

May modifications of human plasma proteins stimulated by homocysteine and its thiolactone induce changes of hemostatic function of plasma *in vitro*?

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Abstract. Homocysteine (Hcys) may be implicated in different diseases, especially in cardiovascular illnesses. The most reactive form of Hcys is its cyclic thioester – homocysteine thiolactone (HTL), which is formed in plasma and represents up to 0.29% of plasma total Hcys. Recently, it has been observed that Hcys and HTL may modify plasma proteins, including albumin, hemoglobin or fibrinogen, but the role of this process is not yet well known. The aim of our study *in vitro* was to investigate the modifications of human plasma total proteins after incubation with the reduced form of Hcys in concentrations 10–100 $\mu\text{mol/l}$, and HTL in concentrations 1–0.1 $\mu\text{mol/l}$, which correspond to levels found in human plasma during hyperhomocysteinemia *in vivo*. The aim of our study was also to explain the effects of Hcys and HTL on coagulation activity of human plasma. We showed that in model system *in vitro* Hcys and HTL change the level of thiol, amino and carbonyl groups in plasma total proteins. Moreover, our studies reported that not only Hcys (10–100 $\mu\text{mol/l}$), but also HTL (at lower concentrations than Hcys) modulates the coagulation properties of human plasma.

Key words: Homocysteine — Homocysteine thiolactone — Plasma — Proteins

Abbreviations: Hcys, homocysteine; HTL, homocysteine thiolactone; LDL, low density lipoproteins; Lys, lysine.

Introduction

L-Homocysteine (Hcys) is an endogenous amino acid, containing free thiol group, which in healthy cells is involved in methionine and cysteine synthesis/resynthesis. Indirectly, Hcys participates in methyl, folate, and cellular thiols metabolism (D'Angelo and Selhub 1997). Approximately 80% of plasma Hcys is protein bound, and only a small amount exists as a free reduced Hcys (about 100 nmol/l). The majority of the unbound portion of Hcys is oxidized to form dimers (homocystine) or combined with cysteine to form mixed disulphides (Mansoor et al. 1992; Ramakrishnan et al. 2006). The excess of Hcys concentration is described in many human pathologies, including neurodegenerative diseases (Ravaglia et al. 2005), thrombosis (Harpel et al. 1996)

and thrombosis-related diseases, like stroke (Boysen et al. 2003; Li et al. 2003).

Recently, two main pathways of Hcys biotoxicity are discussed: 1) Hcys-dependent oxidative stress and 2) Hcys-induced protein structure modifications named homocysteinylolation. In the first case, oxidative stress is generated during oxidation of free thiol group of Hcys, when Hcys binds *via* disulphide bridge with plasma proteins – mainly albumin, or with another low-molecular plasma thiols or with second Hcys molecule. Oxidation of Hcys may induce in further oxidation of proteins, lipids and nucleic acids (Zou and Banerjee 2005). Accumulation of oxidized biomolecules alters functions of many cellular pathways. In the second case, two main types of homocysteinylolation exist, which can be considered as posttranslational protein modifications: S-homocysteinylolation and N-homocysteinylolation. S-homocysteinylolation occurs when Hcys reacts by its free thiol group or with another free thiol derived from cysteine of protein molecules. These changes can alter thiol-dependent redox status of protein (Sengupta et al. 2001). N-homocysteinylolation

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tion take places after acylation of free ϵ -amino group of protein by Hcys, or the most reactive form of Hcys – its cyclic thioester (Hcys thiolactone (HTL)), which represent up to 0.29% of plasma total Hcys (Jakubowski 1999; Perla-Kajan et al. 2007).

In human blood N-homocysteinylated (N-Hcys-protein) or S-homocysteinylated proteins (S-Hcys-protein) were described as N-Hcys-hemoglobin, N-(Hcys-S-S-Cys)-albumin, S-Hcys-albumin, and cysteinylhomocysteine (Cys-Hcys) (Jakubowski 2002, 2006; Chwatko and Jakubowski 2005a,b; Perla-Kajan et al. 2007).

The aim of this study *in vitro* was investigate the modifications of human plasma total proteins after incubation with L-Hcys and HTL in different times (5–60 min). We checked changes in thiol, amino and carbonyl groups content as a sign of S-, N-homocysteinylated and oxidative damages, respectively. We used the reduced form of Hcys in concentrations 10–100 $\mu\text{mol/l}$, and HTL in concentrations 1–0.1 $\mu\text{mol/l}$, which correspond to levels found in human plasma during hyperhomocysteinemia *in vivo*. Moreover, the aim of our study was to explain the effects of Hcys and HTL on coagulation activity of human plasma.

Material and Methods

Materials

Reduced form of L-Hcys and L-homocysteine thiolactone were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were provided by commercial suppliers.

Isolation of plasma

Blood samples were taken from 8 healthy volunteers (aged between 26–32 years (mean 28.7 ± 2.1)) without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, untreated with drugs. Healthy subjects did not use addictive substances and antioxidant supplementation, their diet was balanced (meat and vegetables), lived in similar socio-economic conditions. Subjects with significant medical illness were excluded. They were no smokers.

Human blood was collected into sodium citrate (5 mmol/l final concentration) and immediately centrifuged ($2000 \times g$, 15 min) to get plasma. The natural concentration of total Hcys in plasma was 8–14 $\mu\text{mol/l}$. The endogenous concentration of reduced form of Hcys and HTL was about 100 nmol/l and 0–35 nmol/l, respectively. The classical technique HPLC has been used to analysis of Hcys or HTL in human plasma. The HPLC analysis was performed with a Hewlett-Packard 1100 Series system according to Głowacki et al. (2001) and

Bald et al. (2004). Human plasma was incubated with the reduced form of L-Hcys (at final doses of 10–100 $\mu\text{mol/l}$) or with L-Hcys HTL (0.1–1 $\mu\text{mol/l}$) for following times: 5, 30 and 60 min, at 37°C.

Detection of thiol groups in plasma proteins

The amount of free thiol groups was estimated with 5,5'-dithio-bis(2-nitro-benzoic acid) (Ando and Steiner 1973a,b) in plasma diluted 5 times with 0.9% NaCl. Amount of free thiol groups was estimated using molar extinction coefficient ($13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$).

Detection of ϵ -amino groups of lysine (Lys) in plasma proteins

Free ϵ -amino groups of Lys in plasma proteins were measured spectrophotometrically after reaction with 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) reagent according to Sashidhar et al. (1994). 250 μl of 4% NaHCO_3 and 250 μl TNBS were added to 250 μl of plasma. Mixture was incubated for 2 hours at 42°C. After termination of incubation, 250 μl of 10% SDS and 125 μl 1 mol/l HCl were added. Absorbance was measured at 335 nm. Number of free amino groups of Lys was estimated on the basis of standard curve made for Lys.

Detection of carbonyl groups in plasma proteins

Detection of carbonyl groups by spectrophotometric method in plasma (control, Hcys and HTL) after reaction with 2,4-dinitrophenylhydrazine (DNPH) was carried out according to Levine et al. (2000), and carbonyl groups content was estimated on the basis of molar extinction coefficient ($22\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$).

Measurements of thrombin and prothrombin time

The thrombin and prothrombin time were determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland).

Data analysis

The statistical analysis was done by several tests. All the values in this study were expressed as mean \pm SD. In order to eliminate uncertain data, Grubbs test or Q-Dixon test were performed. The difference between variations and means were assessed by applying the Fisher-Snedecor test and the unpaired Student's *t*-test, respectively. The statistical analysis was also performed with ANOVA I test using StatSoft Inc. "Statistica" v. 6.0. In the present study we accepted the differences with $p < 0.05$ as statistically significant.

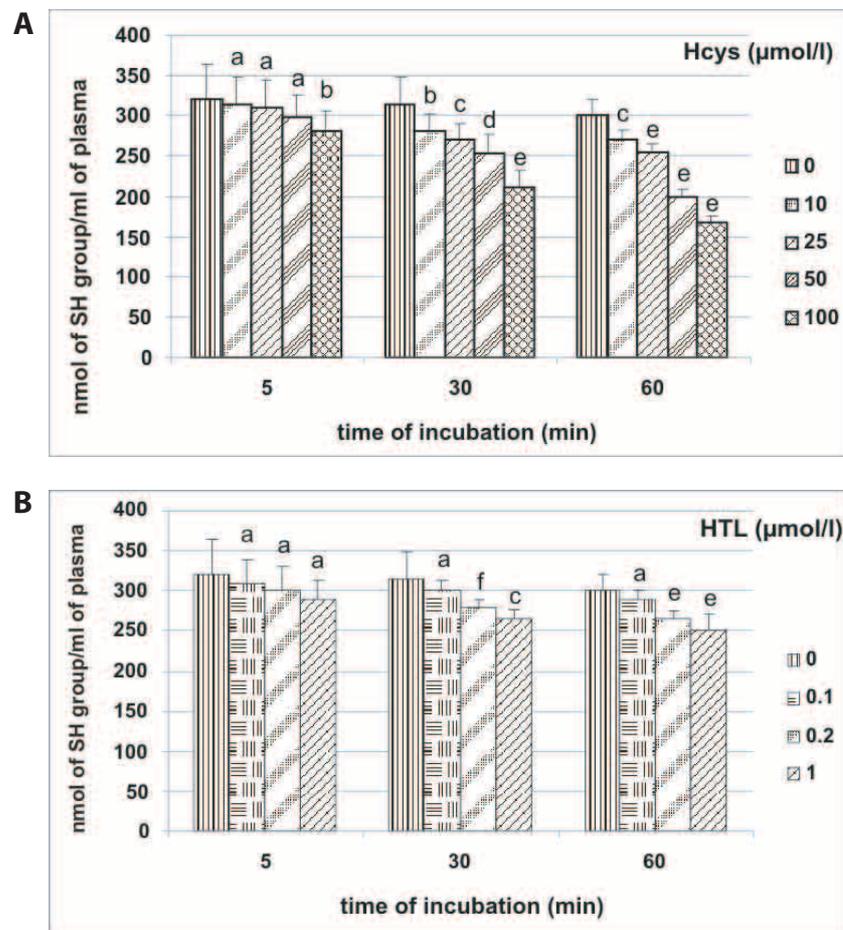


Figure 1. The effects of Hcys (A) and HTL (B) (5, 30, 60 min; 37°C) on the level of thiol groups in plasma total proteins. Data represent means \pm SD of eight experiments. The statistically significant differences were performed with one-way ANOVA and the significance level was $p < 0.05$. The effects were also statistically significant according to the unpaired Student's *t*-test: ^a $p > 0.05$; ^b $p < 0.05$; ^c $p < 0.01$; ^d $p < 0.002$; ^e $p < 0.001$; ^f $p < 0.02$; compared to control (plasma untreated with Hcys or HTL).

Results

Incubation of human plasma with Hcys (10–100 $\mu\text{mol/l}$) resulted in the changes of plasma proteins (Fig. 1A–3A). The level of thiol groups in total proteins after incubation of plasma (30 and 60 min) with Hcys at the tested concentrations (10–100 $\mu\text{mol/l}$) was reduced (Fig. 1A). Figure 1A shows the dose-dependent decrease of thiol groups in plasma total proteins treated with Hcys (for the incubation time 30 and 60 min). The exposure of plasma (30 and 60 min) to HTL resulted also in significant decrease of thiol amount in proteins (Fig. 1B). In the presence of HTL (at the highest tested concentration 1 $\mu\text{mol/l}$, after incubation time 60 min) the decrease of thiol groups was about 16% ($p < 0.001$) (Fig. 1B).

The comparative studies demonstrated the reduction of free ϵ -amino groups of Lys in plasma proteins after 5, 30 or 60 min incubation of plasma with Hcys and HTL, and the ef-

fects were dose-dependent (Fig. 2A and B). The longest used incubation time (60 min) of plasma with Hcys (at the highest tested concentration 100 $\mu\text{mol/l}$) induced a decrease of free ϵ -amino groups of Lys in proteins about 25% ($p < 0.001$) (Fig. 2A). We observed the same process, when plasma was treated with HTL (1 $\mu\text{mol/l}$, after incubation time 60 min) ($p < 0.001$) (Fig. 2B).

We showed that Hcys or HTL (for the incubation time 60 min and at all tested concentrations) *in vitro* induced a decrease of carbonyl groups in plasma proteins, and the effect was dose-dependent (Fig. 3A and B). The highest tested concentration of Hcys (100 $\mu\text{mol/l}$, 60 min) or HTL (1 $\mu\text{mol/l}$, 60 min) caused the decrease of carbonyl groups in plasma proteins that reached about 24% ($p < 0.05$) and 25% ($p < 0.05$), respectively (Fig. 3A and B).

Table 1 shows that the prothrombin time was prolonged in plasma treated with Hcys or HTL when compared with the

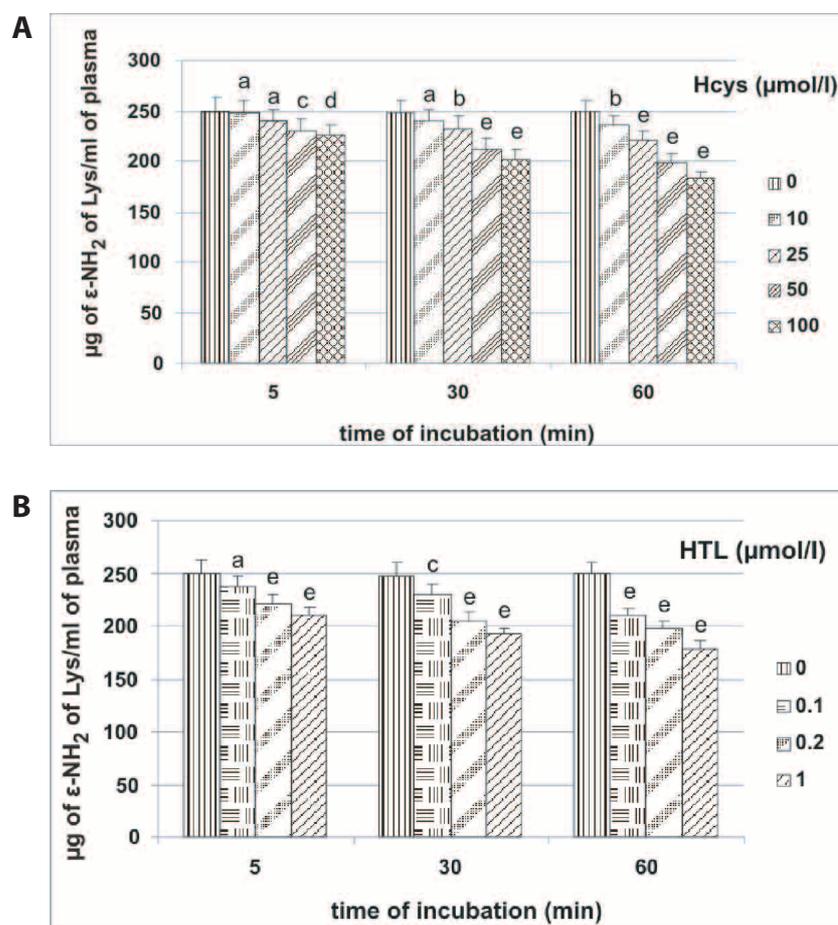


Figure 2. The effects of Hcys (A) and HTL (B) (5, 30, 60 min; 37°C) on the level of ϵ -amino groups of Lys in plasma total proteins. Data represent means \pm SD of eight experiments. The statistically significant differences were performed with one-way ANOVA and the significance level was $p < 0.05$. The effects were also statistically significant according to the unpaired Student's t -test: ^a $p > 0.05$; ^b $p < 0.05$; ^c $p < 0.01$; ^d $p < 0.002$; ^e $p < 0.001$; compared to control (plasma untreated with Hcys or HTL).

control. Hcys (at all tested concentrations) had no effect on thrombin time, but HTL prolonged the thrombin time.

Discussion

Hcys can be incorporated into protein molecules on one of two pathways: 1) translational pathway or 2) posttranslational pathway. In the first case, S-nitrosohomocysteine is necessary as an intermediate, which than is incorporated in polyamino acid chain. In the second case, conversion of Hcys to HTL occurs as a preliminary step to acylation of free ϵ -amino groups in proteins (Jakubowski 2001). ϵ -amino group of Lys is predominant N-homocysteinylated place; however it could be also discovered on the C-terminal carboxy group or N-terminal amino group of protein (Hop and Bakhtiar 2002). If N-homocysteinylated indeed takes place, reduction

Table 1. The prothrombin and thrombin time of plasma treated with Hcys and HTL (30 min; 37°C).

	c_s (μmol/l)	Prothrombin time (s)	Thrombin time (s)
control	–	18.9 \pm 0.7	59.1 \pm 1.2
Hcys	10	23.2 \pm 0.9*	60.6 \pm 1.2**
	25	24.4 \pm 1.2*	60.4 \pm 1.3**
	50	25.6 \pm 1.0*	61.0 \pm 1.0**
	100	22.7 \pm 1.3*	60.5 \pm 0.6**
HTL	0.1	23.4 \pm 0.7*	65.4 \pm 1.0*
	0.2	23.8 \pm 1.7*	65.0 \pm 0.9*
	1	21.8 \pm 0.9*	61.8 \pm 1.2*

Data represent means \pm SD of eight experiments. The effect of four different concentrations of Hcys (10, 25, 50 and 100 μmol/l) was statistically significant according to ANOVA I test, $p < 0.05$ (for prothrombin time), $p > 0.05$ (for thrombin time). The effect of three different concentrations of HTL (0.1, 0.2 and 1 μmol/l) was statistically significant according to ANOVA I test, $p < 0.05$ (for prothrombin time and thrombin time). c_s , concentration of substance, control, without Hcys or HTL; Student's t -test (Hcys and/or HTL-treated plasma vs. control): * $p < 0.001$; ** $p > 0.05$.

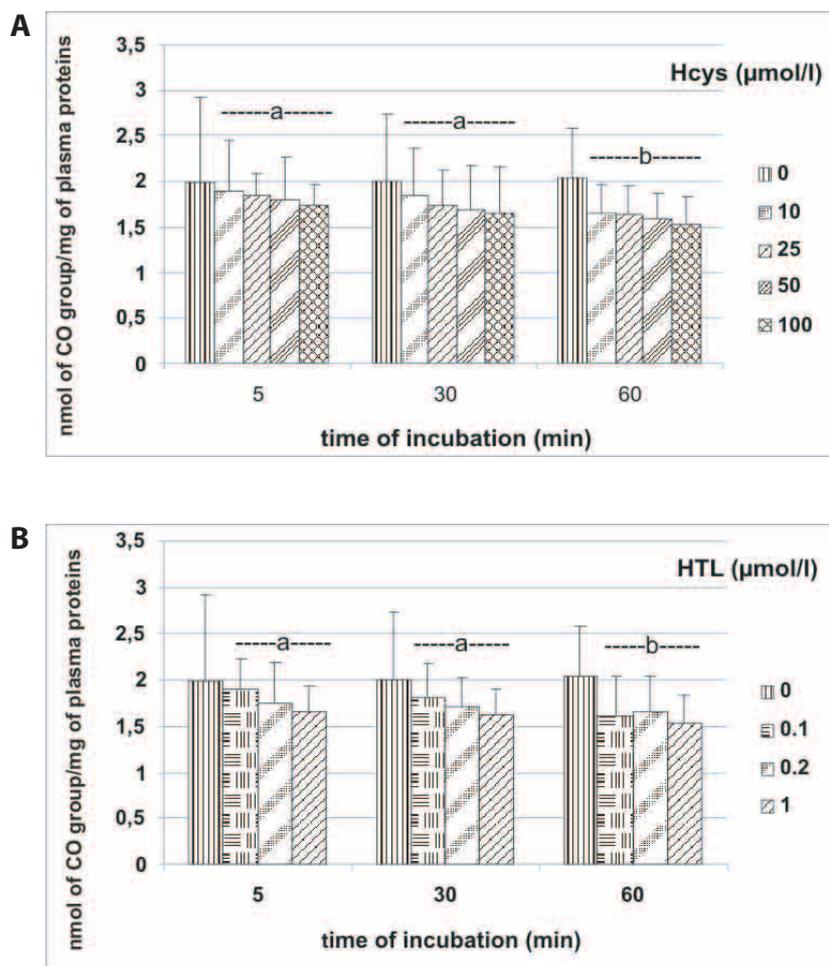


Figure 3. The effects of Hcys (A) and HTL (B) (5, 30, 60 min; 37°C) on the level of carbonyl groups in plasma total proteins. Data represent means \pm SD of eight experiments. The statistically significant differences were performed with one-way ANOVA and the significance level was $p < 0.05$. The effects were also statistically significant according to the unpaired Student's t -test: ^a $p > 0.05$; ^b $p < 0.05$; compared to control (plasma untreated with Hcys or HTL).

in the number of free ϵ -amino groups should be expected. Here, we observed such reduction giving an evidence of N-homocysteinylation of plasma proteins, during incubation of plasma not only with HTL, but also with the reduced form of Hcys (5–60 min) (Fig. 2A and B).

Mass spectrometric analysis of plasma fibrinogen, which is the main substrate for coagulation cascade and form a polymerized fibrin clot, treated with Hcys revealed twelve lysines that were homocysteinylation (Sauls et al. 2003, 2007; Undas et al. 2006; Sauls et al. 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 fibrinolytic enzyme binding and activity could lead to increased resistance to fibrinolysis – the process by which a fibrin clot is dissolved. Modification of fibrinogen induced by Hcys or HTL may be dependent on the alteration of its structure associated with changes of its haemostatic functions and the pathogenesis of different cardiovascular disorders, including thrombosis and atherosclerosis.

Next to acylation on $-\text{NH}_2$ moiety induced by Hcys/HTL, second posttranslational protein modification, named S-homocysteinylation, exists. It is result of disulphide bond formation between free thiol groups derived from protein cysteine or Hcys. It is known that such reaction occurs *in vivo* in humans and decreases the level of free thiols,

reducing in that way a pool of reduced thiols (Rossi et al. 2008). The major components of the S-homocysteinylated protein pool in human plasma are albumin and γ -globulin (Sengupta et al. 2001). Binding of Hcys to plasma proteins is biphasic. First reaction, involving displacement of cysteine from plasma proteins, is rapid and oxygen independent, while the second reaction constitutes slower, oxygen-dependent thiol oxidation (Togawa et al. 2000). Our present results showed that not only the reduced form of Hcys, but also its cyclic thioester may diminish the amount of free protein thiol concentration, especially when blood plasma is exposed for a long incubation time (60 min) (Fig. 1A and B). However, Ferretti et al. (2006) observed increase in thiol content in LDLs (low density lipoproteins) taken from diabetic patients, but in their experiments hyperglycemia state could be responsible for this inconsistency. Moreover, we investigated total plasma proteins without any plasma fractionation.

For the first time, we found that Hcys and HTL reduce carbonyl concentration in total proteins of plasma (Fig. 3A and B). It may be probably due to direct reaction between thiol and carbonyl groups, because it was earlier shown *in vitro* that 1,3-thiazane-4-carboxylic acid is probably synthesized as a result of this reaction. Results of Wriston and Mackenzie (1957) and Jakubowski (2006) showed this product, which may be further metabolized (Wolen and Wriston 1963), but the properties of Hcys or HTL adducts with carbonyls have been unknown. We suggest that the reaction between Hcys/HTL and carbonyl groups in proteins and this process may be a new protein modification. Moreover, Hcys or HTL adducts with carbonyls may change protein structure with unknown physiological consequences. Our results that Hcys and HTL decrease the level of carbonyl groups are in agreement with other studies, where this process was observed in a model system (Jakubowski 2006), in human endothelial cells (Jakubowski et al. 2000), and in human blood platelets (Olas et al. 2008, 2009).

Modifications of many of the plasma proteins induced by Hcys or HTL may stimulate various functional changes, including enzyme inactivation, altered ligand binding, modified susceptibility to proteolytic degradation. For example, homocysteinylated molecules gain biotoxic features like LDL molecules, which after interaction with HTL can induce endothelial lipid peroxidation (Ferretti et al. 2004) or peroxynitrite production by human aortic endothelial cells (Ferretti et al. 2006). Coagulation factor Va is also S-homocysteinylated (Undas et al. 2001) and this modification could play a role in the modulation of hemostasis process. Clots formed from human plasma incubated *in vitro* with Hcys (at the high concentration – 3 mmol/l) have been more compact structure, with shorter and more frequently branched fibers, than those formed in the absence of Hcys (Lauricella et al. 2002, 2006). Harpel et al. (1992)

observed that Hcys (< 8 μ mol/l) enhances the binding of lipoprotein(a) to fibrin, and this results may suggest a biochemical relationship between thiol compound metabolism, thrombosis and atherogenesis. Our present studies reported that not only the reduced form of Hcys (10–100 μ mol/l), but also HTL (at lower concentrations than Hcys) modulates the coagulation properties of human plasma (Table 1). The prolonged prothrombin time may imply that HTL decreased the coagulation properties of plasma proteins (Table 1). Moreover, Table 1 shows that the thrombin time was prolonged in plasma treated with HTL when compared with control. The obtained results are consistent with other experiments, in which rabbit plasma was treated with Hcys (Sauls et al. 2003). In this context, this process may suggest that Hcys and HTL may affect proteins of prothrombinase system and modulate the structure of coagulation factors: V, VII, X and prothrombinase. 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 HTL have no effect on factor V activation by thrombin. Factor V incubated with [35 S]Hcys (10–450 μ M) 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). Our earlier results indicate also that in blood platelets, which are involved in hemostasis process, the decrease of thiol groups in proteins treated with Hcys and HTL occurred (Olas et al. 2009). On the basis of these observations, we proposed that Hcys or HTL may act as an oxidant, because in our earlier other studies we have observed that platelet aggregation (induced by Hcys HTL) was associated with free radical production (Olas et al. 2008).

Moreover, there are not pharmacologically active compounds protecting plasma proteins from investigated modifications. Different compounds (folic acid, vitamins: B₆ and B₁₂) present in human diet may have promising effect on the concentration of Hcys in human plasma. However, the results of Bogers et al. (2007) showed that the increased fruit and vegetable consumption may be insufficient to change plasma Hcys concentration.

In conclusion, our results strongly suggest that Hcys and HTL induced modifications of total plasma proteins. The consequence of these modifications (N- and S-homocysteinylated) may be the alteration in plasma protein structure associated with changes of biological functions, and further studies should be carried out to investigate the possible role of these modifications in different diseases.

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

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Received: November 4, 2009

Final version accepted: February 5, 2010