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Homocysteine-Induced Endothelial Dysfunction

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Key Words
Endothelial dysfunction · Nitric oxide · Hyperhomocysteinemia · Oxidative stress

Abstract
This review discussed and in particular emphasis the potential cellular pathways and the biological processes involved that lead to homocysteine-induced endothelial dysfunction, in particular in the impaired endothelial dependent dilatation aspect. Hyperhomocysteinemia is an independent cardiovascular risk factor that has been associated with atherosclerotic vascular diseases and ischemic heart attacks. The potential mechanisms by which elevated plasma homocysteine level leads to reduction in nitric oxide bioavailability include the disruptive uncoupling of nitric oxide synthase activity and quenching of nitric oxide by oxidative stress, the enzymatic inhibition by asymmetric dimethylarginine, endoplasmic reticulum stress with eventual endothelial cell apoptosis, and chronic inflammation/prothrombotic conditions. Homocysteine-induced endothelial dysfunction presumably affecting the bioavailability of the potent vasodilator 'nitric oxide', and such dysfunction can easily be monitor by flow-mediated dilation method using ultrasound. Understanding the mechanisms by which plasma homocysteine alter endothelial nitric oxide production is therefore essential in the comprehension of homocysteine-induced impairment of endothelial dependent dilatation, and its association of cardiovascular risk and its pathophysiology.

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Introduction

Hyperhomocysteinemia is defined as a pathological condition of excessive plasma homocysteine level (normal homocysteine levels ranges from 4 to 12.3 μmol/l) [1]. In 1969, when children with homocysteinuria were observed to have a high susceptibility to premature death due to severe atherosclerosis and thrombotic occlusions, McCully et al. [2] proposed the notion of hyperhomocysteinemia as an independent cardiovascular risk factor. Since then, evidence continuously emerged that an elevated plasma homocysteine level in the range of 15–25 μmol/l correlated with coronary heart disease [3], stroke [4], peripheral artery stenosis [5] venous thrombosis [6], and dietary folate deficiency [7]. Likewise, a meta-analysis study has presented that for every 5 μM increase in plasma, homocysteine could increase the danger of coronary heart disease by 60% for adult males and 80% for adult females [8]. Another similar meta-analysis study also reported a 7-fold increase in the mortality rate in hyperhomocysteinemic patients when compared to normal subjects [9]. In addition, a large prospective cohort study (3,056 consecutive patients undergoing coronary angiography) also demonstrated that a high level of plasma homocysteine was associated with the extent of coronary atherosclerosis prevalence in Italy. Furthermore, several intervention studies demonstrated that folic acid supplementation in patients with asymptomatic hyperhomocysteinemia can improve endothelial-dependent dilatation [10–12]. In 2015, McCully et al. [13] further supple-

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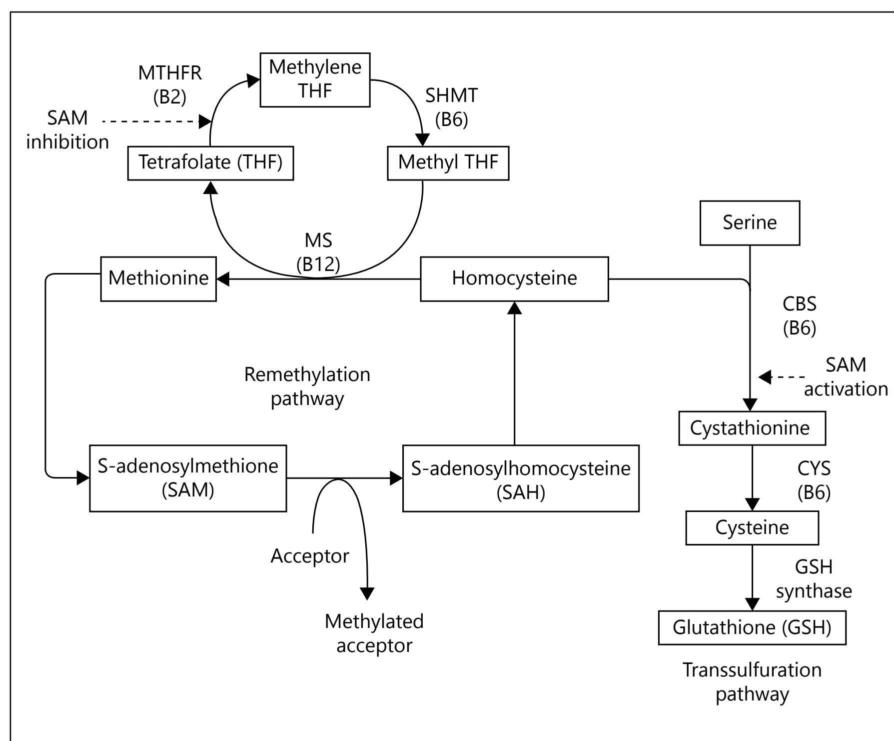


Fig. 1. The metabolism pathway of homocysteine. The fate of homocysteine: it is either re-methylated into methionine by N-5-methyltetrafolate to begin another methyl-group transfer cycle; or combine with serine and irreversibly trans-sulfurized by the enzyme cystathionine beta-synthase to form cystathionine, and cystathionine are subsequently metabolized to cysteine, a precursor to the GSH. CBS = Cystathione-beta-synthase; CYS = cystathione-gamma-lysase; MS = methione synthase; MTHFR = methylenetrahydrofolate reductase; SHMT = serine hydroxymethyltransferase.

mented his view in an expert review that an elevation of plasma homocysteine could lead to atherosclerosis in the general population. Nevertheless, there are many clinical trials today that contend that plasma homocysteine is merely a risk factor for cardiovascular disease. For instance, a large-scale double blind randomized controlled trial indicated a moderate reduction of total homocysteine after an incident of non-disabling cerebral infarct shown no improvement in vascular outcomes [14]. The failure of these clinical trials may be due to numerous factors including age, baseline homocysteine levels, vitamin B12 status, renal function and medications that affect the effectiveness of B vitamin therapy on homocysteine-induced vascular risk [15].

The Homocysteine Metabolic Pathway

Homocysteine is a byproduct of numerous biological processes. The methyl-group rich amino acid methionine is first activated to form S-adenosylmethionine (SAM), which serves as a methyl donor in over 115 methyl transfer reaction [7]. The loss of the methyl group results in the residual product S-adenosylhomocysteine (SAH), and hydrolyzing of SAH yields homocysteine [16]. The homocysteine generated is then metabolized through two pathways subsequently: it is either re-methylated into methionine by N-5-methyltetrafolate to begin another methyl-group transfer cycle; or combine with serine and irreversibly trans-sulfurized by the enzyme cystathionine beta-synthaseto form cystathionine, and cystathionine are subsequently metabolized to cysteine, a precursor to the antioxidant glutathione (GSH) [17]. This trans-sulfuration turnover of homocysteine is predominantly carried out in the liver, kidneys, pancreas, and small intestines (fig. 1).

The activation of the re-methylation pathway and trans-sulfuration pathway that dictates the fate of the homocysteine is regulated in part by methionine intake in the diet. It has been shown that halving the methionine intake will double the average amount of re-methylation cycles gone through per homocysteine molecule [9]. SAM intrinsically mediates this shunting of homocysteine to maintain a stable re-methylation cycle. SAM has been shown to activate cystathionine beta-synthase while simultaneously inhibiting methylenetetrahydrofolatereductase, an enzyme responsible for N-5-methyltetrafolate synthesis [18]. Thus, a high level of methionine with its concurrent raised level of SAM will promote the trans-sulfuration pathway and suppress re-methylation, lowering the methionine and homocysteine back to the basal level. Any blemishes in either pathways will result in an accumulation of homocysteine that is subsequently exported into the blood stream, resulting in hyperhomocys-

teinemia [19]. However, both myocardial and vascular cells, unlike most other tissues, are inherently deficient in the enzyme cystathionine beta-synthase [20]. Thus, cardiovascular cells are unable to stabilize the excess homocysteine through the trans-sulfuration pathway, and are restricted to the re-methylation pathway of homocysteine metabolism. This hypothesis therefore accounts for the high susceptibility of the cardiovascular system to homocysteine toxicity [21].

The Vascular Endothelium Functionality

Endothelial dysfunction can be described as an imbalance between vasodilator and vasoconstrictor produced by the endothelium, and it has been regarded as the core systemic pathological status in the process of atherosclerosis and CVD. Endothelium is composed by a single layer of endothelial cell, which lines the interior surface of vascular lumen, between blood and vascular smooth muscle cells (VSMC) of all kinds of blood vessels and the whole circulatory system. It has many vital functions, including the regulation of vascular tone and inflammatory balance (table 1). The vascular dilatation in response to shear stress of blood flow depends in part on the endothelium-derived relaxing factor – nitric oxide (NO) [22]. NO is majorly synthesized by the endothelial isoform of NO synthase (e-NOS) in response to the vasodilation stimulus. Endothelial NO diffuses across to VSMC where it activates cytosolic guanylylcyclase, increases cyclic GMP production, and leads to vascular smooth muscle relaxation [23]. Therefore, the loss of endothelial-mediated vasodilatory ability that characterized by the tipping of the vascular balance toward an abnormally constrictive, inflammatory and prothrombombic state is considered to be one of the earliest manifestations of cardiovascular damage and precedes the formation of atherosclerotic plaques.

Extensive experimental studies, both in vivo and in vitro, have produced conclusive evidence that high homocysteine levels significantly impaired endothelial-dependent vasodilation and resulted in a concurrent attenuated NO bioavailability in response to dilatory stimulus such as acetylcholine, suggesting that homocysteine-induced endothelial dysfunction at least partly stems from the loss of endothelium-dependent relaxing factor [20]. Also, the observed atherosclerotic changes in hyperhomocysteinemic patients were found related to blood platelet and coagulation activation, and impaired fibrinolysis and chronic inflammation [24]. Nevertheless, the knowledge regarding the pathways by which homocysteine causes reduced NO synthesis and reduced endothelial-dependent dilatation remains fragmented and incomplete. Evidence from numerous previous studies suggests that homocysteine does not directly suppress e-NOS [25–27]. Immunoblot assay in human umbilical vein endothelial cell cultures (HUVEC) has shown that various concentrations of homocysteine have no effect on the gene expression of e-NOS. Thus, it appears that homocysteine intervenes in the production of NO and reduces NO bioavailability in endothelial cells through other indirect mechanisms, leading to the impairment of endothelial-dependent dilatation. Therefore, this review article aims at summarizing the possible biological events leading to homocysteine-induced impairment of endothelial-dependent dilatation, with particular focus on the use of a well-developed and noninvasive ultrasound method, that is, the flow-mediated dilatation (FMD) method [28].

Table 1. A summary of the major roles of endothelium and the associated chemical mediators in vascular function

Role of endothelium	Associated chemical mediators
Vasoconstriction	Angiotensin II Endothelin-1 Thromboxane A2 Prostacyclin H2
Vasodilation	NO Bradykinin Hyperpolarizing factor
Growth stimulation	Platelet growth-derived factor Fibroblast growth factor Insulin-like growth factor-1 Endothelin Angiotensin II
Growth inhibition	Nitric oxide Prostacyclin
Proinflammatory	Vascular cell adhesion molecule Intracellular adhesion molecule
Antithrombotic	Prostacyclin Tissue plasminogen activator
Prothrombotic	Type 1 plasminogen activator inhibitor

Homocysteine and Impaired Endothelial-Dependent Dilatation

From the literature and based on several assumptions and hypotheses in endothelium autoregulation mechanism, we summarized 3 potential mechanisms by which

hyperhomocysteinemia could lead to an impairment in endothelial-dependent dilatation: (1) oxidative stress: the disruptive uncoupling of NO synthase activity, quenching of NO, and enzymatic inhibition, and (2) endoplasmic reticulum stress with eventual endothelial cell apoptosis, and (3) chronic inflammation/prothrombotic conditions.

Homocysteine-Induced Oxidative Stress Condition

NADPH Oxidase Up-Regulation

Incubation of aortic endothelial cells in physiological level of homocysteine (1 mM) has been shown to result in a marked increase in superoxide production [29]. Since antioxidants have proven effective in reducing homocysteine-induced endothelial dysfunction, it has been suggested that oxidative stress may play a role in the detrimental effects of homocysteine on endothelial function. This is supported by the observation that superoxide reduces NO bioavailability by reacting with it to form peroxynitrite.

Homocysteine contains a highly reactive sulfhydryl (-SH) group. The sulfhydryl group readily self-oxidizes to form disulfide linkage with other free thiols, along with the generation of superoxide radicals as a byproduct [29]. A previous study reported that more than 98% of plasma homocysteine existed as an oxidized state, either in the form of homocysteine disulfide dimer or mixed disulfides with proteins [30]. One of the earlier studies has thus attributed the observed superoxide production to the result of this potential thiol self-oxidation [31]. This, however, fails to explain why cysteine, which is another thiol in far more abundance, does not induce marked oxidative stress and apparently does not damage the vascular endothelium [30].

More recent researches have shown that the observed oxidative stress may well have been partly generated through the upregulation of the enzyme 'nicotinamide adenine dinucleotide phosphate-oxidase' (NADPH oxidase), which generates superoxide through the reaction:

$$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$$

There are multiple isoforms of NADPH oxidase (NOX), with endothelial cell mostly exhibiting the isoform NOX2. Homocysteine increased endothelial cells NADPH oxidase expression in a time- and dose-dependent manner [32]. HUVEC incubated with homocysteine for 6 h demonstrated a significant increase in NOX2 gene expression, phox47 (a protein subunit of NOX2) accumulation in the nucleus, and peri-nuclear localization of NOX4 isoform [33, 34]. On the contrary, NADPH oxidase inhibitor apocynin almost completely suppressed any homocysteine-induced reactive oxygen species (ROS) generation in HUVEC. The increase in NADPH oxidase activity, however, by far fails to account for the vast quantity of ROS generated. This is suggestive that the up-regulated NADPH oxidase likely represents an initiating source of oxidative stress in endothelial cells that triggers other dormant ROS producers in hyperhomocysteinemia. In another human study, a better FMD was found in patients with hereditary deficiency of NOX2 [35].

Homocysteine-Induced Reduction in System y+ CAT L-Arginine Cellular Transport

The generation of NO by e-NOS requires nitrogen atoms, the source of which comes from the amino acid L-arginine. In e-NOS, L-arginine reacts with oxygen radicals reduced by NADPH to form NO radicals and a byproduct L-citrulline [25]. Thus, a constant supply L-arginine is important in maintaining sufficient NO production. Recent evidence points to a reduced NO synthesis due to the interference of L-arginine transport by homocysteine. L-Arginine uptake in endothelial cells is mediated by the system y+ cationic amino acid transporter (CAT). Jin et al. [25] has shown that long-term incubation (>24 h) with the clinically relevant level of homocysteine (0.5–2.5 mM) in bovine aortic endothelial cells resulted in a dose-dependent reduction in CAT-1 isoform expression. This is followed by a subsequent reduction in trans-membrane L-arginine transport, reduced total NO production, and suppressed vasodilation in response to acetylcholine [26]. While it remains uncertain why homocysteine suppresses CAT-1 L-arginine transport, it has been suggested that homocysteine-induced oxidative stress plays a role because the functionality of system y+ can be improved by the addition of antioxidants such as ascorbic acid [25].

e-NOS Uncoupling and Peroxynitrite Production

The depletion of L-arginine supply not only resulted in depleted NO synthesis, but also in another phenomenon called e-NOS uncoupling [34]. e-NOS exists as a heterodimer comprising of 2 reductase domains 'coupled' to another pair of oxygen domains. In the reductase domain, electrons from NADPH reduce oxygen with heme as a cofactor and the resultant ferrous-dioxygen complex subsequently oxidizes L-arginine to produce NO in the oxygenase domain [36, 37].

As in figure 2, in the absence of sufficient L-arginine, the oxygen domain needed for NO production is impaired, but the reductase domain continues the reduction of oxygen, resulting in superoxide production [25, 36].

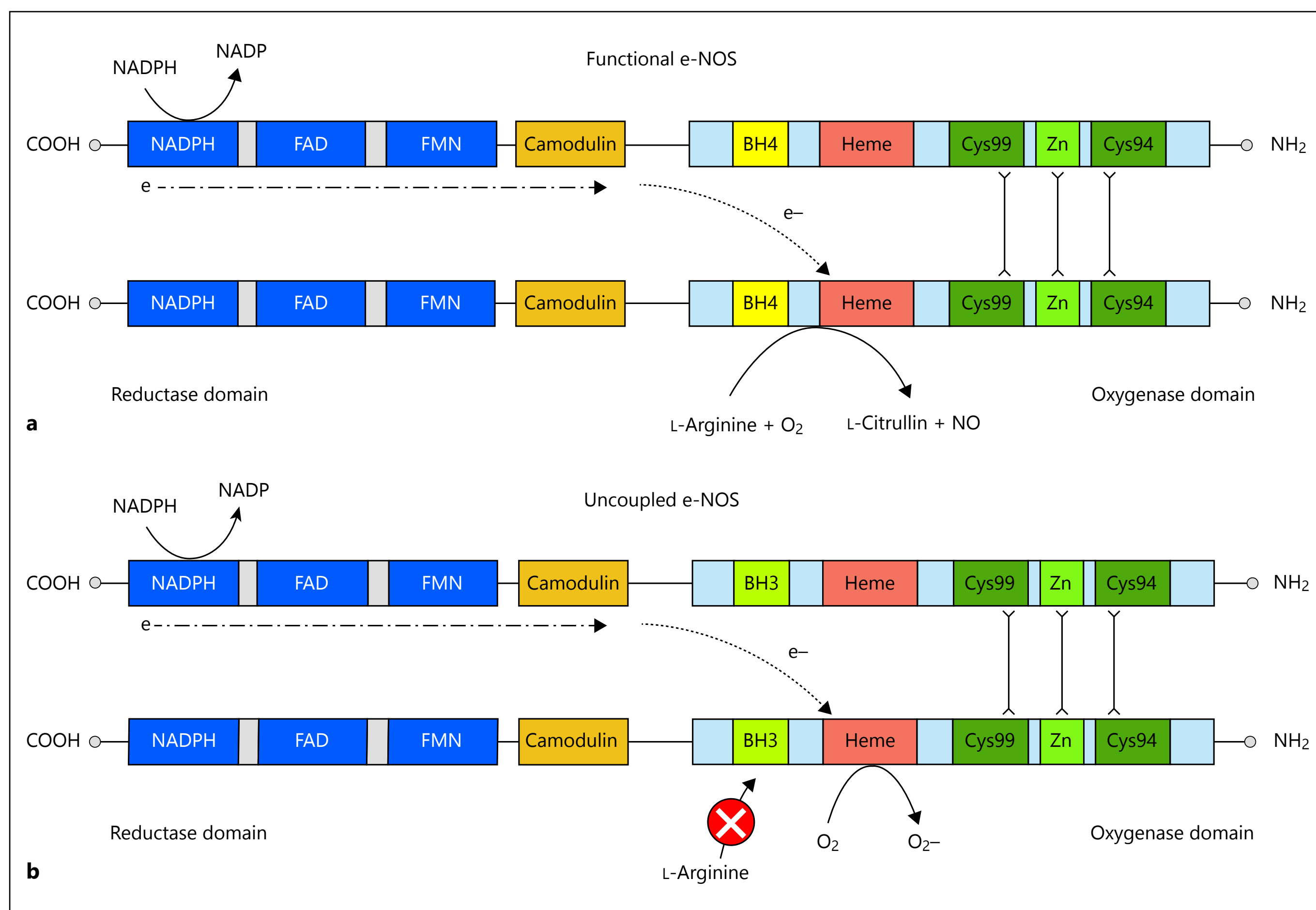


Fig. 2. e-NOS uncoupling due to BH4 oxidation and superoxide generation. **a** e-NOS in its functional conformation exists as a dimer with each monomer featuring a C-terminal reductase domain and an N-terminal oxygenase domain. The 2 monomers are bound at the Cys99, Cys94 and zinc motif on the oxygenase domain. Electrons from NADPH generated in the reductase domain of one monomer is subsequently passed to the ferric heme group on the opposite monomer. The heme group then hydrolyses L-arginine and oxidizes it into L-citrullin and nitric oxide. **b** In the absence of sufficient L-arginine substrate, the e-NOS is uncoupled and results in production of superoxide radicals instead of nitric oxide. Oxidative stress from peroxynitrite oxidizes the BH4 cofactor into the inactive form BH3. In such circumstances restoration of the L-arginine supply is unable to reverse the uncoupling of e-NOS and its superoxide production.

Hence, e-NOS is now enzymatically 'uncoupled' and is diverted from a NO producing enzyme to a producer of ROS [37]. The presence of e-NOS uncoupling in homocysteine exposed HUVEC has been well documented in ex vitro experiments, where the generation of ROS was largely proportional to e-NOS activity, and intracellular BH4 availability has been shown to be decreased by up to 80% in homocysteine-treated endothelial cells [26]. The superoxide from both the uncoupled e-NOS and the upregulated NADPH oxidase quenches the remaining NO from still-functional e-NOS and results in the end-product peroxynitrite, which is an oxidizing agent that has been shown to avidly oxidize and deplete the key cofactor of the e-NOS oxygenase domain 'tetrahydrobiopterin' (BH4) [37–39]. The progressive loss of BH4 due to peroxynitrite production could in turn trigger a vicious cycle of further aggravated e-NOS uncoupling and BH4 destruction [40].

Fluorescence studies indicate that homocysteine-induced ROS generation in HUVEC could be attenuated by 70% when treated with e-NOS inhibitor L-NAME, indicating that uncoupled e-NOS is indeed as a major contributor to oxidative stress [37]. On the contrary, infusion of both peroxynitrite scavenger 'ebselen' [41] and BH4 regenerator ascorbic acid [26] restores NO production in homocysteine-treated endothelial cells. This in turns sup-

ports the notion that the generation of peroxynitrite and loss of BH4 mediates the uncoupling of e-NOS. It is therefore viable that upregulation of NOX, a simultaneous homocysteine-induced impairment in L-arginine transport and a consequential e-NOS uncoupling due to oxidative stress on BH4 is responsible for the observed increase in oxidative stress, and contributes to the observed decrease in endothelial NO production in the case of hyperhomocysteinemia. It is also worth noticing that homocysteine can downregulate glutathione peroxidase, a key generator of intracellular antioxidant pool, suggesting that homocysteine contributes to oxidative stress not only through the increased ROS generation but also involves the suppression of antioxidant capability of endothelial cells [42].

Loss of DDAH Function and Accumulation of ADMA

Another proposed mechanism of homocysteine-induced impaired endothelial-dependent dilatation involves the chemical molecule asymmetric dimethylarginine (ADMA) [38]. ADMA is synthesized when L-arginine incorporated into proteins are methylated by the enzyme 'protein-arginine methyltransferase' during post-translational protein modification [43]. When such methylated proteins are hydrolyzed during protein turnover, ADMA residues will be released. Due to its close structural proximity to L-arginine, ADMA can act as an endogenous competitive inhibitor for e-NOS [44] (fig. 3).

Such notion is supported by observations in chemiluminescence experiments that ADMA is capable of reducing endothelial nitrogen oxide species elaboration, but such reduction is reversible with the infusion of L-arginine [44]. ADMA has long been documented to be a major cardiovascular risk factor. Clinical studies have indicated that an elevated level of ADMA is more strongly correlated to endothelial dysfunction than the cholesterol level in hypercholesterolemia patients [45], and ADMA is rated as the second-most important predictor of cardiovascular event for people with chronic renal failure [43]. While the counterpart of ADMA-'symmetric dimethylarginine' is solely excreted in urine, ADMA can further stabilize to citrulline by the enzyme-dimethylarginine dimethylaminohydrolase (DDAH) [42, 44]. Pharmacological studies using endothelial cell cultures infused with DDAH inhibitor 4124W butanoicacid [43] and animal study using DDAH transgenic mice [46] both demonstrated that the level of ADMA was inversely related to DDAH activity. The implication there is that the

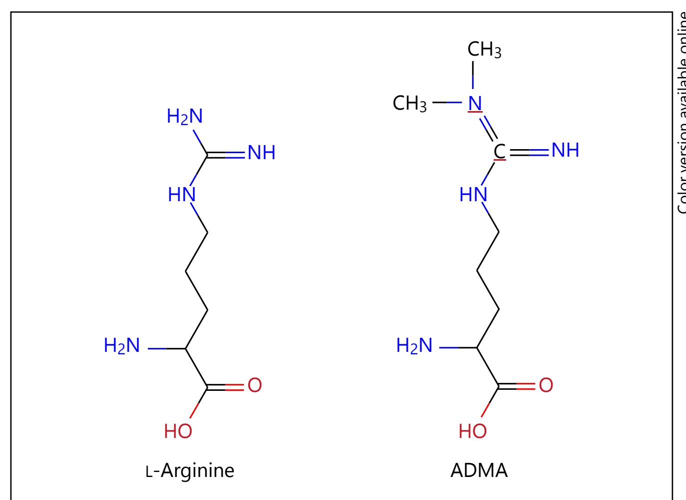


Fig. 3. Molecular structures of L-arginine and ADMA. Note the structural proximity between L-arginine and ADMA. ADMA acts as a competitive inhibitor for e-NOS.

inhibition of DDAH could cause an accumulation of endogenous ADMA and a reduction of NO synthesis.

The possibility that homocysteine could serve as an inhibitor of DDAH was reported in an enzyme kinetics study by Stuhlinger et al. [45]. They found that the DDAH enzyme activity could be suppressed by the addition of pathophysiologically relevant level of homocysteine (from 0 to 300 µM), resulting in a dose-dependent intracellular accumulation of ADMA in the cultured bovine aortic endothelial cells. Such inhibiting actions of DDAH, however, could be reversed by multiple thiol-based antioxidants, suggesting that the inhibition of DDAH could be due to the direct oxidative attack on the enzyme. The sulfhydryl groups of the four-cysteine groups in DDAH are considered vital in the catalytic ability of DDAH because sulfhydryl inhibiting agents are strong inhibitors of DDAH [45]. Whether the reduced DDAH activity involves the reduction of DDAH synthesis is subjected to more debate. Tyagi et al. have shown decreased DDAH mRNA expression in homocysteine-treated (0–100 µM) microvascular endothelial cells [16], and Jia et al. [47] have shown downregulation of DDAH-2 expression by homocysteine through increased DNA methyltransferase expression and consequential hypermethylation of DDAH-2 promoter gene. However, a study by Stuhlinger failed to demonstrate any effects on DDAH-1 isoform gene expression in bovine aortic endothelial cells by homocysteine [45]. Inconsistent results may be due to the use of distinct endothelial cell lines or difference in the target DDAH isoform tested. DDAH-2 is the principal

isoform found in vasculature endothelium, whereas DDAH-1 is predominantly found in kidney proximal tubules [48]; thus, the expression of the two DDAH isoforms may react differently to homocysteine. The loss of DDAH catalytic ability, accumulation of ADMA, and the subsequent suppression of e-NOS likely contribute in part to homocysteine-induced endothelial dysfunction.

Homocysteine-Induced Endoplasmic Reticulum Stress and Unfolded Protein Response Conditions

CHOP/GADD153 Activation and TDAG51 Detachment-Mediated Apoptosis

Circulating apoptotic endothelial cells have been observed in the bloodstream of severe hyperhomocysteinemia patients, suggesting that endothelial dysfunction in hyperhomocysteinemia may not only have stemmed from the loss of NO bioavailability, but also from the actual loss of an intact endothelium. A pathologically relevant level of homocysteine can induce apoptosis in cultured endothelial cells mediated by endoplasmic reticulum (ER) stress and unfolded protein response (UPR) [49].

ER is the site where newly synthesized proteins undergoing posttranslational modifications fold into the correct conformation. To prevent chaotic folding, this process is mainly assisted by two classes of proteins: foldase (enzymes involved in catalyzing protein folding) and chaperons (molecular proteins to prevent the undesirable interaction between unfolded protein domains and neighboring proteins) [50]. Only correctly folded proteins are exported to the Golgi apparatus, whereas any misfolded proteins are retained in the ER for refolding or destined to the ER associated degradation. Any accumulation of unfolded and aggregated proteins results in ER stress and triggers a cascade of signaling pathway collectively named UPR [50].

UPR mitigates the misfolded protein burden by upregulating ER protein folding capacity through an increased production of chaperones and downregulating the ER protein load by suppressing protein transcription and translation [50, 51]. Three parallel signal transduction pathways mediate such protective response: inositol requiring enzyme 1 (IRE1), RNA-activated protein kinase like ER kinase (PERK) and activating transcription factor 6. Prolonged activation of the aforementioned UPR effector pathways, however, has been known to cause the pathways to switch from a protective response into apoptosis [51]. Persistent activation is indicative of the cells that the ER stress cannot be resolved by UPR and elimination of the damaged cells is more desirable in such circumstances.

Homocysteine is an elicitor of ER stress. Studies using both Western blot and Northern blot analysis have reported that homocysteine, similar to other known ER stress inducers 'tunicamycin' and 'thapsigargin', elevated the level of UPR response molecules, including GRP78 and GRP94 in endothelial cell culture in a dose-dependent manner [49, 52, 53]. The homocysteine-induced apoptotic pathway has also been demonstrated to be dependent on the UPR effector pathway: IRE1 and PERK of ER stress response.

Homocysteine-induced cytotoxicity involves at least two apoptotic genes. The first is the activation of CHOP/GADD153 (growth arrest- and DNA-damage inducible gene 153). The second is the activation of TDAG51 (T-cell death associated gene 51) and the initiation of detachment-mediated apoptosis [49, 54]. CHOP/GADD153 belongs to the CCAAT enhancer binding protein family [55]. In quiescent cells, the CHOP/GADD153 expression is kept at a very low level. Overexpression of CHOP/GADD153 will cause cell cycle arrest and cell apoptosis, though the exact downstream mechanism remains poorly understood [52, 55]. Homocysteine was found to increase the CHOP/GADD153 expression in a dose-dependent manner, but in cells with either mutated IRE1 gene or deleted PERK genes, both the CHOP/GADD153 induction and cell death were significantly attenuated. This indicates that homocysteine-induced CHOP/GADD153 apoptosis is a downstream response of ER stress [52]. The activation of the second apoptotic gene TDAG51 has been shown to be dependent on eIF2 alpha phosphorylation, a key signal transducer in the PERK pathway. PERK pathway is activated when eIF2 alpha is phosphorylated by the ER-stress sensing EIF2AK3 trans-membrane kinase [51]. Therefore, TDAG51 is also classified as an ER stress response gene. TDAG51 triggers endothelial cell death through detachment-mediated apoptosis (anoikis). Overexpression of TDAG51 in endothelial cells has been shown to cause cell rounding up, extension of cell pseudopods, dramatic loss of cell adhesion, and caspase-dependent cell death. Caspase activation is likely downstream of cell detachment because caspase inhibition with ZVAD inhibits endothelial cell apoptosis but not its loss of cell adhesion [54]. Given TDAG51 contains a cytoskeletal organizing plekstrin motif, it is conceivable that overexpression of TDAG51 causes cell detachment by disruption of endothelial cytoskeleton. Hossain et al. [54] demonstrated that 0.05–10 mM homocysteine increased TDAG51 mRNA and protein expression. This suggests TDAG51 activation and consequential anoikis could have in part accounted for homocysteine-induced shedding of

Fig. 4. Formation of homocysteine thiolactone due to MetRS misactivation. Misactivated L-homocysteine, causing the thiol group and carboxyl group of homocysteine to join together by thioester linkage, resulting in the generation of cyclic derivative homocysteine thiolactone.

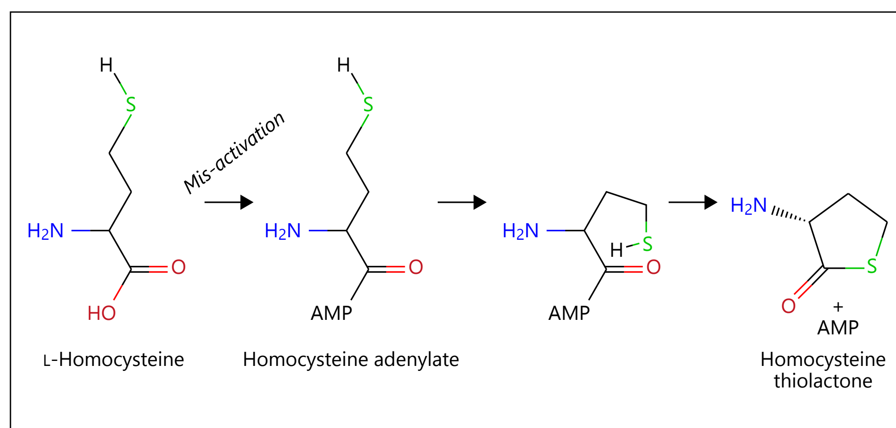
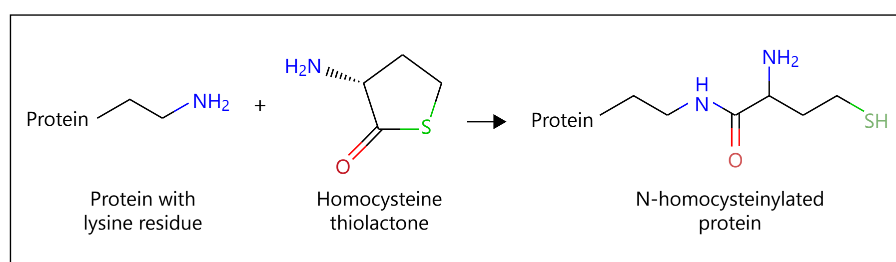


Fig. 5. Protein N-homocysteinylation by homocysteine thiolactone.



endothelial cells and endothelial dysfunction as observed in hyperhomocysteinemia [34, 49, 54]. Both the induction of CHOP/GADD153 and TDAG51 genes are homocysteine specific. The addition of neither superoxide scavengers nor catalase fails to inhibit their expression and the consequential homocysteine-induced apoptosis, indicating that the homocysteine-induced ER stress and the associated apoptotic cell death are independent of the effect of homocysteine thiol group or oxidative stress [52, 54].

Error Editing and Homocysteine Thiolactone

While the cause of homocysteine-induced ER stress remains obscure, protein modification by homocysteine thiolactone has surfaced as one potential mediator. Homocysteine thiolactone is the result of error editing [56]. When the ratio of intracellular homocysteine to methionine is high, the enzyme that links methionine to its conjugate t-RNA 'methionyl-tRNA synthase (MetRS)' will misactivate homocysteine instead of methionine, causing the thiol group and carboxyl group of homocysteine to join with each other by thioester linkage [54] and resulting in the generation of cyclic derivative homocysteine thiolactone [57, 58] (fig. 4).

The thioester group of thiolactone combines avidly with any lysine residues in proteins to form amide bonds in a process known as N-homocysteinylation [59], and the rate of N-homocysteinylation proportionally increases with the amount of lysine residues [42, 58] (fig. 5). This incorporation of thiolactone into functional protein alters protein conformation and is potentially detrimental to protein function. N-homocysteinylated proteins have a tendency to aggregate, and studies have shown that multiple lysine-rich proteins including fibrinogen [58, 59], high-density lipoprotein [60], lysine oxidase [59] and cytochrome c [61] experience loss of functionality after such post-translational incorporation of homocysteine thiolactone.

Homocysteine thiolactone induces apoptosis in endothelial cell cultures in vitro and causes endothelial desquamation in baboons. L-Methione is essential to the human body and is the precursor of L-homocysteine. The chief function of L-methione includes synthesis of L-cysteine and various protein molecules. Deficiency of L-methionine can lead to depression and allergies. Notwithstanding, when L-methane is transformed to L-homocysteine stereoisomer, it becomes cytotoxic. This is concordant with the observation that MetRS, which converts homocysteine to thiolactone can bind only with L-homocysteine but not with its withisomer counterpart – D-homocysteine. This stereoisomer specificity suggests

that the cytotoxic effect of homocysteine stems from its conversion to homocysteine thiolactone. It has thus been proposed that in homocysteine incubated endothelial cell cultures; the observed homocysteine-induced ER stress and apoptosis were attributed to the raised level of intracellular homocysteine thiolactone and protein N-homocysteinylation that caused widespread intracellular protein unfolding. In humans with normal homocysteine metabolism, the level of plasma homocysteine thiolactone was in the range from 0 to 24.8 nM [56]. Subjects with congenital hyperhomocysteinemia were found to have 59–72 times increased levels of plasma homocysteine thiolactone when compared to subjects with normal homocysteine metabolism [59]. Even in physiologically low concentration (less than 10 nM) of homocysteine, thiolactone is enough to cause significant N-homoycsteinylation [57]; extensive protein N-homocysteinylation would thus occur within the endothelial cells in the hyperhomocysteinemic patients. The hypothesis of homocysteine thiolactone cytotoxicity also accounts for the specificity in homocysteine-induced apoptosis because homocysteine thiolactone can be synthesized only in in vivo from homocysteine but not from other sulfhydryl containing amino acids such as cysteine.

Homocysteine-Induced Inflammatory/Prothrombotic Conditions

Hyperhomocysteinemia is associated with increased aggregation of platelets and enhanced vascular inflammation [62]; an enhanced expression of receptor for advanced glycation end products, vascular cell adhesion molecule-1, tissue factor, and MMP-9 was found in a mouse model with methionine-induced hyperhomocysteinemia [63]. Also, the oxidative stress induced by hyperhomocysteinemia could further lead to a cascade in inflammation. In a human study, the inflammatory activity and plasma homocysteine levels were found increased in a group of type 2 diabetic patients with overt atherosclerotic vascular disease [64]. In another animal study, hyperhomocysteinemia was found to inhibit the endothelial-dependent dilatation of isolated porcine retinal arterioles by the production of vascular NAD(P)H oxidase via p38 kinase [65]. However, contradicting results from a previous study have indicated that the inflammatory response induced by homocysteine occurred in VSMC and not at the endothelium, by stimulating CRP production, which is mediated through the NMDAr-ROS-ERK1/2/p38-NF-κB signal pathway [66]. Another study also demonstrated that an improved homocysteine concentration by means of 1-year folic acid supplementation does not influence C-reactive protein, soluble intracellular adhesion molecule-1, and oxidized low-density lipoprotein [67]. Nevertheless, all these observations may suggest to a certain extent that a chronic inflammatory condition induced by homocysteine could lead to impairment of endothelial-dependent dilatation. Further studies are warranted.

Hyperhomocysteinemia also induces a prothrombotic condition [68] including enhanced platelet activation, enhanced coagulation [69], and attenuated fibrinolysis, resulting from posttranslational modification of fibrinogen by homocysteinylation [70]. In an animal study, short-term treatment with folic acid markedly increased plasma folate levels and significantly suppressed laser-induced thrombus formation in Apolipoprotein E and low-density lipoprotein receptor double deficient mice [71]. Also, vitamin B6 and folic acid treatment was found associated with a decreased occurrence of abnormal exercise electrocardiography tests after a 2-year, double-blind, randomized, placebo-controlled therapeutic trial in 158 healthy siblings of hyperhomocysteinemic patients with premature atherothrombotic disease [72]. Although the exact mechanisms responsible for accelerated thrombosis are poorly understood, one of the possible mechanisms is the impaired bioavailability of NO that leads to subsequent enhanced platelet activation [73].

Conclusion

A major role of the endothelium is to regulate the vessel tone through autoregulation, and endothelial dysfunction is regarded as the leading step in atherosclerosis. It has been widely accepted that elevated homocysteine level is associated with increased cardiovascular risks, and extensive experimental evidence suggested that the involvement of oxidative and ER stress was the critical part in the impairment of endothelial-dependent dilatation. Many review papers have already given insightful discussions on the role of homocysteine in plaque stability and/or thrombogenicity. However, can the absence of hypercholesterolemia or other cardiovascular risk factors, including vitamin B supplements protect against cardiovascular disease among hyperhomocysteinemic patients is a question that remains to be answered.

In summary, we have discussed the potential cellular mechanisms that lead to the impairment of endothelial function, in particular, the involvement of NO by homocysteinemia, suggesting that multiple pathways may likely be involved (fig. 6). There is evidence that

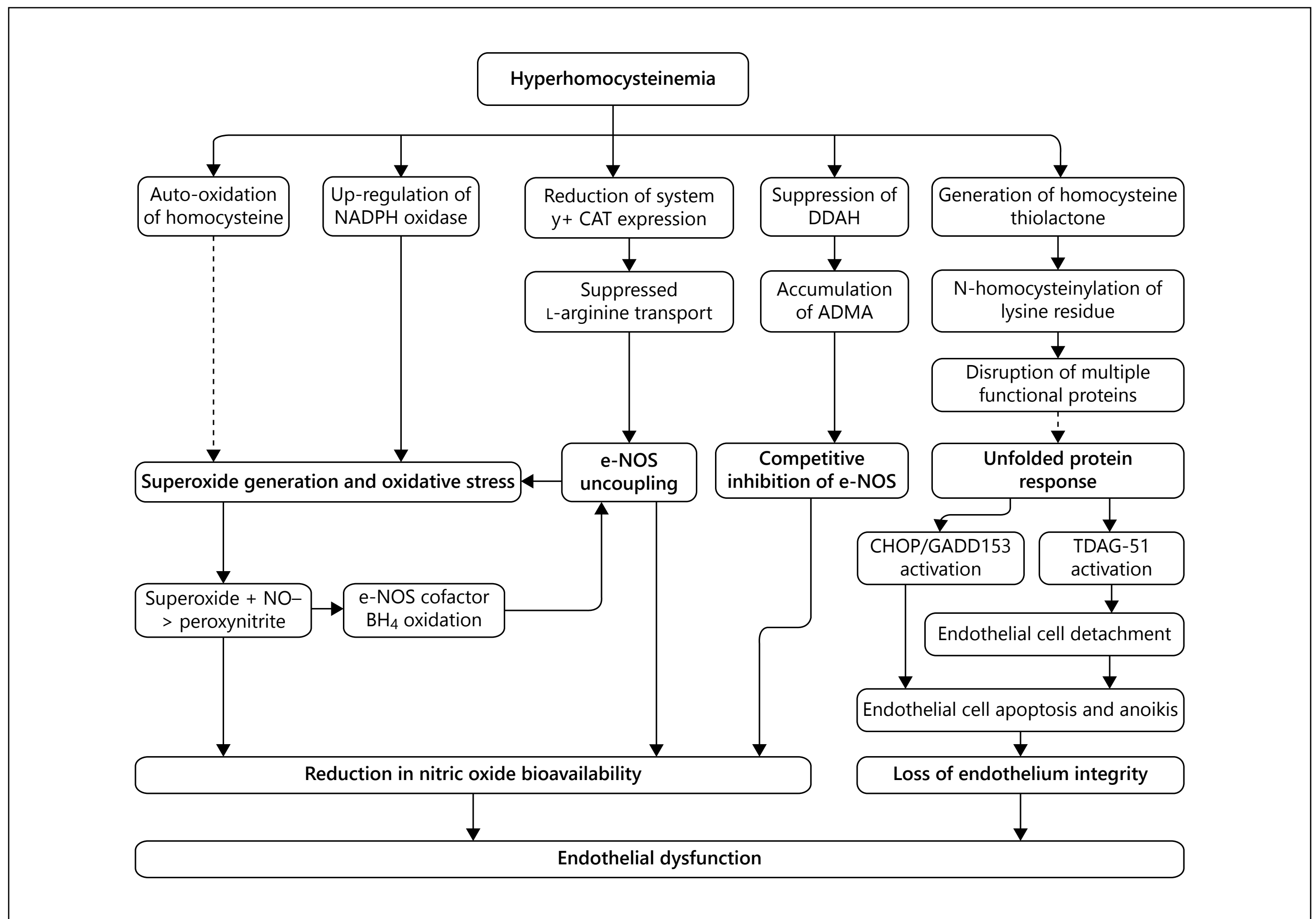


Fig. 6. Potential mechanisms of homocysteine-induced endothelial dysfunction. Multiple pathways involved in homocysteine-induced endothelial dysfunction.

homocysteine-induced impairment in the transportation of NO precursor L-arginine into endothelial cells and up-regulates ROS generation by NADPH oxidase, possibly derailing the NO generation reaction and generating further ROS in a process called e-NOS uncoupling. The vicious cycle of further uncoupling and destruction of e-NOS lead to the reduction of NO bioavailability and inflammation. Homocysteine also degrades the enzyme DDAH and results in an accumulation of e-NOS inhibitor ADMA. In another separate reaction, homocysteine generates homocysteine thiolactone that attacks lysine-rich proteins and potentially trigger ER-stress-related endothelial apoptotic response. All these negative effects of homocysteine on NO bioavailability and endothelial cell viability might help explain some of the pathological effects of homocysteine-induced endothelial dysfunction.

To conclude, understanding the mechanisms by which plasma homocysteine alter endothelial NO production is essential in our comprehension of homocysteine-induced impairment of endothelial-dependent dilatation and the association of cardiovascular risk and its pathophysiology. While a growing body of research has helped to cultivate a foundation of understanding, the knowledge remains incomplete to a large extent. The measurement of endothelial function using FMD method is noninvasive, cheap, and dependable, and thus this technique can open more opportunities for researchers to inspect whether an elevated plasma level of homocysteine is a risk factor or risk marker in further drug intervention and longitudinal projects. New and efficient pharmacological approaches to the prevention and treatment of endothelial dysfunction are also anticipated.

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