Background and Purpose—p53-upregulated modulator of apoptosis (PUMA), a BH3-only member of the Bcl-2 protein family, is required for p53-dependent and -independent forms of apoptosis. PUMA localizes to mitochondria and interacts with antiapoptotic Bcl-2 and Bcl-XL or proapoptotic Bax in response to death stimuli. Although studies have shown that PUMA is associated with pathomechanisms of cerebral ischemia, clearly defined roles for PUMA in ischemic neuronal death remain unclear. The purpose of this study was to determine potential roles for PUMA in cerebral ischemia.

Methods—Five minutes of transient global cerebral ischemia (tGCI) were induced by bilateral common carotid artery occlusion combined with hypotension.

Results—PUMA was upregulated in vulnerable hippocampal CA1 neurons after tGCI as shown by immunohistochemistry. In Western blot and coimmunoprecipitation analyses, PUMA localized to mitochondria and was bound to Bcl-XL and Bax in the hippocampal CA1 subregion after tGCI. PUMA upregulation was inhibited by pifithrin-H9251, a specific inhibitor of p53, suggesting that PUMA is partly controlled by the p53 transcriptional pathway after tGCI. Furthermore, reduction in oxidative stress by overexpression of copper/zinc superoxide dismutase, which is known to be protective of vulnerable ischemic hippocampal neurons, inhibited PUMA upregulation and subsequent hippocampal CA1 neuronal death after tGCI.

Conclusions—These results imply a potential role for PUMA in delayed CA1 neuronal death after tGCI and that it could be a molecular target for therapy. (Stroke. 2009;40:618-625.)

Key Words: PUMA • cerebral ischemia, global • apoptosis • superoxide dismutase • oxidative stress

The Bcl-2 protein family is a principal regulator of mitochondrial membrane integrity and function and is classified into 3 subgroups according to structural homology (Bcl-2 homology [BH] domains): the antiapoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, BCL-W), the proapoptotic proteins (Bax, Bak), and the BH3-only proteins (Bim, Bad, Bid, Bik, p53-upregulated modulator of apoptosis [PUMA], NADPH oxidase activator, Hrk). BH3-only proteins share sequence homology with other Bcl-2 proteins in the BH3 region only and are involved in the mechanisms of cytochrome c release in neuronal apoptosis.

According to a recent hierarchy model, BH3-only proteins are subdivided into activator or inactivator proteins. Activator BH3-only proteins can interact with both antiapoptotic Bcl-2 proteins and proapoptotic Bcl-2 proteins. Among these activator proteins, PUMA was initially identified as a gene activated by p53 in cells undergoing p53-induced apoptosis. In p53-induced cell death, PUMA was shown to localize to mitochondria; interact with Bcl-2, Bcl-XL, and Bax; and induce cytochrome c release, thereby activating caspases-9 and -3. However, the roles of PUMA in cerebral ischemia remain unclear.

To determine these roles, we used as our model 5 minutes of transient global cerebral ischemia (tGCI), which induces delayed neuronal death in the hippocampal CA1 subregion in rats. We investigated expression of PUMA and the interaction between PUMA and Bcl-XL, Bcl-2, and Bax in the hippocampal CA1 subregion after tGCI. To investigate the regulation of PUMA by p53 after tGCI, we administered pifithrin-α (PFT), a specific inhibitor of p53. To demonstrate the effects of oxidative stress on PUMA expression after tGCI, we used copper/zinc superoxide dismutase (SOD1) transgenic (Tg) rats, which have neuroprotection against ischemia because of reduced oxidative stress.

Materials and Methods

Global Cerebral Ischemia

Five minutes of tGCI was induced by bilateral common carotid artery occlusion combined with hypotension according to a...
method described previously,7 with some modifications.6 Male Sprague-Dawley rats (300 to 350 g) were anesthetized with 2.0% isoflurane in 70% nitrous oxide and 30% oxygen via face mask. Rectal temperature was controlled at 37°C during surgery with a homeothermic blanket. The femoral artery was catheterized with a PE-50 catheter to allow continuous recording of arterial blood pressure. After heparinization, blood was quickly withdrawn via the jugular vein. When the mean arterial blood pressure became 30 mm Hg, both common carotid arteries were clamped with surgical clips. Blood pressure was maintained at 30 mm Hg during the ischemic period. After 5 minutes of ischemia, the clips were removed and the blood was reinfused. Regional cerebral blood flow was monitored by laser Doppler flowmetry as previously described.7 Sham-operated animals underwent exposure of the vessels without blood withdrawal or clamping of the carotid arteries. The animals were maintained at 20°C with ad libitum access to food and water. All animals were treated in accordance with Stanford University guidelines, and the animal protocols were approved by Stanford University’s Administrative Panel on Laboratory Animal Care.

Drug Treatment
To examine the effect of a specific p53 inhibitor on PUMA expression after tGCI, we administered PFT (P4359; Sigma-Aldrich, St. Louis, Mo), dissolved in dimethyl sulfoxide and phosphate-buffered saline (PBS). This drug (4 mg/kg in dimethyl sulfoxide in PBS) or vehicle (dimethyl sulfoxide in PBS) was injected via the left jugular vein just after reperfusion as described previously.8

SOD1-Tg Rats
Heterozygous SOD1-Tg rats with a Sprague-Dawley background carrying human SOD1 genes were derived from founder stock and were further bred with wild-type (Wt) Sprague-Dawley rats to generate heterozygous rats, as previously described.7 The phenotype of the SOD1-Tg rats was identified by isoelectric focusing gel electrophoresis as described.7 There were no observable phenotypic differences in brain vasculature between the SOD1-Tg rats and their Wt littermates.7

Western Blot Analysis
The hippocampal CA1 subregion was removed after 1, 4, 24, or 72 hours of reperfusion. Protein extraction of the cytosolic, mitochondrial, and nuclear fractions was performed with a multiple centrifugation method as described previously.9 Equal amounts of samples were loaded per lane and analyzed by sodium dodecyl sulfate–polyacrylamide-gel electrophoresis on a 10% to 20% Tris-glycine gel (Invitrogen, Carlsbad, Calif) and then dodecyl sulfate–polyacrylamide-gel electrophoresis on a 10% to 20% gel. Immunoblotting was performed with anti–Bcl-2 (#610538, BD Biosciences), or anti-Bax (#2772, Cell Signaling Technology) and protein G–Sepharose for 2 hours at 4°C. The negative control was prepared with protein G–Sepharose without an antibody. Whole-brain extract was included as a positive control. The 14 000g pellets were washed 3 times and analyzed as the samples bound to each antibody by Western blotting with anti-PUMA (1:1000, #4976; Cell Signaling Technology), anti–Bcl-XL (1:1000), anti–Bcl-2 (1:1000), or anti-Bax (1:1000).

Cresyl Violet Staining and Immunohistochemistry of PUMA
Anesthetized animals were perfused with 10 U/mL heparin saline and subsequently with 4% formaldehyde in PBS after 1, 4, 24, or 72 hours of reperfusion. Sections were removed, postfixed for 24 hours, and sectioned at 50 μm with a Vibratome. For histologic assessment, the sections were stained with cresyl violet. For immunohistochemistry of PUMA, sections were reacted with anti-PUMA (1:50, #4976; Cell Signaling Technology). Immunohistochemistry was performed with the avidin-biotin technique, and nuclei were counterstained with methyl green solution.

Immunofluorescence Staining
To evaluate colocalization of PUMA and COX IV with neuron-specific nuclear protein (NeuN), Bcl-XL, or Bax, we performed double immunofluorescence. For double immunofluorescence of PUMA and COX IV or Bax, the sections were reacted with anti–COX IV (1:100, #4844; Cell Signaling Technology) or anti–Bax (1:50, sc-526; Santa Cruz Biotechnology), followed by fluorescein isothiocyanate–conjugated anti-rabbit monoclonal Fab fragments of a secondary antibody (Jackson ImmunoResearch, West Grove, Pa) for labeling and blocking of COX IV or Bax. Then the sections were incubated with anti–PUMA (1:50, #4976; Cell Signaling Technology) followed by Texas Red–conjugated anti-rabbit IgG (Jackson ImmunoResearch). For double immunofluorescence of PUMA and NeuN or Bcl-XL, sections were immunostained with anti–PUMA (Cell Signaling Technology) followed by Texas Red–conjugated anti-rabbit IgG. The sections were then incubated with anti–NeuN (1:50, MAB377; Chemicon International, Temecula, Calif) or anti–Bcl-XL (1:50, #610209; BD Biosciences), followed by fluorescein isothiocyanate–conjugated antitohus monoclonal IgG (Jackson ImmunoResearch). The sections were covered with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif) and examined under an LSM510 confocal laser scanning microscope or an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY).

In Situ Detection of Superoxide Anion Production
Early production of superoxide anions after tGCI was investigated with the use of hydroethidine as previously described.10 Hydroethidine is diffusible into the central nervous system parenchyma after intravenous injection and is selectively oxidized to ethidium by superoxide anions. Hydroethidine solution (200 μL of 1 mg/mL in 1% dimethyl sulfoxide with saline) was administered intravenously 15 minutes before ischemia induction. A sample was prepared as described in the immunohistochemistry method. For fluorescent double staining of the ethidium signal and PUMA, sections were incubated with anti-PUMA (1:50, #4976; Cell Signaling Technology), followed by fluorescein isothiocyanate–conjugated anti-rabbit IgG (Jackson ImmunoResearch). Slides were covered with DAPI (Vector Laboratories) and observed with a fluorescence microscope.

Cell Death Assay
For quantification of apoptosis-related DNA fragmentation, we used a commercial enzyme immunoassay to determine cytosplasmic histone–associated DNA fragments (1774425; Roche Molecular Biochemicals, Mannheim, Germany) and to detect apoptotic but not necrotic cell death.11 A sample was prepared as described in the Western blotting method. A cytosolic volume containing 20 μg of...
protein was used for the ELISA, according to the manufacturer’s protocol.

Statistical Analysis
Comparisons among multiple groups were performed with ANOVA followed by a Scheffé post hoc analysis (SigmaStat; Systat Software, San Jose, Calif). Comparisons between 2 groups were achieved with a Student unpaired t test. Data are expressed as mean±SD, and significance was accepted with \( P<0.05 \).

Results
PUMA Induction and Selective Neuronal Death After tGCI
One, 4, or 24 hours after 5 minutes of tGCI, there was no neuronal degeneration in the hippocampal CA1 subregion, as confirmed by cresyl violet staining (Figure 1A). However, >80% of the CA1 neurons were degenerated 72 hours after tGCI, which was compatible with our previous reports.6,7 In contrast, neurons in the hippocampal CA3 subregion were spared even at 72 hours. PUMA immunoreactivity increased after tGCI, peaked at 4 hours, and then started to decline at 24 hours. At 72 hours, the CA1 neurons degenerated, and immunoreactivity could not be evaluated. C indicates control. Scale bar=300 \( \mu \)m (hippocampus), 50 \( \mu \)m (CA1). B, Representative photomicrographs of fluorescent double staining of PUMA (red) and NeuN (green) in the hippocampal CA1 subregion 4 hours after tGCI. Nuclei were counterstained with DAPI (blue). NeuN immunoreactivity showed the distribution of neurons. Overlapped image demonstrates that PUMA-positive cells in the hippocampal CA1 subregion colocalized with neurons. Scale bar=50 \( \mu \)m.
Mitochondrial Localization of PUMA and Subsequent Cytochrome c Release in the Hippocampal CA1 Subregion After tGCI

Western blotting showed that PUMA immunoreactivity was evident as a single band of molecular mass of 19 kDa (Figure 2A). In cytosolic samples from the hippocampal CA1 subregion, PUMA immunoreactivity was slightly detectable and showed no significant change after tGCI. In contrast, PUMA expression was significantly increased in mitochondrial samples, peaking at 4 hours and then decreasing by 72 hours after tGCI. It was barely detectable in the sham-operated brains (Figure 2A, n=4, P<0.05).

To confirm the upstream pathway of PUMA, we investigated nuclear p53 upregulation. Western blotting showed that nuclear p53 significantly increased 4 hours after tGCI (Figure 2B), presenting a pattern similar to that of mitochondrial PUMA upregulation. To confirm activation of the mitochondrial apoptotic pathway after tGCI, we examined cytochrome c release and caspase-9 activation. Cytosolic cytochrome c and cleaved caspase-9 significantly increased at 24 hours (Figure 2C, n=4, P<0.05), which suggests cytochrome c release to the cytosol and subsequent caspase chain reaction after tGCI. These results indicate that PUMA increases in the mitochondria before cytochrome c release and caspase-9 activation.

For further investigation of mitochondrial localization of PUMA after ischemia, we performed double immunofluorescence for PUMA and COX IV, which was used as a mitochondrial marker. Double immunofluorescence demonstrated that PUMA colocalized with COX IV in the hippocampal CA1 subregion 4 hours after tGCI (Figure 3).

Interaction Between PUMA and Bcl-XL or Bax After tGCI

To investigate potential direct interactions between PUMA and Bcl-XL, Bcl-2, or Bax, we performed coimmunoprecipitations in the mitochondrial fraction from the hippocampal CA1 subregion. With Western blot analysis, Bcl-XL, Bcl-2, and Bax immunoreactivity was evident as bands of 30, 26, and 20 kDa, respectively, and showed no significant change at any time point (data not shown). PUMA expression precipitated by Bcl-XL increased time-dependently and significantly increased at 24 and 72 hours (Figure 4A, n=4, P<0.05). PUMA expression precipitated by Bax also increased 24 hours after tGCI (Figure 4B, n=4, P<0.01). In contrast, PUMA expression precipitated by Bcl-2 showed no significant difference at any time point, although it tended to increase after tGCI (data not shown).

For further investigation of direct interaction between PUMA and Bcl-XL or Bax, we performed double immunofluorescence, which demonstrated that PUMA-positive cells colocalized with Bcl-XL-positive cells (Figure 4C) or Bax-positive cells (Figure 4D) in the hippocampal CA1 subregion 24 hours after tGCI. In combination with the coimmunoprecipitation data, these results indicate that PUMA directly interacts with Bcl-XL and Bax in the hippocampal CA1 subregion after tGCI.
PFT Administration
To investigate the regulation of PUMA by p53, we intravenously administered 4 mg/kg of PFT just after reperfusion. Our previous study indicated that this dose was effective in inhibiting PUMA expression. Western blot analysis showed that PUMA expression in the mitochondrial fraction from the hippocampal CA1 subregion was significantly decreased in PFT-treated animals compared with vehicle-treated animals 4 hours after tGCI (Figure 5A, n=4, P<0.01). Bax, Bcl-2, and Bcl-XL expression levels showed no differences between vehicle-treated and PFT-treated rats (data not shown). An immunofluorescence study showed that PUMA expression was decreased in the hippocampal CA1 subregion of the PFT-treated animals compared with the vehicle-treated animals 4 hours after tGCI, which was compatible with the result of the Western blot study (Figure 5B). These results indicate that PFT administration inhibits PUMA upregulation after tGCI.

SOD1 Overexpression
To confirm that superoxide production is associated with PUMA induction, we performed double immunofluorescence of ethidium and PUMA. In the hippocampal CA1 pyramidal neurons, ethidium signals were shown as small particles in the cytosol of the nonischemic brains in both the Wt and SOD1-Tg rats (Figure 6A). Four hours after ischemia, the hippocampal CA1 neurons showed a marked increase in punctate and diffuse signals for both ethidium and PUMA in the Wt rats. However, the increase in signal was less noticeable in the SOD1-Tg rats. The Western blot analysis indicated that PUMA expression was significantly decreased 4 hours after tGCI in the SOD1-Tg rats compared with the Wt rats (Figure 6B, n=4, P<0.05). Bax, Bcl-2, and Bcl-XL expression levels showed no differences between the Wt and Tg rats (data not shown).

We then examined apoptosis-related DNA fragmentation after tGCI to investigate neuroprotection of SOD1 overexpression. DNA fragmentation in the hippocampal CA1 subregion at 72 hours was significantly decreased in the hippocampal CA1 subregion of PFT-treated animals compared with vehicle-treated animals 4 hours after tGCI (Figure 5A, n=4, P<0.01). Bax, Bcl-2, and Bcl-XL expression levels showed no differences between vehicle-treated and PFT-treated rats (data not shown). An immunofluorescence study showed that PUMA expression was decreased in the hippocampal CA1 subregion of the PFT-treated animals compared with the vehicle-treated animals 4 hours after tGCI, which was compatible with the result of the Western blot study (Figure 5B). These results indicate that PFT administration inhibits PUMA upregulation after tGCI.
SOD1-Tg rats compared with the Wt rats at the same time point, which was compatible with the results of a counting study of cells positive for terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (Figure 6C, n=4, P<0.05). In combination with the results of immunofluorescence and Western blotting, these results indicate that SOD1 overexpression reduces superoxide production, PUMA upregulation, and subsequent hippocampal CA1 neuronal death after tGCI.

Discussion

Important roles for PUMA in apoptosis have been explored under various conditions. Although the role of PUMA in cerebral ischemia is unresolved, our results suggest an important role, through cytochrome c release and caspase activation. We base this conclusion on the following findings. First, PUMA increased in mitochondria of vulnerable hippocampal CA1 neurons after tGCI. Second, PUMA induction temporally preceded cytochrome c release and caspase-9 activation. Third, it localized to mitochondria and interacted with Bcl-XL and Bax. Fourth, PUMA upregulation was inhibited by PFT administration or SOD1 overexpression, both of which have neuroprotective effects against cerebral ischemia through inhibition of cytochrome c release and caspase activation.7,12,13 Finally, reduction in oxidative stress by SOD1 overexpression decreased not only PUMA upregulation but also neuronal death in the hippocampal CA1 subregion after tGCI. Our findings are supported by studies reporting that PUMA is extremely effective in inducing apoptosis. In an in vitro study, PUMA expression induced rapid apoptosis,5 and PUMA suppression by an antisense oligonucleotide reduced apoptosis.4 Furthermore, PUMA induces apoptosis through cytochrome c release and caspase activation.4,5 PUMA also plays an important role in neuronal apoptosis. PUMA-nullizygous neurons are protected against araC-induced apoptosis,14 and forced expression of PUMA was sufficient to induce apoptosis in primary neurons.15 PUMA regulated oxidative stress–induced neuronal apoptosis through cytochrome c release and caspase activation in a primary mouse neuron culture.16 It was also necessary for camptothecin-induced neuronal death in a primary culture of mouse neurons.17 In our study, PUMA expression was upregulated after tGCI as previously described.18 PUMA expression was inhibited by PFT, which can inhibit p53 transcriptional activity and prevent DNA damage–induced apoptosis.19 Its expression was also inhibited in SOD1-Tg rats, resulting in significant neuroprotection. Finally, these results indicate that PUMA has important roles in delayed and selective CA1 neuronal death after tGCI. Although our results of PFT administration demonstrated that PUMA was regulated at least in part by p53, this finding is controversial. PUMA was first identified as a direct target

![Figure 6. Effect of SOD1 overexpression on PUMA expression, ethidium signals, and DNA fragmentation after tGCI. A, Representative photomicrographs show fluorescent double staining of PUMA (green) and ethidium (red) in the hippocampal CA1 subregion. Nuclei were counterstained with DAPI (blue). Ethidium signals were seen as small particles in the cytosol in nonischemic brains of both the Wt and Tg rats. Four hours after tGCI, hippocampal CA1 neurons showed a marked increase in ethidium signals in the Wt rats. However, the signal increase was less noticeable in the Tg rats 4 hours after tGCI. In the Wt rats, the signals for PUMA increased dramatically at 4 hours compared with the sham-operated rats and overlapped with the ethidium signals. In the Tg rats, PUMA expression was less strong than in the Wt rats at 4 hours. C indicates control. Scale bar=50 μm. B, Western blot analysis showed that PUMA expression decreased significantly in the Tg rats 4 hours after tGCI (n=4, *P<0.05). COX IV analysis is shown as an internal control. C, Apoptosis-related DNA fragmentation assay. DNA fragmentation at 72 hours was significantly decreased in the hippocampal CA1 subregion of the Tg rats compared with the Wt rats (n=4, *P<0.05).]
of the p53 oncogene with 2 putative p53 binding sites.4,5 Gene-knockout studies revealed that DNA damage-induced p53-dependent apoptosis was severely diminished in PUMA-deficient cells in vitro.20,21 In neuronal cell death, PUMA was shown to be associated with p53. PUMA-deficient neurons are resistant to p53-induced neuronal apoptosis.15 However, several studies have reported that PUMA could also be induced by a p53-independent mechanism.16,20,22 PUMA mRNA was induced by p53-independent apoptotic stimuli, including dexamethasone treatment of thymocytes and serum deprivation of tumor cells.22 Moreover, PUMA induction directly links the endoplasmic reticulum stress response to the mitochondrial apoptosis pathway in neurons after tGCI.18

In our study, PUMA induction after ischemia was significantly inhibited by administration of PFT. Although we cannot exclude the possibility that upregulation of PUMA after ischemia is facilitated by other mechanisms, such as endoplasmic reticulum stress after ischemia,18 these results suggest that PUMA is controlled at least in part by the p53 transcriptional pathway in CA1 neurons after tGCI.

Recent reports have demonstrated that the potency of PUMA in apoptosis induction is related to its interaction with anti- or pro-apoptotic proteins.3–5,16,23 The BH3 domain of PUMA can promiscuously interact with multiple antiapoptotic Bcl-2 family members.23 PUMA was shown to localize to mitochondria and to bind to Bcl-XL or Bcl-XL through a BH3 domain.4,5 Furthermore, PUMA could interact with Bax as well as Bcl-XL or Bcl-2.16,23 In the present study, coimmunoprecipitation showed that PUMA bound to Bcl-XL and Bax in the mitochondrial fraction after tGCI. A double immunofluorescence study demonstrated that these protein interactions occurred in vulnerable CA1 neurons. Binding of PUMA to Bcl-2 also tended to increase, but not significantly. These results suggest that the interaction between PUMA and Bcl-XL or Bax in the mitochondrial fraction is associated with ischemic neuronal death and that this interaction is associated with ischemic neuronal death requires further investigation.

Reactive oxygen species play important roles in the pathogenesis of central nervous system injury. We have reported that SOD1 is a crucial endogenous enzyme responsible for eliminating superoxide and that overexpression of SOD1 reduces superoxide production and protects neurons from death after transient focal cerebral ischemia24 and tGCI.7 Thus, SOD1-Tg animals are very useful tools for investigating the relationship between oxidative stress and ischemic neuronal death. In our study, superoxide production and neuronal death in the hippocampal CA1 subregion after tGCI were prevented in the SOD1-Tg rats, results consistent with those of our previous report in the same tGCI model.25 Furthermore, PUMA upregulation after ischemia was significantly decreased in the SOD1-Tg rats compared with the WT rats, suggesting that reduction in oxidative stress by SOD1 overexpression could modulate PUMA upregulation.

In conclusion, PUMA has potential roles in delayed CA1 neuronal death after tGCI and can hypothetically be a molecular target for therapy, although our study may lack direct evidence. To confirm the role of PUMA, PUMA-knockout mice or an RNA interference technique should be used in future studies.

Acknowledgments

We thank Liza Reola and Bernard Calagui for technical assistance, Cheryl Christensen for editorial assistance, and Elizabeth Hoyte for figure preparation.

Source of Funding

This study was supported by National Institutes of Health grants P50 NS014543, R01 NS025372, R01 NS036147, and R01 NS038653.

Disclosures

None.

References


Potential Role of PUMA in Delayed Death of Hippocampal CA1 Neurons After Transient Global Cerebral Ischemia

Kuniyasu Niizuma, Hidenori Endo, Chikako Nito, D. Jeannie Myer and Pak H. Chan

Stroke. 2009;40:618-625; originally published online December 18, 2008;
doi: 10.1161/STROKEAHA.108.524447
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/40/2/618

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke 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 Stroke is online at:
http://stroke.ahajournals.org//subscriptions/