Glutathione Peroxidase-3 Deficiency Promotes Platelet-Dependent Thrombosis In Vivo

Richard C. Jin, BS; Christopher E. Mahoney, BS; Laura (Coleman) Anderson, BS; Filomena Ottaviano, PhD; Kevin Croce, MD, PhD; Jane A. Leopold, MD; Ying-Yi Zhang, PhD; Shiw-Shih Tang, PhD; Diane E. Handy, PhD; Joseph Loscalzo, MD, PhD

Background—Glutathione peroxidase-3 (GPx-3) is a selenocysteine-containing plasma protein that scavenges reactive oxygen species in the extracellular compartment. A deficiency of this enzyme has been associated with platelet-dependent thrombosis, and a promoter haplotype with reduced function has been associated with stroke risk.

Methods and Results—We recently developed a genetic mouse model to assess platelet function and thrombosis in the setting of GPx-3 deficiency. The GPx-3(-/-) mice showed an attenuated bleeding time and an enhanced aggregation response to the agonist ADP compared with wild-type mice. GPx-3(-/-) mice displayed increased plasma levels of soluble P-selectin and decreased plasma cyclic cGMP compared with wild-type mice. ADP infusion-induced platelet aggregation in the pulmonary vasculature produced a more robust platelet activation response in the GPx-3(-/-) than wild-type mice; histological sections from the pulmonary vasculature of GPx-3(-/-) compared with wild-type mice showed increased platelet-rich thrombi and a higher percentage of occluded vessels. Cremaster muscle preparations revealed endothelial dysfunction in the GPx-3(-/-) compared with wild-type mice. With a no-flow ischemia-reperfusion stroke model, GPx-3(-/-) mice had significantly larger cerebral infarctions compared with wild-type mice and platelet-dependent strokes. To assess the neuroprotective role of antioxidants in this model, we found that manganese(III) meso-tetrakis(4-benzoic acid)porphyrin treatment reduced stroke size in GPx-3(-/-) mice compared with vehicle-treated controls.

Conclusions—These findings demonstrate that GPx-3 deficiency results in a prothrombotic state and vascular dysfunction that promotes platelet-dependent arterial thrombosis. These data illustrate the importance of this plasma antioxidant enzyme in regulating platelet activity, endothelial function, platelet-dependent thrombosis, and vascular thrombotic propensity. (Circulation. 2011;123:1963-1973.)

Key Words: antioxidants ■ GPx-3 ■ reactive oxygen species ■ stroke ■ thrombosis

Glutathione peroxidase-3 (GPx-3), a member of the selenocysteine-containing GPx family, is a major antioxidant enzyme in plasma that scavenges reactive oxygen species (ROS) arising from normal metabolism or after oxidative insult, thereby maintaining the vasorelaxant and antithrombotic properties of the vascular endothelium. Of the 5 known GPx isoforms, GPx-3 is the only one found in the extracellular space. Recent studies have demonstrated a correlation between decreased GPx-3 activity and arterial thrombosis in human subjects. Glutathione peroxidase-3 attenuates oxidant stress by reducing hydrogen peroxide and organic hydroperoxides to their corresponding alcohols. In contrast, a deficiency of GPx-3 impairs the reductive metabolism of ROS, leading in part to a decrease in nitric oxide (NO) bioavailability, thereby potentially impairing the inhibitory effect of extracellular (plasma-borne) NO on platelets. An impairment of normal platelet inhibitory mechanisms results in hyperreactive platelets and in a prothrombotic state. To understand better the relationship of GPx-3 with platelet-dependent thrombosis, we recently developed a genetic model of GPx-3 deficiency in which to assess platelet function in hemostasis and thrombosis. We hypothesized that GPx-3 deficiency induces platelet activation in vivo and thereby promotes platelet-dependent arterial thrombosis.

Editorial see p 1923
Clinical Perspective on p 1973

Methods

Glutathione Peroxidase-3 Knockout Mice
To disrupt the GPx-3 gene, a targeting construct was prepared in the pNTVK targeting vector with a 3.57-kb BglII/KpnI, 5’ arm, which contains intron 1 and a portion of exon 2 of the GPx-3 gene, and a 3.81-kb EcoRI fragment comprising the 3’ arm, which contains a portion of exon 5 and the 3’ flanking region of the GPx-3 gene. The
neomycin cassette replaced the 3’ end of exon 2 through the 5’ end of exon 5 (Figure 1A). Recombinants were selected in ES cells and used to produce chimeric mice. Male chimeras were bred with C57Bl/6 females. Brown mice from these matings were genotyped, and mice that were positive for the knockout locus were used in subsequent breeding. Each male chimera showed germline transmission to offspring. Heterozygous knockout mice were backcrossed 10 generations to nC57/Bl/6 mice.

Screening of Knockout Mice

An ~600-bp probe (EcoRV 5’ probe) was generated with the primers GP3EF (CTAAGCTACATTCCCAGTTG) and GP3EB (GGTTCACTCCTAGACTTG) and used to screen for the knockout loci by Southern blot hybridizations after EcoRV digestion of high-molecular-weight DNA from mouse tails. The EX4F/EX5R combination yields a fragment of 660 bp (wild type [WT]), and the KOF/EX5R combination produces a fragment of 323 bp (knockout). WT mice were identified by an exclusive 485-bp polymerase chain reaction (PCR) product; GPx-3(-/-) mice were identified by an exclusive 323-bp PCR product; and GPX-3(-/-/-) mice were identified by having both a 323-bp PCR product and a 485-bp PCR product (D; RV indicates right ventricular).

Genotype Determination

DNA was obtained by extraction from tail snips. To identify the GPx-3 gene, we used the forward primer 5’-TATCACCCCTACTG CAGACGTAA-3’ and the reverse primer 5’-AATGTGGCTCTCCCTCCTGAGAGTC-3’ to amplify a 485-bp fragment. To detect the neomycin resistance gene cassette present in the GPx-3(-/-) mice, we used the forward primer 5’-CCTACCGTGGATGTGGAATG TG-3’ to amplify a 323-bp fragment. Wild-type mice were identified by having both a 323-bp PCR product and a 485-bp PCR product; GPx-3(-/-) mice were identified by an exclusive 485-bp PCR product; and GPX-3(-/-/-) mice show no overt phenotype compared with WT mice.
identified by an exclusive 323-bp PCR product; and GPx-3(−/−) mice were identified by having both a 323-bp PCR product and a 485-bp PCR product (Figure 1D).

Glutathione Peroxidase-3 Expression via Quantitative Reverse-Transcription Polymerase Chain Reaction
Mice tissues were homogenized followed by total RNA extraction using the RNeasy kit (Qiagen). Total RNA (3 µg) was reverse transcribed with murine Moloney leukemia virus reverse transcriptase (Clontech). Murine GPx-3 cDNA was amplified with TaqMan probes. The expression of GPx-3 was normalized to GAPDH expression by means of the comparative Ct method (2−ΔΔCt; Applied Biosystems).

Glutathione Peroxidase Activity Assay
The GPX activity was measured indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced on reduction of hydrogen peroxide or an organic hydroperoxide (t-butyl-hydroperoxide) by GPx-3, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm that is proportional to GPX-3 activity.

Bleeding Time
Bleeding time was measured through a real-time determination of hemoglobin concentration. Mouse tails were cut and bled into tubes filled with the Drabkin reagent prewarmed at 37°C at 15-second intervals. Aliquots were then measured spectrophotometrically at 540 nm. The bleeding time was determined by taking the intersection of the initial slope and the plateau of the plot of hemoglobin concentration versus time.

Soluble P-Selectin Assay
After the mice were euthanized, plasma was obtained by centrifugation of blood collected in tubes containing 0.1 mol/L (final concentration) EDTA. Samples were then stored at −80°C until analysis. Plasma was diluted 1:50, and P-selectin was measured with a commercially available immunoassay (MPS00; R&D Systems).

cGMP Assay
After the mice were euthanized, plasma was obtained by centrifugation of blood collected in tubes containing 0.1 mol/L (final concentration) EDTA. Samples were then stored at −80°C until analysis. Plasma cGMP was measured with a commercially available immunoassay (Cayman Chemical 581021).

Platelet Preparation
After anesthesia (90 mg/kg ketamine, 10 mg/kg xylazine), mouse blood was drawn via cardiac puncture with ACD (65 mmol/L trisodium citrate, 70 mmol/L citric acid, 100 mmol/L dextrose, pH 6.5) as the anticoagulant. The blood was diluted with an equal volume of modified Tyrode buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl2, 12 mmol/L NaHCO3, 0.4 mmol/L Na2HPO4, 0.35% bovine serum albumin, 10 mmol/L Heps, 5.5 mmol/L glucose), pH 7.4, and centrifuged at 1200 g for 15 minutes at 22°C to obtain platelet-rich plasma. The platelet-rich plasma was further centrifuged at 1200g to isolate the platelet pellet and washed with a Heps-saline buffer containing 1% prostaglandin E1 (. The washed platelet pellet was reconstituted in modified Tyrode buffer and volume of modified Tyrode buffer were combined with 150 µL plasma from mice (WT or GPx-3(−/−)). After stirring for 1 minute, 20 µmol/L ADP was added to the platelet/plasma mixture, and aggregation was recorded for 4 minutes.

Assessment of Endothelial Function
Male mice were anesthetized (90 mg/kg ketamine, 10 mg/kg xylazine), and the cremaster muscle was isolated in situ to assess endothelial function. Bright-field microscopy was used to obtain images. Methacholine (10−10 to 10−6 mol/L) or bradykinin (10−10 to 10−6 mol/L) was superinfused onto the cremaster muscle, and changes in vessel diameter were recorded. Diethylenetriamine NONOate (10−11 to 10−7 mol/L) was also superfused as an endothelium-independent vasodilator control.

Pulmonary Thromboembolism Model
Mice were anesthetized with a ketamine (80 mg/mL)/xylazine (10 mg/mL) mixture. An incision was made on the ventral side of the neck to expose the right jugular vein. An Ultra-Miniature Mikro-Tip Pressure Transducer 1.4F catheter (Millar Instruments) was inserted into the right jugular vein and advanced into the right ventricle. Right ventricular pressures were measured for ~5 minutes with the MPVS 400 system (Millar Instruments) to establish the baseline. The right jugular vein was exposed, and a cannula was inserted and secured in the vein. Increasing doses of ADP (0.1 to 10 µmol/L administered at 80 µL/min at 3-minute intervals between doses) were administered while the dose-dependent changes in right ventricular pressure to the platelet agonist were monitored.

Lung Histology
Lung sections were processed and stained with an anti-P-selectin (C20; Santa Cruz Biotechnology) antibody, 1:50 dilution, followed by the goat ABC staining system (sc-2023; Santa Cruz Biotechnology).

Middle Cerebral Artery Occlusion Model
Mice were anesthetized with 1.5% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide. A flexible 0.5-mm fiberoptic probe was affixed to the skull over the brain cortex supplied by the middle cerebral artery (MCA) for relative cerebral blood flow measurements by laser Doppler flowmetry (Perimed). Baseline cerebral blood flow values measured before internal carotid artery occlusion were defined as 100% flow. An incision was made in the common carotid artery, and a silicon-covered 8–0 nylon monofilament (Doccol) was advanced through internal carotid artery to the origin of the anterior cerebral artery to occlude the MCA and posterior communicating artery. Middle cerebral artery occlusion (MCAO) was documented as a decrease in the laser Doppler signal to <20% of control values, after which the monofilament was secured in place. Mice were subjected to 1 hour of ischemia followed by removal of the filament after 1 hour. Mice were allowed to recover from surgery and then were evaluated for neurological deficits; they were euthanized 23 hours later for brain histology.

Neurological Scoring
Twenty-four hours after MCAO, mice were analyzed for neurological deficits with a 5-point scale. Normal motor function was scored as 0, flexion of the contralateral torso and forelimb by lifting the mouse by the tail as 1, circling to the contralateral side but normal posture at rest as 2, leaning to the contralateral side at rest as 3, and no spontaneous motor activity as 4.

Measurement of Infarct Volume
Infarct size was determined by staining with 2,3,5-triphenyltetrazolium chloride. Brains were cut into 2-mm-thick coronal sections and stained with 1% 2,3,5-triphenyltetrazolium chloride at 37°C for 1 hour. Sections were then analyzed with ImageJ, and infarct sizes were determined with the indirect method, which corrects for edema and volume loss from (putrefaction) necrosis.
Pharmacological Interventions in the Middle Cerebral Artery Occlusion Model

Mice were injected with 30 mg/kg clopidogrel hydrogen sulfate (Sigma) intraperitoneally 1 hour before the MCAO ischemia/reperfusion procedure. Mice were injected with 30 mg/kg manganese(III) meso-tetrakis(4-benoic acid)porphyrin (MnTBAP), a superoxide dismutase and peroxidase mimetic, intraperitoneally 30 minutes before the MCAO ischemia/reperfusion procedure.

Statistics

All results are expressed as mean±SE. Statistical analysis was performed with the Student t test method to analyze GPx-3 mRNA expression, GPx-3 activity, bleeding times, plasma P-selectin, plasma cGMP, and quantification of histological analysis. In some cases, statistical analysis was performed with 1-way ANOVA, 2-way ANOVA, or repeated measures ANOVA and appropriate posthoc tests (Student-Newman Keuls, Kruskal-Wallis, Tukey). Differences of P<0.05 were considered significant. Linear regression was performed to analyze the association between stroke infarct volume and neurological deficit score.

All experiments were approved by the Harvard Medical School Institutional Animal Care and Use Committee. Experimental success rates (defined as completion of each protocol) for the bleeding times, endothelial function, pulmonary thromboembolism, and MCAO models were 100%, 75%, 80%, and 80%, respectively.

Results

Glutathione Peroxidase-3 mRNA Expression

Glutathione peroxidase-3 mRNA expression was measured in mouse tissues via quantitative reverse-transcription PCR. In the WT mouse, GPx-3 was most highly expressed in the kidney, with the lowest expression levels in the liver (Figure 2A). The GPx-3−/− mice showed an ~50% reduction in renal expression compared with WT mice, whereas GPx-3+/− mice had virtually undetectable levels of renal expression (P<0.05; Figure 2B).

Plasma Glutathione Peroxidase Activity

The level of GPx-3 activity in the plasma from GPx-3−/− mice or WT littermates was measured by a coupled spectrophotometric assay with H2O2 and GSH (reduced glutathione) as cosubstrates. The GPx-3 activity was significantly decreased in plasma samples from GPx-3−/− mice (~20% residual activity, which likely reflects peroxidase activity from a non–GPx-3 source), and GPx-3+/− mice (~50%) compared with WT littermates (P<0.001; Figure 2C).

Bleeding Times

The GPx-3−/− mice showed an attenuated bleeding time compared with WT mice (94.5±14.4 versus 156.6±11.2 seconds, P<0.05), with GPx-3+/− mice having intermediate values (101.2±10.6 seconds; P<0.05; Figure 3A).

Plasma Concentrations of Soluble P-selectin

Soluble P-selectin, a marker of platelet and prothrombotic activity, was measured in the plasma from GPx-3−/− mice or GPx-3+/− littermates by ELISA. The GPx-3−/− mice showed significantly higher plasma soluble P-selectin levels compared with the WT littermates (137.8±12.3 versus 101.5±8.8 ng/mL plasma; P<0.05; Figure 3B).

Plasma cGMP

Cyclic GMP, a key second messenger molecule and marker for activation of soluble guanylyl cyclase by nitric oxide, was measured in plasma from GPx-3−/− or GPx-3+/− mice by ELISA. The plasma from GPx-3−/− mice had significantly lower cGMP levels compared with the WT littermates (5.38±1.75 versus 23.67±3.59 pmol/mL plasma; P<0.001; Figure 3C).

Platelet Aggregation Response to Agonist

To assess the ability of GPx-3 to inhibit platelet aggregation, washed platelets were added to platelet-poor plasma from either GPx-3−/− or from GPx-3+/− mice. The agonist ADP 20 μmol/L was added to the mixture, and aggregation was measured via light transmittance aggregometry. The GPx-3−/− mice had an average maximal extent of aggregation of...
42±2% compared with 24±1% for GPx-3(+/-) mice (Figure 4A; P<0.001). The GPx-3(−/−) mice had an average maximal extent of aggregation of 34±2% compared with 24±1% for GPx-3(+/-) mice (Figure 4A; P<0.001).

Endothelial Function
To assess endothelial function, the cremaster muscle was isolated in anesthetized mice, and methacholine was superfused at increasing concentrations while the dilatory response was measured via light microscopy. The GPx-3(+/-) mice showed a 9.7±2.3% increase in vessel diameter normalized to baseline at 1×10−7 mol/L methacholine compared with a 38.0±9.5% decrease in vessel diameter in GPx-3(−/−) mice (P<0.05; Figure 4B). At a concentration of 1×10−6 mol/L methacholine, GPx-3(+/-) mice showed a 11.2±2.8% increase in vessel diameter normalized to baseline compared with a 47.4±4.7% decrease in vessel diameter in GPx-3(−/−) mice (P<0.05; Figure 4B). Bradykinin also caused dose-dependent (10−10 to 10−6 mol/L) vasodilation in GPx-3(+/-) mice (maximal, 9.8±0.8% increase in vessel diameter normalized to baseline at 10−7 mol/L) compared with a decrease in vessel diameter in GPx-3(−/−) mice (maximal, 23.2±4.2% decrease at 10−7 mol/L; P<0.05; data not shown). In contrast, diethylenetriamine NONOate (10−11 to 10−6 mol/L) yielded equivalent responses in GPx-3(+/-) and GPx-3(−/−) mice over the full concentration range studied (data not shown).

Thromboembolic Response In Situ
To determine whether GPx-3 deficiency would contribute to platelet-dependent thromboembolism in vivo, ADP was infused in the left jugular vein while right ventricular systolic pressure was simultaneously recorded in the mice. The results of this experiment showed the highest increase in pulmonary artery pressure normalized to baseline in GPx-3(−/−) mice (Figure 5B) compared with GPx-3(+/-) mice (Figure 5A) at the peak ADP infusion concentration (0.1 μmol/L) (29.3±5.1% versus 3.68±8.06%; P=0.028; Figure 5C), suggesting a more robust platelet activation response in the GPx-3−/− deficient mice. The GPx-3−/− mice had a higher mortality rate compared with the GPx-3(+/-) mice (36% versus 9%), resulting in part from enhanced platelet-dependent thrombosis (Figure 5D).

Histological and Immunohistochemical Analyses of Thromboembolism Formation
Histological sections from the pulmonary vasculature of GPx-3(−/−) (Figure 6B) compared with GPx-3(+/-) mice (Figure 6A) showed significantly increased thrombi per 7.5 mm2 lung section on the basis of staining intensity for P-selectin (1.7±0.4 versus 1.0±0.1; P<0.001; Figure 6D), in addition to a significantly higher percentage of occluded vessels (82±16% versus 54±21%; P<0.05; Figure 6C).

Middle Cerebral Artery Occlusion Ischemia/Reperfusion
The middle cerebral artery was occluded in mice for 1 hour followed by 23 hours of reperfusion to determine the effects of GPx-3 deficiency on stroke size (Figure 7). The GPx-3(+/-) mice had an average infarct volume of 19.35±2.09 mm3 (Figure 7A and 7J) compared with 97.29±13.27 mm3 in GPx-3(−/−) mice (Figure 7D and 7J) and 117.48±7.99 mm3 in GPx-3(+/−) mice (P<0.001, 1-way ANOVA; Figure 7G and 7J).

Neurological Deficit Scoring
Neurological deficit scoring was conducted after the MCAO ischemia/reperfusion procedure to determine the extent of neurological injury. The GPx-3(+/-) mice had an average neurological score of 1.7±0.3; GPx-3(−/−) mice, 3.0±0.4; and GPx-3(+/−) mice, 3.4±0.3 (P=0.014, 1-way ANOVA; Figure 7K). The GPx-3(−/−) mice pretreated with MnTBAP had a score of 1.8±0.3 (P=0.025; Figure 6K).

Effect of Platelet Inhibition on Stroke Size and Neurological Deficit
To determine the role that platelets may play in these strokes, we pretreated mice with clopidogrel, a thienopyridine inhibi-
Figure 4. Gene dose response of glutathione peroxidase-3 (GPx-3) on platelet aggregation and endothelial function. A. To determine the effects of GPx-3 deficiency on platelet aggregation, platelet-poor plasma was isolated from each genotype and mixed with washed platelets from wild-type litter mates. The platelet agonist ADP (20 μmol/L) was then added to the mixture, and the maximal extent of aggregation was measured in 5 to 12 mice for each genotype using light transmittance aggregometry at 4 minutes. B. To assess the consequences of GPx-3 deficiency on endothelial function, the cremaster muscle was isolated in situ in mice, and methacholine was superinfused onto the preparation while the response was measured via light microscopy. The percent change in vessel diameter reported has been normalized to their respective measurements at baseline for 3 mice of each genotype.

Antioxidant Treatment on Stroke Size and Neurological Deficit

To test the potential benefit of reducing ROS generation pharmacologically on stroke size, MnTBAP, a superoxide dismutase and peroxidase mimetic, was injected into mice 30 minutes before the MCAO ischemia/reperfusion was performed. The GPx-3(+/−) mice pretreated with MnTBAP had an average infarct volume of 14.09±5.27 mm³ (Figure 7E and 7J) compared with 97.29±13.27 mm³ in vehicle-treated GPx-3(+/−) mice (P<0.001; Figure 7D and 7J). The GPx-3(−/−) mice pretreated with MnTBAP had an average infarct volume of 17.9±3.63 mm³ (Figure 7G and 7J) compared with 117.48±7.99 mm³ in vehicle-treated GPx-3(−/−) mice (P<0.001; Figure 7H and 7J). The GPx-3(+/−) mice pretreated with MnTBAP had a neurological deficit score of 1.8±0.3 (P=0.025) compared with GPx-3(+/−) mice (Figure 7K).

Discussion

In this study, we developed a mouse model that recapitulated the phenotype reported by our group in 2 brothers with GPx-3 deficiency who had arterial thrombosis and stroke syndromes. These patients were found to have an 3-fold increase in plasma H₂O₂ levels, decreased GPx-3 activity, and an attenuation of the normal platelet inhibitory response to NO as a result of impaired metabolism of ROS. The GPx-3 activity was reduced in the plasma of the 2 brothers compared with age-matched control subjects, and the attenuation of normal platelet inhibition was normalized by the addition of exogenous GPx. The data suggested that enhanced ROS flux impairs normal platelet inhibition.

Although we did not unequivocally demonstrate the specific ROS mechanism(s) responsible for enhanced platelet-dependent thrombosis in these early studies, several possibilities should be considered. Platelets generate superoxide, as well as hydrogen peroxide and lipid peroxides, on activation, which can promote platelet activation and impair the inhibitory action of NO on platelets. In support of these proposed mechanisms, we have previously shown that lipid peroxides enhance platelet activation, and that they decrease bioactive NO and platelet cGMP accumulation, and that GPx prevents these effects. Superoxide can directly react with NO to form peroxynitrite; however, neither lipid peroxides nor hydrogen peroxide can directly react with NO to inactivate it. Derivative peroxyl or hydroxyl radicals can do so and are likely to be generated from the reaction of parent peroxides with redox-active iron generated during platelet activation and aggregation. Furthermore, oxidation of tetrahydrobiopterin by ROS may also lead to endothelial dysfunction as manifested by attenuated NO synthesis and uncoupled NO synthase-dependent superoxide generation. The extent to which each or any of these reactions accounts for enhanced platelet activation in vivo is difficult to ascertain owing to limitations of measuring intermediates, the complexity and multiplicity of the reactions that occur in the intracellular environment, and the uncertainty of the mi-
croenvironmental concentrations of coreactants in the extracellular space in the immediate vicinity of an aggregated platelet.

From these data and our initial observations, we concluded that GPx-3 deficiency impairs the metabolism of ROS, ultimately resulting in a prothrombotic state. Subsequent follow-up studies conducted in our laboratory identified a link between a dysfunctional promoter haplotype in the GPx-3 gene and arterial ischemic stroke, as well as cerebral venous thrombosis, both in large unrelated cohorts. Produced primarily in the kidney, GPx-3 is the only isoform in the GPx family that is exclusively found in the extracellular compartment. Although its specific activity is only 10% that of the ubiquitously expressed cellular isoform GPx-1, it is the most important antioxidant enzyme in the extracellular compartment. To investigate further the link between GPx-3 deficiency and stroke, we created a GPx-3 knockout mouse model. We hypothesized that GPx-3 deficiency promotes platelet-dependent thrombosis after vascular injury and tested this hypothesis using several experimental approaches.

The tissue and vasculature of GPx-3–null mice are 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 illustrated by the increase in basal levels of soluble P-selectin and the decrease in cGMP in the GPx-3(-/-) plasma compared with WT animals. The low-level oxidant stress occurring throughout the lifetime of these knockout animals may result in increased susceptibility to platelet activation and aggregation, predisposing to thrombus formation. This point is evident in the bleeding time assay in which GPx-3(-/-) mice showed attenuated bleeding times compared with WT animals. Platelet aggregation studies also revealed hyperreactivity to ADP of WT platelets suspended in plasma taken from GPx-3(-/-) mice compared with GPx-3(+/+) mice, which recapitulated the findings in the original human study. We next administered ADP into the pulmonary vasculature to test the ability of GPx-3 or the lack thereof to maintain inhibition.

Figure 5. Thromboembolic response of glutathione peroxidase-3 (GPx-3) deficiency to ADP infusion. ADP was infused at increasing concentrations into the left jugular vein while changes in right ventricular pressure to the platelet agonist were simultaneously recorded. The right ventricular pressure tracing in A is representative of GPx-3(+/-) mice, whereas that in B is representative of the response seen in GPx-3(-/-) mice. C, Further analysis showed a greater increase in right ventricular pressure normalized to baseline in GPx-3(-/-) mice (n=4) compared with GPx-3(+/-) mice (n=4) (C). D, The mortality rate was also higher in GPx-3(-/-) mice (n=4) compared with GPx-3(+/-) mice (n=3). WT indicates wild type.
of platelet activation in response to the stress of a platelet agonist. Consequently, we observed higher right ventricular systolic pressures in GPx-3+/−/+ mice compared with GPx-3+/+/+ mice in response to ADP administration. Histological analysis revealed a significantly greater number and percentage of occluded pulmonary vessels in lung sections stained with P-selectin in GPx-3+/−/+ mice compared with GPx-3+/+/+ mice, consistent with a platelet-dependent acute thrombotic process. This platelet-dependent thrombosis, coupled with the potential for pulmonary vascular endothelial dysfunction (as suggested by the cremaster muscle vascular experiments), offers a ready explanation for the increased mortality in the GPx-3+/−/+ mice compared with the WT mice.

Perhaps the most relevant findings of this study are the effects of GPx-3 deficiency on cerebral infarction via transient ischemia/reperfusion in the MCA. A GPx-3 deficiency resulted in significantly larger strokes compared with GPx-3+/+/+ mice, which may be a consequence of both enhanced platelet activation and impaired endothelial function in the setting of increased oxidant stress accompanying vascular occlusion and reperfusion injury. The larger strokes seen in the GPx-3−/− mice correlated with higher (worse) neurological scores. The pretreatment of GPx-3−/− mice with the platelet inhibitor clopidogrel clearly showed the platelet-dependent nature of the strokes: GPx-3−/− mice treated with clopidogrel had relatively small, focal infarcts associated with lower (better) neurological scores, whereas GPx-3−/− mice treated with vehicle (saline) had strokes encompassing major portions of the affected hemisphere associated with higher (worse) neurological scores.

Prior studies have demonstrated an association between decreased plasma GPx-3 activity and arterial thrombosis.17,18 During platelet activation, ROS are generated in abundance and facilitate recruitment of platelets to the growing platelet plug (thrombus). Hydrogen peroxide (H2O2) production as a result of collagen-stimulated platelet activation has been shown to achieve concentrations up to 1 mmol/L,19 and this peroxide increase can have other (non–NO-dependent) effects on platelet signaling that enhance aggregation. Redondo and colleagues20 determined that H2O2 mediates changes in intracellular Ca2+ levels and affects Ca2+-related mechanisms through sulfhydryl oxidation–dependent and –independent mechanisms within platelets. The modulation of intracellular Ca2+ levels by H2O2 occurs through stimulatory Ca2+ release from the dense tubular system and from mitochondria within the platelets.21,22 In addition, H2O2 inhibits Ca2+ reuptake into the dense tubular system, mediated by the sarcoendoplasmic reticulum Ca2+ ATPase.23 Plasma membrane Ca2+ ATPase, which is responsible for the extrusion of Ca2+ from the platelet, is significantly attenuated when exposed to H2O2.24 Therefore, the combined effects that H2O2 exerts on platelets result in an overall increase in platelet activation.

Pretreatment of GPx-3−/− mice with MnTBAP, a superoxide dismutase and peroxidase (as well as peroxynitrate reductase)25 mimetic, reduced stroke size. By preventing the accumulation of ROS (including H2O2 and lipid peroxides), GPx-3 limits further platelet activation and maintains the bioavailability of NO, further limiting platelet thrombus expansion. Thus, a deficiency of GPx-3 impairs the metabolism of ROS, leading to enhanced platelet activation and endothelial dysfunction by a variety of potential mechanisms and perhaps impairing neurocytoprotection. Taken together, these abnormalities in the vascular redox environment result in hyperreactive platelets and the potential for platelet-dependent thrombosis (Figure 8).

The balance between NO generation and ROS production helps maintain normal platelet inhibition without compromising protective hemostasis. Platelet-derived NO regulates platelet activation and aggregation by maintaining platelets in their resting state. Superoxide, produced in activated platelets by NADPH oxidase, increases both platelet adhesion and aggregation,26 in part by reacting with NO to form peroxyni-
trite, resulting in decreased NO bioavailability. Other platelet-derived ROS include OH⁻ and \( \text{H}_2\text{O}_2 \), both generated by resting and activated platelets. This endogenous formation of ROS suggests that they may have both autocrine and paracrine effects on platelet activation, aggregation, and ultimately thrombus formation.

NADPH oxidase consists of membrane-bound components (gp91-phox and p22-phox), cytosolic proteins (p47-phox, p67-phox, and p40-phox), and Rac, a small GTP-binding protein. Seno and colleagues first showed evidence of NADPH oxidase activity in platelets by detecting p22-phox and p67-phox in platelet lysates. Krotz and coworkers

Figure 7. Gene dose response of glutathione peroxidase-3 (GPx-3) deficiency to middle cerebral artery occlusion (MCAO) ischemia/reperfusion. MCAO ischemia/reperfusion was performed to analyze the gene dose effect of GPx-3 on stroke size and the effects of anti-platelet and antioxidant treatments. Cor- onal sections (2 mm) were stained to determine the extent of stroke. Sections are representative for all mice (n=3 to 4 in each group): A, GPx-3(+/−); B, GPx-3(+/−)+manganese(III) meso-tetrakis(4-benzoic acid)porphyrin (MnTBAP); C, GPx-3(+/−)+clopidogrel; D, GPx-3(+/−); E, GPx-3(+/−)+MnTBAP; F, GPx-3(+/−)+clopidogrel; G, GPx-3(+/−)+H, GPx-3(+/−)+MnTBAP; and I, GPx-3(+/−)+clopidogrel. Cerebral infarction volumes are shown in J with corresponding neurological deficit scores in K. Linear regression analysis reveals a strong association between infarct volume and neurological deficit score (L).

Figure 8. Effect of glutathione peroxidase-3 (GPx-3) on platelets. Glutathione peroxidase-3 (GPx-3) is an enzyme that protects cells from oxidative damage via the reduction of hydrogen peroxide and organic hydroperoxides. By pre-venting the accumulation of reactive oxygen species (ROS), GPx-3 limits their effects on platelet function and maintains the bioavailability of nitric oxide (NO); thus, a deficiency of GPx-3 impairs the metabolism of ROS, leading to their poten- tiation of platelet activation and to a decrease in NO bioavailability both by decreasing tetrahydrobiopterin (BH₄) levels (which uncouples endothelial nitric oxide synthase [eNOS]) and by oxidatively inactivating NO (eg, via superoxide and lipid per- oxy radical formation). These effects impair nor- mal platelet inhibitory mechanisms, leading to hyperreactive platelets and the potential for plate-let-dependent thrombosis.
showed that NADPH oxidase–dependent platelet superoxide release increases platelet aggregation; these investigators also showed that superoxide generated from NADPH oxidase enhanced thrombus formation by inhibiting NTPDase1, resulting in an increase in platelet agonist ADP bioavailability. Another mechanism by which superoxide contributes to thrombus formation is by disrupting the redox potential-dependent regulation (GSH/GSSG ratio) of the platelet fibrinogen receptor (glycoprotein IIb/IIIa). Superoxide can also directly react with growth-stimulating hormone, increasing GSSG production and superoxide generation. Other studies have shown that superoxide reduces the threshold for platelet activation to thrombin, collagen, and ADP and may even induce spontaneous aggregation. Thus, GPx-3 can be viewed as an important determinant of vascular redox homeostasis crucial to modulation of the susceptibility of platelets to activation and aggregation.

Conclusions
Glutathione peroxidase-3 deficiency results in enhanced ROS flux and platelet activation, in part owing to decreased NO bioavailability in the plasma. A GPx-3 deficiency results in attenuated bleeding times and a more robust platelet activation response, as evidenced by higher levels of aggregation in GPx-3–deficient mouse plasma compared with WT control plasma, as well as higher right ventricular systolic pressures and more thrombi present in the pulmonary vasculature in response to ADP infusion. Endothelial function studies revealed dysfunction in the GPx-3–deficient mice compared with WT controls. The MCAO ischemia/reperfusion experiments showed larger strokes in GPx-3–deficient mice compared with WT controls. The reduction in stroke size with the administration of clopidogrel, an antiplatelet agent, showed that the cerebral infarcts created were platelet dependent and supports the role of GPx-3 in maintaining normal platelet inhibition and endothelial function. The reduction in stroke size with MnTBAP supports the importance of oxidant stress in platelet-dependent thrombosis and cerebral infarction in this model. We conclude that GPx-3 deficiency promotes platelet-dependent thrombosis and enhances arterial thrombotic risk.

Acknowledgments
We would like to thank Stephanie Tribuna for expert secretarial assistance, Eric Brunskill for his assistance in helping create the GPx-3 knockout mice, and Dmitriy Atochin and Jonathan Walsh for their assistance with the middle cerebral artery occlusion procedure.

Sources of Funding
This work was supported by National Institutions of Health grants HL081587, HL061795, HL070819, HL089734 (to Dr Loscalzo), HL081587, HL061795, HL070819, HL089734 (to Dr Loscalzo), HL086672, and a Harris Family Foundation Award (to Dr Croce).

Disclosures
None.

References

**CLINICAL PERSPECTIVE**

The identification of genetic risk factors for thrombotic stroke is a field in its infancy. This study presents a unique mouse model of thrombotic risk in which the extracellular antioxidant enzyme, glutathione peroxidase-3 (GPx-3), is eliminated by targeted gene disruption. This enzyme, the most important antioxidant enzyme in plasma and the extracellular space, eliminates peroxides from those compartments. This model was developed because earlier work from our group showed that a deficiency of this enzyme in plasma is an independent risk factor for thrombotic stroke in young individuals. Here, we show that mice deficient in glutathione peroxidase-3 have heightened platelet activation in the basal state, and, with provocation, have an increased propensity to thrombosis in the pulmonary and cerebral circulations. Furthermore, thrombotic strokes in glutathione peroxidase-3–deficient mice are much more severe than in wild-type mice; the size and severity of these strokes can be attenuated by inhibiting platelets and reactive oxygen species pharmacologically. These results demonstrate the importance of this key antioxidant enzyme in modulating platelet activation and thrombotic responses, and, together with prior genetic epidemiological studies from our group and others, suggest that glutathione peroxidase-3 is a potential marker of and therapeutic target for thrombotic stroke.
Glutathione Peroxidase-3 Deficiency Promotes Platelet-Dependent Thrombosis In Vivo
Richard C. Jin, Christopher E. Mahoney, Laura (Coleman) Anderson, Filomena Ottaviano, Kevin Croce, Jane A. Leopold, Ying-Yi Zhang, Shiw-Shih Tang, Diane E. Handy and Joseph Loscalzo

Circulation. 2011;123:1963-1973; originally published online April 25, 2011;
doi: 10.1161/CIRCULATIONAHA.110.000034
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/123/18/1963

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/