Chapter 2
An Overview of Homocysteine Metabolism

Mammals, in contrast to bacteria and plants, cannot make their own methionine (Met). Thus, in humans and animals, Met is an essential amino acid that is provided in the form of proteins ingested as food. In our digestive tract dietary proteins are hydrolyzed to amino acids. Met released from dietary proteins is taken up by the epithelium of the digestive tract and transported in the blood to cells of various organs. In every cell of the body Met is metabolized by two major pathways (Fig. 1.1): (1) as a building block to make new proteins in the ribosomal protein biosynthetic apparatus and (2) as a precursor of *S*-adenosylmethionine (AdoMet), a universal donor that provides *methyl groups* for biological methylation reactions and *propyl groups* for polyamine biosynthesis (both derived from Met). In metabolic pathways (1) and (2), Met is activated by reactions with ATP, albeit in a pathway-specific manner: the carboxyl group of Met is activated in pathway (1), while the thioether sulfur atom is activated in pathway (2).

It is the Met utilization pathway (2) that eventually leads to Hcy [20] (Fig. 1.1). As a result of the transfer of its methyl group to an acceptor, AdoMet is converted to *S*-adenosylhomocysteine (AdoHcy). *The reversible enzymatic hydrolysis of AdoHcy is the only known source of Hcy in the human or animal body*. Levels of Hcy are regulated by remethylation to Met, catalyzed by the enzyme MS, and a two-step transsulfuration to cysteine, the first step of which is catalyzed by the enzyme CBS, while the second step is catalyzed by cystathionine γ-lyase (CSE). The remethylation catalyzed by MS requires vitamin B_{12} and 5,10-methyltetrahydrofolate (CH_3-THF), generated by MTHFR [31] (Fig. 1.1). An alternative remethylation pathway is catalyzed by betaine–Hcy methyltransferase (BHMT) [80]. The transsulfuration requires vitamin B_6. While remethylation by MS occurs in every organ of the body, remethylation by BHMT and transsulfuration by CBS are limited to the liver and kidneys. Hcy can also react oxidatively with other thiols, mainly sulfhydryl groups of extracellular proteins, to form mixed disulfides (*S*-Hcy-protein) [78, 79, 81].

A fraction of Hcy is also metabolized by methionyl-tRNA synthetase (MetRS in Fig. 1.1) to the thioester Hcy-thiolactone [68, 69, 82, 83]. The flow through the Hcy-thiolactone pathway is increased by a high-Met diet (the ultimate precursor of

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Hcy), inadequate supply of CH_3-THF, or impairment of remethylation or transsulfuration reactions by genetic alterations of enzymes, such as CBS, MS, or MTHFR. Hcy-thiolactone is neutral at physiological pH ($pK_a = 6.67$, [84]) and thus accumulates mostly in the extracellular fluids.

Hcy-thiolactone is metabolized by two pathways: (1) a hydrolytic pathway that affords Hcy—this pathway is catalyzed by intracellular and extracellular Hcy-thiolactonases, otherwise known as bleomycin hydrolase (Blmh) [85] and paraoxonase 1 (Pon1) [81], respectively; and (2) a synthetic pathway in which Hcy-thiolactone reacts chemically with protein lysine residues, forming *N*-Hcy-protein [78, 79, 81] (Fig. 1.1).

Proteolytic degradation of *N*-Hcy-protein affords *N*ε-Hcy-Lys (Fig. 1.1), first discovered in a biological system when *N*-Hcy-hemoglobin was incubated with liver extracts [72]. The isopeptide *N*ε-Hcy-Lys is present in human and mouse plasma. Its levels increase in renal disease and CBS deficiency in humans. Genetic deficiencies in folate and Hcy metabolism and a hyperhomocysteinemic high-Met diet cause elevation of *N*ε-Hcy-Lys in mice. The plasma levels of *N*ε-Hcy-Lys are positively correlated with the nitric oxide synthase inhibitor asymmetric dimethylarginine [86], which is consistent with their common origin as products of protein turnover [72].

2.1 Homocysteine Metabolite Levels

In addition to the free thiol (reduced) form, i.e., free Hcy-SH, several other Hcy species are present in human and animal plasma (Table 2.1) [87]. These include the disulfide homocystine Hcy-S-S-Hcy identified in the urine of patients with homocystinuria [88]; a mixed disulfide of Hcy and Cys, Hcy-S-S-Cys, first described in patients with cystinuria [89] and then in patients with homocystinuria [90]; and a mixed disulfide of Hcy with plasma protein, *S*-Hcy-protein [91, 92], as well as species with substituted carboxyl groups, such as Hcy-thiolactone [64, 76, 93–95], *N*-Hcy-protein [76, 79, 96], and *N*ε-Hcy-Lys [72]. Other known Hcy metabolites include AdoHcy, cystathionine [97, 98], homocysteine sulfinic acid, and homocysteic acid (Table 2.1) [20, 99].

S-Nitroso-Hcy is a potential metabolite that has been first detected in bovine aortic endothelial cells incubated with exogenous Hcy [100]. Treatment of these cells with the nitric oxide synthase inhibitor *N*-nitro-L-Arg prevents nitric oxide generation and the formation of *S-nitroso*-Hcy. Increasing nitric oxide generation by overexpression of the inducible nitric oxide synthase in endothelial cells results in a significant increase in the formation of *S-nitroso*-Hcy [101]. That human endothelial cells produce *S-nitroso*-Hcy has also been inferred from its ability to mediate translational incorporation of Hcy into protein [75] that is observed in these cells [68, 76, 83]. However, the presence of *S-nitroso*-Hcy in humans or animals remains to be documented.

Table 2.1 Concentrations of Hcy metabolites in normal human and mouse plasma and urine

Hcy metabolite	Concentration (nM) in human		Concentration (nM) in mouse	
	Plasma	Urine	Plasma	Urine
tHcy	12,000	2,500	3,000	45,000
Hcy	250			
Hcy-S-S-Hcy + Hcy-S-S-Cys	1,940			
S-Hcy-protein	9,810			
Hcy-thiolactone	0.2	168	3.7	136
N-Hcy-protein	490		1,900	
*N*ε-Hcy-Lys	<100	<100	400	<100
AdoHcy	15	490		
Homocysteic acid	186			
Homocysteine sulfinic acid	677			
Cystathionine	126	350–49,900	2,479	

Concentration values are from references discussed in the text

2.1.1 *"Total Homocysteine"*

The sum of free thiol (reduced) and disulfide-bound Hcy (oxidized) species, obtained by the treatment of a sample with a reducing agent, is called "total Hcy" (tHcy) and serves as a plasma marker in clinical studies of relationships between Hcy metabolism and human disease [102]. Unfortunately, the term "total Hcy" is a misnomer because it does not include a host of important Hcy congeners (discussed in the following sections). Normal tHcy levels of about 10 μM increase to several hundred μM in genetic deficiencies in Hcy, folate, or vitamin B_{12} metabolism, both in mice and humans. In normal human plasma, *S*-Hcy-protein comprises ~80 % of tHcy, homocystine Hcy-S-S-Hcy and homocysteine-cysteine mixed disulfide Hcy-S-S-Cys comprise 20 %, while free thiol Hcy comprises just ~1 % of tHcy (Table 2.1) [87].

In healthy humans, tHcy concentration in plasma (~10 μM) is ~twofold higher than in urine [95]. In mice, however, plasma tHcy (3.5 μM) is 15-fold lower than urinary tHcy (Table 2.1) [93]. While in humans urinary tHcy clearance is insignificant (~1 %), in mice a significant fraction of tHcy (~38 %) is eliminated by urinary excretion [93]. It should be noted, however, that because the reduction of the disulfide bonds in the commonly used tHcy assays is carried out at alkaline pH, one of them—Hcy-thiolactone—most likely contributes to the tHcy pool.

2.1.2 S-*Homocysteinyl-Protein*

Plasma protein *S*-linked Hcy is carried on albumin [103] and γ-globulin [79] and in smaller quantities on transthyretin [104, 105], α_1-acid glycoprotein, and high-density lipoprotein (HDL) [106]. Each of those proteins, except ApoA1 in HDL,

possesses a cysteine residue with free –SH group that is capable of forming a disulfide bond with a low molecular thiol.

In addition to *S*-linked Hcy, albumin, γ-globulin [107–109], transthyretin [104, 105, 110], α_1-acid glycoprotein, and HDL [106] are known to carry *S*-linked cysteine, CysGly, and glutathione (GSH) in vivo. In normal human plasma the concentration of protein *S*-linked Hcy (10 μM) is lower than the concentration of protein *S*-linked Cys (165 μM) and CysGly (17 μM), but higher than the concentration of protein *S*-linked GSH (1.5 μM) [111]. In erythrocytes the bulk of blood *S*-GSH-protein occurs as *S*-GSH-hemoglobin, which, with a concentration of 130 μM, comprises 14 % of erythrocyte GSH content [112].

The presence of *S*-Hcy-protein has also been reported in the liver, kidney, and brain tissues from a patient with MTHFR deficiency at autopsy, but not in tissues from three subjects who died of unrelated causes [91]. Much higher levels of *S*-Hcy-protein accumulate in the brain (403.1 pmol/mg protein) than in the liver (76.1 pmol/mg protein) and kidney (55.2 pmol/mg protein) of the MTHFR-deficient patient. The level of liver *S*-Hcy-protein in the MTHFR-deficient patient (76.1 pmol/mg protein) [91] is lower than the level of liver tHcy in *Mthfr*$^{-/-}$ mice (184 ± 71 μM, $n = 7$) [113].

In rodents, tHcy concentrations in tissues and plasma are similar, in the micromolar range [114]. Significant pools of Hcy bound to intracellular proteins by disulfide linkages (*S*-Hcy-protein) exist in rodent tissues and plasma [114]. For example, *S*-Hcy-protein comprises ~42 % of tHcy in rat liver, kidney, heart, and lung. In rat cerebrum and cerebellum *S*-Hcy-protein constitutes 30.4 % and 5.3 %, respectively, of tHcy.

2.1.3 *Homocysteine-Thiolactone*

In normal humans and mice, plasma Hcy-thiolactone concentrations are in the subnanomolar to low nanomolar range, whereas urinary Hcy-thiolactone concentrations are ~100-fold higher (Table 2.1) [93–95]; >95 % of the filtered plasma Hcy-thiolactone is cleared by the kidney [93, 95]. Plasma Hcy-thiolactone is elevated 59- to 237- and 72-fold in human MTHFR and CBS deficiency, respectively, and 14-fold in mice fed with a high methionine diet.

2.1.4 N-*Homocysteinyl-Protein*

N-linked Hcy is carried on all blood proteins that have been examined, including hemoglobin, albumin, γ-globulin, fibrinogen, transferrin, antitrypsin, LDL, and HDL [79]. In normal human and mouse blood, *N*-Hcy-protein concentrations are in the submicromolar to low micromolar range (Table 2.1) and increase 10–20-fold in genetic or dietary hyperhomocysteinemia [79, 113, 115]. The levels of *N*-Hcy-protein

in mouse liver are 50–160 pmol/mg protein and increase 4- and 12-fold in $Pcft^{-/-}$ and $Cbs^{-/-}$ animals [113].

2.1.5 Nε-*Homocysteinyl-Lysine*

The isopeptide *N*ε-Hcy-Lys, discovered in vitro as a product of a facile reaction of Hcy-thiolactone with lysine [73], forms in vivo during proteolytic turnover of *N*-Hcy-protein [72]. *N*ε-Hcy-Lys is undetectable in normal human plasma (<0.1 μM) and increases to 0.17 and 0.42 μM in patients with renal disease and CBS deficiency, respectively. In normal mouse plasma, *N*ε-Hcy-Lys level is 0.4 μM (Table 2.1) and increase up to tenfold in hyperhomocysteinemic animals.

2.1.6 S-*Adenosylhomocysteine*

AdoHcy, an immediate precursor of Hcy (Fig. 1.1), occurs in human cells at micromolar concentrations, while in plasma, it is present at nanomolar concentrations (Table 2.1) [116, 117]. AdoHcy concentrations are 33-fold higher in human urine than in plasma, consistent with its significant fractional excretion (39 %) [117].

2.1.7 *Cystathionine*

Normal human plasma level of cystathionine, an intermediate in the transsulfuration pathway converting Hcy to Cys, is 126 nM. Cystathionine concentrations are much higher in urine, reaching millimolar range (Table 2.1). The urinary clearance of cystathionine is 50.9 % of the clearance of creatinine [98]. In mice, normal plasma cystathionine concentration is 2,479 nM [118], 20-fold higher than in normal human plasma.

2.1.8 *Homocysteic Acid and Homocysteine Sulfinic Acid*

Other oxidized Hcy metabolites have been reported in humans and experimental animals: homocysteine sulfinic acid (HSA) and homocysteic acid (HCA). However, the mechanism of biological formation of HSA and HCA is not known. Normal human plasma concentration is 677 ± 210 nM for HSA and 186 ± 38 nM for HCA (Table 2.1) [99]. Elevation of Hcy and HCA occurs in cerebrospinal fluid of

children after methotrexate treatment [119]. HSA and HCA are both identified in urine from CBS-deficient patients but not in urine from normal individuals [120].

Extremely high HCA concentrations are reported to be present in plasma (up to 23 μM) and in urine (up to 25 mM) of Alzheimer's disease patients, whereas in control individuals, the HCA levels are somewhat lower (by 40 %) [121]. Such HCA concentrations are 100–10,000-fold higher than HCA values reported by other investigators. Unfortunately, Hasegawa et al. [121] do not discuss these discrepancies and give only a very limited experimental detail for their assay, which prevents any rational explanation of their data.

In rats, oral administration of Hcy increases plasma HCA from an undetectable level to 2.0 μM [122]. Methotrexate treatment elevates both HSA and HCA in rat cerebrospinal fluid [123]. HCA is also reported to be present in mouse brain and urine, at 2.3 μM and 22.5 μM, respectively [124].

HCA has been reported to arise by spontaneous oxidation of Hcy [125]. It can also form by the radiolytic degradation of methionine and methionine-containing peptides [126] and from photooxidation of methionine peptides induced by hydrogen peroxide and UV laser light [127]. Analysis of archived samples from a previously unreported 1958 Stanley Miller's origin of life experiment shows that HCA and other sulfur amino acids are generated in spark discharge experiments designed to imitate primordial environments [128]. Prebiotic formation of methionine in spark discharges in a simulated primitive earth atmosphere containing CH_4, N_2, NH_3, H_2O, and H_2S or CH_3SH has been reported in 1972 [129].

2.2 Toxicity of Homocysteine Metabolites

Acute exposure of living cells and organisms to excess Hcy is known to cause cellular toxicity [7, 68]. The presence of multiple molecular Hcy species raises a question regarding whether any of those species could be more harmful than the other. Numerous clinical and animal studies have established that chronic elevation of plasma tHcy is associated with cardiovascular and neurological abnormalities [34, 36, 37], most severely manifested in genetic deficiencies in Hcy (CBS) [20], folate (MTHFR, PCFT) [31, 130], and cobalamin (cblC) [131, 132] metabolism. However, it should be noted that tHcy is a composite marker, comprised of at least five different Hcy species [87, 102], each of which could exert a distinct biological effect. Moreover, tHcy does not encompass other Hcy metabolites present in the human blood, such as Hcy-thiolactone [94, 95], *N*-Hcy-protein [79, 115], *N*ε-Hcy-Lys [72], AdoHcy, cystathionine, and homocysteic acid [68]. Thus, a contribution of an individual Hcy species to cardiovascular risk or mortality is likely to be obscured by using tHcy as a marker [133]. Indeed, in a few studies that addressed this issue, plasma AdoHcy [116] and anti-*N*-Hcy-protein autoantibodies [134, 135] turned out to be much more sensitive indicators of human cardiovascular disease than plasma tHcy.

2.2.1 *Free Reduced Homocysteine*

There is some evidence suggesting that the free thiol Hcy (reduced form) is more harmful than the disulfide Hcy species (oxidized forms). For example, in two studies that examined the toxicity of individual Hcy species, the free thiol Hcy was associated with endothelial dysfunction in humans, whereas much more abundant disulfide Hcy species were not [136, 137]. The toxicity of free thiol Hcy most likely results from its metabolic conversion to Hcy-thiolactone, which occurs in cultured human and rodent cells [68, 73, 77, 138, 139] and in human [64, 76, 94, 95] and mouse bodies [93, 113]. Furthermore, Hcy-thiolactone is known to be more toxic than Hcy to cultured human endothelial cells [61] and to mice [140, 141]. That the metabolic conversion of Hcy to Hcy-thiolactone is responsible for Hcy toxicity is further supported by studies with rat embryos, which show that L-Hcy-thiolactone, D- Hcy-thiolactone, and L-Hcy are toxic, whereas metabolically inactive D-Hcy (which is not recognized by methionyl-tRNA synthetase and thus cannot be metabolized to Hcy-thiolactone) is not [142]. Plasma levels of the free thiol Hcy are maintained low by its oxidation to disulfides, mostly with the major plasma proteins albumin [81, 103] and γ-globulins [79, 106].

2.2.2 S-*Homocysteinyl-Protein*

There is no evidence that any of the disulfide species of Hcy that occur in the plasma is harmful to the human body [143]. In fact, only free reduced Hcy, comprising 1–2 % of tHcy, but not any of the more abundant disulfide forms of Hcy, including *S*-Hcy-protein, comprising 80 % of tHcy, is associated with diminished vascular function as measured by flow-mediated dilatation [136, 137]. However, that some *S*-Hcy-proteins may be harmful is suggested by findings showing that treatments of human endothelial cells with in vitro-prepared *S*-Hcy-ApoB-100 reduce cell proliferation and viability [144].

In a mouse model of dietary hyperhomocysteinemia, Hcy appears to inhibit vascular fibrinolysis in vivo by forming a disulfide bond with annexin A2 thiol [145]. Annexin A2, an endothelial cell surface co-receptor for plasminogen and tissue plasminogen activator, accelerates the catalytic activation of plasmin, the major fibrinolytic agent in mammals. Thus, derivatizing annexin A2 by Hcy contributes to prothrombotic effects of hyperhomocysteinemia [145].

Whether the formation of *intracellular S*-Hcy-protein in humans could be harmful has been addressed in an ex vivo study, which suggests that Hcy binding to metallothionein, an intracellular zinc-binding protein, causes elevation of intracellular free zinc levels and oxidative stress in cultured human endothelial cells treated with exogenous Hcy [146]. Unfortunately, whether the proposed Hcy binding involves a disulfide bond formation with metallothionein has not been confirmed with purified protein. Although these effects were ascribed to the formation of a putative *S*-Hcy-metallothionein disulfide, the authors' observations that

Hcy binding is not affected by 10 mM glutathione and that cysteine does not form a disulfide with metallothionein [146] suggest that Hcy may bind to metallothionein by another mechanism, possibly *N*-homocysteinylation. In fact, Hcy-thiolactone and protein *N*-linked Hcy are major Hcy species formed in human endothelial cells [74]. Similar to other proteins [78], metallothionein contains numerous lysine residues and is expected to be a target for *N*-homocysteinylation by Hcy-thiolactone.

2.2.3 Homocysteine-Thiolactone

Because of its high solubility, relative stability, and odorless character, Hcy-thiolactone has been often used instead of Hcy to produce experimental hyperhomocysteinemia in animal models. Early studies, beginning in 1974, well before biological significance of Hcy-thiolactone was established, have shown that *chronic* treatments of animals with Hcy-thiolactone cause pathophysiological changes akin to those observed in human genetic hyperhomocysteinemia. For example, continuous Hcy-thiolactone infusions in baboons for 3 months produced patchy desquamation of vascular endothelium, appearance of circulating endothelial cells, and arterial thrombosis [24]. All Hcy-thiolactone-infused animals developed typical arteriosclerotic or preatherosclerotic intimal lesions composed of proliferating smooth muscle cells averaging 10–15 cell layers surrounded by large amounts of collagen, elastic fibers, glycosaminoglycans, and sometimes lipid [147]. (Note: That Hcy-thiolactone has been used in Harker et al.'s experiments [24, 147] is clarified in Mudd et al. [148].) More recent experiments show that Hcy-thiolactone-supplemented diet for 7 weeks produces atherosclerosis in rats [149].

In contrast, rabbits are resistant to the detrimental effects of Hcy-thiolactone infusions [150, 151]. The inability to generate atherosclerosis in rabbits is most likely due to their high levels of serum Hcy-thiolactonase/PON1, ~tenfold higher than in humans or other animals. Thus, rabbits turn over serum Hcy-thiolactone much more efficiently than other animals [81, 152, 153]. Hcy-thiolactone infused into rabbits is quickly cleared from the blood (within $<$15 min) [150].

Chronic treatments with Hcy-thiolactone cause developmental abnormalities in chick embryos [154]. Remarkably, these abnormalities include optic lens dislocation [155] that is identical to the ocular phenotype (ectopia lentis) prevalent in human CBS deficiency [20, 29, 30]. This finding suggests that ectopia lentis is caused by elevations of Hcy-thiolactone that are in fact observed in CBS-deficient patients [93].

Acute treatments with Hcy-thiolactone are also known to cause toxicity in experimental animals. For example, intraperitoneal infusions of Hcy-thiolactone into mice or rats, which are used as a model to study mechanisms of epilepsy, cause seizures and death within minutes [156–159]. Exposure of mouse [160] or rat [142] embryos to Hcy-thiolactone causes lethality, growth retardation, and

developmental abnormalities. In one study Hcy-thiolactone was reported to be non-teratogenic in mouse embryos, but the maximum dose used in that study [160] was lower than those used in other studies, so that a teratogenic dose has not been reached.

Rigorous studies in mice using the intraperitoneal injection model show that plasma Hcy-thiolactone is cleared with a half-life of 5.0 min [140, 141]. Studies with *Blmh*$^{-/-}$ and *Pon1*$^{-/-}$ mouse models that have diminished ability to hydrolyze Hcy-thiolactone led to a conclusion that bleomycin hydrolase (Blmh) and paraoxonase 1 (Pon1) protect the animals against neurotoxicity induced by intraperitoneal injections of L-Hcy-thiolactone [140, 141]. Similar to other harmful products of normal metabolism, endogenous Hcy-thiolactone is also eliminated by urinary excretion, as demonstrated both in humans and mice [93, 95].

Treatments with Hcy-thiolactone inhibit proliferation and can be toxic to cultured human and animal cells [143]. For instance, short-term pretreatment (10 min) of cultured rat hepatoma cells with Hcy-thiolactone leads to the inhibition of insulin signaling by preventing tyrosine phosphorylation of the β-subunit of the insulin receptor and its substrates IRS-1 and Sam68 [161]. Furthermore, pretreatment with Hcy-thiolactone inhibits the interactions of the insulin receptor β-subunit and its substrates with the regulatory subunit p85 of phosphatidylinositol 3-kinase, which in turn inhibits the kinase and blocks the insulin-stimulated glycogen synthesis [161]. Hcy-thiolactone also prevents phosphorylation of GSK-3 and p70S6K catalyzed by mitogen-activated protein kinase, as well as insulin-mediated growth and proliferation [162]. The addition of GSH 5 min before the pretreatment with Hcy-thiolactone restores insulin signaling, suggesting that Hcy-thiolactone-induced oxidative stress might be involved [161, 162]. Much longer (24 h) treatments with higher Hcy concentrations (0.1–1 mM) induce insulin resistance by reducing tyrosine phosphorylation of insulin receptor and IRS-1 in rat adipocytes [163]. Treatments with 0.3–1 mM Hcy (lower concentration is ineffective) also induce resistin, a peptide hormone linked to insulin resistance, after 8–24 h (no induction after 4 h) [163]. Taken together, these findings suggest that Hcy-thiolactone is more effective than Hcy in inducing insulin resistance in cultured cells and thus is likely to contribute of insulin resistance associated with hyperhomocysteinemia in humans and experimental animals.

Hcy-thiolactone is also known to induce endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in retinal epithelial cells [60], as well as apoptotic death in cultured human vascular endothelial cells [61, 164], promyeloid cells [165], and placental trophoblasts [166]. For example, human umbilical vein endothelial cells (HUVECs) treated with 50–200 μM D,L-Hcy-thiolactone for 24 h show 15–40 % apoptotic cell death quantified after labeling with the fluorescent probe DiOC6(3) and flow cytometry analysis (Table 2.2) [154]. The magnitude of apoptosis in HUVECs treated with 200 μM D,L-Hcy-thiolactone corresponds to about 2/3 of apoptosis observed in the cells treated with 5 μg/mL staurosporine or serum-free medium as positive controls (Table 2.2). In contrast to apoptosis induced by staurosporine, apoptotic cell death induced by Hcy-thiolactone in HUVECs is not accompanied by the induction of caspase activity and is not prevented by caspase

Table 2.2 Hcy-thiolactone induces apoptosis in human endothelial cells (adapted from [154])

Treatment (24 h, 37 °C)	Apoptotic cells (%)
D,L-Hcy-thiolactone (200 μM)	30 ± 5
D,L-Homocysteine (200 μM)	1 ± 2
D,L-Homocystine (200 μM)	3 ± 4
L-Cysteine (200 μM)	4 ± 2
Staurosporine (5 μg/mL) (positive control)	58 ± 20

Confluent human umbilical vein endothelial cells (HUVECs) in complete M199 growth medium are treated with indicated thiocompounds or staurosporine. The cells are labeled with the fluorescent probe $DiOC_6(3)$ (10 μg/mL) and analyzed by flow cytometry to quantify apoptotic cells

inhibitors. However, the cell death induced by Hcy-thiolactone is accompanied by phosphatidylserine exposure in cell membranes, characteristic of apoptosis [154]. Control experiments show that 200 μM D,L-Hcy, D,L-homocystine, or L-Cys do not induce apoptosis in HUVECs (Table 2.2), thus indicating that the induction of apoptosis is specific to D,L-Hcy-thiolactone. However, Hcy-thiolactone does not induce apoptosis in transformed cell lines such as HL60 leukemic cells and EA.hy 926 endothelial cells, indicating that the sensitivity to Hcy-thiolactone is cell type dependent [154].

Although the treatment with Hcy, similar to the treatment with Hcy-thiolactone, induces apoptosis, much higher Hcy concentrations are required [58, 61], suggesting that Hcy-thiolactone is more potent than Hcy in causing apoptotic cell death [60, 61, 164]. These findings confirm early studies in which treatments with Hcy-thiolactone caused greater cytotoxicity to endothelial cells than treatments with Hcy [167]. In those studies the toxicity of Hcy-thiolactone is manifested as gross changes in endothelial cell morphology and lysis of 42 % cells during a 16-h exposure. Similar exposure to Hcy produces no changes in cell morphology and only 2 % cell lysis [167].

2.2.4 N-*Homocysteinyl-Protein*

Plasma *N*-Hcy-protein levels are elevated in patients with CBS or MTHFR deficiency [115], or coronary artery disease (CAD) patients, compared with normal healthy individuals [168]. Preponderance of evidence links *N*-Hcy-protein with the pathology of these diseases. As will be discussed in a greater detail in the following sections of this book, *N*-Hcy-protein is harmful because of its ability to induce inflammation [169], cell death [170, 171], and an autoimmune response [134, 135, 172, 173] and to interfere with blood clotting [174, 175].

2.2.5 Nε-*Homocysteinyl-Lysine*

There is no direct evidence that *N*ε-Hcy-Lys is toxic. However, similar to other Hcy metabolites, *N*ε-Hcy-Lys levels are elevated under pathological conditions, such as human CBS deficiency, renal disease, cardiovascular disease, as wells as in $Cbs^{-/-}$ and $Mthfr^{-/-}$ mice [72, 86]. Plasma *N*ε-Hcy-Lys levels are higher in mice than in humans, most likely reflecting higher Hcy-thiolactone [93] and protein *N*-linked Hcy [113] levels in mice compared with humans.

2.2.6 S-*Adenosylhomocysteine*

Experimental evidence suggests that the accumulation of AdoHcy could be harmful. For example, treatments with Hcy and adenosine deaminase inhibitors elevate intracellular AdoHcy and inhibit cell growth in cultured human endothelial cells [176]. Human uremic patients have hyperhomocysteinemia and elevated AdoHcy levels. The elevated AdoHcy exerts epigenetic effects on gene expression by interfering with cellular methylation reactions and changing DNA methylation patterns in peripheral mononuclear cells [177]. Folate therapy, a common method to reduce hyperhomocysteinemia, lowers AdoHcy, restores DNA methylation to normal levels, and corrects the patterns of gene expression. Plasma AdoHcy is highly correlated with plasma tHcy, whereas increased plasma and lymphocyte AdoHcy levels are associated with decreased DNA methylation [177, 178]. Furthermore, an increase in plasma AdoHcy is a much more sensitive indicator of human cardiovascular disease than an increase in plasma tHcy [116].

In mice, effects of elevated AdoHcy on DNA methylation appear to be tissue specific. $Cbs^{+/-}$ mice fed with a high-methionine diet develop hyperhomocysteinemia, elevated levels of AdoHcy, and lower AdoMet/AdoHcy ratios ($p < 0.001$) in the liver and brain [179]. Plasma tHcy correlates positively with AdoHcy in most tissues, except the kidney [180], and negatively with DNA methylation, as one would expect, in the liver. On the other hand, paradoxical *increases* in DNA methylation occur in the brain and the aorta of hyperhomocysteinemic animals [179].

Methylation patterns of the imprinted genes H19 and insulin-like growth factor 2 (Igf2), important for their regulation, are affected by hyperhomocysteinemia in a tissue-specific manner. For example, hyperhomocysteinemic $Cbs^{+/-}$ mice have decreased H19 methylation in the liver, whereas in the brain increased H19 methylation and decreased ratios of H19/Igf2 transcripts are observed. In the aorta, hyperhomocysteinemia produces an increase in H19 methylation and a 2.5-fold increase in expression of H19 transcripts. Levels of H19 transcripts in aorta correlate positively with plasma tHcy concentration. These findings suggest that factors other than the methylation capacity can influence DNA methylation patterns.

2.2.7 Cystathionine

There is no indication that cystathionine is toxic [20]. Although it accumulates under pathological conditions, such as cystathionase deficiency or cancer, cystathionine seems to be benign. However, the *lack* of cystathionine synthesis has been shown to be responsible for liver steatosis and kidney injury in mouse models [181].

2.2.8 Homocysteic Acid

HCA is an analog of the neurotransmitter glutamate. Because of this, HCA is a selective agonist of glutamate receptors [182]. In tissue cultures, supplementation with HCA is toxic to human [183] and animal [184] neuronal cells.

Elevation of HCA from undetectable levels to 119 ± 20 μM that occurs in cerebrospinal fluid of children after methotrexate treatment is associated with neurological toxicity [119]. Persistent cognitive deficits in rats are induced by intrathecal methotrexate treatment and are associated with elevated concentrations of HCA (and HSA) in the cerebrospinal fluid [123]. These deficits are reversed by intraperitoneal injections of dextromethorphan, a noncompetitive antagonist of the *N*-methyl-D-aspartate receptor (NMDA). These findings are consistent with a known agonist role of HCA at glutamate receptors. Overstimulation of the NMDA receptor by HCA has been suggested to contribute to the central nervous system pathology observed in patients with homocystinuria [185]. Consistent with this suggestion, HCA is present in homocystinuric patients (at ~7 μM in plasma [186] and in urine [120]). However, it is not known whether HCA accumulates in CNS of these patients.

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Homocysteine in Protein Structure/Function and Human Disease

Chemical Biology of Homocysteine-containing Proteins

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