Mechanism action of homocysteine and its thiolactone in haemostasis system

KAMIL KAROLCZAK, BEATA OLAS*

Department of General Biochemistry, Institute of Biochemistry, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

* corresponding author

Beata Olas

e-mail: olasb@biol.uni.lodz.pl

Summary

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

Key words: homocysteine, homocysteine thiolactone, haemostasis

Introduction

Haemostasis is a complex process that regulates *in vivo* the flowing properties of blood. Classical primary and secondary haemostasis only comprised the sequential formation of the white and red thrombus, but there is growing awareness that haemostasis 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 haemostasis 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). Haemostatic abnormalities can lead to excessive bleeding, thrombosis or other cardiovascular diseases. Recently, it have shown that homocysteine (Hcy), which is an intermediate formed during the catabolism of the essential dietary amino acid methionine induces changes in haemostasis, including blood clotting and fibrinolysis (Perla-Kajan *et al*. 2007). Elevated level of Hcy 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 haemostasis process.

Chemical structure and function of homocysteine and its metabolites

Homocysteine is involved in convertions of methionine (Met) and cysteine (Cys). The immediate precursor of Hcy 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, Hcy enters the transulfuration 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 Hcy 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, Jakubowski 2004, Perla-Kajan *et al.* 2007). The chemical structure of Hcy and its thiolactone is presented on Figure 1. Homocysteine metabolism depends on the level of vitamins (folic acid, vitamin B_6 and B_{12}) as cofactors for the enzymes involved in Hcy turnover. In human blood homocysteine may exist in free or protein bound forms as N-homocysteinylated (N-Hcy-protein) or S-homocysteinylated proteins (S-Hcy-protein), that were described as N-Hcy-hemoglobin, N-(Hcy-S-S-Cys)-albumin, S-Hcy-albumin, and cysteinylhomocysteine (Cys-Hcy)) (Jakubowski 2002, Chwatko and Jakubowski 2005A Chwatko and Jakubowski 2005B, Jakubowski 2005, Jakubowski 2006, 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, Jakubowski 2004, Jakubowski 2005, Jakubowski 2006), whereas S-homocysteinylation is induced by Hcy (Fig. 1). In human plasma some various haemostatic 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-Hcy-proteins are also found in different haemostatic 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 Hcy is oxidized to form dimers (homocystine) or combined with cysteine to form mixed disulphides (Jakubowski 2002, Chwatko and Jakubowski 2005A, Chwatko and Jakubowski 2005B, Jakubowski 2005, Jakubowski 2006, Perla-Kajan *et al.* 2007). Plasma Hcy level is determined by multiple factors, like genetic, demographic, acquired, and lifestyle determinants. Total plasma Hcy concentration for healthy adults is in the range of 5-15 μM. Patients with mild hyperhomocysteinemia have 15-25 μM Hcy (Perla-Kajan *et al.* 2007). In urine after oxidation of Hcy is referred to as homocystinuria. Thus, for abnormal metabolism of Hcy, the blood can be analyzed. Mild hyperhomocysteinemia is an independent risk factor for atherosclerotic disease, deep vein thrombosis and thromboembolism. Elevated Hcy levels promote thrombosis, although the mechanism by which Hcy 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 haemostasis. Endothelial cells are very sensitive even to a mild increase of 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 homocysteine catabolism through transsulfuration pathway (Jacobsen 1998). Elevated level of Hcy 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

glycosaminogycan-antithrombin III interaction. Results of Rodgers *et al.* (1986) showed that after incubation of endothelium with Hcy activity of coagulation factor V increases. Coagulation factor V in Hcy-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, Hcy and its thiolactone have no effect on factor V activation by thrombin. It was showed that factor V incubated with [^{35}S]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) suggest 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 Hcy on activity of thrombomodulin, which is involved in blood clotting.

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

Homocysteine and nitric oxide

Nitric oxide ($^{\bullet}$NO) plays an important role in a number of physiological processes. It is well known, that vascular endothelial cells produce $^{\bullet}$NO. However, other cells, which are involved in haemostasis - blood platelets may also synthesize $^{\bullet}$NO and platelet $^{\bullet}$NO synthase (NOS) has been described and identified during the mid 90s (Muruganadam and Mutus 1994). Two main NOS isoforms have been isolated: calcium independent inducible NOS (iNOS) and a calcium/calmodulin dependent endothelium type NOS (eNOS) (Mehta et al. 1995). $^{\bullet}$NO is released by NOS-es action from the guanidyno moiety of L-arginine, yielding NO and cytruline. $^{\bullet}$NO is very simple free radical which play important regulatory role in many tissues, but originally $^{\bullet}$NO was identified as a factor influenced mainly human vascular system. $^{\bullet}$NO has been implicated in a number of cardiovascular diseases and every risk factor for these appears to be associated with a reduction in endothelial generation of $^{\bullet}$NO.

Evidences of some cooperation between homocysteine and $^{\bullet}$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 tendency to higher Hcy concentration in the blood. Mentioned tendency was especially distinct in subpopulation of *NOS3* 894TT patients who had low foliate levels. However, there was no strictly correlation between *NOS3* 894TT genotype and Hcy plasma levels (Brown *et al.* 2003). The question is how Hcy can modulate $^{\bullet}$NO synthase. First thought is that Hcy may influence on NOS activity. Transformation of radiolabelled arginine to cytruline catalyze by nitric oxide synthase is unchanged after renal arterial endothelium incubation with Hcy (40 μM) in comparison with control cells. Also after incubation of endothelial cells with Hcy (10, 20 and 50 μM during 24 hours) NOS activity was untouched (Fatini *et al.* 2005). Despite the fact that NOS activity seemed to be normal also during

hyperhomocysteinemia *in vitro,* level of $^{\bullet}NO$ under such conditions is definitely lower and this effect is directly proportional to Hcy plasma level (Becker *et al.* 2005). One of possible way of NOS activity regulation by Hcy 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 hiperhomocysteinemia 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 Hcy (24 hours) can be stopped by using S-adenosylhomocysteine – methylation inhibitor.

The main molecules responsible for decrease of $^{\bullet}NO$ level in hyperhomocysteinemic patients are probably reactive oxygen species (ROS). Hcy can enhance ROS production (Li *et al.* 2002, Fischer *et al.* 2003, Fatini *et al.* 2005) – the generation of superoxide anion ($O_2^{-\bullet}$) in reaction catalyzed by NADPH oxidase (Becker *et al.* 2005). Results of Fischer *et al.* (2003) showed the increased of protein nitration (induced by peroxynitrite, which is produced in the reaction of $^{\bullet}NO$ with $O_2^{-\bullet}$.

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 responses of platelet to agonists, which is named platelet activation, include 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, Ryningen 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 Hcy levels is associated with more potent platelets aggregation (Rajkumar *et al.* 1999). Signorello *et al.* (2007) observed that Hcy induces oxidative stress, inhibits nitric oxide formation in platelets from type 2 diabetic patients, and may promote platelet hyperactivity and various cardiovascular diseases. Moreover, Hcy stimulates the calcium mobilization in platelets from 2 diabetic patients (Alexandru *et al.* 2007). The study of Mohan *et al.* (2008) suggest that platelet activation and hypercoagulability occur after exposure to Hcy, especially in patients with critical limb ischemia. Hcy 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 then in control animals (Durand *et al.* 1996). The direct action of Hcy 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 and 2007) and protein tyrosine phosphorylation in blood platelets (Leoncini *et al.* 2006). Results of Undas *et al.* (2007) demonstrated that elevated total Hcy 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 Hcy slightly stimulated platelet aggregation induced by a physiological agonist - thrombin (Olas *et al.* (in press)). We also observed that HTL alone do not induce platelet aggregation, but HTL, like Hcy increased platelet aggregation induced by thrombin (Olas *et al.* (in press)). Platelets aggregation induced by HLT is followed by thromboxane A_2 synthesis and secretion (McCully and Carvalho 1987). McGarrigle *et al.* (2006) showed that Hcy and HTL promote activation of platelet integrin $\alpha_{IIb}\beta_3$, that is involved in platelet adhesion or aggregation. On the other hand, Luo *et al.* (2006) observed that Hcy 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 Hcy is the CD40/CD40L system in platelets; and upregulation of CD40/CD40L signaling may represent a link between hyperhomocyteinemia 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. In blood platelets are postulated several sources of ROS (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 xantine oxidase (Miller *et al*. 1993). Our recent data, where we used in our experiments the method of cytochrome c reduction for the estimation of the level of $O_2^{-\bullet}$ showed that not only Hcy, but

also HTL induces the production of $O_2^{-\bullet}$ in resting and thrombin - activated platelets (Olas *et al.* (in press)).

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, in our study, we observed that reduced form of Hcy and HTL distinctly reduced nitration of platelet proteins (Olas *et al.* (in press)). It is suggested that the nitrosation of homocysteine or its metabolite – HTL in blood platelets may exist. Nitrosation of Hcy may be responsible for a decrease in the level of $^{\bullet}NO$, because Hcy reacts with $^{\bullet}NO$ to form S-nitroso-homocysteine (Ignarro and Gruetter 1980). Signorello *et al.* (2007) showed that in type 2 diabetic patients high plasma Hcy is associated with increased platelet ROS level and reduced $^{\bullet}NO$ formation in blood platelets. On the other hand, Hcy induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite and can activate key signaling cascades in the myocardium (Levrand *et al*. 2007). Moreover, Erol *et al.* (2007) have showed that Hcy increases $^{\bullet}NO$ release from stimulated coronary mircovascular endothelial cells without affecting basal $^{\bullet}NO$ production, which is probably accompanied by increased production of reactive oxygen species. It can be postulated that endothelial cells generate $^{\bullet}NO$ in order to

minimize the damage caused by Hcy. There is 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 on the same proteins occurs. Probably 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 $^{\bullet}NO$ on platelet adhesion. Some results showed that homocysteine stimulates the tyrosine phoshorylation and activation of platelet phospholipase Cγ2 (Leoncini *et al.* 2003) and Src kinase (Luo *et al.* 2006). The stimulation of this pathway by Hcy requires signals through oxygen free radicals and thromboxane A_2. 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 nonidentical polypeptide chains, Aα, Bβ 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 Hcy 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 Hcy revealed twelve lysines that were homocysteinylated. Several of these are close to tissue plasminogen activator (tPA) and plasminogen binding sites. Moreover, lysines are major binding sites for fibrinolystic enzymes and are also sites of plasmin cleavage (Sauls *et al.* 2003, 2005, 2006 and 2007). Sauls *et al.* (2007) suggest that homocysteinylation of lysine residues in fibrinogen may be linked to three important functional consequences. First, modification in the αC 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 fibrinolitic 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 Hcy-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 and 2006) observed that clots formed from human plasma incubated *in vitro* with Hcy have been more compact structure, with shorter and more frequently branched fibers, than those formed in the absence of Hcy. Harpel *et al.* (1992) showed that Hcy 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 dysfibirinogemia (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, Souls *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 coagulation factor VIII levels. Moreover, Ebbesen and 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. Contrary, Al-Obaidi *et al.* (2000) observed that levels of coagulation factor VIIa and Hcy correlate in plasma of acute coronary syndrome patients. This changes may enhanced 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 Hcy and HTL action on coagulation and fibrinolysis process are presented in Figure 2.

Conclusion

The mechanism of homocysteine and its thiolactone action on haemostasis process is complex and still unclear. Hcy 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 $^{\bullet}NO$) and may be responsible for the modification of haemostasis induced by these compounds (Fig. 3). The biological significance of haemostatic protein modification (fibrinogen and other coagulation factors) induced by Hcy or HTL is not well known, although especially N-homocysteinylation

induced by HTL may play an important role in different pathophysiological consequences leading to cardiovascular diseases.

References

AKHAND AA, PU M, SENGA T, KATO M, SUZUKI H, MIYATA T, HAMAGUCHI M, NAKASHIMA I: Nitric oxide controls src kinase activity through a sulfhydryl group modification-mediated Tyr-527-independent and Tyr-416-linked mechanism. *J Biol Chem* **274**: 25821-25826, 1999.

ALEXANDRU N, JARDIN I, POPOV D, SIMIONESCU M, GARCIA-ESTAN J, SALIDO GM, ROSADO JA: Effect of homocysteine on calcium mobilisation and platelet function in type 2 diabetes mellitus. *J Cell Mol Med* **14:** 2007.

AL-OBAIDI MK, PHILIPPOU H, STUBBS PJ, ADAMI A, AMERSEY R, NOBLE MM, LANE DA: Relationships between homocysteine, factor via, and thrombin generation in acute coronary syndromes. *Circulation* **101**: 372-377, 2000.

BECKER A, VAN HINSBERGH VW, KOSTENSE PJ, JAGER A, DEKKER JM, NIJPELS G, HEINE RJ, BOUTER LM, STEHOUWER CD: Serum homocysteine is weakly associated with von Willebrand factor and soluble vascular cell adhesion molecule 1, but not with C-reactive protein in type 2 diabetic and non-diabetic subjects - the Hoorn study. *Eur J Clin Invest* **30**: 763-770, 2000.

BECKER JS, ADLER A, SCHNEEBERGER A, HUANG H, WANG Z, WALSH E, KOLLER A, HINTZE TH: Hyperhomocysteinemia, a cardiac metabolic disease: Role of nitric oxide and the p22phox subunit of NADPH oxidase. *Circulation* **111**: 2112-2118, 2005.

BEGONJA AJ, GAMBARYAN S, GEIGER J, AKTAS B, POZGAJOVA M, NIESWANDT B, WALTER U: Platelet NAD(P)H oxidase-generated ROS production regulates {alpha}IIb{beta}3 integrin activation independent of the NO/cGMP pathway. *Blood* **106:** 2757-2760, 2005.

BROSNAN JT, JACOBS RL, STEAD LM, BROSNAN ME: Methylation demand: a key determinant of homocysteine metabolism. *Acta Biochim Pol* **51:** 405-413, 2004.

BROWN KS, KLUIJTMANS LA, YOUNG IS, WOODSIDE J, YARNELL JW, MCMASTER D, MURRAY L, EVANS AE, BOREHAM CA, MCNULTY H, STRAIN JJ, MITCHELL LE, WHITEHEAD AS: Genetic evidence that nitric oxide modulates homocysteine: The nos3 894tt genotype is a risk factor for hyperhomocystenemia. *Arterioscler Thromb Vasc Biol* **23**: 1014-1020, 2003.

CHWATKO G, JAKUBOWSKI H: The determination of homocysteine-thiolactone in human plasma. *Anal Biochem* **337**: 271-277, 2005A.

CHWATKO G, JAKUBOWSKI H: Urinary extraction of homocysteine thiolactone in humans. *Clin Chem* **51:** 408-415, 2005B.

DUAN J, DAI S, FANG CX, SUN R, SHAVALI S, SHARMA SK, EBADI M, REN J: Phytoestrogen alpha-zearalanol antagonizes homocysteine-induced imbalance of nitric oxide/endothelin-1 and apoptosis in human umbilical vein endothelial cells. *Cell Biochem Biophys* **45**: 137-145, 2006.

DURAND P, PROST M, BLACHE D: Pro-thrombotic effects of a folic acid deficient diet in rat platelets and macrophages related to elevated homocysteine and decreased n-3 polyunsaturated fatty acids. *Atherosclerosis* **121**: 231-243, 1996.

EBBESEN LS, INGERSLEV J: Folate deficiency-induced hyperhomocysteinemia attenuates, and folic acid supplementation restores, the functional activites of rat coagulation factors XII, X, and II. *J Nutr* **135:** 1836-1840, 2005.

EROL A, CINAR MG, CAN C, MURAT O, ULKER S, KOSAY S: Effect of homocysteine on nitric oxide production in coronary microvascular endothelial cells. *J Endothel Cell Res* **14:** 157-161, 2007.

FATINI C, SOFI F, GORI AM, STICCHI E, MARCUCCI R, LENTI M, CASINI A, SURRENTI C, ABBATE R, GENSINI GF: Endothelial nitric oxide synthase -786T>C, but not 894G>T and 4a4b, polymorphism influences plasma homocysteine concentrations in persons with normal vitamin status. *Clin Chem* **51**: 1159-1164, 2005.

FISCHER PA, DOMINGUEZ GN, CUNIBERTI LA, MARTINEZ V, WERBA JP, RAMIREZ AJ, MASNATTA LD: Hyperhomocysteinemia induces renal hemodynamic dysfunction: Is nitric oxide involved? *J Am Soc Nephrol* **14**: 653-660, 2003.

FORDE RC, FITZGERALD DJ: Reactive oxygen species and platelet activation in reperfusion injury. *Circulation* **95:** 787-789, 1997.

GOPALAKRISHNA R, JAKEN S: Protein kinase C signaling and oxidative stress. *Free Rad Biol Med* **28:** 1349-1361, 2000.

GUGLIUCCI A: Antithrombin activity is inhibited by acrolein and homocysteine thiolactone: protection by cysteine. *Life Sci* **82:** 413-418, 2008.

HARPEL PC, CHANG VT, BORTH W: Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: a potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism. *Proc Natl Acad Sci USA* **89:** 10193-10197, 1992.

HERNANDEZ-HERNANDEZ A, ANCHEZ-YAGUE J, MARTIN-VALMASEDA EM, LLIANILLO M: Oxidative inactivation of human and sheep platelet membrane-associated phosphotyrosine phosphatase activity. *Free Rad Biol Med* **26:** 1218-1230, 1999.

IGNARRO LJ, GRUETTER CA: Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. *Biochim Biophys Acta* **631:** 221-231, 1980.

ISCHIROPOULOS H: Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem Biophys Res Commun* **305:** 776-783, 2003.

JACOBSEN DW. Homocysteine and vitamins in cardiovascular disease. *Clin Chem* **44:** 1833-1843, 1998.

JAHN B, HANSCH GM: Oxygen radical generation in human platelets: depence of 12-lipoxygenase activity and on the glutathione cycle. *Int Arch Allergy Appl Immunol* 93: 73-79, 1990.

JAKUBOWSKI H, FERSHT AR: Alaternative pathway for editing noncognate amino acids by aminoacyl-tRNA synthases. *Nucleic Acids Res* **9:** 3105-3117, 1981.

JAKUBOWSKI H: Anti-N-homocysteinylated protein autoantibodies and cardiovascular disease. *Clin Chem Lab Med* **43:** 1011-1014, 2005.

JAKUBOWSKI H: Homocysteine is a protein amino acid in humans. Implications for homocysteine-linked disease. *J Biol Chem* **277:** 30425-30428, 2002.

JAKUBOWSKI H: Homocysteine-thiolactone and S-nitroso-homocysteine mediate incorporation of homocysteine into protein in humans. *Clin Chem Lab Med* **41:** 1462-1466, 2003.

JAKUBOWSKI H: Molecular basis of homocysteine toxicity in humans. *Cell Mol Life* **61:** 470-487, 2004.

JAKUBOWSKI H: Pathophysiological consequences of homocysteine excess. *J Nutr* **136:** 1741S-1749S, 2006.

JAKUBOWSKI H, ZHANG L, BARDEGUEZ A, AVIV A: Homocysteine thiolactone and protein homocysteinylation in human endothelial cells: implications for atherosclerosis. *Circ Res* **87:** 45-51, 2000.

KROTZ F, SOHN HY, POHL U: Reactive oxygen species, players in the platelet game. *Arterioscler Thromb Vasc Biol* **24:** 1988-1996, 2004.

LAURICELLA AM, QUINTANA I, CASTANON M, SASSETTI B, KORDICH L: Influence of homocysteine on fibrin network lysis. *Blood Coagul Fibrinolysis* **17**: 181-186, 2006.

LAURICELLA AM, QUINTANA IL, KORDICH LC: Effects of homocysteine thiol group on fibrin networks: Another possible mechanism of harm. *Thromb Res* **107**: 75-79, 2002.

LENTZ SR, PIEGORS DJ, FERNANDEZ JA, ARNING EE, MALINOW MR, GRIFFIN JH, BOTTIGLIERI T, HAYNES WG, HEISTAD DD: Effect of hyperhomocysteinemia on protein C activation and activity. *Blood* **100:** 2108-2112, 2002.

LEONCINI G, BRUZZESE D, SIGNORELLO MG: A role for PLCγ2 in platelet activation by homocysteine. *J Cell Biochem* **100:** 1255-1265, 2007.

LEONCINI G, BRUZZESE D, SIGNORELLO MG: Activation of p38MAPKinase/cPLA$_2$ pathway in homocysteine treated platelets. *J Thromb Haemos* **4:** 209-216, 2006.

LEONCINI G, PASCALE R, SIGNORELLO MG: Effects of homocysteine on l-arginine transport and nitric oxide formation in human platelets. *Eur J Clin Invest* 33: 713-719, 2003.

LEVRAND S, PACHER P, PESSE B, ROLLI J, FAIHL F, WAEBER B, LIAUDET L: Homocysteine induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite. *Biochem Biophys Res Com* **359:** 445-450, 2007.

LEVY-TOLEDANO S: Platelet signal transduction pathways: Could we organize them into a 'hierarchy'? *Haemostasis* **29**: 4-15, 1999.

LIJFERING WM, VEEGER NJGM, BROUWER JLP, van der MEER J: The risk of venous and arterial thrombosis in hyperhomocysteinemic subjects may be a result of elevated factor VIII levels. *Haematol* **92:** 1703-1706, 2007.

LOW SY, SABETKAR M, BRUCKDORFER KR, NASEEM KM: The role of protein nitration in the inhibition of platelet activation by peroxynitrite. *FEBS Lett* **511:** 59-64, 2002.

LUO F, LIU X, WANG S, CHEN H: Effect of homocysteine on platelet activation induced by collagen. *Nutrition* **22**: 69-75, 2006.

MARCONDES S, CARDOSO MHM, MORGANTI RP, THOMAZZI SM, LILLA S, MURAD F, DE NUCCI G, ANTUNES E: Cyclic GMP-independent mechanisms contribute

to the inhibition of platelet adhesion by nitric oxide donor: a role for α–actinin nitration. *PNAS* **103:** 3434-3439, 2006.

MC CULLY KS, CARVALHO AC: Homocysteine thiolactone, N-homocysteine thiolactonyl retinamide, and platelet aggregation. *Res Commun Chem Pathol Pharmacol* **56**: 349-60, 1987.

MC DONALD L, BRAY C, FIELD C, LOVE F, DAVIES B: Homocystinuria, thrombosis and the blood platelets. *Lancet* **1:** 745-746, 1964.

MCGARRIGLE SA, WALSH GM, O'NEILL S, MORAN N, COLLINS PB: Homocysteine and its thiolactone derivative promote activation of the platelet integrin αIIbβ3. *8th UK Platelet Meeting*, Reading , 49, 2006.

MEHTA JL, CHEN LY, KONE CB, MEHTA P, TURNER P: Identification of constitutive and inducible forms of nitric oxide synthase in human platelets. *J Lab Clin Med* **125**: 87-96, 1995.

MILLER DM, GROVER TA, NAYINI N, AUST SD: Xantine-oxidase- and iron-dependent lipid peroxidation. *Arch Biochem Biophys* **301:** 1-7, 1993.

MOHAN IV, JAGROOP IA, MIKHAILIDIS DP, STANSBY GP: Homocysteine activates platelets in vitro. *Clin Appl Thromb Hemost* **14**: 8-18, 2008.

MONDORO TH, SHAFER BC, VOSTAL JG: Peroxynitrite-induced tyrosine nitration and phosphorylation in human platelets. *Free Rad Biol Med* **22:** 1055-1063, 1997.

MURUGANANDAM A, MUTUS B: Isolation of nitric oxide synthase from human platelets. *Biochim Biophys Acta* **1200:** 1-6, 1994.

OLAS B, KEDZIERSKA M, WACHIOWICZ B: Comparative studies on homocysteine and its metabolite – homocysteine thiolactone action in blood platelets in vitro. *Platelets* (in press)

OLAS B, WACHOWICZ B: Role of reactive nitrogen species in blood platelet functions. *Platelets* **23:** 1-11, 2007.

PERŁA-KAJAN J, TWARDOWSKI T, JAKUBOWSKI H: Mechanisms of homocysteine toxicity in humans. *Amino Acids* **32:** 561-572, 2007.

PIGNATELLI P, PULCINELLI FM, LENTI L, GAZZANIGA PP, VIOLI F: Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* **91:** 484-490, 1998.

PRONTERA C, MARTELLI N, EVANGELISTA V, D'URBANO E, MANARINI S, RECCHIUTI A, DRAGANI A, PASSERI C, DAVI G, ROMANO M: Homocysteine modulates the CD40/CD40L system. *J Am Coll Cardiol* **49:** 2182-2190, 2007.

RAJKUMAR V, RAGATZKI P, SIMA A, LEVY J: Enhanced platelet aggregation, high homocysteine level, and microvascular disease in diabetic muscle infarctions: Implications for therapy. *Endocrine* **11**: 57-60, 1999.

RAPOSO B, RODRIGUEZ C, MARTINEZ-GONZALES J, BADIMON L: High levels of homocysteine inhibit lysyl oxidase (LOX) and downregulate LOX expression in vascular endothelial cells. *Atheroscl* **177:** 1-8, 2004.

RIBA R, NICOLAOU A, TROXLER M, HOMER-VANIASINKAM S, NASEEM KM: Altered platelet reactivity in peripheral vascular disease complicated with elevated plasma homocysteine levels. *Atherosclerosis* **175**: 69-75, 2004.

RODGERS GM, KANE WH: Activation of endogenous factor V by a homocysteine-induced vascular endothelial cell activator. *J Clin Invest* **77**: 1909-1916, 1986.

RYNINGEN A, HOLMSEN H: In Platelet Physiology and Pharmacology. (Gundu H. and Rao R. eds.) Kluwer Academic Publishers, Norwell, 1-22, 1999.

SAULS DL, ARNOLD EK, BELL CW, ALLEN JC, HOFFMAN M: Pro-thrombotic and pro-oxidant effects of diet-induced hyperhomocysteinemia. *Thromb Res* **120:** 117-126, 2007.

SAULS DL, LOCKHART E, WARREN ME, LENKOWSKI A, WILHELM SE, HOFFMAN M: Modification of fibrinogen by homocysteine thiolactone increases resistance to

fibrinolysis: a poptential mechanism of the thrombotic tendency in hyperhomocysteinemia. *Biochem* **45:** 2480-2487, 2006.

SAULS DL, WOLBERG AS, HOFFMAN M: Elevated plasma homocysteine leads to alterations in fibrin clot structure and stability: Implications for the mechanism of thrombosis in hyperhomocysteinemia. *J Thromb Haemost* **1**: 300-306, 2003.

SIGNORELLO MG, PASCALE R, LEONCINI G: Effect of homocysteine on arachidonic acid release in human platelets. *Eur J Clin Invest* **32**: 279-284, 2002.

SIGNORELLO MG, VIVIANI GL, ARMANI U, CERONE R, MINNITI G, PIANA A, LEONCINI G: Homocysteine, reactive oxygen species and nitric oxide in type 2 diabetes mellitus. *Thromb Res* **120:** 607-613, 2007.

STAMLER JS, OSBORNE JA, JARAKI O, RABBANI LE, MULLINS M, SINGEL D, LOSCALZO J: Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* **91:** 308-318, 1993.

THAMBYRAJAH J, TOWNEND JN: Homocysteine and atherothrombosis – mechanisms for injury. *European Heart Journal* **21:** 967-974, 2000.

UHLEMANN ER, TENPAS JH, LUCKY AW, SCHULMAN JD, MUDD SH, SHULMAN NR: Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. *N Engl J Med* **295:** 1283-1286, 1976.

UNDAS A, BROZEK J, JANKOWSKI M, SIUDAK Z, SZCZEKLIK A, JAKUBOWSKI H: Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. *Arterioscler Thromb Vasc Biol* **26**: 1397-1404, 2006.

UNDAS A, STEPIEN E, PLICNER D, ZIELINSKI L, TRACZ W: Elevated total homocysteine is associated with increased platelet activation at the site of microvascular injury: effects of folic acid administration. *J Thromb Haemost* **5**: 1070-1072, 2007.

UNDAS A, WILLIAMS EB, BUTENAS S, ORFEO T, MANN KG: Homocysteine inhibits inactivation of factor Va by activated protein c. *J Biol Chem* **276**: 4389-4397, 2001.

WACHOWICZ B, OLAS B, ZBIKOWSKA HM, BUCZYŃSKI A: Generation of reactive oxygen species in blood platelets. *Platelets* **13:** 175-182, 2002.

WU KK: Platelet activation mechanisms and markers in arterial thrombosis. *J Intern Med* **239**: 17-34, 1996.

YANG X, GAO Y, ZHOU J, ZHEN Y, YANG Y, WANG J, SONG L, LIU Y, XU H, CHEN Z, HUI R: Plasma homocysteine thiolactone adducts associated with risk of coronary heart disease. *Clin Chim Acta* **364:** 230-234, 2006.

Acknowledgement

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

Legend to Figures

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

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

Fig. 3. Proposed model for the regulatory role of Hcy and HTL in vascular well (smooth muscle, endothelial cells) and in blood platelets. Abbreviations: Hcy – homocysteine, HTL – homocysteine thiolactone, LDL – low density lipoproteins, Hcy-LDL – homocysteinylated LDL, ox-LDL – oxidized LDL, ROS – reactive oxygen species, $^{\bullet}NO$ – nitric oxide, $ONOO^-$ - peroxynitrite, NO-LDL- nitrated LDL, TXA_2 – thromboxane A_2

Fig. 2

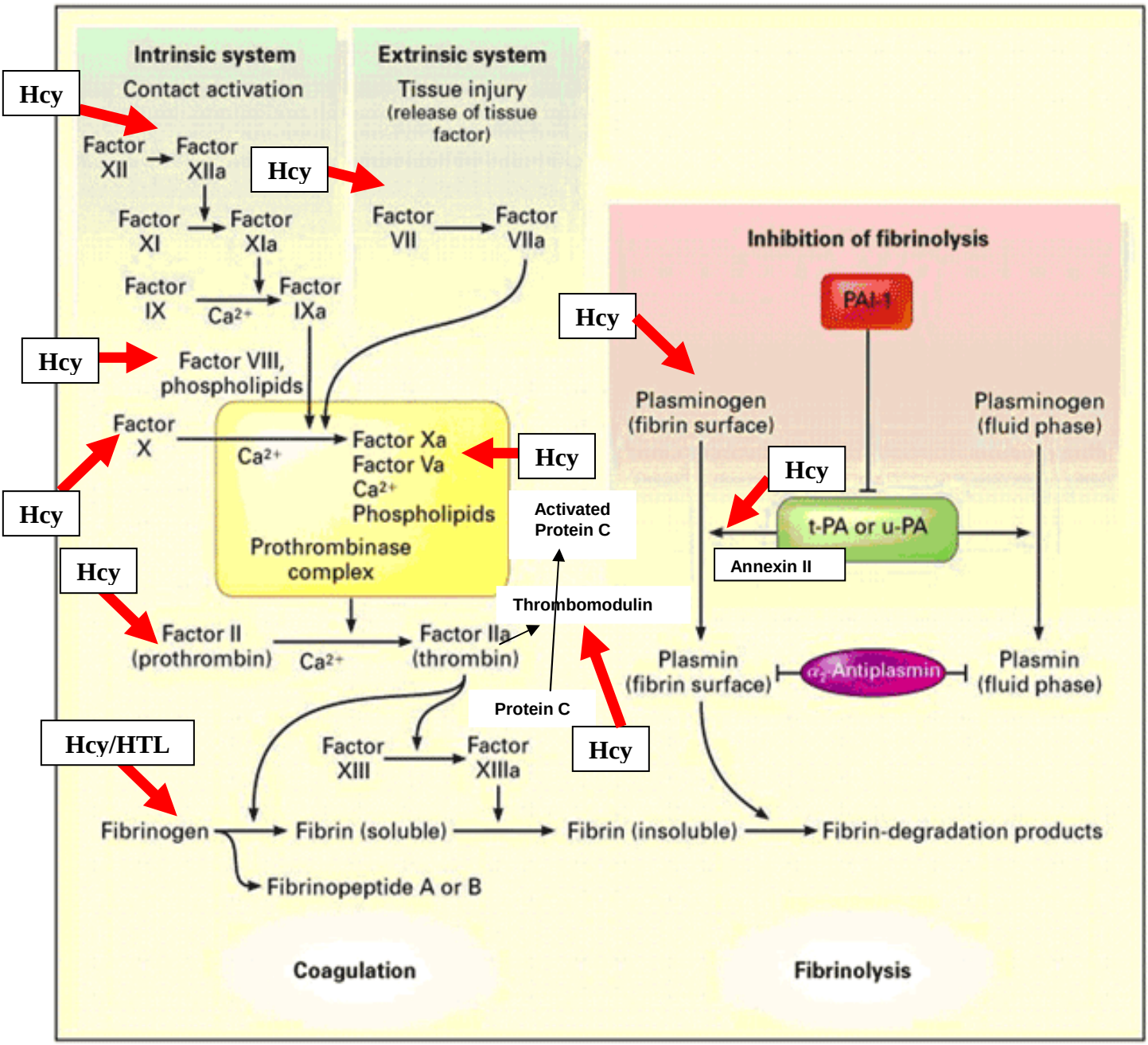

Intrinsic system
Contact activation
Extrinsic system
Tissue injury
(release of tissue factor)
Hcy
Factor XII
Factor XIIa
Factor XI
Factor XIa
Factor IX
Ca2+
Factor IXa
Factor VII
Factor VIIa
Factor VIII, phospholipids
Factor X
Ca2+
Factor Xa
Factor Va
Ca2+
Phospholipids
Prothrombinase complex
Activated Protein C
Thrombomodulin
Protein C
Factor II (prothrombin)
Ca2+
Factor IIa (thrombin)
Factor XIII
Factor XIIIa
Fibrinogen
Fibrin (soluble)
Fibrin (insoluble)
Fibrin-degradation products
Fibrinopeptide A or B
Hcy/HTL
Inhibition of fibrinolysis
PAI-1
Plasminogen (fibrin surface)
Plasminogen (fluid phase)
t-PA or u-PA
Annexin II
Plasmin (fibrin surface)
α2-Antiplasmin
Plasmin (fluid phase)
Coagulation
Fibrinolysis

Fig. 3

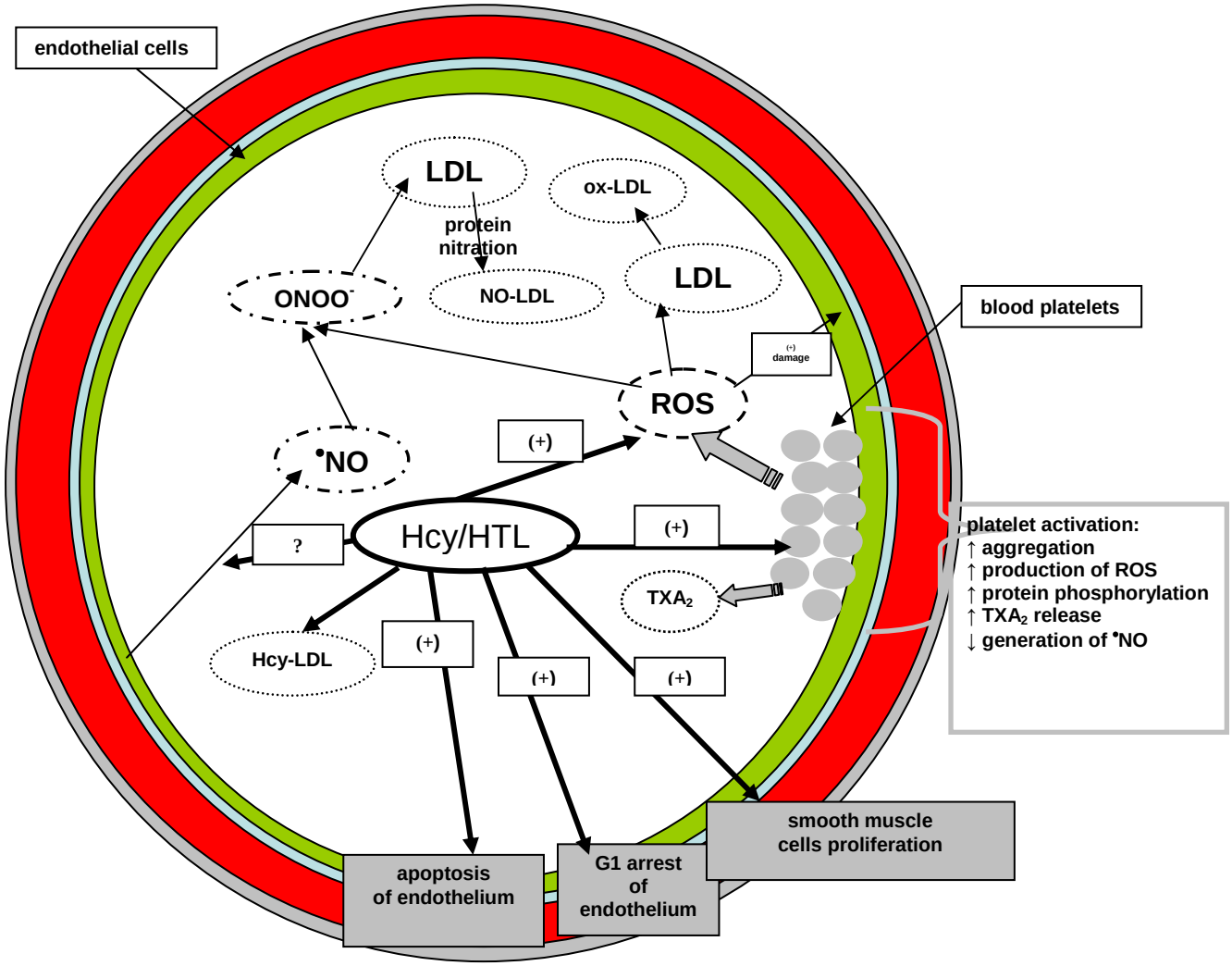

endothelial cells
LDL
ox-LDL
protein nitration
ONOO-
NO-LDL
LDL
blood platelets
(+) damage
ROS
•NO
(+)
?
Hcy/HTL
(+)
platelet activation:
↑ aggregation
↑ production of ROS
↑ protein phosphorylation
↑ TXA2 release
↓ generation of •NO
TXA2
Hcy-LDL
(+)
(+)
(+)
smooth muscle cells proliferation
apoptosis of endothelium
G1 arrest of endothelium

Fig. 1(B)

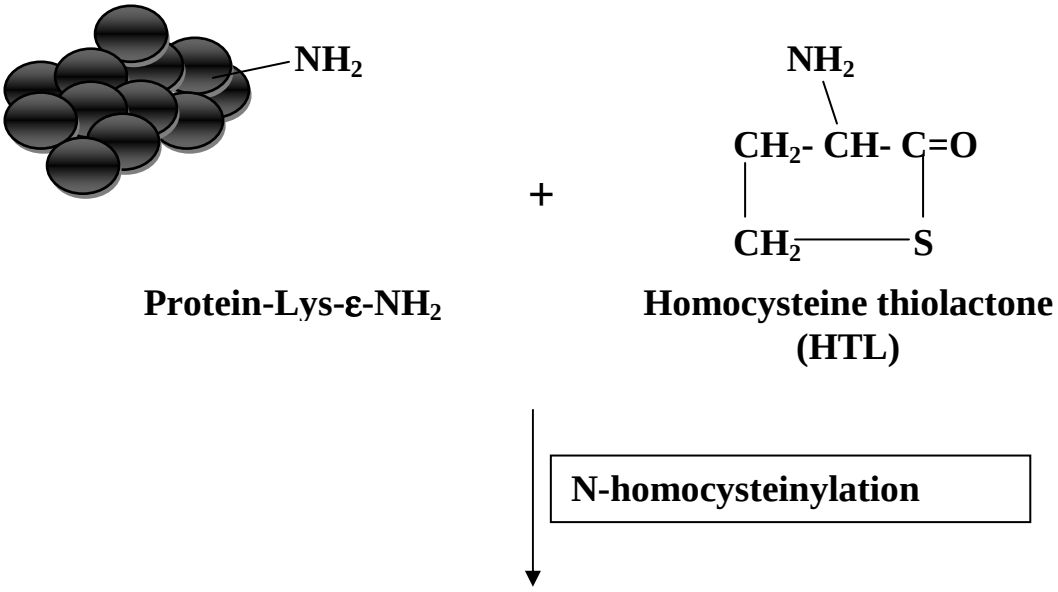
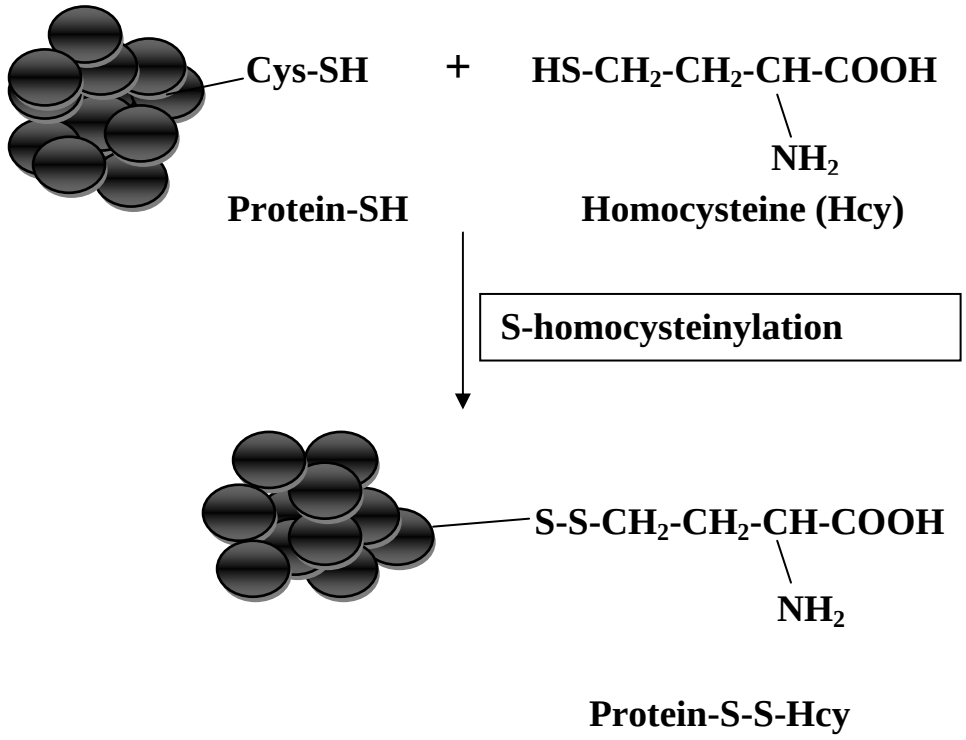

NH2
+
NH2
CH2- CH- C=O
CH2
S
Protein-Lys-ε-NH2
Homocysteine thiolactone (HTL)
N-homocysteinylation
Lys-ε–NH-C-CH-CH2-CH2-SH
O NH2
Protein-Lys-ε-N-Hcy

Fig. 1(A)


Cys-SH
+
HS-CH2-CH2-CH-COOH
NH2
Protein-SH
Homocysteine (Hcy)
S-homocysteinylation
S-S-CH2-CH2-CH-COOH
NH2
Protein-S-S-Hcy