Mechanisms of homocysteine toxicity in humans

Review Article

J. Perła-Kaján[1,*], **T. Twardowski**[1], and **H. Jakubowski**[1,2]

[1] Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland
[2] UMDNJ-New Jersey Medical School, Newark, NJ, U.S.A.

Summary. Homocysteine, a non-protein amino acid, is an important risk factor for ischemic heart disease and stroke in humans. This review provides an overview of homocysteine influence on endothelium function as well as on protein metabolism with a special respect to posttranslational modification of protein with homocysteine thiolactone. Homocysteine is a pro-thrombotic factor, vasodilation impairing agent, pro-inflammatory factor and endoplasmatic reticulum-stress inducer. Incorporation of Hcy into protein via disulfide or amide linkages (S-homocysteinylation or N-homocysteinylation) affects protein structure and function. Protein N-homocysteinylation causes cellular toxicity and elicits autoimmune response, which may contribute to atherogenesis.

Keywords: Homocysteine – Homocysteine thiolactone – Protein N-homocysteinylation – Toxicity – Autoantibodies – Protein S-homocysteinylation

Abbreviations: APC, activated protein C; BLH, bleomycin hydrolase; ER, endoplasmatic reticulum; HDL, high density lipoprotein; HTL, homocysteine thiolactone; LDL, low density lipoprotein; MetRS, methionyl-tRNA synthetase; MS, methionine synthase; PON, paraoxonase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TPA, tissue plasminogen activator; UPR, unfolded protein response; VEGF, vascular endothelial growth factor

Introduction

Hyperhomocysteinemia has been recognized as a risk factor for a number of human diseases including cardiovascular diseases (Anderson et al., 2000; Cavalca et al., 2001; Knekt et al., 2001), stroke (Yoo and Lee, 2001), peripherial arterial occlusive disease (Kang et al., 1992) and ve-

nous thrombosis (den Heijer et al., 1996). Elevated level of homocysteine (Hcy) plays also an important role in neural tube defects (Mills et al., 1996), the development of pregnancy complications (Nelen et al., 1997) and neurodegenerative diseases (Seshadri et al., 2002). Cardiovascular diseases are a major cause of mortality in developed countries. In recent years a number of studies were undertaken to understand homocysteine metabolism and mechanisms of its toxicity. Studies that provide insight into the metabolic pathways of homocysteine, regulation strategies and negative effects of elevated level of homocysteine, are crucial for the development of a new diagnostic and therapeutic methods.

Homocysteine is involved in convertions of methionine (Met) and cysteine (Cys) (Fig. 1). The immediate precursor of homocysteine is S-adenosylhomocysteine (SAH), which is hydrolyzed by SAH hydrolase (EC 3.3.1.1) to homocysteine and adenosine. In the next step homocysteine is remethylated to methionine by methionine synthase (EC 2.1.1.13) or betaine:homocysteine methyltransferase (EC 2.1.1.5). The first step of transmethylation reactions is the activation of methionine to S-adenosylmethionine (SAM) catalyzed by methionine adenosyltransferase (EC 2.5.1.6). Methyl group of SAM is subsequently transferred onto acceptor molecule and SAH is formed.

Homocysteine enters the transsulfuration pathway and is converted into cysteine by cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1). The transsulfuration pathway is present only in the liver, kidney, pancreas and small intestine (Brosnan et al., 2004).

* Present address: Department of Biochemistry and Biotechnology, Agricultural University, 60637 Poznań, Poland; E-mail: asiape1@yahoo.com
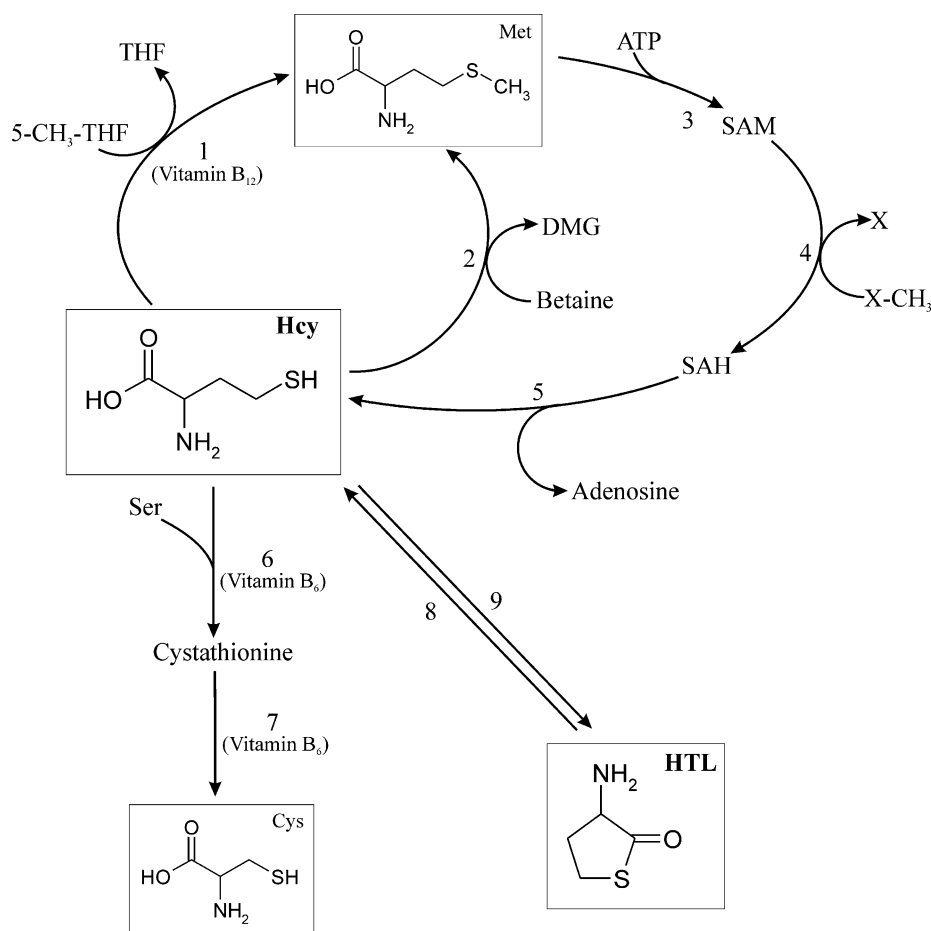
Fig. 1. Homocysteine metabolism. *1* – Methionine synthase, *2* – Betaine:homocysteine methyltransferase, *3* – Methionine adenosyltransferase, *4* – Methyltransferase, *5* – SAH hydrolase, *6* – Cystathionine β-synthase, *7* – Cystathionine γ-lyase, *8* – Thiolactonase, *9* – Met-tRNA synthetase, DMG – Dimethylglycine, THF – Tetrahydrofolate, 5-CH₃-THF – 5-Methyltetrahydrofolate

Homocysteine can also enter the first step of protein biosynthesis. Because of structural similarity to methionine, homocysteine can be recognized and activated by methionyl-tRNA synthetase (MetRS). However, error-editing activity of MetRS does not allow homocysteine to be incorporated into protein. As a product of the editing reaction homocysteine thiolactone (HTL) is formed (Jakubowski and Fersht, 1981; Jakubowski, 2003, 2004). Subsequently, HTL may be hydrolyzed by thiolactonases to homocysteine (Jakubowski, 2000a; Perdziak et al., 2005; Zimny et al., 2005, 2006).

Homocysteine metabolism depends on the level of vitamins, which are required as cofactors by the enzymes involved in homocysteine turnover. Methionine synthase contains cobalamin (Vitamin B_{12}) as a prosthetic group and uses a folic acid derivative as a methyl group donor. Each of the transsulfuration pathway enzymes, cystathionine β-synthase and cystathionine γ-lyase, contain pyridoxal phosphate (Vitamin B_6) as a prosthetic group. De-

ficiencies of any of these vitamins are associated with hyperhomocysteinemia (Brosnan et al., 2004).

In human blood homocysteine exists in free or protein bound form. It may be either oxidized or reduced (Jacobsen, 1998) (Fig. 2). A major fraction of Hcy exists as protein N-linked homocysteine, with N-Hcy-hemoglobin and N-Hcy-albumin accounting for 75 and 22%, respectively, of the total N-Hcy-protein present in the human blood (Jakubowski, 2002b, 2005, 2006). Another homocysteine metabolite, HTL, represents up to 0.29% and up to 28% of plasma and urinary total homocysteine, respectively (Chwatko and Jakubowski, 2005a, b). A small fraction of homocysteine is also found as a free, reduced form. Most of oxidized form of homocysteine is bound to the protein, and the great bulk of this is linked to cysteine 34 (Cys34) of albumin. The remainder occurs as disulfide, homocystine (Hcy-S-S-Hcy) and cysteinylhomocysteine (Cys-S-S-Hcy) (Jacobsen, 1998; Mudd et al., 2000). The term "total homocysteine" ("tHcy") is commonly used to describe the pool
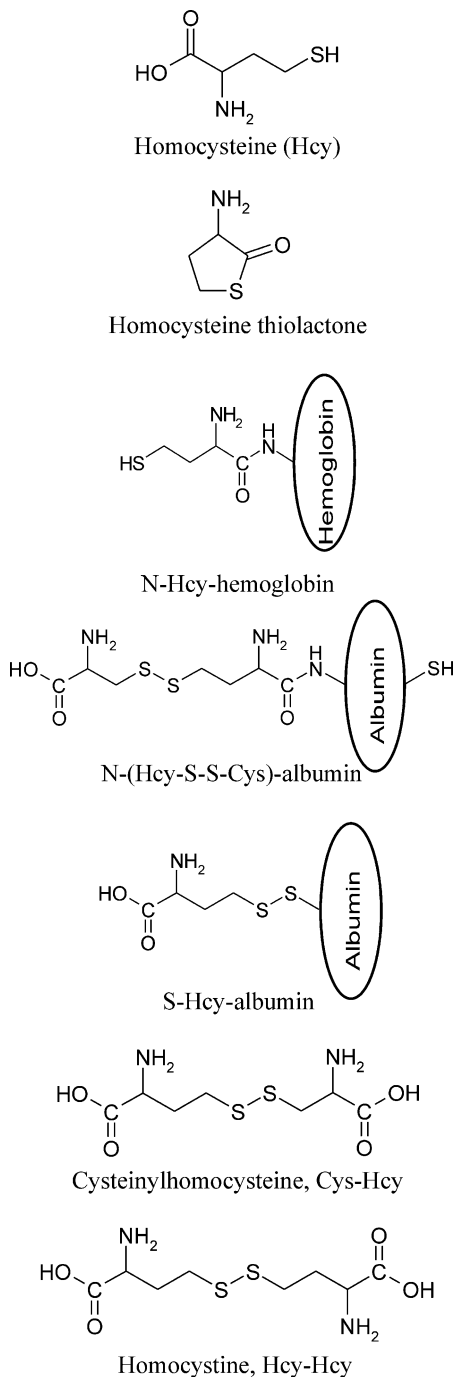
Fig. 2. Species of homocysteine present in the human blood

of homocysteine released by reduction of all disulfide bonds in the sample. This pool, however, does not include homocysteine bound to the protein by an amide bond (Mudd et al., 2000), or HTL (Chwatko and Jakubowski, 2005b).

Homocysteine toxicity

Hyperhomocysteinemia, as defined by Mudd et al. is the presence of an abnormally elevated concentration of plas-

ma or serum "tHcy" (Mudd et al., 2000). Plasma homocysteine level is determined by multiple factors, among them are genetic, demographic, acquired, and lifestyle determinants. Plasma tHcy concentration for "healthy adults" is in the range of 5–15 μM. Patients with mild hyperhomocysteinemia have 15–25 μM tHcy. Usually, they suffer from coronary artery, cerebrovascular, and peripheral vascular diseases. Intermediate concentrations of tHcy (25–50 μM) are found in subjects with impaired renal function and with end-stage renal disease. Individuals with inborn errors of homocysteine metabolism have severe hyperhomocysteinemia (50–500 μM).

The problem of Hcy toxicity has attracted a great deal of interest. At molecular level several potential mechanisms were proposed, including mechanisms involving formation of reactive oxygen species (McDowell and Lang, 2000), hypomethylation (Hultberg et al., 2000), induction of unfolded protein response and protein N-homocysteinylation. At cellular level pathological role of Hcy seems to be associated with an alteration of endothelial cells function. Endothelial cells play an important role in regulating and maintaining the health of the vascular system (McDowell and Lang, 2000) and are very sensitive even to a mild increase in Hcy concentration. This sensitivity may be explained by the fact, that human endothelial cells do not express active form of cystathionine β-synthase and consequently can not initiate Hcy catabolism through transsulfuration pathway (Jacobsen, 1998).
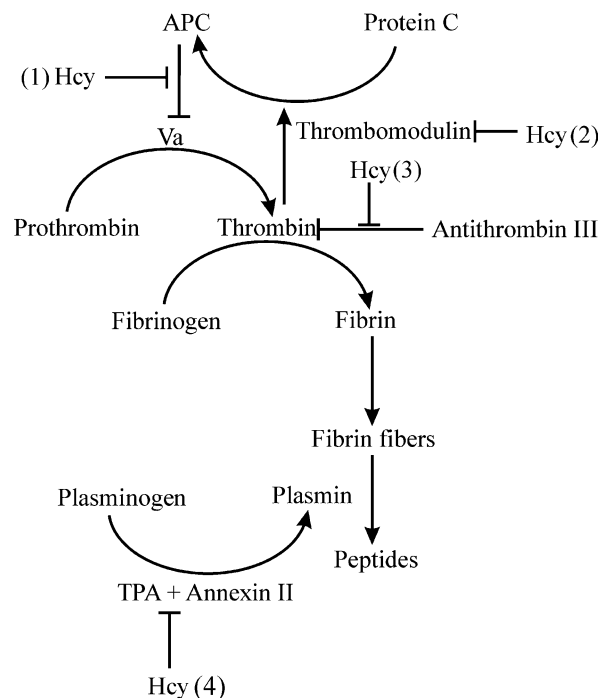

Fig. 3. Homocysteine effects on blood clotting and fibrinolysis

Hemostasis

Elevated level of homocysteine may disrupt functions of the vascular endothelium, changing the character of its surface from anticoagulant to procoagulant (Jacobsen, 1998). Based on *in vitro* studies results, Undas et al. (2001) suggest that the prothrombotic tendency in hyperhomocysteinemia may be related to impaired inactivation of S-homocysteinylated factor Va by activated protein C (Fig. 3 (1)).

Another study conducted on endothelial cell cultures has shown that homocysteine inhibits cell-surface thrombomodulin expression and irreversibly inactivates thrombomodulin and protein C in a sulphydryl-dependent process. By inhibiting both thrombomodulin surface expression and protein C activation, homocysteine may contribute to the development of thrombosis (Fig. 3 (2)) (Lentz and Sadler, 1991).

However, *in vivo* studies did not support the hypothesis that elevated homocysteine level has a detrimental effect on protein C activation by thrombin or on inactivation of factor V by activated protein C. Lentz et al. (2002) have shown that sensitivity of plasma factor V to human activated protein C was identical in monkeys with mild hyperhomocysteinemia, cystationine β-synthase deficient mice and in humans with acute hyperhomocysteinemia. Moreover two large clinical studies also failed to demonstrate an association between hyperhomocysteinemia and activated protein C resistance (Podda et al., 2003; Zarychanski and Houston, 2004).

Another endothelial anticoagulant pathway is based on heparin-like glycosaminoglycan-antithrombin III interaction. Nishinaga et al. have shown that homocysteine, cysteine and 2-mercaptoethanol reduce antithrombin binding activity of endothelial cells. This effect might be caused by generation of hydrogen peroxide (Fig. 3 (3)) (Nishinaga et al., 1993).

Endothelial cells modulate fibrinolysis by synthesis and secretion of tissue plasminogen activator and its inhibitor, plasminogen activator inhibitor-1. Treatment of cultured endothelial cells with homocysteine results in selective reduction in cellular binding sites for tissue plasminogen activator (Hajjar, 1993). Homocysteine blocks the tissue plasminogen activator binding domain of annexin II, by binding to Cys9 of annexin II (Hajjar et al., 1998) (Fig. 3 (4)).

Hypothesis that impaired endothelial cell function is associated with hyperhomocysteinemia was confirmed by *in vivo* research conducted on animals and humans. Van den Berg et al. (1995) studied endothelial function in mildly hyperhomocysteinemic patients with peripheral arterial occlusive disease. Levels of von Willebrand factor and thrombomodulin were elevated but decreased after treatment with pyridoxine.

Clots from plasma or fibrinogen of homocysteinemic rabbits are composed of thin, tightly packed fibers that are more resistant to lysis by plasmin than control clots. The formation of such clots could directly contribute to the increased risk of thrombosis in hyperhomocysteinemia (Sauls et al., 2003). In humans, plasma homocysteine affects clot permeability and resistance to lysis, most likely by a mechanism involving fibrinogen modification by HTL (Undas et al., 2006) (see below).

Vascular relaxation

Hyperhomocysteinemia in humans is associated with impaired endothelium-dependent relaxation (Tawakol et al., 1997; Chambers et al., 1999). Homocysteine can alter properties of cultured endothelial cells by impairing the production or bioavailability of vasoactive mediators such as endothelin-1 (Demuth et al., 1999), nitric oxide (Upchurch et al., 1997) and prostacycline (Wang et al., 1993). Endothelial cells incubated with Hcy (0.05–1.00 mM) produce less endothelin-1, and the effect is mediated through oxidative products (Drunat et al., 2001).

Inflammation

Atherosclerosis is an inflammatory disease (Ross, 1999), in which critical role is played by proinflammatory cytokines. Pathophysiological concentrations of homocysteine upregulate monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) expression and secretion in cultured human endothelial cells. This suggests that homocysteine may influence vascular disease by promoting leukocyte recruitment (Poddar et al., 2001). Induction of MCP-1 expression is also observed upon treatment of smooth muscles cells (Wang et al., 2000) and monocytes (Wang and O, 2001) with homocysteine. It has been shown that homocysteine induces expression of MCP-1 and IL-8 through activation of NF-κB, a transcription factor known to stimulate the production of chemokines, cytokines, hemopoetic growth factors, and leukocyte adhesion molecules, which are thought to be involved in atherogenesis and vascular inflammation (Berliner et al., 1995; Ross, 1999).

Endoplasmatic reticulum stress and unfolded protein response

One of proposed homocysteine toxicity mechanisms includes endoplasmatic reticulum stress and unfolded

protein response. The cellular consequence of protein modification with homocysteine is endoplasmatic reticulum (ER) stress. ER stress is a condition in which unfolded proteins accumulate in the ER (Kaufman, 1999). Disruption of protein folding and maturation activates the unfolded protein response (UPR), a signaling pathway that includes increased expression of UPR responsive genes, reduced global protein translation and unfolded protein degradation. Stress ER activates sensor proteins as IRE-1, ATF-6, and PKP-like ER kinase (PERK) through their dissociation from GRP78. Severe ER stress and lack of functional UPR can lead to apoptotic cell death (Austin et al., 2004).

It was found that homocysteine decreases extracellular superoxide dismutase (EC-SOD) (glycoprotein that protects the vascular wall from oxidative stress) mRNA expression and protein secretion. Moreover, homocysteine induces the expression of GRP78 mRNA and activates PERK in vascular smooth muscle cells, responses observed during ER stress (Nonaka et al., 2001).

Vascular endothelial growth factor (VEGF) expression is increased by exposure to chemical inducers of ER stress (Abcouwer et al., 2002). Homocysteine increases VEGF expression 4.4-fold due to ATF4-dependent activation of VEGF transcription in the retinal-pigmented epithelial cell line ARPE-19. HTL and DTT induce VEGF expression 7.9 and 8.8-fold, respectively (Roybal et al., 2004).

Protein S-homocysteinylation

Homocysteine forms stable disulfide bonds with protein cysteine residues, which may alter or impair the function of the protein (Jacobsen et al., 2005). Homocysteine exhibits the greatest (comparing to cysteine and glutathione) tendency to generate disulfide bonds with protein thiol groups. Protein thiol groups are involved in the function of many enzymes, structural proteins and receptors, thus interaction with them might disrupt cellular metabolism (Hultberg et al., 1998). Binding of homocysteine to plasma protein is biphasic. First reaction, involving displacement of cysteine from plasma protein, is rapid and oxygen independent, while the second reaction constitutes slower, oxygen dependent thiol oxidation (Togawa et al., 2000).

S-homocysteinylated proteins (S-Hcy-proteins) are present in human plasma. Major components of the S-Hcy-protein pool in human plasma are albumin, containing about 1 homcysteine molecule per 100 protein molecules, and γ-globulin, containing about 3.4 molecules per 100 protein molecules. Other proteins contain >10-fold less S-Hcy-protein than albumin (Jakubowski, 2002b).

Human serum albumin is the major plasma protein, making up more than 50% of the total plasma protein. Cys34 of albumin does not participate in interchain disulfide bonds and accounts for the bulk of free thiol in plasma (Peters, 1996). Because of abnormally low pK_a ($\sim$5) of the thiol group of Cys34, at physiological pH albumin exists primarily as thiolate anion, highly reactive with metals, thiols and disulfides. One-third of the plasma albumin molecules has Cys34 disulfide bound with thiols (e.g. homocysteine or cysteine) (Carter and Ho, 1994).

Studies *in vitro* and *in vivo* have shown, that post-translational homocysteine incorporation into protein via S-homocysteinylation may lead to impairment of protein function (Hajjar et al., 1998; Undas et al., 2001; Majors et al., 2002; Lim et al., 2003; Roda et al., 2003; Sass et al., 2003) (Table 1).

Table 1. Protein S-homocysteinylation, examples and consequences

Protein	Hcy incorporation position	Protein function	Effect of S-homocysteinylation	References
Annexin II	Cys9	Receptor for tissue-type plasminogen activator	Inhibition of the tissue plasminogen activator binding	Hajjar et al. (1998)
Fibronectin	5 homocysteine molecules per fibronectin dimer, mainly in the C-terminal region, within and adjacent to the fibrin-binding domain	Cell adhesion, cell migration, embryogenesis, hemostasis, thrombosis, wound healing, tissue remodeling	Impairing of fibronectin – fibrin interaction	Majors et al. (2002)
Transthyretin	Cys10	Binding and transport of thyroid hormones	Possible role in pathology of amyloid diseases	Lim et al. (2003) Sass et al. (2003)
Factor Va	Cys539, Cys585, Cys1085, Cys1960, Cys2113	Essential cofactor for prothrombin complex, which converts prothrombin into thrombin	Inhibition of factor Va inactivation by activated protein C	Undas et al. (2001)

Homocysteine thiolactone

Homocysteine is also metabolized by methionyl-tRNA synthetases (MetRS) to homocysteine thiolactone (HTL) (Jakubowski and Fersht, 1981). This reaction occurs in all so far investigated organisms, including human. In the first step of the conversion of homocysteine to HTL, MetRS misactivates homocysteine with formation of homocysteinyl-adenylate. In the next step homocysteine side chain thiol group reacts with homocysteine activated carboxyl group and HTL is produced. Homocysteine side chain, missing the methyl group of methionine, reacts with specificity subsite of MetRS much less strongly than methionine. This allows homocysteine side chain to react also with thiol-binding subsite of MetRS, which facilitates thioester bond formation during error-editing reaction (Kim et al., 1993).

The level of HTL synthesis in cultured cells depends on homocysteine and methionine levels. In yeasts and normal mammalian cells, homocysteine is converted into cysteine in transsulfurylation pathway, which leads to relatively low Hcy/Met ratios and decreased HTL synthesis. High Hcy/Met ratio results in excessive production of homocysteine thiolactone by MetRS (Jakubowski and Goldman, 1993). Human cell lines with disrupted homocysteine metabolism (e.g. mutation in cystationine β-synthase gene) and cancer cells produce more homocysteine thiolactone than normal cells (Jakubowski, 1997).

Human body developed an enzymes involved in hydrolysis of homocysteine thiolactone. First such enzyme was discovered in human serum and identified as a 45-kDa protein component of high density lipoprotein (HDL) that requires calcium for its activity. N-terminal sequence of thiolactonase is identical with that of human serum paraoxonase, whose natural substrate and function were unknown (Jakubowski, 2000a). Human serum paraoxonase is a product of polymorphic gene *PON1* (James et al., 2000). Two of genetic polymorphisms of paraoxonase gene result in amino acid residue substitution at position 55 and 192 in protein PON1 (Humbert et al., 1993). High homocysteine thiolactone hydrolase (HTase) activity is associated with L55 and R192 alleles, more prevalent in blacks than in whites. High activity form of HTase gives a better protection against protein homocysteinylation than the low activity form (Jakubowski et al., 2001). Lacinski et al. (2004) studied HTase activities and PON1 genotypes in a group of subject with coronary artery disease (CAD) and healthy controls. The hydrolytic activities of the serum PON1 protein towards HTL and paraoxon substrates were strongly correlated. Mean HTase activities were similar in CAD subjects and in healthy controls. However, the fre-quency of the PON1-192-RR genotype tended to be lower in CAD patients than in controls (2% vs 10%, $p = 0.057$) (Lacinski et al., 2004).

Recently, intracellular enzymes hydrolyzing HTL were also discovered in yeast (Zimny et al., 2005) and in human placenta (Perdziak et al., 2005). Mass spectrometry analysis identified both proteins as bleomycin hydrolase (BLH) (Perdziak et al., 2005; Zimny et al., 2005). BLH belongs to a family of evolutionarily conserved cysteine aminopeptidases and its only known biologically relevant function was deamidation of the anticancer drug bleomycin. Recombinant yeast and human BLH, expressed in *E. coli* hydrolyze HTL to homocysteine (Zimny et al., 2005). Active site mutations, C73A for human BLH and H369A for yeast BLH, inactivate Hcy-thiolactonase activities. Yeast *blh1* mutants are deficient in Hcy-thiolactonase activity *in vitro* and *in vivo*, produce more Hcy-thiolactone, and exhibit greater sensitivity to Hcy toxicity than wild type yeast cells. The data suggest that BLH protects cells against Hcy toxicity by hydrolyzing intracellular Hcy-thiolactone.

Human body eliminates homocysteine thiolactone also by urinary excretion. Using a sensitive HPLC method with postcolumn derivatization and fluorescence detection, it was found that urinary concentrations of HTL (11–485 nmol/L; $n = 19$) were approximately 100-fold higher than those in plasma (<0.1–22.6 nmol/L; $n = 20$). Urinary homocysteine thiolactone accounted for 2.5–28.3% of urinary total Hcy, whereas plasma HTL accounted for <0.002–0.29% of plasma total Hcy (Chwatko and Jakubowski, 2005b).

Urinary excretion of HTL, the existence of inter- and extracellular enzymes hydrolyzing HTL, as well as the reactivity toward proteins, explain relatively low level of HTL in healthy human plasma.

Effect of homocysteine thiolactone on cells

HTL induces endothelial cell apoptosis in endothelial cells independently of the caspase pathway (Mercie et al., 2000). HTL also induces induced cell death and features of apoptosis in human promyeloid HL-60 cells. Those features include increased apoptotic cells with hypoploid DNA contents, increased phosphotidylserine exposure on the membrane surface, and internucleosomal DNA fragmentation. Genotoxic effect of HTL on HL-60 cells is exerted through caspase 3 mediated apoptotic pathway, induced by an increase in intracellular hydrogen peroxide (Huang et al., 2001). HTL enhances apoptosis also in cultured human trophoblast, and the effect can be

limited by antioxidants, such as vitamin C and N-acetyl-cysteine. Apoptosis in cultured trophoblasts was induced by increased expression of p53 and Bak, but not of Bcl-2 or Bax (Kamudhamas et al., 2004).

In retinal-pigmented epithelial cell line HTL elevates vascular endothelial growth factor expression (VEGF). VEGF mRNA induction parallels that of the ER-stress gene GRP78 and GADD153 (Roybal et al., 2004).

Inhibition of lysyl oxidase by homocysteine thiolactone

Downregulation of lysyl oxidase (LOX) (EC 1.4.3.13), an enzyme involved in extracellular matrix maturation, impairs endothelial barrier function. HTL is an irreversible inhibitor of lysyl oxidase. Inhibition is likely to happen by derivatization and reduction of the carbonyl cofactor in active site of the enzyme (Liu et al., 1997). Raposo et al. (2004) analyzed the effect of homocysteine and HTL on LOX regulation in vascular endothelial cells. Both compounds inhibited LOX activity through oxidative stress. Higher doses of Hcy (200 μM) decreased LOX mRNA levels and LOX promoter activity.

Protein N-homocysteinylation

HTL produced within the cell may be secreted, hydrolyzed, but also incorporated into intra- and extracellular proteins. Intramolecular thioester bond of HTL is susceptible to reactions with nucleophiles, especially with free amino groups of protein Lys residues (Jakubowski, 1997, 1999). The mechanism of homocysteine incorporation into proteins (N-homocysteinylation) involves acylation of Lys ε-amino group by the activated carboxyl group of HTL (Fig. 4) (Jakubowski, 2003, 2004, 2005, 2006).

Protein N-homocysteinylation was first discovered in cell cultures (Jakubowski, 1997, 1999; Jakubowski et al., 2000), and then in human body (Jakubowski, 2000b, 2001, 2002b). HTL (Jakubowski, 2001, 2002a; Daneshvar et al., 2003), as well as N-homocysteinylated protein (N-Hcy-protein) (Jakubowski, 2000b, 2002b; Uji et al., 2002) are present in the human blood (Jakubowski, 2002b). N-Hcy-protein represents from 0.3 to 23% of total homocysteine in human plasma. Human serum albumin and γ-globulin contain about 0.36% N-Hcy-protein. Other proteins (LDL, HDL, transferrin, antitrypsin and fibrinogen) contain 0.04–0.1% N-Hcy-protein (Jakubowski, 2002b, 2004, 2005, 2006).

The rate of N-homocysteinylation is proportional to the number of protein lysine residues. N-Homocysteinylation changes the Lys charge, because Lys ε-amino group (pK = 10.5) is more basic than Hcy α-amino (pK = 7.1). Protein N-homocysteinylation leads to the addition of new thiol groups, which increase protein's susceptibility to oxidation, intramolecular disulfide bonds formation, and multimerisation (Jakubowski, 1999).

In endothelial cell cultures, intensity of protein N-homocysteinylation increases with higher Hcy concentration and drops with elevated levels of folic acid and HDL. In the majority of examined serum samples the level of N-Hcy-protein is positively correlated with serum Hcy level, and negatively correlated with Met, folic acid and HDL (Jakubowski et al., 2000). Results of a recent study conducted on uremic hemodialysis patients revealed significantly higher protein N-homocysteinylation, as well as protein S-homocysteinylation in patients vs. controls. Folate therapy normalized levels of N-Hcy-protein and reduced, but did not normalize, levels of S-Hcy-protein (Perna et al., 2006). Yang et al. also found that in patients with coronary heart disease the plasma level of N-Hcy-protein is significantly higher than in controls. The authors did not, however, find a correlation between plasma levels of N-Hcy-protein and plasma concentration of Hcy (Yang et al., 2006).

The effects of N-homocysteinylation on the function and structure of a number of proteins were studied (Table 2). It was shown that this modification leads to protein damage and loss of function (Jakubowski, 1999). Most of N-Hcy-protein in human blood is present in
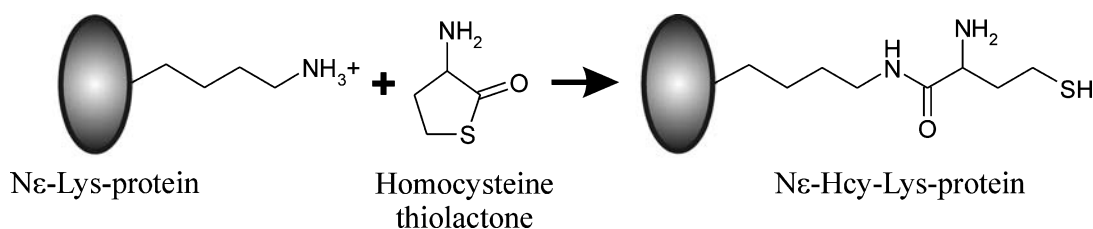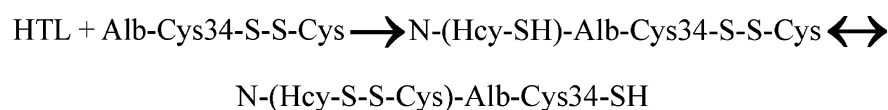

Fig. 4. N-homocysteinylation of protein amino groups

Table 2. Protein N-homocysteinylation, examples and consequences

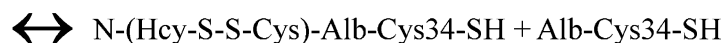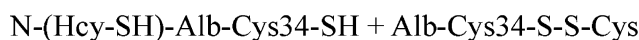| Protein	Function	Effect of N-homocysteinylation	References
Albumin	Maintenance of the blood within the vascular system, transport of metabolic products, regulatory mediators, nutrients and proteins, neutralization of endogenous or exogenous toxins	Structural changes, influence the function of Cys34, increase of susceptibility to proteolysis, diminished binding capacity for diazepin	Jakubowski (1999, 2000b) Perla et al. (2004) Undas et al. (2004, 2005) Perna et al. (2006)
High denstity lipoprotein – Paraoxonase	Protection against lipid peroxidation of LDL and HDL	A decrease in the activity of the enzyme HDL-PON, resulting in diminished protection against oxidative damage and toxicity of homocysteine thiolactone	Ferretti et al. (2003)
Low density lipoprotein*	Transport of cholesterol to tissues and regulation of cholesterol synthesis	Protein aggregation and spontaneous precipitation, higher accumulation of intracellular cholesterol	Naruszewicz et al. (1994)
Fibrinogen	Precursor of fibrin	Protein aggregation, fibers thiner and more resistant to fibrinolysis	Jakubowski (1999) Sauls et al. (2005)
Antitrypsin*	Proteases inactivation	Recognized by human N-Hcy-protein specific antibodies	Perla et al. (2004)
Trypsin	Cleavage of amide and ester bonds of Arg and Lys	Complete inactivation when ∼88% lysine residues modified	Jakubowski (1999)
Methionyl-tRNA synthetase	Aminoacylation of tRNA^{Met} with methionine	Complete inactivation when ∼33% lysine residues modified	Jakubowski (1999)
Cytochrome c	Electron transport	Protein aggregation	Jakubowski (1999)
Hemoglobin*	Oxygen transport	Protein aggregation	Jakubowski (1999, 2004) Perla et al. (2004) Undas et al. (2004)
Myoglobin	Oxygen transport	Protein aggregation	Jakubowski (1999)
γ-Globulin	Antibodies	Protein aggregation	Jakubowski (1999)
Transferrin*	Iron transport	Protein aggregation	Jakubowski (1999) Perla et al. (2004)
RNase A [14]	RNA hydrolysis	Protein aggregation	Jakubowski (1999)
α2-macroglobulin	Proteases inactivation	Unknown	Jakubowski (2000)
Crystalin	Eye structural protein	Unknown	Jakubowski (2000)
DNase I	DNA hydrolysis	Unknown	Jakubowski (2000)

* N-Homocysteinylated forms are recognized by human autoantibodies

HTL + Alb-Cys34-S-S-Cys ⟶ N-(Hcy-SH)-Alb-Cys34-S-S-Cys ⟷

N-(Hcy-S-S-Cys)-Alb-Cys34-SH

Reaction 1

HTL + Alb-Cys34-SH ⟶ N-(Hcy-SH)-Alb-Cys34-SH

Reaction 2

N-(Hcy-SH)-Alb-Cys34-SH + Alb-Cys34-S-S-Cys

⟷ N-(Hcy-S-S-Cys)-Alb-Cys34-SH + Alb-Cys34-SH

Reaction 3

Fig. 5. Mechanism of albumin N-homocysteinylation by homocysteine thiolactone

hemoglobin, albumin and γ-globulins. Small amounts of N-Hcy-protein are found also in other blood proteins (e.g., LDL, HDL, antitrypsin, transferrin, fibrinogen) (Jakubowski, 2002b).

About 0.36% albumin molecules in human serum contains N-linked homocysteine (Jakubowski, 2002b). The predominant site of albumin N-homocysteinylation in human serum albumin *in vitro* and *in vivo* is Lys525. The rate of N-homocysteinylation depends on the status of albumin Cys34. The disulfide forms of albumin are N-homocysteinylated faster than mercaptoalbumin. N-Homocysteinylation of albumin-Cys34-S-S-Cys may cause regeneration of free thiol at Cys34 as a result of thiol-disulfide exchange between a free thiol of incorporated Hcy and the disulfide at Cys34 (Fig. 5). N-Homocysteinylated fibrinogen, antitrypsin, hemoglobin, myoglobin, and cytochrome c, also remove Cys from albumin-Cys34-S-S-Cys with the liberation of thiol group at Cys34. N-Homocysteinylation changes the susceptibility of albumin to oxidation and proteolysis.

N-(Hcy-S-S-Cys)-Albumin-Cys34-SH is more sensitive than N-(Hcy-SH)-albumin-Cys34-SH to proteolysis. This suggests that the disulfide bond at Cys34 facilitates proteolytic turnover of N-homocysteinylated albumin (Glowacki and Jakubowski, 2004).

Fibrinogen undergoes facile N-homocysteinylation by HTL *in vitro* (Jakubowski, 1999) and *in vivo* in the human body (Jakubowski, 2002b). Clots formed from HTL-treated normal human plasma lyse more slowly than clots from untreated control plasma and the magnitude of this effect depends on the concentrations of HTL used (Sauls et al., 2005). Purified human fibrinogen modified with HTL forms clots with characteristics similar to fibrinogen from hyper-homocysteinemic rabbits. Clots formed from N-Hcy-fibrinogen are more resistant to lysis than control clots from native fibrinogen. Proteomic analysis of N-Hcy-fibrinogen shows 10 different Nε-Hcy-Lys residues in the D and αC domains. Some of those residues are close to tPA and plasminogen binding or plasmin cleavage, which can explain abnormal clot characteristics. Detrimental effects of elevated plasma homocysteine on clot permeability and resistance to lysis in humans are consistent with a mechanism involving fibrinogen modification by homocysteine thiolactone (Undas et al., 2006). These results suggest that N-homocysteinylation of fibrinogen can lead to abnormal resistance of fibrin clots to lysis and contribute to increased risk of cardiovascular disease in hyperhomocysteinemia (Jakubowski, 1999; Sauls et al., 2005; Undas et al., 2006).

Immunogenicity of N-homocysteinylated proteins

LDL and albumin with modified lysine residues (e.g. carbamylated, acetylated, ethylated, methylated) become immunogenic for experimental animals and induce production of antibodies directed almost exclusively against the derivatized lysine residue. Such immunoglobulins react equivalently with other modified proteins that contained the same lysine derivative (Steinbrecher et al., 1984). HTL modified proteins may also be physiologically harmful and induce immune response (Jakubowski, 2005, 2006).

Ferguson et al. (1998) were the first to show that N-homocysteinylated LDL is immunogenic in the rabbit. Rabbit anti-N-Hcy-LDL antibodies recognize N-homocysteinylated rabbit LDL, hemoglobin and albumin with 40% Lys residues modified.

Antibodies directed against HTL-modified proteins may be used for detection of N-Hcy-proteins and serve as diagnostic tool in case of hyperhomocysteinemia-related diseases. A new procedure for the preparation of polyclonal anti-N-Hcy-protein antibodies was developed in our laboratory. As a source of anti-N-Hcy-protein antibodies we used rabbits immunized with N-Hcy-keyhole limpet hemocyanin and an affinity matrix, Nω-Hcy-aminohexyl-Agarose, was used for the antibody purification. Immunoglobulins retained on the affinity matrix contained N-Hcy-protein-specific antibody, as shown by competitive ELISA experiments (Perla et al., 2004).

As discussed in the preceding sections, N-Hcy-albumin, N-Hcy-hemoglobin, N-Hcy-LDL and other N-Hcy-proteins are present in human blood (Jakubowski, 2002b). If N-homocysteinylated proteins induce immune response, specific auto-antibodies should also be detected. In fact, Undas et al. (2004) have shown that auto-antibodies that specifically recognize epitop Nε-Hcy-Lys on N-Hcy-proteins are present in human blood. Levels of anti-Nε-Hcy-Lys-protein immunoglobulins in serum positively correlate with the level of total Hcy, but not of Cys and Met. In a group of male, but not female, subjects with stroke, mean level of anti-Nε-Hcy-Lys-protein autoantibodies was approximately 50% higher than in a group of healthy subjects. These findings support a hypothesis that Nε-Hcy-Lys-protein is a neoself antigen, which may contribute to immune activation (Undas et al., 2004), an important modulator of atherogenesis (Binder et al., 2002). Mean levels of antibodies against N-Hcy-albumin are also significantly higher in males with coronary artery disease (CAD) than in healthy controls. However serum levels of IgG antibodies against Nε-Hcy-albumin were not associated with traditional risk factors, and there was only

weak correlation between the autoantibodies and plasma total homocysteine. Stronger correlation was observed between anti-Nε-Hcy-albumin antibodies with C-reactive protein levels. Seropositivity to anti-Nε-Hcy-albumin antibodies showed no association with the MTHFR C677T polymorphism (Undas et al., 2005).

Conclusions

Elevated level of Hcy is a risk factor for cardiovascular disease. Several mechanisms of Hcy toxicity were proposed. Many potentially detrimental effects of homocysteine were characterized *in vitro* or in cell cultures. These studies face certain limitations. Most of them were carried out with homocysteine concentrations exceeding even pathophysiological levels. Moreover, homocysteine added to cell cultures is subjected to enzymatic and nonenzymatic reactions; the metabolites of these processes in most cases were not analyzed. Most of homocysteine effects were repeated also by other thiol compounds, e.g. cysteine, which is not regarded as atherogenic.

Hypothesis concerning HTL modified proteins are especially appealing because of the following reasons: i. this modification occurs at physiological concentrations of HTL in human body; ii. N-homocysteinylated proteins contain major pool of Hcy, larger than "tHcy"; iii. N-homocysteinylation causes severe pathophysiological consequences, such as cellular toxicity and the activation of immune response. There is a growing number of examples of detrimental effect of N-homocysteinylation on protein structure and function.

Modification of lipoprotein, especially LDL plays an important role in atherogenesis. Covalent changes incorporated into LDL increase lipoprotein uptake and accumulation of intracellular cholesterol (Naruszewicz et al., 1994). Moreover, oxidized or glycated LDL induce lipid peroxidation in cultured cells (Pirillo et al., 2000; Salvayre et al., 2002). N-Homocysteinylation could be another atherogenic modification of LDL (Naruszewicz et al., 1994). N-Homocysteinylation may also result in decreased protective function of HDL against oxidative damage and against toxicity of HTL (Ferretti et al., 2003). N-Hcy-Fibrinogen forms clots with increased resistance to lysis, which might contribute to the increased risk of cardiovascular disease related to hyperhomocysteinemia (Sauls et al., 2005; Undas et al., 2006). Elucidation of specific roles of Hcy metabolites, such as HTL and N-Hcy-protein, in pathophysiology will require further investigation.

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Authors' address: Hieronim Jakubowski, UMDNJ-New Jersey Medical School, 225 Warren Street, Newark, NJ 07101-1709, U.S.A., Fax: +1-973 972-8982, E-mail: jakubows@umdnj.edu