5th Amino Acid Assessment Workshop

Pathophysiological Consequences of Homocysteine Excess

Hieronym Jakubowski

Department of Microbiology & Molecular Genetics, UMDNJ-New Jersey Medical School, International Center for Public Health, Newark, NJ 07101, and Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznań, Poland

ABSTRACT Elevated level of the nonprotein amino acid homocysteine (Hcy) is a risk factor for cardiovascular diseases, neurodegenerative diseases, and neural tube defects. However, it is not clear why excess Hcy is harmful. To explain Hcy toxicity, the “Hcy-thiolactone hypothesis” has been proposed. According to this hypothesis, metabolic conversion of Hcy to a chemically reactive metabolite, Hcy-thiolactone, catalyzed by methionyl-tRNA synthetase is the first step in a pathway that contributes to Hcy toxicity in humans. Plasma Hcy-thiolactone levels are elevated in human subjects with hyperhomocysteinemia caused by mutations in CBS or MTHFR genes. Plasma and urinary Hcy-thiolactone levels are also elevated in mice fed a high-methionine diet. Hcy-thiolactone can be detrimental because of its intrinsic ability to form N-Hcy-protein adducts, in which a carboxyl group of Hcy is N-linked to ε-amino group of a protein lysine residue. This article reviews recent studies of Hcy-thiolactone and N-Hcy-protein in the human body, including their roles in autoimmune response, cellular toxicity, and atherosclerosis. Potential utility of Hcy-thiolactone, N-Hcy-protein, or anti-N-Hcy-protein autoantibodies as markers of Hcy excess is discussed. J. Nutr. 136: 1741S–1749S, 2006.

KEY WORDS: • homocysteine-thiolactone • N-homocysteineylated protein • cellular toxicity • autoimmune response • cardiovascular disease

Early animal studies have suggested that high-protein diets can be harmful. Subsequent examinations of individual dietary amino acids have led to the conclusion that methionine, ingested in excess, is the most toxic amino acid (1, 2). For example, in female rats fed diets containing 5% methionine, there were no successful pregnancies (3), whereas animals fed high-protein or high-methionine diets for 2 y developed hyperhomocysteinemia and evidence of vascular disease (4). Excess methionine is toxic because it leads to elevation of homocysteine (Hcy) in body tissues, particularly when intake of folate, vitamin B-12, or vitamin B-6 is inadequate. Hcy is also elevated in genetic disorders of methionine metabolism, first reported in 1963 (5), which are associated with severe pathologies affecting multiple organs and lead to premature death from vascular complications (6). McCully observed advanced arterial lesions in children with inborn errors of methionine metabolism and in 1969 proposed

the hypothesis that Hcy causes vascular disease in humans (7). High-methionine or low-folate diets are often used in studies of experimental hyperhomocysteinemia and atherosclerosis in animal models (8).

The only known source of Hcy in the human body is dietary protein methionine. After meal ingestion, methionine is liberated from dietary protein in the digestive system. Free methionine is transported in the blood to body organs and is taken up by cells (Fig. 1). Inside cells, methionine is used for synthesis of new proteins and S-adenosylmethionine (AdoMet), a universal methyl donor. As a result of biological methylation reactions, AdoMet is converted to AdoHcy, which is subsequently hydrolyzed to adenosine and Hcy. Under normal circumstances, most, but not all, of the Hcy formed in transmethylation reactions is remethylated back to methionine or converted into cysteine in transsulfuration reactions.

Hcy is also metabolized to the cyclic thioester Hcy-thiolactone (9, 10). The Hcy-thiolactone pathway becomes predominant when remethylation or transsulfuration reactions are impaired by genetic alterations of enzymes involved in Hcy metabolism, such as cystathionine β-synthase (CBS), methionine synthase (MS), or methylenetetrahydrofolate reductase (MTHFR) or by inadequate supply of folate, vitamin B-12, or vitamin B-6 (Fig. 1). Hcy-thiolactone is formed by methionyl-tRNA synthetase (MetRS) in an error-editing reaction in protein biosynthesis when Hcy becomes mistakenly selected in protein biosynthesis when Hcy becomes mistakenly selected in

...
Levels of Hcy-thiolactone in the human body

Although Hcy-thiolactone has been routinely assayed in other biological systems, methods to assay Hcy-thiolactone in human body fluids, such as plasma and urine, have been developed only recently (Table 1). Our cation-exchange HPLC method (18–20) exploits unique physicochemical properties of Hcy-thiolactone to achieve its separation, identification, and fluorescence detection and quantification by postcolumn derivatization with o-phthalaldehyde (21). This method is highly selective and sensitive and has a detection limit of 0.36 nmol/L. As little as 25 fmol Hcy-thiolactone in a sample can be detected and quantified (19,20).

We found that normal human plasma levels of Hcy-thiolactone vary from 0 to 34.8 nmol/L and account for 0.002–0.3% plasma total Hcy (tHcy) (19). In 29 of the 60 human plasma samples analyzed, Hcy-thiolactone was below the detection limit. There was no correlation between plasma Hcy-thiolactone and plasma tHcy. Although somewhat surprising, this finding suggests that Hcy is not a major determinant of plasma Hcy-thiolactone in humans. Other possible determinants of plasma Hcy-thiolactone, such as folic acid, methionine, HDL-associated Hcy-thiolactonase, MetRS (or other aminoacyl-tRNA synthetases), or renal function may also be important.

Men tended to have higher plasma Hcy-thiolactone concentrations than women, but the differences were not statistically significant. There was a weak correlation between plasma Hcy-thiolactone concentration and age in men, but it was not significant. There was no correlation between Hcy-thiolactone and age in a group of women.

We found that, in contrast to tHcy, Hcy-thiolactone is efficiently eliminated by urinary excretion (20). Urinary concentrations of Hcy-thiolactone are ~100-fold higher than those found in plasma. Normal urinary Hcy-thiolactone levels vary from 11 to 485 nmol/L. Urinary Hcy-thiolactone accounts for 2.5–28% of urinary tHcy and thus contributes significantly to urinary tHcy pools. Whereas relative renal
Assay methods for the determination of Hcy-thiolactone in human body fluids

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC (cation exchange) with UV multiwavelength detection</td>
<td>(18)</td>
</tr>
<tr>
<td>HPLC (cation exchange) with postcolumn α-phthalaldehyde derivatization</td>
<td>(19,20)</td>
</tr>
<tr>
<td>HPLC (C30 reverse phase) with postcolumn α-phthalaldehyde derivatization</td>
<td>(21)</td>
</tr>
<tr>
<td>GC/MS with precolumn derivatization with heptafluorobutyric acid anhydride</td>
<td>(23)</td>
</tr>
</tbody>
</table>


clearance of tHcy is only about 0.001 – 0.003, the clearance of Hcy-thiolactone is 0.2–7.0 of creatinine clearance. This suggests that in some individuals Hcy-thiolactone is not only filtered in the glomeruli but also secreted into the tubular lumen. In contrast, high local intrarenal synthesis of Hcy-thiolactone cannot be excluded. However, this is unlikely given a significant positive correlation between urinary and plasma Hcy-thiolactone concentrations. Interestingly, urinary Hcy-thiolactone correlates negatively with urinary pH, although there is no correlation between urinary tHcy and urinary pH. This suggests that the ionization status of the α-amino group of Hcy-thiolactone (pK ≈ 7.1) affects its urinary excretion. An uncharged form of Hcy-thiolactone is excreted in the urine, where it gains a positive charge as a result of acidification. The positively charged form of Hcy-thiolactone is not reabsorbed in the tubules. Urinary acidification apparently maintains a low fractional concentration of the uncharged Hcy-thiolactone inside the tubular lumen, thus enabling continuous diffusion of the uncharged form of Hcy-thiolactone from the tubular cells into the lumen.

Although it contributes very little to the daily flux of tHcy in a healthy individual (22), renal excretion removes a large fraction of Hcy-thiolactone (20), which would otherwise cause protein N-homocysteinylatation. Our data suggest that urinary excretion is an important route of Hcy-thiolactone elimination from the human body and that intact renal function is important for Hcy-thiolactone detoxification. Urinary excretion is also consistent with a view that Hcy-thiolactone is a toxic metabolite in humans. It is likely that urinary Hcy-thiolactone can serve as a potentially useful marker of tHcy excess in the human body.

Two other Hcy-thiolactone assays have been developed, but their utility for analysis of human body fluids has not been fully demonstrated. One of these assays involves a C30 reverse-phase HPLC with postcolumn derivatization with α-phthalaldehyde followed by fluorescence detection and has a limit of detection of 200 fmol Hcy-thiolactone. So far, this assay has been used only for the determination of Hcy-thiolactone in cultures of human hepatoma Hep G2 cells (21).

Another method is based on GC/MS and involves precolumn derivatization with heptafluorobutyric anhydride. The derivative is analyzed and quantified by GC/MS using deuterated Hcy-thiolactone as internal standard. The limits of detection and quantification are 1.7 nmol/L and 5.2 nmol/L for Hcy-thiolactone, respectively. So far, plasma samples from only 2 human subjects were analyzed by this method and were found to have 18 nmol/L and 25 nmol/L Hcy-thiolactone, respectively, about 0.2% relative to plasma tHcy (23).

**CBS- and MTHFR-deficient patients have elevated plasma Hcy-thiolactone.** Plasma tHcy levels are elevated in subjects with hyperhomocysteinemia secondary to genetic deficiencies in CBS or MTHFR genes (3–9). We have found that plasma Hcy-thiolactone is also elevated in these human subjects. For example, mean Hcy-thiolactone levels in 14 CBS-deficient patients were 15 ± 30.7 nmol/L and represented up to 0.6% of plasma tHcy (H. Jakubowski, G. Boers, and G. Chwatko, unpublished data). Hcy-thiolactone levels are higher in MTHFR-deficient homozygotes (15.0 ± 21.6 nmol/L, n = 4) than in MTHFR-deficient heterozygotes (0.30 ± 0.29 nmol/L, n = 6) or control subjects (0.20 ± 0.14 nmol/L, n = 9) (H. Jakubowski, K. Strauss, and G. Chwatko, unpublished data).

**High-methionine diet leads to the elevation of Hcy-thiolactone in the mouse.** In mice fed a normal nonpurified diet, plasma concentration of Hcy-thiolactone is 3.0 nmol/L and accounts for 0.08% of plasma tHcy (Table 2). In mice, as in humans, Hcy-thiolactone is eliminated by urinary excretion. Urinary concentrations of Hcy-thiolactone in the mouse are 136 nmol/L, 37-fold higher than those found in mouse plasma. Because mice, in contrast to humans, eliminate significant amounts of tHcy in the urine, urinary Hcy-thiolactone accounts only for 0.3% of urinary tHcy in the mouse.

Feeding mice with a high-methionine diet results in elevations of Hcy-thiolactone and tHcy in body fluids (Table 2). Plasma and urinary Hcy-thiolactone are elevated 3.7- and 25.7-fold, respectively, in mice fed a high-methionine diet, compared to mice fed a normal diet. Plasma and urinary tHcy are elevated 17.3- and 30.2-fold, respectively, in mice fed a high-methionine diet compared to mice fed a normal diet (H. Jakubowski, D. Shih, and G. Chwatko, unpublished data). Thus, mice appear to eliminate excess Hcy-thiolactone and excess tHcy by urinary excretion.

**Homocysteine-thiolactone is cytotoxic.** Early evidence suggesting that Hcy-thiolactone is cytotoxic to the cardiovascular system was obtained in the 1970s, well before metabolism of

| TABLE 1 |

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC (cation exchange) with UV multiwavelength detection</td>
<td>(18)</td>
</tr>
<tr>
<td>HPLC (cation exchange) with postcolumn α-phthalaldehyde derivatization</td>
<td>(19,20)</td>
</tr>
<tr>
<td>HPLC (C30 reverse phase) with postcolumn α-phthalaldehyde derivatization</td>
<td>(21)</td>
</tr>
<tr>
<td>GC/MS with precolumn derivatization with heptafluorobutyric acid anhydride</td>
<td>(23)</td>
</tr>
</tbody>
</table>

**TABLE 2**

Urinary and plasma concentrations of Hcy-thiolactone and tHcy in the mouse

<table>
<thead>
<tr>
<th>7-wk diet, n = 4</th>
<th>Mean ± SD in urine</th>
<th>Mean ± SD in plasma</th>
<th>Ratio urine/plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hcy-thiolactone, nmol/L</td>
<td>tHcy, μmol/L</td>
<td>Hcy-thiolactone, nmol/L</td>
</tr>
<tr>
<td>Control</td>
<td>136 ± 22</td>
<td>45 ± 14</td>
<td>3.7 ± 2.1</td>
</tr>
<tr>
<td>High Met</td>
<td>3490 ± 3780</td>
<td>1360 ± 840</td>
<td>13.0 ± 4.8</td>
</tr>
</tbody>
</table>

Hcy-thiolactone in humans was deciphered and its physiological significance established. Infusions with Hcy-thiolactone have been used as an early model of clinical homocystinuria. For instance, baboons chronically infused with Hcy-thiolactone developed patchy desquamation of vascular endothelium and atherosclerosis (24). Similar vascular changes occurred in baboons in response to infusions of Hcy (25), which, as we now know, is metabolically converted to Hcy-thiolactone (9–13). However, infusions with Hcy-thiolactone failed to induce atherosclerosis in rabbits (26,27) and pigs (28). Although unexplained at that time, these differences in sensitivity to Hcy-thiolactone were most likely related to differences in Hcy-thiolactone metabolism among animal species. We now know that Hcy-thiolactone is hydrolyzed to Hcy by serum Hcy-thiolactonase/paraoxonase carried on HDL (29) and that rabbits have about 10 times higher Hcy-thiolactonase activity than an average human being does (30,31), which could explain the greater resistance of rabbits than primates to Hcy-thiolactone.

Hcy-thiolactonase activity of the intracellular enzyme bleomycin hydrolase (32,33) (Fig. 1) is also likely to vary between species and contribute to their sensitivity to Hcy-thiolactone.

Hcy-thiolactone is also known to be acutely toxic to the central nervous system in experimental animals (34–36). The toxicity of Hcy-thiolactone to the central nervous system cannot result from its metabolism to homocysteic acid, a potent neurotransmitter (37). Metabolism to homocysteic acid can occur only after the hydrolysis of Hcy-thiolactone to Hcy. However, Hcy, which is also neurotoxic to cortical cultures of mixed neurons and glia from embryonic rats, is not metabolized to homocysteic acid in these cultures (38). Other cell culture and animal studies have shown that Hcy induces cell death and potentiates amyloid β-peptide toxicity in neurons (39). As in other cellular systems, Hcy is most likely metabolized to Hcy-thiolactone also in neurons; however, this has not been examined.

Hcy-thiolactone, injected intravenously in 1 dose into mice and rats as a possible radioprotectant in studies of tumor therapy, is extremely neurotoxic (36). For example, at 200 mg/kg Hcy-thiolactone, many mice developed immediate seizures followed by death within minutes. At 350 mg/kg, all animals developed seizures and died. At doses of 100 mg/kg or below, mice developed only mild somnolence, and no long-term effects were observed within 30 d.

Exposure of mouse (40), rat (41), or chicken (42) embryos to Hcy-thiolactone (0.5 mmol/L and above) causes increased lethality, growth retardation, blister, and abnormalities of somite development. In 1 study Hcy-thiolactone was reported to be nonteratogenic in mouse embryos, but the maximum dose used in that study (43) was lower than those used in other studies (40–42), so that an embryotoxic dose has not been reached.

That the conversion of Hcy to Hcy-thiolactone is most likely responsible for Hcy toxicity to rat embryos is suggested by experiments utilizing L- and D-stereoisomers. For example, only L-Hcy, but not the D-form, was found to be toxic to rat embryos. In contrast, both L- and D-forms of Hcy-thiolactone are toxic (41). The stereospecific embryotoxicity of L-Hcy is consistent with the stereospecificity of MetRS, which converts only L-Hcy to L-Hcy-thiolactone. On the other hand, embryotoxicities of both L- and D-forms of Hcy-thiolactone are consistent with identical chemical reactivity of each stereoisomer of Hcy-thiolactone toward proteins. Toxicity of Hcy to chicken embryos (42) can also be caused by its conversion to Hcy-thiolactone.

In fact, Hcy-thiolactone, which is unlikely to be efficiently hydrolyzed to Hcy in the chicken because of lack of Hcy-thiolactonase (29,31), is also toxic to chicken embryos (42). These observations suggest that chemical reactivity toward cellular components is responsible for the toxicity of Hcy-thiolactone.

Tissue culture studies show that Hcy-thiolactone induces apoptotic death in human vascular endothelial cells (44), promyeloid HL-60 cells (45), and placental trophoblasts (46), and inhibits insulin signaling in HTC rat hepatoma cells transformed with insulin receptor (47). Apoptosis is also induced by Hcy, but at much higher concentrations (3 mmol/L) (48) than Hcy-thiolactone (0.05 mmol/L) (44). Hcy-thiolactone has also been demonstrated to be more effective than Hcy in inducing endoplasmic reticulum (ER) stress in a retinal pigmented epithelial cell line (49). Because Hcy is metabolized to Hcy-thiolactone in endothelial cell cultures (13,18), it is likely that Hcy toxicity is caused by its conversion to Hcy-thiolactone.

Toxicity has usually been observed after acute exposure to Hcy-thiolactone concentrations far exceeding the concentrations that are present in humans or rodents in vivo. Thus, it might be unclear whether the acute Hcy-thiolactone toxicity, observed within hours or days, is relevant for chronic hyperhomocysteinemia in humans, whose detrimental effects are manifested after decades of exposure. However, it is likely that the small amount of damage caused by mild elevations in Hcy-thiolactone levels could accumulate to harmful levels over the extended periods of time required for the development of atherosclerosis or Alzheimer’s disease.

**N-linked protein Hcy in human blood.** Standard Hcy assay measures only so-called “total” Hcy (tHcy), that is, Hcy present after reductive cleavage of disulfide bonds in a sample (50) (Fig. 2). Methods allowing assays of N-linked protein Hcy in plasma or individual proteins were developed only recently (Table 3). A protein sample is first processed to remove tHcy, and N-linked Hcy is then released from the sample by acid hydrolysis at elevated temperature (15). The hydrolysis is carried out under reducing conditions, which leads to the liberation of N-linked protein Hcy in the form of Hcy-thiolactone, which is then quantified by cation-exchange HPLC (15). Sensitivity of our method allows detection of as little as 0.04 mol % N-linked Hcy in a protein.

Using these methods, we found that N-linked protein Hcy constitutes a significant pool of Hcy in the human body. Human plasma levels of N-linked protein Hcy are much higher than the plasma levels of Hcy-thiolactone. This finding may not be surprising because Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinilation. The concentrations of plasma N-linked protein Hcy vary from 0.1 μmol/L to 13 μmol/L and comprise up to 25% of plasma tHcy (15). Plasma

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay methods for the determination of N-linked Hcy in human proteins</strong></td>
</tr>
</tbody>
</table>

| Acid hydrolysis under reducing conditions, extraction of liberated Hcy-thiolactone, and quantitation by cation-exchange HPLC with UV/multilength detection (15) | Derivatization with 4-fluoro-7-sulfamyl-benzofurazan (ABD-F), acid hydrolysis, extraction of liberated ABD-Hcy, and quantitation by C18 reverse-phase HPLC with fluorescence detection (51) |

Downloaded from https://academic.oup.com/jn/article-abstract/136/6/1741S/4664476 by guest on 27 March 2019
N-linked protein Hcy correlates positively with plasma tHcy, which suggests that plasma tHcy is a determinant of plasma N-Hcy-protein. In some subjects, plasma N-Hcy-protein is lower than expected from their tHcy content; this suggests that factors other than tHcy affect N-linked protein Hcy (15). A likely candidate for a determinant of plasma N-Hcy-protein is Hcy-thiolactonase/paraoxonase (29), which has been shown to affect the formation of N-Hcy-protein in cultured endothelial cells (13) and in human serum in vitro (30).

In another study, 0.51 μmol/L N-Hcy-protein has been detected in healthy adults, about 4% relative to plasma tHcy. Hemodialysis patients had 0.74 μmol/L N-Hcy-protein, about 2% relative to plasma tHcy (51).

N-Linked Hcy is also present in plasma protein from mouse, rat, or chicken serum and in albumins isolated from other animals such as rabbit, pig, and sheep (15).

In vitro, Hcy thiolactone can modify essentially all plasma proteins, as well as other proteins that have been examined, at rates proportional to protein’s concentration and lysine content (14). Thus, one can expect to find many proteins containing N-linked Hcy in the human body. Indeed, we found that each human blood protein examined, such as hemoglobin, albumin, γ-globulin, fibrinogen, LDL, HDL, transferrin, and antitrypsin, contains from 0.04 to 0.6 mol % of N-linked Hcy (15). The levels of N-linked Hcy present in individual human blood proteins are roughly proportional to the proteins’ abundance. Thus, most of N-linked Hcy is carried on hemoglobin (75%), albumin (22%), and γ-globulin (2%) in the human blood. All other blood proteins contain about 1% of N-linked Hcy. In human plasma, most of N-linked Hcy, 90%, is carried on albumin. The absence of N-linked Hcy in transferrin reported by Sass et al. (52) is most likely a result of inadequate sensitivity of their methods.

On the basis of the normal blood concentrations of hemoglobin, the concentration of N-linked Hcy carried on hemoglobin is 12.7 μmol/L (15), which is greater than the concentration of tHcy (50) (Fig. 2). The concentration of N-linked protein Hcy carried on albumin in plasma is about 2.8 μmol/L, which is 25% relative to plasma tHcy (15). We estimate the concentration of N-linked protein Hcy in normal human blood to be about 16 μmol/L. In other words, most (about 70%) of the Hcy present in human blood circulates in the form of N-linked protein Hcy, and about 30% circulates as tHcy (Fig. 2).

N-Homocysteinylolation by Hcy-thiolactone causes protein damage. The substitution of the ε-amino group of a protein lysine residue with an Hcy residue containing a free thiol group is expected to affect protein structure and function. The immediate result of protein N-homocysteinylolation is a decrease of the net positive charge on a protein because the highly basic ε-amino group of a protein lysine residue (pK_a = 10.5) is replaced by a less basic α-amino group of N-linked Hcy (estimated pK_a ~ 7). Protein N-homocysteinylolation leads to secondary structural changes. For example, after incorporation of just one N-linked Hcy/mol protein, N-Hcy-cytochrome c becomes prone to aggregation as a result of intermolecular disulfide bond formation (14), whereas N-Hcy-hemoglobin (9) and N-Hcy-albumin (16), in contrast to corresponding unmodified proteins, are susceptible to further irreversible damage by oxidation. N-homocysteinylolation can affect protein’s susceptibility to proteolysis, as demonstrated for N-Hcy-albumin (16).

The most detailed studies of structural alterations caused by N-homocysteinylolation have been carried out with human serum albumin (16), a known target for N-homocysteinylolation in the human body (15). These studies have led to the discovery of a novel molecular form of albumin and provided a paradigm illustrating how the function of a protein thiol can be affected by N-homocysteinylolation. Of the 2 major physiological forms of human albumin (Fig. 3), albumin-Cys^34-S-S-Cys (containing cysteine in a disulfide linkage with Cys^34 of albumin) is N-homocysteinylolated faster than albumin-Cys^34-SH (mercaptoprotein, containing Cys^34 with a free thiol). The reactivity of Lys^325, a predominant site of N-homocysteinylolation, is about 2-fold greater in albumin-Cys^34-S-S-Cys than in mercaptoprotein. These observations are consistent with a structural transition in albumin that is known to occur depending on the status of the Cys^34 residue (53). N-Homocysteinylolation of albumin-Cys^34-S-S-Cys and albumin-Cys^34-SH yield 2 different primary products, N-(Hcy-SH)-albumin-Cys^34-SH and N-(Hcy-SH)-albumin-Cys^34-S-S-Cys, respectively (Fig. 3). However, subsequent thiol-disulfide exchange reactions result in the formation of a single product, N-(Hcy-S-S-Cys)-albumin-Cys^34-SH (Fig. 3), which is more sensitive to proteolysis than N-homocysteinylolated mercaptoprotein, N-(Hcy-SH)-albumin-Cys^34-SH. Among many possible sites in albumin, Lys^325 is a predominant site of N-homocysteinylolation in vitro and in vivo. Taken together, our data identify a novel form of albumin, N-(Hcy-S-S-Cys)-albumin-Cys^34-SH, and suggest that a disulfide at Cys^34, a conserved residue in albumins from various organisms, promotes conversion of N-(Hcy-SH)-albumin-Cys^34-SH to a proteolytically sensitive form, N-(Hcy-S-S-Cys)-albumin-Cys^34-SH, which would facilitate clearance of the N-homocysteinylolated form of mercaptoprotein. Our data also suggest that N-homocysteinylolation interferes with the structural transition in albumin dependent on the status of the conserved Cys^34 residue (Fig. 3).

Fibrinogen is known to undergo facile N-homocysteinylolation by Hcy-thiolactone in vitro (14). The presence of small amounts of N-linked Hcy in native human fibrinogen suggests that it is a target for the modification by Hcy-thiolactone in the human body (15). Clots formed from Hcy-thiolactone-treated
normal human plasma lyse more slowly than clots from untreated control plasma, and the magnitude of this effect depends on the concentrations of Hcy-thiolactone used (54). Purified human fibrinogen modified with Hcy-thiolactone forms clots with characteristics similar to fibrinogen from hyperhomocysteinemic rabbits. Clots formed from N-Hcy-fibrinogen are more resistant to lysis than control clots from native fibrinogen. Mass spectrometric analysis of N-Hcy-fibrinogen reveals the presence of 10 different N\(^{\text{Hcy}}\)-Lys residues in the D and E domains. Some of these residues are close to tPA and plasminogen binding or plasmin cleavage sites, which can explain abnormal clot characteristics. These results suggest that N-homocysteinylated fibrinogen can lead to abnormal resistance of fibrin clots to lysis and contribute to increased risk of cardiovascular disease in hyperhomocysteinemia (14,54).

Incorporation of multiple N-linked Hcy residues has been shown to be detrimental to the function of other proteins. For example, complete loss of enzymatic activity occurs after N-homocysteinylated of 8 lysine residues in MetRS (33% of total lysine residues) or 11 lysine residues in tryptophan (88% of total lysine residues) (14). Extensively N-homocysteinylated proteins, such as myoglobin, transferrin, globulins, fibrinogen, RNase A, and trypsin are prone to multimerization and undergo gross structural changes that lead to their denaturation and precipitation (14). Chicken egg lysozyme is also denatured by extensive N-homocysteinylation (55).

N-Homocysteinylation may also be detrimental to the normal function of LDL. For example, N-homocysteinylated LDL, in which 10% or 25% of lysine residues have been modified (i.e., containing 36 and 89 mol N-linked Hcy/mol LDL), is taken up and degraded by human monocyte-derived macrophages significantly faster than native LDL (56). However, less extensively N-homocysteinylated LDL (8 molecules of N-linked Hcy/mol LDL) is taken up and degraded by leukemic L2C guinea pig lymphocytes cells in vitro to the same extent as native LDL via the high-affinity LDL-specific receptor pathway (57). It has been suggested that additional thiol groups present in N-Hcy-LDL may protect LDL lipids against oxidation (58).

Hcy-thiolactone also inactivates enzymes by other mechanisms. For example, lysine oxidase, an important enzyme responsible for posttranslational collagen modification essential for the biogenesis of connective tissue matrices, is inactivated by Hcy-thiolactone, which derivatizes the active site tyrosine-quinone cofactor with a half-life of 4 min (59). The inactivation of lysine oxidase by Hcy-thiolactone might play a role in skeletal abnormalities seen in homocystinuric children (6). Hcy-thiolactone has also been shown to decrease enzymatic activity of lysine oxidase in cultured porcine aortic endothelial cells (60). These observations can account for the reduced number of collagen cross-links observed in patients with homocysteinuria (61). Inactivation of the lysine oxidase gene in a mouse model leads to aortic aneurisms, cardiovascular dysfunction, and premature death (62).

**Pathophysiologival responses to protein N-homocysteinylatation:** Anti-N-Hcy-Lys-protein autoantibodies. Injections of rabbits with proteins, such as rabbit LDL (63) or keyhole limpet hemocyanine (64,65) modified with Hcy-thiolactone, induce essentially identical immune responses in these animals, suggesting that any N-Hcy-protein can be immunogenic. Because endogenous N-Hcy-proteins are present in the human body (9,10,15–17,51), we hypothesized that they would be recognized as neo-self antigens and induce an autoimmune response. Indeed, we found that each human serum tested showed some titer of IgG (64) and IgM (J. Perla, T. Twardowski, H. Jakubowski, unpublished data) autoantibodies against N\(^{\text{Hcy}}\)-hemoglobin or N\(^{\text{Hcy}}\)-albumin. The antigen specificity tests using structural analogs of the N\(^{\text{Hcy}}\)-Lys epitope as competitors and a variety of N-Hcy-proteins as antigens show that the human IgG autoantibody recognizes the N\(^{\text{Hcy}}\)-Lys epitope on Hcy-thiolactone-modified proteins. These specificity tests suggest that any protein containing N-linked Hcy is likely to be autoimmunogenic in humans. Consistent with this suggestion is our finding that an antibody raised against N-Hcy-keyhole limpet hemocyanine in rabbits has antigen specificity identical to that of human anti-N-Hcy-protein autoantibody (64,65). The high specificity of the human autoantibody is illustrated by our findings that N\(^{\text{Hcy}}\)-N\(^{\text{acetyl}}\)-Lys competes with the human IgG binding to an N\(^{\text{Hcy}}\)-Lys-protein antigen, whereas structural analogs, including N\(^{\text{Hcy}}\)-acetyl-N\(^{\text{acetyl}}\)-Hcy-Lys in which Hcy is attached to the α-amino (instead of the ε-amino) group of lysine, do not compete. We also found that serum levels of anti-N\(^{\text{Hcy}}\)-Lys-protein IgG significantly correlate with plasma tHcy levels (Fig. 4); however, there was no correlation with plasma cysteine (Fig. 4) or methionine levels (64). These findings support a mechanistic link between Hcy and the anti-N\(^{\text{Hcy}}\)-Lys-protein IgG: Hcy is first metabolized to Hcy-thiolactone, which subsequently converts into N\(^{\text{Hcy}}\)-Lys-protein, which in turn induces an autoimmune response (Fig. 5).

An important question is whether the levels of anti-N\(^{\text{Hcy}}\)-Lys-protein IgG are associated with atherosclerosis. We examined this question with groups of stroke patients, patients with coronary artery disease (CAD), and corresponding controls. We found that male stroke patients have significantly higher serum levels of anti-N\(^{\text{Hcy}}\)-Lys-protein IgG than healthy male controls (64). Higher levels of anti-N\(^{\text{Hcy}}\)-Lys-protein autoantibodies most likely reflect higher levels of tHcy observed in these patients. Plasma levels of tHcy and anti-N\(^{\text{Hcy}}\)-Lys-protein IgG autoantibody in female stroke patients were similar to corresponding levels in female controls. There were no differences in

**FIGURE 4** In healthy human subjects (n = 70), serum levels of anti-N\(^{\text{Hcy}}\)-Lys-protein IgG are positively correlated with plasma tHcy levels (r = 0.50, P < 0.001) (A). There is no correlation between anti-N\(^{\text{Hcy}}\)-Lys-protein IgG and cysteine (r = 0.14) (B). Data from (64).
The Hcy-thiolactone hypothesis: possible mechanism underlying the involvement of Hcy in atherosclerosis.

plasma cysteine or methionine concentrations between stroke patients and controls, both for men and women.

We also found that male patients with angiographically documented CAD have higher serum levels of anti-\( N^\text{e}\)-Hcy-Lys-protein IgG and tHcy than healthy male controls (66). Although the levels of anti-\( N^\text{e}\)-Hcy-Lys-protein IgG were not associated with traditional risk factors, there was a weak positive correlation between the autoantibodies and plasma tHcy. Our findings that the levels of anti-\( N^\text{e}\)-Hcy-Lys-protein IgG are elevated in CAD and stroke patients, compared to controls, suggest that the enhanced formation of these antibodies is a common feature of atherosclerosis (Fig. 5). Induction of autoimmune response explains why relatively small amounts of \( N \)-Hcy protein occurring in the human body can be detrimental. Our findings also suggest that anti-\( N^\text{e}\)-Hcy-Lys-protein IgG autoantibodies may be a useful marker of Hcy excess in the human body.

**Cytotoxicity of \( N\)-Hcy-proteins.** The deposition in tissue of protein aggregates is associated with human degenerative conditions, including Alzheimer’s disease, light-chain amyloidosis, and the spongiform encephalopathies. These aggregates are inherently cytotoxic. However, even aggregates of proteins not associated with disease can be inherently cytotoxic (67). Trace amounts of aggregates of a variety of proteins might occur spontaneously or as a result of protein modification, particularly during aging, and could account for subtle impairments of cellular function even in the absence of an evident amyloid phenotype. Thus, the observations that \( N \)-Hcy-proteins occur in the human body (9,10,15–17,51) and tend to form aggregates in vitro (14,16) raises an interesting question of whether such aggregates can be cytotoxic. Answers to this question are beginning to emerge. For example, small amounts of \( N \)-linked Hcy, known to be present in human LDL (15), may be cytotoxic, as the results of Ferretti et al. suggest (68). Ferretti et al. demonstrated that N-Hcy-LDL (prepared in vitro) causes toxicity in endothelial cells (68). They also showed that cell viability is negatively correlated with the extent of LDL \( N \)-homocysteinylation and the levels of hydroxyperoxides, suggesting that oxidative damage is involved. Other studies have shown that endothelial cells have the ability to synthesize Hcy-thiolactone and \( N \)-Hcy-proteins (9,10,13,18) and that Hcy-thiolactone is toxic to endothelial cells (44). However, the Ferretti et al. study (68) is the first to demonstrate the toxicity of \( N \)-Hcy-LDL to endothelial cells. The mechanism underlying \( N \)-Hcy-LDL toxicity may involve a decrease in endothelial \( Na^+\)-\( K^+\)-ATPase activity, leading to an overload with sodium and, subsequently, with calcium. This in turn causes reduced production of nitric oxide and generation of peroxynitrate, a highly reactive nitrogen metabolite (69). Taken together, these observations suggest that protein \( N \)-homocysteinylation may contribute to endothelial dysfunction, a key event initiating the development of atherosclerotic plaque. An important question remaining to be answered is whether other \( N \)-Hcy-proteins present in human circulation (15) are cytotoxic.

**Does \( N \)-homocysteinylation cause ER stress?** A possible mechanism contributing to vascular injury by Hcy involves endoplasmic reticulum (ER) stress and activation of the unfolded protein response (8,48,49,70). Exactly how Hcy causes protein unfolding is not clear. One possibility is that Hcy is metabolized to Hcy-thiolactone, which then causes protein \( N \)-homocysteinylation in the ER, leading to damage to secretory proteins. Hcy itself could participate in disulfide exchange reactions with ER proteins. Those 2 reactions could lead to the formation of misfolded proteins such as thrombomodulin and von Willebrand factor (8). ER stress is manifested by dysregulation of lipid metabolism, activation of inflammatory pathways, impaired insulin signaling, and possibly cell death. Both Hcy-thiolactone and \( N \)-Hcy-proteins are known to be formed in human cells, and the magnitude of their synthesis depends on the concentration of Hcy (9,12,13,18,27). Hcy-thiolactone has been demonstrated to be more effective than Hcy in inducing ER stress (49) and apoptosis (44) in human cells, suggesting that \( N \)-homocysteinylation may be a predominant pathway contributing to protein misfolding in the ER.

**Conclusions**

At the nutritional level, there is little doubt that excess Hcy is a direct consequence of excess intake of protein methionine. The condition of excess Hcy can be exacerbated by inadequate intake of folic acid, vitamin B-12, and vitamin B-6 as well as by the allelic variation in genes encoding enzymes participating in Hcy metabolism. There is also little doubt that excess Hcy is harmful to the human body. However, there is an ongoing debate as to why excess Hcy is harmful. Given that excess Hcy is detrimental to most organs in the human body, one has to conclude that fundamental processes, common to all organs, are affected by excess Hcy. Data from our laboratory, in conjunction with the work of other investigators, suggest that metabolic conversion of Hcy to Hcy-thiolactone followed by subsequent spontaneous protein \( N \)-homocysteinylation by Hcy-thiolactone may contribute to Hcy toxicity in humans (Fig. 5). Pathophysiological effects of protein \( N \)-homocysteinylation recognized so far include immune activation and cellular toxicity, possibly through ER stress, activation of unfolded protein response, and enhanced protein degradation. Induction of an autoimmune response explains why relatively small amounts of \( N \)-Hcy protein occurring in the human body can be detrimental. Molecular participants in these pathways, such as Hcy-thiolactone, specific \( N \)-Hcy-proteins, and/or anti-\( N \)-Hcy-protein autoantibodies may provide useful markers of Hcy excess in the human body.

**LITERATURE CITED**


