Mechanisms of Homocysteine Toxicity on Connective Tissues: Implications for the Morbidity of Aging

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ABSTRACT It is proposed that chronic moderate hyperhomocysteinemia has a causal role in a number of common diseases of late life, including occlusive vascular disease, cognitive decline, senile osteoporosis and presbyopia. These diseases are seen as clinical counterparts of the main manifestations of homocystinuria (vascular occlusions of arteries and veins, mental retardation, osteoporosis and ectopia lentis, respectively) that develop only after many years of exposure to moderately elevated homocysteine (Hcy) levels. The multisystem toxicity of Hcy is attributed to its spontaneous chemical reaction with many biologically important molecules, primarily proteins. The formation of these Hcy-adducts is dependent on time and Hcy concentration and leads to loss or diminution of function of the derivatized molecules. Irreversible homocysteinylination of long-lived proteins should lead to cumulative damage and progressive clinical manifestations. Fibrillin 1 is seen as the paradigm of extracellular connective tissue proteins that are specially susceptible to Hcy (and presumably Hcy thiolactone) attack. The prominent presence of epidermal growth factor (EGF)-like domains in fibrillin and in many other extracellular proteins of the coagulation, anticoagulation, and lipoprotein transport pathways, all of which malfunction in hyperhomocysteinemia, suggests that EGF-like domains may be preferential sites of homocysteinylination. J. Nutr. 130: 365S–368S, 2000.

KEY WORDS: homocysteine  homocysteine thiolactone  protein homocysteinylination  aging  fibrillin-1  EGF-like domains

Under normal conditions, the mean fasting concentration of total homocysteine in plasma (tHcy) is \( \sim 10 \) \( \mu \text{mol/L} \) (Refsum et al. 1998). Higher levels define hyperhomocysteinemia which, if severe (>10 times normal) as in the rare inborn error of metabolism homocystinuria, is accompanied by a plethora of serious clinical manifestations occurring early in life. Primarily affected are the eye and three organ systems, i.e., the skeletal, vascular and central nervous systems. Severe myopia and dislocation of the lens, osteoporosis together with skeletal malformations, often lethal thromboembolic vascular occlusions of arteries and veins, and mental impairment develop in childhood or young adulthood (Mudd et al. 1995). A large body of epidemiologic evidence now supports the notion that similar clinical counterparts develop many years later in normal individuals as part of the aging process.

Moderate hyperhomocysteinemia (tHcy up to \( 30 \) \( \mu \text{mol/L} \)) (Kang et al. 1992), a common condition, is a major independent risk factor for a number of diseases characteristic of old age, primarily occlusive vascular disease (coronary, cerebral and peripheral) (Boushey et al. 1995) cognitive decline, including Alzheimer's disease (Clarke et al. 1998), and possibly, senile osteoporosis (Miyao et al. 1998) and presbyopia (Krumdieck, unpublished). These disorders, which together account for much of the morbidity and mortality in the aged, are strikingly similar in all aspects but time of onset, to the main manifestations of homocystinuria and could be considered as the clinical signs of a single disorder of late life, i.e., chronic moderate hyperhomocysteinemia. The contribution of chronic moderate hyperhomocysteinemia to diseases of old age may have gone unrecognized because we are conditioned to accept them as inescapable consequences of growing old. The possibility of lessening the effect of hyperhomocysteinemia as a determinant of premature aging by appropriate nutritional interventions known to lower tHcy, i.e., supplemental folate, vitamins B-6 and B-12, and reducing the intake of methionine, could change this perception.

The role of Hcy in aging is supported by the experimental studies of Orentreich et al. (1993) and Richie et al. (1994) on the extension of life span in rats brought about by diets severely restricted in methionine and devoid of cysteine. A 42% increase in mean life span and a 44% increase in maximum life span was observed in these rats compared with controls. Under these dietary conditions, the only route to cysteine synthesis in mammals, the transulfuration of Hcy and serine through the cystathionine \( \beta \) synthase reaction, and the reactions of regeneration of methionine by remethylation of...
Hcy catalyzed by methionine synthase and by betaine-homocysteine methyltransferase, would be completely de-repressed (Mudd et al. 1995). All Hcy formed in these rats must have been converted immediately to cysteine or remethylated to methionine, resulting in extremely low levels of tHcy in circulation. Unfortunately, tHcy was not reported and the authors did not discuss the possible contribution of the lowering of tHcy to the observed effects on life span.

As aptly put by Mudd et al. (1995), "... no aspect... has remained so obscure as the steps by which hyperhomocysteinemia leads to the clinical manifestations associated with it." No single mechanism that by itself explains the multisystem toxicity of hyperhomocysteinemia has been proposed, maybe because no single mechanism can. Ironically, it may be that its baffling multiplicity of actions contains a valuable hint to the mechanism of Hcy toxicity. That is, Hcy reacting with proteins by one or a few reactions, may damage the components of many metabolic pathways, thus acquiring pleiotropic toxicity.

Homocysteine, a short-lived highly reactive thiol-containing amino acid formed as an obligatory intermediate in the metabolism of methionine, belongs to the most chemically active group of compounds found in the animal organism (Jocelyn 1972). It is capable of reacting specifically, and often quantitatively, with a number of thiol-containing groups, many of which are present in proteins and other biologically important molecules. In addition, Hcy cyclizes readily with the formation of Hcy thiolactone (Hcytl), an "activated" intramolecular thioester with its own unique repertoire of chemical reactions (Dudman et al. 1991). It has been proposed that the harmful effects of Hcy and Hcytl are due to their spontaneous (i.e., nonenzymatic) chemical reactions with, and inactivation of proteins and other biologically important molecules. Free Hcy readily adds to free, solvent-accessible, cysteinyl residues in proteins (thiol-thiol interaction) and can cleave solvent-accessible disulfide bridges (thiol-disulfide exchange) with damage to the folding pattern of the protein. Homocysteine thiolactone, whose long-questoned formation in vivo has now been elegantly demonstrated by Jakubowski (1997 and 2000), reacts through its carboxyl group with the ε-NH₂ group of lysyl residues with the irreversible formation of homocystamide derivatives, as first shown more than 40 years ago by Benesch and Benesch (1956). All of these reactions of protein homocysteinylation can lead to loss or degradation of the biological function of multiple enzymes, receptors, growth factors and structural proteins and can be envisioned as analogous to protein glycation, the reaction of proteins with glucose in protracted hyperglycemia, believed to cause many of the complications of diabetes (Brownlee 1992). It should be noted that protein homocysteinylation occurs normally in vivo at physiological concentrations of tHcy, as first shown for albumin by Kang et al. (1979) and recently by Hajjar et al. (1998) and Ling and Hajjar (2000) who also demonstrated a concomitant loss of biological activity of the derivatized protein, annexin II.

As is true for any other chemical reaction, the extent of formation of Hcy and Hcytl protein derivatives is dependent on time and concentration. The longer the duration of exposure and the higher the concentrations of Hcy or Hcytl, the greater the biochemical damage inflicted. Furthermore, if the molecules attacked are long-lived and the derivation reactions irreversible (i.e., formation of homocystamide), the harmful effects will be cumulative and the clinical consequences progressive.

It is of fundamental importance to investigate which proteins are particularly susceptible to Hcy and Hcytl attack. A good criterion to select likely targets for homocysteinylation is to focus on proteins found in structures singularly affected in homocystinuria. One such structure is the ciliary zonule (the suspensory ligament of the lens), a small anatomical structure which, if damaged, results in dislocation of the lens. This rare clinical manifestation occurs mainly in homocystinuria and in Marfan's syndrome (Pyeritz 1993), a disorder of connective tissue which, aside from lens dislocation, has skeletal and cardiovascular abnormalities similar to those of hyperhomocysteinemia. The zonule is formed solely by elastic microfibrils (Cleary and Gibson 1996), which forces the conclusion, verified by histopathologic demonstration of fraying and disruption of the zonula fibers (Mudd et al. 1995), that it is these fibers and hence their constituent proteins that must be damaged in both homocystinuria and Marfan's syndrome. Focusing on one zonular protein was made possible by the recent identification of fibrillin-1 as the main component of elastic microfibrils (Sakai et al. 1991). Fibrillin-1, a large rod-like glycoprotein with an exceptionally high cysteine content (~14%) much of which (~33%) appears in the free reactive sulphydryl form, is also found in the medial layer of all elastic arteries, in the heart, bone, periostium, cartilage, skin and lung, all structures that are compromised in both Marfan's syndrome and homocystinuria. Recently, mutations in the fibrillin-1 gene have been shown to cause Marfan's syndrome (Dietz et al. 1991, Lee et al. 1991), supporting the assumption that fibrillin-1 is an important target of homocysteinylation and that the resulting post-translational damage to its structure is responsible for many of the abnormalities that severe hyperhomocysteinemia has in common with Marfan's syndrome.

Fibrillin-1 consists largely of 56 cysteine-rich imperfect repeat domains, 47 of which show significant homology to a motif originally found in epidermal growth factor (EGF), the "EGF-like" repeats (Cleary and Gibson 1996). These repeats are characterized by six predictably spaced cysteine residues that interact to form three highly critical disulfide bonds. Not conserved hydrophobic residues are required to maintain the EGF-like domain fold; it is stabilized by the three disulfide bonds, which form in a 1–3, 2–4, 5–6 pattern (Bork et al. 1996). The biological significance of these cysteine residues is demonstrated by the fact that many of the mutations causing Marfan's syndrome substitute one of the highly conserved cysteinyl residues, add a new one, or alter their relative spacing. Many of the EGF-like domains of fibrillin-1 contain a consensus for Ca²⁺ binding (cb), a property that may contribute to stabilize the disulfide bonds of these cb-EGF-like modules. Free homocysteine may react with some of the numerous free cysteinyl sulphydryl (SH) groups in fibrillin-1 or may disrupt critical Cys-Cys disulfide bridges in EGF-like domains with the formation of protein mixed disulfides via thiol-disulfide exchange reactions.

Fibrillin-1 may also be irreversibly homocysteinylated at lysyl residues, many of which are concentrated in a long (17,587-Da) region at the carboxyl end of the protein, which is conspicuously lacking in cysteinyl residues. This region may be involved in intermolecular fibrillin aggregation. Its homocysteinylation may therefore impede the formation of microfibrils.

Alternatively, the insertion of an amide-linked homocysteine introduces a new free SH group that can react with an adjacent disulfide bridge in an intramolecular thiol-disulfide exchange reaction. A native disulfide bridge could be cleaved and a mixed disulfide bridge formed between one of the cysteinyl residues and the aberrant Hcy-lysyl-amide side chain. A similar reaction involving a free cysteine SH group has been described in albumin (Jocelyn 1972 and references therein).
Dissulfide cleavage, with the major disruption in folding it brings about, may thus result from the introduction of an amide-linked homocysteinyl residue.

It is worth noting that fibrillin-1 is a long-lived protein, which in some locations, such as the ciliary zonule, may be as old as the individual. In addition, the zonule, bathed by the essentially albumin-free aqueous humor, may be devoid of the protection against homocysteinylation reactions conferred in other sites by the presence of albumin, which, with its highly reactive free cysteine 34 and its high proportion of lysyl residues, may act as an efficient scavenger of both free Hcy and Hcyt.

Further support for the proposed mechanism of protein damage involving cleavage of disulfide bridges is provided by the similarity of the clinical manifestations of severe hyperhomocysteinemia with those of Sulfite Oxidase and Molybdenum Cofactor deficiencies. In these two rare disorders, the sulfite anion (SO3H) accumulates and reacts with cysteine to form sulfocysteine. As in severe hyperhomocysteinemia, ectopia lentis, skeletal malformations and vascular occlusions develop early in life (Johnson and Wadman 1989). Accumulation of either homocysteine or SO3H, both of which react avidly with thiols and cleave disulfides bridges by thiol-disulfide exchange and sulfitolysis, respectively, produces similar symptoms and discloses commonality of pathogenetic mechanisms.

The hypothesis that the EGF-like domains of fibrillin-1 are specially vulnerable sites of homocysteinylation suggests that other extracellular proteins with similar regions could also be particularly susceptible to Hcy attack. The presence of EGF-like domains in many proteins involved in the pathways of coagulation (factors V, VII, IX, X, thrombin receptor, thrombin activatable fibrinolysis inhibitor), anticoagulation (protein C, thrombomodulin, protein S, antithrombin III), thrombolysis (plasminogen activator) and lipoprotein translocation (several lipoprotein receptor molecules) is highly significant because thrombophila and premature arteriosclerosis are prominent manifestations of hyperhomocysteinemia. Of particular importance the protein C/thrombomodulin/protein S system is made up of proteins that contain EGF-like modules. Their susceptibility to inactivation by Hcy was demonstrated in vitro as far back as 1991 (Lentz and Sadler 1991). These authors showed that free Hcy, but not homocysteine, irreversibly destroys the biological activity of both thrombomodulin and protein C, preventing the activation of the latter by thrombomodulin-modified thrombin. The normal degradation of activated coagulation factors Va and VIIIa by activated protein C, which prevents the catastrophic progression of the coagulation cascade, would fail to occur, leading to thrombus formation (Dahlback 1995). Several other anti- and procoagulant proteins such as antithrombin III, plasminogen activator, thrombin receptor and Hageman factor (the latter activated by Hcy) also contain EGF-like domains and hence are suspected homocysteinylation targets.

It is of great interest that the amino-terminal region of the LDL receptor (LDLR) consists of seven tandem repeated cysteine-rich modules of ~40 amino acids (the LDL-A modules) each of which contains six cysteine residues disulfide-bonded in a manner similar to the EGF-like domains (Brown et al. 1997, Fass et al. 1997). Reduction of these disulfides destroys the structure and abolishes binding of the LDL particle to the receptor (Brown et al. 1997). Genetic mutations leading to this condition cause familial hypercholesterolemia with its accompanying arteriosclerosis. The structure of the LDL-A modules is stabilized not only by the disulfide bridges but also by a single atom of Ca2+ contained in an octahedral cage. In this manner the LDL-A modules resemble also the calcium-binding EGF-like domains of the fibrillins. The important notion is that homocysteinylation of LDLR may lead to inactivation of the receptor and provide an explanation for the propensity to develop arteriosclerosis in hyperhomocysteinemia.

It is important to also consider the effect that different amino acid sequences will have on the susceptibility to Hcy or Hcyt attack of different proteins. It can be expected that some EGF-like domains will be more susceptible to attack than others. Genetic polymorphisms of EGF-like domains or other regions of susceptible proteins will determine their liability to attack and help explain the association between risk of pathology and a wide range of Hcy levels without a clear cut-off point below which there is no increased risk (Verhoeft et al. 1997).

In addition to protein homocysteinylation, Hcy modifies the release or activity of small molecules of endothelial origin, primarily nitric oxide, that have pronounced vasoactive effects in response to blood flow (Bellamy and McDowell 1997, McDowell and Lang 2000) or that can modulate cell proliferation and differentiation in blood vessels (Dalton et al. 1997). Oxidative damage produced by homocysteine-mediated generation of free radicals is yet another possible mechanism of Hcy toxicity. None are mutually exclusive and, indeed, all may be involved simultaneously.

Finally, a word of caution regarding the methodologies for determination of homocysteine status. Current procedures for measuring tHcy rely on the assumption that all forms of protein-bound Hcy in circulation are liberated after reduction. This is incorrect; amide-linked Hcy is completely stable to reductive cleavage, and no method for the routine quantitation of this adduct in plasma or in tissues is currently available. Given the potential pathologic significance of protein homocysteinylation at lysyl residues discussed before and exemplified by the irreversible inhibition of lysyl oxidase by Hcyt (Liu et al. 1997), every effort should be directed to the development of the required methodologies.

**LITERATURE CITED**


