Glutathione Peroxidase-1 Plays a Major Role in Protecting Against Angiotensin II–Induced Vascular Dysfunction

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Abstract—Levels of reactive oxygen species, including hydrogen peroxide increase in blood vessels during hypertension and in response to angiotensin II (Ang II). Although glutathione peroxidases are known to metabolize hydrogen peroxide, the role of glutathione peroxidase during hypertension is poorly defined. We tested the hypothesis that glutathione peroxidase-1 protects against Ang II–induced endothelial dysfunction. Responses of carotid arteries from Gpx1-deficient (Gpx1+/− and Gpx1−/−) and Gpx1 transgenic mice, and their respective littermate controls, were examined in vitro after overnight incubation with either vehicle or Ang II. Under control conditions, relaxation to acetylcholine (ACh; an endothelium-dependent agonist) was similar in control, Gpx1+/−, and Gpx1 transgenic mice, whereas in Gpx1−/− mice, responses to ACh were impaired. In control mice, ACh-induced vasorelaxation was not affected by 1 nmol/L of Ang II. In contrast, relaxation to ACh in arteries from Gpx1−/− mice was inhibited by ~60% after treatment with 1 nmol/L of Ang II, indicating that Gpx1 haploinsufficiency markedly enhances Ang II–induced endothelial dysfunction. A higher concentration of Ang II (10 nmol/L) selectively impaired relaxation to ACh in arteries from control mice, and this effect was prevented in arteries from Gpx1 transgenic mice or in arteries from control mice treated with polyethylene glycol-catalase (which degrades hydrogen peroxide). Thus, genetic and pharmacological evidence suggests a major role for glutathione peroxidase-1 and hydrogen peroxide in Ang II–induced effects on vascular function. (Hypertension. 2008;51:872-877.)

Key Words: oxidative stress ■ hydrogen peroxide ■ genetically altered mice ■ carotid arteries ■ endothelium

During hypertension, cells within the vessel wall produce a variety of reactive oxygen species, including hydrogen peroxide (H2O2).1–3 Increased levels of reactive oxygen species contribute to vascular pathophysiology.4 In addition to its rate of production by superoxide dismutases (SODs), steady-state levels of H2O2 are also determined by the activity of glutathione peroxidases (GPxs), which metabolize H2O2 to water.5–7 Increasing evidence suggests that H2O2 may play diverse and important roles in vascular biology.5,8 For example, H2O2 can cause vasoconstriction or vasodilation (by both endothelium-dependent and endothelium-independent mechanisms).5,7,8 H2O2 can also impair endothelium-dependent responses to other stimuli.5 Studies using mice deficient in the expression of Gpx1 or exogenous application of H2O2 suggest that H2O2 can impair NO-mediated signaling in blood vessels.10–12 This effect may occur through several mechanisms, including stimulation of NADPH oxidases, a major source of superoxide.5,8,13

The ren-angiotensin system plays a major role in vascular biology and contributes importantly to changes in vascular structure and function in pathophysiological conditions, including hypertension, atherosclerosis, and diabetes.14,15 It is well established that angiotensin II (Ang II) produces oxidative stress in blood vessels, because both superoxide and H2O2 increase in blood vessels in models of hypertension and in response to Ang II.1,3,16,17 Ang II impairs endothelium-dependent relaxation,1,16,17 whereas superoxide can impair endothelial function via its well-described interaction with NO.7 H2O2 may impair vascular function by other, more complex mechanisms.5 Little is known regarding the role of H2O2 in vascular function in models of hypertension.

Expression and activity of the various GPx isoforms are presumably key determinants of oxidative stress, and vascular expression and activity of GPx may change in models of hypertension.1–2 The functional importance of GPx in response to Ang II is unknown, however. In addition to genetic factors, activity of GPx-1 may be inhibited by oxidative stress.20 Thus, the functional importance of GPx-1 in blood vessels is largely unknown and difficult to predict. Interestingly, the rate of cardiovascular events in patients with atherosclerosis is inversely related to activity of GPx in erythrocytes,21 suggesting a key role for H2O2 in vascular disease.
Thus, the goal of these experiments was to examine the functional importance of GPx-1 and the role of H$_2$O$_2$ in Ang II–induced endothelial dysfunction. We tested the hypotheses that genetic deficiency in Gpx1 would promote, and overexpression of Gpx1 would protect against, Ang II–induced endothelial dysfunction. As part of these studies, we also examined whether polyethylene glycol (PEG) catalase (which degrades H$_2$O$_2$) protects against Ang II–induced endothelial dysfunction.

Methods

Experimental Animals

Male and female Gpx1-deficient mice$^{22}$ were derived from breeding pairs of heterozygous Gpx1 mice. We studied heterozygous and homozygous Gpx1 deficient (Gpx1−/− and Gpx1−−) mice and their wild-type (Gpx1+/+) littermates. Gpx1 transgenic mice (Gpx1 Tg)$^{23}$ were derived from breeding pairs of C57Bl/6 and Gpx1 Tg mice. Gpx1 Tg and their nontransgenic littermates (non-Tg) were studied. For studies investigating effects of PEG catalase, C57Bl/6 mice were used.

All of the breeding was performed in a virus- and pathogen-free barrier facility at the University of Iowa. The genotype of Gpx1-deficient mice and their wild-type littermates was performed by PCR.$^{12}$ Genotyping for the presence of Gpx1 transgene was performed by real-time quantitative PCR using the comparative threshold method.$^{24}$ Genomic DNA obtained from tail biopsies was used for templates, and values were normalized to GAPDH. All of the experimental protocols and procedures conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of the University of Iowa.

General Preparation

Methods used to study carotid arteries in mice have been described.$^{18}$ Each segment of carotid artery was placed in individual wells using cell culture dishes as described.$^{18}$ Vessels were then incubated with Krebs buffer, loose connective tissue was removed, and vessels were cut into rings (3- to 4-mm in length). Each segment of carotid artery was placed in individual wells using cell culture dishes as described.$^{18}$ Vessels were then incubated with either vehicle (double-distilled H$_2$O) or Ang II (1 or 10 nmol/L) for 22 hours at 37°C.$^{18}$ After incubation, vascular rings were connected to force transducers to measure isometric tension in an organ bath maintained at 37°C. Resting tension was increased stepwise to reach a final tension of 0.25 g, and the rings were allowed to equilibrate for 45 minutes.

Experimental Protocols

Concentration-response curves to serotonin (10$^{-8}$ to 10$^{-3}$ mol/L) in carotid arteries were obtained at the beginning of each experiment. Relaxation of carotid arteries in response to acetylcholine (an endothelium-dependent agonist) and nitroprusside (an endothelium-independent agonist) were measured after submaximal precontraction using the thromboxane analog U46619 (9,11-dideoxy-11a,9a-epoxy-methanoprostaglandin-F$_{20}$). Using pharmacological approaches and gene-targeted mice, we have shown that responses of the carotid artery to acetylcholine are mediated by endothelial NO synthase.$^{25}$ At the end of each experiment, we obtained a full concentration response curve to U46619 (0.03 to 3.00 μg/mL).

To examine the role of H$_2$O$_2$ in endothelial dysfunction in response to Ang II, responses to acetylcholine and nitroprusside were examined in arteries from C57Bl/6 mice incubated with vehicle or Ang II (10 nmol/L) in the absence and presence of PEG catalase (1000 U/mL). PEG catalase was incubated with 10 nmol/L of Ang II for 22 hours at 37°C, as described above.

Real-Time PCR

Levels of mRNA for Gpx1 and Gapdh were measured by quantitative real-time RT-PCR. Total RNA was isolated from aorta using TRIZOL reagent (Invitrogen). RNA (2 μg) was then reverse transcribed using Taqman reverse transcriptase and random hexamer primers as described previously.$^{26}$ PCR primers and 6-carboxy fluorescein-labeled probes for Gapdh (Mm99999915_g1) and Gpx1 were purchased from Applied Biosystems. Reverse-transcribed cDNA was incubated with Taqman Universal PCR mix (Applied Biosystems) and PCR primers and probes at 50°C for 2 minutes and then at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using the Applied Biosystems 7700 sequence detection system. Amplicon-specific standard curves generated by serial dilutions of cloned cDNA were used to quantify the amount of Gpx1 and Gapdh cDNA in each sample. Data were analyzed using sequence detection software version 1.6.3 (Applied Biosystems) and expressed as a ratio to levels of Gapdh mRNA.

Drugs

Acetylcholine, Ang II, nitroprusside, serotonin, and PEG catalase were obtained from Sigma, and all were dissolved in saline. U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilutions being made with saline.

Statistical Analysis

All of the data are expressed as means±SEs. No differences were noted in data from male and female mice, so the data from both groups were pooled. Relaxation to acetylcholine and nitroprusside is expressed as a percentage of relaxation to U46619-induced contraction. Contraction to serotonin and U46619 is presented in grams of tension. Comparisons of relaxation and contraction were made using ANOVA, followed by Tukey-Kramer posthoc test. Statistical significance was accepted at $P<0.05$.

Results

Deficiency in Gpx1 Selectively Impairs Endothelial Function in Carotid Artery

In wild-type and Gpx1−/− mice, acetylcholine produced relaxation that was similar in arteries incubated with vehicle (Figure 1; $P>0.05$). These findings suggest that loss of a single copy of the Gpx1 gene does not alter endothelial function under basal conditions. In contrast, complete deficiency in Gpx1 produced impairment of vascular responses to acetylcholine (Figure 1; $P<0.05$ versus Gpx1+/+ and Gpx1−/−). There was no effect of Gpx1 deficiency on responses to nitroprusside (Figure 2; $P>0.05$).

Endothelial Dysfunction in Response to Ang II Is Enhanced in Gpx1+/− Mice

Acetylcholine produced relaxation that was similar in carotid arteries from wild-type mice incubated with vehicle or 1 nmol/L of Ang II (Figure 1; $P>0.05$), as we have shown previously.$^{18}$ In contrast, 1 nmol/L of Ang II produced marked impairment of the response to acetylcholine in the carotid artery from Gpx1+/− mice (Figure 1; $P<0.05$). These findings suggest that Gpx1 haploinsufficiency markedly enhances Ang II–induced endothelial dysfunction at a concentration of Ang II that has no effect in arteries from wild-type mice. In Gpx1+/− mice, responses to acetylcholine were greatly impaired under basal conditions, and 1 nmol/L Ang II had no additional effect (Figure 1; $P>0.05$).

Relaxation of the carotid artery to nitroprusside (Figure 2; $P>0.05$), as well as contraction to U46619 (3 μg/mL after Ang II treatment: Gpx1+/−=0.31±0.03 g, n=7; Gpx1+/−=
0.37±0.04 g, n=6; Gpx1−/−=0.29±0.03 g, n=6; P>0.05) and serotonin (10 μmol/L after Ang II treatment: Gpx1+/+ = 0.08±0.01 g, n=7; Gpx1−/− =0.07±0.02 g, n=6; Gpx1−/− = 0.06±0.01 g, n=6; P<0.05) were similar in wild-type, Gpx1+/+, and Gpx1−/− mice, suggesting that the effect of Ang II is selective for endothelium.

**Ang II–Induced Endothelial Dysfunction Is Prevented by PEG Catalase**

In contrast to 1 nmol/L of Ang II, 10 nmol/L of Ang II produced marked impairment of acetylcholine-induced relaxation in arteries from control mice (Figure 3; P<0.05). To examine the role of H2O2 in Ang II–induced endothelial dysfunction, vascular responses were examined after treatment with 10 nmol/L of Ang II plus PEG catalase. Coincubation of PEG catalase with 10 nmol/L of Ang II almost completely prevented the inhibitory effect of Ang II on endothelium-dependent responses (Figure 3; P<0.05 versus Ang II). PEG catalase had no effect on relaxation to nitroprusside (Figure 3; P>0.05). These data provide pharmacological evidence that Ang II produces endothelial dysfunction, which depends on formation of H2O2.

**Ang II–Induced Endothelial Dysfunction Is Prevented in Mice Overexpressing Gpx1**

In aorta from non-Tg and Gpx1 Tg mice, mRNA for Gpx1 was present (please see supplemental Figure S1, available at http://hyper.ahajournals.org). Levels of mRNA are expressed relative to total RNA and Gapdh (see Figure S1) to examine whether the increase in Gpx1 mRNA levels is dependent on the method of data presentation. Importantly, we found that Gpx1 expression was increased by ≈3-fold in Tg mice compared with non-Tg mice. In non-Tg and Gpx1 Tg mice, carotid arteries treated with vehicle relaxed in a similar manner to acetylcholine and nitroprusside (Figure 4), suggesting that overexpression of Gpx1, per se, does not alter vascular responses.

In non-Tg mice, 10 nmol/L of Ang II impaired responses of carotid arteries to acetylcholine (Figure 4; P<0.05) but not nitroprusside (Figure 4; P>0.05). In contrast, overexpression of Gpx1 completely prevented inhibition of acetylcholine responses by Ang II (Figure 4; P<0.05). Responses to nitroprusside (Figure 4; P<0.05), U46619 (3 μg/mL after Ang II treatment: non-Tg =0.41±0.03 g, n=9; Gpx1 Tg =0.41±0.04 g, n=10; P>0.05) and serotonin (10 μmol/L after Ang II treatment: non-Tg =0.09±0.01 g, n=9; Gpx1 Tg =0.06±0.01 g, n=10, P>0.05) were similar in non-Tg and Gpx1 Tg mice. These findings suggest that overexpression of Gpx1 prevents endothelial dysfunction caused by Ang II.

**Discussion**

Our study had several major findings. First, we found that heterozygous Gpx1 deficiency does not alter endothelium-dependent relaxation under baseline conditions. In contrast, complete deficiency in Gpx1 produced marked impairment of endothelium-dependent relaxation in the carotid artery. Second, a low concentration of Ang II (1 nmol/L), which had no effect in wild-type mice, produced marked endothelial dysfunction in Gpx1−/− mice, suggesting that loss of a single copy of the Gpx1 gene (haploinsufficiency) greatly increases sensitivity to Ang II–induced endothelial dysfunction. We also show that overexpression of Gpx1 protects against...
endothelial dysfunction in response to a higher concentration of Ang II (10 nmol/L) and that Ang II–induced endothelial dysfunction could be prevented by treatment with PEG catalase. Thus, both genetic and pharmacological evidence suggest that Ang II–induced vascular dysfunction is mediated by H₂O₂. Taken together, these results provide direct evidence that Gpx1 plays an essential role in protecting the vasculature against endothelial dysfunction caused by Ang II. Because these studies were performed in vitro, these changes reflect direct effects of Ang II on the vessel wall independent of changes in arterial pressure or other systemic effects of Ang II.

Genetic Deficiency in Gpx1 Produces Endothelial Dysfunction

Levels of reactive oxygen species are regulated, in part, by activity of an array of antioxidant enzymes, the importance of which is poorly defined in vascular cells. These enzymes include GPxs, part of a family of selenium-dependent enzymes. In the absence of GPx activity, subcellular concentrations of H₂O₂ rise. Similar to the SODs, there are multiple isoforms of GPxs. GPx is expressed in vascular cells and blood vessels (present study and References 1, 2, 30–32) and is expressed in relatively high levels in endothelium. Although GPx isoforms are presumably key determinants of H₂O₂ levels and overall oxidative stress, very little is known about the functional importance of GPx isoforms in disease states for any blood vessel.

Studies using exogenous application of H₂O₂ or mice deficient in expression of GPx suggest that H₂O₂ can impair NO-mediated signaling in blood vessels. For example, H₂O₂ may impair endothelium-dependent relaxation after conversion to hydroxyl radical. H₂O₂ can stimulate NADPH oxidase in vascular cells, as well as reduce levels of tetrahydrobiopterin, and, thus, may promote uncoupling of endothelial NO synthase, further increasing superoxide. Such increases in superoxide presumably result in a feedforward mechanism that further amplifies oxidative stress.

We found that loss of a single copy of the Gpx1 gene, which produces an ≈50% reduction in Gpx-1 activity, had no effect on the response of the carotid artery to acetylcholine. In Gpx1−/− mice, activity of GPx-1 is undetectable, and we observed marked impairment of endothelial function in the carotid artery. Our findings are consistent with previous work in this area. Specifically, endothelial function was impaired in Gpx1−/− mice in both aorta and mesenteric arterioles. Such findings suggest an absence in compensatory increases in activity of other antioxidant enzymes, such as SODs and catalase, in Gpx-deficient mice. Overall, these findings suggest that complete genetic deletion of Gpx1 produced marked impairment of endothelial function, consistent with the concept that Gpx-1 plays a major role in protecting the vasculature.

Gpx1 Deficiency Enhances Ang II–Induced Endothelial Dysfunction

Compared with other antioxidant enzymes, such as SODs, relatively little is known regarding the functional importance of GPx-1 in the vasculature. In relation to disease, it has been shown previously that GPx-1 protects against oxidative stress and endothelial dysfunction during hyperhomocysteinemia and atherosclerosis. In the present study, we examined whether Ang II–induced endothelial dysfunction is enhanced in Gpx1−/− or Gpx1−/− mice. Studies involving heterozygous-deficient mice are of particular interest in relation to genetic polymorphisms and disease states that produce reduced activity of GPx-1. For example, a genetic polymorphism in Gpx1, which reduces its activity, is associated with carotid artery thickening in patients with diabetes.

Perhaps the most important finding of the present study was the observation that a very low concentration of Ang II,
which had no effect on endothelial responses in wild-type mice, produced marked endothelial dysfunction in Gpx1−/− mice. These data provide direct evidence that both copies of the Gpx1 gene are required to fully protect blood vessels from Ang II–induced endothelial dysfunction. Although our findings related to Gpx-1 are new, the data support the overall concept that oxidative stress plays a key role in mechanisms that produce vascular dysfunction in response to Ang II.

In contrast to Gpx1−/− mice, Ang II did not produce significant additional impairment of endothelial function in Gpx1−/− mice. Gpx1 deficiency, per se, did not appear to produce major alterations in Ang II–mediated signaling, because Gpx1−/− mice were very responsive to the peptide. In other models of oxidative stress, receptors for Ang II and Ang II–mediated signaling are generally intact or increased (eg, see References 37,38). We assume that Ang II did not have a large effect in Gpx1−/− mice, because responses to acetylcholine were already markedly impaired in the absence of Ang II.

Overexpression of Gpx1 Prevents Ang II–Induced Endothelial Dysfunction

Our data with real-time RT-PCR indicate that expression of Gpx1 mRNA is increased several-fold in the vasculature of Gpx1 Tg mice. Importantly, overexpression of Gpx1 was very effective in attenuating Ang II–induced endothelial dysfunction, because the higher concentration of Ang II (10 nmol/L), which produced marked inhibition of acetylcholine–induced relaxation in non-Tg mice, had no effect on this response in Gpx1 Tg mice. We and others have shown previously that Ang II increases superoxide levels and causes superoxide- and NADPH oxidase-dependent endothelial dysfunction in this model.18,39,40 We also found that copper-zinc SOD overexpression protects against Ang II–induced endothelial dysfunction.15 The findings that both SOD18,41 and catalase or GPx-1 (present study) protect the vasculature from Ang II–induced vascular dysfunction is not surprising. Both SOD and GPx protect blood vessels against elevated homocysteine, which produces oxidative stress in the vasculature.12 Within the vasculature, there are examples in which both scavengers of superoxide and catalase protect vascular cells.13,42 This combination of effects may reflect increased superoxide generation via activation of NADPH oxidase,5,13 and/or promotion of endothelial NO synthase uncoupling, by H2O2.16

In addition to studies using Gpx1 Tg mice, we found that treatment with PEG catalase prevented Ang II–induced vascular dysfunction. Thus, both genetic and pharmacological evidence suggest that H2O2 plays a key role in mediating vascular dysfunction in response to Ang II. Taken together, the data suggest that overexpression of Gpx1 protects against, whereas deficiency in Gpx1 promotes, Ang II–induced endothelial dysfunction.

Perspectives

Reactive oxygen species are thought to play a major role in the biology of vascular disease. The present study supports the concept of GPx-1–specific vascular protection and provides the first evidence that GPx-1 protects against Ang II–induced endothelial dysfunction. These studies support the concept that H2O2 is a key player in mechanisms that promote vascular disease.5,8 Multiple lines of evidence suggest that Ang II promotes oxidative stress and vascular disease during hypertension. Thus, the present findings support this concept but also have broader implications, because Ang II may contribute to vascular disease in conditions such as atherosclerosis, diabetes, and aging.

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Disclosures

None.

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