Cellular Redox State and Endothelial Dysfunction in Mildly Hyperhomocysteinemic Cystathionine $\beta$-Synthase–Deficient Mice

Norbert Weiss, Stanley Heydrick, Ying-Yi Zhang, Charlene Bierl, André Cap, Joseph Loscalzo

Abstract—Previous in vitro experiments have shown that hyperhomocysteinemia leads to oxidative inactivation of nitric oxide, in part by inhibiting the expression of cellular glutathione peroxidase (GPx-1). To elucidate the role of intracellular redox status on homocysteine-induced endothelial dysfunction and oxidant stress, heterozygous cystathionine $\beta$-synthase–deficient (CBS$^{+/+}$) and wild-type (CBS$^{++}$) mice were treated with the cysteine donor L-2-oxothiazolidine-4-carboxylic acid (OTC). CBS$^{+/+}$ mice had significantly lower GPx-1 activity compared with their CBS$^{++}$ littermates, and OTC treatment led to a modest increase in tissue GPx-1 activity and significant increases in total thiols and in reduced glutathione levels in both CBS$^{+/+}$ and CBS$^{++}$ mice. Superfusion of the mesentery with $\beta$-methacholine or bradykinin produced dose-dependent vasodilation of mesenteric arterioles in CBS$^{+/+}$ mice and in CBS$^{+/+}$ mice treated with OTC. In contrast, mesenteric arterioles from CBS$^{++}$ mice manifested dose-dependent vasoconstriction in response to both agonists. OTC treatment of CBS$^{+/+}$ mice restored normal microvascular vasodilator reactivity to $\beta$-methacholine and bradykinin. These findings demonstrate that mild hyperhomocysteinemia leads to endothelial dysfunction in association with decreased bioavailable nitric oxide. Increasing the cellular thiol and reduced glutathione pools and increasing GPx-1 activity restores endothelial function. These findings emphasize the importance of intracellular redox balance for nitric oxide bioactivity and endothelial function. (Arterioscler Thromb Vasc Biol. 2001;2:34-41.)

Key Words: homocysteine | endothelial function | oxidant stress | nitric oxide | P-selectin

Mild hyperhomocysteinemia, ie, an elevation of the plasma levels of homocysteine, homocystine, or homocystine–mixed disulfides, has been shown to be a risk factor for atherosclerotic vascular disease and its thrombotic complications. Population-based, nested case-control studies demonstrate that a 5 $\mu$mol/L increase in plasma homocysteine concentrations leads to a 30% increase in cardiovascular risk (reviewed in the first 5 references). In addition, several prospective studies indicate that the increased risk is higher during short-term follow-up and declines after 3 to 4 years. Hyperhomocysteinemia is an even stronger predictor of cardiovascular risk in patients with preexisting conditions, such as chronic renal failure, coronary heart disease, diabetes mellitus, peripheral arterial occlusive disease, systemic lupus erythematosus, or venous thromboembolism. In accordance with these observations, elevated homocysteine levels are more strongly associated with recurrent cardiovascular events than with first stroke or myocardial infarction. These data suggest that homocysteine promotes acute thrombotic events in the presence of preexisting vascular lesions rather than induces atherosclerotic lesions de novo.

The mechanisms by which elevated homocysteine levels alter the vascular environment to facilitate the development and progression of vascular lesions and the occurrence of acute thrombotic events have not been fully elucidated. Homocysteine influences several vascular responses, including coagulation, fibrinolysis, platelet function, vascular smooth muscle cell proliferation, and endothelial function. Endothelial dysfunction appears to play a key role in homocysteine-induced vascular pathophysiology. Animal models of mild hyperhomocysteinemia, induced by vitamin deficiency, heterozygous disruption of the cystathionine $\beta$-synthase (CBS) gene, or the combination of both, show an impairment of endothelium-dependent vasoreactivity and regulation of blood flow. Humans with either acutely elevated total plasma homocysteine levels after a methionine challenge or chronic, mild hyperhomocysteinemia also show impaired endothelium-dependent vasodilator function but preserved endothelium-independent vasodilator responses. In accordance with these in vivo findings, homocysteine has been shown to decrease the production and/or bioactivity of nitric oxide (NO) and $S$-nitrosothiols by cultured endothelial cells.
One of the mechanisms suggested for the adverse effects of homocysteine on endothelial function involves oxidant stress, with a resulting depletion of bioavailable NO. When added to plasma, homocysteine, like other thiol-containing amino acids, undergoes auto-oxidation, which is accompanied by the generation of reactive oxygen species such as hydrogen peroxide (H₂O₂) or superoxide anion. Superoxide anion can react with NO to form peroxynitrite, which impairs its biological activity. The role of superoxide formation in homocysteine-induced endothelial dysfunction is underscored by the demonstration of greater superoxide production in aortic tissue from mildly hyperhomocysteinemic mice than from wild-type mice and the finding that superoxide dismutase can reverse the decreased cerebrocortical blood flow during superfusion with homocysteine-containing buffer.

Earlier investigations have supported a role for H₂O₂ in homocysteine-induced endothelial toxicity in vitro because catalase was found to inhibit the homocysteine-induced lysis of endothelial cells in the presence of transition metals or ceruloplasmin. More recently, chronic exposure of endothelial cells to homocysteine was observed to accelerate the rate of endothelial senescence and to increase the amount of telomere length lost per population doubling; these effects could be prevented by catalase.

Homocysteine-induced vascular oxidant stress may be additionally aggravated by a homocysteine-mediated decrease in the expression of the cellular isoform of glutathione peroxidase (GPx-1), as recently shown in vitro and in vivo. This key enzyme for the cellular defense against peroxides to their respective alcohols and may also act as a peroxynitrite reductase.

H₂O₂ decomposes to the toxic oxygen species hydroxyl radical, which is highly reactive and causes lipid peroxidation, and hydroxide, which promotes alkaline tissue damage. Elevated levels of lipid peroxides lead to an increase in peroxyl radicals that can inactivate NO through the formation of lipid peroxynitrites. Peroxynitrite may further react with cellular tyrosine residues to form nitrosated end products or with thiols to form S-nitrosothiols. Because reduced glutathione (GSH) represents the most abundant and one of the most important intracellular antioxidants and primarily serves as a cosubstrate for cellular GPx-1, we sought to elucidate further the role of the intracellular redox state, which is mainly determined by the intracellular glutathione concentration, on homocysteine-induced endothelial dysfunction and oxidant stress. Specifically, we examined the effect of treating mildly hyperhomocysteinemic, heterozygous CBS-knockout mice with the intracerebral cysteine donor 1,2-oxothiazolidine-4-carboxylic acid (OTC), a drug known to increase cellular glutathione concentration, on endothelium-dependent and -independent vasodilator function and on the expression of adhesion molecule markers of endothelial cell activation.

**Methods**

**Animal Model, Genotyping, and OTC Protocol**

Mice heterozygous for disruption in the CBS gene were obtained from the Jackson Laboratory (Bar Harbor, Me) and subsequently bred at our institution. Mice had been bred back into the C57BL/6J mouse strain for >10 generations. Heterozygous CBS-deficient mice (CBS−/−) and littermate, wild-type control mice (CBS+/+) were used at 10 to 12 weeks of age. Genotyping for the targeted CBS allele was performed in each mouse by polymerase chain reaction on genomic DNA obtained from tail biopsies. The animals were fed standard chow ad libitum (LabDiet 5001, PMI Nutrition International), a diet sufficient in folic acid (0.59 mg folic acid per 100 g chow), pyridoxine (0.60 mg/100 g chow), and vitamin B₁₂ (0.022 µg/100 g chow).

L-OTC (Sigma Chemical Co) was administered to the animals in water bottles at a concentration of 2 µmol·g⁻¹·d⁻¹ for 7 days, a dose known to affect hepatic GSH levels in glutathione-depleted mice. Actual ingestion was determined by weighing the drinking bottles every other day. The animals were handled according to National Institutes of Health guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Boston University Medical Center.

**Determination of Total Plasma Homocysteine Concentrations**

At the time the animals were humanely killed as previously described, blood was drawn from the inferior vena cava into a syringe containing 1/10 vol CPD (10 mmol/L citric acid, 90 mmol/L sodium citrate, 15 mmol/L Na₂HPO₄, and 142 mmol/L dextrose, pH 7.35) and immediately centrifuged at 10 000 g for 10 minutes. The plasma was separated, divided into aliquots, snap-frozen in liquid N₂, and stored at −80°C until analysis. Plasma homocysteine was measured by a fluorescence polarization immunoassay. In this method, dithiothreitol (DTT) is used to liberate protein-bound homocysteine and to reduce all homocysteine and homocysteine–mixed disulfide species to homocysteine. S-Adenosyl-homocysteine hydrolase and excess adenosine are then used to convert all homocysteine to S-adenosyl-homocysteine. After completion of this reaction, excess adenosine is removed by adenosine deaminase treatment. Subsequently, the competition between S-adenosyl-homocysteine in the sample and a fluoresceinated S-adenosyl-homocysteine analogue for binding to a monoclonal antibody against S-adenosyl-homocysteine is used for quantification of homocysteine. The assay was performed on an Abbott IMX analyzer (Abbott).

**Cellular GPx-1 Activity**

After blood collection at the time the mice were humanely killed, livers were perfused with normal saline through the portal vein and then harvested. Liver samples were snap-frozen in liquid N₂ and stored at −80°C until analysis. Approximately 500 mg tissue was homogenized in an-ice cold buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA (pH 8), and 1 mmol/L DTT. The homogenate was centrifuged at 10 000 g for 20 minutes at 4°C. GPx-1 activity was then determined from the supernatant by coupling the reduction of peroxides and the oxidation of glutathione with a molar extinction coefficient for NADPH of 6220 (mol/L·cm⁻¹) and normalized to protein concentration measured by the Bradford dye-binding procedure with a commercially available kit.

**Tissue Glutathione and Total Thiol Concentrations**

Total thios were determined in tissue homogenates by measuring the absorbance of 5-thio-2-nitrobenzoic acid, the reaction product of sulphydryl groups with 5,5'-dithiobis-2-nitrobenzoic acid (Ellman’s reagent). An equal volume of 10% metaphosphoric acid was added to the samples, the resulting precipitated proteins were pelleted by centrifugation, and the supernatant was neutralized with 50 µL/L metaphosphoric acid and the resulting precipitated proteins were pelleted by centrifugation, and the supernatant was neutralized with 50 µL/L metaphosphoric acid until analysis. Plasma homocysteine was measured by a fluorescence polarization immunoassay. In this method, dithiothreitol (DTT) is used to liberate protein-bound homocysteine and to reduce all homocysteine and homocysteine–mixed disulfide species to homocysteine. S-Adenosyl-homocysteine hydrolase and excess adenosine are then used to convert all homocysteine to S-adenosyl-homocysteine. After completion of this reaction, excess adenosine is removed by adenosine deaminase treatment. Subsequently, the competition between S-adenosyl-homocysteine in the sample and a fluoresceinated S-adenosyl-homocysteine analogue for binding to a monoclonal antibody against S-adenosyl-homocysteine is used for quantification of homocysteine. The assay was performed on an Abbott IMX analyzer (Abbott).
Tissue concentrations of glutathione (total, reduced, and oxidized) were measured in tissue homogenates (10% wt/vol) after deproteinization with metaphosphoric acid in an enzymatic recycling method with glutathione reductase, as provided by a commercially available assay (Cayman Chemical Co). Values were normalized to protein concentration in the homogenate.

Mesenteric Microvascular Reactivity
Vascular reactivity in the mesenteric circulation in response to β-methacholine (BMC), bradykinin (BK), or sodium nitroprusside was assessed in vivo by using videomicroscopy as previously described. During the procedure, mean arterial pressure was maintained in the 85 to 95 mm Hg range, and arterial blood oxygen was maintained in the 80 to 100 mm Hg range in all cases.

Aortic Endothelial NO Synthase Expression
Expression of endothelial NO synthase (eNOS) in aortic tissue was assessed by Western blot analysis. Thoracic aortas were homogenized in 50 mM/L Tris-HCl buffer (pH 7.5) in the presence of EDTA (2 mM/L) and proteinase inhibitors (aprotinin 1 µg/mL, pepstatin 10 µg/mL, leupeptin 10 µg/mL, benzamidine 1 mM/L, and PMSF 1 mM/L), and adjusted to equal protein concentrations in the same buffer. Samples were reduced and denatured by adding DTT (0.05 mol/L final concentration) and lithium dodecyl sulfate sample buffer (Invitrogen) and boiling for 5 minutes. Samples of 10 µg protein each were electrophoresed through Bis-Tris-HCl-buff ered (pH 6.4) 12% polyacrylamide gels (NuPAGE, Invitrogen) and blotted on nitrocellulose filters. Blots were blocked in 5% skimmed milk in PBS-T (1× PBS and 0.05% Tween) for at least 15 minutes, followed by an overnight incubation with a monoclonal antibody to eNOS (1:1000, Signal Transduction Laboratories). Blots were washed 3 times for at least 15 minutes each in PBS-T and then incubated with a peroxidase-conjugated secondary antibody for 1 hour (1:2500, Signal Transduction Laboratories). Immunoblots were developed with the ECL detection system (Amersham Pharmacia Biotech). Equal loading of protein was confirmed by staining either parallel gels with Coomassie brilliant blue or with Ponceau S.

Plasma Levels of Soluble P-Selectin and Immunostaining for P-Selectin
Plasma levels of soluble P-selectin were determined by a sandwich ELISA developed in the laboratory. Ninety-six–well microtiter plates were coated with a polyclonal goat anti-mouse CD62P antibody (5 µg/mL in 1× PBS and 0.01% NaCl, at 4°C for 16 to 20 hours; Research Diagnostics), washed with 1× PBS, and blocked with 1% bovine serum albumin in PBS-T for 3 hours at room temperature. Plasma samples were thawed on ice, centrifuged at 10,000g for 10 minutes at 4°C, diluted 1:5 in 0.5% bovine serum albumin in PBS-T, and incubated for 3 hours at room temperature. Incubations with bovine serum albumin without mouse plasma were used for the determination of nonspecific binding. After another washing step, a biotinylated monoclonal rat anti-mouse CD62P antibody (400 ng/mL in PBS-T, Pharmingen) was added and incubated for 3 hours at room temperature. Finally, streptavidin–horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was added for another 3 hours at room temperature, and after repeated washes, 3,3′,5,5′-tetramethylbenzidine was added as a substrate and developed with agitation for 1 hour. The reaction was stopped by adding 0.5N H2SO4, and absorption was read at 450 nm on a microplate reader (ThermoMax, Molecular Devices). Plate blanks and values for nonspecific binding were subtracted from the reading. The linearity of readings was determined by incubating serial dilutions of pooled plasma. Values are expressed as relative optical density units at 450 nm and represent the mean of triplicates. The coefficient of variance between triplicates was <10%.

Endothelial P-selectin expression was assessed by immunostaining of aortic sections with a P-selectin antibody. Aortas of mice were collected at the time the animals were humanely killed, snap-frozen in liquid N2, and stored at −80°C. Tissue samples were thawed, fixed in 10% formalin, embedded in paraffin, and cut in transverse sections. The sections were deparaffinized (1× toluene, 2× xylene,
glutathione increased to a more reduced state. The total thiols and glutathione levels were more variable in hepatic tissue and did not differ significantly between CBS/+/+ and CBS−/− mice nor between untreated and OTC-treated mice (Table 2).

**Hepatic Cellular GPx-1 Activity**

As shown previously in 20-week-old CBS−/− mice, 31 10- to 12-week-old CBS−/− mice had significantly lower hepatic cellular GPx-1 activity compared with their CBS+/+/+ littermates. One week of treatment with OTC led to a 20% and 30% increase in cellular GPx-1 activity in both CBS+/+/+ and CBS−/− mice, respectively. After OTC treatment, the enzyme activity was not different between CBS+/+/+ and CBS−/− mice (Figure 1).

**Mesenteric Microvascular Reactivity**

Superfusion of the mesentery with BMC produced dose-dependent vasodilation of mesenteric arterioles in CBS+/+/+ mice and in CBS+/− mice treated with OTC, with a maximal increase in arteriolar diameter of 16.1±2.3% and 14.5±3.4%, respectively, at a BMC concentration of 10^{-4} mol/L (Figure 2A). Superfusion with BK had the same effect (Figure 2B). In contrast, mesenteric arterioles from CBS−/− mice showed dose-dependent vasoconstriction in response to BMC or BK, with a maximal change in arteriolar diameter of 12.5±4.3% at a BMC concentration of 10^{-4} mol/L (P<0.001 vs all other groups) and of 11.2±2.13% at a BK concentration of 10^{-5} mol/L (P<0.001 vs all other groups). After 1 week of treatment with OTC, mesenteric arterioles of CBS−/− mice showed dose-dependent vasodilation in response to superfusion with BMC or BK, with a maximal increase in diameter of 11.6±2.8% (BMC 10^{-4} mol/L) and 16.1±1.9% (BK 10^{-5} mol/L) (Figures 2B and 2C). The vascular reactivity of

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**Table 2. Hepatic Total Thiols and Total, Reduced, and Oxidized Glutathione Levels**

<table>
<thead>
<tr>
<th></th>
<th>No OTC</th>
<th>OTC, 2 μmol·g⁻¹·d⁻¹</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CBS+/+</td>
<td>CBS−/−</td>
</tr>
<tr>
<td>Total thiols, nmol/mg protein</td>
<td>37.22±3.25</td>
<td>54.08±8.46</td>
</tr>
<tr>
<td>Total glutathione, nmol/mg protein</td>
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<td>33.78±3.66</td>
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<tr>
<td>GSH, nmol/mg protein</td>
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<td>28.42±8.07</td>
</tr>
<tr>
<td>Oxidized glutathione, nmol/mg protein</td>
<td>4.39±0.51</td>
<td>5.37±0.44</td>
</tr>
</tbody>
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Values are mean±SEM; n=6 per group.
OTC-treated CBS+/− mice was not different from that observed in CBS−/− mice without and with OTC treatment. Mesenteric arteriolar relaxation to sodium nitroprusside was similar in CBS+/− and CBS−/− mice, without and with OTC treatment (Figure 2C). Baseline vessel diameters were not significantly different among groups: 0.0291±0.0028 mm in CBS+/−, 0.0285±0.0036 mm in CBS−/−, 0.0253±0.0029 mm in CBS+/− plus OTC, and 0.0291±0.0030 mm in CBS−/− plus OTC.

### Aortic eNOS Expression

Western blot analysis of aortic eNOS protein levels did not show any appreciable difference between CBS+/− and CBS−/− mice, without or with OTC treatment (n=5 per group; data not shown).

### Discussion

The major findings of this study are that chronic, mild hyperhomocysteinemia due to heterozygous CBS deficiency leads to endothelial dysfunction/activation, as evidenced by impaired endothelium-dependent vasodilator function in mesenteric arterioles and increased expression of P-selectin. Increasing the cellular thiol and glutathione pools in vascular tissue restores endothelial vasodilator function in CBS−/− mice and normalizes elevated plasma levels of P-selectin. There is some evidence that increased vascular oxidant stress may contribute to a depletion of bioactive NO and that an impairment of the activity of cellular GPx-1 in hyperhomocysteineemic mice contributes to increased oxidant stress. Thus, increasing the cellular thiol and glutathione pools in vascular tissue and increasing cellular GPx-1 activity restore endothelial function in CBS+/− mice and normalize elevated plasma levels of P-selectin.

Glutathione is the most abundant low-molecular-weight thiol and plays a key role in the cellular defense against oxidative and nitrosative stress and reactive electrophiles. Glutathione reacts rapidly and nonenzymatically with hydroxyl radicals, the cytotoxic end products of the Fenton reaction, and with NO and peroxynitrite, cytotoxic products formed by the reaction of NO with O2 and superoxide, respectively. Together with the different isoforms of GPx-1, glutathione participates in the detoxification of hydrogen peroxides and lipid peroxides. Thus, we hypothesized that an increase in the intracellular thiol and/or glutathione pool would counteract the homocysteine-induced endothelial dysfunction, which is believed to be at least partly mediated by increased oxidative and nitrosative stress.

OTC-treated CBS+/− and CBS−/− mice with OTC significantly increased total cardiac thiol and glutathione levels but did not increase total hepatic thiol or glutathione levels. This tissue-specific effect can be explained by the fact that OTC has been shown to increase glutathione levels only in hepatic tissue that has been depleted of glutathione, either by pretreatment with buthionine sulfoximine or diethyl maleate, after hepatotoxic doses of acetaminophen known to decrease glutathione levels; cysteine and glutathione levels in vascular tissue, plasma, and lymphocytes; and the blood cysteine concentrations. Because OTC serves as a substrate of dihydroxybenzoate, after hepatotoxic doses of acetaminophen known to decrease glutathione levels; cysteine and glutathione levels in vascular tissue, plasma, and lymphocytes; and the blood cysteine concentrations.
ized tissue, is a shift in the cellular redox state to a more reduced environment.

OTC treatment also increased the cellular GPx-1 activity in both CBS−/− and CBS+/− mice. Previous studies had shown an increase in cellular GPx-1 activity in cultured macrophages with OTC treatment. This effect might be due to an induction of enzyme synthesis by increased availability of the cosubstrate glutathione or through preservation of enzyme activity by preventing the oxidation of selenocysteine in the catalytic center by the increased intracellular thiol pool. However, because we could not demonstrate an increase in total hepatic thiol or glutathione levels by OTC treatment, the increase in hepatic cellular GPx-1 activity cannot be explained by either of these mechanisms.

CBS+/− mice demonstrated paradoxical vasoconstriction of the mesenteric microcirculation to BMC and BK as a manifestation of endothelial dysfunction, confirming our previous findings. Muscarinic agonists typically evoke endothelium-dependent vasodilation in the mesenteric arteries of rodents, which is inhibited by NOS inhibitors. This constriction effect of BMC and BK is likely secondary to the direct actions of these agonists on vascular smooth muscle unopposed by endothelial NO or to the direct effect of reactive oxygen species on vascular tone. This effect of homocysteine is presumably due to a decrease in bioavailable NO, since vasoreactivity is impaired only in response to endothelium-dependent agonists known to stimulate endothelial NO release but not to the endothelium-independent vasodilator sodium nitroprusside in hyperhomocysteinemic animals. Furthermore, cGMP accumulation in aortas from CBS−/− mice tended to be decreased compared with that of CBS+/− mice.

Treating CBS+/− mice with OTC reversed the pathological vasoconstriction of mesenteric arteries in response to superfusion with BMC or BK. This response supports an increase in bioavailable NO as an underlying mechanism. Previous studies in patients with coronary artery disease had shown an improvement in NO-dependent brachial artery responses to shear stress, as measured by ultrasound after OTC treatment, supporting this conclusion.

CBS+/− mice also had significantly higher plasma levels of soluble P-selectin and increased P-selectin expression in aortic tissue and in platelets adherent to the aortic vessel wall, as shown by immunostaining. P-selectin is expressed on activated/dysfunctional endothelial cells and on activated platelets. Circulating, soluble P-selectin, the molecular weight of which is slightly lower than that of native P-selectin, is derived from an alternatively spliced mRNA that codes for an isoform of P-selectin that lacks the transmembrane-anchoring domain. There is evidence that most of the circulating P-selectin is derived from this pool and that the plasma concentration of soluble P-selectin is correlated with the cell-surface expression of native P-selectin. Individuals with atherosclerotic risk factors, including hypercholesterolemia, hypertension, and diabetes mellitus, have been shown to have higher plasma levels of soluble P-selectin than those without these risk factors. Increased lipid peroxidation, which induces endothelial dysfunction and persistent activation of platelets, leads to an increase of soluble P-selectin. NO is known to be a potent inhibitor of platelet adhesion and aggregation and of leukocyte adhesion to the microvascular endothelium. The latter effect of NO is, at least in part, mediated by inhibition of the expression of P-selectin on endothelial cells. The pharmacological inhibition of eNOS or the targeted disruption of endothelial or neuronal NOS genes leads to increased expression of P-selectin and to increased leukocyte rolling and adhesion to the microvascular endothelium. The increased plasma levels of soluble P-selectin and the increased aortic P-selectin expression in CBS−/− mice, therefore, support the hypothesis derived from functional studies of vascular reactivity in animal models and in human subjects that elevated homocysteine levels lead to endothelial dysfunction, at least in part, by decreasing bioavailable NO. OTC-treated CBS−/− had normal plasma levels of soluble P-selectin, indicating that OTC treatment can restore endothelial dysfunction presumably by increasing bioavailable NO. Immunoblot analysis of aortic eNOS expression in CBS−/− and CBS+/− mice indicates that the endothelial dysfunction in hyperhomocysteinemia is not caused by a decrease in eNOS expression. This finding is in accordance with the hypothesis that reactive oxygen species oxidatively inactivate NO. Increasing the cellular thiol and glutathione levels also did not influence eNOS expression but nevertheless restored NO bioactivity.

There are several possible explanations for the increase in NO bioavailability in OTC-treated hyperhomocysteinemic mice that need further evaluation. Shifting the cellular redox state to a more reduced environment, together with an increase in GPx-1 activity, might detoxify reactive oxygen species formed under hyperhomocysteinemic conditions, thereby reducing the oxidative inactivation of NO. In addition, GPx-1 seems to function not only as a peroxodase but also as a peroxynitrite reductase. GSH is used in this reaction in the regeneration cycle of the enzyme. Increasing its activity and cofactor availability by OTC treatment might, therefore, restore NO bioavailability by reducing peroxynitrite and thereby detoxifying it. Finally, it has been shown that GSH and other low-molecular-weight thiols like cysteine and DTT increase NOS stability and l-arginine turnover by reduction of essential NOS protein thiols and an increased affinity of NOS for its essential redox cofactor tetrahydrobiopterin. Cysteine appears to be crucial for tetrahydrobiopterin-dependent NOS stability and activity. An increase in the cellular thiol pool by OTC treatment might, therefore, increase eNOS action, which might overcome NO inactivation by increased vascular oxidant stress.

In conclusion, these data show that mildly elevated plasma total homocysteine levels lead to endothelial dysfunction and activation, which might promote atherothrombotic vascular complications. Treatment with OTC, which increases total thiol and glutathione levels in vascularized tissue, shifts the cellular redox state to a more reduced environment and increases GPx-1 activity, thereby restoring endothelial function. These findings underscore the importance of cellular redox balance for the maintenance of endothelial function.

**Acknowledgments**

This study was supported in part by National Institutes of Health (Bethesda, Md) grants HL 55993, HL 58976, and HL 61795 to J.L. and Deutsche Forschungsgemeinschaft grant WE 1984/2–1 to N.W. The authors wish to thank Stephanie Tribuna for excellent secretarial assistance.
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doi: 10.1161/hq1201.100456

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