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Forum Review

Homocysteine and Glutathione Peroxidase-1

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ABSTRACT

Mildly elevated homocysteine levels (Hcy) increase the risk for atherothrombotic vascular disease in the coronary, cerebrovascular, and peripheral arterial circulations. The molecular mechanisms responsible for decreased bioavailability of endothelium-derived nitric oxide (NO) by Hcy involve an increase of vascular oxidant stress and inhibition of important antioxidant capacity. Glutathione peroxidase-1 (GPx-1), a selenocysteine-containing antioxidant enzyme, may be a key target of Hcy's deleterious actions, and several experimental and clinical studies have demonstrated a complex relationship between plasma total homocysteine (tHcy), GPx-1, and endothelial dysfunction. Hcy may promote endothelial dysfunction, in part by decreasing GPx-1 expression; however, there is evidence to suggest that overexpression of GPx-1 can compensate for these effects. This review summarizes the current knowledge of the metabolism of Hcy, the effects of hyperhomocysteinemia observed in *in vitro* and *in vivo* models that lead to endothelial dysfunction and the possible mechanisms for these actions, and the role of GPx-1 in the pathogenesis of Hcy-induced cardiovascular disease (CVD). *Antioxid. Redox Signal.* 9, 1923–1940.

INTRODUCTION

Hyperhomocysteinemia has long been recognized as a risk factor for cardiovascular disease and venous thrombosis (50, 55). McCully (130), who noted a link between atherothrombotic changes at autopsy and levels of homocysteine (Hcy) in severe hyperhomocysteinemic and homocystinuric patients with different underlying genetic defects, first raised the hypothesis that elevations of plasma Hcy may contribute to atherosclerosis. As with many other cardiovascular risk factors, hyperhomocysteinemia may produce endothelial dysfunction, which is likely a consequence of oxidative inactivation of endothelium-derived nitric oxide (NO) (108, 120).

Auto-oxidation of Hcy *in vitro* generates reactive oxygen species (ROS), and treatment of cultured endothelial cells with Hcy decreases bioavailabe NO (200, 231) and produces cytotoxicity mediated by hydrogen peroxide (180). Alterations in cellular redox status may contribute to alterations in gene expression, endoplasmic reticulum (ER) stress, and activation of cholesterol biosynthesis.

Hcy may also cause oxidative stress, in part by inhibiting the expression of antioxidant enzymes, such as cellular glutathione peroxidase-1 (GPx-1), an effect that may lower the threshold for the cytotoxic effects of Hcy-derived hydrogen peroxide and lipid peroxides and/or augment the accumulation of ROS (145, 200). The accumulation of oxidants in hyperhomocysteinemia also suggests that restoration of the antioxidant capacity may prevent some of Hcy's damaging effects. In fact, impaired endothelium-dependent vasodilatory responses in hyperhomocysteinemic mice can partially be restored by administration of the thiol antioxidant L-2-oxothiazolidine-4-carboxylic acid (OTC), a precursor of glutathione, or by transgenic overexpression of GPx-1 (212, 215).

In clinical studies, Hcy and GPx-1 are among the strongest univariate predictors of future cardiovascular events, even after adjustment for other cardiovascular risk factors. In patients with GPx-1 activity below the median value, plasma Hcy levels above the median are associated with a three-fold increase in cardiovascular risk, whereas Hcy loses its independent risk prediction in individuals with increased antioxidant capacity, as

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reflected by high GPx-1 activity (164). These and other findings suggest threshold levels of GPx-1 activity are necessary for optimal protection against Hcy or other sources of oxidant stress.

This review summarizes the current knowledge of factors that may influence plasma Hcy levels, provides an overview of epidemiological studies on Hcy and cardiovascular disease risk, and discusses the effectiveness of folate in reducing Hcy levels and reducing cardiovascular risk. It also discusses the role of endothelial dysfunction in hyperhomocysteinemia, and presents mechanisms by which Hcy may limit the bioavailability of NO and alter cellular redox status. GPx-1 plays an important role in the pathogenesis of Hcy-induced vascular dysfunction, and this review will provide an overview of relevant experimental and clinical data that illustrate Hcy's negative effect on GPx-1 expression and the importance of GPx-1 in cardiovascular protection.

HOMOCYSTEINE AND HOMOCYSTEINE METABOLISM

Hcy is a sulfur-containing amino acid that functions as a key intermediate in methionine metabolism (Fig. 1). It is produced as a byproduct of methyl transfer reactions (58), which are important for the methylation of nucleic acids, proteins (including proteins methylated on arginine residues), neurotransmitters, and phospholipids (167). In the methionine cycle, methionine is converted to *S*-adenosylmethionine (SAM), which serves as a methyl donor for methyl transferases. The major product of these transfer reactions is *S*-adenosylhomocysteine (SAH), which is rapidly hydrolyzed to Hcy and adenosine in a reversible reaction by adenosylhomocysteinase. Importantly, accumulation of SAH can inhibit many transmethylases. The fates of intracellular Hcy are remethylation to methionine, transsulfuration to cystathionine, or transport from the cell.

In most tissues, the primary remethylation pathway for Hcy is catalyzed by the vitamin B_{12}-dependent enzyme methionine synthase (MS). This reaction utilizes the folate-containing methyl donor, 5-methyltetrahydrofolate, which is generated by 5,10-methylenetetrahydrofolate reductase (MTHFR). In the liver and kidney, a second remethylation pathway, using betaine (trimethylglycine) as a methyl donor for the enzyme betaine-homocysteine methyltransferase (BHMT), is present and dimethylglycine (DMG) is its metabolic product (167). In severe hyperhomocysteinemic patients, betaine is commonly used to increase Hcy remethylation and, thereby, therapeutically decrease Hcy (220).

Trans-sulfuration is an irreversible process that results in the production of cysteine and other sulfur-containing compounds, including glutathione, and involves two vitamin B_6-containing enzymes, cystathionine-β-synthase (CBS) and γ-cystathionase. This pathway is an important pathway in liver, which is the ma-

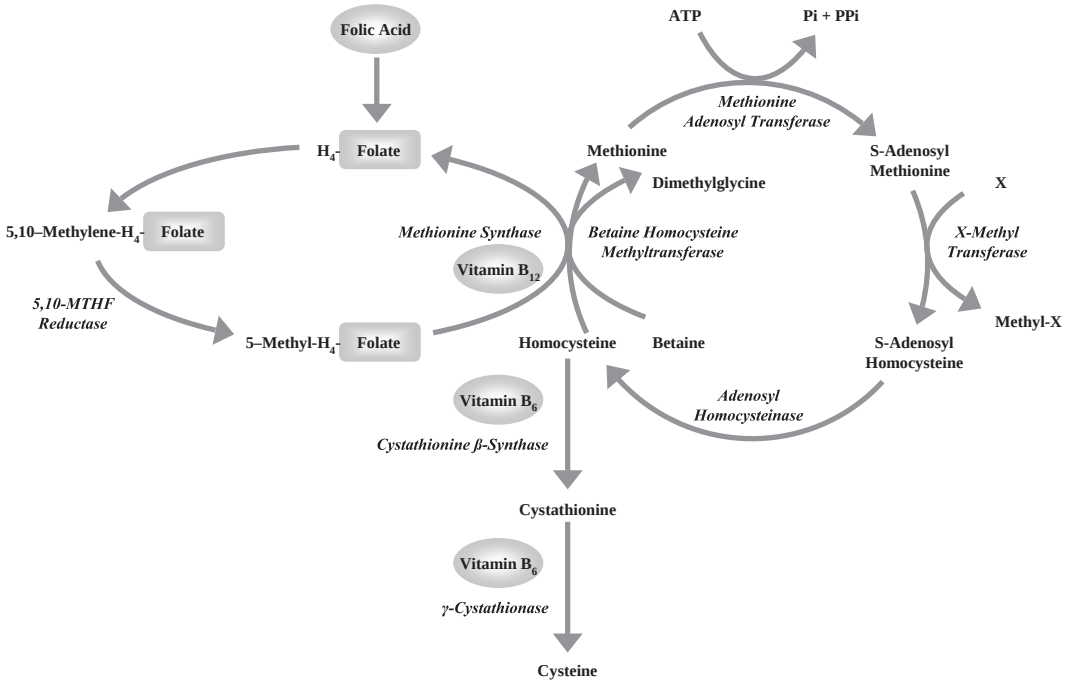

FIG. 1. Homocysteine metabolism. Homocysteine is produced as a byproduct of methylation pathways that use *S*-adenosylmethionine (SAM) as a methyl donor to produce *S*-adenosylhomocysteine (SAH), which is rapidly hydrolyzed to form homocysteine. Homocysteine is remethylated to methionine either by methionine synthase (MS) or by betaine-homocysteine methyltransferase (BHMT), using the methyl donors 5-methyltetrahydrofolate (5-methyl-H_4-folate) or betaine, respectively. The latter enzyme is only found in liver and kidney. MS is a vitamin B_{12}-dependent enzyme. Methylenetetrahydrofolate reductase (MTHFR) is involved in the synthesis of 5-methyl-H_4-folate. Homocysteine is further catabolized to form cysteine and other sulfur-containing compounds, including glutathione, in the transsulfuration pathway. The enzymes cystathionine β-synthase (CBS) and γ-cystathionase are both vitamin B_6-containing enzymes in the transsulfuration pathway.

jor source of circulating Hcy (134). Recent evidence indicates that the trans-sulfuration pathway may also be present in some other cell types (149, 157).

Hcy also can be exported into extracellular fluid and blood where it may undergo oxidation or disulfide exchange reactions with plasma proteins (170). Plasma total homocysteine (tHcy) is a mixture of reduced (free) Hcy and oxidized Hcy, which includes homocystine or mixed disulfides (cysteine-homocysteine or glutathione-homocysteine) (20–30%) and protein-bound disulfides (70–80%) (127, 135, 197). Only ~1% or less of tHcy species consists of free Hcy under physiological conditions.

HYPERHOMOCYSTEINEMIA

In humans, normal tHcy levels range from 5 to 15 μM (87, 110, 197); mildly elevated plasma levels of Hcy (>12 μM) (169) are an independent risk factor for atherothrombotic vascular disease. Causes of intermediate or severe hyperhomocysteinemia include renal insufficiency and genetic defects in the metabolism of Hcy or methionine, which lead to tHcy levels of 30–50 μM in the former and 100–300 μM in severe forms of the latter (68, 93). Hcy levels may also be increased in men compared with women and increase with age, menopause, alcoholism, or hypothyroidism, as well as in those taking a variety of medications, including agents that inhibit folate uptake and metabolism, those that promote methylation reactions, and other agents (*e.g.*, methotrexate, theophylline, isoniazid, cholestyramine, metformin, fenofibrate, levodopa, niacin, L-arginine, phenytoin, nitrous oxide, and trimethoprim) (4, 11, 110, 165, 181, 217). Importantly, elevated tHcy levels are associated with an increase in the prevalence of coronary artery disease, suggesting that the therapeutic use of drugs or supplements that raise tHcy levels may have undesirable cardiovascular consequences (159).

GENETIC DEFECTS THAT CAUSE HYPERHOMOCYSTEINEMIA AND CARDIOVASCULAR DISEASE

Many environmental and genetic factors can influence Hcy concentrations (165, 167). Severe hyperhomocysteinemia is inherited as a recessive disorder and is most commonly caused by mutations in the CBS gene, in which over 130 mutations have been characterized (updated list found at http://www.uchsc.edu/sm/cbs/, accessed June 1, 2007) (101). Other rare mutations in genes involved in Hcy metabolism may also cause severe hyperhomocysteinemia (66).

In comparison to the rare mutations causing severe hyperhomocysteinemia, a common polymorphism in the MTHFR gene (*C677T*) produces a thermolabile enzyme with reduced activity caused by a valine substituted for an alanine (94). This polymorphic form can be stabilized with high folate levels, and in clinical studies, a gene-diet interaction between the *C677T* mutant and folate has been shown to influence Hcy levels. Individuals with low folate and the less prevalent polymorphism have even higher levels of Hcy; by contrast, in the presence of adequate folate, the polymorphism has no effect on Hcy levels (88).

In 1995, Frosst and co-workers (64) suggested that the common *C677T* polymorphism is a likely risk factor for cardiovascular disease (CVD); however, opposite results were published about the association between the *TT* genotype and CVD risk (29, 97, 122). The controversy about the influence of the MTHFR genotype on CVD is still unresolved, but one explanation for the lack of association in some studies may be adequate folate status in the study population offsetting the effect of the polymorphism on tHcy levels. Based on this hypothesis, a meta-analysis performed by the MTHFR Studies Collaboration Group (96) found that individuals with folate levels below the median and the *TT* genotype have a greater risk for coronary heart disease (CHD) (OR 1.44, 95% CI 1.12–1.83) than individuals with the *CC* genotype and folate levels above the median.

HYPERHOMOCYSTEINEMIA AND ATHEROTHROMBOSIS: EPIDEMIOLOGICAL EVIDENCE

In 1969, McCully first proposed that hyperhomocysteinemia is a risk factor for CVD based on the finding of atherosclerotic plaque at autopsies of young people with homocystinuria and severe hyperhomocysteinemia caused by inborn errors of methionine metabolism (130). This hypothesis was later modified to include a broader population, positing that mild hyperhomocysteinemia caused by dietary deficiencies of the vitamin cofactors required for the metabolism of Hcy—folic acid, vitamin B_{12}, and vitamins B_6—is a risk factor for atherothrombosis.

Several epidemiological studies demonstrated that a mild to moderate elevation of tHcy (15–50 μM) is an independent risk factor for arterial and venous diseases (26, 28, 42, 123, 137, 158, 196, 221), which include peripheral vascular disease (38), venous thrombosis (49), coronary artery disease (42, 141), and cerebrovascular disease (1, 147, 209). In 1995, a meta-analysis by Boushey and colleagues (28) of 27 observational studies determined that a 5 μM elevation in tHcy increases CAD risk as much as a 20 mg/dL increase in cholesterol. The increased risk for CAD was 1.6–fold (95% CI 1.4–1.7) in men and 1.8–fold (95% CI 1.3–1.9) in women; in peripheral arterial disease, 6.8–fold (95% CI 2.9–15.8); and in cerebrovascular disease, 1.5–fold (95% CI 1.3–1.9). These finding set the stage for large, retrospective case-control studies, such as the European Concerted Action Project (68), which reported significantly higher levels of tHcy in patients with atherosclerosis compared to control subjects.

The results from prospective studies, however, have been less consistent. For example, the Physician's Health Study 5-year update (179) reported an increase in relative risk for myocardial infarction (MI) in individuals with elevated tHcy, whereas the 7.5–year update (37) failed to find a significant association between elevated tHcy and CVD events. In other recent studies of high-risk CHD patients (2, 5, 143, 184, 218), modest elevations in tHcy (cutoffs as low as 14.1 μM) correlated with

increased mortality or an increased risk of MI. The Homocysteine Studies Collaboration found in a meta-analysis of prospective studies of first events that a 25% lower tHcy (a reduction of ~3 μM) is associated with an 11% lower risk of ischemic heart disease (OR 0.89, 95% CI 0.83–0.96) and a 19% lower risk of stroke (OR 0.81, 95% CI 0.69–0.95). They also reported a modest 16% increase in risk for CVD in patients with no history of CVD who are in the top quintile of baseline tHcy, after combining data from prospective studies published between 1966 and 1999 (1). The results of prospective studies of recurrent cardiovascular events are more consistent than those for first events; for a recurrent event, the hazard ratio increases by 16% with each increase of 5 μM in the serum Hcy concentration (209). Similar results have been reported in a meta-analysis of prospective studies with no significant heterogeneity, with a significant average relative risk for elevated tHcy and CHD of 1.34 (95% CI, 1.17–1.54) (13). Several large, population-based, prospective studies, such as the Framingham Heart Study, also found that modest hyperhomocysteinemia significantly increases the risk for all-cause mortality and CVD mortality after adjustments for other cardiovascular risk factors (27). AFCAPS/TexCAPS and the Women's Health Study (156) reported that modest elevations of tHcy can increase the risk of CVD even in otherwise healthy individuals.

Several studies have documented an association between mortality in patients with pre-existing coronary (98, 99) or peripheral vascular disease and plasma Hcy levels, independent of traditional risk factors (3, 5, 40, 81, 141, 182, 191), or with cerebrovascular events in patients with significant stenosis of the carotid artery (183), suggesting that Hcy may promote acute thrombotic events leading to cardiac death or stroke. Plasma Hcy levels were related to the extent of atherosclerosis in coronary and peripheral arteries (36, 166, 203, 205, 208) and to greater carotid artery intimal-medial wall thickness in cross-sectional studies of subjects with subclinical vascular disease (124, 168, 222). Taken together, the findings that elevated Hcy may promote CVD suggests the importance of understanding the underlying mechanisms to develop new targeted therapies that compensate for Hcy's adverse effects. A unique target for preventive approaches is based on the hypothesis that lowering serum Hcy levels with the use of inexpensive, naturally occurring cofactors can reduce the risk of cardiovascular events. In several clinical studies, including recent, randomized and placebo-controlled intervention trials, supplementation with folic acid, vitamin B_6, and vitamin B_{12} was used to reduce tHcy, and in most patients, tHcy was decreased by folic acid with or without various vitamin B supplements (41). Recent large studies, however, showed no clinical benefit of the use of folic acid and vitamin B_{12} (with or without the addition of vitamin B_6) treatments in patients with established vascular disease (25, 119, 194), regardless of the Hcy lowering effect of these treatments.

Explanations for the fact that folic acid and vitamin B supplementation do not improve cardiovascular risk regardless of the consequences of folic acid fortification on Hcy levels are not entirely clear; however, there are potential mechanisms that warrant consideration. Folic acid promotes cell proliferation, and a recent study of patients who had undergone angioplasty and were treated with folic acid, vitamin B_{12}, and vitamin B_6, showed worsening rates of in-stent-restenosis in patients treated with vitamins compared to those treated with placebo (104). Based on the relation of Hcy to the methylation cycle, high Hcy concentrations are associated with a reduced methylation potential, an effect that folic acid and vitamin B_{12} can reverse. Alteration of the methylation potential may change cell proliferation rates, modify gene expression (227), and affect endothelial function by modulating the activity of nitric oxide synthase (NOS) (23). Despite the fact that there is no clinical benefit from the use of folic acid, long-term treatment with folic acid (5 mg) in individuals with high-normal Hcy levels have been found to improve endothelial function (17, 193). One possible explanation for these results is that folic acid may also have antioxidant properties that can improve vascular function through mechanisms independent of its effect on tHcy levels (6, 52). For a better understanding of the relations in this complicated metabolic pathway and their association with atherothrombotic mediators, further exploration will be needed and alternative approaches to reducing Hcy levels should be considered.

IN VITRO AND *IN VIVO* MODELS OF HYPERHOMOCYSTEINEMIA AND ENDOTHELIAL DYSFUNCTION

Impairment of endothelium-dependent relaxation of blood vessels with normal endothelium-independent vasodilation, commonly referred to as endothelial dysfunction (160), is associated with many cardiovascular risk factors, such as hypertension, hypercholesterolemia, and diabetes mellitus (30). Endothelial dysfunction is not merely a marker of CVD, but also has prognostic implications for adverse clinical outcomes and is associated with a poor outcome in the setting of existing CAD, predicting coronary disease progression and cardiovascular event rate (132, 163, 188). Endothelial dysfunction is characterized by decreased vasodilator capacity and NO insufficiency, which may contribute to activation of circulating leukocytes and platelets, activation of prothrombotic and inhibition of fibrinolytic mechanisms, and stimulation of vascular smooth muscle cell proliferation (61). It also facilitates the interactions between modified oxidized lipoproteins and monocyte-derived macrophages and between T-cells and the arterial wall, inciting early and promoting late atherosclerotic processes. All of these effects participate in the initiation and progression of atheroma formation and thrombus development (214).

Hcy (10 μM to 5 mM), but not cysteine (20 μM to 5 mM), has been shown to decrease the production and/or bioactivity of NO and of S-nitrosothiols in cultured endothelial cells (200, 215, 231). Nitric oxide production from endothelial cells is dose-dependently decreased by Hcy as measured by photolysis-chemiluminescence (200), by an NO-selective electrode (231), or with a recently developed fluorescent dye (207). In patients with hyperhomocysteinemia, flow-mediated endothelium-dependent vasodilatation (16, 32, 33, 92, 153, 201), as well as vasodilation induced by acetylcholine, are impaired (114). Acute elevation of tHcy after a methionine challenge (16, 34, 35, 76, 201), or chronic mild hyperhomocysteinemia (190, 223) impairs endothelium-dependent vasodilator function induced by shear stress, acetylcholine, or bradykinin, while re-

sponses to sodium nitroprusside or nitroglycerin (endothelial-independent vasodilation) are generally preserved. Additionally, plasma levels of the NO-derived endproducts, nitrite and nitrate, are significantly lower in hyperhomocysteinemic subjects than in healthy controls, suggesting that Hcy reduces bioavailable NO (80). Recent studies with human subjects have demonstrated that administration of antioxidant vitamins, such as ascorbate and α-tocopherol, can attenuate impairment of endothelium-dependent vasodilation during acute hyperhomocysteinemia produced by oral methionine loading (33, 92, 139, 153), indicating that oxidative mechanisms are involved in the Hcy-induced attenuation of endothelial function.

Impairment of endothelium-dependent relaxation of blood vessels is a consistent finding observed in various animal models of hyperhomocysteinemia, such as diet-induced hyperhomocysteinemia in cynomolgus monkeys (111, 114) or rats, (10, 152, 199, 230), heterozygous CBS ($CBS^{+/-}$) deficiency in mice, or the combination of genetic and dietary abnormalities (32, 45, 46, 54, 109). Eberhardt and colleagues (54) observed that in $CBS^{+/-}$ mice, there was paradoxical vasoconstriction of the mesenteric arteries in response to the vasodilators methacholine or bradykinin. These $CBS^{+/-}$ mice had tHcy levels two-fold higher than wild-type mice. Paradoxical vasoconstriction to cholinergic stimulation is known to occur in atherosclerotic coronary arteries (121), and is a hallmark of endothelial dysfunction. In $CBS^{+/-}$ mice, vascular dysfunction occurred without a change in endothelial-independent vasodilator responses and without alteration in endothelial nitric oxide synthase (eNOS) protein levels, suggesting that the lack of bioavailable NO may be caused by NO inactivation. In support of this hypothesis, $CBS^{+/-}$ mice have a deficiency in the acetylcholine-stimulated production of cyclic guanosine monophosphate (cGMP), the second messenger of NO-mediated vasodilation, in aortic rings in association with an increase in markers of oxidant stress, including increased immunodetectable aortic 3-nitrotyrosine and increased F_2-isoprostane levels. Increased endothelial activation was also observed in $CBS^{+/-}$ mice as indicated by increased immunodetectable P-selectin in aortic sections (54, 212, 215).

In other studies, Lentz and colleagues (45, 109) manipulated Hcy levels by dietary restriction of folate or methionine supplementation in $CBS^{+/-}$ and wild-type $CBS^{+/+}$ mice. In these studies, maximal relaxation of aortic rings and cerebral arterioles to the endothelium-dependent dilator acetylcholine was significantly impaired in $CBS^{+/-}$ mice fed the low folate or high methionine diets (45, 46, 109), whereas relaxation in response to the endothelium-independent vasodilator nitroprusside was unaffected. Similar findings were obtained in MTHFR-deficient and MS-deficient mice (45, 51). In some of these studies, plasma tHcy levels in $CBS^{+/-}$ and $CBS^{+/+}$ mice were nearly identical, and dietary manipulation was necessary to promote vascular dysfunction concurrent with increases in Hcy levels to 20–30 μM in $CBS^{+/-}$ mice. Interestingly, dietary treatments induced impairment of endothelium-dependent dilatation of cerebral arterioles not only in $CBS^{+/-}$ mice with moderate hyperhomocysteinemia (plasma tHcy $\sim$20 μM) but also in $CBS^{+/+}$ mice with mild hyperhomocysteinemia (plasma tHcy $\sim$8 μM) (45). This observation contrasts with previous findings from this group using aortic rings in which only vessels from $CBS^{+/-}$ mice with plasma tHcy $>$ 20 μM exhibited impaired relaxation responses to acetylcholine (109). In the study by Eberhardt and colleagues (54), endothelial dysfunction was detected in mesenteric arterioles and acetylcholine-induced cGMP production in aortic rings was reduced in $CBS^{+/-}$ mice that had Hcy levels of $\sim$9.2 μM. One possible explanation for these differences is that other factors influence vasodilation in aortic rings in addition to cGMP-mediated downstream effects on vascular tone; differences in basal diets used in different labs may also contribute to alterations in basal Hcy levels in $CBS^{+/-}$ mice and differences in vascular function. Another possibility is that different vascular beds are more sensitive to the effects of mild hyperhomocysteinemia than others and that arterioles from cerebral beds or mesenteric beds are more responsive than the aorta (54, 206, 215). In support of this interpretation, hypertrophy of cerebral arterioles has been observed in $CBS^{+/+}$ and $CBS^{+/-}$ mice with mild hyperhomocysteinemia (plasma tHcy $>$8 μM) (12), whereas no structural changes were reported in aorta from $CBS^{+/-}$ mice in studies in which endothelial stimulated-cGMP production was reduced. Overall plasma levels of tHcy in mice are generally lower than plasma tHcy levels in humans.

MECHANISM OF HOMOCYSTEINE-INDUCED OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

The major functional abnormality underlying hyperhomocysteinemia-induced endothelial dysfunction appears to be decreased bioavailability of endothelium-derived NO (57), which may reflect an absolute deficit of NO, impaired availability of bioactive NO, or enhanced NO inactivation. As endothelium-independent vasodilation is normal, the responsiveness of vascular smooth muscle to NO is relatively unaffected (54). NO is a potent vasodilator that is produced by eNOS in response to physiological stimuli such as acetylcholine, thrombin, bradykinin, or shear stress, and is a major mediator of endothelium-dependent relaxation in both large arteries and small resistance vessels (30). NO may function as an endogenous anti-atherogenic molecule by maintaining low arterial tone at rest, inhibiting leukocyte-endothelial interactions, attenuating platelet activation, and inhibiting smooth muscle cell proliferation (138). Thus, decreased bioavailability of NO may contribute to a pro-atherogenic state.

One mechanism for endothelial dysfunction during hyperhomocysteinemia is based on the oxidative stress hypothesis (Table 1) (120, 213), and evidence in support of this mechanism has been obtained in animals using both pharmacological

TABLE 1. MECHANISMS THAT INDUCE OXIDANT STRESS DURING HYPERHOMOCYSTEINEMIA

- NAD(P)H oxidase activation
- Uncoupled eNOS
- ER stress and protein misfolding
- Autooxidation
- Alterations in redox capacity (thiol oxidation)
- Reduction of GPx-1

(33, 54, 92, 198, 212) and genetic approaches (47, 215). Hyperhomocysteinemia-induced oxidant stress not only leads to biological inactivation of NO (54, 57), but also may lead to lipid peroxidation at the cell surface and direct oxidation of low-density lipoprotein (77, 79, 224), and may indirectly contribute to oxidative modification of low-density lipoprotein mediated by vascular cells *in vitro* (77, 79, 176, 224). Oxidation of Hcy may directly lead to ROS production, or Hcy may promote ROS and reactive nitrogen species (RNS) generation by several indirect mechanisms (Fig. 2). The mechanisms by which oxidative stress contribute to endothelial dysfunction are discussed below.

Theoretically, accumulation of hydrogen peroxide may lead to inactivation of NO. Hydrogen peroxide (180), which is produced from superoxide via superoxide dismutase, decomposes via a reaction catalyzed by transition metal ions (iron and copper) to hydroxyl radical, a toxic ROS (77, 91), which is highly reactive and causes lipid peroxidation. Elevated levels of lipid peroxides lead to an increase in peroxyl radicals that can inactivate NO through the formation of lipid peroxynitrites (142, 161). Evidence for a role of hydrogen peroxide in Hcy-induced endothelial toxicity *in vitro* has been found, as catalase inhibits Hcy-induced lysis of endothelial cells in the presence of transition metals or ceruloplasmin (180, 210), but this lytic effect is only observed at concentrations of Hcy far exceeding those observed in the most severe cases of hyperhomocysteinemia. An increase in the fluorescence of a hydrogen peroxide-sensitive 2′,7′-dichlorofluorescein probe (DCF) has been observed in cultured endothelial cells (39, 231) and cultured vascular smooth muscle cells exposed to Hcy (140), thus providing evidence of ROS accumulation by Hcy.

Superoxide anion (103) can react with NO in a diffusion-limited reaction to form peroxynitrite (151), which does not induce vasorelaxation (70) and is itself highly reactive. Peroxynitrite may further react with cellular tyrosine residues to form nitrosated end-products (15, 72). An increase in immunodetectable 3–nitrotyrosine has been found in aortic tissue from $CBS^{+/-}$ mice and in protein lysates from aortic rings cultured in Hcy-containing media (54, 136). This marker of protein oxidation can alter protein function and is associated with vascular diseases (150). Generation of superoxide during hyperhomocysteinemia may result from activation of vascular NAD(P)H oxidases (14, 21, 31, 46, 69, 175, 198, 204), a large enzyme composed primarily of two membrane-bound subunits ($p22^{phox}$ and $gp91^{phox}$), three cytosolic subunits ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$), and guanosine triphosphate (GTP)-binding proteins (such as rac-1) (8), or uncoupling of eNOS (187).

Uncoupling of eNOS can be caused by oxidation of its cofactor tetrahydrobiopterin, resulting in the preferential production of superoxide rather than NO by the enzyme. Uncoupling of eNOS during hyperhomocysteinemia may be mediated by peroxynitrite (106), as uric acid (45), an inhibitor of peroxynitrite-mediated oxidation (177), decreases dihydroethidium fluorescence, in $CBS^{+/-}$ mice fed a high methionine diet. Pharmacological inhibition of eNOS by L-nitroarginine methyl ester (L-NAME) supports the hypothesis that Hcy causes uncoupling of eNOS and contributes to Hcy-induced production of superoxide in cultured endothelial cells (78) and hyperhomocysteinemic mice (45). L-NAME blocks the Hcy-dependent stimulation of ROS formation and abrogates both the Hcy-dependent increases in isoprostane $F_2\alpha$-III formation and DCF fluorescence.

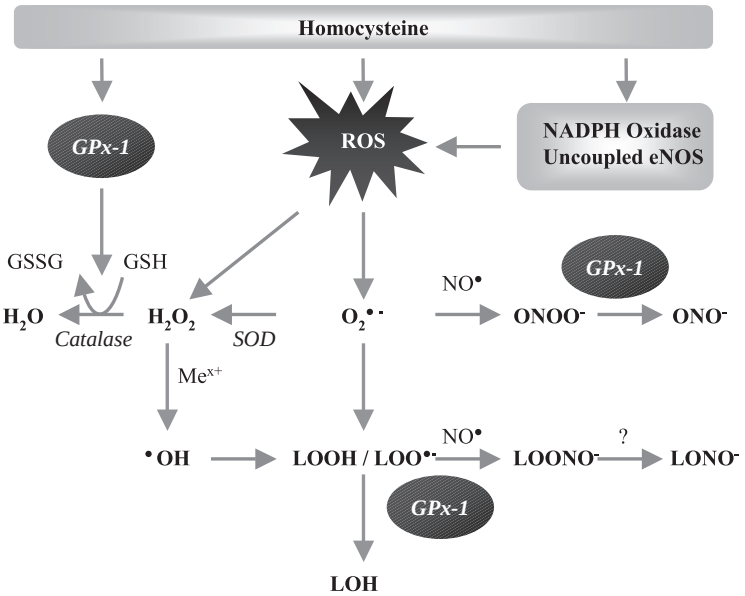

FIG. 2. Homocysteine augments reactive oxygen species production. Auto-oxidation of homocysteine to homocystine may directly lead to reactive oxygen species (ROS) production. Generation of superoxide anion ($O_2^{\cdot -}$) may result from activation of vascular NADPH oxidases or uncoupling eNOS caused by hyperhomocysteinemia. Hydrogen peroxide (H_2O_2) can also be produced from superoxide via superoxide dismutase (SOD) and can be reduced to water by catalase or glutathione peroxidase-1 (GPx-1). During this process, reduced glutathione (GSH) becomes oxidized glutathione (GSSG). In the presence of transition metals such as copper or iron (Me^{x+}), H_2O_2 may react to form a hydroxyl radical ($^{\cdot}OH$) which leads to the generation of lipid peroxides (LOOH). The hydroxyl radical may also promote the generation of lipid peroxyl radicals ($LOO^{\cdot}$), and both superoxide and lipid peroxyl radicals may react rapidly with endothelium-derived nitirc oxide ($NO^{\cdot}$) to produce peroxynitrite ($ONOO^-$) or lipid peroxynitrite ($LOONO^-$), respectively. This may lead to NO inactivation, tyrosine nitrosation and lipid oxidation. The protective role of GPx-1 may be mediated by reduction of peroxides and lipid peroxides to their respective alcohols (LOH). GPx-1 can also convert peroxynitrite to nitrite (ONO^-) and may reduce lipid peroxynitrites. A decrease in GPx-1 activity may augment homocysteine's damaging effects.

Another possible mechanism by which elevated Hcy uncouples eNOS is the inhibition of endogenous NOS by asymmetric dimethylarginine (ADMA) (185, 202). ADMA is derived from protein methylation reactions (131) that produce Hcy as a byproduct, and are either excreted in the urine or metabolized to citrulline by the enzyme dimethylarginine diemethylaminohydrolase. Hcy inhibits this enzyme by directly binding to it covalently, forming a mixed disulfide with the active site cysteine; this adverse effect can be prevented by thiol antioxidants and dithiothreitol (DTT) (186), as well as by increased oxidant stress (86). In addition, physiological levels of Hcy can interfere with L-arginine uptake by the Y^+ transporter [at least in platelets (116)] resulting in decreased eNOS activity and uncoupling owing to limited substrate availability. In cynomolgus monkeys fed a methionine-rich, folic acid-depleted diet free of choline, and in healthy humans after an oral methionine challenge, plasma levels of ADMA increase (23, 24). Similary, ADMA is increased in a dose- and time-dependent fashion in endothelial cells or nonvascular cells exposed to Hcy or to its precursor methionine. Reduction of eNOS activity in these cases would also enhance superoxide formation; however, one recent study suggests that eNOS activation (and expression) are altered by hyperhomocysteinemia in $CBS^{+/-}$ mice and in cultured endothelial cells, primarily through protein kinase C (PKC) activation, and not by an oxidant mechanism as the antioxidant enzymes superoxide dismutase, catalase, and GPx-1 did not compensate for the adverse effect of Hcy (89). This result is in direct contrast to prior studies that reported no changes in eNOS expression in $CBS^{+/-}$ mice (54).

Regardless of the source of superoxide, its role in Hcy-induced endothelial dysfunction is underscored by the demonstration of greater superoxide production in aortic tissue from mildly hyperhomocysteinemic $CBS^{+/-}$ mice compared with wild-type mice (54). Similar results were demonstrated in cultured endothelial cells incubated with Hcy (103). Enhanced superoxide and/or hydrogen peroxide formation seems to be a key mediator of Hcy-induced endothelial dysfunction as scavenging of superoxide anion by either superoxide dismutase or 4,5-dihydroxybenzene-1,3-disulfonate (tiron) reversed the effect. In other studies, superoxide dismutase reversed the paradoxical vasoconstriction of mesenteric arterioles of $CBS^{+/-}$ mice in response to stimulation with β-methacholine (54) and reversed the decreased cerebral (cortical) blood flow during superfusion with Hcy-containing buffer (215). The superoxide scavenger tiron has also been shown to inhibit the effect of Hcy on acetylcholine- and A23187-induced relaxation of rabbit aortic rings, (103) and can partially restore vasodilation of cerebral arterioles to acetylcholine in $CBS^{+/+}$ mice or $CBS^{+/-}$ mice fed a methionine-rich diet (46). Another recent study found that superoxide and not other ROS principally involved in lipid peroxidation: overexpression of Cu, Zn superoxide dismutase blocked the effect of Hcy on endothelial cell lipid peroxidation (78). Importantly, in this study neither the addition of extracellular catalase nor loading cells with catalase had any effect on lipid peroxidation, indicating that hydrogen peroxide was not responsible for cellular lipid peroxidation.

Further support for the link between Hcy-dependent endothelial dysfunction and oxidative stress has been established by the fact that the impaired endothelium-dependent vasodilatory response in mesenteric arterioles of $CBS^{+/-}$ mice can be restored toward normal by administration of the thiol antioxidant OTC (212) or by transgenic overexpression of GPx-1 (215). Bagi and colleagues (9) also demonstrated that administration of ascorbate prevents endothelial dysfunction in gracilis muscle arterioles of hyperhomocysteinemic rats. In addition, several clinical studies showed that treatment with antioxidant vitamins prevents endothelial and platelet dysfunction associated with hyperhomocysteinemia induced by an oral methionine challenge (33, 92, 139, 153).

Another possible mechanism of Hcy actions is via activation of the endoplasmic reticulum (ER) stress pathway. Hcy contains a reactive thiol group that can undergo disulfide exchange reactions (170) and disrupt the folding and processing of newly synthesized proteins in the ER leading to the cellular unfolded protein response (UPR) (107), which ultimately alters the translational program of cells (112, 113, 162). In binding to free thiols in proteins, Hcy may have no effect on protein function or may alter protein function by interfering with protein folding, protein-protein interactions, or other protein activities that require reactive cysteinyl residues. Many of the cell culture experiments on ER stress pathways and Hcy used superphysiological levels of Hcy suggesting some of these effects may be due to the reductive effects of Hcy rather than oxidative effects. Nevertheless, mild hyperhomocysteinemia has been shown to promote the expression of some ER stress-response genes (145, 216), including those that mediate cell growth and differentiation (*e.g.*, GADD45, GADD153, Id-1, cyclin D1, and FRA-2) *in vitro* and *in vivo* (145). ER stress and oxidative stress may thus be linked in some way with one augmenting the other in hyperhomocysteinemia, leading to apoptosis and cell death (233). GPR78 mRNA is also elevated in the livers of $CBS^{+/-}$ mice that have mild hyperhomocysteinemia (144), and overexpression of GRP78 by Hcy may protect cells from apoptosis (155). The cellular consequence of ER stress include dysregulation of lipid metabolism, activation of inflammatory pathways, increased proteolysis of misfolded proteins, and apoptotic cell death in endothelial cells (7, 229), findings that support the observation that Hcy causes a dose-dependent decrease in DNA synthesis and proliferation of endothelial cells (145, 195). Through reprogramming cellular translation in ER stress, Hcy can also be involved in cholesterol biosynthesis, as studies have been shown that (millimolar concentrations of) Hcy increases the expression of the sterol response-binding protein (SREBP), a nuclear factor causing upregulation of mRNA levels of genes involved in cholesterol biosynthesis, such as HMG-CoA reductase, isopentenyl diphosphate (IPP) isomerase, and farnesyl diphosphate (FPP) synthase in hepatic cells, human endothelial cells, and human aortic smooth muscle cells (216).

In addition to promoting NADPH oxidase activation or eNOS uncoupling, Hcy has classically been thought to autooxidize to form homocystine, oxidize other thiols such as cysteine and glutathione to form mixed disulfides, or oxidize cysteinyl residues on proteins and peptides to form mixed disulfides. Thus, Hcy may significantly alter cellular redox balance, a concept supported by the decreased ratio of reduced-to-total aminothiols in plasma in experimental hyperhomocysteinemia after a methionine challenge (71, 126, 127) and in hyperhomocysteinemic patients (5, 125, 128, 129).

The intracellular redox buffer system consisting of reduced glutathione and glutathione disulfide, together with glutathione reductase and glutathione peroxidase, plays a central role in the cellular defense against oxidant stress and has an important role in maintaining endothelial function. This point is underscored by the finding that NO synthesis is impaired in glutathione-depleted human endothelial cells and, conversely, bolstering cellular glutathione content with glutathione monoethyl ester results in enhanced NO production (67). The role of the cellular redox state in Hcy-induced endothelial dysfunction is emphasized by the observation that treatment of $CBS^{+/-}$ mice with the intracellular cysteine donor L-2-oxo-4-thiazolidine carboxylate increases intracellular levels of reduced glutathione, shifting the cellular redox state to a more reduced environment and resulting in restoration of endothelial function (212).

The cellular defense system against ROS includes several antioxidant enzymes and nonenzymatic antioxidants, such as α-tocopherol, ascorbic acid, β-carotene, and glutathione (Fig. 3). Hcy has been shown, in particular, to decrease the normal function and expression of GPx-1, the important cellular antioxidant enzyme. Hcy, but not other low-molecular-weight thiols, decreases both the expression and specific activity of GPx-1 as shown *in vitro* and *in vivo* (83, 145, 200, 212, 215). This key enzyme for the cellular defense against oxidants uses glutathione to reduce hydrogen peroxide to water and lipid peroxides to their respective alcohols (59), and may also act as a peroxynitrite reductase (174). Treatments that enhance GPx-1 activity have been shown to improve NO production and vascular responsiveness.

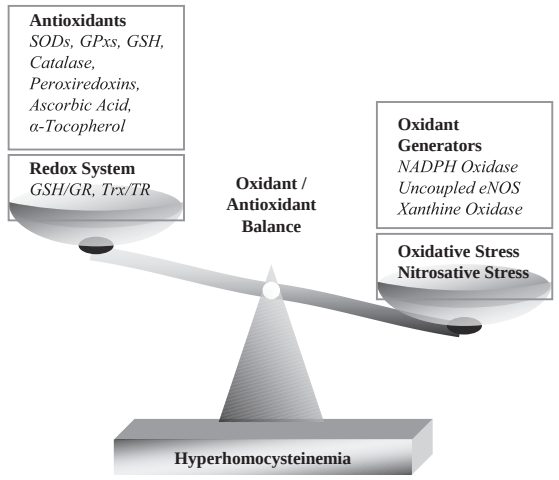

FIG. 3. The oxidant/antioxidant balance. The cellular defense system against reactive oxygen species (ROS) includes several antioxidant enzymes and nonenzymatic antioxidants, such as α-tocopherol, ascorbic acid, and glutathione. Homocysteine has been shown, in particular, to decrease the normal function and expression of GPx-1, an important cellular antioxidant enzyme. Hyperhomocysteinemia may also activate NADPH oxidase and uncouple eNOS. Increased ROS from these and other sources (*e.g.*, xanthine oxidase) may increase oxidative and nitrosative stress.

GLUTATHIONE PEROXIDASE-1: A POTENTIAL UNIFYING MECHANISM

First characterized in red blood cells (146), the glutathione peroxidases comprise a group of selenocysteine-containing proteins, and cellular glutathione peroxidase, GPx-1, is one of the major intracellular antioxidant enzymes (53). In 1979, Perona and colleagues (148) presented evidence that neonatal deficiency of the selenoenzyme GPx-1 may result from 'selenium imbalance' during pregnancy. Subsequent studies with selenium deficiency in animals and in patients on long-term total parenteral alimentation found that glutathione peroxidase protein (189) and activity in erythrocytes, granulocytes, and platelets is low. Replacement with intravenous selenous acid results in slow recovery of glutathione peroxidase activity in erythrocytes over a 3-month period as recovery occurs only in cells generated in the presence of selenium (43).

Selenium is necessary for the translation of glutathione peroxidases in the form of selenocysteine, an amino acid that is structurally similar to cysteine with selenium in place of sulfur and occurs at the catalytic site of GPx-1 (residue 48) (Fig. 4). A specialized mechanism of selenocysteine incorporation involves decoding of the UGA codon, normally a nonsense or stop codon in translation, as a selenocysteine codon (Fig. 5) (53, 117, 171, 178). This translational process requires other *cis*-elements in the mRNA, notably the selenocysteine incorporation sequence (SECIS) element that forms a putative stem-loop structure in the 3′-untranslated region (UTR). Transfer of the SECIS element to the UTR of heterologous mRNAs has been shown to promote recognition of a UGA embedded in the protein-coding region of a transcript as a site for selenocysteine incorporation (115, 118). An important requirement for read-through includes insertion of the SECIS element downstream of the protein-coding region in the proper orientation. Selenocysteine incorporation requires the formation of a stable complex of the tRNA specific for the selenocysteine amino acid ($tRNA^{SEC}$) that has an anticodon which recognizes the UGA, the elongation factor for selenocysteine incorporation (eF^{SEC}), and selenocysteine-specific mRNA at the ribosome (19, 53, 228). Additional translational cofactors that bind RNA, such as SBP2, may serve to stabilize this complex (53, 117, 171, 178). This specialized process is less efficient than insertion of the other common amino acids, and selenoprotein expression may be affected by availability of selenium as well as other factors that influence translation and/or the stability of selenoprotein encoding RNAs (18, 20, 74, 232).

GENETIC VARIANTS OF GLUTATHIONE PEROXIDASE-1

GPx-1 gene is located on chromosome 3p21.3 (90, 95, 219), contains two exons (85), and has common polymorphisms, one of which is caused by different numbers of an inframe *GCG* trinucleotide repeat (172). To date, these allelic variants have not been associated with diminished enzyme activity. Another common genetic variant that causes a proline-to-leucine substitution has been identified near the carboxyterminus (62) in the GPx-1 gene. In cell culture studies, the leucine allele ap-

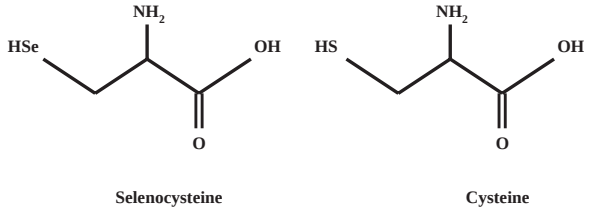

FIG. 4. Structural formula of selenocysteine and cysteine. Selenium, in the form of selenocysteine, is necessary for the translation of glutathione peroxidases. Selenocysteine is an amino acid that is structurally similar to cysteine with selenium in place of sulfur, and it is present at the catalytic site of GPx-1 (residue 48).

pears to be less responsive to stimulation of enzyme activity during selenium supplementation than the more common proline allele (82). The leucine allele has also been associated with increased risk of lung (154), bladder (84), and breast (82) cancer in some studies, although two other studies found no association with breast cancer risk (44, 100). Other mutations, which are associated with increased intima media thickness (IMT) of carotid arteries and risk of cardiovascular and peripheral vascular diseases in type 2 diabetic patients, have been reported in the GPx-1 promoter and untranslated regions (73, 133).

GLUTATHIONE PEROXIDASE-1 AND ATHEROTHROMBOSIS: A REVIEW OF THE CLINICAL EVIDENCE

In the Athero*Gene* study, a large prospective cohort of patients with angiographically documented CAD (median follow-up time 4.7 years), GPx-1 activity has been identified as a powerful biomarker that is significantly lower among those who died from cardiac causes or had a nonfatal myocardial infarction than among those who did not (45.3 ± 12.9 vs. 49.8 ± 11.3 U/g of Hb, $p < 0.001$). In this cohort, GPx-1 activity is associated with sex (lower in males) and smoking status (146). In contrast, no association has been found between the risk of cardiovascular events and the activity of another antioxidant, superoxide dismutase (22). After adjustment for other common risk factors, a low level of GPx-1 activity has been shown to be independently associated with an increased risk of future fatal and nonfatal myocardial infarction, suggesting this measurement has prognostic value in addition to that of traditional risk factors (22). Furthermore, these data raise the intriguing possibility that increasing GPx-1 activity might lower risk of cardiovascular events (22, 102).

In the same cohort, an analysis of the extent of atherosclerosis showed that those patients with multivessel atherosclerosis had the lowest GPx-1 activities (56). This result supports the hypothesis that oxidative mechanisms are involved in atherosclerotic processes in the arterial vessel, and that GPx-1 is protective against vascular oxidative stress. The event rate is inversely associated with GPx-1 activity, and when the extent of atherosclerotic vascular involvement is taken into account, the hazard ratio is highest in patients with multivessel disease and low GPx-1 activity. These data suggest GPx-1 activity combined with knowledge of the extent of vascular involvement may predict high-risk populations.

GLUTATHIONE PEROXIDASE-1 *IN VITRO* AND *IN VIVO*

Studies have shown that GPx-1 activity is decreased in atherosclerotic plaque excised from the carotid artery (105). In fact,

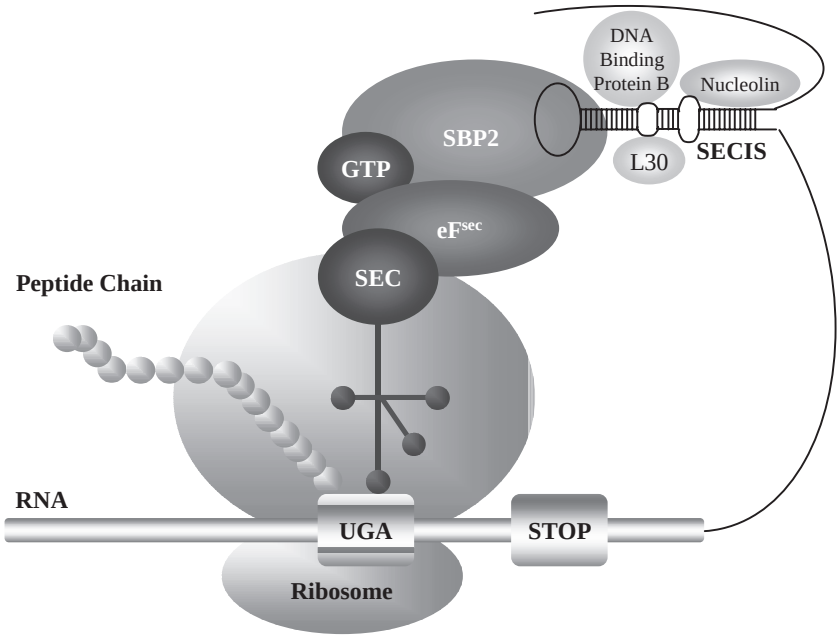

FIG. 5. Factors involved in selenocysteine incorporation. The selenocysteine incorporation sequence (SECIS) element in the 3' untranslated region of the mRNA (stem loop) (UTR) recruits SBP2, which binds to an elongation factor for selenocysteine incorporation (eF^{Sec}) and the tRNA specific for the selenocysteine amino acid ($tRNA^{Sec}$). Other nucleic acid binding factors, such as DNA binding protein B, nucleolin, and L30, may play a role in selenocysteine incorporation and transcript stability. The complex interacts at the ribosome to decode UGA as selenocysteine.

several studies have found that levels of GPx-1 increase during ischemia/reperfusion (192), and increased expression of GPx-1 in transgenic mice decreases tissue damage after cerebral or myocardial ischemia/reperfusion (211, 225). A recent study in homozygous deficient GPx-1 (GPx-$1^{-/-}$) mice indicates that a lack of GPx-1 may reduce neovascularization following ischemia owing to impaired ability to increase endothelial progenitor cell (EPC) levels in response to ischemic injury or subcutaneous administration of vascular endothelial growth factor protein. EPCs isolated from GPx-$1^{-/-}$ mice have a reduced ability to neutralize oxidative stress, leading to impaired migration toward vascular endothelial growth factor and increased sensitivity to ROS-induced apoptosis. These data suggest that EPC dysfunction is a mechanism by which elevated levels of ROS can contribute to vascular disease (65).

GPx-1 transcription is induced by oxidant stress (48), and studies have demonstrated that GPx-$1^{-/-}$ mice are highly sensitive to the oxidant paraquat with lethality within 24 h after exposure to the oxidant, at doses as low as one-seventh the LD_{50} of wild-type controls (48). Thus, a deficiency of GPx-1 may lead to an increase in ROS and lipid hydroperoxides, and a reduced capacity to handle acute oxidant insults, such as observed during inflammation, ischemia-reperfusion, and direct oxidant administration. GPx-1 deficiency may also lead to enhanced peroxynitrite or lipid peroxynitrite formation either by loss of GPx's peroxynitrite reductase activity or by accumulaton of ROS, the end-result of which is a decrease in bioavailable NO (Fig. 6). In support of this hypothesis, previous studies have found that GPx-$1^{-/-}$ mice have endothelial dysfunction, decreased bioavailable endothelium-derived NO, increased oxidative stress, and increased lipid generation as measured by increased plasma and aortic levels of the isoprostane $F_{2\alpha}$-III and increased hepatic phospholipid hydroperoxide levels. Importantly, many of these changes can be found in mice lacking only one copy of the GPx-1 gene (GPx-$1^{+/-}$), suggesting minimal levels of GPx-1 are needed to maintain oxidant/antioxidant balance in cells. Evidence for increased nitrosative stress has also been found in GPx-$1^{-/-}$ mice indicated by increased immunostaining for 3-nitrotyrosine in aortic tissue compared to wild-type mice. Deficiency in GPx-1 also causes structural changes in the coronary vasculature indicative of increased perivascular matrix deposition, increased advential fibroblasts, and intimal thickening. These findings support the hypothesis that a change in the function of GPx-1 causes vascular oxidant stress, endothelial dysfunction, and vascular and cardiac abnormalities that can promote atherosclerosis and predispose to cardiac dysfunction (60, 61).

In contrast to the negative effects of GPx-1 deficiency, GPx-1 overexpressing mice are comparatively protected against insults that promote oxidant stress. GPx-1 overexpressing mice have an increased survival rate with decreased left ventricular dilatation, dysfunction, and end-diastolic pressure compared to wild-type mice after induction of myocardial infarction with comparable infarct size (173). The improvement in left ventricular function is accompanied by a decrease in myocyte hypertrophy, apoptosis, and interstitial fibrosis in the noninfarcted left ventricle. Overexpression of GPx-1 protects the heart against postmyocardial infarction remodeling and heart failure in mice. Ongoing work indicates that increasing GPx-1 expression in endothelial cells protects against oxidant injury and preserves bioavailable NO, whereas inhibition of GPx-1 expression augments oxidative damage and cell death (215, 232).

HOMOCYSTEINE AND GLUTATHIONE PEROXIDASE-1

Several studies have shown that elevated Hcy, but not cysteine, alters the expression of several genes and gene products including GPx-1 in cultured bovine endothelial cells (145, 200) and in mildly hyperhomocysteinemic mice *in vivo* (54, 200) in association with a reduction of bioavailable NO. A decrease in GPx-1 activity has been also found in hepatic tissue of folate-depleted, hyperhomocysteinemic rats (83). These data are in agreement with earlier studies in endothelial cells showing that

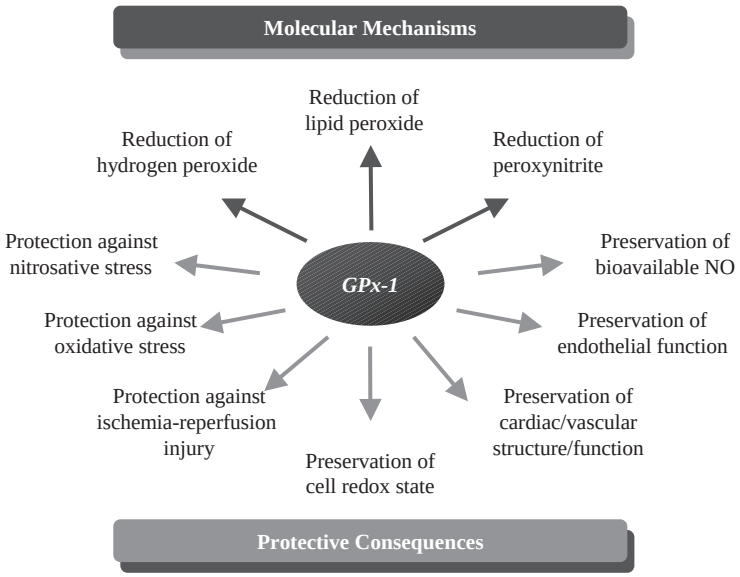

FIG. 6. Molecular mechanisms and protective consequences of glutathione peroxidase-1. Glutathione peroxidase-1 (GPx-1), a key enzyme for the cellular defense against oxidants, uses glutathione to reduce hydrogen peroxide to water and lipid peroxides to their respective alcohols, and may also act as a peroxynitrite reductase. By reducing intracellular oxidants, GPx-1 protects against ischemia–reperfusion injury and cardiac or vascular structural changes, preserves endothelial function and bioavailable nitrix oxide (NO), and helps to maintain the cellular redox state.

high, supraphysiological (5 m*M*) concentrations of Hcy dramatically decrease GPx-1 mRNA (145, 200). Importantly, in animal models and in other cell culture systems (145, 214), GPx-1 activity is diminished by only physiological (10–50 μM) or pathological (100–300 μM) concentrations of Hcy. In fact, in cultured cells, low micromolar concentrations of Hcy did not affect GPx-1 mRNA levels or GPx-1 promoter activity, although this treatment reduced immunodetectable GPx-1 protein and GPx-1 enyzme activity (75). As GPx-1 mRNA was not altered by treatments that modestly increased cellular Hcy production, a reporter gene system was used to determine whether selenoprotein translation is inhibited by Hcy. These data showed that treatments based on folate restriction in the presence of methionine supplementation, which alter intracellular production of Hcy, reduce selenium-dependent readthrough of a UGA-codon, suggesting Hcy reduces GPx-1 activity by interfering with GPx-1 translation (75).

In accordance with this observation is the finding that impairment of aorta relaxation was observed in GPx-$1^{-/-}$ mice treated with methionine-induced hyperhomocysteinemia. In this study, no difference in vasorelaxation to nitroprusside or papaverine was observed between GPx-$1^{+/+}$ and GPx-$1^{-/-}$ mice fed either a control or high methionine diet, indicating that endothelium-independent vasodilator responses were preserved. In addition, dihydroethidium fluorescence was elevated in GPx-$1^{-/-}$ mice fed the high methionine diet compared to GPx-$1^{+/+}$ mice fed the control diet (47).

To analyze the protective effects of GPx-1 against Hcy, crosses were made between heterozygous $CBS^{+/-}$ mice and mice that overexpress GPx-1 (GPx-$1^{(tg+)}$) mice. GPx-1 activity was 28% lower in $CBS^{+/-}$GPx-$1^{(tg+)}$ mice than in $CBS^{+/+}$GPx-$1^{(tg+)}$ littermates (215), showing that mild hyperhomocysteinemia reduces GPx-1 expression as in the $CBS^{+/-}$ mice without a GPx-1 transgene. In this model, GPx-1 activity levels in the $CBS^{+/-}$GPx-$1^{(tg+)}$ and the $CBS^{+/+}$GPx-$1^{(tg+)}$ mice were only 1.5-fold higher than in the $CBS^{+/-}$ or $CBS^{+/+}$ mice with no GPx-1 transgene (GPx-$1^{(tg+)}$). Thus, Hcy lowers GPx-1 levels but not to the levels found in $CBS^{+/-}$ without a GPx-1 transgene. As in previous studies, mesenteric arterioles of $CBS^{+/-}$GPx-$1^{(tg-)}$ mice had paradoxical vasoconstriction to superfusion with β-methacholine and bradykinin ($p < 0.001$ versus all other groups), whereas overexpression of GPx-1 in hyperhomocysteinemic ($CBS^{+/-}$GPx-$1^{(tg+)}$) mice restored the normal endothelium-dependent vascular reactivity response found in nonhyperhomocysteinemic ($CBS^{+/+}$GPx-$1^{(tg-)}$ and $CBS^{+/+}$GPx-$1^{(tg+)}$) mice. These data suggest a minimal level of GPx-1 is necessary to maintain a favorable balance between oxidant and antioxidant effects. Below this threshold level, the oxidant effects of agents such as Hcy will predominate; above this threshold level, the protective effects of the antioxidant GPx-1 will predominate. Additional support for this hypothesis comes from cell culture experiments involving transfection with GPx-1 and incubation with Hcy. Hcy incubation decreased GPx-1 activity and decreased NO release in sham-transfected bovine aortic endothelial cells (BAEC) but not in GPx-1-transfected cells. Hcy also reduced cGMP accumulation in bovine aortic vascular smooth muscle cells (BASMC) cocultured with bradykinin-stimulated BAEC. GPx-1 overexpression in BAEC also compensated for this deficiency. These data suggest that the vascular pathophysiology of Hcy is at least partly mediated by decreased bioavailable NO owing to oxidative inactivation by ROS, as increasing the GPx-1 antioxidant capacity restores the normal phenotype (215). An alternative possibility may involve GPx-1-mediated catabolism of *S*-nitrosothiols (such as *S*-nitrohomocysteine) (63) as a mechanism to prolong the action of NO. Other studies of platelet activity reported that addition of purified GPx-1 potentiated *S*-nitrosothiol-induced inhibition of platelet aggregation and increased *S*-nitrosothiol-induced cGMP accumulation in platelets in an ex vivo system, suggesting GPx-1 can increase the potency of NO; however, this platelet study did not distinguish between the role of GPx-1 in reducing oxidants that can inactivate NO and the role of GPx-1 in *S*-nitrosothiol catabolism. It is an interesting, although unproven, concept that liberation of NO from *S*-nitrosohomocysteine might explain the apparent protective effects of GPx-1 overexpression in endothelial cells and in transgenic mice. *S*-nitrosohomocysteine is known to have a longer biological half-life than free NO. NO is relative lipophilic, modestly reactive, and can tranverse cell membranses freely. Cellular entry and bioactivity of NO may be regulated by transnitrosation reactions, and it has been suggested that cell-surface protein disulfide isomerase catalyzes transnitrosation reactions and regulates intracellular transfer of NO from extracellular *S*-nitrosothiols (226). Overall, these data show that deficiency of GPx-1 may augment Hcy's effects on vascular function and ROS accumulation, whereas increasing levels of GPx-1 protect against these adverse effects.

HOMOCYSTEINE AND GLUTATHIONE PEROXIDASE-1 IN CLINICAL STUDIES

Consistent with the idea that GPx-1 plays a crucial role in oxidant/antioxidant balance and cell protection, a recent clinical study involving the Athero*Gene* cohort analyzed the interaction between GPx-1 activity levels and Hcy. These data indicate that patients with low GPx-1 activity below the median value have a three-fold increase in cardiovascular risk (death from cardiovascular causes and nonfatal MI) if their Hcy level is above the median value. In patients with high GPx-1 activity, the effect associated with elevated Hcy is decreased by ~50%. Although this difference is not significant owing to a small sample size, it suggests that patients with higher GPx-1 levels are relatively protected against adverse oxidative effects induced by hyperhomocysteinemia. These results also suggest that simultaneous assessment of both biomarkers provides additional information for cardiovascular risk stratification, and that interpretation of the Hcy levels without knowledge of the GPx-1 activity may be misleading (164).

CONCLUSIONS

Important progress has been made over the past several years toward understanding mechanisms by which Hcy contributes to CVD, endothelial dysfunction, and atherothrombogenesis. The consequence of a lack of bioavailable NO caused by oxidative stress is an increase in expression of inflammatory mediators

and monocyte binding to the endothelium, oxidation of lipids and proteins, and vascular dysfunction. GPx-1, the ubiquitous intracellular isoform of this peroxidase family and a key antioxidant enzyme within many cells, plays a central role in protection against Hcy-induced oxidative stress, and this role has been confirmed in experimental and clinical studies. Future studies should focus on the molecular mechanisms by which Hcy and GPx-1 act together to modulate vascular redox state and influence the course of vascular disease.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ADMA, asymmetric dimethylarginine; BAEC, bovine aortic endothelial cells; CAD, coronary artery disease; CBS, cystathionine-β-synthase; CHD, coronary heart disease; cGMP, cyclic guanosine monophosphate; CVD, cardiovascular disease; DCF, 2′,7′-dichlorofluorescein; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; ER, endoplasmic reticulum; GPx-1, glutathione peroxidase-1; Hcy, homocysteine; MS, methionine synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; NO, nitric oxide; NOS, nitric oxide synthase; odds ratio; OR; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SECIS, selenocysteine incorporation sequence; tHcy, total homocysteine.

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