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Forum Review

Molecular Targeting of Proteins by L-Homocysteine: Mechanistic Implications for Vascular Disease

ALLA V. GLUSHCHENKO[1] and DONALD W. JACOBSEN[1,2]

ABSTRACT

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease, complications of pregnancy, cognitive impairment, and osteoporosis. That elevated homocysteine leads to vascular dysfunction may be the linking factor between these apparently unrelated pathologies. Although a growing body of evidence suggests that homocysteine plays a causal role in atherogenesis, specific mechanisms to explain the underlying pathogenesis have remained elusive. This review focuses on chemistry unique to the homocysteine molecule to explain its inherent cytotoxicity. Thus, the high pKa of the sulfhydryl group (pKa, 10.0) of homocysteine underlies its ability to form stable disulfide bonds with protein cysteine residues, and in the process, alters or impairs the function of the protein. Studies in this laboratory have identified albumin, fibronectin, transthyretin, and metallothionein as targets for homocysteinylation. In the case of albumin, the mechanism of targeting has been elucidated. Homocysteinylation of the cysteine residues of fibronectin impairs its ability to bind to fibrin. Homocysteinylation of the cysteine residues of metallothionein disrupts zinc binding by the protein and abrogates inherent superoxide dismutase activity. Thus, *S*-homocysteinylation of protein cysteine residues may explain mechanistically the cytotoxicity of elevated L-homocysteine. *Antioxid. Redox Signal.* 9, 1883–1898.

INTRODUCTION

It is well accepted that hyperhomocysteinemia (elevated blood levels of homocysteine) increases the risk of coronary artery disease, cerebrovascular disease and peripheral vascular occlusive disease (13, 91). Elevated homocysteine is also associated with Alzheimer's disease and other disorders of cognitive function (18, 114). Furthermore, hyperhomocysteinemia is an emerging risk factor for neural tube defects and other complications of pregnancy (20, 126). Recently, hyperhomocysteinemia has been identified as a risk factor for osteoporosis and hip fracture (81, 125). High homocysteine is a strong predictor of cardiovascular events and is a risk factor for mortality in patients with coronary artery disease (90), peripheral artery disease (118), renal failure (8), venous thromboembolism (21), and diabetes (116).

Homocysteine is continuously formed in the ubiquitous methionine cycle by the hydrolysis of *S*-adenosylhomocysteine, which is the product of *S*-adenosylmethionine–dependent methyltransferase activity (23). Remethylation of homocysteine back to methionine completes the cycle. This reaction is catalyzed by cobalamin-dependent methionine synthase (E.C. 2.1.1.13), which uses N^5-methyltetrahydrofolate as co-substrate. In liver and kidney, betaine can serve as the methyl donor for the remethylation of homocysteine using the enzyme betaine-homocysteine methyltransferase (E.C. 2.1.1.5). Alternatively, homocysteine, a branch-point metabolite, is converted to cystathionine in a β-condensation reaction with serine cat-

[1]Department of Cell Biology, Lerner Research Institute, Cleveland Clinic; and [2]Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, Ohio.

alyzed by pyridoxal phosphate-dependent cystathionine β-synthase (E.C. 4.2.1.22). Cystathionine is then converted to cysteine and α-ketobutyrate by the pyridoxal phosphate-dependent enzyme γ-cystathionase (E.C. 4.4.1.1). The distribution of this two-enzyme transsulfuration pathway is largely limited to liver, kidney, and possibly brain. In liver and kidney, ~50% of intracellular homocysteine is used for the production of cysteine (23). The adult cardiovascular system does not express cystathionine β-synthase and is dependent on cysteine delivered *via* the circulation (15). Hyperhomocysteinemia is caused by deficiencies or impaired activities of the enzymes that metabolize homocysteine as a result of genetic mutations. If the affected individual is homozygous for mutations in cystathionine β-synthase, methionine synthase, or methylenetetrahydrofolate reductase (E.C. 1.5.1.20), a serious disease known as homocystinuria occurs, and patients excrete large quantities of homocystine in the urine. Patients with homocystinuria often have total plasma homocysteine levels approaching 500 μM (normal concentrations range between 5 and 12 μM) (76). Acquired deficiencies of folate and cobalamin will also elevate blood levels of homocysteine, and in some subjects, severe hyperhomocysteinemia (>100 μM) is the result.

Does homocysteine play a causal role in cardiovascular disease, cognitive dysfunction, complications of pregnancy, and osteoporosis? The question remains largely unanswered. In homocystinuric patients, who have deficiencies of key enzymes in homocysteine metabolism, reasonable evidence supports a causal role for homocysteine in the etiology of premature thromboembolic disease (83). However, in cardiovascular patients with mild hyperhomocysteinemia, a causal role for homocysteine in atherogenesis has not been established. Considerable evidence suggests that homocysteine causes vascular dysfunction [reviewed in (1, 82, 129)], and in the apoE-deficient mouse model of atherogenesis, hyperhomocysteinemia dramatically accelerates atherogenesis (41, 127, 140). This review focuses on a new hypothesis, the *molecular targeting hypothesis,* as a mechanistic explanation for homocysteine-induced cellular dysfunction in the cardiovascular system and perhaps in other organ and tissue systems as well.

Molecular targeting of proteins is possible with homocysteine and with homocysteine thiolactone, which has a five-member ring structure containing a thioester and activated carbonyl group. Molecular targeting by L-homocysteine results in the formation of stable covalent disulfide bonds with protein cysteine residues *via* thiol-disulfide exchange reactions and is called "*S*-homocysteinylation." Molecular targeting by homocysteine thiolactone leads to the formation of stable covalent amide-bond–linked homocysteine ("*N*-homocysteinylation"). The activated carbonyl group of homocysteine thiolactone can react with the ε-amino group of lysine residues on protein targets, as described by Jakubowski (47). The possible pathophysiologic consequences of homocysteine thiolactone have been recently reviewed (48). It is interesting to note that *N*-homocysteinylation has been implicated in resistance to clot fibrinolysis (110, 122). Homocysteine that is amide-bonded to protein cannot be determined by conventional plasma total homocysteine assays, all of which use reducing agents that break disulfide bonds and release free homocysteine. Amide-bonded homocysteine can be released from protein only by acid hydrolysis (47). Although this review focuses on *S*-homocysteinylation, the reader should be aware of the many studies, particularly those of Jakubowski and colleagues (47, 48), that document thiolactone-mediated *N*-homocysteinylation.

PATHOGENESIS OF HOMOCYSTEINE-INDUCED CELLULAR DYSFUNCTION

Although our understanding of homocysteine-induced cellular dysfunction is still mechanistically incomplete, a plethora of proposals have been put forward [reviewed in (1, 47, 61)]. Homocysteine is thought to have prooxidant activity. Like most thiols, homocysteine can undergo autooxidation in the presence of molecular oxygen and a transition metal catalyst. The products of autooxidation are homocystine, the disulfide dimer of homocysteine, and hydrogen peroxide. Indeed, elevated homocysteine and oxidative stress are often associated, but the mechanism is unlikely to involve autooxidation (46). Homocysteine appears to alter the normally anticoagulant phenotype of blood vessels to a procoagulant phenotype, along with the activation of platelets. In homocystinuric patients with untreated hyperhomocysteinemia, thromboembolic disease occurs in roughly 50% of the patients before they reach the age of 30 years (83). Homocysteine may also be a pro-inflammatory molecule. The migration of monocytes into the neo-intima of developing atherosclerotic lesions may be stimulated by homocysteine-induced expression of monocyte chemoattractant protein 1 in the endothelium (97). Homocysteine appears to be mitogenic to smooth muscle cells that invade the neo-intima of developing atherosclerotic lesions (120). Recent studies from several laboratories show that hyperhomocysteinemia in humans and animals causes endothelial cell dysfunction, manifested by impaired relaxation of blood vessels (60). Although the mechanism is poorly understood, studies suggest that homocysteine limits the bioavailability of endothelium-derived nitric oxide, a potent vasodilator, because of the ability of homocysteine to generate reactive oxygen species (61).

MOLECULAR TARGETING OF PLASMA PROTEINS

The molecular-targeting hypothesis was initially based on early reports that described the formation of stable disulfide complexes between homocysteine and plasma proteins in circulation (50, 51, 101, 109, 135). The free forms of plasma homocysteine account for only 20–30% of plasma total homocysteine. Protein-bound homocysteine accounts for 70–80% of plasma total homocysteine in healthy individuals (101) and in patients with end-stage renal disease (115). In the early clinical assays for homocysteine, plasma protein (and protein-bound homocysteine) was removed by precipitation, and "free soluble homocysteine" in the supernatant was then determined by amino acid analysis. The free forms that could be detected included homocystine and homocysteine-cysteine mixed disulfide, but not homocysteine itself (133). The elegant studies of Mansoor and colleagues (74–76) have given us a detailed quan-

titative picture of protein-bound homocysteine as well as protein-bound cysteine, cysteinylglycine, and glutathione in health and disease. In the current assays for plasma total homocysteine, strong reducing agents are used to reduce all forms of disulfide-linked homocysteine, thereby generating free reduced homocysteine, which is determined directly by electrochemical detection or indirectly after conversion to a second adduct (102).

Albumin

The first report that albumin might be a carrier of disulfide-bonded homocysteine in circulation by Refsum *et al*. (101) appeared in 1985. Although the fractions from their gel-filtration procedure included proteins other than albumin (*e.g.,* immunoglobulins), it is clear from more recent studies that albumin is the major *S*-homocysteinylated protein in circulation. Albumin is the most abundant plasma protein, with a concentration range of 34–48 mg/ml (512–723 μM). The albumin molecule (molecular mass, 66.4 kDa) contains 585 amino acids, 35 of which are cysteine residues. Thirty-four of these cysteine residues form intrachain disulfide bonds, which are generally buried within the globular structure of the molecule (39). The cysteine at position 34 sits in a 10-Å hydrophobic crevice on the surface of albumin, and because the pKa of the sulfhydryl group is abnormally low (pKa ~5), Cys^{34} exists as a thiolate anion at physiologic pH (96). Christodoulou *et al*. (16, 17) proposed that Cys^{34} exists in exposed and buried forms, as shown in Fig. 1. The Cys^{34} thiolate anion is normally buried and in close proximity to His^{39}, allowing it to form a salt bridge for stabilization. Disulfide bond formation of Cys^{34} with homocysteine or cysteine would favor the formation of the exposed form. Thus, when the exposed Cys^{34} thiolate anion participates in thiol/disulfide exchange reactions to form disulfide-bonded products, the equilibrium shifts in favor of the exposed form (17).

Albumin-Cys^{34}-S-S-homocysteine (Alb-S-S-Hcy) is formed by thiol/disulfide exchange reactions based on *in vitro* modeling studies in this laboratory with human crystalline albumin, D,L-^{35}S-homocysteine, albumin-Cys^{34}-S-S-cysteine (Alb-S-S-Cys) and homocysteine-cysteine mixed disulfide (HCMD or Hcy-S-S-Cys) at physiological and pathophysiologic concentrations (up to 500 μM for L-homocysteine) (112). When homocysteine enters circulation from cells and tissues, less than 1% of its sulfhydryl group undergoes proton dissociation because of the high pKa of this reaction:

$$\text{Hcy-SH} \leftrightarrows \text{Hcy-S}^- + \text{H}^+,\ \text{pKa} = 10.0$$

However, the homocysteine thiolate anion (Hcy-S^-) is a very reactive nucleophile and undergoes thiol/disulfide exchange with Alb-S-S-Cys to form the mixed disulfide and albumin thiolate anion (Alb-S^-) (112):

$$\text{Hcy-S}^- + \text{Alb-S-S-Cys} \leftrightarrows \text{Hcy-S-S-Cys} + \text{Alb-S}^-$$

The concentration of Alb-S-S-Cys in human plasma ranges from 124 to 178 μM for females and from 151 to 201 μM for males (75), making it an abundant target for Hcy-S^- attack. That the attack occurs on the cysteine sulfur of Alb-S-S-Cys can be explained by thermodynamic considerations and relative stabilities of the leaving groups (112). Albumin thiolate anion can now attack Hcy-S-S-Cys to form Alb-S-S-Hcy:

$$\text{Alb-S}^- + \text{Hcy-S-S-Cys} \leftrightarrows \text{Alb-S-S-Hcy} + \text{Cys-S}^-$$

This attack occurs on the homocysteine sulfur (≥80%), as shown by *in vitro* studies with synthetic Hcy-S-S-Cys (10, 112). Cysteine thiolate anion (pKa = 8.3) is a much better leaving group and thermodynamically more stable than homocysteine thiolate anion (pKa = 10.0). Albumin thiolate anion can also attack homocystine (Hcy-S-S-Hcy) to form Alb-S-S-Hcy:

$$\text{Alb-S}^- + \text{Hcy-S-S-Hcy} \leftrightarrows \text{Alb-S-S-Hcy} + \text{Hcy-S}^-$$

The formation of Hcy-S-S-Hcy in circulation also involves thiol/disulfide exchange mechanisms, as shown by our *in vitro* studies (113):

$$\text{Hcy-S}^- + \text{Hcy-S-S-Cys} \leftrightarrows \text{Hcy-S-S-Hcy} + \text{Cys-S}^-$$

$$\text{Hcy-S}^- + \text{Alb-S-S-Hcy} \leftrightarrows \text{Hcy-S-S-Hcy} + \text{Alb-S}^-$$

It is estimated that only 20% of the homocysteine entering circulation undergoes autooxidation, and it appears that copper bound to albumin catalyzes this reaction. In contrast, the autooxidation of cysteine in circulation appears to be catalyzed by ceruloplasmin copper (113). The studies on the mechanism of the formation of Alb-S-S-Hcy serve as a paradigm for investigating other potential molecular targets for homocysteine. A model describing the biochemistry of homocysteine in circulation, which includes the formation of albumin-Cys^{34}-S-S-homocysteine, is shown in Fig. 2.

Transthyretin (prealbumin)

In human plasma, transthyretin exists as a homotetramer and serves as a carrier protein for the hormone thyroxine and the retinol-binding protein–retinal complex (5). Monomeric

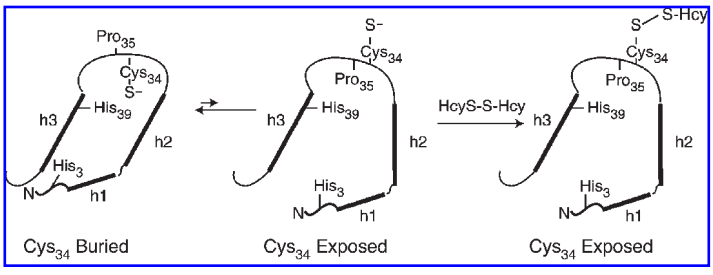

FIG. 1. Buried and exposed conformations of albumin-Cys^{34} thiolate anion. Cys^{34} exists in buried and exposed conformations, as proposed by Christodoulou *et al*. (16). When albumin-Cys^{34} thiolate anion (exposed) attacks homocystine (Hcy-S-S-Hcy), the homocysteinylated product is stabilized in the exposed conformation. Modified from Christodoulou *et al*. (16). Reprinted by permission from Sengupta *et al*. (112).

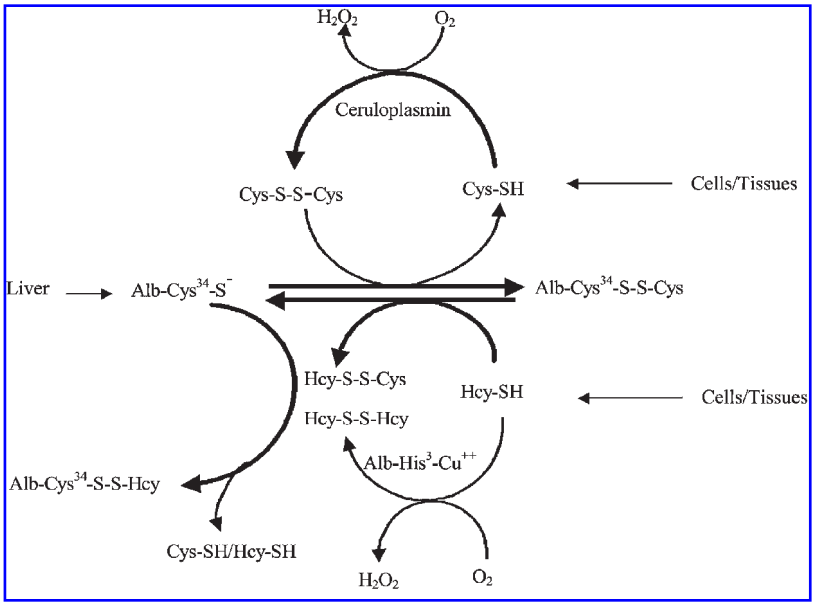

FIG. 2. Model for the formation of homocystine, homocysteine-cysteine mixed disulfide (HCMD), cystine, and albumin-bound homocysteine in circulation. Free reduced cysteine (Cys-SH) entering circulation is autooxidized by ceruloplasmin to cystine (Cys-S-S-Cys). Albumin thiolate anion enters the circulation and reacts with cystine to form albumin-Cys^{34}-S-S-cysteine and cysteine. Free reduced homocysteine (Hcy-SH) entering circulation then attacks albumin-Cys^{34}-S-S-cysteine toform HCMD (Hcy-S-S-Cys) and albumin thiolate anion. Albumin thiolate anion then reacts with HCMD, preferentially forming albumin-Cys^{34}-S-S-homocysteine and cysteine thiolate anion. A small amount of homocysteine is also autooxidized to homocystine (Hcy-S-S-Hcy) by the copper attached to His^3 of albumin. Albumin thiolate anion also reacts with homocystine to form albumin-Cys^{34}-S-S-homocysteine. *Thick arrows*, the major reactions. Reprinted by permission from Sengupta *et al.* (113).

transthyretin (13.8 kDa) has a single cysteine residue at position 10, and, in the normally folded tetrameric protein, the Cys^{10} residues are in exposed sites at the start of helical regions. Previous work had shown that Cys^{10} residues form covalent disulfide complexes with cysteine, glutathione, and cysteinylglycine (54, 119). In a series of *in vitro* studies using physiologic and pathophysiologic concentrations of homocysteine, Lim *et al.* (67) demonstrated that purified transthyretin and transthyretin in normal human plasma is homocysteinylated by ^{35}S-D,L-homocysteine, as shown in Fig. 3. Low levels of endogenous homocysteinylated transthyretin have been identified in normal human plasma (67). Much higher concentrations of homocysteinylated transthyretin have been found in the plasma of patients with end-stage renal disease and homocystinuria, as shown in Fig. 4 (67). Lim *et al.* demonstrated that the ratio of transthyretin-Cys^{10}-S-S-homocysteine to that of unmodified transthyretin increased with increasing concentrations of plasma total homocysteine. Thus, homocysteinylated transthyretin is a novel indicator of plasma homocysteine burden in subjects with hyperhomocysteinemia and homocystinuria.

Transthyretin is an amyloid protein and has been implicated in the formation of amyloid deposits in familial transthyretin amyloidosis and senile systemic amyloidosis (5). The latter is a nonhereditary disease that affects ~25% of individuals older than 80 years, primarily in the cardiovascular system (19). The concentration of plasma total homocysteine increases with increasing age but it remains to be seen whether posttranslational modification of transthyretin by homocysteine plays a role in amyloid deposition.

Fibronectin

Human plasma contains soluble fibronectin, a 440-kDa glycoprotein consisting of two similar but nonidentical subunits. Two disulfide bridges hold the subunits together, and each subunit contains >60 cysteine residues, most of which are in the form of intrachain disulfides. Fibronectin plays a key role in cell migration, cell adhesion, hemostasis, thrombosis, wound healing, and tissue remodeling. The fibronectin molecule has distinct domains for interactions with collagen, fibrin, heparin, and DNA. Majors *et al.* (73) showed that ^{35}S-D,L-homocysteine (≤500 μM) reacts with fibronectin in human plasma and with purified human fibronectin, as shown in Fig. 5. When the incubation was carried out with ^{35}S-L-cysteine, thiolation of plasma fibronectin and purified fibronectin was not observed

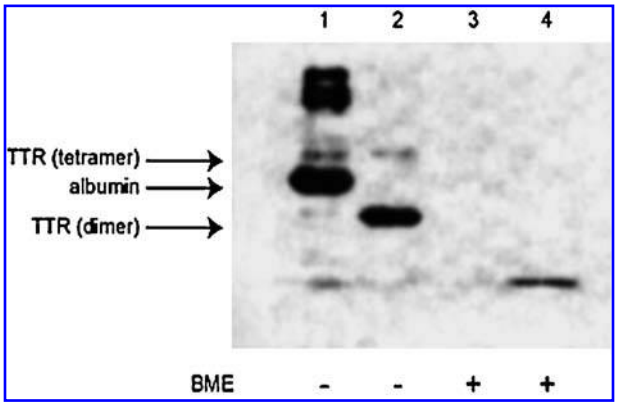

FIG. 3. Phosphorimage analysis of the binding of ^{35}S-D,L-homocysteine to albumin and transthyretin. Human plasma from a healthy donor (lanes 1 and 3) and purified transthyretin (lanes 2 and 4) were incubated with ^{35}S- D,L-homocysteine for 5 h at 37°C. The samples, before (lanes 1 and 2) and after (lanes 3 and 4) treatment with 2-mercaptoethanol (BME), were subjected to SDS-PAGE followed by phosphorimage analysis. Reprinted by permission from Lim *et al.* (67).

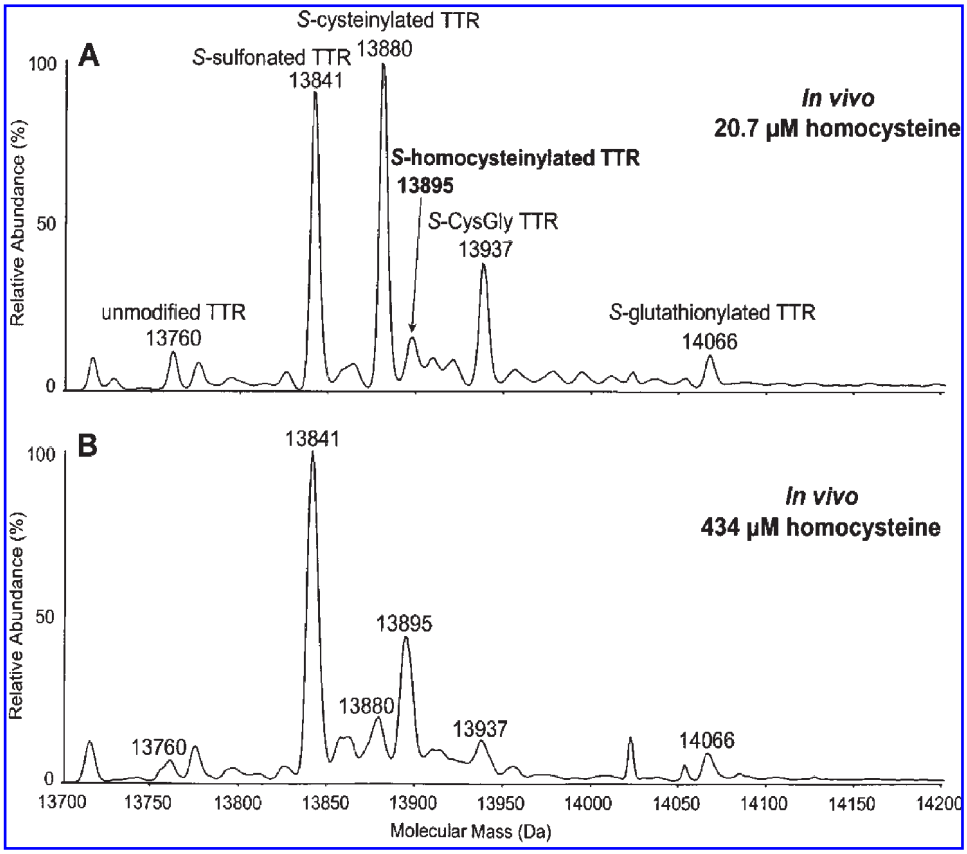

FIG. 4. Binding of L-homocysteine to transthyretin (*in vivo*). Deconvoluted electrospray ionization mass spectra of transthyretin, immunoprecipitated and HPLC-purified, from (**A**) the plasma of a patient with end-stage renal disease (20.7 μM total plasma homocysteine) and (**B**) from the plasma of a patient with homocystinuria (434 μM total plasma homocysteine) are shown. Reprinted by permission from Lim *et al*. (67).

(see Fig. 5). Because 2-mercaptoethanol can remove fibronectin-bound homocysteine, the interaction involves disulfide bond formation. Approximately five homocysteine molecules bind to each 440-kDa dimer. The mechanism of fibronectin targeting by L-homocysteine, be it by thiol/disulfide exchange, or by oxidative processes, has not been established. Moreover, *S*-homocysteinylated fibronectin remains to be demonstrated *in vivo*. The interaction of fibronectin with fibrin is impaired after fibronectin is homocysteinylated (73). This could lead to prolonged recovery from a thrombotic event and contribute to prolonged vascular occlusion (73).

Factor Va

The prothrombinase complex, which includes cofactor factor Va, converts prothrombin to thrombin during blood clotting. Human factor V (330 kDa) is activated by α-thrombin cleavages at Arg^{709}, Arg^{1018}, and Arg^{1545} to produce factor Va. The clotting process is downregulated by proteolytic cleavage of factor Va by activated protein C. Undas *et al*. (123) showed that factor V is homocysteinylated *in vitro* and that the resulting homocysteinylated factor Va is resistant to proteolytic inactivation by activated protein C. Factor V (600 n*M*) was treated with 450 μM ^{35}S-D,L-homocysteine for 2 h at 25°C and then treated with α-thrombin (12 n*M*) for 10 min. [Note: the method used for the preparation of radiolabeled homocysteine produces D,L-^{35}S-homocysteine, not L-^{35}S-homocysteine]. Factor Va was then subjected to proteolysis by activated protein C (6 n*M*) for 5–180 min. The proteolytic fragments were resolved by nonreducing SDS-PAGE and then subjected to autoradiography. ^{35}S-D,L-homocysteine was associated with both the heavy and light chains of factor Va. Label was also observed in the fragments containing cysteine produced by activated protein C proteolysis. Treatment of ^{35}S-D,L-homocysteinylated-factor V with 2-mercaptoethanol resulted in the loss of label, suggesting that disulfide bonds were responsible for adduct formation (123). Thiol specificity was demonstrated in this study in that neither cysteine nor homocysteine thiolactone treatment of factor V affected activated protein C inactivation of derived factor Va. Impaired inactivation of the prothrombinase complex by homocysteine may explain the thrombotic tendency of patients with hyperhomocysteinemia, especially those with the most severe form found in homocystinuric patients. However, homocysteinylated-factor V (Va) has yet to be identified in the plasma of patients with hyperhomocysteinemia, and Lentz *et al*. (62) could find no evidence for the impairment of protein C activation by thrombin or the inactivation of factor Va by activated protein C in animal models of hyperhomocysteinemia and in normal human subjects with acute hyperhomocysteinemia after methionine loading.

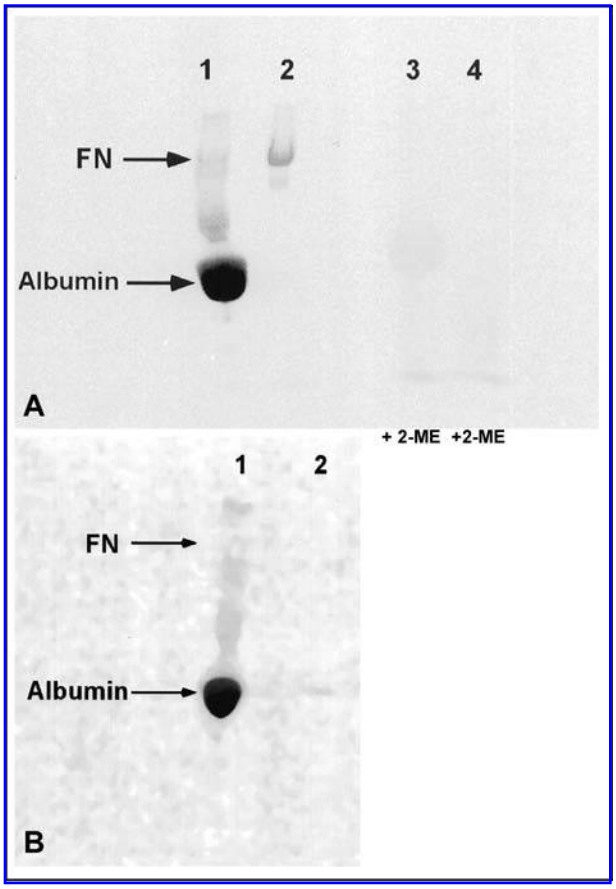

FIG. 5. The binding of homocysteine and cysteine to plasma proteins and purified fibronectin. ^{35}S-D,L-Homocysteine (**A**) and ^{35}S-L-cysteine (500 μM final concentration) (**B**) were incubated with 50% human plasma in 0.05 *M* TES buffer (pH 7.4) and with human fibronectin (1 mg/ml in 0.05 *M* TES buffer, pH 7.4) for 5 h at 37°C. After the incubation, protein was precipitated with 1.5 *M* perchloric acid and solubilized with SDS-PAGE sample buffer. Aliquots were subjected to analysis by SDS-PAGE. The gels were stained with Coomassie blue, dried, and subjected to phosphorimaging. (**A**) Binding of ^{35}S-D,L-homocysteine. Lanes are as follows: 1 and 3, human plasma; 2 and 4, human plasma fibronectin. Lanes 1 and 2 are without reduction, and lanes 3 and 4 are with reduction by 2-mercaptoethanol (2-ME). (**B**) Binding of ^{35}S-L-cysteine. Lanes are as follows: 1, human plasma; 2, human plasma fibronectin. Reprinted by permission from Majors *et al*. (73).

Lipoprotein(a)

Lipoprotein(a) [Lp(a)] is an independent risk factor for cardiovascular disease (24, 40, 134), and the risk may be associated with the ability of Lp(a) to compete with plasminogen for binding sites on fibrin in clots, delaying the fibrinolysis. Lp(a) has two lipoprotein components: apo(a) and apoB-100, which are linked covalently by a single disulfide bond. In addition to the covalent disulfide bond, non-covalent contacts are found between the two proteins, which result in reversible interactions of association and disassociation. The molecular weight of the apo(a) component ranges from 280,000 to 800,000 because of a variable number of K-IV–like kringle repeats. The single disulfide bond between apo(a) and apoB-100 may be a target for homocysteine and other sulfhydryl compounds *in vivo*. *In vitro* treatment of Lp(a) with these reagents results in enhanced binding to plasmin-modified fibrin, as shown by Harpel *et al*. (36). Dithiothreitol (1.0 m*M*) was more effective than 2.0 m*M* D,L-homocysteine, L-cysteine, *N*-acetylcysteine, or glutathione in promoting the reaction. Molecular targeting by L-homocysteine could occur by two mechanisms, depending on which sulfur atom is attacked in Lp(a):

$$\text{Hcy-S}^- + \text{Lp(a)} \leftrightarrows \text{apo(a)-Cys-S-S-Hcy} + \text{apoB-100-Cys-S}^-$$

or

$$\text{Hcy-S}^- + \text{Lp(a)} \leftrightarrows \text{apo(a)-Cys-S}^- + \text{apoB-100-Cys-S-S-Hcy}$$

The course of the reaction will be determined by the pKa values of the cysteine residue–leaving groups. Homocysteine thiolate anion attack will occur on the sulfur with the higher pKa. Herrmann *et al*. (40) found five- to seven-fold elevations in free apo(a) in patients with nephrotic syndrome and in patients receiving peritoneal dialysis compared with normal controls but could not demonstrate a correlation between apo(a) and plasma total homocysteine. Because apo(a) binds more tightly to fibrin than does Lp(a), an increase in the plasma concentration of apo(a), for whatever reason, would further delay clot lysis. It is also possible that homocysteinylation of either apo(a) or apoB-100 would block the biosynthesis of Lp(a) and thus increase the concentration of free apo(a). *In vivo* evidence for these homocysteinylated forms of either apoB or apo(a) in patients with hyperhomocysteinemia has not been reported.

MATRIX AND MEMBRANE-ASSOCIATED PROTEINS

Collagen

Several observations suggest that homocysteine affects collagen synthesis and metabolism, but no direct evidence exists that homocysteine can form stable disulfide complexes with the molecule. Majors *et al*. (71, 72) showed that pathophysiologic concentrations of L-homocysteine (up to 300 μM) stimulated collagen synthesis and accumulation in cultured aortic smooth muscle cells. L-Cysteine (600 μM) also stimulated collagen synthesis in cultured aortic smooth muscle cells but to a lesser extent. Increased collagen synthesis and accumulation has also been observed *in vivo* in animal models of hyperhomocysteinemia (68, 140). Interestingly, increased collagen deposition has been observed on postmortem examination of hyperhomocysteinemic patients with acute coronary syndrome (11).

Fibrillin-1

Fibrillins (fibrillin-1, -2, and -3) are extracellular calcium-binding matrix proteins with high cysteine content (12–13% of all amino acids). The cysteine residues are thought to form intramolecular disulfide bonds, which stabilize domain architecture. Fibrillins form the backbone of microfibrils and serve as

a scaffold for the deposition of elastin and assembly of elastin fibers. The autosomal dominant disorder called Marfan syndrome is caused by mutations in the gene for fibrillin-1, and the phenotype of affected patients includes cardiovascular disease, skeletal abnormalities, and ocular problems (98). Patients with homocystinuria due to cystathionine β-synthase deficiency, an autosomal recessively inherited condition, have a similar phenotype in many respects (84), and it has long been thought that excess homocysteine interacts with matrix proteins and impairs their functions.

Recently, Hubmacher *et al*. (44) provided strong evidence that fragments of recombinant human fibrillin-1 undergo *S*-homocysteinylation in the presence of 300 μM L-homocysteine and that homocysteinylated fibrillin-1 fragments have loss of function. Evidence for the disruption of secondary structural integrity in the *S*-homocysteinylated-protein was provided by circular dichroism. Calcium-binding activity of the homocysteine-modified fragments was completely abolished, along with greater susceptibility to proteolytic degradation. Double immunofluorescence studies on the matrix of cultured dermal fibroblasts showed that L-homocysteine-α-amino-fluorescein isothiocyanate–labeled matrix protein(s) by thiol/disulfide exchange and that the label colocalized with antibodies specific for fibrillin-1 (44). The effect of other thiols (*e.g*., L-cysteine) on the structure and function of recombinant fibrillin-1 fragments was not reported. These studies suggest that L-homocysteine may interact with fibrillin-1 by thiol/disulfide exchange in patients with homocystinuria, leading to *S*-homocysteinylation of fibrillin-1 and loss of function of this essential matrix protein.

Metalloproteinases

In severe hyperhomocysteinemia, a striking remodeling of the extracellular matrix in arterial walls occurs, and elastolysis mediated by metalloproteinases appears to be involved. McCully (80) first observed fragmentation of the medial elastic lamina and of the internal elastic lamina in patients with homocystinuria. Metalloproteinase 2 (MMP-2; gelatinase A) is a member of a family of elastolytic proteinases and has been studied extensively by Charpiot's group (6). ProMMP-2, the latent form of the enzyme, contains a cysteine residue in the propeptide. The sulfhydryl group of this cysteine coordinates to zinc in the catalytic domain, keeping the enzyme inactive. The proenzyme can be activated by proteolytic cleavage of the propeptide to give active MMP-2. Alternatively, D,L-homocysteine and L-homocysteine thiolactone but not L-cysteine, L-methionine, or L-homocystine, can convert proMMP-2 to an active enzyme without loss of the propeptide (6). The activation of proMMP-2 by D,L-homocysteine and L-homocysteine thiolactone occurs at a low molar ratio of D,L-homocysteine and L-homocysteine thiolactone to proenzyme, with maximal activation occurring at 1:1 and 10:1, respectively. At a molar ratio of 1,000:1, both D,L-homocysteine and L-homocysteine thiolactone are inhibitory. The mechanism of activation and inhibition of proMMP-2 by D,L-homocysteine implies that homocysteine is attacking the propeptide cysteine residue to activate the proenzyme, but at higher concentrations, D,L-homocysteine may be attacking the active-site zinc to inhibit enzyme activity (6, 86). Confirmation of this mechanism must await the isolation of homocysteinylated-proMMP-2 and identification of the homocysteine-binding sites.

Annexin II

The anticoagulant phenotype (thromboresistance) of the vascular endothelium is likely to be maintained by its ability to generate plasmin at the luminal cell surface (31). Plasmin generation is mediated by the calcium-dependent, phospholipid-binding protein annexin II, which serves as a docking protein for plasminogen and tissue plasminogen activator. The tripartite complex results in a 60-fold increase in catalytic efficiency of plasmin generation from plasminogen (30). Hajjar *et al*. (32) discovered that L-homocysteine (0–50 μM) decreased in the ability of annexin II to bind tissue plasminogen activator by 60–66%. L-Cysteine (0–50 μM) had little or no effect on the binding of tissue plasminogen activator to annexin II. Other studies using LC/MS/MS demonstrated that the Cys^9 residue of annexin II became homocysteinylated. In an attempt to see if annexin II could be homocysteinylated at the cellular level, cultured human umbilical vein endothelial cells were labeled with ^{35}S-D,L-homocysteine for 18 h, and lysates were prepared. Whole lysates and immunoprecipitated annexin II were run on nonreducing and reducing SDS-PAGE. Prominent 36-kDa bands corresponding to labeled annexin II were present on the nonreduced gels but were absent on the reduced gels, suggesting that homocysteine had indeed formed a stable disulfide link with annexin II in cell culture (32). This is one of the first *in vitro* studies to demonstrate the concept of homocysteine targeting at the molecular and cellular level. These studies also suggest a mechanism for the prothrombotic effects of hyperhomocysteinemia. It will be difficult to observe directly the homocysteinylation of annexin II *in vivo,* but a functional consequence would be higher circulating levels of tissue plasminogen activator.

MOLECULAR TARGETING OF INTRACELLULAR PROTEINS

Are the cysteine residues of *intracellular* proteins homocysteinylated, and, if so, will homocysteinylation impair or alter function? It could be argued that high concentrations of intracellular glutathione favor a reducing environment, which might prevent or disrupt protein-S-S-homocysteine bond formation. However, *S*-thiolation of intracellular proteins, primarily by glutathione, is common when cells are subjected to oxidative stress. Because of the high pKa of the sulfhydryl group of L-homocysteine (*vide infra*), it is likely that *S*-thiolation with L-homocysteine will result in the formation of mixed disulfide adducts that are more stable than the mixed disulfide adducts with glutathione. Evidence now suggests that intracellular targets are homocysteinylated and that the functions of at least one of these proteins targeted by L-homocysteine are severely impaired. *S*-Thiolation of protein cysteine residues by L-homocysteine could be a major cause of cellular dysfunction.

Metallothionein

Is it possible that intracellular proteins with an unusually high abundance of cysteine residues are more likely to be targets of

L-homocysteine than proteins with the usual low abundance of cysteine? Cysteine residues account for 35% of the amino acids in metallothionein (MT), a 6-kDa Zn-binding protein. MT appears to be capable of detoxifying heavy metals, regulating zinc/copper homeostasis, and scavenging reactive oxygen species (33, 66, 95). The protein plays critical roles in the regulation of cellular redox, nitric oxide signaling, and zinc homeostasis (45, 49, 78, 79). Although MT has a high affinity for zinc, it can be released during nitric oxide signaling and by oxidized glutathione (45, 49, 78, 87). Because of the importance of zinc in enzymatic function and signal transduction, impaired zinc binding could have deleterious consequences across multiple cellular processes.

Recent work from this laboratory has shown that MT is *S*-homocysteinylated in cultured human aortic endothelial cells (HAECs) (3). Cells were cultured to 80% confluence and incubated with 50 μM ^{35}S-D,L-homocysteine for 12 h. Proteins in cell lysates were resolved by SDS-PAGE with and without 2-mercaptoethanol, transferred to polyvinylidene fluoride membranes, Western blotted, and then analyzed by phosphorimaging. As shown in Fig. 6A, Western blotting reveals ~10-kDa MT bands in both the reducing and nonreducing lanes. However, phosphorimage analysis of the Western blot (Fig. 6B) shows a ^{35}S-radioactive band only in the nonreducing lane. These studies show that intracellular MT was homocysteinylated in cultured HAECs. The observation that homocysteine was removed from the protein by β-mercaptoethanol suggests that homocysteinylation occurred by disulfide bond formation. When similar lysates from cultured HAEC were treated with 10 m*M* reduced glutathione and analyzed as described earlier, ^{35}*S*-homocysteinylated-MT was again identified by phosphorimage analysis, suggesting that, even in the presence of high physiologic concentrations of reduced glutathione, the mixed disulfide bond of MT-S-S-homocysteine is stable and remains intact (3).

L-Homocysteine, in a dose-dependent manner, caused a rapid increase in intracellular free zinc in cultured HAECs previously loaded with Zinquin-AM (3). After incubation with 50, 100, and 500 μM L-homocysteine for 60 min, intracellular free zinc increased to 34 ± 5.1, 130 ± 15.3, and 1,208 ± 118.4 n*M*, respectively. L-Cysteine, at similar concentrations, was unable to elicit a dose-dependent increase in intracellular free zinc, thus demonstrating thiol specificity (3). Sudden increases in intracellular free zinc induce the expression of immediate-early genes such as early growth response 1 (Egr-1) (4, 108). As shown in Fig. 7, treatment of cultured HAECs with 50 μM L-homocysteine resulted in the rapid increase in intracellular free zinc within 30 min and a transient expression of Egr-1 protein occurring in 60 min but disappearing after 120 min. Reactive oxygen species began to appear slowly after 180 min but rapidly increased after 12 h, as detected with the fluorescent dye 5-(and 6)-chloromethyl-2′,7′-dichlorodi-hydrofluorescein diacetate (CM-H_2DCFDA). Finally, the ability of MT to scavenge superoxide anion radicals was severely impaired by L-homocysteine (3).

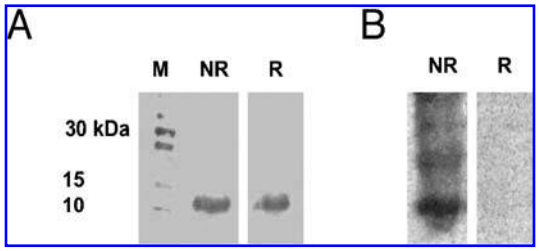

FIG. 6. Identification of ^{35}*S*-D,L-homocysteinylated-metallothionein (MT) in human aortic endothelial cells (HAECs). (**A**) Western blot of HAEC lysate probed with anti-MT antibody in the absence (NR, nonreducing) and presence (R, reducing) of β-mercaptoethanol (BME). (**B**) Phosphorimage of the same Western blot depicted in (A), demonstrating ^{35}*S*-homocysteinylated proteins. Several bands including the one corresponding to MT are present in the NR lane but not in the R lane. Reprinted with permission from Barbato *et al*. (3).

The pathologic consequence of molecular targeting of an intracellular zinc chaperon and antioxidant defense molecule by L-homocysteine is a novel mechanism for homocysteine-mediated vascular damage. The identification of MT as a target of homocysteine in HAECs represents the first endogenous intracellular protein to be homocysteinylated in an intact cellular system. The transient expression of Egr-1 within 1 h of homocysteine incubation is significant, because consensus sequences for Egr-1 are found in the promoters of various mediators of atherosclerosis, including monocyte chemoattractant protein 1, tumor necrosis factor-α, and intracellular adhesion molecule 1. Moreover, the upregulation of Egr-1 by homocysteine could potentially explain the downstream activation of monocyte chemoattractant protein 1 within 2–3 h of homocysteine incubation, as previously reported by our laboratory (97).

Glutathione peroxidase

Cellular glutathione peroxidase (GPx-1) is a selenocysteine-containing antioxidant defense enzyme that reduces hydrogen peroxide and organic hydroperoxides. Accumulating evidence indicates that homocysteine decreases GPx-1 activity, but the precise mechanism(s) remains unclear. A possible consequence of homocysteine-induced inactivation of GPx-1 would be an increase in reactive oxygen species (ROS), a decrease in the bioavailability of nitric oxide, and endothelial dysfunction. Mild hyperhomocysteinemia associated with heterozygous cystathionine β-synthase deficiency in mice or with folate deficiency in rats decreases hepatic GPx-1 activity (43, 130). It is interesting to note that overexpression of GPx-1 in heterozygous cystathionine β-synthase–deficient mice can rescue the endothelial dysfunction caused by hyperhomocysteinemia in these animals (131).

Homocysteine, in a dose-dependent manner, inhibited GPx-1 activity in cultured rat aortic smooth muscle cells and bovine aortic endothelial cells (89, 124). Conflicting reports are found as to whether the observed decrease in cellular GPx-1 activity is due to direct inhibition of the enzyme by homocysteine. Upchurch *et al*. (124) found no evidence for direct inhibition but did find that homocysteine decreased the steady-state level of GPx-1 mRNA. Conversely, Nishio and Watanabe (89) observed direct inhibition of purified GPx-1 by homocysteine in a dose-dependent manner. Recently, Handy *et al*. (34) provided evidence that homocysteine inhibits the translation of GPx-1 by a mechanism involving a selenocysteine-incorporation sequence, or SECIS element in the 3′-untranslated region of the GPx-1

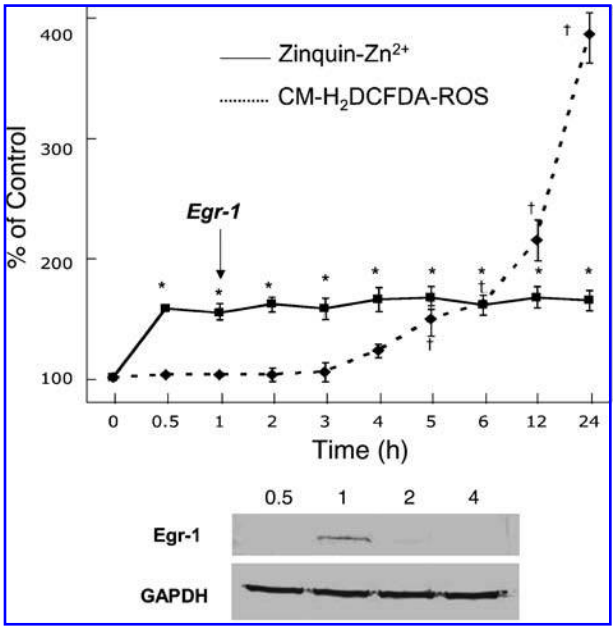

FIG. 7. Intracellular free Zn^{2+}, ROS, and Egr-1 protein expression in HAECs as a function of time exposed to L-homocysteine. *Upper panel: Solid line*, the percentage change in intracellular free Zn^{2+} in HAECs incubated with 50 μM L-homocysteine. *Dashed line*, the percentage change in ROS as measured with CM-H_2DCFDA–loaded HAECs incubated with 50 μM L-homocysteine. *Lower panel:* Western blots probed with anti-Egr-1 and GAPDH antibodies at 0.5-, 1-, 2-, and 4-h points. *Arrow*, Egr-1 protein expression. Error bars represent ±SD. *Statistical significance compared with Zinquin-loaded cells at the zero time point ($p < 0.01$). †Statistical significance compared with CM-H_2DCFDA–loaded cells at the zero time point ($p < 0.01$). Reprinted with permission from Barbato *et al.* (3).

mRNA and other factors that allow a read-through at the UGA-stop codon and incorporation of selenocysteine into the protein. All of this work suggests that homocysteine is targeting one or more factors essential for GPx-1 expression. The factor(s) involved could be at the level of transcription or translation, or it could be the enzyme itself.

Dimethylarginine dimethylaminohydrolase

The plasma concentrations of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) are elevated in an animal model of hyperhomocysteinemia and hypercholesterolemia (7). Arginine residues on proteins undergo *S*-adenosylmethionine–dependent methylation, and when proteins are degraded, ADMA is released into the circulation. It can be eliminated in the urine of healthy individuals, but in chronic renal failure, ADMA accumulates. ADMA can also be hydrolyzed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which is found in two isoforms. In tissues expressing neuronal nitric oxide synthase, DDAH-1 is found, and in tissues expressing endothelial nitric oxide synthase, DDAH-2 is found. Stühlinger *et al.* (117) were the first to obtain evidence that homocysteine could target DDAH directly and inhibit its activity. They first showed that D,L-homocysteine, in a dose-dependent manner (0–300 μM), induced the accumulation of ADMA in the culture media of ECV304 cells and that the thiol antioxidant pyrrollidine dithiocarbamate could block the effect. They then showed that DDAH activity was inhibited in lysates of ECV304 cells incubated with D,L-homocysteine (0–1.0 m*M*) for 24 h. Using purified recombinant DDAH, they were able to show a dose-dependent inhibition of enzyme activity by D,L-homocysteine. Finally, they demonstrated that biotinylated-homocysteine but not biotinylated-methionine or biotinylated-cysteine bound directly to recombinant DDAH and that the binding could be blocked by pyrrollidine dithiocarbamate (117). To our knowledge, this is the first demonstration of *in vitro* molecular targeting by homocysteine of an intracellular enzyme. However, it remains to be seen whether homocysteinylated-DDAH can be isolated from cell lysates. Recently, Frey *et al.* (25) determined the crystal structure of DDAH-1 with L-homocysteine "trapped" in the active site of bovine brain enzyme. However, electron-density evidence to support the presence of a disulfide bond between homocysteine and a protein cysteine residue was not observed.

HOMOCYSTEINE AND THE ENDOPLASMIC RETICULUM

Studies dating back to the early 1990s suggested that elevated concentrations of homocysteine alter the functions of the endoplasmic reticulum (ER) (37, 63, 64). Polyribosomes associate with the cytoplasmic side of the ER plasma membrane ("rough ER") and translate mRNAs for secretory and membrane-associated proteins. Nascent polypeptides enter the lumen of the ER, where processing machinery assures folding and post-translational modification (28, 53, 99). Within the lumen of the ER are chaperone proteins that prevent aggregation of nascent polypeptide chains and assist in the proper folding of these client proteins. One of the most abundant ER chaperones is the 78-kDa glucose-regulated protein/immunoglobulin chain–binding protein (GRP78/BiP), a member of heat-shock protein 70 family. GRP78 promotes folding and prevents aggregation of proteins within the ER using energy from ATP hydrolysis (27, 52, 59). The ER lumen also provides an oxidizing environment, and enzymes such as protein disulfide isomerase assure that intramolecular disulfide bonds are formed correctly within newly synthesized proteins (9, 138). Properly folded proteins are exported from the ER lumen to the Golgi apparatus, the plasma membrane, lysosomes and other organelles, or to the cell exterior. However, misfolded proteins are retrotranslocated from the ER and degraded by the cytoplasmic ubiquitin-proteasome system (52, 53, 70, 99).

Changes in the normal physical environment of the cell can lead to the accumulation of misfolded proteins and cause ER stress. The hallmark of the ER stress response is the coordinate transcriptional upregulation of resident ER proteins that include molecular chaperones and folding enzymes (59, 70, 77). The other important function of the ER stress response, also known as the unfolded protein response (UPR), is that global translation is blocked *via* phosphorylation of eIF2α by PKR-like ER kinase (PERK). ER stress is an imbalance between the cellular demand for ER function and ER capacity (111, 139). Prolonged

ER stress can lead to cell death by inducing the programmed cell death pathway (137).

Alterations in the biosynthesis of proteins, in the oxidizing potential of the ER, and in calcium homeostasis produce stress signals that activate the UPR and multiple signal transduction pathways as shown in Fig. 8 (35, 94, 138). These intracellular signaling pathways are essential for survival of cells undergoing ER stress as a result of the accumulation of unfolded and misfolded proteins. UPR is mediated *via* the ER-resident transmembrane proteins: a type-1 ER transmembrane protein kinase (IRE1), the activating transcription factor 6 (ATF6), and PERK.

ER stress can activate these three sensors through dissociation from GRP78, an ER-resident molecular chaperone responsive to ER stress that interacts with unfolded proteins (52, 57, 59, 106). GRP78 associates with the three sensors under normal conditions; however, after the accumulation of misfolded proteins, GRP78 dissociates from these sensors to bind to the accumulating unfolded proteins. Dissociation of GRP78 from these sensors is the trigger for UPR activation. Activation of these sensors increases the transcriptional activation of UPR-responsive genes, including GRP78, GRP94, growth-arrest and DNA damage–inducible gene 153 (GADD153), and homocysteine-induced ER protein (Herp) (55). PERK also inhibits the global protein translation by phosphorylating eIF2α. This prevents the synthesis of additional nascent proteins that would otherwise cause further stress to the ER (57).

Elevated levels of homocysteine can induce ER stress in cultured cells, tissues, and *in vivo* (37, 42, 55, 56, 63, 64, 92, 93, 132, 137), leading to activation of the UPR (1, 42). Lentz and Sadler (64) reported on the effect of homocysteine on thrombomodulin expression and protein C activation in cultured human umbilical vein endothelial cells (HUVECs) and CV-1(18A) monkey kidney cells that express recombinant human thrombomodulin. A high concentration of homocysteine (5.0 m*M*) prevented thrombomodulin export and cell-surface expression, whereas protein C was irreversibly inactivated. Hayashi *et al*. (37) reported that homocysteine inhibited the cofactor activity of thrombomodulin on the surface of endothelial cells and in the whole cells. Homocysteine enhanced thrombomodulin expression within HUVECs. Also, homocysteine inhibited the processing and secretion of von Willebrand factor in HUVECs (63), enhanced the expression of tissue factor and factor V (26, 105), reduced the production of activated protein C (104), inhibited tissue plasminogen activator binding to the cell (29), and decreased antithrombin III binding to anticoagulant heparan sulfate on the cell (88). All of these changes result in a procoagulant phenotype and may contribute to the development of thrombosis.

In addition, evidence exists about the association between ER stress and lipid metabolism, which is based on the activation of the sterol regulatory element–binding proteins (SREBPs) and overproduction of lipid components such as cholesterol and triglycerides through the activation of the UPR. The result is an accumulation of lipids in hepatocytes, macrophages, and smooth muscle cells (57, 132). ER stress also induced cleavage of membrane-bound ATF6 by the same proteases that activate SREBP (136).

ER chaperones play very important roles in contributing to all of the major functions of the ER. They control folding and assembly of secretory-pathway proteins and identify the misfolded or unfolded proteins and target them for degradation (69). One of the ER chaperones, GRP78, behaves like a sensor in the ER environment and forms stable complexes with misfolded or incompletely assembled proteins. As mentioned previously, GRP78 normally associates with the sensors; however, on accumulation of misfolded proteins, GRP78 dissociates from the sensors to the unfolded proteins, which triggers the UPR (100, 128). In previous investigations (55–57, 92, 93,

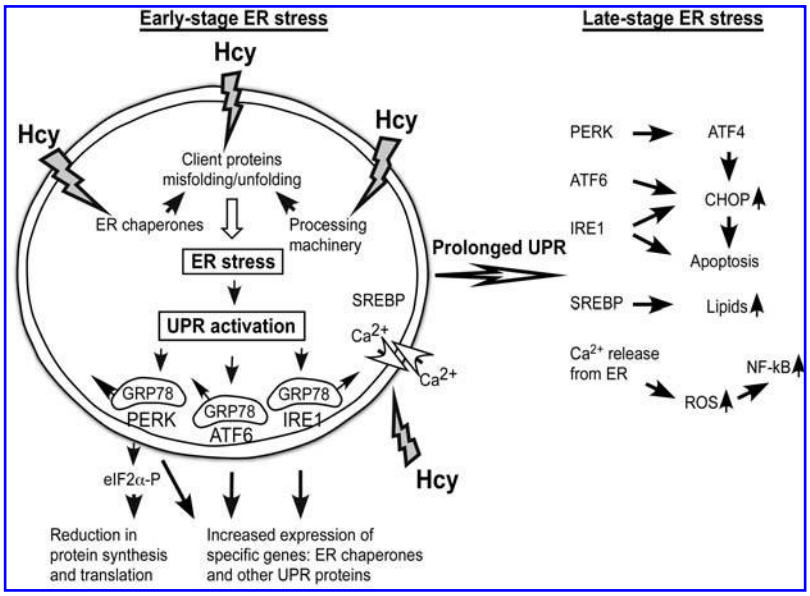

FIG. 8. Direct frontal attack of homocysteine (Hcy) on the ER. Molecular targeting of the ER plasma membrane, ER chaperones (*e.g*., GRP78), ER processing machinery, or client proteins by homocysteine may induce ER stress. ER stress results in the activation of the intracellular signaling pathway, called the unfolded protein response (UPR). Genes encoding ER chaperones and processing enzymes become transcriptionally upregulated. In eukaryotes, the UPR is regulated by the proximal sensors PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and type-I ER transmembrane protein kinase (IRE1). Activation of these sensors leads to increased expression of ER-resident chaperones (GRP78) and the other UPR proteins. ER stress leads to a decrease in protein synthesis and translation through PERK. In late-stage ER stress, PERK, ATF6, and IRE1 upregulate CHOP (C/EBP homologous protein), promoting cell death (apoptosis). SREBP (sterol regulatory element–binding protein) upregulates lipid synthesis. Prolonged UPR leads to Ca^{2+} release from the ER, causing production of ROS, which may lead to activation of NF-κB. Activation of these downstream pathways after ER stress can have direct effects on atherosclerotic lesion development and thrombogenicity.

132), it was reported that elevated levels of intracellular homocysteine increased the expression of several ER stress-response genes, including GRP78, GRP94, and Herp. Kokame *et al*. (56) identified six upregulated (GRP78, NMDMC, ATF4, HUMORF12, and three novel genes) and one downregulated gene under homocysteine treatment of cultured HUVECs. Upregulation of ATF4 may reflect the stress-inducing effects of homocysteine because ATF4 mRNA is known to be induced by increasing intracellular Ca^{2+} concentration (121) and by anoxia (22). ATF4 is also a downstream target of PERK activation. It is interesting to note that ATF4 mRNA is translated under conditions of ER stress, even when global protein synthesis is inhibited by eIF2α phosphorylation. This is because the ATF4 mRNA transcript contains internal ribosomal entry–binding sites.

Another stress-response gene induced by thiol-reducing agents that affect ER function is GADD153. Homocysteine can induce the expression of this gene, and it can adversely affect the function of the ER because GADD153 is considered to be proapoptotic; some suggest that expression of GADD153 links apoptotic cell death with ER stress. GADD153 may be involved in linking ER stress to alterations in the expression of genes responsible in cell growth and proliferation (92). Hossain *et al*. (42) showed that homocysteine induces the expression of T-cell death–associated gene 51 (TDAG51). This overexpression changes the cell morphology, decreases cell adhesion, and promotes detachment-mediated programmed cell death or apoptosis. These changes contribute to atherosclerotic lesion development observed in hyperhomocysteinemia. The UPR is activated in homocysteine-induced apoptosis (12, 137). Increasing the expression of TDAG51 was specific for homocysteine and not for the other structurally similar amino acids, such as methionine, cysteine, and homoserine. The steady-state mRNA levels of TDAG51 were increased and correlated with an increase in the steady-state mRNA levels of GRP78. The expression of TDAG51 was mediated by eukaryotic initiation factor-2α phosphorylation as well as UPR activation of PERK. The induction of TDAG51 by homocysteine involves ER stress and may contribute to the development of atherosclerosis (42). Homocysteine activates programmed cell death (apoptosis) through induction of the UPR that is signaled through IRE1. Induction of C/EBP homologous protein (CHOP), the non–ER-resident transcription factor, may be more closely related to homocysteine-induced cell death than GRP78/GRP94 (137). Herp, a novel integral membrane protein, was found to be upregulated in response to ER stress, including that induced by homocysteine (55, 65).

The ER is the major site of intracellular calcium storage, which appears to play an important role in apoptosis. High ER calcium storage leads to more calcium release and a stronger apoptotic signal. ER stress induces loss of calcium from the ER (69, 103). Alterations in ER calcium levels have profound effects on protein folding in this compartment (107). Homocysteine appears to induce calcium mobilization from the ER of cultured vascular smooth muscle cells (85), which can lead to the generation of ROS through the activation of cyclooxygenases and lipoxygenases (94). Thus, hyperhomocysteinemia appears to be associated with two independent cellular stress states: ER stress and oxidative stress. How homocysteine initiates stress pathways is the subject of considerable research in many laboratories.

Although substantial work has been done on the perturbation of the ER by homocysteine, a mechanistic understanding of homocysteine-induced ER stress remains elusive. Does homocysteine directly target cysteine residues of plasma membrane proteins associated with this organelle, or is homocysteine imported into the lumen of the ER, where it targets cysteine residues of chaperones, processing enzymes, or client proteins themselves? In either case, we propose that L-homocysteine is capable of launching a direct frontal assault on the ER (Fig. 8). The homocysteinylation of specific proteins that make up the luminal processing machinery could disrupt critical steps in

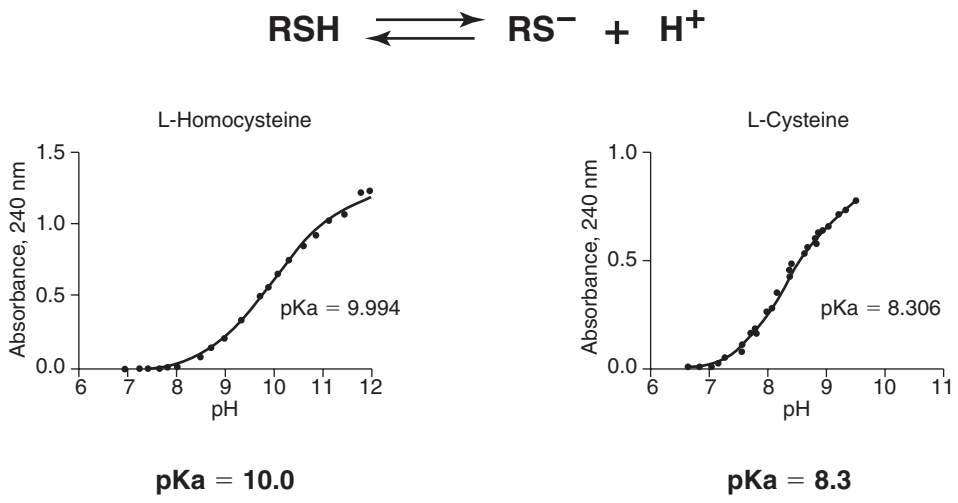

FIG. 9. Determination of the macroscopic sulfhydryl group pKa values for L-homocysteine (left) and L-cysteine (right). Absorbance of the thiolate anion (RS^-) was measured at 240 nm at the indicated pH in physiologic saline and buffer. The final concentration of L-homocysteine and L-cysteine was 250 μM. (Jacobsen, Catanescu, and Abramczyk, unpublished observations).

client protein folding. However, no evidence exists to support transport (uptake) of L-homocysteine by the ER. For that matter, the import and export of reduced and oxidized glutathione remains controversial (2, 14, 38, 58). If homocysteine gains entry to the lumen of the ER, will it target the cysteine residues of specific client proteins, or the cysteine residues of the proteins that make up the processing machinery? Studies to address these important questions are under way in this laboratory using high-specific-activity ^{35}S-L-homocysteine (100% optically pure) to identify *S*-homocysteinylated ER proteins in both *in vitro* and *in vivo* settings.

CHEMICAL BASIS FOR MOLECULAR TARGETING BY L-HOMOCYSTEINE

Homocysteine has the ability to form stable disulfide bonds with protein cysteine residues, even in the presence of other thiols. A case in point is homocysteinylated-albumin (Alb-Cys^{34}-S-S-Hcy), described earlier. In the circulation of healthy individuals, the concentration of Alb-Cys^{34}-S-S-Hcy ranges from 3 to 12 μM. The concentration of reduced thiols in circulation, which is the sum of cysteine, cysteinylglycine, and glutathione, is at least twofold higher, yet there appears to be no displacement of homocysteine from its albumin-binding site. Therapeutic approaches to lower protein-bound homocysteine in circulation with high concentrations of exogenous thiols have, in general, met with little success.

The stability of the protein-S-S-homocysteine disulfide bond is related to the high intrinsic pKa of its sulfhydryl group ($\sim$10.0). In contrast, the pKa of the sulfhydryl group of cysteine is only 8.3. Because of conflicting literature values for the –SH pKa values of cysteine and homocysteine, we recently redetermined them in this laboratory, as shown in Fig. 9.

The higher pKa of the sulfhydryl group of homocysteine has several consequences: (a) the thiolate anion will be at relatively low concentration at physiologic pH; (b) although $<$1% of homocysteine will be in the thiolate anion form, it is a highly reactive nucleophile, as our studies with Alb-Cys^{34}-S-S-Cys have demonstrated (112, 113); (c) the protein-S-S-homocysteine disulfide bond will be stronger than those with other thiols having lower sulfhydryl group pKa values; and (d) as a consequence, displacement of homocysteine from protein-S-S-homocysteine by other thiols will be limited.

SUMMARY AND CONCLUSIONS

The molecular-targeting hypothesis for homocysteine-induced pathogenesis has been presented in this review. Several extracellular protein targets have been identified and partially characterized. Although several possible intracellular proteins may be targeted by homocysteine, these are less well characterized, and much additional work is needed. This review has focused only on S-homocysteinylation of protein cysteine residues. It must be emphasized that homocysteine thiolactone carbonyl targeting of protein lysine residues is also important as a potential mechanism of homocysteine-induced vascular pathogenesis. Also not discussed in this review is the targeting of small molecules by homocysteine. It is well known that homocysteine will react with nitric oxide in the presence of a transition metal catalyst to form the nitrosothiol derivative of homocysteine. However, the direct interaction of homocysteine itself with nitric oxide is unlikely to account for the limited bioavailability of this signaling molecule in mild hyperhomocysteinemia. Finally, the molecular targeting of protein cysteine residues by homocysteine offers a new paradigm with which to consider vascular pathogenesis due to hyperhomocysteinemia.

ABBREVIATIONS

ADMA, asymmetric dimethylarginine; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; CM-H_2DCFDA, 5-(and -4)-chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate acetyl ester; DDAH, dimethylarginine dimethylaminohydrolase; Egr-1, early growth response 1; ER, endoplasmic reticulum; GADD153, growth arrest– and DNA damage–inducible gene 153; GPx-1, cellular glutathione peroxidase; GRP78/BiP, glucose-regulated protein 78/immunoglobulin chain–binding protein; HAECs, human aortic endothelial cells; HCMD, homocysteine-cysteine mixed disulfide; Herp, homocysteine-induced endoplasmic reticulum protein; HUVECs, human umbilical vein endothelial cells; IRE1, type-I ER transmembrane protein kinase; Lp(a), lipoprotein(a); MMP-2, metalloproteinase 2; MT, metallothionein; PERK, PKR-like ER kinase; ROS, reactive oxygen species; SREBP, sterol regulatory element–binding protein; TDAG51, T-cell death-associated gene 51; UPR, unfolded protein response.

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Address reprint requests to:
Donald W. Jacobsen, Ph.D.
Department of Cell Biology, NC-10
Lerner Research Institute
Cleveland Clinic
9500 Euclid Ave.
Cleveland, OH 44195

E-mail: jacobsd@ccf.org

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