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Review

Homocysteine and vitamin therapy in stroke prevention and treatment: a review

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Homocysteine (Hcy), a sulfur amino acid, is the only direct precursor for L-methionine synthesis through a reaction that requires vitamin B_{12}, representing a connection with "one-carbon" units metabolism. Hcy catabolism requires vitamin B_6 and as a consequence, alteration in folic acid and B vitamins status impairs Hcy biotransformation. Numerous studies have indicated that Hcy is an independent risk factor for cardio- and cerebrovascular diseases. In the last decade, several clinical trials have investigated the possible correlation between the use of folic acid and vitamins B_6 and B_{12} for lowering Hcy plasma concentration and the reduced risk of stroke or its recurrence. This review is aimed to present some aspects of Hcy biochemistry, as well as the mechanisms through which it exerts the toxic effects on the vascular endothelium. We also discuss the results of some of the clinical trials developed to investigate the beneficial effects of vitamin therapy in the prevention and management of stroke.

Keywords: homocysteine, stroke, folic acid, vitamin B_6, vitamin B_{12}, vitamin therapy

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INTRODUCTION

Stroke is a major cause of morbidity and mortality worldwide. Many studies indicate a plethora of conventional risk factors for stroke (i.e., hypertension, dyslipidemia, smoking, diabetes mellitus, obesity, and family history). Nevertheless, cerebrovascular events can occur sometimes in individuals without any of the previously mentioned risk factors. As a consequence, it is very likely that other risk factors exist. Hyperhomocysteinemia, defined as an elevated plasma total homocysteine (tHcy) concentration (>10 μM), is one such factor. There is epidemiological evidence suggesting a relationship between hyperhomocysteinemia and increased risk of a broad range of neurodegenerative conditions, including Alzheimer and Parkinson disease, and stroke (Mattson & Shea, 2003; Zou & Banerjee, 2005).

METABOLIC PATHWAYS OF HOMOCYSTEINE

In mammalian cells, Hcy metabolism involves one pathway for biosynthesis and three Hcy consuming pathways (Fig. 1). There is also a strong connection between some of these metabolic pathways and the metabolism of „one carbon" donors like *S*-adenosyl methionine (SAM) and N^5-methyl tetrahydrofolate (N^5-methyl THF).

Demethylation of the essential, diet-derived amino acid methionine (L-Met) represents the only reaction through which Hcy is synthesized in humans. This conversion involves two enzymes, SAM synthetase/L-Met adenosyltransferase (EC 2.5.1.6) and *S*-adenosyl homocysteine (SAH) hydrolase (EC 3.3.1.1). SAM synthetase activates L-Met in a reaction with ATP leading to SAM synthesis. SAM is used as a methyl donor in a variety of cellular biosyntheses (i.e., creatine, epinephrine, carnitine, phospholipids, proteins, nucleic acids and polyamines). Demethylation of SAM during the synthesis of the aforementioned compounds generates SAH, which undergoes hydrolysis mediated by SAH hydrolase. Despite its reversibility, the latter reaction is responsible for SAH hydrolysis *in vivo*, due to the rapid removal of its two products. One consequence of an increased intracellular Hcy concentration is the accumulation of SAH through the reverse reaction catalyzed by SAH hydrolase. When present in excess, SAH acts as an inhibitor for SAM-dependent methylation reactions (Cantoni, 1985).

Resynthesis of L-Met from Hcy occurs *via* two reactions catalyzed by methionine synthase (EC 2.1.1.13) and betaine-Hcy methyltransferase (EC 2.1.1.5). Methionine synthase is one of the two enzymes that use vitamin B_{12} as coenzyme. The second substrate required by methionine synthase is N^5-methyl THF. The reaction catalyzed by this enzyme sets a link between Hcy and "one carbon" units' metabolism (Castro *et al.*, 2006). N^5-Methyl THF is synthesized from $N^{5,10}$-methylene THF through a reaction catalyzed by $N^{5,10}$-methylene THF reductase (MTHFR) (EC 1.1.1.68). This reaction requires NADH, being regulated by SAM and SAH as a negative and positive regulator, respectively.

Hcy is also the source of a highly reactive compound, Hcy-thiolactone. The synthesis of Hcy-thiolactone is associated with the misactivation of Hcy by the methionyl-tRNA synthetase (Jakubowski, 1997; Jakubowski, 2008a).

Liver, kidney and lens cells have the ability to convert Hcy to L-Met through a B_{12}-independent reaction catalyzed by betaine-Hcy *S*-methyltransferase (BHMT) (EC

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Abbreviations: BHMT, betaine-Hcy *S*-methyltransferase; Hcy, homocysteine; HDL, high density lipoprotein; LDL, low density lipoprotein; PON, paraoxonase; tHyc, total homocysteine

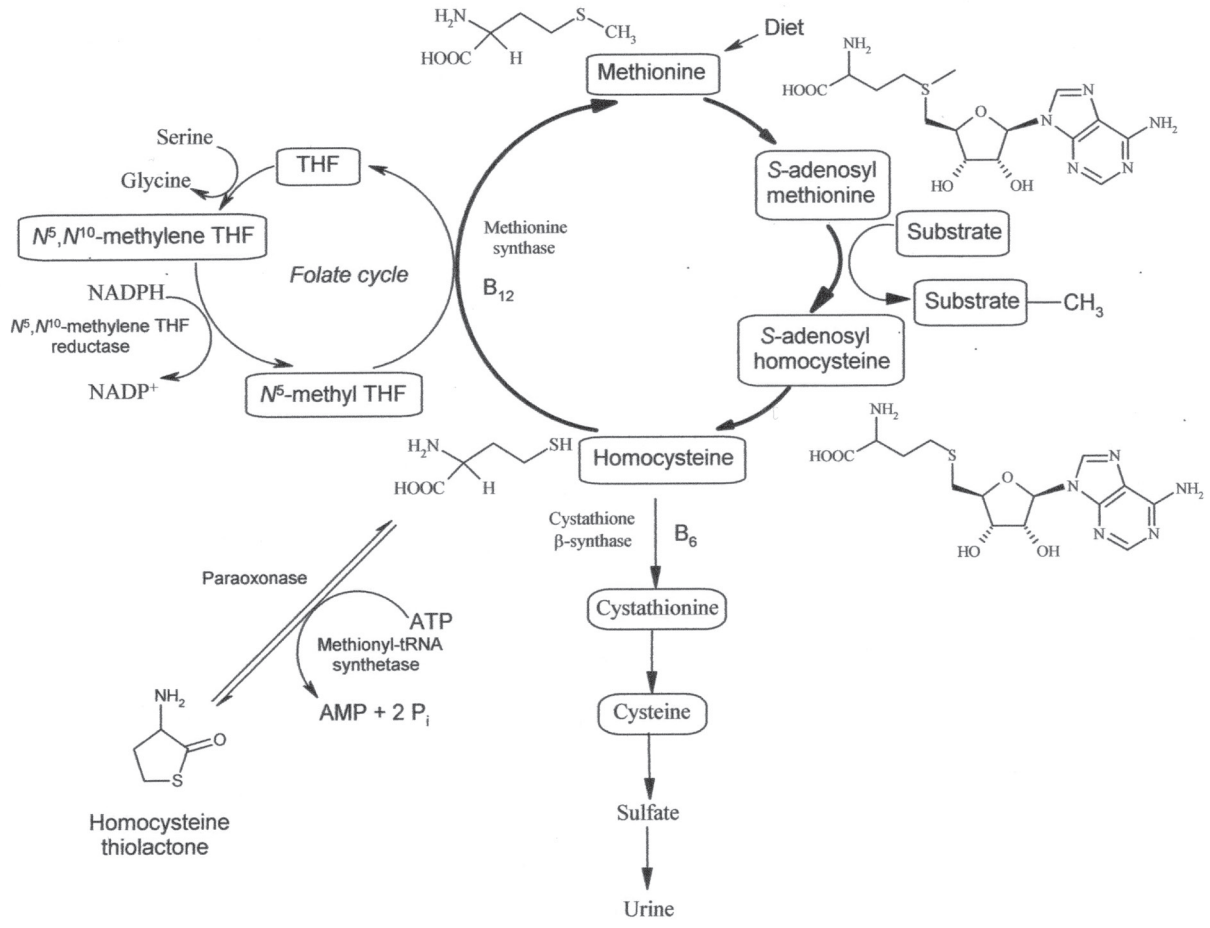

Figure 1. Schematic overview of homocysteine metabolism and its relationship with folic acid and vitamins B_6 and B_{12} (modified from Lentz & Haynes, 2004)

Table 1. Methods in use for total plasma homocysteine assay

Method	Observations	References
Chromatography	Liquid chromatography assays	
	HPLC, fluorescence detection HPLC, UV spectrophotometric detection HPLC, colorimetric detection HPLC, electrochemical detection LC-MS/MS	(Sawula *et al.*, 2008; Dai *et al.*, 2002) (Glowacki & Bald, 2009) (Badiou *et al.*, 2009) (Zhang & Pfeiffer, 2004) (Kuhn *et al.*, 2006)
	Gas chromatography assays	
	GC-MS, electron ionization mode GC-MS, flame photometric detection	(Windelberg *et al.*, 2005) (Kataoka *et al.*, 1995)
Enzymatic assays	assay using cystathionine β-synthase assay using methionine γ-lyase from *E. coli* assay using coupled enzymatic reactions (cystathionine β-lyase, lactate dehydrogenase) homocysteine α,γ-lyase releases H_2S which is converted to a fluorescent chromophore through reaction with *N,N*-dibutyl phenylene diamine	(Martens *et al.*, 2008) (Chan *et al.*, 2005) (Roberts & Roberts, 2004) (Tan & Hoffman, 2008)
Combined assays	conversion of Hcy to SAH followed by binding of SAH to a monoclonal anti-SAH antibody: fluorescence detection (Abbott ImxTM), colorimetric detection using a second antibody coupled with peroxidase (Bio-Rad EIA)	(Donnelly & Pronovost, 2000) (Frantzen *et al.*, 1998)
Capillary electrophoresis	assay using laser induced fluorimetric detection	(Bayle *et al.*, 2002)
Chemosensors	complex of Ir (III); color change from orange to yellow; luminescence change from deep red to green, complex of Pt (II); luminescence change from green to orange	(Chen *et al.*, 2007) (Huang *et al.*, 2009)

Table 2. Methods in use for the assessment of Hcy-thiolactone in different biological samples (modified from Jakubowski, 2006)

Method	Detection system	Characteristics of the method	Reference
HPLC	UV	cation exchange/reverse phase	(Jakubowski, 2002b)
	fluorescence	cation exchange derivatization with OPA	(Chwatko & Jakubowski, 2005)
	fluorescence	reverse phase derivatization with OPA	(Mukai *et al.*, 2002)
GC/MS	negative chemical ionization	derivatization with heptafluorobutyric anhydride	(Daneshvar *et al.*, 2003)

OPA, *o*-phthalaldehyde

2.1.1.5) (Purohit *et al.*, 2007). This reaction requires dietary betain or betain synthesized from dietary or endogenous choline (Stipanuk, 2004). Central nervous system lacks BHMT, being thus dependent on folate and vitamin B_{12} for the conversion of Hcy to L-Met (Sunden *et al.*, 1997).

The main disposal pathway of Hcy is its transsulfuration to L-Cys. This process takes place primarily in the liver, kidney, small intestine and pancreas. The first reaction is a condensation between Hcy and L-Ser leading to cystathionine which is further hydrolyzed to L-Cys and α-ketobutyrate. These two reactions are catalyzed by the B_6-dependent enzymes cystathionine-β-synthase (EC 4.2.1.22) and cystathionine-γ-lyase (EC 4.4.1.1), respectively. α-Ketobutyrate undergoes oxidative decarboxylation to propionyl~CoA, which is converted to succinyl~CoA entering in the Krebs cycle. The transsulfuration pathway is responsible for both L-Met catabolism and sulfur atom transfer from L-Met to L-Ser, yielding L-Cys. L-Cys is a precursor for the synthesis of proteins, coenzyme A, and glutathione. Further catabolism of L-Cys gives rise to taurine and inorganic sulfate, both excreted through urine.

In conclusion, Hcy is central for some metabolic pathways: (i) resynthesis of SAH through reversal of SAH hydrolysis, (ii) remethylation to L-Met, and (iii) conversion to cystathionine.

ASSESSMENT OF HOMOCYSTEINE AND HCY-THIOLACTONE IN BIOLOGICAL SAMPLES

There are several species of plasma Hcy: (i) free Hcy, (ii) protein-bound Hcy (S-linked, and N-linked), (iii) oxidized forms, and (iv) Hcy-thiolactone (Syardal *et al.*, 1986; Jakubowski, 2002a; 2008b). Under physiological conditions, less than 1% of total Hcy is present in a free reduced form. About 10–20% of total Hcy is present in different oxidized forms such as Hcy-Cys and homocystine, the Hcy dimer. The great majority (80–90%) of plasma Hcy is N-linked and S-linked to serum albumin and γ-globulins (Jakubowski, 2002a).

Total plasma Hcy (tHcy) is defined as the pool of free homocysteine, homocystine, Hcy-S-S-Cys disulfide, as well as protein N- and S-linked Hcy. Total Hcy is used as a predictive risk factor for cardiovascular events, the post stroke evolution, screening for inborn errors of methionine metabolism, and as a supplementary test for vitamin B_{12} deficiency.

Hcy can be assessed in blood collected in either the fasting or nonfasting state. A standard dinner (about 50 g protein content) induces a 1.15-fold increase of plasma Hcy concentration (Guttormsen *et al.*, 2004). Total Hcy concentration can be assessed in both plasma and serum samples, despite the fact that the latter give slightly higher values (Rasmussen & Moller, 2000). Plasma has to be isolated very quickly to avoid Hcy leakage from erythrocytes and leukocytes or else blood samples should be treated with a preservative (i.e., sodium fluoride, 3-deazaadenosine, citric acid) (Salazar *et al.*, 1999; Hansrani & Stansby, 2007), albeit preservatives may influence the assay of total Hcy (Refsum *et al.*, 2004).

Assessment of total Hcy concentration requires pretreatment of samples with a reducing agent (i.e., sodium borohydride, dithioerythritol, tris(2-carboxyethyl)-phosphine) to reduce all disulfide bonds involving Hcy. This treatment does not release the N-linked Hcy, i.e. Hcy residues bound to ε-amino groups of protein lysine residues. This can be achieved only by protein hydrolysis in an acidic environment and at high temperature (Jakubowski, 2008b).

The methods used to assess Hcy level in different biological samples can be classified into chromatographic methods, enzymatic assays, and combined assays (enzymatic reaction followed by an immunoassay) (Refsum *et al.*, 2004). Plasma tHcy concentration can also be assessed through capillary electrophoresis with a suitable detection system. Very recently, assays based on addition of transition-metal complexes to carbonyl groups were developed (Chen *et al.*, 2007; Hung *et al.*, 2009). Most of the chromatographic methods require sample deri-

Table 3. Methods in use for the assessment of protein-linked Hcy

Method	Derivatization agent	Detection system	Reference
HPLC (C_{18} RP)	2-chloro-1-methylpyridinium iodide	UV	(Bald *et al.*, 2000)
HPLC (C_{18} RP)	4-fluoro-7-sulfamoyl-benzofurazan	Fluorescence	(Uji *et al.*, 2002)
HPLC (CE)	–	UV multiwavelength	(Jakubowski, 2002a)
HPLC (CE)	*o*-phthaldialdehyde	Fluorescence	(Jakubowski, 2008b)
HPLC	7-fluoro-1,3-benzoxadiazole-4-sulfonate	Fluorescence	(Hortin *et al.*, 2006)
HPLC	7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate	Fluorescence	(Perna *et al.*, 2006)

RP, reverse phase; CE, cation exchange

Table 4. Causes of increased plasma tHcy (modified from Lentz & Haynes, 2004; Brosnan *et al.*, 2004; Pezzini *et al.*, 2007; Bottiglieri, 2005)

Plasma Hcy concentration	Deficiencies
Severe hyperhomocysteinemia (>100 μM)	genetic deficiency of cystathionine β-synthase (Hcy≈500 μmol/L) genetic deficiency of $N^{5,10}$-methylene tetrahydrofolate reductase genetic defect impairing vitamin B_{12} absorption
Moderate hyperhomocysteinemia (30–100 μM)	dietary moderate vitamin B_{12} deficiency dietary severe folate deficiency genetic deficiency of $N^{5,10}$-methylene tetrahydrofolate reductase renal failure atrophic gastritis medication (nitric oxide, L-DOPA)
Mild hyperhomocysteinemia (10–30 μM)	genetic deficiency of $N^{5,10}$-methylene tetrahydrofolate reductase (polymorphism C677T) dietary vitamin B_6 deficiency diabetes, hypothyroidism, malignancies, atrophic gastritis, renal transplantation daily habits (smoking, alcohol and coffee consumption) medication (niacin, fibrates, methotrexate, isoniazid, levodopa, theophylline, phenytoin, nitric oxide, trimethoprim, anticonvulsants, metformine, azauridine, androgens, cyclosporin A)

vatization. The derivatization is made either before the passage of the sample through the column, or after this step. The derivatization agents are classified depending on the detection system used: (1) 2-chloro-1-methylpyridinium iodide (UV spectrophotometric detection), (2) ninhydrin, 2-chloro-1-methylquinolinium tetrafluoroborate (visible spectrophotometric detection), (3) monobromobimane, *o*-phthaldialdehyde, halogensulfonyl benzofurazans, iodoacetamidofluoresceine (spectrofluorimetric detection) (Bald *et al.*, 2000; Ubbink, 2000; Fermo & Paroni, 2000; Bayle *et al.*, 2002; Glowacki & Bald, 2009; Ichinose *et al.*, 2009). Alternatively, an electrochemical detection system, not needing derivatization, can be used (Martin *et al.*, 1999; Houze *et al.*, 2006). Table 1 summarizes aspects concerning the methods used to assess total plasma Hcy concentration. Different methods are available for the assessment of Hcy-thiolactone in biological samples (plasma, urine) (Table 2). The assessment of protein-linked Hcy requires also a different approach (Table 3).

DETERMINANTS OF PLASMA tHcy CONCENTRATIONS

Plasma tHcy levels are influenced by genetic, physiologic (age, sex) and lifestyle factors, and various pathologic conditions (De Bree *et al.*, 2002). The physiological range of plasma total Hcy is defined as the 2.5th to 97.5th percentile interval for the analyzed group (Lentz & Haynes, 2004). This corresponds to a lower limit of 5 μM, while the upper limit varies considerably between laboratories and different populations. As in the case of other biochemical parameters, for Hcy also its own reference interval should be established taking into account the influence of different non-modifiable and modifiable factors. The relationship between different factors and plasma Hcy concentration is beyond the aim of this review as there are several excellent reviews dealing with this subject (De Bree *et al.*, 2002; Pezzini *et al.*, 2007).

HYPERHOMOCYSTEINEMIA

The term "hyperhomocysteinemia" does not define a pathological condition. It is rather used to describe a biochemical abnormality which can be a direct consequence of various pathological conditions. Plasma Hcy concentration could represent a gradual indicator of the risk for cardiovascular disease (Lentz & Haynes, 2004). Thus, every increase of 2.5 μM in plasma Hcy can be associated with an increase of stroke risk of about 20% (Clarke *et al.*, 2002). Moreover, plasma tHcy levels above 20 μM are associated with a nine-fold increase of the myocardial infarction and stroke risk when compared to concentrations below 9 μM (Nygard *et al.*, 1997).

Certain drugs (adenosine analogues, D-penicillamine, *N*-acetylcysteine, estrogens, tamoxifen, betaine) decrease blood Hcy concentration through different mechanisms (i.e., remethylation stimulation, inhibition of SAH hydrolase activity) (Pezzini *et al.*, 2007).

MECHANISMS OF HOMOCYSTEINE TOXICITY

Over the years, several theories concerning the toxicity of Hcy have been elaborated, but despite the efforts, none does to completely explain the toxicity of this compound.

One of the first hypotheses suggested that hydrogen peroxide formed as a byproduct in metal ion-catalyzed redox reactions involving the thiol group of Hcy was responsible for the toxicity of this compound. The major drawback of this hypothesis was the fact that L-Cys is not a risk factor for vascular disease, despite its 20- to 30-fold higher concentration than that of Hcy (Jacobsen, 2000). Moreover, the amount of hydrogen peroxide produced with the participation of Hcy is very low and its formation is possible only in the presence of transition metal ions as catalysts (Zappacosta *et al.*, 2001).

A more recent theory suggests that Hcy toxicity is a consequence of covalent binding of this compound to proteins, thus modifying their functions (Fig. 2). The process is called homocysteinylation and can be either S-homocysteinylation, when Hcy binds through a disulfide bond to a free protein sulfhydryl residue, or N-homocysteinylation, when Hcy binds through an amide bond to an ε amino group of a protein lysine residue.

Hcy binds through a disulfide bond to fibronectin, annexin II, and intracellular metallothionein, among other intracellular proteins (Majors *et al.*, 2002; Hajjar *et al.*, 1998; Barbato *et al.*, 2007). S-Homocysteinylation of the first two proteins results in inhibition of fibronectin-fibrin interaction and annexin II–tissue plasminogen activator interaction, respectively. One of the plasma proteins that is subject to modification through homocysteinylation is transthyretin (Hanyu *et al.*, 2009). This modification could be responsible for the denaturation and deposition of this protein, as seen in familial amyloid polyneuropathy. Homocysteinylation of metallothionein

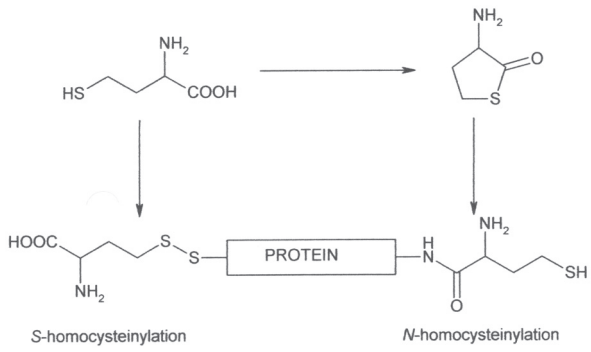

Figure 2. *S*- and *N*-homocysteinylation of proteins by homocysteine and homocysteine thiolactone, respectively

could be responsible for redox imbalance. Generally, S-homocysteinylation alters the function of biomolecules through: (i) inactivation of potentially active free thiol groups, and (ii) alteration of redox potential of a protein molecule.

N-Homocysteinylation is a consequence of the high reactivity of Hcy-thiolactone towards free amino functions, especially the ε amino group of lysine residues from proteins (Jakubowski, 1997; 1999). *In vivo*, Hcy-thiolactone targets and modifies blood albumin, hemoglobin, immunoglobulins, LDL, HDL, transferrin, antitrypsin, and fibrinogen (Jakubowski, 2002a; 2008a). Also, Hcy-thiolactone acts as an inhibitor of Na^+/K^+-ATP-ase from the cortex, hippocampus, and brain stem of rats, affecting the membrane potential with deleterious effects for neurons (Rasic-Markovic *et al.,* 2009).

Elevated plasma levels of both Hcy-thiolactone and proteins modified through N-homocysteinylation are a direct consequence of either genetic defects in Hcy metabolism or a methionine-rich diet (Brosnan *et al.*, 2004; Jakubowski *et al.,* 2009). Spiroski *et al.* (2008) showed that methylenetetrahydrofolate reductase (*MTHFR-677* and *MTHFR-1298*) genotypes and haplotypes can be correlated with increased tHcy plasma levels in patients with occlusive artery disease and deep venous thrombosis.

Plasma concentration of proteins modified through N-homocysteinylation ranges between 0.35 and 0.51 μM (Jakubowski *et al.,* 2008). However, in pathological conditions the plasma concentration of these proteins ranges from a two-fold elevation (renal failure) up to a 24–30-fold elevation (genetic defects of *MTHFR* and cystathionine β-synthase) (Perna *et al.*, 2006).

Generally, N-homocysteinylation alters the function of proteins through (i) introduction of new free thiol groups, and (ii) inactivation of free amino groups, affecting the overall redox potential of proteins. Moreover, it has been found that proteins modified through N-homocysteinylation can act as neoantigens, triggering activation of the inflammatory response, a key component in atherogenesis, atherothrombosis and stroke etiology (Undas *et al.*, 2004). Also, these neoantigens induce an autoimmune response, the concentration of autoantibodies being higher in some pathological conditions (i.e., cerebrovascular disease, renal failure) than in normal subjects (Undas *et al.*, 2007). This situation could explain, at least in part, the association of hyperhomocysteinemia with certain stroke subtypes such as small vessel disease, large vessel disease, and primary intracerebral hemorrhage (Eikelboom *et al.*, 2000; Li *et al.*, 2003; Perini *et al.*, 2005). It has been hypothesized that N-homocysteinylated proteins exposed at the luminal face of endothelial cells are recognized by specific antibodies, the neoantigen-autoantibody interaction leading to activation of circulating macrophages, which become responsible for repeated vascular endothelium damage. Moreover, Hcy-thiolactone impairs the ability of the vascular endothelium to regenerate itself through direct inhibition of lysyl oxidase, which is responsible for the correct cross-linking of collagen and elastin in the arterial wall (Liu *et al.*, 1997; Raposo *et al.*, 2004).

One mechanism of protection from N-homocysteinylation is through the action of the enzyme called paraoxonase 1 (PON1, EC 3.1.8.1). PON1, a calcium-dependent enzyme synthesized by the liver, is a major component of plasma HDL particles, responsible for the antioxidant protection of LDL particles. For PON1, three enzymatic activities have been described: paraoxonase, arylesterase and lactonase (Billecke *et al.*, 2000). The paraoxonase activity is the ability of the enzyme to hydrolyze different organophosphate compounds (paraoxon, diazoxon, soman, sarin). The arylesterase activity is evaluated using phenylacetate as a substrate, while lactonase activity is evaluated using different aromatic lactones (i.e., dihydrocoumarin, 2-coumaranone, homogentisic acid lactone). It is believed that under physiological conditions, the lactonase activity prevails, one of the enzyme's *in vivo* substrate being Hcy-thiolactone. PON1 has the ability to hydrolyze Hcy-thiolactone, this activity being a major determinant of plasma N-homocysteinylated protein concentration (Jakubowski, 2000; Perla-Kajan & Jakubowski, 2010). It was found that both *in vitro* and *in vivo* the lactonase activity of PON1 negatively correlates with N-homocysteinylated protein concentration. Impairment of the PON1 Hcy-thiolactonase activity is responsible, at least in part, for the severity of cardiovascular disease in subjects with coronary artery disease (Koubaa *et al.*, 2009). Thus, due to the lactonase/Hcy-thiolactonase activity of PON1, HDL particles could prevent post-translational modification of LDL apoproteins through N-homocysteinylation.

Another report suggests that Hcy exerts its toxicity through induction of endoplasmic reticulum (ER) stress. Increased intracellular Hcy concentration is associated with both alteration of redox balance and post-translational protein modifications through N- and S-homocysteinylation. ER is very sensitive to intracellular Hcy or Hcy metabolites' accumulation due to a local redox imbalance and Ca^{2+} release. As a consequence of the local redox imbalance, proteins misfold, initiating an ER stress response (Chigurupati *et al.*, 2009) that triggers the expression of different chaperone proteins, growth arrest and apoptosis (Outinen *et al.*, 1999; Zhang *et al.*, 2001). Intracytoplasmic accumulation of calcium has deleterious effects leading to excessive ATP consumption as cells try to pump Ca^{2+} out, and also activates a series of enzymes such as calpain, phospholipase A_2, nitric oxide synthase etc.

The effect of high plasma Hcy concentration on gene expression is dual. Transcription of the gene coding for glutathione peroxidase-1 (GPx1) is down regulated by Hcy (Lubos *et al.*, 2007). Moreover, Hcy downregulates endothelial heparane sulfate with subsequent decrease of extracellular membrane-bound superoxide dismutase present at the luminal face of the endothelium and as a consequence, the endothelium becomes exposed to deleterious effects of $O_2^{\bullet-}$ (Yamamoto *et al.*, 2000). Other studies suggest that Hcy induces the expression of superoxide dismutase in endothelial cells leading to con-

sumption of NO• and thus impairing endothelial vasorelaxation (Hucks *et al.*, 2004).

HOMOCYSTEINE AS AN AGGRESSION FACTOR AGAINST THE ENDOTHELIUM

Hcy is an important endothelial aggression factor promoting endothelial dysfunction through different mechanisms, leading to atherosclerotic plaque formation. Hcy (i) inhibits the growth of endothelial cells, (ii) induces an imbalance between $O^{\bullet-}$ and NO• with deleterious effects on the vascular physiology, (iii) induces the expression of different adhesion molecules, and (iv) promotes the formation of modified LDL particles, which are important players in the etiology of atherosclerotic plaque formation (Fig. 3).

Hcy activates, through different pathways, NAD(P)H oxidase and xanthine oxidase, two important pro-oxidant enzymes that generate $O_2^{\bullet-}$ in the endothelial cells.

Using human umbilical vein endothelial cells (HUVEC) it was found that Hcy activates the NADPH oxidase complex through plasma membrane translocation of the cytoplasmic subunit $p47^{phox}$ (Carluccio *et al.*, 2007). The fact that Hcy-dependent activation of NAD(P)H oxidase plays a central role in endothelial redox imbalance was proved by the ability of the NAD(P)H oxidase inhibitor apocynin which restores both $O_2^{\bullet-}$ concentration and vascular tonus to control Hcy levels in hyperhomocysteinemic rats (Edirimanne *et al.*, 2007).

Another important source of $O_2^{\bullet-}$ in the vascular endothelium is xanthine oxidase (Bagi *et al.*, 2002). Administration of the xanthine oxidase inhibitor oxypurinol prevented flow-induced constriction of arterioles from rats with hyperhomocysteinemia.

$O_2^{\bullet-}$ can interact with NO•, synthesized by the constitutive endothelial nitric oxide synthase (eNOS), producing the peroxynitrite anion ($ONOO^-$). Consumption of NO• through this reaction is responsible for alteration of the vascular tonus. Moreover, $ONOO^-$ is responsible for the induction of thromboxane A_2 synthesis in both endothelial cells and platelets, leading to vasoconstriction (Ungvari *et al.*, 2000; Bagi *et al.*, 2002). This is probably achieved through peroxynitrite-dependent inactivation of prostaglandin I_2 synthase, arachidonic acid becoming thus available for thromboxane A_2 synthesis (Zou *et al.*, 1999).

Free radicals induce the conversion of tetrahydrobiopterin (BH_4), one of the eNOS cofactors, to trihydrobiopterin ($BH_3^{\bullet}$) leading to eNOS uncoupling (Patel *et al.*, 2002). There is evidence for a direct involvement of Hcy in free radical-induced eNOS uncoupling (Topal *et al.*, 2004). Decreased bioavailability of BH_4 for eNOS changes the activity of this enzyme from NO• to $O_2^{\bullet-}$ production. Moreover, in animal models for hyperhomocysteinemia, administration of an $O_2^{\bullet-}$ scavenger (e.g., superoxide dismutase, 4,5-dihydroxybenzene 1,3-disulfonate) reverses endothelial dysfunction, suggesting that at least in part, the Hcy-dependent endothelial dysfunction is mediated by $O_2^{\bullet-}$ (Weiss *et al.*, 2003).

The plasma concentration of ADMA (N^{ω},N^{ω}-dimethyl-L-arginine), an endogenous inhibitor of NOS isoforms, is controlled by two dimethylarginine dimethylaminohydrolases (DDAH-1 and DDAH-2), both hydrolyzing ADMA to L-citrulline and dimethylamine. The Hcy-NO• adduct is a selective inhibitor for the human DDAH-1, preventing ADMA hydrolysis and leading to inhibition of NOS enzymes (Hong & Fast, 2007).

Moreover, Hcy-induced overproduction of $O_2^{\bullet-}$ is responsible, at least in part, for the activation of the nuclear factor κB (NF-κB), a key regulator of gene expression (Au-Yeung *et al.*, 2004). NF-κB induces the expression of genes coding for pro-inflammatory proteins like monocyte chemoatractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, and receptor for advanced glycation end products (RAGE) in endothelial cells (Poddar *et al.*, 2001; Hofmann *et al.*, 2001; Carluccio *et al.*, 2007; Hwang *et al.*, 2008). Thus, Hcy enhances monocyte binding to vascular endothelium, a key event in the process of atherosclerotic plaque formation. *In vitro* studies using endothelial cells, monocytes and macrophages exposed to different concentrations of Hcy for different times, have revealed different degrees of induc-

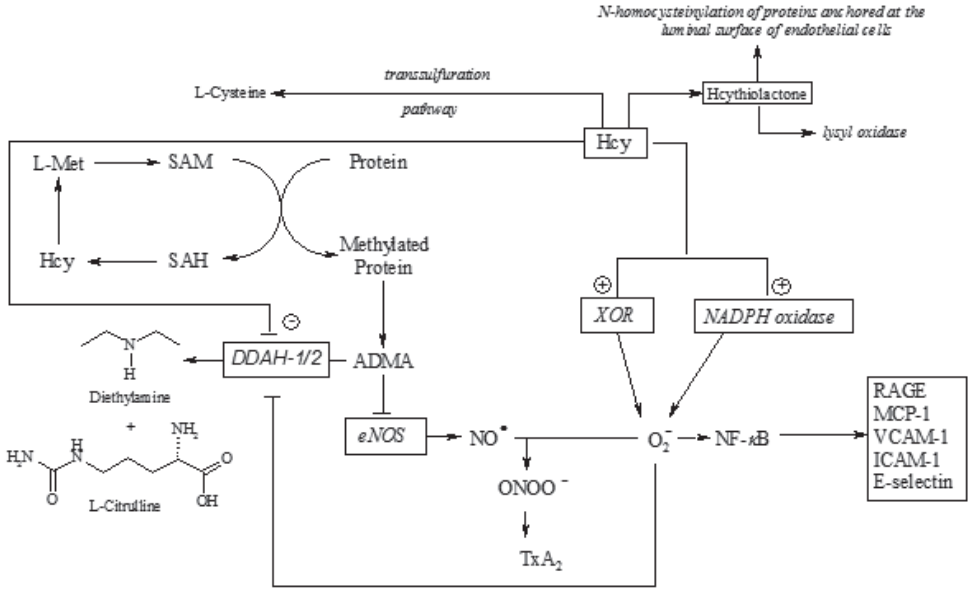

Figure 3. Some of the biological effects of Hcy at endothelial level.

tion of pro-inflammatory cytokines (MCP-1, IL-1β, IL-6, IL-8, and TNF-α) (Dalal *et al.*, 2003; Zeng *et al.*, 2003). Moreover, Hcy induces selective differentiation of Ly-6C^{hi} and Ly-6C^{mid} inflammatory monocyte subsets, followed by their retention in atherosclerotic lesions (Zhang *et al.*, 2009). Also, the interaction between Hcy-activated endothelial cells and monocytes leads to increased expression of CD36 scavenger receptor by monocytes (Thampi *et al.*, 2008). This process could represent another link between hyperhomocysteinemia and atherosclerotic plaque formation and progression.

A metabolic peculiarity of endothelial cells that renders them very susceptible to Hcy toxicity is their inability to metabolize Hcy through the transsulfuration pathway (Jacobsen *et al.*, 1995). Hcy alters gene expression in endothelial cells through different mechanisms: (i) inhibition of the transcription of the *cyclin A* gene, (ii) inhibition of cyclin A messenger RNA expression, and (iii) transcriptional repression of the gene coding for FGF2 (fibroblast growth factor 2) (Wang *et al.*, 2002; Jamaluddin *et al.*, 2007; Chang *et al.*, 2008). These effects are the result of alteration of the methylation patterns of the promoters of the *cyclin A* and *FGF2* genes.

In vitro studies suggested an additional mechanism for Hcy toxicity through post-translational modifications of LDL particles. Hcy promotes both oxidation of LDL particles by ceruloplasmine's oxidase activity, and nitration (Exner *et al.*, 2002; Griffiths *et al.*, 2006). Modified LDL particles are highly pro-aterogenic promoting the formation of foam cells through uptake by scavenger receptors expressed by monocytes.

Finally, another mechanism that in conjunction with the aggression effect upon endothelium could explain the proatherosclerotic effect of Hcy and Hcy-thiolactone is the ability of the latter compound to covalently modify serum fibrinogen. It was found that clots containing N-homocysteinylated fibrinogen are more resistant to fibrinolysis, leading to increased risk of thrombotic events (Jakubowski *et al.*, 2008; 2009).

These processes could provide an important link between hyperhomocysteinemia and atherosclerotic aggression on endothelium.

HOMOCYSTEINE AS A NEUROTOXIN

Both *in vitro* and *in vivo* studies have indicated that Hcy exerts neurotoxic effects inducing neuronal damage and cell loss through excitotoxicity and apoptosis. This could be, at least in part, a direct consequence of the inability of cerebral tissue to metabolize Hcy through the betaine and transsulfuration pathways, favoring Hcy accumulation in the nervous system (Finkelstein, 1998).

Stroke patients are a particular risk group as disruption of the blood-brain barrier exposes nervous tissue to plasma Hcy concentration for prolonged periods (Lindgren *et al.*, 1995). Moreover, it was found that Hcy itself is able to induce blood-brain barrier (BBB) disruption (Kamath *et al.*, 2006). The disruption of BBB is the consequence of at least two different processes. On the one hand, Hcy induces an imbalance between the activity of matrix metalloproteinase 9 (MMP-9) and the level of tissue inhibitor of metalloproteinase-4 (TIMP-4), through increasing MMP-9 activity and decreasing TIMP-4 level (Moshal *et al.*, 2006; Tyagi *et al.*, 2009). As a consequence, MMP-9 acts upon different components of the BBB leading to disruption of this structure. On the other hand, Hcy acts as an excitatory neurotransmitter through γ-aminobutyric acid (GABA) receptors A/B, leading to increased vascular permeability (Tyagi *et al.*, 2005; 2007).

Hcy acts as an agonist for both group I and group III metabotropic glutamate receptors as well as for *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainite ionotropic glutamate receptors (Boldyrev and Johnson, 2007; Bleich *et al.*, 2004; Ho *et al.*, 2002). Overstimulation of these receptors triggers cytoplasmic calcium pulses, overproduction of free radicals, and activation of caspases leading to apoptosis. Using spontaneously hypertensive stroke-prone rats, it was found that exposure of nervous tissue to Hcy during stroke is followed by secretion of excitatory amino acids, i.e., glutamate and aspartate, which are responsible for further excitotoxic effects on neurons (Ganguly *et al.*, 2008). Activation of neuronal ERK2 (extracellular signal-regulated kinase 2) is a direct consequence of Hcy-induced NMDA receptor stimulation (Poddar & Paul, 2009). Also, after a transient activation of the pro-survival transcription factor CREB (cAMP response-element binding protein) by ERK2, CREB becomes inhibited, favoring neuronal apoptotic death.

Expression of NMDA receptor is not confined to neurons. Other cells, including endothelial cells from cerebral tissue, can express this receptor. Free radicals induce up-regulation of the NR1 subunit of the NMDA receptor, increasing the susceptibility of cerebral endothelial cells to excitatory amino acids, favoring BBB disruption (Betzen *et al.*, 2009).

Another consequence of the nervous tissue exposure to Hcy is the activation of poly-ADP-ribose polymerase (PARP) in neurons leading to consumption of ATP and NAD^+ with deleterious effects upon energetic metabolism, followed by free radical production (Kruman *et al.*, 2000). It was also found that Hcy is able to inhibit neuronal DDHA activity leading to accumulation of ADMA with subsequent inhibition of neuronal NOS (Selley, 2004).

Not only neuronal cells are subject to toxic effects of Hcy, but also microglia, which are brain-resident macrophages. Hcy promotes activation of these cells through induction of NAD(P)H oxidase (Zou *et al.*, 2009). Also, *in vivo* studies indicated that Hcy exerts toxic effects on endothelial cells from cerebral vasculature through mechanisms previously presented (Dayal *et al.*, 2004). Another consequence of hyperhomocysteinemia is represented by Hcy-induced toxic effect on endothelial progenitor cells (Alam *et al.*, 2009). Exposure of these cells to high levels of Hcy is followed by induction of their apoptosis through caspase-3 and -8 activation and cytochrome *c* release from mitochondria.

HOMOCYSTEINE, STROKE AND VITAMIN THERAPY

Different clinical trials have recognized Hcy as an independent cardio- and cerebrovascular risk factor. One such trial enrolled 5661 monitored in time (Perry *et al.*, 1995). During the follow-up period 141 cerebrovascular events (stroke) were recorded. Using a study group of 107 from among those that developed stroke and a control group of 118, it was found that the geometric mean of plasma Hcy concentration was significantly increased in the study group (13.7 μM *vs.* 11.9 μM, $P=0.004$).

Due to the dependence of Hcy metabolism on folic acid and vitamins B_6 and B_{12} it was suggested that administration of these vitamins could reduce plasma Hcy concentration, lowering the risk of stroke and stroke re-

currence. Indeed, many trials have indicated that administration of folic acid and vitamins B_6 and B_{12} decreased serum Hcy concentration. However, some of these studies failed to show a direct correlation between vitamin intake and lowered risk for cardio- and cerebrovascular events (Clarke *et al.*, 2002). Some potential causes responsible for the lack of consensus between these trials could be (i) differences between study groups in respect to baseline Hcy concentrations, (ii) inclusion of patients from countries that do not have regulations regarding food enrichment with folic acid, (iii) trial period, (iv) composition of vitamin formulations, (v) gender distribution, and (vi) patients' medication. Also, patients' compliance is an important factor for the success of trials that investigate the impact of some drugs on the course of a pathological condition.

The VISP (Vitamin Intervention for Stroke Prevention) trial (double-blind, randomized, controlled study) showed no significant reduction in stroke recurrence between the group that received a low-dose vitamin formulation (200 μg B_6, 6 μg B_{12}, 20 μg folic acid) and the group that received a high-dose vitamin formulation (25 mg B_6, 0.4 B_{12}, 2.5 mg folic acid) ($P=0.80$). There was also a modest difference in the reduction of Hcy plasma level between the two groups (2 μM) (Toole *et al.*, 2004). One explanation for the failure of that study was the very low baseline level of plasma B_{12} vitamin. Excluding the patients that received monthly vitamin B_{12}, the patients with high plasma vitamin B_{12} concentration (> 637 pmol/L) and those with renal failure generated a new group of 2155 persons. Statistical analysis performed with this new group indicated that stroke incidence in the new high-dose vitamin formulation group was almost 21% lower than in the other group, suggesting a beneficial role of high doses of vitamin B_{12} ($P=0.049$) (Spence *et al.*, 2005). At the same time, it was noted that a better capacity of vitamin B_{12} absorption associated with the high-dose vitamin B_{12} therapy decreased the incidence of cerebrovascular events.

HOPE-2 (Heart Outcomes Prevention Evaluation 2) and NORVIT (Norwegian Vitamin) trials indicated a beneficial effect of folic acid and vitamins B_6 and B_{12} on plasma Hcy concentration (Bønaa *et al.*, 2006; Saposnik *et al.*, 2009). One major difference between the HOPE-2 and NORVIT trials on the one hand and VISP trial on the other was their duration. The follow-up period for the first two studies was 5 years and 3.5 years, respectively, while the follow-up period for the VISP trial was 2 years.

The HOPE-2 trial indicated that the overall stroke incidence was lower in the vitamin group when compared to the placebo group ($P=0.03$, Saposnik *et al.*, 2009). Also the incidence of the two main types of stroke was lower in the vitamin group when compared to the control group (ischemic stroke, 30.62% *vs.* 37.98%; hemorrhagic stroke, 3.1% *vs.* 3.87%). By contrast, full recovery at day 7 or at discharge was more frequent, but not significantly, in the vitamin group (vitamin group *vs.* placebo group, 15.7% *vs.* 9.6%). In the period 24 h from the moment of stroke onset there was no significant improvement of the neurological deficit between the two groups. The results of HOPE-2 trial indicated that vitamin therapy could be beneficial for some particular categories: persons younger than 70 years (untreated dyslipidemia, no medication for coagulopathies), patients with hyperhomocysteinemia, and people from countries where there is no regulation regarding food enrichment with folic acid.

Another conclusion of the VISP, HOPE-2 and NORVIT trials was that correction of vitamin B_{12} status in the elderly, in conjunction with folic acid therapy, could decrease stroke risk.

A recent meta-analysis study of several prospective trials from 2007 until 2009 has offered some possible directions for future research in this area (Lee *et al.*, 2010). It was found that Hcy-lowering therapy could be beneficial in patients with vascular disease in early stages. Also, such an approach could be more successful in men than in women. This study suggested that only clinical trials with follow-up periods of 3 years or more report decreased stroke incidence, probably because the biological effect only then becomes apparent.

The supplementation of grain products with folic acid was implemented mandatory by 1998 in the USA and Canada. If high homocysteine concentrations are an independent risk factor for stroke, one might expect a reduction in stroke mortality following folic acid fortification. Indeed, a recent population-based study showed that the decline in stroke mortality accelerated in 1998–2002 in nearly all population in the USA and Canada (Wang *et al.*, 2007).

CONCLUSIONS

Despite the beneficial effects of vitamin therapy on plasma Hcy concentration, there are no unequivocal data regarding the impact of lowering Hcy level on the incidence and/or recurrence of cerebrovascular events. This situation could be solved by using larger population trials. Also, the trial period could be an important factor for the success of such studies, enabling the beneficial effects to become apparent. Stroke incidence and recurrence should be investigated in relation with gender, as men have higher incidence of cerebrovascular events than women, along with a plethora of secondary pathologies. Another issue that should be better addressed is the subclinical vitamin B_{12} deficiency that affects a large number of elderly people. Correction of vitamin B_{12} status, along with administration of the other vitamins required for Hcy metabolism could lead to a decrease in the frequency of cerebrovascular events.

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