

Mechanism of Action of Homocysteine and Its Thiolactone in Hemostasis System

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Received March 27, 2008

Accepted September 8, 2008

On-line November 4, 2008

Summary

In the article, the actions of homocysteine (Hcys) and its metabolite - cyclic thioester – homocysteine thiolactone (HTL) on complex process of hemostasis, which regulates the flowing properties of blood, are described. Possible interaction of Hcys and HTL with endothelial cells, blood platelets, plasmatic fibrinogen and plasminogen, as the important major components of hemostasis are also discussed. The modification of hemostatic proteins (N-homocysteinylated or S-homocysteinylated proteins) induced by Hcys or its thiolactone, and links of homocysteine or homocysteine thiolactone to *NO metabolism seem to be the main reason of biotoxicity of homocysteine in cardiovascular diseases.

Key words

Homocysteine • Homocysteine thiolactone • Hemostasis

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Introduction

Hemostasis is a complex process that regulates the flowing properties of blood *in vivo*. Classical primary and secondary hemostasis only comprised the sequential formation of the white and red thrombus, but there is growing awareness that hemostasis is intimately coupled to fibrinolytic processes, inflammatory reactions as well as to initiation of angiogenesis and wound healing. Basically, three components need to interact to assure

effective primary and secondary hemostasis together with the normal sequelae, fibrinolysis and tissue repair: 1) the vascular wall (smooth muscle, matrix and connective tissue, endothelial cells), 2) formed elements of the blood (blood platelets, granulocytes, monocytes, lymphocytes), and 3) the plasmatic clotting and fibrinolytic systems (Becker *et al.* 2000). Hemostatic abnormalities can lead to excessive bleeding, thrombosis or other cardiovascular diseases. Recently, it has been shown that homocysteine (Hcys), which is an intermediate formed during the catabolism of the essential dietary amino acid methionine induces changes in hemostasis, including blood clotting and fibrinolysis (Perla-Kajan *et al.* 2007). Elevated level of Hcys may disrupt functions of the vascular endothelium, changing the character of its surface from anticoagulant to procoagulant (Jacobsen 1998, Perla-Kajan *et al.* 2007). This review describes the chemical structure and biological activities of homocysteine and its cyclic thioester – homocysteine thiolactone (HTL) and their effects on hemostasis process.

Chemical structure and function of homocysteine and its metabolites

Homocysteine is involved in conversions of methionine (Met) and cysteine (Cys). The immediate precursor of Hcys is S-adenosylhomocysteine (SAH), which is hydrolyzed by SAH hydrolase to homocysteine and adenosine. In the next step homocysteine is remethylated to methionine by methionine synthase or betaine:homocysteine methyltransferase. Moreover, Hcys enters the transsulfuration pathway and is converted into

cystathionine β -synthase and cystathionine γ -lyase. This process is present only in the liver, kidney, pancreas and small intestine (Brosnan *et al.* 2004, Perla-Kajan *et al.* 2007). Homocysteine may also enter the first step of protein synthesis. Because structural similarity of Hcys to Met, homocysteine may be recognized and activated by methionyl-tRNA synthetase. However, error-editing activity of methionyl-tRNA synthetase does not allow homocysteine to be incorporated into proteins. As a product of the editing reaction homocysteine thiolactone is formed (Jakubowski and Fersht 1981, Jakubowski 2003, 2004, Perla-Kajan *et al.* 2007). The chemical structure of Hcys and its thiolactone is presented in Figure 1. Homocysteine metabolism depends on the level of vitamins (folic acid, vitamin B₆ and B₁₂) as cofactors for the enzymes involved in Hcys turnover. In human blood homocysteine may exist in free or protein bound forms as N-homocysteinylated (N-Hcys-protein) or S-homocysteinylated proteins (S-Hcys-protein), that were described as N-Hcys-hemoglobin, N-(Hcys-S-S-Cys)-albumin, S-Hcys-albumin, and cysteinylhomocysteine (Cys-Hcys) (Jakubowski 2002, 2005, 2006, Chwatko and Jakubowski 2005a,b, Perla-Kajan *et al.* 2007). Mechanism of N-homocysteinylation involves acylation of Lys ϵ -amino group by the activated carboxyl group of HTL (Jakubowski 2003, 2004, 2005, 2006), whereas S-homocysteinylation is induced by Hcys (Fig. 1). In human plasma some various hemostatic proteins are S-homocysteinylated, for example coagulation factor Va (Undas *et al.* 2001). N-homocysteinylated proteins in human plasma represent from 0.3 to 23 % of total homocysteine. Small amounts of N-Hcys-proteins are also found in different hemostatic proteins (antitrypsin, fibrinogen) (Jakubowski 2002). These modifications may lead to impairment of protein functions. Approximately 80 % of plasma homocysteine is protein bound, and only a small amount exists as a free reduced homocysteine. The majority of the unbound portion of Hcys is oxidized to form dimers (homocystine) or combined with cysteine to form mixed disulphides (Jakubowski 2002, 2005, 2006, Chwatko and Jakubowski 2005a,b, Perla-Kajan *et al.* 2007). Plasma Hcys level is determined by multiple factors, like genetic, demographic, acquired, and lifestyle determinants. Total plasma Hcys concentration for healthy adults is in the range of 5-15 μ M. Patients with mild hyperhomocysteinemia have 15-25 μ M Hcys (Perla-Kajan *et al.* 2007). In urine after oxidation of Hcys is referred to as homocystinuria. Thus, for abnormal metabolism of Hcys, the blood can be analyzed. Mild

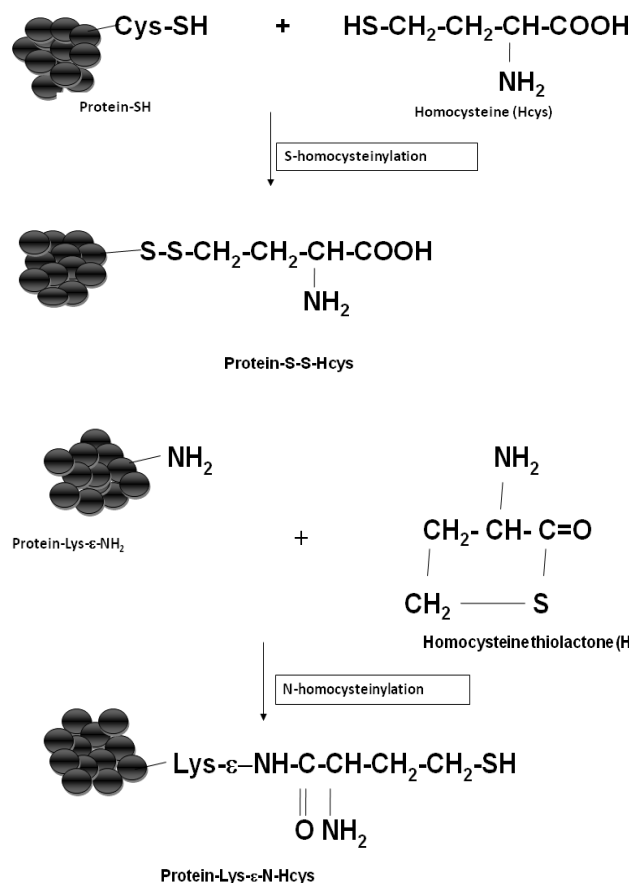


Fig. 1. The chemical structure of homocysteine (Hcys) and its thiolactone (HTL), mechanism of N- and S-homocysteinylation of proteins.

hyperhomocysteinemia is an independent risk factor for atherosclerotic disease, deep vein thrombosis and thromboembolism. Elevated Hcys levels promote thrombosis, although the mechanism by which Hcys exerts its prothrombotic effect remains unclear. Moreover, homocysteine metabolites (thiolactone) and protein homocysteinylation formed in plasma, are implicated in different cardiovascular diseases (Thambyrajah *et al.* 2000, Yang *et al.* 2006).

The effect of homocysteine and its thiolactone on endothelial cells

At cellular level pathological role of homocysteine seems to be associated with an alteration of endothelial cells, which play an important role in hemostasis. Endothelial cells are very sensitive even to a mild increase of Hcys 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 homocysteine catabolism

through transsulfuration pathway (Jacobsen 1998). Elevated level of Hcys may modulate functions of the vascular endothelium, changing the character of its surface from anticoagulant to procoagulant (Jacobsen 1998). Endothelial anticoagulant pathway is based on heparin-like glycosaminoglycan-antithrombin III interaction. Rodgers *et al.* (1986) showed that after incubation of endothelium with Hcys the activity of coagulation factor V increases. Coagulation factor V in Hcys-modified endothelium is cleaved in fragments different than those obtained after factor V cleavage by thrombin or coagulation factor Xa (Rodgers *et al.* 1986). Moreover, in hyperhomocysteinemia the prothrombotic tendency may be related to impaired inactivation of S-homocysteinylated coagulation factor Va by activated protein C (Undas *et al.* 2001). However, Hcys and its thiolactone have no effect on factor V activation by thrombin. It was showed that factor V incubated with [³⁵S]homocysteine (10-450 μM) incorporated label within 5 min, which was found only in those fragments that contained free sulfhydryl groups: the light chain (Cys-1960, Cys-2113), the B region (Cys-1085), and the 26/28-kDa (residues 507-709) activated protein C cleavage products of the heavy chain (Cys-539, Cys-585). On the other hand, Lentz *et al.* (2002) suggested that activation of protein C by thrombin and inactivation of factor Va by activated protein C are not impaired during moderate hyperhomocysteinemia *in vivo* in monkeys. Another studies showed the effect of Hcys on activity of thrombomodulin, which is involved in blood clotting.

It has been demonstrated that not only Hcys, but also HTL may modulate properties and functions of endothelial cells. Raposo *et al.* (2004) indicated that both compounds – Hcys and its thiolactone inhibit the activity of lysyl oxidase (an enzyme involved in extracellular matrix maturation) in vascular endothelial cells. Jakubowski (2000) showed that N-homocysteinylated exists in endothelial cell proteins, and this process depends on the concentration of Hcys. Modification of endothelial cell proteins may cause different pathophysiological consequences, such as modulation of hemostasis system, which may contribute to cardiovascular diseases.

Homocysteine and nitric oxide

Nitric oxide (•NO) plays an important role in a number of physiological processes. It is well known that vascular endothelial cells produce •NO. However, other cells, which are involved in hemostasis – blood platelets

may also synthesize •NO and platelet •NO synthase (NOS) has been identified by Muruganadam and Mutus (1994). Two main NOS isoforms have been isolated: calcium-independent inducible NOS (iNOS) and a calcium/calmodulin-dependent endothelial NOS (eNOS) (Mehta *et al.* 1995). •NO is released by NOS action from the guanidine moiety of L-arginine, yielding NO and citrulline. •NO is very simple free radical which play important regulatory role in many tissues, but originally •NO was identified as a factor influenced mainly human vascular system. •NO has been implicated in a number of cardiovascular diseases and every risk factor appears to be associated with a reduction in endothelial generation of •NO.

Evidences of some cooperation between homocysteine and •NO in vascular system pathogenesis originate from physiological, cellular and genetic reports. Patients from population with *NOS3* 894TT genotype (mutation in gene for nitric oxide synthase) have a tendency to higher Hcys concentration in the blood. Mentioned tendency was especially distinct in subpopulation of *NOS3* 894TT patients who had low foliate levels. However, there was no close correlation between *NOS3* 894TT genotype and Hcys plasma levels (Brown *et al.* 2003). The question is how Hcys can modulate •NO synthase. First thought is that Hcys may influence on NOS activity. Transformation of radiolabeled arginine to citrulline catalyzed by nitric oxide synthase is unchanged after renal arterial endothelium incubation with Hcys (40 μM) in comparison with control cells. Also after incubation of endothelial cells with Hcys (10, 20 and 50 μM for 24 h) NOS activity was untouched (Fatini *et al.* 2005). Despite the fact that NOS activity also seemed to be normal during hyperhomocysteinemia *in vitro*, level of •NO under such conditions is definitely lower and this effect is directly proportional to Hcys plasma level (Becker *et al.* 2005). One of possible way of NOS activity regulation by Hcys is action through direct dimethylarginine dimethylaminohydrolase (DDAH) binding *via* disulfide bounds and further asymmetric dimethylarginine (ADMA) accumulation, what disrupts NOS activity (Stühlinger *et al.* 2001). Similar observations confirming ADMA accumulation during hyperhomocysteinemia has been done in animal models (Akhand *et al.* 1999). The same authors have observed *in vitro* that ADMA secretion by human endothelial cells after stimulation by Hcys can be stopped (for 24 h by using S-adenosyl-homocysteine methylation inhibitor).

The main molecules responsible for decrease of $\bullet\text{NO}$ level in hyperhomocysteinemic patients are probably reactive oxygen species (ROS). Hcys can enhance ROS production (Li *et al.* 2002, Fischer *et al.* 2003, Fatini *et al.* 2005) – the generation of superoxide anion ($\text{O}_2^{\bullet-}$) in reaction catalyzed by NADPH oxidase (Becker *et al.* 2005). Fischer *et al.* (2003) showed the increased of protein nitration (induced by peroxyntirite, which is produced in the reaction of $\bullet\text{NO}$ with $\text{O}_2^{\bullet-}$).

The effect of homocysteine and its thiolactone on blood platelets

Blood platelets are multiresponding cells, both with respect to the number of agonists and number of responses. They can be activated by different compounds including coagulation factors (thrombin), hormones (epinephrine, vasopresin), low-molecular-weight substances (serotonin, adenosine diphosphate (ADP)), lipid derivatives (platelet aggregating factor (PAF), thromboxane A_2 (TXA_2)), and other protein substances (collagen or immune complexes). The response of platelet to agonists, which is called platelet activation, includes mainly adhesion (to foreign surfaces such as collagen or glass), shape change, aggregation and secretion of active compounds from three different storage granules (dense granules, α -granules and lysosomes), shedding of microvesicles, formation of platelet procoagulant activity and retraction of fibrin clots (Wu 1996, Levy-Toledano 1999, Rynningen and Holmsen 1999). Increased platelet activation with hyperaggregability is one of the risk factors in pathogenesis of different cardiovascular disease. Blood platelets obtained from patients with peripheral occlusive arterial disease, with associated hyperhomocysteinemia, are far more reactive and sensitive for agonists, but also far less sensitive for inhibitors (Riba *et al.* 2004). In diabetic patients a high level of Hcys levels is associated with more potent platelets aggregation (Rajkumar *et al.* 1999). Signorello *et al.* (2007) observed that Hcys induces oxidative stress, inhibits nitric oxide formation in platelets from type 2 diabetic patients, and may promote platelet hyperactivity and various cardiovascular diseases. Moreover, Hcys stimulates the calcium mobilization in platelets from type 2 diabetic patients (Alexandru *et al.* 2008). Mohan *et al.* (2008) suggested that platelet activation and hypercoagulability occur after exposure to Hcys, especially in patients with critical limb ischemia. Hcys concentrations of approximately 50 μM appear to be the

level at which these changes occur *in vitro*, and this effect on platelets appears to be indirect. In animal model with hyperhomocysteinemia (induced by diet poor of folic acid), the aggregation of platelets stimulated by ADP or thrombin is higher than in control animals (Durand *et al.* 1996). The direct action of Hcys on blood platelets is unknown and sometimes controversial. Some studies demonstrated that homocysteine promotes arachidonic acid release, thromboxane A_2 formation (Signorello *et al.* 2002, 2007) and protein tyrosine phosphorylation in blood platelets (Leoncini *et al.* 2006). Undas *et al.* (2007) demonstrated that elevated total Hcys is associated with increased platelet activation at the site of microvascular injury. McDonald *et al.* (1964) showed increased platelet adhesion in homocysteinuric patients. However, there is no evidence of a direct *in vitro* effect of homocysteine on platelet adhesion (Uhlemann *et al.* 1976) or aggregation (Stamler *et al.* 1993). Our preliminary studies have reported that reduced form of Hcys slightly stimulated platelet aggregation induced by a physiological agonist – thrombin (Olas *et al.* 2008). We also observed that HTL alone did not induce platelet aggregation, but HTL, similarly as Hcys, increased platelet aggregation induced by thrombin (Olas *et al.* 2008). Platelet aggregation induced by HLT is followed by thromboxane A_2 synthesis and secretion (McCully and Carvalho 1987). McGarrigle *et al.* (2006) showed that Hcys and HTL promote activation of platelet integrin $\alpha_{\text{IIb}}\beta_3$, that is involved in platelet adhesion or aggregation. On the other hand, Luo *et al.* (2006) observed that Hcys potentiates collagen type I induced-platelet activation through signaling components of glycoprotein VI and integrin $\alpha_2\beta_1$ pathway. Prontera *et al.* (2007) indicate that a potential molecular target of Hcys is the CD40/CD40L system in platelets; and upregulation of CD40/CD40L signaling may represent a link between hyperhomocysteinemia and an increased risk of cardiovascular diseases.

Blood platelets, in analogy to other circulating blood cells, can generate different reactive oxygen/nitrogen species (ROS/RNS) that may behave as second messengers and may regulate platelet functions. Several sources of ROS are postulated in blood platelets (Pignatelli *et al.* 1998, Wachowicz *et al.* 2002, Krotz *et al.* 2004). Intracellular sources of reactive oxygen species in platelets are arachidonic acid pathway (*via* cyclooxygenase or 12-lipoxygenase) stimulated by different agonists, the glutathione cycle (Jahn and Hansch 1990, Pignatelli *et al.* 1998) and metabolism of phosphoinositides (Gopalakrishna *et al.* 2000,

Wachowicz *et al.* 2002). ROS are generated in platelets mostly by activation of NAD(P)H oxidase (Forde and Fitzgerald 1997, Krotz *et al.* 2004, Begonja *et al.* 2005) and xanthine oxidase (Miller *et al.* 1993). Our recent data, when we used the method of cytochrome c reduction for the estimation of the level of O_2^{\bullet} showed that not only Hcys, but also HTL induces the production of O_2^{\bullet} in resting and thrombin-activated platelets (Olas *et al.* 2008).

In blood platelets peroxynitrite may be formed (Lufrano and Balazy 2003). It has been demonstrated that reaction of protein with ONOO⁻ resulted in the nitration and oxidation of some amino acid residues (Hernandez-Hernandez *et al.* 1999, Olas and Wachowicz 2007). Free and protein-bound 3-nitrotyrosine, a stable product of tyrosine nitration, may be measured as a biomarker of protein damage induced by peroxynitrite and other reactive nitrogen species (Forde and Fitzgerald 1997, Begonja *et al.* 2005), since peroxynitrite nitrates tyrosine residues in different proteins *in vivo* and *in vitro* (Miller *et al.* 1993). During platelet activation spontaneous nitration of proteins may also take place, without the addition of exogenous peroxynitrite (Muruganandam and Mutus 1994). When proteins from activated and resting platelets were separated on gels and immunoblotted with antinitrotyrosine antibodies, a number of proteins in the low molecular weight region were nitrated. Moreover, we observed that reduced form of Hcys and HTL distinctly reduced nitration of platelet proteins (Olas *et al.* 2008). It is suggested that the nitrosation of homocysteine or its metabolite – HTL in blood platelets may exist. Nitrosation of Hcys may be responsible for a decrease in the level of [•]NO, because Hcys reacts with [•]NO to form S-nitroso-homocysteine (Ignarro and Gruetter 1980). Signorello *et al.* (2007) showed that in type 2 diabetic patients high plasma Hcys is associated with increased platelet ROS level and reduced [•]NO formation in blood platelets. On the other hand, Hcys induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite and can activate key signaling cascades in the myocardium (Levrant *et al.* 2007). Moreover, Erol *et al.* (2007) have showed that Hcys increases [•]NO release from stimulated coronary microvascular endothelial cells without affecting basal [•]NO production, which is probably accompanied by increased production of reactive oxygen species. It can be postulated that endothelial cells generate [•]NO in order to minimize the damage caused by Hcys. There is a possibility that nitration of tyrosine may directly inhibit the tyrosine

phosphorylation of proteins, which is well-recognized mechanism of signal transduction in blood platelets (Ischiropoulos 2003). It was showed that the addition of peroxynitrite to platelets, at relatively high concentrations, brought about the nitration of proteins and a rapid increase in the phosphorylation of tyrosine residues (Mondoro *et al.* 1997). However, if platelets were activated by the low doses of thrombin, the amount of phosphorylation was decreased; if high doses of thrombin were used, peroxynitrite caused the increase of the phosphorylation. There is no direct evidence that phosphorylation and nitration occur on the same proteins. Probably the relationship between nitration and tyrosine phosphorylation is a competitive process (Low *et al.* 2002). Marcondes *et al.* (2006) suggested that nitration of α -actinin interferes with its phosphorylation and contributes consequently to the inhibitory role of [•]NO on platelet adhesion. Some results showed that homocysteine stimulates the tyrosine phosphorylation and activation of platelet phospholipase C γ 2 (Leoncini *et al.* 2003) and Src kinase (Luo *et al.* 2006). The stimulation of this pathway by Hcys requires signals through oxygen free radicals and thromboxane A₂. It is possible that the same mechanism may exist when blood platelets are treated with thiolactone homocysteine.

Homocysteine, coagulation and fibrinolysis

Fibrinogen is the main substrate for coagulation cascade and form a polymerized fibrin clot. Fibrinogen is a 340 kDa glycoprotein, consisting of three pairs of non-identical polypeptide chains, A α , B β and γ , interconnected by disulfide bonds. In the course of blood coagulation, fibrinogen, after the thrombin-induced cleavage of short fibrinopeptides A and B from the amino termini of α and β chains, is converted to fibrin monomers. The monomers thereafter interact spontaneously and form half-staggered protofibrils flowed by the laterally associated fibers. The initial clot is stabilized by the formation of covalent cross-links in a reaction catalyzed by activated coagulation factor XIII. Fibrinogen and other plasma proteins can be covalently modified by Hcys or its thiolactone. Lysine homocysteinylation is a plausible mechanism for protein modification *in vivo*; and is likely an important pathogenic mechanism. Mass spectrometric analysis of fibrinogen treated with Hcys revealed twelve lysines that were homocysteinylated. Several of them are close to tissue plasminogen activator (tPA) and plasminogen

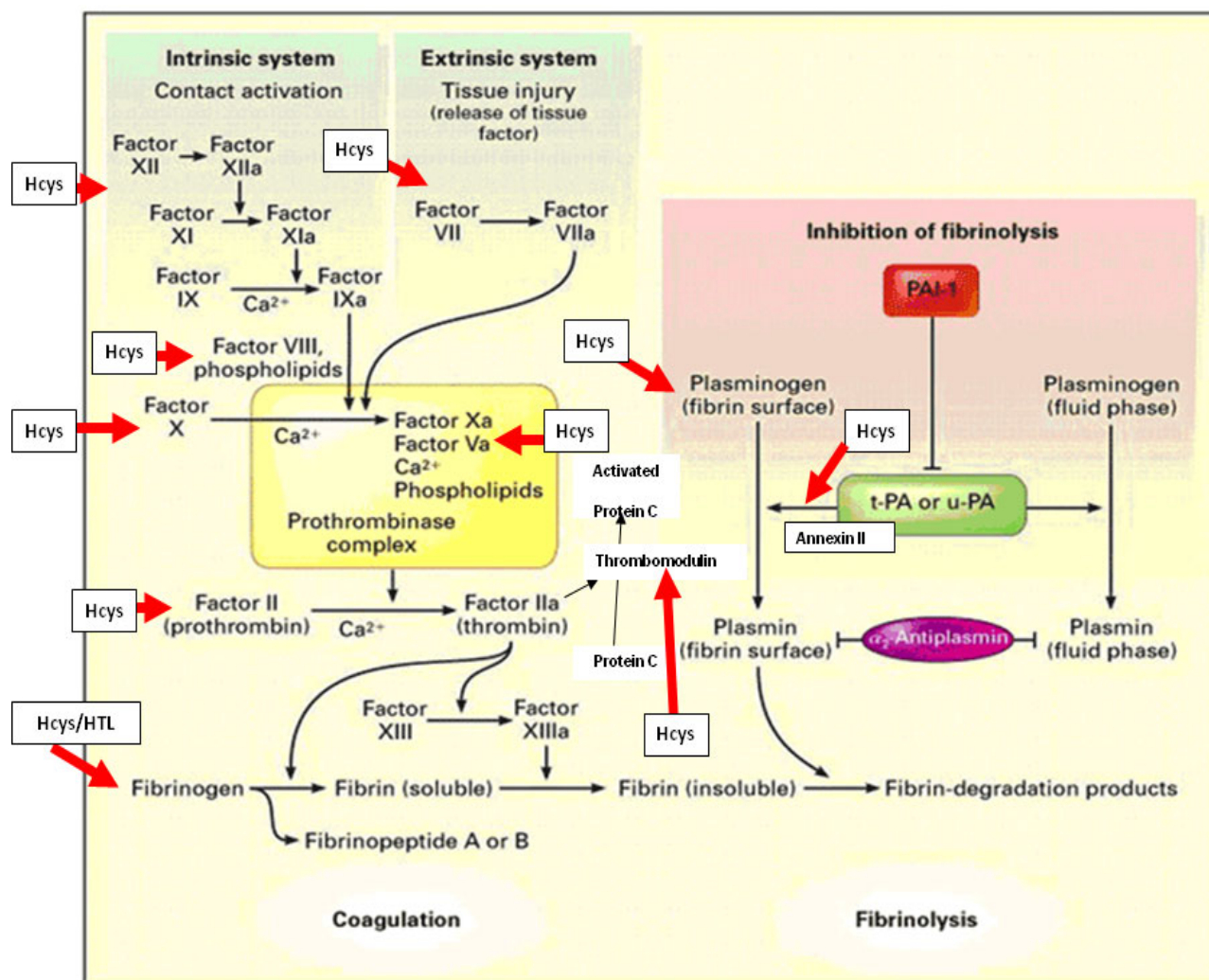


Fig. 2. Proposed targets of homocysteine (Hcys) and its thiolactone (HTL) action on coagulation and fibrinolysis.

binding sites. Moreover, lysines are major binding sites for fibrinolytic enzymes and are also sites of plasmin cleavage (Sauls *et al.* 2003, 2005, 2006, 2007). Sauls *et al.* (2007) suggest that homocysteinylated lysine residues in fibrinogen may be linked to three important functional consequences. First, modification in the α C domain could alter the lateral association of fibers and thereby alter clot structure. Second, the alteration of the protein conformation may interfere with calcium binding, which could contribute to alterations in fibrin clot structure. Third, modification of lysine sites that are directly involved in fibrinolytic enzyme binding and activity could lead to increased resistance to fibrinolysis – the process by which a fibrin clot is dissolved. Recent reports showed that compounds with thiol groups enhance plasma factor XIII-mediated fibrinogen cross-linking. Since Hcys-SH is involved in oxide-reduction reactions or disulfide exchange reactions, it is possible that some components of fibrin formation such as

fibrinogen and coagulation factor XIII can be altered. Lauricella *et al.* (2002, 2006) observed that clots formed from human plasma incubated *in vitro* with Hcys have a more compact structure, with shorter and more frequently branched fibers, than those formed in the absence of Hcys. Harpel *et al.* (1992) showed that Hcys enhances the binding of lipoprotein(a) to fibrin, and this results may suggest a biochemical relationship between thiol compound metabolism, thrombosis and atherogenesis.

In mildly hyperhomocysteinemic human subjects dysfibrinogenemia (which is characterized by formation of clots composed of abnormally thin, tightly packed fibers with an increased resistance to fibrinolysis) could play a role in the increased risk of atherothrombotic disease (Undas *et al.* 2006, Sauls *et al.* 2007). On the other hand, Lijfering *et al.* (2007) showed that the increased risk of cardiovascular diseases in hyperhomocysteinemia is mainly related to elevated levels of coagulation factor VIII. Moreover, Ebbesen and

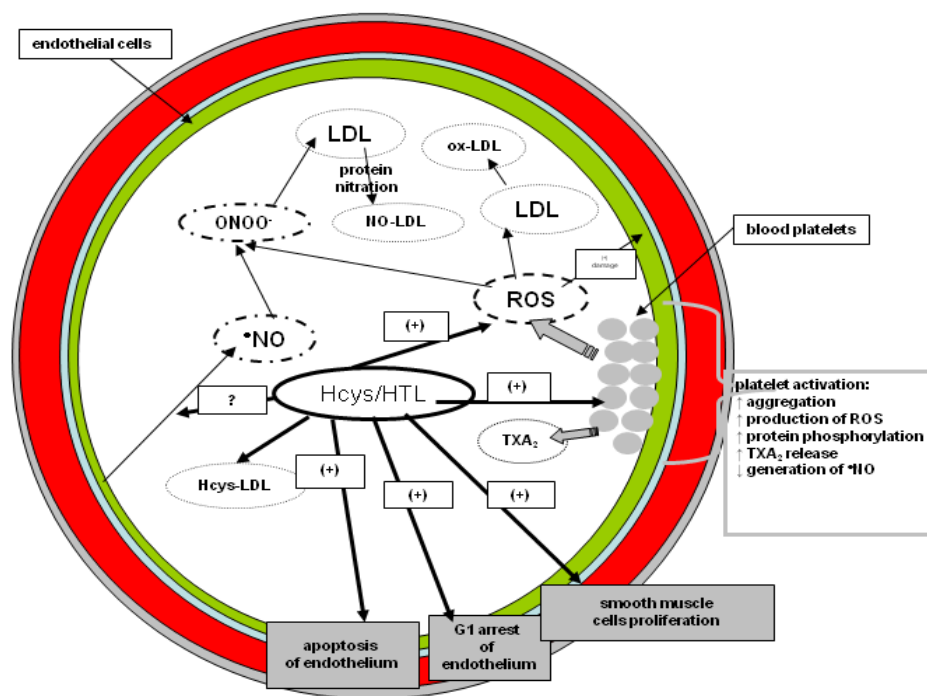


Fig. 3. Proposed model for the regulatory role of Hcys and HTL in vascular wall (smooth muscle, endothelial cells) and in blood platelets.

Hcys – homocysteine
 HTL – homocysteine thiolactone
 LDL – low density lipoproteins
 Hcys-LDL – homocysteinylated LDL
 ox-LDL – oxidized LDL
 ROS – reactive oxygen species
 *NO – nitric oxide
 ONOO[•] – peroxynitrite
 NO-LDL – nitrated LDL
 TXA₂ – thromboxane A₂

Ingerslev (2005) found reduced functional activities of coagulation factors XII, X and II in hyperhomocysteinemia-induced by folate deficiency in rat, whereas the functional factor VII activity was unchanged. On the contrary, Al-Obaidi *et al.* (2000) observed that levels of coagulation factor VIIa and Hcys correlate in plasma of patients with acute coronary syndrome. These changes may enhance risk of thrombotic events in hyperhomocysteinemic patients. Recent reports showed that activity of antithrombin, which is one of the most important inhibitors of blood coagulation, is inhibited by HTL (Gugliucci 2008). Proposed targets of Hcys and HTL action on coagulation and fibrinolysis process are presented in Figure 2.

Conclusions

The mechanism of homocysteine and its thiolactone action on hemostasis process is complex and still unclear. Hcys or HTL may modulate the signal

transduction in different cells and sometimes act in opposite ways. Homocysteine and its metabolite cause the changes in the level of reactive oxygen species and reactive nitrogen species (special *NO) and may be responsible for the modification of hemostasis induced by these compounds (Fig. 3). The biological significance of hemostatic protein modification (fibrinogen and other coagulation factors) induced by Hcys or HTL is not well known, although especially N-homocysteinylated induced by HTL may play an important role in different pathophysiological consequences leading to cardiovascular diseases.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by grant 506/810 from University of Lodz.

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