Heterozygous Cellular Glutathione Peroxidase Deficiency in the Mouse

Abnormalities in Vascular and Cardiac Function and Structure

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Background—Oxidant stress has been implicated in the pathogenesis of atherothrombosis and other vascular disorders accompanied by endothelial dysfunction. Glutathione peroxidases (GPx) play an important role in the cellular defense against oxidant stress by utilizing glutathione (GSH) to reduce lipid hydroperoxides and hydrogen peroxide to their corresponding alcohols. Cellular GPx (GPx-1) is the principal intracellular isoform of GPx. We hypothesized that GPx-1 deficiency per se induces endothelial dysfunction and structural vascular abnormalities through increased oxidant stress.

Methods and Results—A murine model of heterozygous deficiency of GPx-1 (GPx-1<sup>-/-</sup>) was investigated to examine this hypothesis. Mesenteric arterioles in GPx-1<sup>-/-</sup> mice demonstrated vasoconstriction to acetylcholine compared with vasodilation in wild-type mice (maximal change in vessel diameter, −13.0±2.8% versus 13.2±2.8%, P<0.0001). We also noted an increase in the plasma and aortic levels of the isoprostane iPF<sub>2α</sub>−III, a marker of oxidant stress, in GPx-1<sup>-/-</sup> mice compared with wild-type mice (170.4±23 pg/mL plasma versus 98.7±7.1 pg/mL plasma, P<0.03; 11.7±0.87 pg/mg aortic tissue versus 8.2±0.55 pg/mg aortic tissue, P<0.01). Histological sections from the coronary vasculature of GPx-1<sup>-/-</sup> mice show increased perivascular matrix deposition, an increase in the number of adventitial fibroblasts, and intimal thickening. These structural abnormalities in the myocardial vasculature were accompanied by diastolic dysfunction after ischemia-reperfusion.

Conclusions—These findings demonstrate that heterozygous deficiency of GPx-1 leads to endothelial dysfunction, possibly associated with increased oxidant stress, and to significant structural vascular and cardiac abnormalities. These data illustrate the importance of this key antioxidant enzyme in functional and structural responses of the mammalian cardiovascular system. (Circulation. 2002;106:1154-1158.)

Key Words: endothelium ■ vasculature ■ antioxidants ■ oxygen ■ nitric oxide

Nitric oxide (NO) synthesized by the endothelium represents an important regulatory determinant of vascular function. This short-lived endothelial product promotes vasodilation, impairs platelet activation and leukocyte adherence to the endothelium, preserves the barrier function of the endothelium, and acts as an antioxidant. Oxidant stress, associated with an increase in reactive oxygen species in the vasculature, promotes NO insufficiency and the ensuing endothelial dysfunction.

Reduced glutathione (GSH) plays an important role in the cellular defense against oxidant stress. GSH is an essential cosubstrate for the glutathione peroxidases (GPx), selenocysteine-containing proteins that reduce lipid peroxides (LOOH) and hydrogen peroxide to their corresponding alcohols. Peroxyl radicals generated from LOOH can inactivate NO through the formation of lipid peroxynitrites. GPxs exist in at least 4 different isoforms. GPx-1 is the most abundant and ubiquitous intracellular isoform, and the expression of this enzyme is significantly suppressed by a known risk factor for atherothrombosis, ie, homocysteine. Because GSH represents one of the most important intracellular antioxidants, primarily in its role as a cosubstrate for GPx-1, we propose that this antioxidant system plays a central role in protecting the vasculature in states of increased oxidant stress.

Recently, targeted gene disruption of GPx-1 has been achieved in the mouse. To assess the vascular consequences of oxidant stress per se on vascular homeostasis, we investigated the effect of heterozygous GPx-1 deficiency on endothelium-dependent vascular function, oxidant stress, and cardiovascular function and structure in this murine model.
Methods

Reagents and Animals

β-Methacholine (BMC), bradykinin, and sodium nitroprusside (SNP) were obtained from Sigma Chemical Co. Mice homozygous for disruption of the GPx-1 gene were kindly provided by Y. Ho at Wayne State University (Detroit, Mich.) and subsequently bred at our institution.

GPx-1 was inactivated by insertion of a neomycin resistance gene cassette into an EcoRI site located in exon 2 of the GPx-1 gene, which was then inserted into mouse embryonic stem cells, as previously described.1 Heterozygous (+/−) GPx-1−/− deficient male mice and littermate, wild-type (+/+) (WT) control male mice were used at 40 to 60 weeks of age for the experiments assessing function. The animals were fed standard chow and handled secondary to National Institutes of Health guidelines. The Institutional Animal Care and Use Committee at Boston University Medical Center approved the procedures.

Genotype Determination

DNA was obtained by extraction from mouse tail snips. One microliter of DNA was amplified in 50-μL polymerase chain reaction (PCR) containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.01% gelatin, 0.1 mmol/L dNTPs, and 0.18 μmol/L of each primer. To identify the WT GPx-1 gene, we used the forward primer FinN (5′-GTACCTGGTGCAGATCGT-TCG-3′) and the reverse primer R3 N(5′-CAACCTGATTGCCAG-3′) to amplify a 293-bp fragment. To detect the neomycin resistance gene cassette present in GPx-1−/− mice, we used FinN and RpgkN (5′-GTACCTGGTGCAGATCGT-TCG-3′) as the reverse primer R.N (5′-TGGACCTGACTTTGA-GGAAT-3′) to amplify a 293-bp fragment. To detect the neomycin resistance gene cassette present in GPx-1−/− mice, we used FinN and RpgkN (5′-CATTTGTCACGTCCTGCAC-3′) as the reverse primer to amplify a 509-bp fragment in the neomycin resistance cassette. Reaction products were analyzed by electrophoresis on a 1% agarose gel. GPx-1−/− mice were identified by having both a 509-bp PCR product and a 293-bp PCR product; WT mice were identified by an exclusive 293-bp PCR product. Genotype was correlated with phenotype by measuring GPx-1 activity in cardiac or vascular tissue extracts, as previously described.11 GPx-1−/− mice had 48±4% of the activity of GPx-1+/− mice.

Mesenteric Microcirculation Studies

Mice were anesthetized with pentobarbital (0.04 mg/kg) by intraperitoneal injection, then intubated and ventilated with 100% oxygen. Vascular reactivity in the mesenteric circulation was assessed in vivo using videomicroscopy,1 as previously described. The mesentry was placed on a heated microscope stage and superfused with saline, β-methacholine, bradykinin, or sodium nitroprusside, and the mesenteric arteriolar response to these agents was monitored by quantitative videomicroscopic measurement. In some experiments, Cu,Zn superoxide dismutase was superfused at 150 U/ml to evaluate the effect of this agent on endothelial dysfunction in response to 10−6 mol/L methacholine.

Measurement of Isovolumic Contractile Performance

Hearts were isolated and perfused in the Langendorff model, as previously described, with minor modifications.14 Briefly, mice were perfuncted with Krebs-Henseleit buffer (in mmol/L NaCl 118, KCl 4.7, CaCl2 1.9, KH2PO4 1.2, MgSO4 1.2, NaHCO3 24.0, and glucose 10.0) equilibrated with 95% O2 and 5% CO2 yielding a pH of 7.4. The pulmonary artery was cannulated with a thin polyethylene tube (PE-50) for collection of coronary effluent. A thin cannula was pierced through the apex of the left ventricle (LV) to vent Thebesian drainage. A small balloon, custom-made from polyvinyl chloride film and connected to 5-cm polyethylene tubing (PE-50; internal diameter 0.58 mm), was inserted into the LV through the mitral valve via an incision in the left atrium. The balloon was specifically designed to ensure that the unstressed volume and inherent compliance of the balloon were greater than those of the ventricle for accurate assessment of ventricular pressure. No discernible pressure drop was detected over the length of the tubing. The balloon was connected to a pressure transducer (Statham P23Db, Gould) for recording LV pressures. Previous experiments have determined no discernible damping of the pressure signal with balloon-derived ventricular pressures relative to direct transducer-tipped catheter-derived ventricular pressures, over heart rates from 0 to 7 Hz and over a range of ventricular pressures from 0 to 100 mm Hg. The balloon was inflated with H2O to adjust the end-diastolic pressure (EDP) at 10 mm Hg (balloon volume, 35±8.15 μL and 36.5±1.1 μL for WT and GPx-1−/− mice, respectively), and the balloon volume was held constant for the duration of the experiment. Hearts were paced (Gross Instruments) throughout platinum wires placed on the epicardial surface of the right ventricle. Coronary perfusion pressure (CPP) was monitored via a sidearm of the aortic cannula connected to a pressure transducer (Statham P23Db, Gould). An inline ultrasonic flow probe (Transonics, Inc.) was positioned immediately above the aortic cannula to measure coronary blood flow. Systolic pressure and EDP, heart rate, CPP, and coronary blood flow were collected online at rates of 400 samples/sec using a commercially available data acquisition system (MacLab ADInstruments). Developed pressure (DevP), ie, the difference between systolic and diastolic pressures, was calculated offline. DevP was used as an index of contractile performance and EDP as an index of diastolic function.

After instrumentation, all hearts were stabilized for 15 minutes at 37°C at a CPP of 80 mm Hg and paced at 7 Hz. Throughout the duration of the experiment, myocardial temperature was held constant at 37°C via careful temperature regulation of the myocardial perfusate and submersion of the heart in Krebs-Henseleit buffer warmed by a water-jacketed chamber. Hearts were then subjected to 15 minutes of zero-flow ischemia followed by 30 minutes of reperfusion. The pacer was turned off during ischemia and turned on again 15 minutes into reperfusion.

Isoprostane Determination

Mice were sacrificed and plasma and thoracic aortas were obtained by centrifugation of blood collected in tubes containing 0.1 mol/L (final concentration) EDTA. Samples were then snap-frozen in liquid nitrogen and stored at −80°C. Plasma was diluted 1:15 in ultrapure water (Cayman Chemical Co), applied to a reverse-phase C-18 column (Alltech), pH 3.0, and eluted with 50/50% (vol/vol) ethyl acetate/hexane. The eluent was then additionally purified on a silica column (Alltech) and eluted with 50/50% (vol/vol) ethyl acetate/hexane. Measurement of iPF2-III was then made using a commercially available immunoassay (Cayman Chemical Co). Aortic tissue was minced, and iPF2-III was extracted as previously described.11

cGMP Determination

cGMP content was measured in isolated thoracic aortas, as previously described.11

Histological Assessment

Tissue, including heart, aorta, and mesentery, was excised from 6 GPx-1−/− and 6 WT mice between 40 to 60 weeks of age immediately after the animals were euthanized. The tissues were fixed in 4% formalin and embedded in paraffin, and the sections were stained with hematoxylin and eosin as well as Masson trichrome.

Statistics

Data were analyzed using the Students’ t test or (repeated measures) ANOVA with post-hoc comparisons where appropriate. P<0.05 was considered significant.

Results

Mesenteric Vascular Reactivity

Superfusion of BMC produced dose-dependent vasodilatation of mesenteric arterioles in WT mice (Figure 1). There was a
maximal 13.2±2.8% increase in arteriolar diameter while superfusing BMC 10⁻⁴ mol/L. In contrast, mesenteric arterioles from GPx-1⁻/⁻ mice manifested dose-dependent vasoconstriction to BMC. There was a maximal 13±2.8% decrease in vessel diameter while superfusing BMC at 10⁻⁴ mol/L, which was significantly different compared with the response of WT mice (P<0.0001). This paradoxical vasoconstriction to BMC is indicative of impaired endothelium-dependent vasodilation in GPx-1⁻/⁻ mice involving the microcirculation, consistent with a depletion of bioavailable NO. Similar results were obtained using bradykinin as another endothelium-dependent agonist, i.e., paradoxical vasoconstriction in GPx-1⁻/⁻ mice compared with dose-dependent vasodilation in WT mice (data not shown).

Superfusion of 150 U/mL Cu,Zn superoxide dismutase converted a -7.2% (vasoconstriction) response into a +5.5% (vasodilator) response at 10⁻⁶ mol/L methacholine. Superfusion of SNP resulted in dose-dependent vasodilation in both GPx-1⁻/⁻ and WT mice (Figure 2), with no significant difference in the response between the two groups. Therefore, endothelium-independent vasodilation in the microcirculation of GPx-1⁻/⁻ mice is preserved.

Figure 1. Mesenteric microvascular response to superfusion of p-methacholine in WT mice (■) (n=6) and GPx-1⁻/⁻ mice (●) (n=4) at 40 to 60 weeks of age. §P<0.0001 for total response; *P<0.001 for individual doses by post-hoc Newman-Keuls comparisons.

Aortic cGMP Levels
Aortic tissue from GPx-1⁻/⁻ mice accumulated less cGMP with 1 μmol/L acetylcholine stimulation for 1 minute compared with aortic tissue from WT mice (0.50±0.02 versus 0.90±0.10 pmol/mg tissue, P<0.01).

Cardiac Function
Figure 3A shows the developed pressure (DevP) during the course of the ischemia-reperfusion protocol. At baseline, DevP was similar between GPx-1⁻/⁻ and WT hearts. At the onset of zero-flow ischemia, DevP dropped quickly to zero in both groups. Under reperfusion, there was a gradual recovery of DevP with no difference in recovery between groups during the course of reperfusion. These data demonstrate that partial inactivation of GPx-1 did not result in any worse recovery of contractile performance after ischemia-reperfusion compared with WT hearts.

With global (zero-flow) ischemia, Figure 3B shows that EDP gradually rose during the course of the ischemic period. Immediately on reperfusion, EDP increased to a maximum of 81.5±4.3 mm Hg in WT and 99.7±6.6 mm Hg in GPx-1⁻/⁻ hearts (P<0.05). EDP gradually decreased during the course of reperfusion in both groups; however, GPx-1⁻/⁻ hearts exhibited worse diastolic function, as shown by a greater EDP compared with WT hearts. Total coronary flow was comparable between groups at baseline and reperfusion (baseline WT, 3.3±0.1 mL/min; GPx⁻/⁻, 2.8±0.4 mL/min; reperfusion WT, 1.3±0.1 mL/min; and GPx⁻/⁻, 1.3±0.1 mL/min). These data suggest that GPx-1⁻/⁻ hearts are more susceptible to diastolic dysfunction secondary to ischemia-reperfusion injury.

iPF₂-III Levels
There was a 172% increase in plasma levels of iPF₂-III in GPx-1⁻/⁻ mice (170.4±23.0 pg/mL, n=5) compared with
We noted striking changes in the vasculature of GPx-1/- mice compared with WT mice, as illustrated in Figure 4. Both interstitial and perivascular fibrosis were observed in the myocardium of GPx-1/- mice (Figures 4A and 4B). Abundant periadventitial inflammation was also noted (Figure 4C), as was neointima formation (Figures 4D and 4E). Extensive collagen deposition was observed surrounding the epicardial coronary arteries, as well (Figure 4F), implicating the presence of abundant fibroblasts of a synthetic phenotype. No such changes were apparent in WT mice (data not shown).

**Histological Assessment**

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**Discussion**

Previous studies have supported a relationship between decreased GPx activity and vascular injury. GPx-1 activity is decreased in atherosclerotic plaques excised from patients with carotid artery disease, and hyperhomocysteinemia is associated with a significant reduction in GPx-1 expression, indicating that a change in the expression of this enzyme may contribute to vascular oxidant stress and the progression of atherothrombosis. Pharmacological inhibition of GPx-1 by 50% is associated with an increased flux of reactive oxygen species and low-density lipoprotein oxidation. Selenium deficiency is associated with a decrease in GPx-1 activity in rats, and dietary selenium supplementation increases GPx-1 activity in the aorta and liver of rats. Patients with selenium deficiency have an increased incidence of coronary artery disease and a decrease in GPx-1 activity. These data are consistent with the requirement for dietary selenium in the synthesis of selenocysteine-containing proteins such as GPx-1. Taken together, these observations suggest that the selenocysteine-containing protein GPx-1 serves an important role in protecting the vasculature from oxidant stress. Our findings of endothelial dysfunction, increased oxidant stress, and abnormal vascular structural changes in older mice with heterozygous deficiency of GPx-1 support this hypothesis.

Mesenteric vessels from mice with heterozygous GPx-1 deficiency demonstrate paradoxical vasoconstriction to methacholine and bradykinin superfusion. In addition, cGMP levels in response to stimulation with a muscarinic agonist were significantly lower in GPx-1/- mice compared with WT mice. This result suggests that the endothelial dysfunction develops as a result of a deficiency of bioactive NO in the presence of GPx-1 deficiency. Importantly, there was no difference in the arteriolar response to SNP, a direct vasodilator, between WT and GPx-1/- mice, indicating that the endothelial dysfunction in GPx-1/- mice is not a result of nonspecific impairment of vascular smooth muscle function.

Oxidant stress plays an important role in the development and progression of atherothrombosis. Isoprostanes are formed by the nonenzymatic reaction of free radicals with arachidonic acid. The isoprostane iP2>III has been identified as a marker of increased oxidant stress in several conditions associated with atherothrombosis, including diabetes mellitus, cigarette use, and hypercholesterolemia. The increase in iP2>III in the plasma and aortas of GPx-1/- mice suggests that increased oxidant stress occurs as a result of a deficiency of this important antioxidant enzyme. Although there are some data in vitro to support the production of iP2>III in a cyclooxygenase-dependent fashion, this mechanism is not supported by recent patient data. Taken together with the improvement in endothelial function after mesenteric superfusion with Cu,Zn superoxide dismutase, these data showing impaired endotheli um-dependent NO-mediated endothelial function in the setting of increased oxidant stress in the vasculature suggest that bioactive NO may be decreased by interaction with reactive oxygen species, whose inactivation is limited by GPx-1 deficiency.

Heterozygous deficiency of GPx-1 is also associated with impaired cardiac function after ischemia-reperfusion injury. In contrast to a prior published report, we failed to find any abnormality in contractile performance after ischemia-reperfusion injury; however, we report for the first time an abnormality in diastolic dysfunction accompanying recovery from no-flow ischemia. This abnormal diastolic function may be a consequence of intramyocardial vascular dysfunction, cardiomyocyte dysfunction, regional differences in the severity of
ischemia, changes in myocardial turgor, or a combination of these effects resulting from inadequate intracellular GPx-1 pools. That GPx-1 is present in much higher concentrations in the heart than is catalase, has a lower Michaelis constant for hydrogen peroxide than catalase, and reduces lipid peroxides as well as hydrogen peroxide\(^2\) suggests that this enzyme is critical for the protection of the myocardium against oxidant stress.

The heart and vasculature of GPx-1\(^{-/-}\) animals is subject to greater injury and dysfunction, not only by the profound oxidant stress of acute ischemia-reperfusion injury but also by low-level oxidant stress occurring throughout the lifetime of these animals. This point is clearly illustrated by the increase in basal levels of F\(_2\)-isoprostanes as well as the profound histologic changes observed in the myocardium and myocardial vasculature.

Histological sections from coronary arteries and aorta from these older GPx-1\(^{-/-}\) mice show increased perivascular matrix deposition and adventitial fibroblasts, as well as inflammatory cells and intimal thickening, when compared with WT mice. These vascular lesions may be indicative of the damage caused to the vasculature by the increased oxidant stress accompanying lifelong partial GPx-1 deficiency. These lesions are reminiscent of changes noted in the aging process, but are significantly more marked, suggesting that the GPx-1\(^{-/-}\) mouse may serve as a model of aging for cardiovascular studies and supporting a relationship between the cumulative effects of free radical-based oxidant injury and the aging process.\(^{29,30}\)

In summary, heterozygous GPx-1 deficiency results in impaired endothelium-dependent vascular relaxation in resistance vessels and abnormal diastolic function in hearts subject to ischemia-reperfusion injury. GPx-1 deficiency causes increased oxidative stress, as measured by elevations of plasma IPF\(_2\)-III. The vasculature and myocardium of these animals demonstrate increased perivascular matrix deposition and fibrosis, as well as intimal thickening. We conclude that GPx-1 deficiency per se causes increased oxidant stress, which impairs endothelial and cardiomyocyte function and leads to structural vascular and cardiac abnormalities.

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