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Review

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Protein homocysteinylation: a new mechanism of atherogenesis?

Modyfikacja białek przez tiolakton homocysteiny – nowy mechanizm powstawania miażdżycy?

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Summary

An increased concentration of homocysteine is an important risk factor of atherosclerosis; however, the mechanism of the proatherogenic effect of this amino acid is not yet known. Studies performed during the last two decades suggest that the atherogenic effect of homocysteine may be accounted for by homocysteine thiolactone (HCTL). Homocysteine is nonspecifically activated by methionyl-tRNA synthetase; however, it is not transferred to tRNA and incorporated into proteins, but is transformed to a cyclic thioester, homocysteine thiolactone. HCTL is highly reactive and acylates free amino groups of protein lysine residues, the process referred to as protein N-homocysteinylation. Various plasma proteins are homocysteinylated *in vitro* and *in vivo.* Homocysteinylation results in the incorporation of additional thiol groups which may alter the physicochemical properties and biological activity of proteins. In particular, homocysteinylation of low-density lipoproteins (LDLs) increases their susceptibility to oxidation and accelerates their uptake by macrophages. In addition, homocysteinylated LDL elicit humoral immune response. Anti-homocysteinyllysine antibodies are detected in plasma of healthy humans and their titer is elevated in patients with ischemic heart disease or ischemic cerebral stroke. Homocysteine thiolactone is hydrolyzed to homocysteine by paraoxonase (PON), a calcium-dependent esterase synthesized in the liver and contained in plasma high-density lipoproteins (HDLs). Protein homocysteinylation may contribute to accelerated atherogenesis in individuals with hyperhomocysteinemia.

Key words: **homocysteine • homocysteine thiolactone • protein homocysteinylation • atherosclerosis • paraoxonase**

Streszczenie

Podwyższone stężenie homocysteiny jest ważnym czynnikiem ryzyka miażdżycy, jednak mechanizm miażdżycorodnego działania tego aminokwasu nie został dotychczas wyjaśniony. Wyniki badań przeprowadzonych w ciągu ostatnich kilkunastu lat sugerują, że związkiem, który może stwarzać molekularne podstawy miażdżycy jest tiolakton homocysteiny (HCTL). Homocysteina jest nieswoiście aktywowana przez syntetazę metionylo-tRNA, ale następnie nie jest przenoszona na cząsteczkę tRNA, lecz przekształcana w cykliczny tioester, tiolakton homocysteiny. Jest to związek wysoce reaktywny, który reaguje z wolnymi grupami aminowymi reszt lizyny białek. Wykazano, że różne białka osocza ulegają modyfikacji przez HCTL zarówno *in vitro*, jak i *in vivo.* Skutkiem tej modyfikacji jest wbudowanie do cząsteczki białka dodatkowych grup tiolowych, co może zmieniać jego własności fizykochemiczne oraz aktywność biologiczną. W szczególności reakcja lipoprotein osocza o małej gęstości (LDL) z HCTL zwiększa ich podatność na utlenianie oraz przyspiesza wychwytywanie ich przez makrofagi. Ponadto, LDL zmodyfikowa-

ne przez HCTL indukują humoralną odpowiedź immunologiczną. Przeciwciała przeciw homocysteinylolizynie występują w osoczu zdrowych osób, a ich miano jest podwyższone u pacjentów z chorobą niedokrwienną serca lub udarem niedokrwiennym mózgu. Tiolakton homocysteiny jest hydrolizowany do homocysteiny przez paraoksonazę (PON) – wapniowozależną esterazę syntetyzowaną w wątrobie i zawartą w lipoproteinach osocza o dużej gęstości (HDL). Modyfikacja białek przez HCTL może się przyczyniać do przyspieszonego rozwoju miażdżycy u osób z podwyższonym stężeniem homocysteiny.

Słowa kluczowe: **homocysteina • tiolakton homocysteiny • modyfikacja białek przez tiolakton homocysteiny • miażdżyca, paraoksonaza**

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Abbreviations: **ADMA** – asymetryczna dimetyloarginina (asymmetric dimethylarginine); **CBS** – β-syntaza cystationiny (cystathionine β-synthase); **CSE** – γ-liaza cystationiny (cystathionine γ-lyase); **DDAH** – dimetyloaminohydrolaza dimetyloargininowa (dimethylarginine dimethylaminohydrolase); **HCTL** – tiolakton homocysteiny (homocysteine thiolactone); **Hcy** – homocysteina (homocysteine); **HDL** – lipoproteiny o dużej gęstości (high-density lipoprotein); **LDL** – lipoproteiny o małej gęstości (low-density lipoprotein); **LOX** – oksydaza lizynowa (lysyl oxidase); **MTHFR** – reduktaza metylenotetrahydrofolianowa (methylenetetrahydrofolate reductase); **NO** – tlenek azotu (nitric oxide); **PON** – paraoksonaza (paraoxonase); **SAH** – S-adenozylohomocysteina (S-adenosylhomocysteine); **SAM** – S-adenozylometionina (S-adenosylmethionine); **SMHT** – tiolakton S-metionylohomocysteiny (S-methionylhomocysteine thiolactone)

Introduction – an overview of homocysteine metabolism

Homocysteine (Hcy) is a non-protein sulfur-containing amino acid (Figure 1) which is an intermediate product of methionine metabolism (Figure 2). Adenosine is transferred from ATP to methionine to form S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyltransferase (SAM synthetase). SAM is a major donor of methyl groups for various methylation reactions. When a $–CH_3$ group is transferred by methyltransferases to the respective acceptor, SAM is converted to S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed by SAH hydrolase to adenosine and homocysteine (Figure 2). Homocysteine is metabolized by two alternative pathways. First, it may be remethylated to methionine by methionine synthase, which uses 5-methyltetrahydrofolate and vitamin B_{12} as cofactors (the remethylation pathway). Second, in the transsulfuration pathway catalyzed by cystathionine β-synthase (CBS), Hcy undergoes condensation with serine to form cystathionine. Cystathionine is then converted to cysteine and α-ketobutyrate by cystathionine γ-lyase (CSE). Both CBS and CSE use pyridoxal 5-phosphate (an active form of vitamin B_6) as a cofactor. Homocysteine circulates in the blood at a concentration of ~10 μM. In plasma, about ²/₃ of total Hcy is bound to protein cysteine residues by disulfide bridges (Hcy-protein disulfides) and most of the remaining ¹/₃ circulates in oxidized form as low-molecular-weight disulfides, homocystine (Hcy-S-S-Hcy), or Hcy-S-S-cysteine. Only about 1% of total Hcy circulates in free reduced form [4,60].

It was noted in the 1960s that the risk of atherosclerosis is markedly increased in patients with homocystinuria, the inherited disease resulting from homozygous CBS deficiency and characterized by episodes of thromboembolism, mental retardation, lens dislocation, hepatic steatosis, and osteoporosis. In these patients, plasma Hcy usually exceeds 100 μM. Much more frequent (5–10% of the general population) is mild or moderate hyperhomocysteinemia (plasma Hcy 15–40 μM), which also increases the risk of atherosclerosis. The most common causes of hyperhomocysteinemia include heterozygous CBS deficiency, a thermolabile variant of methylenetetrahydrofolate reductase (MTHFR) resulting from C677T polymorphism of MTHFR gene, deficiency in vitamins involved in Hcy metabolism (folate, B_6 and B_{12}), renal impairment, and probably high methionine intake [4].

Apart from atherosclerosis, homocysteine is also associated with several other health burdens, including Alzheimer's disease and other forms of dementia, cancer, as well as some complications of pregnancy such as intrauterine growth restriction, preeclampsia, miscarriages, and neural tube defects in the neonate. However, the mechanism through which homocysteine contributes to these abnormalities is not entirely clear. Many potentially harmful effects of homo-

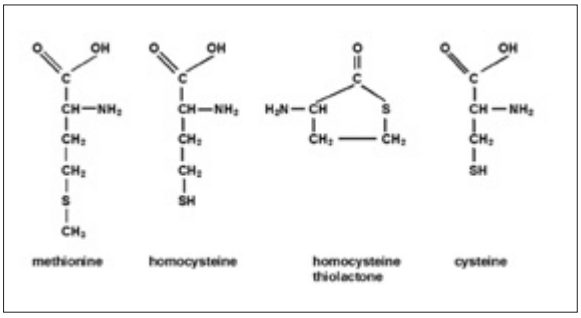

Fig. 1. Structure of physiologically relevant sulfur-containing amino acids.

cysteine have been characterized *in vitro*, such as stimulation of oxidative stress, scavenging of nitric oxide, enhanced coagulation, and apoptosis. However, most of these effects were observed at high Hcy concentrations, far exceeding values observed under physiological or even pathological conditions. In addition, most of them were reproduced by other thiol-containing compounds such as cysteine, whereas there is no evidence that cysteine is atherogenic. Thus these effects may not explain the pathogenesis of hyperhomocysteinemia-associated complications. Studies performed by Hieronim Jakubowski [31,34,43] suggest that unfavorable effects of Hcy, including accelerated atherosclerosis, may be mediated by one of its metabolites, homocysteine thiolactone (HCTL), which specifically damages proteins by reacting with free $–NH_2$ groups of lysine residues; the process referred to as protein N-homocysteinylation. The purpose of this paper is to summarize the current knowledge about the synthesis, metabolism, and physiologic effects of homocysteine thiolactone and its possible implications for the harmful consequences of hyperhomocysteinemia.

Physicochemical properties of homocysteine thiolactone

Homocysteine thiolactone was first synthesized in 1934 by boiling methionine with hydriodic acid (HI) [2]. HCTL may also be synthesized from homocysteine by heating it in an acidic environment [69]. Homocysteine thiolactone is a cyclic thioester in which the –SH group is condensed with the carboxyl group (Figure 1). Like other thioesters, HCTL absorbs ultraviolet light with a maximum at 240 nm. In physiological conditions (aqueous solutions, pH 7.4, 37ºC) homocysteine thiolactone slowly hydrolyzes to homocysteine with a half-life of about 25 hours. In contrast, in alkaline solutions the hydrolysis occurs rapidly. For example, in 0.1 M NaOH the thiolactone is completely converted to homocysteine within 15 min. In comparison with amino groups of other amino acids, the $–NH_2$ group of homocysteine thiolactone has an unusually low pKa (≈7.1). Therefore, unlike the amino groups of other amino acids positively charged at physiological pH, HCTL is mostly neutral, which allows it to permeate plasma membranes freely. In contrast, most of the HCTL is positively charged at pH<6.

The esterified carboxyl group of HCTL is highly reactive and especially tends to react with ε-NH_2 groups of protein lysine residues. Its reactivity toward the α-NH_2 group of free lysine is about threefold lower than toward the ε-NH_2 group. On the other hand, the $–NH_2$ group of HCTL easily

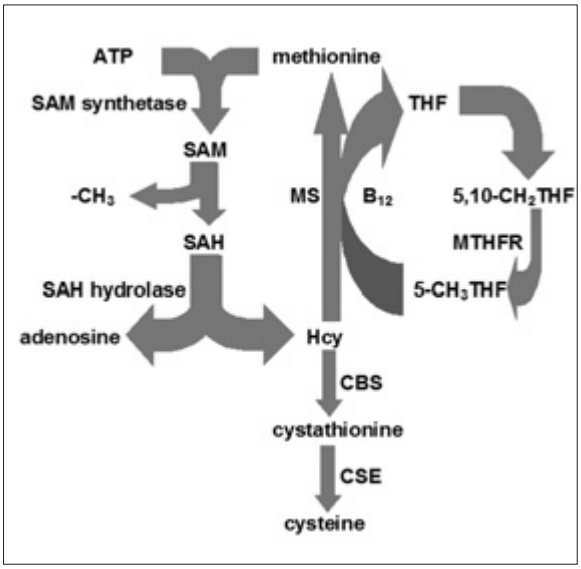

Fig. 2. Homocysteine metabolism. MS – methionine synthase, THF – tetrahydrofolate, 5,10-CH_2THF – 5,10-methylenetetrahydrofolate, 5-CH_3THF – 5-methyltetrahydrofolate.

reacts with aldehydes such as the streptomycin contained in most tissue culture media.

Biochemistry of HCTL synthesis in bacteria

That HCTL may be synthesized *in vivo* was first suggested more than 30 years ago [77]; however, this was probably due to analytical errors because the concentrations reported were unexpectedly high. That homocysteine thiolactone is indeed produced in biological systems was definitely established only during the last two decades. The synthesis of HCTL is associated with the activation of the amino acids by aminoacyl-tRNA synthetases (AARS).

The activation of the amino acids is a two-step reaction. In the first step, the amino acid (AA) is bound by the enzyme using ATP-derived energy and pyrophosphate (PPi) is liberated:

$$AARS + AA + ATP \rightarrow AARS\text{-}AA\sim AMP + PPi$$

Subsequently, the amino acid is transferred to the respective tRNA, thus forming aminoacyl-tRNA, used for ribosomal protein synthesis:

$$AARS\text{-}AA\sim AMP + tRNA \rightarrow AA\text{-}tRNA + AMP$$

Synthesis of a protein with the appropriate amino acid sequence requires a high specificity of these reactions, i.e. a given amino acid should be activated by the respective aminoacyl-tRNA synthetase, bound to the tRNA molecule containing the corresponding anticodon, and then incorporated specifically into the specific place of the polypeptide chain. The estimated frequency of errors is 10^{-3}–10^{-4}, i.e. one wrong amino acid is incorporated into the protein chain per 1000–10,000 correct residues. Most errors occur at the level of translation (an incorrect aminoacyl-tRNA is used). The overall selectivity of aminoacyl-tRNA synthetases toward an amino acid is 10^{-5}–10^{-4}, i.e. one aminoacyl-tRNA containing an incorrect amino acid is formed per 10,000–100,000 correct ones [40].

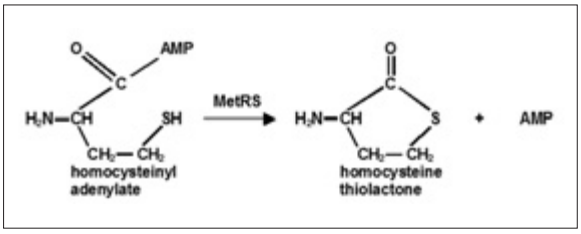

Fig. 3. Editing of homocysteinyl adenylate to homocysteine thiolactone.

It was first noted in 1981 [39] that *Escherichia coli* methionyl-tRNA synthetase (MetRS) can nonspecifically activate homocysteine *in vitro*:

$$\text{MetRS} + \text{Hcy} + \text{ATP} \rightarrow \text{MetRS-Hcy}\sim\text{AMP} + \text{PPi}$$

However, Hcy is never transferred to tRNA. Instead, its terminal thiol group displaces AMP, thus forming the high-energy thioester bond of homocysteine thiolactone (Figure 3). This reaction is called "editing" and is catalyzed by methionyl-tRNA synthetase. The misactivation and editing of Hcy was subsequently observed in *E. coli in vivo* [25]. This "proofreading" reaction protects against incorporation of the non-cognate amino acid homocysteine into the polypeptide chain in places normally occupied by methionine. In bacterial cells, the Hcy concentration is relatively high (comparable to methionine) and it was calculated that in *E. coli* one Hcy molecule is misactivated per each 100 molecules of methionine. This suggests that the affinity of MetRS to Hcy is about 100 times lower than its affinity to methionine. Since the rate of errors of aminoacyl-tRNA synthesis is at least two orders of magnitude lower than the rate of initial Hcy misactivation, the editing process markedly improves the overall specificity of methionyl-tRNA synthetase. Early studies have demonstrated that *in vitro* Hcy may be misactivated and subsequently edited also by isoleucyl-, valyl-, lysyl-, and leucyl-tRNA synthetases [39]; however, these enzymes seem to play no role in HCTL synthesis in intact cells.

Later studies [29] revealed that if methionine is bound by MetRS, the editing reaction may also occur. In this case, methionine is cyclized to S-methionylhomocysteine thiolactone (SMHT). Unlike in the case of Hcy, the formation of SMHT is usually preceded by the transfer of methionine from MetRS-bound methionyl-adenylate to tRNA (Figure 4). Although methionyl-adenylate may be directly converted to SMHT, this occurs much less efficiently. Thus, MetRS may also edit its cognate amino acid. It was demonstrated that methionyl-tRNA synthetase possesses two sites which compete for a side-chain of the bound amino acid [48]. The "editing subsite" binds Hcy much more efficiently than methionine, whereas the "synthetic subsite" binds almost exclusively the side chain of methionine. Therefore, most homocysteine is edited to thiolactone, whereas only a small portion of activated methionine is edited to SMHT instead of being incorporated into proteins. SMHT may be used as a donor of methyl groups, but may also be hydrolyzed to free methionine by esterases. Since MetRS demonstrates some sequence homology with S-adenosylmethionine synthetase, it is suggested that both enzymes occurred during evolution by duplication of an ancestral gene encoding an archetypal enzyme

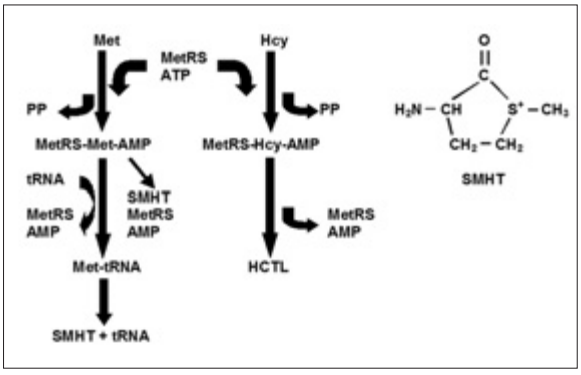

Fig. 4. Editing of methionine (Met) by methionyl-tRNA synthetase (MetRS) to S-methionylhomocysteine thiolactone (SMHT). PPi – inorganic pyrophosphate.

playing initially two separate roles: formation of methionyl-tRNA for protein synthesis ("synthetic pathway") and formation of a methyl donor, SMHT ("editing pathway"). After duplication, each enzyme evolved to specialize in its own function; therefore, editing of methionyl-tRNA to SMHT by MetRS is much less efficient in currently living organisms. Indeed, in a cell-free system only about 2% of methionyl-tRNA is converted to SMHT. In intact *Escherichia coli* cells, even less methionyl-tRNA is edited (about 3 molecules per 10,000 molecules transferring methionine to proteins, i.e. about 1 molecule per 30 edited molecules of homocysteine).

Activation of non-cognate amino acids by other aminoacyl-tRNA synthetases

Although Hcy is the most frequently misactivated and edited amino acid, the analogous reactions have also been observed for other aminoacyl-tRNA synthetases. For example, lysyl-, valyl-, or isoleucyl-tRNA synthetases may nonspecifically activate homoserine with its subsequent edition to homoserine lactone (Figure 5). Interestingly, acylated homoserine lactone (AHL) derivatives are intercellular signaling molecules in Gram-negative bacteria which allow them to monitor cell population density ("quorum-sensing" mediators) [18]. AHL of bacterial origin may also have some effects on mammalian cells, e.g. it elicits endothelium-independent vasorelaxation [51] and induces apoptosis of macrophages and neutrophils [82].

Isoleucyl-tRNA synthetase (IleRS) may activate valine. In this case, valine itself is recovered in the editing reaction:

$$\text{Val} + \text{IleRS} + \text{ATP} \rightarrow \text{IleRS-Val}\sim\text{AMP} + \text{PPi}$$
$$\text{IleRS-Val}\sim\text{AMP} + \text{H}_2\text{O} \rightarrow \text{IleRS} + \text{Val} + \text{AMP}$$

This catalytic cycle operates as "ATP pyrophosphatase", destroying ATP to AMP and pyrophosphate [3]. However, some aminoacyl-tRNA synthetases are very specific toward their cognate amino acids. For example, tyrosyl-, cysteinyl-, and arginyl-tRNA synthetases activate only their corresponding amino acids. Although cysteinyl-tRNA synthetase recognizes cysteine with high specificity, the cysteinyl-tRNA, apart from being incorporated into the polypeptide chain, may also be edited to cysteine thiolactone [28]. Finally, at least in bacteria, editing reactions may result in

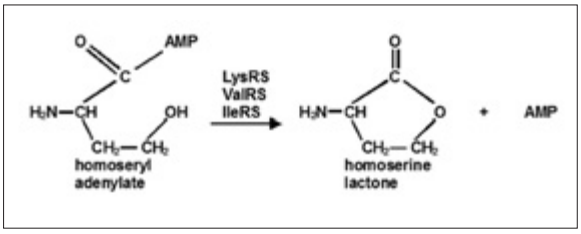

Fig. 5. Editing of homoseryl adenylate to homoserine lactone by lysyl-, valyl-, and isoleucyl-tRNA synthetases.

the formation of intermolecular thioesters. In particular, MetRS may aminoacylate coenzyme A or cysteine, forming aminoacyl-S-coenzyme A thioester or dipeptide aminoacyl-cysteine, respectively [34].

Although Hcy is never transferred by MetRS from homocysteinyl adenylate to tRNA and thus is not incorporated into proteins, bacterial ValRS and LeuRS may synthesize Hcy-tRNA, at least *in vitro* [40]. Other aminoacyl-tRNA synthetases may sometimes synthesize non-cognate aminoacyl-tRNAs and thus contribute to sequence errors in target proteins. For example, valyl-tRNA synthetase synthesizes approximately one isoleucyl-tRNA per 350,000 valyl-tRNA molecules. Thus, editing not always affords complete protection against translation errors [34].

Homocysteine thiolactone in eukaryotes

Although initially described in bacteria, HCTL synthesis by methionyl-tRNA synthetase seems to be the universal mechanism operating in all living cells. Thus, HCTL synthesis has been observed in the yeast *Saccharomyces cerevisiae* [26] and in several types of mammalian cells, including Chinese hamster ovary (CHO) cells, human cervical carcinoma (HeLa) cells, mouse renal adenocarcinoma (RAG) cells, human breast cancer cells, human hepatoma HepG2 cells, CBS-deficient human fibroblasts, and human umbilical vein endothelial cells [30, 41, 43, 62]. Interestingly, HCTL is also synthesized in plants, as demonstrated in seedlings of yellow lupine (*Lupinus luteus*) and for recombinant rice methionyl-tRNA synthetase [42].

The level of free homocysteine thiolactone in human plasma varies between 0 and 35 nM (mean: 2.82±6.13 nM), which is about 0.3% of total plasma homocysteine [35]. In the plasma of 60 healthy individuals, HCTL tended to be higher in men than in women and tended to positively correlate with increasing age, although neither of these effects was statistically significant [8].

Interestingly, although homocysteine concentration in human urine is comparable to that in plasma, urinary HCTL concentration is much higher (median: 145 nM, range: 11–474 nM, i.e. 2.5–28% of urinary Hcy) [9]. Whereas renal clearance of Hcy is only about 0.1–0.3% of creatinine clearance, clearance of HCTL is between 20 and 700% of creatinine clearance. This indicates that, at least in some individuals, HCTL is not only filtered in the glomeruli, but also secreted to the tubular lumen, although high local intrarenal generation of this compound cannot be excluded. There was no correlation between urinary Hcy and HCTL; however, a significant positive correlation was observed between HCTL concentrations in urine and plasma. These data suggest that urinary excretion is an important route of HCTL elimination from the human body. The estimated urinary excretion of HCTL in humans is 300–400 nmol/day. Notably, in contrast to homocysteine, urinary HCTL concentration negatively correlates with urine pH, suggesting that, consistent with the chemical properties of HCTL, urine alkalization may promote spontaneous HCTL hydrolysis. In addition, because the majority of HCTL is positively charged in low pH, urine acidification will maintain a low concentration of uncharged HCTL inside the tubular lumen, thus enabling its continuous passive diffusion from the tubular cells.

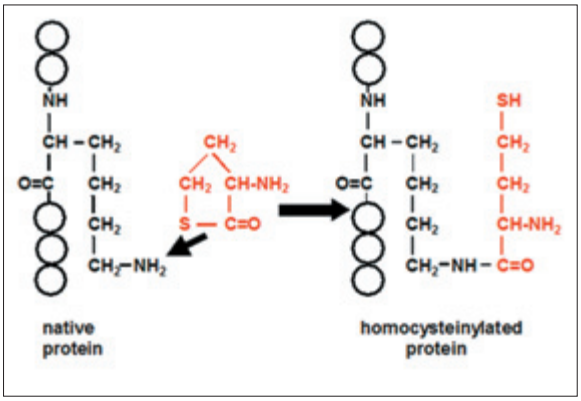

Fig. 6. Reaction of homocysteine thiolactone (red) with ε-amino groups of protein lysine residues.

Protein homocysteinylation

One of the most striking features of HCTL is its ability to acylate free amino groups, especially ε-NH_2 groups of protein lysine residues. This posttranslational modification yields N-Hcy protein adducts (homocystamide) (Figure 6). Interestingly, this reaction was used to titrate immunoglobulins for electron microscopy long before the existence of endogenous HCTL was described [46]. Routine laboratory methods of Hcy assay measure only free Hcy and Hcy liberated from disulfides by thiol-reducing agents. Thus, Hcy bound to proteins by amide bonds is not released. The latter may be liberated only by hydrolysis of proteins in an acidic environment and high temperature. In cultured mammalian cells, homocysteine liberated from hydrolyzed proteins comprises 0.04–0.24% of protein methionine residues [30].

In vitro, HCTL attaches to various proteins, such as serum albumin, fibrinogen, transferrin, α2-macroglobulin, myoglobin, and cytochrome c. The reaction occurs easily under physiological conditions (pH 7.4, 37ºC). The amount of HCTL molecules attached to a given protein increases with increasing number of lysine residues. However, proteins of high molecular weight are homocysteinylated to a lesser degree than would be expected from their lysine content, probably because some lysine residues are not easily accessible. Importantly, protein homocysteinylation occurs even at HCTL concentrations as low as 10 nM [31], much lower than any other toxic effects of homocysteine described so far. Addition of HCTL to the culture medium results in homocysteinylation of proteins in human umbilical vein endothelial cells [43].

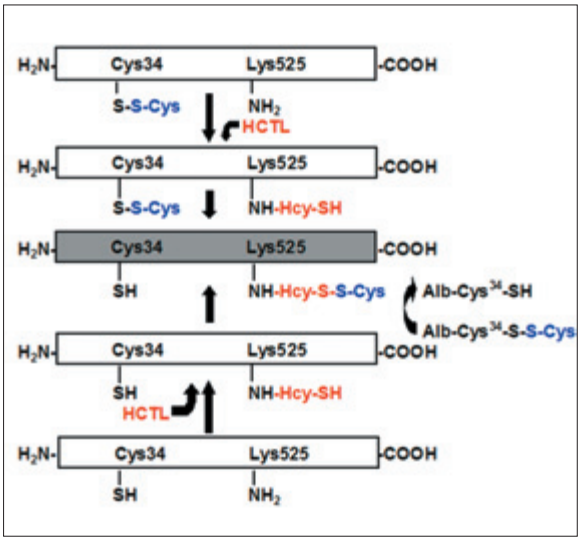

Fig. 7. N-homocysteinylation of two major forms of human plasma albumin [19], albumin-Cys^{34}-S-S-Cys (top) and mercaptoalbumin (bottom), with subsequent thiol-disulfide exchange. A common final product, N-(Hcy-S-S-Cys)-Lys^{525}-Alb-Cys^{34}-SH, is marked in gray.

N-linked homocysteine was identified in proteins isolated from normal human and rat serum [36]. It was estimated that 0.36% of plasma albumin molecules and 0.6% of hemoglobin is homocysteinylated, which corresponds to 1 molecule of Hcy per 1000 molecules of protein methionine. Other proteins, such as γ-globulins, LDL, HDL, antitrypsin, transferrin, and fibrinogen, are also homocysteinylated, but their Hcy content is lower, varying between 0.04 and 0.1% of protein molecules. In plasma or serum, albumin is a main reservoir of N-linked homocysteine, carrying at least 90% of its total amount. Taking into account the normal concentration of albumin, the level of Hcy bound with it in the form of homocystamide is about 2.8 μM, i.e. 25% of total plasma homocysteine. However, if one considers whole blood, hemoglobin is the main carrier of homocystamide; the concentration of hemoglobin-bound homocystamide may be about 12 μM, i.e. more than the classical "total" (free + bound to proteins by disulfide bonds) homocysteine [36]. In whole blood, 75% of homocystamide is bound to hemoglobin, 22% to albumin, and 2% to γ-globulins. The total concentration of N-linked homocysteine in the blood is about 15 μM. In other words, ²/₃ of Hcy present in the blood actually circulates in the form of protein-linked homocystamide and the remaining ¹/₃ is that which is classically referred to as "total" homocysteine [36].

A recent study [19] suggests a relationship between N-homocysteinylation and "S-homocysteinylation" (binding of Hcy to proteins by disulfide bridges), at least in the case of albumin. In human plasma, albumin exists in two major forms: mercaptoalbumin, containing a free thiol group at cysteine 34 (Alb-Cys^{34}-SH), and albumin, with this residue bound to cysteine by a disulfide bond (Alb-Cys^{34}-S-S-Cys); they account for ²/₃ and ¹/₃ of the circulating albumin pool, respectively. A third, small fraction (1–2%) is bound to homocysteine (Alb-Cys^{34}-S-S-Hcy). *In vitro*, Alb-Cys^{34}-S-S-Cys and Alb-Cys^{34}-S-S-Hcy are N-homocysteinylated much faster than mercaptoalbumin. The predominant site of N-homocysteinylation in human plasma albumin is lysine 525. It was demonstrated that after N-homocysteinylation of Alb-Cys^{34}-S-S-Cys at Lys^{525}, thiol-disulfide exchange occurs, i.e. an additional cysteine residue bound to Cys^{34} is transferred to the –SH group of N-linked homocysteine, thus regenerating a free thiol group at Cys^{34} (Figure 7). N-homocysteinylation of mercaptoalbumin yields N-(Hcy-SH)-Alb-Cys^{34}-SH, which undergoes thiol-disulfide exchange with Alb-Cys^{34}-S-S-Cys to form the same final product, N-(Hcy-S-S-Cys)-Alb-Cys^{34}-SH (Figure 7). Because albumin is a predominant serum protein, it is a "sink" for Hcy and HCTL, binding them by S- and N-homocysteinylation, respectively. This may be considered a mechanism protecting against the homocysteinylation of tissue proteins.

However, not all plasma proteins are homocysteinylated. For example, no N-linked homocysteine was found in human plasma transthyretin (prealbumin), even in patients with hyperhomocysteinemia, although this protein contains 7 lysine residues [73].

Consequences of protein homocysteinylation

Homocysteinylation of protein lysine residues results in the incorporation of additional thiol groups, which has several consequences for the physicochemical properties of the affected protein. First, these –SH groups enable the formation of additional intra- and intermolecular disulfide bridges, leading to changes in the protein tertiary structure and protein aggregation, respectively. Aggregation and precipitation of trypsin, RNAase A, myoglobin, and cytochrome c was observed following homocysteinylation of these proteins *in vitro* [31]. Second, new –SH groups may alter the net electric charge of the molecule, thus affecting its reactivity with other proteins or, in the case of enzymes, with the corresponding substrates. Additional –SH groups may also make the protein more susceptible to oxidative damage. Finally, although the total number of $-NH_2$ groups does not change following homocysteinylation, the net positive charge of these groups decreases because the ε-NH_2 groups of lysine residues have higher pK_a (~10.5) and thus are more charged under physiological conditions than the α-NH_2 groups of incorporated homocysteine. [31].

Several enzymes have been demonstrated to be inactivated by homocysteinylation. For example, methionyl-tRNA synthetase is completely inactivated when ¹/₃ of its lysine residues are homocysteinylated. Trypsin is more resistant to HCTL-induced inactivation; it completely loses its activity when almost 90% of its lysine residues are homocysteinylated [31].

It has been demonstrated [19] that *N*-(Hcy-S-S-Cys)-albumin-Cys^{34}-SH is more susceptible to proteolysis than albumin-Cys^{34}-SH. Although *N*-(Hcy-SH)-albumin-Cys^{34}-SH is resistant to proteolytic digestion, this form is unlikely to exist in the circulation because it rapidly undergoes thiol-disulfide exchange to form proteolysis-prone *N*-(Hcy-S-S-Cys)-albumin-Cys^{34}-SH. More rapid proteolysis of N-homocysteinylated albumin, most likely in the liver, may be the mechanism of detoxification of HCTL.

An important consequence of protein homocysteinylation is blockade of the ε-NH_2 groups of the lysine residues in

collagen, which impairs cross-linking of extracellular matrix fibers. Indeed, a reduced number of cross-links was observed in patients with homocystinuria [53]. In addition, lysyl oxidase (LOX), which catalyzes the cross-linking of extracellular matrix proteins, is inactivated by homocysteine thiolactone [52]. In cultured bovine aortic endothelial cells, homocysteine thiolactone decreases LOX gene expression and enzymatic activity, most likely by stimulating oxidative stress [67].

Enzymes involved in the metabolism of homocysteine thiolactone

It was noticed more than 50 years ago that human serum is capable of hydrolyzing the organophosphate compound paraoxon, a metabolite of the insecticide parathion. The enzyme catalyzing this reaction was named paraoxonase (PON), now referred to as paraoxonase 1 (PON1). In 1996, Primo-Parmo and colleagues [66] discovered that PON1 gene is a member of a three-gene family, together with two relatives, PON2 and PON3. In humans, all three genes are located on the long arm of chromosome 7. The respective proteins share about 65% homology in their amino acid sequences. PON1 is the best characterized member of the family; therefore, the terms PON and PON1 are often used as synonyms. In plasma, PON1 circulates attached to high-density lipoproteins (HDLs). PON1 is also detected in various tissues, but at a much lower level than in plasma [70].

Although some studies suggest that PON1 protects against the acute and chronic toxicity of organophosphates, hydrolyzing these artificial substrates is certainly not the major physiological role of PON1. Studies performed during the last decade indicated that PON1 is responsible for the antiatherosclerotic effect of HDL [86]. PON1 knockout mice are more susceptible to diet-induced atherosclerosis than wild-type animals [75], whereas mice overexpressing human PON1 develop less atherosclerotic lesions [87]. In humans, low plasma PON1 activity was an independent predictor of acute cardiovascular events in at least one prospective study [55]. Nevertheless, the mechanism through which PON1 protects against atherosclerosis is not entirely clear. Many studies have suggested that PON1 degrades proinflammatory native and oxidized phospholipids, including platelet-activating factor (PAF), and thus protects plasma lipoproteins and plasma membranes from reactive oxygen species-mediated damage [1,56,71]. However, more recent studies question this possibility by demonstrating that this activity results from trace amounts of platelet-activating factor acetylhydrolase (PAF-AH) contaminating PON1 preparations [58]. Indeed, recombinant PON1 has no activity toward oxidized phospholipids and does not protect low-density lipoproteins from oxidation [10,13,84].

It was noticed in 2000 that PON1 hydrolyzes several synthetic aliphatic and aromatic lactones [5]. Soon thereafter, Jakubowski demonstrated that PON1 hydrolyzes homocysteine thiolactone [32]. Lactone-hydrolyzing activity may be an ancestral function of PON1 which occurred during evolution much earlier than its other activities [47,50]. PON1 is responsible for rapid metabolism of HCTL by mammalian plasma. Like all other PON1 activities, HCTL hydrolysis is Ca^{2+}-dependent and thus is inhibited by EDTA. D-homocysteine thiolactone is hydrolyzed by PON1 about fourfold less efficiently than natural L-isomer [32]. HCTL is not hydrolyzed by chicken serum, which does not contain PON1. Serum obtained from PON1 knockout mice is also unable to degrade HCTL, whereas this compound is rapidly hydrolyzed by rabbit serum, consistently with a tenfold higher level of PON1 in rabbits than in humans [32]. Thus, hydrolyzing homocysteine thiolactone may be an important if not the principal mechanism of atheroprotective activity of PON1.

Two common sequence polymorphisms of the PON1 molecule have been identified: 192 R/Q (arginine/glutamine) and 55 M/L (methionine/leucine). The R/Q polymorphism affects PON1 specific activity (activity per unit of protein mass) toward some synthetic substrates. The R isoform has higher activity toward paraoxon than the Q isozyme, whereas the latter is more efficient in metabolizing some other organophosphates, such as diazoxon, soman, and sarin. Other substrates, such as phenyl acetate, are hydrolyzed by both isoforms with the same efficacy. Some epidemiological studies suggested that ischemic heart disease is more frequent in RR homozygotes than in either QQ homozygotes or in heterozygotes, which was attributed to better protection of LDL from oxidation by the Q isozyme. However, other studies did not confirm this. A recent meta-analysis including many studies and a large number of individuals failed to demonstrate any significant relationship between PON1 genotype and the risk of atherosclerosis [94]. In addition, more careful epidemiological studies clearly indicate that total PON1 concentration and activity (i.e. the so-called "PON1 status") is more important than the genotype in determining susceptibility to vascular disease [44,54]. The 55 M/L polymorphism has no effect on specific enzyme activity toward any known substrate; however, the MM genotype is associated with a lower concentration of PON1 in plasma. This results from the lower stability of the M isoform as well as from a linkage disequilibrium between 55M/L polymorphism and C(-108)T polymorphism within the promoter region of *PON1* gene, which determines the rate of *PON1* gene transcription [11].

It was demonstrated that in healthy volunteers, R192 and L55 alleles are associated with higher HCTL-hydrolyzing activity [38]. The effect of M/L polymorphism is not surprising in view of its association with plasma enzyme concentration. Although the significance of the relationship between R/Q polymorphism and PON1 activity toward HCTL is unclear, the fact that this polymorphism affects activities toward paraoxon and HCTL in the same manner contributes to very strong correlation ($r \approx 0.9$) between activities assayed with both substrates. Thus, the paraoxon-hydrolyzing activity of whole serum is a good estimate of HCTL-hydrolyzing activity, at least in humans [38].

Exogenous HCTL added to human serum is rapidly metabolized, with a $T_{½}$ of about 1 hour. About 50% of the added HCTL is degraded by serum PON1 to homocysteine, which then attaches to proteins by -S-S- bridges. The remaining HCTL acylates amino groups of protein lysine residues [8]. The fraction of HCTL bound by plasma proteins in the form of homocystamide inversely correlates with PON1 activity, confirming that PON1 determines the rate of protein homocysteinylation [36,38,85].

In contrast to PON1, PON2 does not circulate in plasma but is expressed in almost all tissues studied [65]. PON3 is synthesized in the liver and circulates in plasma attached to HDL; however, its level is about two orders of magnitude lower than that of PON1 [12,68]. It is unclear whether PON2 and PON3 hydrolyze homocysteine thiolactone, although this possibility is very likely because both enzymes hydrolyze homoserine lactone, which differs from HCTL only in that it possesses one oxygen atom instead of sulfur (Figure 5) [13,83].

Since all three paraoxonases are expressed in various tissues, HCTL may be metabolized not only in plasma, but also inside the cells. For example, cultured human breast cancer cells metabolize exogenous HCTL with a half-life of about 1 hour [30]. Various cell types metabolize HCTL with different efficacies [30], although it is not clear whether this is determined by the level of PON1 expression.

Growing lupine seedlings are able to hydrolyze HCTL, and HCTL-hydrolyzing activity was detected in their extracts [42]. The enzyme catalyzing this reaction is distinct from paraoxonase because it is not able to hydrolyze PON1's artificial substrates, such as paraoxon, and it does not require Ca^{2+} for its activity. The specific HCTL-hydrolyzing activity of purified plant enzyme is sevenfold higher than that of mammalian paraoxonase.

Factors affecting HCTL synthesis and protein homocysteinylation

Since MetRS is the only enzyme involved in HCTL synthesis *in vivo*, the amount of HCTL produced in a given cell depends on its expression and activity. In addition, because methionine competes with homocysteine for the active site of MetRS, the synthesis of HCTL depends on the ratio between homocysteine and methionine concentrations. A high concentration of Hcy promotes HCTL production. Prokaryotic cells produce much more HCTL than do eukaryotic cells since the former contain more homocysteine. In addition, malignant eukaryotic cells generate more HCTL than normal cells because tumor cells, in general, metabolize Hcy less efficiently [30,95]. The average ratio of Hcy to methionine is about 1 in bacteria, 0.06 in nonmalignant mammalian cells, and 0.15 in malignant mammalian cells. Factors which impair homocysteine metabolism, such as folate deficiency, antifolate drugs, and CBS deficiency, increase HCTL production in cultured cells [30]. However, whereas in cultured fibroblasts the antifolate drug aminopterin increases the level of free HCTL 5–7 times, the degree of protein homocysteinylation increases only by 50% [30]. This suggests that the amount of HCTL incorporated into proteins is not strictly proportional to the free thiolactone level. On the other hand, a high methionine concentration will decrease HCTL synthesis. However, some HCTL is also formed from methionine, which is first metabolized to homocysteine by the SAM-SAH pathway. Indeed, cultured endothelial cells may convert up to 4% of exogenous methionine to HCTL [43].

Although the relationship between Hcy level and protein homocysteinylation *in vitro* is quite well established, the factors which regulate this process *in vivo* are much less understood. Uji et al. [88] demonstrated that although plasma homocysteine is threefold higher in hemodialyzed patients than in control individuals, protein-bound homocystamide in serum is higher by only 50%, i.e. although the absolute level of homocysteinylated proteins increases, the ratio between protein-bound homocystamide and homocysteine is reduced by about 50%. Thus the relationship between homocysteine concentration and protein homocysteinylation is not strictly proportional. This is even more surprising if one considers that renal failure is associated with reduced PON1 activity [74], which should increase the HCTL/Hcy ratio. Unfortunately, the free HCTL level was not examined in that study [88]. In general, very little is known about how various disease states affect HCTL synthesis, metabolism, and protein homocysteinylation. It should be noted that no correlation between plasma homocysteine and free HCTL concentration was observed in 60 healthy individuals [8]. Thus it is unclear how the level of protein homocysteinylation is determined by homocysteine concentration *in vivo*.

Homocysteine thiolactone and atherosclerosis

Oxidative modification of LDL plays an important role in the development of atherosclerosis [78]. This has led to the assumption that homocysteinylation of LDL's integral apolipoprotein, apo-B100, may also contribute to atherogenesis. That homocysteinylation is involved in the proatherogenic effect of homocysteine seems an attractive proposal. First, protein homocysteinylation is observed at low concentrations of HCTL and thus could explain the proatherogenic effect of homocysteine at the physiological level. Second, other thiol-containing compounds, such as cysteine, are not converted to homocysteine thiolactone, which helps to understand why only homocysteine is atherogenic.

There are several possible mechanisms through which HCTL could accelerate atherogenesis. First, it was hypothesized that homocysteinylated LDLs are more susceptible to oxidation than native lipoproteins. A detailed study performed by Ferguson et al. [14] demonstrated that homocysteinylated LDLs are actually more resistant to oxidation *ex vivo*, which is accounted for by the protective effect of additional, HCTL-derived –SH groups. Because homocysteinylated LDLs bind to scavenger receptors rather than LDL receptors, they are taken up by macrophages more rapidly than are native LDLs, leading to intracellular cholesterol accumulation and formation of "foam cells". In addition, homocysteinylated LDL induce oxidative stress in cultured human aortic endothelial cells and reduce cell viability, suggesting that homocysteinylation may contribute to endothelial dysfunction, which is a key event initiating the development of atherosclerotic plaque [17]. Homocysteinylated LDLs decrease endothelial Na^+,K^+-ATPase activity, leading to intracellular overload with sodium and, subsequently, calcium. This is associated with reduced production of nitric oxide (NO) and increased generation of peroxynitrite ($ONOO^-$), a highly reactive nitrogen species originating from NO and superoxide anion radical [92].

Oxidized LDLs, in which apolipoprotein B is modified by reactive oxygen species and/or lipid peroxidation products, induce the humoral immune response. Antibodies against oxidized LDLs are a marker of lipoprotein oxida-

tion *in vivo* and correlate with various indices of atherosclerosis, such as carotid artery intima-media thickness [24]. Analogously, injection of homocysteinylated LDLs induced antibody formation in the rabbit [15]. These antibodies specifically recognize homocystamide-protein adducts because they react with various homocysteinylated rabbit and human proteins but not with native LDLs, oxidized LDLs, or free homocysteine. Immunization of rabbits with homocysteinyllysine, the product of lysine N-homocysteinylation, also yields immunoglobulin formation [90]. In addition, these antibodies are detected in human serum, suggesting that homocysteinylated proteins elicit the immune response *in vivo*. The level of these antibodies varies among individuals; however, it correlates with total plasma homocysteine, suggesting that the formation of antibodies depends on the amount of homocysteinylated proteins. Interestingly, plasma titers of antibodies recognizing homocysteinylated protein lysine residues were 20% higher in males with ischemic cerebral stroke than in healthy controls [90]. A recent study [89] demonstrated that anti-homocysteinyllysine antibodies are also elevated in patients with ischemic heart disease. It is unclear whether these antibodies are directly involved in the pathogenesis of atherosclerosis. Antigen-antibody complexes could be, for example, phagocytozed by macrophages or elicit proinflammatory cytokines. Alternatively, the antibody titers might simply reflect the amount of homocysteinylated proteins. The role of antibodies against oxidized LDLs is also not entirely clear. Some studies suggest that they accelerate atherogenesis by forming immune complexes with their target antigens, whereas others indicate that their protective role is explained by the acceleration of modified lipoprotein clearance [24].

Ferretti et al. [16] demonstrated that incubation of HDLs isolated from healthy subjects with homocysteine thiolactone results in homocysteinylation of these lipoproteins, as evidenced by a progressive increase in the amount of free –SH groups. Homocysteinylation was associated with a decrease in PON1 activity toward paraoxon, whereas the amount of lipid peroxidation products did not change. This study demonstrates that homocysteinylation of PON1 (or other protein components of HDL regulating its activity, such as apolipoprotein AI) leads to the inactivation of the enzyme, thus impairing its pleiotropic antiatherosclerotic activities. In addition, this may initiate a positive feedback mechanism, since reduced PON1 activity will cause further accumulation of HCTL and may augment protein homocysteinylation.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase. Since NO counteracts atherogenesis by reducing platelet aggregation, attenuating the proliferation of vascular smooth muscle cells, and inhibiting the inflammatory reaction, increased ADMA concentration is proatherogenic. Indeed, high ADMA level is an independent predictor of acute cardiovascular events [6]. Several studies have demonstrated that hyperhomocysteinemia is associated with elevated ADMA concentration in animals and humans; however, the underlying mechanism linking these two abnormalities has not been established [7,79,80,81,93]. ADMA is synthesized during methylation of protein arginine residues by S-adenosylmethionine: protein arginine methyltransferases (protein methylases, PRMT) and is then liberated during proteolysis. Free ADMA is not incorporated into proteins, but is metabolized by dimethylarginine dimethylaminohydrolase (DDAH). Reduced DDAH activity is responsible for the elevation of ADMA in various disease states, such as hypercholesterolemia and diabetes mellitus. One of the possibilities through which homocysteine could increase ADMA is homocysteinylation of DDAH and its subsequent inactivation. Various DDAH isoforms contain 7–19 lysine residues, which makes them potentially good substrates for homocysteinylation. In addition, some homocysteinylated proteins are more susceptible to proteolysis, which might result in increased production of ADMA [37].

A HCTL-induced decrease in LOX activity [52,67] may also contribute to atherogenesis. LOX deficient mice are characterized by endothelial dysfunction, vascular damage, and shorter life-span than wild-type animals [57].

It should be noted that infusion of homocysteine thiolactone at a dose of 140 mg/kg/week induces atherosclerosis in rabbits without modulating plasma homocysteine or lipid profile [59]. These data suggest that HCTL may be more atherogenic than homocysteine.

Other effects of HCTL

One important consequence of misactivation of Hcy and its conversion to homocysteine thiolactone is the breakdown of ATP used in the reaction catalyzed by methionyl-tRNA synthetase. It was calculated that in mouse renal adenocarcinoma cells cultured on methionine-containing medium (i.e. under conditions of low HCTL production), 0.6% of ATP used by methionyl-tRNA synthetase is utilized for the activation of Hcy. However, when these cells are cultured in the presence of Hcy, the amount of ATP wasted in this way increases to 12% [27,41]. ATP depletion may explain why some cells are not able to grow on homocysteine-containing media. In addition, the high energy costs of editing may contribute to the proapoptotic effect of homocysteine observed in various cell types.

Homocysteine thiolactone is toxic for rat embryo [91]. Interestingly, both the L- and D-forms of thiolactone were toxic, whereas only the L- but not the D-isomer of homocysteine was toxic. This is consistent with the identical chemical reactivity of L- and D-homocysteine thiolactone (both may equally homocysteinylate proteins) and with the stereospecific activation of L- but not D-homocysteine by methionyl-tRNA synthetase. HCTL is also toxic for chick [72] and mouse embryo [20], although the latter is controversial [21]. *In vitro*, HCTL induces apoptosis of endothelial cells [61] and trophoblast cells [45] at concentrations much lower than homocysteine. In human promyeloid leukemia HL-60 cells, HCTL-induced apoptosis was associated with increased intracellular H_2O_2 production, conversion of inactive procaspase-3 to active caspase-3, and DNA fragmentation [23]. These effects were abolished by catalase as well as by other antioxidants, such as vitamin C, vitamin E, and N-acetylcysteine [22].

In rat hepatoma cells transfected with human insulin receptor, homocysteine thiolactone impairs insulin signaling, as evidenced by reduced tyrosine phosphorylation of

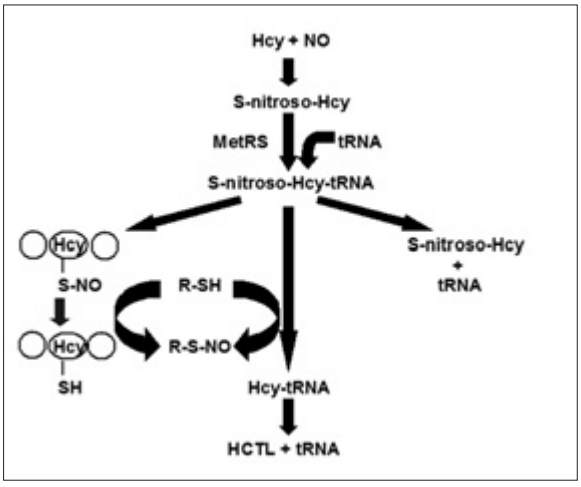

Fig. 8. Mechanism through which homocysteine may be translationally incorporated into proteins in places normally occupied by methionine. NO – nitric oxide, R-SH – thiol group-containing compound, R-S-NO – S-nitrosothiol.

the β-subunit of insulin receptor and of the insulin receptor substrate-1 (IRS-1), attenuation of binding of the regulatory subunit (p85) of phosphatidylinositol-3-kinase (PI3K) to IRS-1, decrease in insulin-stimulated PI3K activity, and glycogen synthesis [63]. These effects are reversed by reduced glutathione, suggesting that they are accounted for by HCTL-induced oxidative stress. In addition, HCTL impairs insulin-stimulated activation of the mitogen-activated protein kinases p44 and p42 (p44/p42 MAPK), protein and DNA synthesis, as well as insulin-activated cell proliferation [64]. Finally, infusion of homocysteine thiolactone induces seizures in rats and mice [76].

It is unclear whether these acute toxic effects of HCTL are accounted for by protein homocysteinylation because they are observed at relatively high thiolactone concentrations and occur within a short time-frame. However, it is likely that lower concentrations of this compound may induce similar changes *in vivo* when acting cumulatively over a longer time period. Nevertheless, it should be mentioned that due to its hydrophobicity, HCTL readily permeates plasma membranes and, inside the cells, may be hydrolyzed to Hcy by PONs or other esterases. Thus, even if HCTL induces some effect in intact cells at a concentration lower than homocysteine, this is not definite evidence that this effect is specific to HCTL.

Translational incorporation of homocysteine into proteins

Recent studies indicate that homocysteine may be incorporated into proteins during translation in a nitric oxide-dependent manner. The free –SH group of homocysteine may react with NO, forming S-nitrosohomocysteine [33]. S-nitroso-Hcy is one of the most stable S-nitrosothiols in plasma, with a half-life of about 1.5 hour. S-nitrosohomocysteine is activated by methionyl-tRNA synthetase; however, it is not edited to homocysteine thiolactone but is transferred to methionine-specific tRNA and then incorporated into the polypeptide chain (Figure 8). Subsequent exchange of NO between S-nitroso-Hcy and other thiols yields a protein in which Hcy is contained in places normally occupied by methionine. S-nitrosohomocysteinyl-tRNA may also spontaneously hydrolyze to S-nitrosohomocysteine or may react with other thiol-containing compounds to form homocysteinyl-tRNA, rapidly edited to homocysteine thiolactone. It should be noted that translationally incorporated Hcy is, similarly to N-linked homocysteine, released during acid hydrolysis of proteins and will thus contribute to the total protein-bound Hcy pool. It is estimated that about 50% of protein-linked Hcy (excluding that bound by –S-S- bonds) is attached to ε–NH_2 groups of lysine residues in HCTL-mediated process and another 50% is translationally incorporated into polypeptide chains [34]. The functional consequences of translational Hcy incorporation are not clear; however, it may be similar to N-homocysteinylation because this process is also associated with the insertion of additional free –SH groups which are not present in methionine residues. Interestingly, S-nitrosohomocysteine was recently identified as a potent inhibitor of DDAH, which might be an additional mechanism through which homocysteine increases ADMA level [49].

Conclusions

Nonspecific activation of homocysteine by methionyl-tRNA synthetase and its subsequent editing is a common mechanism protecting living cells from incorporation of this non-cognate amino acid into proteins. However, the expense of this is synthesis of homocysteine thiolactone, a highly reactive compound which acylates protein lysine residues. N-homocysteinylation is a novel mechanism through which Hcy might exert its deleterious effects, including accelerated atherogenesis. Although the biochemistry of this process is relatively well understood, many points still remain open. First, little is known about the factors which regulate HCTL synthesis and its reaction with proteins. The level of HCTL and protein N-linked homocysteine in pathological conditions has not been, with few exceptions, studied so far. To now it is not known whether intracellular proteins are homocysteinylated *in vivo*, although this possibility is very likely, since HCTL is synthesized intracellularly and PON1 activity in most tissues is much lower than in plasma. It is also unclear whether PON2 and PON3 can hydrolyze HCTL. Finally, although one study demonstrated that homoserine lactone inhibits lysyl oxidase almost as effectively as HCTL [52], it is not known if HSL can attach to proteins in a manner similar to HCTL. Such "protein homoserination" could be an important mechanism affecting host proteins by HSL-producing bacteria. These are most exciting directions for future research in this promising field.

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