Glutathione Peroxidase Overexpression Inhibits Cytochrome c Release and Proapoptotic Mediators to Protect Neurons From Experimental Stroke

B. Hoehn, BA; M.A. Yenari, MD; R.M. Sapolsky, PhD; G.K. Steinberg, MD, PhD

**Background and Purpose**—Ischemic injury and reperfusion increases superoxide (O$_2^-$) production and reduces the ability of neurons to scavenge free radicals, leading to the release of cytochrome c and apoptosis. Here we test whether overexpression with the use of gene therapy of the antioxidant glutathione peroxidase (Gpx), delivered before or after experimental stroke, is protective against ischemic injury.

**Methods**—Sixty-two rats underwent middle cerebral artery occlusion for 1 hour. Defective herpes simplex viral vectors expressing Gpx/lacZ or lacZ alone (control) were delivered into each striatum 12 hours before or 2 or 5 hours after ischemia onset.

**Results**—Striatal neuron survival at 2 days was improved by 36% when Gpx was delivered 12 hours before ischemia onset, 26% with a 2-hour delay, and 25% when delayed 5 hours. After ischemia, Gpx overexpression significantly reduced cytosolic translocation of cytochrome c and increased the proportion of Bcl-2–positive cells compared with cells transfected with control vector. Bax and activated caspase-3, while present in control-transfected neurons after ischemia, were rarely noted in Gpx-transfected cells.

**Conclusions**—Expression from these herpes simplex viral vectors begins 4 to 6 hours after injection, which suggests a 9- to 11-hour temporal therapeutic window for Gpx. This is the first study to show that overexpression of Gpx with the use of gene therapy protects against experimental stroke, even with postischemic transfection, and the neuroprotective mechanism involves attenuation of apoptosis-related events. (*Stroke. 2003;34:2489-2494.*)

**Key Words:** apoptosis ■ cytochrome c ■ gene therapy ■ glutathione peroxidase ■ ischemia ■ neuroprotection

*Reactive* oxygen species (ROS) play a significant role in the development of ischemic damage. ROS may be involved in the damage either directly, through interaction with and destruction of cellular proteins, lipids, and DNA, or indirectly, by affecting normal cellular signaling and gene regulation. Ischemic damage in the brain may be reduced by increasing levels of enzymatic antioxidants, including superoxide dismutase (SOD), which catalyzes the dismutation of superoxide (O$_2^-$), and catalase, which mediates the breakdown of H$_2$O$_2$. It has been suggested that these antioxidants protect mainly by extracellular scavenging of ROS, thus protecting cells from oxidative stress.$^2$ An antioxidant enzyme known to protect cells from oxidative stress, but which has not been as thoroughly investigated, is glutathione peroxidase (Gpx). Gpx is a ubiquitous antioxidant enzyme that, like catalase, facilitates the breakdown of H$_2$O$_2$ into water and oxygen. In in vitro studies, Gpx was shown to confer greater protection against oxidative stress than SOD, catalase, or the combination of SOD and catalase.$^3$ Its efficacy may reflect the fact that it is located in both the mitochondria and the cytosol, and it can use both lipid peroxidase and H$_2$O$_2$ as substrates.

The role of ROS in focal ischemia and cytochrome c release has also been investigated previously.$^{4,5}$ These studies suggest that an increase in O$_2^-$ levels within the mitochondria after focal ischemia releases cytochrome c and results in DNA fragmentation. Recent work shows that overexpression of cytosolic SOD1 can attenuate the early release of cytochrome c from mitochondria and subsequent neuronal apoptosis in transgenic mice after focal ischemia.$^6$ We investigated whether overexpression of Gpx with the use of herpes simplex viral (HSV) vectors protects striatal neurons against transient middle cerebral artery occlusion (MCAO) in rats when given before or after ischemic onset. We also tracked cytochrome c release and subsequent regulators of apoptosis to develop a better understanding of the interrelationship between Gpx and apoptosis.

**Materials and Methods**

**Amplicon Plasmids**

The construction of amplicon plasmids *pa22Bgalα4Gpx* and *pa22Bgalα4s* has been described in detail elsewhere.$^{7,8}$ Amplicon

Received February 3, 2003; final revision received June 9, 2003; accepted June 18, 2003.

From the Departments of Neurosurgery (B.H., M.A.Y., R.M.S., G.K.S.), Neurology and Neurological Sciences (M.A.Y., R.M.S., G.K.S.), and Biological Sciences (R.M.S.), Stanford Stroke Center (B.H., M.A.Y., R.M.S., G.K.S.) and Stanford Neurosciences Program (B.H., M.A.Y., R.M.S., G.K.S.), Stanford University, Stanford, Calif.

Correspondence to Gary K. Steinberg, MD, PhD, R281, Department of Neurosurgery, Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305-5327. E-mail gsteinberg@stanford.edu

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*Stroke* is available at [http://www.strokeaha.org](http://www.strokeaha.org)

DOI: 10.1161/01.STR.0000091268.25816.19
plasmid pao4Gpxα22Bgal was constructed as follows: Human gpx cDNA was isolated from the pbsGpxpβ2α23 plasmid (graciously provided by Michael Kelner) as a ClaI/HindIII fragment. The fragment was then blunt-end ligated into the BamHI site of pG310. This expression vector contains the polyadenylation (polyA) signal (from nucleotides +3270 to +3430) of the human cytomegalovirus 11 gene in a pGEM-2 plasmid background. The Gpx coding sequence together with the polyA tail was then isolated as a BamHI/BglII fragment and blunt-end ligated into the HindIII site of pao22xgal, downstream of the α4 promoter. The control vectors pao22Bgal and pao4s+galler have been described previously.8,9 The pao22Bgal contained the Eschericia coli lacZ gene, a simian virus 40 polyA signal, and α22 of HSV as promoter.10 Because both promoters are immediate early genes of HSV with similar kinetics, we have previously shown that this system results in coexpression of the 2 transgenes of interest.8,9,10 The HSV oriS and the “α” sequence were also included to provide the necessary cis signals for replication and packaging of the ampiclon DNA, pao22Bgalα4s, which lacks the gpx gene and contains a stop codon after the promoter (“α”), was used to produce control vector.

HSV Vector
Protocols for generating viral vectors in this study with the use of the ampiclon system have been previously described.7,8 Briefly, pao22Bgalα4Gpx and pao22Bgal were transfected into E5 cells with the use of lipofectamine according to the manufacturer’s protocol. Twenty-four hours after transfection with the plasmid, cultures were superinfected with helper virus d120 (multiplicity of infection 0.1). These cells were harvested when 100% cytopathic effect was developed. Stocks were further purified by centrifugation and resuspension in PBS. The titers of helper virus were determined on Vero cells by quantifying percentage of infected cells. The percentage of infected cells was determined with the use of X-gal. These cells were harvested when 100% cytopathic effect was developed. Stocks were further purified by centrifugation and resuspension in PBS. The titers of helper virus were determined on E5 cells by plaque assay. The titers of ampiclon vectors were determined on Vero cells by quantifying the number of β-galactosidase (β-gal is the gene product of lacZ) expressing cells. Assays revealed plasmid titers of 3.3 to 4.1 × 107 (vectors per milliliter) and d120 helper virus titers of 4.13 to 6.25 × 107 (plaque-forming units per milliliter). Ratios of plasmid to helper virus were 1:2.

Focal Cerebral Ischemia
The Stanford University Administrative Panel of Laboratory Animal Care approved all experimental protocols. Sixty-two male Sprague-Dawley rats (Charles River, Wilmington, Mass) weighing 280 to 320 g were initially anesthetized with 3% halothane or isoflurane plus oxygen and air (ratio: 0.2/0.8 L/min) by facemask. When the surgical planes of anesthesia were attained, halothane was decreased to 2% to 2.5%. Depth of anesthesia was assessed by hind leg withdrawal every 15 minutes, and temperature, ECG, and respiration rate were monitored and kept in physiological ranges. After the skull was exposed, 2 Burr holes were drilled bilaterally at the coordinates anteroposterior 0 mm, mediolateral 3.5 mm, relative to bregma. The dura was kept intact, and the animal was prepared for MCAO. The common carotid, external carotid, and pterygopalatine arteries were exposed and ligated on the left side. The left internal carotid artery was transiently occluded with a microsurgical clip, and an arteriogram was made in the common carotid artery. A 3.0-monofilament suture (Ethicon) with a rounded tip was inserted into the common carotid artery and advanced through the internal carotid artery to the ostium of the middle cerebral artery (MCA) to occlude the MCA. The suture was left in place for 1 hour and then removed to allow reperfusion for 48 hours. Viral vector was stereotaxically injected into rats 12 hours before, 2 hours after, or 5 hours after MCAO. Vector (2.5 μL) was injected via the Burr holes with a Hamilton syringe (Hamilton Company) lowered to depths of 4.5 and 5.5 mm in both striata. The first injection was on the ischemic side (targeting the core of the infarct with these coordinates), followed by the nonischemic side 15 minutes later. Wounds were then closed, and rats were allowed to recover before transport to the animal intensive care unit for postoperative monitoring.

Histopathology/Cell Counts
At 2 days after ischemia, rats were killed by an overdose of halothane and transcardially perfused with 200 mL of normal saline followed by 200 mL of 3% paraformaldehyde solution. After postfixation in 3% paraformaldehyde/20% sucrose solution for 1 to 2 days, 30-μm frozen sections in the coronal plane were taken at 100-μm increments 1 mm anterior and posterior to the needle track. Slices were stained with X-gal (5’-bromo-4-chloro-3-indoly-β-D-galactopyranoside, Molecular Probes), a chromogenic substrate for β-gal, and counterstained with cresyl violet (Sigma Chemicals) to allow the identification of healthy, intact, and virally targeted neurons. Therefore, cells were counted only if they (1) were contained within the striatum, (2) were X-gal positive, and (3) demonstrated the characteristic neuronal morphology (possessed processes and were larger in size, with cell body diameters of 15 to 25 μm). Brains without infarction were excluded from the analysis. The numbers of positive-staining, teel-colored neurons from 10 consecutive slices (5 anterior to and 5 posterior to the injection site) were counted at ×40 magnification by an investigator blind to treatment groups. Neuron survival was expressed for each animal as the percentage of X-gal-positive neurons in the ischemic striatum relative to the contralateral nonischemic striatum. The protective effect of Gpx was assessed by comparing mean percentage of neurons surviving across groups. Because differences in survival could be influenced by the severity of the ischemia, we also assessed infarct sizes. From the cresyl violet–stained sections, infarct sizes were graded according to a semiquantitative scale, where 0 = no stroke, 1 = stroke in striatum only, 2 = striatum and some cortex, and 3 = complete distribution of MCA. This scale has previously been shown to reasonably reflect the quantitative measurement of infarct size.10,12

Immunofluorescence Staining
Rat brains were fixed 48 hours after ischemia onset and sectioned as described above. Sections were treated with proteinase K (1:2 dilution, Dako) in PBS (10 mmol/L) for 15 minutes and washed in PBS for 3 × 5 minutes. Sections were blocked in PBS containing 5% donkey serum (Sigma), 1% bovine serum albumin, and 0.03% Triton X-100 for 2 hours at room temperature, then incubated in the primary antibodies diluted in blocking solution at 4°C overnight. Sections were washed with PBS and incubated for 2 hours at room temperature (light shielded) in the 2 secondary antibodies diluted in blocking solution. Sections were washed with PBS and coverslipped with the use of propidium iodide mounting medium (Vector Labs) and examined by confocal fluorescence microscopy (LM 530, Carl Zeiss). Negative controls, in which the primary antibodies were omitted, were run in parallel. For double labeling of cytchrome c, Bax, Bcl-2, CM-1 (activated caspase-3), and β-gal and triple labeling with propidium iodide, primary antibodies of purified mouse anti-cytochrome c antibody (1:500; catalog No. 556432, PharMingen), Bax antibody (1:500; catalog No. 554106, PharMingen), Bcl-2 antibody (1:500; catalog No. 554087, PharMingen), CM-1 antibody (1:500; catalog No. 556432, PharMingen), and rabbit anti-β-gal (1:200; code No. 55976, ICN Biomedicals, Inc) were used, respectively. The secondary antibodies were Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch) and fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch). Staining was quantified by analysis of adjacent sections until a total of 20 consecutive β-gal–positive cells were counted in each striatum. Then the proportion that was double labeled was determined with the use of 3 animals per group, with 60 cells counted per group.

Statistical Analysis
Standard statistical methods were used to analyze the data. Differences in survival between groups were determined by the Student t test. Differences in infarct size were determined by the Mann-Whitney test. The Fisher exact was used to determine differences in staining patterns. Statistical significance was determined at the P<0.05 level. All data are presented as mean±SD.
Results
Gpx Overexpression Protects Ischemic Neurons
Gpx overexpression improved striatal neuron survival (representing the core of the infarct in this model) at each time point (Figure 1). When Gpx vectors were delivered 12 hours before ischemia onset, striatal neuron survival was 36% greater relative to animals injected with control vector ($P<0.05$; Figure 1). When vector was delayed by 2 hours, survival was 26% greater than control ($P<0.05$; Figure 1), and after 5 hours survival was 25% greater than control ($P<0.05$; Figure 1).

There was no difference in infarct scores between the treated and untreated groups, implying that the severity of ischemia was similar between groups. In all rats the striatum was infarcted, and a few brains showed infarction in the overlying cortex as well. There were no differences in temperature, respiratory rate, or heart rate between the groups. Several hundred neurons could be transfected by our method with approximately 10% overall efficiency of infection.13 Employing each animal as its own internal control reduced the variability and increased the statistical power of this model.

We previously showed that helper virus injection did not lead to gross neurotoxicity,8 and vector injection did not cause DNA fragmentation.12 We similarly found that terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was absent in brains of vector (Gpx and control) and saline-injected uninjured rats assessed at 1, 3, and 7 days after injection.13a

Gpx Overexpression Prevents Cytochrome c Release
Gpx-targeted neurons in the contralateral striatum showed punctate or no cytochrome $c$ staining (Figure 2A). Figure 2 shows Gpx-transfected (Figure 2C) and $\alpha_{4s}$-transfected (Figure 2B) neurons in the ischemic striatum. Ischemic Gpx-transfected neurons displayed a punctate cytochrome $c$ staining, while ischemic nontransfected and $\alpha_{4s}$-transfected neurons had a diffuse cytochrome $c$ staining pattern. On quantification, 91% of cytochrome $c$/Gpx–positive neurons showed a punctate pattern in the ischemic hemisphere (Figure 3A) compared with 7% of cytochrome $c$/\$\alpha_{4s}$–positive neurons.

Gpx Overexpression Prevents Bax and Caspase-3 Expression
Double labeling showed little colocalization of Gpx with Bax or CM-1 (Figure 2E and 2G). In contrast, many cells transfected with control vector colocalized with these markers (Figure 2D and 2F). Quantitative analysis showed that 10% of Gpx-targeted neurons were positive for Bax, while 91% of control-transfected neurons were Bax positive ($P<0.0001$; Figure 3C). Caspase-3 was colocalized with only 1% of Gpx-targeted neurons in the ischemic hemisphere but was colocalized with 80% of $\alpha_{4s}$-transfected neurons in the ischemic hemisphere ($P<0.0001$; Figure 3B).

Bcl-2 Is Upregulated in Gpx-Targeted Striatal Neurons
Bcl-2 expression was observed in Gpx-transfected neurons after ischemia (Figure 2I). Eighty-two percent of Gpx-transfected cells in the ischemic striatum colocalized with β-gal and Bcl-2. In control-transfected neurons (Figure 2H), only 6% colocalized with Bcl-2 ($P<0.0001$; Figure 3D).

Discussion
Our study is the first to show that overexpression of Gpx using gene transfer protects against experimental stroke, even when delayed, and that this neuroprotection involves attenuation of apoptosis-related events. This emphasizes the importance of Gpx in the regulation of neuron death in response to ischemia/reperfusion injury. We observed increased survival of Gpx-targeted striatal neurons in the core of the infarct, when vectors were delivered 12 hours before as well as 2 and 5 hours after the onset of ischemia. Gpx overexpression also reduced Bax expression and caspase-3 activation and increased Bcl-2 expression. We also observed a punctate cytochrome $c$ pattern in Gpx-targeted neurons, characteristic of cytochrome $c$ retention in the mitochondria, versus a diffuse pattern in control-targeted neurons, indicating cytosolic cytochrome $c$ release. These results suggest that gene transfer of Gpx has possible therapeutic potential not only through its antioxidant properties but also through its ability to inhibit the endogenous apoptotic pathway.

It has been shown previously that Gpx overexpression in transgenic mice protects neurons against experimental stroke,14 and, conversely, Gpx-1 knockout mice show an increase in infarct size after ischemia.15 These studies only examined preischemic modification of Gpx, however, because of the constitutive expression or lack of expression of the gpx gene in their models. Other groups have assessed the effects of postsischemic application of ebelen, a Gpx mimic, in cats16 using models of reperfusion and prolonged ischemia and in rats17 using a model of permanent occlusion without reperfusion. Both groups found a reduction in infarct area and edema. Reperfusion is thought to further enhance the generation of ROS,18 the depletion of Gpx and SOD, and the
induction of apoptosis after stroke. A viral vector system using endogenous gene transfer permits the study of Gpx applied at multiple time points relative to ischemia, thereby providing a more useful assessment of its potential in stroke treatment.

In the present study vector delivery was performed approximately 12 hours before and 2 and 5 hours after stroke onset.

We have previously shown that vector expression begins 4 to 6 hours after injection; therefore, protection was observed in neurons transfected with Gpx.
when vector expression began as early as 8 hours before and as late as 9 hours after ischemia onset. Since endogenous Gpx is expressed constitutively in neurons, gene transfer may have enhanced endogenous neuronal Gpx expression by increasing Gpx synthesis, thus extending its protective effects for several hours. Since astrocytes may also protect neurons against oxidative stress through endogenous astrocyte glutathione-dependent mechanisms, overexpression of neuronal Gpx with gene transfer could have an additive protective effect. This extended time window compares favorably with our observations with Bcl-2 and heat shock protein (HSP) 72,12,22,23 which were protective at 1.5 and 2 hours after ischemia, respectively, but were no longer protective at 5 hours after ischemia. As shown in the present study, Gpx overexpression is associated with increased Bcl-2. We previously showed that HSP70 overexpression also increases Bcl-2, for reasons that are not yet clear.24 The extended temporal therapeutic window for Gpx compared with Bcl-2 and HSP70 might be due not only to the antiapoptotic effects of Bcl-2 but to the antioxidant properties of Gpx as well.

The mechanisms of neuroprotection by Gpx are not fully elucidated. In addition to altering the expression of Bcl-2, Gpx reduces ROS levels, limits nitric oxide–mediated mitochondrial injury, prevents activation of proapoptotic factors such as apoptotic protease activating factor 1 (APAF-1), caspase-9, dATP, and Bax, and decreases cytochrome c release.25–29 Cytochrome c release from mitochondria to cytosol has been demonstrated after focal ischemia.5 This release may be modulated by ROS, since SOD2-knockout animals show increased cytosolic cytochrome c and DNA fragmentation, and SOD1 overexpressing animals show less cytosolic cytochrome c release.30–32 The release of cytochrome c results in further ROS production by inhibition of the respiratory chain.30 These events promote a vicious cycle of increased cytochrome c release followed by increased mitochondrial ROS production, which may be maintained, leading to further activation of the apoptotic cascade. Studies have shown that, in some systems, Gpx can prevent apoptosis.14

Alternatively, Gpx might directly block steps in the apoptotic pathway without altering ROS levels. Our data show an increase in Bcl-2, which is known to reduce cytochrome c release. It is also possible that Gpx directly inhibits Bax expression or caspase-3 activation, which were both decreased by Gpx treatment in our study. During apoptosis (in vitro and in vivo) cytochrome c is released from mitochondria, and this is inhibited in the presence of Bcl-2. Cytosolic cytochrome c forms an essential part of the vertebrate “apoptosome,” which is composed of cytochrome c, APAF-1, and procaspase-9. The result is activation of caspase-9, which then processes and activates caspase-3 to orchestrate the biochemical execution of cells.31 Our data suggest that Gpx prevents apoptosis upstream of cytochrome c, as evidenced by increased Bcl-2 expression and decreased Bax expression.

It has been shown that Bcl-2 may prolong cell survival by inhibiting the formation of the voltage-dependent anion channel. Bax interacts with voltage-dependent anion channel to form a high-conductance pore and cytochrome c release. Our observation of decreased cytochrome c release, caspase-3 activation, and increased neuron survival also implicates apoptosis-related events as a mechanism underlying the neuroprotection of Gpx, possibly because of reduction in H2O2 levels27,32–34 but also potentially because of direct effects on one or more apoptosis-related proteins. Activation of caspase-3–like proteases by H2O2 has been reported in nonneuronal tissue.35

This study is the first demonstration that Gpx can prevent the release of cytochrome c from mitochondria, potentially blocking the formation of a functional apoptosome and subsequent processing of procaspase-9 and -3. Our data are also the first to show that Gpx overexpression reduces caspase-3 activation. It has been shown that caspase-3 has a biphasic time course, peaking at 1 hour and 12 hours after ischemia.36 Elevating levels of Gpx at 9 to 11 hours (5 hours of delay plus 4 to 6 hours until expression begins) after ischemic onset may prevent the second phase of caspase activation and reduce ischemic neuronal death.

Our results suggest a therapeutic temporal window for Gpx overexpression of 9 to 11 hours after ischemia onset. This postinsult neuroprotective effect has implications for future gene therapy with the use of Gpx or other strategies that induce Gpx overexpression.14,19,37 Although gene therapy with Gpx is feasible, there are still limitations to the extent and numbers of neurons that these vectors can infect, as well as the route of administration. Future studies should explore the full temporal therapeutic window of Gpx neuroprotection, as well as methods for increasing transfection.

Acknowledgments

This study was supported by National Institutes of Health, National Institute of Neurological Disorders and Stroke grants PO1 NS37520 (G.K.S., R.M.S.), and RO1 NS27292 (G.K.S.); American Heart Association grant 0060091Y (M.A.Y.); Student Scholars in Cardiovascular Disease and Stroke (B.H.); National Medical Fellowship (B.H.); grant R01 NS40516 (M.A.Y.); and TRDRP of the State of California (R.M.S.). We thank Dr N.A. DeLuca (University of Pittsburgh, Pittsburgh, Pa) for providing HSV d120 and E5 cell lines, Hui Wang and Heng Zhao for vector preparation, Guo Hua Sun, David Kunis, and Danye Cheng for technical support, Beth Hoyte for preparation of the figures, and David Schaal for assistance in preparing the manuscript.

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Stroke. 2003;34:2489-2494; originally published online September 18, 2003; doi: 10.1161/01.STR.0000091268.25816.19

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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