hyperhomocysteinemia, through interactions with other established risk factors such as smoking or hypertension, greatly increases total risk synergistically (116). Simple, cheap, effective and safe treatment of elevated Hcy levels through low-dose folic acid supplementation makes this risk factor a very attractive target over others for risk reduction (117).

In addition to clarifying the potential of cardiovascular prevention in ongoing randomized, prospective Hcy-lowering trials, it is still essential to identify subjects at risk and the ones who may benefit most from Hcy-lowering.

Previous studies have suggested that elevation of plasma Hcy concentration impairs NO-mediated dilation of small coronary arteries (71) and relaxation of peripheral vessels (69) by decreasing bioavailability of NO, an effect that can be reversed by administration of O_2^- scavengers. There is direct generation of ROS by Hcy, but it may be unlikely to have extensive physiological relevance. Rather, it is probable that the oxidative stress in hyperhomocysteinemia is a secondary effect, which may be subject to regulation by NO. Thus, the balance between NO and Hcy may be a key to whether the response is physiological or maladaptive.

Several strategies were considered in restoring deficits in endogenous NO at the endothelial cell interface. NO can be administered directly at low concentrations either inhaled as a gas (118) or infused in solution (119). Another approach is the application of NO donors as with the commonly used nitroglycerine and nitroprusside (120). Organic nitrates including nitroglycerine have been used successfully in the treatment of ischemic heart disease; however, prolonged exposure to nitrates may diminish not only the bioavailabil-

ity but also the efficacy of their active metabolite NO, thus leading to tolerance and limiting their clinical application (121). Both *in vitro* and *in vivo* treatment with ascorbic acid prevented nitrate tolerance (122), confirming the role of increased O_2^- production in the development of end-organ tolerance. Importantly, both NOS dysfunction induced by nitroglycerine and nitrate tolerance were also prevented by short-term oral folic acid supplementation (123, 124), supporting the role of folate as a direct antioxidant with clinical implications. Interesting experiments have demonstrated transfection of the gene for eNOS into the vessel wall with hypothetical restoration of the ability of the endothelium to produce its own NO (125). However, clinical use is as yet far from being established. Another, perhaps the most practicable method, is administration of L-arginine, the precursor of NO biosynthesis (126). Two recent studies show that O_2^- production by NOS is inhibited by BH_4 , but not L-arginine (105, 127). Whereas L-arginine inhibits O_2^- production by preventing uncoupled NADPH oxidation, BH_4 appears to act by directly scavenging O_2^- (105). Supplementation with BH_4 was demonstrated effective in improvement of ED (78). Notably, ascorbic acid increases intracellular BH_4 levels of human EC, and 5-MTHF augments synthesis of BH_4 (107), thus allowing for optimal NOS activity. Indeed, folic acid supplementation effectively improves ED in healthy (hyperhomocysteinemic) (32, 33) and atherosclerotic subjects (30, 31).

Currently ongoing trials will most likely not be able to answer several important questions (128). At the time of conception of these trials it was largely unknown that independent of Hcy-lowering, folic acid may have direct beneficial effects on its own on the endothelium, such as direct antioxidant properties. Accordingly, almost all studies use relatively high doses of folic acid (0.8–5 mg) that may not allow discrimination between the independent effects of folate supply and Hcy-lowering. Understanding of the complex interactions between Hcy, radicals, NO and folate offers promising perspectives in the prevention and treatment of vascular disease. It appears, however, that the individual influence of each component on cell pathology changes during the course of disease progression. Thus, preventive and therapeutic strategies may require a different approach and better discrimination of target groups according to risk and greatest possible efficacy in vasoprotection.

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