Deficiency of Glutathione Peroxidase-1 Sensitizes Hyperhomocysteinemic Mice to Endothelial Dysfunction

Sanjana Dayal, Kara L. Brown, Christine J. Weydert, Larry W. Oberley, Erland Arning, Teodoro Bottiglieri, Frank M. Faraci, Steven R. Lentz

Objective—We tested the hypothesis that deficiency of cellular glutathione peroxidase (GPx-1) enhances susceptibility to endothelial dysfunction in mice with moderate hyperhomocysteinemia.

Methods and Results—Mice that were wild type (Gpx1+/+), heterozygous (Gpx1+/−), or homozygous (Gpx1−/−) for the mutated Gpx1 allele were fed a control diet or a high-methionine diet for 17 weeks. Plasma total homocysteine was elevated in mice on the high-methionine diet compared with mice on the control diet (23±3 versus 6±0.3 μmol/L, respectively; P<0.001) and was not influenced by Gpx1 genotype. In mice fed the control diet, maximal relaxation of the aorta in response to the endothelium-dependent dilator acetylcholine (10−5 mol/L) was similar in Gpx1+/+, Gpx1+/−, and Gpx1−/− mice, but relaxation to lower concentrations of acetylcholine was selectively impaired in Gpx1−/− mice (P<0.05 versus Gpx1+/+ mice). In mice fed the high-methionine diet, relaxation to low and high concentrations of acetylcholine was impaired in Gpx1−/− mice (maximal relaxation 73±6% in Gpx1−/− mice versus 90±2% in Gpx1+/+ mice, P<0.05). No differences in vasorelaxation to nitroprusside or papaverine were observed between Gpx1+/+ and Gpx1−/− mice fed either diet. Dihydroethidium fluorescence, a marker of superoxide, was elevated in Gpx1−/− mice fed the high-methionine diet (P<0.05 versus Gpx1+/+ mice fed the control diet).

Conclusions—These findings demonstrate that deficiency of GPx-1 exacerbates endothelial dysfunction in hyperhomocysteinemic mice and provide support for the hypothesis that hyperhomocysteinemia contributes to endothelial dysfunction through a peroxide-dependent oxidative mechanism. (Arterioscler Thromb Vasc Biol. 2002;22:1996-2002.)

Key Words: endothelium ■ homocysteine ■ nitric oxide ■ peroxide

Hyperhomocysteinemia is an emerging risk factor for cardiovascular events and venous thrombosis,1,2 but the mechanisms responsible for the vascular pathology of hyperhomocysteinemia are still incompletely understood. Like many other cardiovascular risk factors, hyperhomocysteinemia produces endothelial dysfunction, which is possibly due to oxidative inactivation of endothelium-derived NO.3,4 The oxidative stress hypothesis5 is supported by the observation that auto-oxidation of homocysteine in vitro generates reactive oxygen species (ROS), including hydrogen peroxide and superoxide, and promotes oxidation of LDL.6-7 Treatment of cultured endothelial cells with homocysteine decreases bioavailable NO8,9 and produces cytotoxicity mediated by hydrogen peroxide.10 Homocysteine also indirectly contributes to oxidative stress by inhibiting the expression of antioxidant enzymes, such as cellular glutathione peroxidase (GPx-1), an effect that may sensitize one to the toxic effects of homocysteine-derived hydrogen peroxides and lipid peroxides.8,11

However, the role of oxidative stress in the vascular dysfunction of hyperhomocysteinemia in vivo is less clear, and its clinical importance has been questioned.12 Attempts to demonstrate elevated levels of oxidation products in humans with hyperhomocysteinemia have produced conflicting results.13-16 Recent studies with human subjects have demonstrated that administration of antioxidant vitamins such as vitamin C or vitamin E can attenuate impairment of endothelium-dependent vasodilatation during acute hyperhomocysteinemia produced by oral methionine loading.17-19 Perhaps the strongest evidence of an oxidative mechanism for endothelial dysfunction in hyperhomocysteinemia has been obtained from studies in hyperhomocysteinemic mice. Mild to moderate hyperhomocysteinemia (plasma total homocysteine [tHcy] levels of 10 to 30 μmol/L) can be produced in mice by
genetic and/or dietary approaches, and it is associated with impairment of endothelium-dependent relaxation.\textsuperscript{20–24} Impaired endothelium-dependent vasodilatory responses in hyperhomocysteinemic mice can be restored toward normal by administration of the thiol antioxidant t-2-oxothiazolidine-4-carboxylic acid (OTC)\textsuperscript{23} or by transgenic overexpression of GPx-1.\textsuperscript{24} To further explore the role of oxidative stress in the endothelial dysfunction of hyperhomocysteinemia in vivo, we tested the hypothesis that deficiency of GPx-1 increases susceptibility to endothelial dysfunction in mice with moderate hyperhomocysteinemia. Our results show that deficiency of GPx-1 exacerbates endothelial dysfunction in hyperhomocysteinemic mice, which suggests that hyperhomocysteinemia contributes to vascular dysfunction through an oxidative mechanism that involves peroxides.

**Methods**

**Mice**

GPx-1–deficient mice containing a targeted disruption of the Gpx1 gene\textsuperscript{25} were generously provided by Dr Y.S. Ho (Wayne State University, Detroit, Mich.). To minimize the potential influence of differences in genetic background, GPx-1–deficient mice were crossed to C57BL6 mice (The Jackson Laboratory, Bar Harbor, Me) for at least 7 generations and then interbred to generate litters that were wild type (Gpx1\textsuperscript{+/+}), heterozygous (Gpx1\textsuperscript{+/-}), or homozygous (Gpx1\textsuperscript{-/-}) for the mutated Gpx1 allele. Genotyping for the wild-type and mutated Gpx1 alleles was performed by polymerase chain reaction as described by Forgione et al.\textsuperscript{26} At the time of weaning, mice were placed on either a control diet (LM-485 chow, Harlan Teklad) or a high methionine diet (LM-485 chow and drinking water supplemented with 0.5% L-methionine). After 17 weeks of the experimental diet, mice were euthanized with sodium pentobarbital (75 mg IP). Blood was collected by cardiac puncture into EDTA (final concentration 5 mmol/L) for measurement of plasma tHcy and methionine, and the thoracic aorta and liver were removed for ex vivo studies. The experimental protocol was approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees.

**Plasma tHcy and Methionine**

Plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds,\textsuperscript{27} was measured by high-performance liquid chromatography and electrochemical detection.\textsuperscript{28} Plasma methionine was measured by high-performance liquid chromatography coupled to coulometric electrochemical detection. The method was modified from that described by Martin et al\textsuperscript{28} by increasing the electrochemical potential to 1100 mV to detect methionine.

**GPx-1 Activity**

Hepatic GPx-1 activity was measured with hydrogen peroxide as the substrate, as described previously.\textsuperscript{29,30} Liver samples were kept frozen at \(-80^\circ\)C until analysis, and then they were homogenized in 0.05 mol/L potassium phosphate buffer, pH 7.8. Homogenates were assayed in 0.1 mol/L potassium phosphate, pH 7.0, containing 1.0 mol/L EDTA and 1.0 mol/L NaF, (to inhibit catalase), 1.0 mmol/L glutathione, 0.35 U/mL glutathione reductase, 0.15 mmol/L NADPH, and 0.15 mmol/L hydrogen peroxide. GPx-1 activity was determined from the rate of oxidation of NADPH and was measured spectrophotometrically at 340 nm. One unit of GPx-1 activity was defined as the amount of protein required to oxidize 1.0 \mu mol NADPH per minute. The protein concentration of the homogenates was determined by using a modified Bradford assay (Bio-Rad).

**Other Antioxidant Enzyme Assays**

Catalase activity of liver homogenates was determined by monitoring the disappearance of hydrogen peroxide spectrophotometrically at 240 nm\textsuperscript{31} and was expressed in k units per milligram protein, as described previously.\textsuperscript{32} Total superoxide dismutase (SOD) activity of liver homogenates was measured by a modification of the nitro blue tetrazolium method, as described previously.\textsuperscript{32,33} One unit of SOD activity was defined as the amount of protein required to inhibit the reduction of nitro blue tetrazolium by 50%.\textsuperscript{32}

**Vasomotor Responses**

Relaxation of precontracted aortic rings was measured in vitro as described previously.\textsuperscript{21,22} After removal of loose connective tissue, the thoracic aorta was cut into multiple 3- to 4-mm rings that were suspended in an organ chamber containing oxygenated Krebs buffer maintained at 37°C. Rings were contracted submaximally by using the thromboxane analogue U46619, and relaxation dose-response curves were generated by cumulative addition of the endothelium-dependent vasodilator acetylcholine (10\textsuperscript{-8} to 10\textsuperscript{-5} mol/L) or the endothelium-independent vasodilators sodium nitroprusside (10\textsuperscript{-5} to 10\textsuperscript{-2} mol/L) or papaverine (10\textsuperscript{-8} to 10\textsuperscript{-2} mol/L). We have demonstrated previously that responses to acetylcholine in mouse aortas are mediated by NO.\textsuperscript{34,35}

**Dihydrorhodamin Fluorescence**

Dihydrorhodamin, an oxidative fluorescent dye, was used to detect superoxide in segments of carotid artery as described previously.\textsuperscript{36,37} Briefly, fresh unfixed segments of the common carotid artery were frozen in OCT compound, and transverse sections (10 \mu m) were generated with a cryostat and placed on glass slides. Sections were then incubated in a light-protected chamber at room temperature for 30 minutes with 10 \mu mol/L dihydrorhodamin (Molecular Probes). Images were obtained with the use of a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. The excitation wavelength was 488 nm, and emission fluorescence was detected with the use of a 585 nm long-pass filter. Identical laser settings were used for acquisition of images from different groups of mice. Fluorescent images were acquired as 8 bit (256 intensity levels) and were subsequently analyzed with NIH Image software (available online at http://rsb.info.nih.gov/nih-image). Data are reported as the percentage of surface area of carotid sections within the upper 20% of fluorescence intensity.

**Statistical Analysis**

Comparisons between genotypes (Gpx1\textsuperscript{-/-}, Gpx1\textsuperscript{+/-}, and Gpx1\textsuperscript{+/+} mice) were performed by unpaired 2-tailed Student t tests. Responses to vasodilators were analyzed by 2-way repeated-measures ANOVA, with the Tukey post hoc test used for multiple comparisons. Correlation coefficients were calculated by the Pearson method. A value of P<0.05 was used to define statistical significance. Values are reported as mean±SE.

**Results**

**Plasma Levels of tHcy and Methionine**

Mice were studied after 17 weeks on a control or high-methionine diet (ie, at 20 weeks of age). Body weight did not differ between diets or between Gpx1 genotypes (data not shown). Plasma tHcy was elevated on the high-methionine diet (23.3±3.1 \mu mol/L) compared with the control diet (6.1±0.3 \mu mol/L, P<0.001) and was not influenced by Gpx1 genotype (Figure 1A). Plasma methionine was elevated to a similar extent in Gpx1\textsuperscript{-/-}, Gpx1\textsuperscript{+/-}, and Gpx1\textsuperscript{+/+} mice that were fed the high-methionine diet (P<0.05 versus control diet, Figure 1B). The overall correlation between plasma tHcy and plasma methionine was strongly positive (r=0.72, P<0.0001).
GPx-1 Activity
Hepatic GPx-1 activity in Gpx1+/+ mice tended to be slightly lower for the high-methionine diet (299±63 mU/mg) than the control diet (347±47 mU/mg), although this difference did not reach statistical significance (P>0.05, Figure 2). Compared with Gpx1+/+ mice, Gpx1−/− mice had hepatic GPx-1 activity that was decreased by ~50% on either the control (177±42 mU/mg) or high-methionine (186±49 mU/mg) diet. Hepatic GPx-1 activity was undetectable in Gpx1−/− mice fed either diet.

Activity of Catalase and SOD
Hepatic catalase activity and SOD activity were similar in mice fed control or high-methionine diets and were not influenced by Gpx1 genotype (Table).

Vasomotor Responses
Aortic rings from all groups of mice contracted similarly to the thromboxane analogue U46619 (data not shown). For mice fed the control diet, maximal relaxation to the endothelium-dependent vasodilator acetylcholine (10−5 mol/L) was similar in Gpx1+/+, Gpx1−/−, and Gpx1−/− mice, but relaxation to lower concentrations of acetylcholine was impaired in Gpx1−/− mice (P<0.05 versus Gpx1+/+ mice, Figure 3A). No significant differences in relaxation to the endothelium-independent vasodilators nitroprusside or papaverine were observed between Gpx1+/+, Gpx1−/−, and Gpx1−/− mice fed the control diet (Figure 3B and 3C).

For mice fed the high-methionine diet, impairment of relaxation to acetylcholine was evident in Gpx1−/− mice at both high and low concentrations of acetylcholine (Figure 4A). Maximal relaxation to 10−5 mol/L acetylcholine was significantly decreased in Gpx1−/− mice (73±6%) compared with Gpx1+/+ mice (90±2%) (P<0.05). No differences in relaxation to nitroprusside or papaverine were observed between Gpx1+/+, Gpx1−/−, and Gpx1−/− mice fed the high-methionine diet (Figure 4B and 4C).

Dihydroethidium Fluorescence
Dihydroethidium, an oxidative fluorescent dye, was used to detect superoxide in segments of carotid artery, as described previously.35-37 Dihydroethidium is freely permeable to cells and is oxidized to the fluorescent dye ethidium bromide in the presence of superoxide.36 Dihydroethidium fluorescence was detected at similar levels in Gpx1+/+ and Gpx1−/− mice fed the control diet (Figure 5A and 5B) and in Gpx1+/+ mice fed the high-methionine diet (Figure 5C) but was elevated ~3-fold in

<table>
<thead>
<tr>
<th>Gpx1+/+</th>
<th>Gpx1−/−</th>
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<tr>
<td>Catalase, k units/mg</td>
<td>0.20±0.01 (n=8)</td>
</tr>
<tr>
<td>SOD, U/mg</td>
<td>501±69 (n=6)</td>
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Values are mean±SE; n indicates the number of mice in each group.
Gpx1/H11002 mice fed the high-methionine diet (P<0.05 versus Gpx1/H11001/H11001 mice fed the control diet, Figure 5D and 5E).

Discussion
The major finding of the present study is that deficiency of GPx-1 produces impairment of endothelium-dependent relaxation of the aorta in mice with moderate hyperhomocysteinemia. GPx-1–deficient mice also had impaired relaxation responses to low concentrations of acetylcholine in the absence of hyperhomocysteinemia, but maximal relaxation to acetylcholine was impaired only when GPx-1–deficient mice were fed the hyperhomocysteinemic diet. Deficiency of GPx-1 did not affect vascular responses to the endothelium-independent vasodilators nitroprusside and papaverine, either in the absence or presence of hyperhomocysteinemia. These findings suggest that hyperhomocysteinemia contributes to endothelial dysfunction through a peroxide-dependent oxidative mechanism.

At least 4 isoforms of glutathione peroxidase have been described, but GPx-1 is the major cellular isoform in most tissues.38 GPx-1 reduces hydrogen peroxide to water and can also reduce lipid peroxides to their corresponding alcohols.38 The GPx-1–deficient mice used in the present study were generated by targeted homologous recombination of the Gpx1 gene.25 Gpx1−/− mice are overtly healthy, but they have enhanced susceptibility to several forms of oxidative injury, including myocardial reperfusion injury,29 virus-induced myocarditis,40 peroxide-induced DNA strand breakage,41 and neutrophil-mediated hepatic injury during endotoxemia.42 Our present results indicate that they also have increased sensitivity to endothelial dysfunction, especially when they are fed a hyperhomocysteinemic diet. These findings, together with a recent study in which transgenic overexpression of Gpx-1 was found to rescue heterozygous cystathionine β-synthase (CBS) mice (CBS+/− mice) from endothelial dysfunction,24 establish a critical role for GPx-1 in protecting endothelium from oxidative damage during moderate hyperhomocysteinemia in vivo.

Normal relaxation responses to acetylcholine in mouse aortas are dependent on endothelium-derived NO.34,35 Expression of endothelial NO synthase (eNOS) appears to be unaffected in hyperhomocysteinemia, but the bioavailability of endothelium-derived NO is decreased.20 Hyperhomocysteinemia may promote the oxidative inactivation of NO and thereby provide sensitization to endothelial dysfunction through several different mechanisms (Figure 6). Auto-oxidation of homocysteine in vitro produces hydrogen per-
oxide and, in the presence of transition metals such as copper or iron, can also generate more highly reactive ROS, such as superoxide and hydroxyl radical. If auto-oxidation of homocysteine occurs during hyperhomocysteinemia in vivo, excessive production of hydroxyl radicals may cause lipid peroxidation, and both superoxide and lipid peroxyl radicals may react rapidly with endothelium-derived NO to produce peroxynitrite and lipid peroxynitrites. In support of this mechanism, elevated levels of superoxide and 3-nitrotyrosine, a product of protein modification by peroxynitrites, have been detected in the aortas of hyperhomocysteinemic mice. Whether or not homocysteine auto-oxidation is a major mechanism for the generation of ROS in vivo is uncertain. Conversion of homocysteine to its disulfide forms in plasma is mediated mainly by thiol-disulfide exchange reactions rather than by copper-dependent oxidation, which suggests that homocysteine is unlikely to be a major source of hydrogen peroxide in vivo. Therefore, it is perhaps more likely that indirect mechanisms are responsible for the oxidative stress of hyperhomocysteinemia (Figure 6). Indirect oxidative effects of hyperhomocysteinemia may include generation of superoxide from xanthine oxidase or uncoupled eNOS, downregulation of antioxidant enzymes, and depletion of intracellular glutathione. Another potential source of superoxide is vascular NAD(P)H oxidase, which appears to contribute to endothelial dysfunction in hypertension and diabetes but may be less important in hyperhomocysteinemia. Each of these indirect effects is consistent with increased dihydroethidium fluorescence in Gpx1−/− mice fed the high-methionine diet (Figure 5). Regardless of whether hyperhomocysteinemia promotes oxidative stress through direct or indirect mechanisms, the protective role of GPx-1 is presumably mediated by the reduction of hydrogen peroxide and lipid peroxides (Figure 6). GPx-1 may also provide protection from the toxic effects of peroxynitrite through its peroxynitrite reductase activity.

Our results confirm the findings of Forgione et al that deficiency of GPx-1 leads to impairment of endothelium-dependent vasodilator function in mice with normal plasma levels of tHcy. Forgione et al observed endothelial dysfunction with paradoxical vasoconstriction to endothelium-dependent agonists in mesenteric arterioles of Gpx1−/− mice fed standard chow. In contrast, the degree of impairment of endothelium-dependent relaxation of the aortas of Gpx1−/− mice that we observed in the absence of hyperhomocysteinemia was relatively mild and was apparent only with submaximal concentrations of acetylcholine. Similar differences in the magnitude of endothelial dysfunction between the aorta and mesenteric arterioles of Gpx1−/− mice fed standard chow, or between the aorta and cerebral arterioles (S.R. Lentz, F.M. Faraci, unpublished data, 2002) have been observed in CBS-deficient mice with mild hyperhomocysteinemia. Further work will be required to determine whether these observations reflect differences in experimental methods, genetic background, or inherent differences between large arteries and small arterioles in susceptibility to oxidative stress.

We did not detect significant endothelial dysfunction in the aortas of Gpx1−/− or Gpx1+/− mice that were fed the high-methionine diet despite the fact that these mice had moderately elevated plasma levels of tHcy. This result is consistent with a previous study in which the high-methionine diet produced endothelial dysfunction in CBS−/− mice but not in their CBS+/− littermates. Plasma tHcy levels obtained in mice fed the high-methionine diet in the present study (23 ± 3 μmol/L) were somewhat higher than those obtained in the previous study (13 ± 2 μmol/L), which probably reflects differences in the timing of sample collection. Plasma samples in the present study were drawn from nonfasted mice, whereas they were drawn after 4 to 6 hours of fasting in the previous study. This interpretation is consistent with the higher plasma levels of methionine in the present study (57 ± 8 μmol/L) compared with mice fed the same diet in the previous study (28 ± 4 μmol/L). Plasma levels of tHcy exhibit a marked diurnal variation in mice, especially when they are fed a methionine-enriched diet (Jonathan Smith, oral communication, April 2002). Therefore, it is likely that mice...
fed the high-methionine diet were exposed to a similar degree of chronic hyperhomocysteinemia in both studies. These levels are within the pathophysiological range of plasma tHcy seen in most patients with moderate hyperhomocysteinemia.

In summary, we have demonstrated that that deficiency of GPx-1 exacerbates endothelial dysfunction in mice with moderate hyperhomocysteinemia. These in vivo findings provide support for the hypothesis that vascular damage in hyperhomocysteinemia is mediated through a peroxide-dependent oxidative mechanism. Although the present findings do not exclude the possibility that hyperhomocysteinemia also may produce endothelial dysfunction through additional mechanisms, they do establish a critical role for GPx-1 in providing protection from endothelial dysfunction in the face of diet-induced hyperhomocysteinemia.

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Figure 6. Possible mechanisms for protection from hyperhomocysteinemia-induced endothelial dysfunction by GPx-1. Auto-oxidation of homocysteine may produce hydrogen peroxide (H₂O₂). Indirect oxidative effects of hyperhomocysteinemia may include generation of superoxide from uncoupled eNOS, xanthine oxidase, or NADPH oxidase, downregulation of antioxidant enzymes, and depletion of intracellular glutathione. In the presence of transition metals such as copper or iron, H₂O₂ and superoxide (O₂⁻) may react to form a hydroxyl radical (·OH). The hydroxyl radical may promote the generation of lipid peroxyl radicals (LOO·), and both superoxide and lipid peroxyl radicals may react rapidly with endothelium-derived NO (·NO) to produce peroxynitrite (ONO'O) or lipid peroxynitrite (LOONO), respectively. The protective role of GPx-1 may be mediated by reduction of hydrogen peroxide, lipid peroxides (LOOH), and peroxynitrite. ONOO' indicates nitrite.


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