Mevastatin, an HMG-CoA Reductase Inhibitor, Reduces Stroke Damage and Upregulates Endothelial Nitric Oxide Synthase in Mice

Sepideh Amin-Hanjani, MD; Nancy E. Stagliano, PhD; Masaru Yamada, MD; Paul L. Huang, MD, PhD; James K. Liao, MD; Michael A. Moskowitz, MD

**Background and Purpose**—The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) lower serum cholesterol and decrease the incidence of stroke and cardiovascular disease. There is growing evidence that statins exert some of their beneficial effects independent of cholesterol lowering. Indeed, we have previously demonstrated that chronic simvastatin administration upregulates endothelial nitric oxide synthase (eNOS), resulting in more functional protein, augmentation of cerebral blood flow, and neuroprotection in a murine model of cerebral ischemia. In this report we examined whether another member of the statin family shared these effects and whether eNOS upregulation is sustained with longer treatment.

**Methods**—Mevastatin (2 mg/kg or 20 mg/kg per day) was administered to 18- to 22-g male mice for 7, 14, or 28 days before 2-hour middle cerebral artery occlusion with the use of the filament model (n=9 to 12). Neurological deficits and cerebral infarct volumes were assessed at 24 hours. Arterial blood pressure and gases, relative cerebral blood flow, and blood cholesterol levels were monitored in a subset of animals (n=5). Absolute cerebral blood flow was measured by the [14C]iodoamphetamine indicator fractionation technique (n=6). eNOS mRNA and protein levels were determined.

**Results**—Mevastatin increased levels of eNOS mRNA and protein, reduced infarct size, and improved neurological deficits in a dose- and time-dependent manner. Greatest protection was seen with 14- and 28-day high-dose treatment (26% and 37% infarct reduction, respectively). Cholesterol levels were reduced only after 28 days of treatment and did not correlate with infarct reduction. Baseline absolute cerebral blood flow was 30% higher after 14-day high-dose treatment.

**Conclusions**—Chronic prophylactic treatment with mevastatin upregulated eNOS and augmented cerebral blood flow. These changes occurred in the absence of changes in serum cholesterol levels, were sustained for up to 1 month of treatment, and resulted in neuroprotection after middle cerebral artery occlusion. (*Stroke. 2001;32:980-986.*)

**Key Words:** cerebral ischemia ■ endothelial nitric oxide synthase ■ HMG-CoA reductase inhibitors ■ mice
lersterol. The mechanism is eNOS dependent because the statins do not reduce tissue injury in eNOS-deficient mice.

In the present study we used mevastatin to show that the statins as a general class increase eNOS mRNA and protein levels and protect against stroke damage. We also show that mevastatin demonstrates a different potency compared with previously reported statins. By varying the duration of treatment and dosage we were able to establish a drug treatment window, and we determined that tachyphylaxis does not develop after 1 month of daily mevastatin administration. Finally, we determined that enhanced eNOS mRNA and protein expression corresponded to the protective actions of mevastatin in ischemic brain.

**Materials and Methods**

**Drug Preparation**

Mevastatin (Compactin, Sigma) was chemically activated by alkaline hydrolysis, as previously described. Mevastatin powder was prepared by vacuum centrifugation; the drug was reconstituted in 0.1 mol/L phosphate buffer, and pH was adjusted to 7.4.

**Drug Administration**

Wild-type 129-SV/eVTAcBr male mice (Taconic Farms; weight, 18 to 22 g) and eNOS-deficient male mice9 (weight, 18 to 22 g) were treated with either mevastatin at a dose of 2 or 20 mg/kg per day or a corresponding concentration of vehicle for 7, 14, or 28 days. The drug was delivered via 7- or 14-day ALZET miniosmotic pumps (Alza Scientific Products) implanted subcutaneously. Pumps were replaced once for 28-day treatments.

**Focal Cerebral Ischemia Model**

Animals were subjected to transient 2-hour middle cerebral artery occlusion (MCAO) with the use of the intraluminal filament method previously described. Briefly, mice were anesthetized with 2% halothane and maintained on a mixture of 1% halothane, 70% nitrous oxide, and 30% oxygen via face mask. An 11-mm silicone-coated 8-0 nylon monofilament was introduced into the external carotid artery, navigated into the internal carotid artery, and advanced to the anterior cerebral artery, occluding the origin of the middle cerebral artery (MCA). Laser-Doppler flowmetry (Perimed) at the core of the ischemic territory was used to assess changes in regional CBF (rCBF). After 2 hours in a small animal incubator, the animals were reanesthetized, and the filament was removed. During the surgical procedure, mice were maintained at a core body temperature of 36°C to 37°C via a temperature control unit (FHC). For a subset of animals the left femoral artery was cannulated for arterial blood pressure measurements and arterial blood gas analysis (Corning 178, CIBA-Corning Diagnostics). For these animals, rCBF was monitored throughout ischemia and for 15 minutes after reperfusion.

**Neurological Deficits**

Mice were tested for neurological deficits on a scale of 0 (no deficit) to 3 (severe deficit), as previously described, at 2 hours of ischemia and at 22 hours of reperfusion by an observer blinded to the treatment group.

**Infarction Assessment**

Twenty-two hours after reperfusion, mice were killed, and brains were rapidly removed. Two-millimeter-thick coronal sections of the forebrain were prepared with the use of a mouse brain matrix (RBH-2000C; Harvard Apparatus). Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma) dissolved in phosphate-buffered saline 0.1 mol/L, pH 7.4, for 15 minutes at room temperature, and fixed in 10% buffered formalin overnight. The infarct volumes were quantified with the use of an image analysis system (M4, Imaging Research) by an experimenter blinded to the treatment. Infarct volumes were calculated directly by summing the infarct volume of each section or indirectly to correct for brain edema by subtracting the volume of the undamaged ipsilateral hemisphere from the contralateral hemisphere.

**Absolute CBF Measurements**

Given that it was previously shown that the statins have an effect on CBF in mice, we studied a small group of mice for this effect after chronic treatment. A subset of mice (n = 6 per group) treated with mevastatin (20 mg/kg) or vehicle was used for the determination of baseline absolute CBF by the [14C]iodoamphetamine indicator fractionation technique. Mice were anesthetized, ventilated, and monitored as described. Thirty minutes after anesthetic stabilization, CBF was determined as described.

**Cholesterol Measurement**

Serum total cholesterol levels were quantified from blood drawn from the orbital plexus of mice just before euthanasia with the use of the Sigma Diagnostics Cholesterol kit (procedure No. 352, Sigma Diagnostics) and the recommended cholesterol calibrator (No. C 0534) in a spectrophotometric assay.

**Semiquantitative Reverse Transcription–Polymerase Chain Reaction**

Aortas were rapidly harvested from the same group of animals used for infarct measurements, frozen in liquid nitrogen, and stored at −80°C until use. Aortas were collected because increases in aortic eNOS levels have previously been shown to correlate with increases in brain eNOS levels. Total RNA isolation, reverse transcription (RT), and semiquantitative competitive polymerase chain reaction (PCR) for eNOS were performed as previously described. The sense (5′-TCCCGCTCCACGCTGTTAAGAGG-3′) and antisense (5′-AACATATGTCCTGGTCGAACGA-3′) primers amplified a 340-bp fragment of murine eNOS.

**Immunoblot Analysis**

Aortas were rapidly harvested from mice after euthanasia, frozen in liquid nitrogen, and stored at −80°C. Pooled aortas (n = 2) from mevastatin- (20 mg/kg) or vehicle-treated animals were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer and assayed for total protein. Twenty micrograms of total protein for each sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (BioRad) and blocked overnight at 4°C in 5% milk/Tris-buffered saline/0.05% Tween 20. eNOS protein was detected with the use of a polyclonal antibody (Transduction Laboratories) at a concentration of 1:200. A chemiluminescent system (ECL, Amersham Pharmacia Biotech) was used to expose Kodak autoradiographic film. To ensure equivalent protein loading across samples, the membranes were reprobed with a monoclonal antibody to α-tubulin.

**Statistical Analysis**

Data are expressed as mean±SEM. Unpaired 2-tailed Student’s t test or ANOVA with Bonferroni post hoc comparisons (when >2 groups were involved) was used for statistical analysis. Probability values of <0.05 were considered statistically significant.

**Results**

**Cerebral Infarct Reduction**

Dosage and duration of statin treatment were varied to assess the effects on brain injury. At a dose of 2 mg/kg, mevastatin failed to significantly decrease infarct volume after 14 or 28 days of treatment (Figure 1). At 20 mg/kg, both 14 and 28 days of treatment successfully reduced injury by 26% and 37%, respectively, compared with vehicle (Figure 1); in
contrast, 7-day pretreatment was not sufficient to reduce infarct size. Correction for brain edema did not alter the results (indirect; 25% and 36% decrease). Since it was previously reported that simvastatin treatment failed to protect eNOS-deficient mice from stroke damage,8 we administered only the maximal dose to mutants (20 mg/kg mevastatin). Unlike the experience in wild-type mice, no reduction in infarct size was evident after pretreatment.

Neurological Deficits
After 2 hours of ischemia, vehicle-treated animals showed moderate to severe neurological deficits such as circling and loss of the righting reflex; by 24 hours, mild to moderate deficits persisted. Animals treated with 20 mg/kg mevastatin showed better neurological scores at 2 and 24 hours than vehicle-treated mice (P < 0.05).

Physiological Parameters
Physiological parameters were assessed in animals treated with 20 mg/kg mevastatin or vehicle for 14 days. No significant differences in blood pressure, PaCO2, PaO2, or pH were apparent between drug-treated and vehicle groups (Table 1). To reduce the likelihood that any differences in histological outcome were due to alterations in the degree of ischemic insult, rCBF was monitored before, during, and after ischemia. Intraischemic rCBF reductions and postischemic reperfusion levels were comparable between vehicle and treated groups, indicating an equivalent relative depth and duration of ischemia.

Serum Cholesterol Levels
Total serum cholesterol levels were reduced after 28 days of treatment with either the 2- or 20-mg/kg dose. Serum cholesterol did not decrease, however, by 7 or 14 days of treatment (Table 2). Prior reports have also demonstrated lack of effect on cholesterol levels in experimental animals after 2 weeks of statin administration but a reduction of serum cholesterol levels with a longer (4-week) treatment course.6,14

eNOS Upregulation
The effect of mevastatin on eNOS messenger RNA and protein was assessed by RT-PCR and Western blotting. An increase in eNOS mRNA after 14 days with daily 20 mg/kg treatment was observed (Figure 2A and 2B). To determine the time dependence of this effect, eNOS mRNA was compared at 7, 14, and 28 days after administration of 20 mg/kg per day. eNOS mRNA significantly increased at 14 and 28 days (P < 0.05) (Figure 2C and 2D). In addition, a 2-fold increase in eNOS protein was detected by immunoblotting after 14 days of 20 mg/kg treatment (Figure 3).

Absolute CBF
Chronic mevastatin treatment (14 days at 20 mg/kg) raised CBF by 30% to 82 ± 6 mL/100 g per minute compared with basal CBF in vehicle-treated animals (63 ± 3 mL/100 g per minute) (P < 0.05).

Discussion
The chemical class statins increase eNOS mRNA and protein levels, decrease infarct volume, and reduce neurological deficits in nonatherogenic, normocholesterolemic mice. Mevastatin (chemical name compactin), the family member used in the present experiments, protected the brain when given daily for 14 days at 20 mg/kg. Whenever neuroprotection was achieved, eNOS mRNA levels were increased. Moreover, after 28 days of daily treatment, mevastatin continued to increase eNOS expression and protected ischemic brain, thereby arguing against the development of tachyphylaxis. In fact, infarct reduction may have been enhanced. However, mevastatin given at a lower dose (2 mg/kg per day) did not increase eNOS or protect against ischemic injury regardless of the treatment duration.

Serum cholesterol lowering was not necessary for acute stroke protection by statins. Lower serum cholesterol was detected after 28-day pretreatment, whereas infarct reduction was detected after 14 days, at a time when eNOS was upregulated (Figure 1). Furthermore, eNOS-deficient mice, refractory to statin-induced stroke protection, still showed
TABLE 1. Physiological Parameters Before, During, and After 2-Hour Ischemia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle (n=5)</th>
<th>Mevastatin 20 mg/kg (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCBF, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>6.2±1.2</td>
<td>11.2±2.0</td>
</tr>
<tr>
<td>After</td>
<td>81.2±3.7</td>
<td>109.3±13.8</td>
</tr>
<tr>
<td>MABP, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>90.0±2.4</td>
<td>95.6±5.3</td>
</tr>
<tr>
<td>During</td>
<td>92.2±4.4</td>
<td>87.2±6.7</td>
</tr>
<tr>
<td>After</td>
<td>86.8±4.5</td>
<td>89.2±4.7</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>290.4±61</td>
<td>363.3±51</td>
</tr>
<tr>
<td>During</td>
<td>423.8±74</td>
<td>390.4±43</td>
</tr>
<tr>
<td>After</td>
<td>366.8±70</td>
<td>362.7±69</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>36.4±0.1</td>
<td>36.3±0.1</td>
</tr>
<tr>
<td>During</td>
<td>37.1±0.2</td>
<td>36.9±0.1</td>
</tr>
<tr>
<td>After</td>
<td>36.9±0.1</td>
<td>36.8±0.1</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.30±0.00</td>
<td>7.30±0.10</td>
</tr>
<tr>
<td>During</td>
<td>7.30±0.00</td>
<td>7.30±0.00</td>
</tr>
<tr>
<td>After</td>
<td>7.30±0.10</td>
<td>7.30±0.00</td>
</tr>
<tr>
<td>Pao2, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>122.4±13.1</td>
<td>134.8±14.7</td>
</tr>
<tr>
<td>During</td>
<td>118.3±10.1</td>
<td>122.8±12.4</td>
</tr>
<tr>
<td>After</td>
<td>151.1±3.4</td>
<td>146.9±5.7</td>
</tr>
<tr>
<td>Paco2, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>49.5±1.2</td>
<td>53.2±3.9</td>
</tr>
<tr>
<td>During</td>
<td>39.1±1.9</td>
<td>44.9±2.7</td>
</tr>
<tr>
<td>After</td>
<td>40.2±1.2</td>
<td>43.5±3.0</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Physiological parameters were measured in a subset of mice pretreated with 20 mg/kg mevastatin daily for 14 days. MABP (mean arterial blood pressure), heart rate, rectal temperature, and blood gas determinations (pH, Pao2, Paco2) were measured at baseline (15 minutes before ischemia), during (1 hour after onset of ischemia), and after (15 minutes after reperfusion) ischemia. rCBF was measured by laser-Doppler flowmetry and is expressed as percentage of baseline. There were no significant differences in any of the variables examined.

TABLE 2. Serum Cholesterol Levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pretreatment, d</th>
<th>Cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type SV/129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mevastatin 2 mg</td>
<td>28</td>
<td>104±3*</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>120±6</td>
</tr>
<tr>
<td>Mevastatin 20 mg</td>
<td>7</td>
<td>114±9</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>129±16</td>
</tr>
<tr>
<td>Mevastatin 20 mg</td>
<td>14</td>
<td>116±11</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>128±7</td>
</tr>
<tr>
<td>Mevastatin 20 mg</td>
<td>28</td>
<td>108±8*</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>148±14</td>
</tr>
<tr>
<td>eNOS−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mevastatin 20 mg</td>
<td>28</td>
<td>123±10*</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td>164±4</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Serum cholesterol level as a function of dose and duration of mevastatin pretreatment is shown. Wild-type SV/129 mice or eNOS-null mice (eNOS−/−) were treated with either 2 or 20 mg/kg per day of mevastatin or vehicle for varying intervals (n=6–9 animals per group). Significant reductions in total cholesterol were observed with 2 and 20 mg/kg per day mevastatin only when it was administered for 28 days. *P<0.05.

Amin-Hanjani et al An HMG-CoA Reductase Inhibitor Reduces Stroke and Upregulates eNOS

protective effect. Other statin actions may also be relevant, such as upregulation of the fibrinolytic system, enhanced tissue plasminogen activator and decreased plasminogen activator inhibitor mRNA within endothelial cells, and reduced monocyte chemotaxis and inflammatory responses. The present study, however, argues in favor of a predominant role for eNOS upregulation within the vascular endothelium, at least in this experimental ischemia model. Accordingly, animals deficient in eNOS did not show infarct protection even after 28 days of mevastatin treatment in the presence of lower serum cholesterol.

The statin effect may not be unique to the cerebrovasculature since they may also target the coronary arteries and protect against myocardial infarction. Within weeks of administration, statins enhance responsivity of coronary vessels to acetylcholine and increase tissue plasminogen activator activity in endothelial cells, actions that precede any effect on serum cholesterol levels. In cultured endothelial cells, statins upregulate eNOS mRNA and protein and protect against hypoxic insults, indicating that the newly synthesized protein is functionally active. The mechanism of upregulation appears to be posttranscriptional, secondary to enhanced mRNA stability.

Our study highlights that all statins are not equipotent in upregulating eNOS and protecting brain against ischemic insults, despite similar K, values against HMG-CoA reductase in vitro. The differences between statins may relate to variable drug penetration into endothelial cells based on differences in lipophilicity. For example, the active form of mevastatin is approximately 8 times less lipophilic than simvastatin, and lovastatin is less lipophilic than simvastatin. Both are consistent with observations that mevastatin and lovastatin were less potent than simvastatin against ischemic injury. Hence, statins with the highest lipophilicity
and potency provide the greatest degree of eNOS upregulation and infarct protection.

Inhibition of the enzyme HMG-CoA reductase depletes downstream isoprenoids such as geranylgeranyl pyrophosphate and farnesyl pyrophosphate. These isoprenoids not only serve as intermediates for cholesterol biosynthesis but modify proteins to facilitate their attachment to cell membranes. For example, HMG-CoA inhibition blocks geranylgeranylation of G-proteins such as Rho GTPases, thereby inhibiting GTPase activity and causing disruption of actin stress fibers. This mechanism has been shown to increase NOS expression in culture and to be relevant to ischemic data in vivo.

In conclusion, we demonstrated that mevastatin, a member of the class of drugs that inhibit HMG-CoA reductase, increases eNOS mRNA and protein levels, augments CBF, and reduces cerebral injury after murine MCAO in a cholesterol-independent manner. The relevant target for HMG-CoA reductase inhibition appears to be within the endothelium rather than the liver, as it is for cholesterol reduction. These findings underscore the importance of developing lipophilic enzyme inhibitors that penetrate the endothelium to block downstream steps in the mevalonic pathway.

Acknowledgments

This work was supported by the National Institutes of Health–sponsored Stroke Program Project 5-P50-NS10828 and National Institutes of Health grant 1-RO1-HL62602. We thank Matthias Endres, Zihong Huang, and Dao-Shan Chui for technical assistance.

References


Clinical trials with HMG-CoA reductase inhibitors (statins) have demonstrated a significant reduction in the incidence of coronary events and coronary mortality in patients at risk for coronary disease.1,2 Meta-analysis of statins trials has shown a lower risk of ischemic stroke in patients with history of coronary artery disease with average and/or elevated serum cholesterol levels.3 Statins have also been shown to attenuate the development of atherosclerosis in both coronary and carotid arterial beds.4 Subgroup analysis of the major statin trials suggests that some of the effects might be due to mechanisms other than cholesterol reduction.5

Moskowitz’s group was among the first to demonstrate that statin may be neuroprotective by causing an increase in eNOS activity in an animal stroke model.6 This statin action is independent of its cholesterol-lowering effect.7 The study in the accompanying article, led by Amin-Hanjani and Stagliano in the Moskowitz laboratory, showed that the infarct volume was reduced in animals pretreated for 2 weeks with mevastatin. The observed effect of mevastatin was closely linked to its ability to increase the eNOS content in the aorta. This additional information strengthens the original contention that the neuroprotective effect of statin action is independent of the cholesterol-lowering effect. The authors reaffirmed that the protective role of mevastatin was absent in mice that are deficient in eNOS.

An excessive amount of NO accumulates in the ischemic brain. The exact role of NO in brain injury has been confounded by the existence of 3 different NOS isoforms that may have different roles in cerebral ischemia. eNOS, through the work of the Moskowitz laboratory, has been shown to be salutary,7 in contrast to the detrimental roles of neuronal NOS8,9 or inducible NOS10 in acute cerebral ischemia. The present study, which uses a lipophilic statin to increase endogenous eNOS, is an elegant way to demonstrate that eNOS is an endogenous protective mechanism in acute cerebral ischemia. Statins selectively increase eNOS activity and may also suppress the activity of other NOS isoforms such as inducible NOS. Thus, statin treatment may be a more desirable therapeutic strategy than other putative neuroprotective agents, such as NO donors or NOS inhibitors, to alter the NO content in the ischemic brain. An important lesson regarding the application of statins derived from the results of this study, in which different dosing schedules were used, is that pretreatment with mevastatin is necessary. Mevastatin required a period of 2 weeks to increase aortic eNOS levels and confer a neuroprotective role. Statins may be promising as prophylactic agents for ischemic stroke and are less likely to be efficacious in the acute setting.

In the present study mevastatin increased the basal CBF by approximately 30%, presumably through increasing eNOS activity in the endothelium. However, the reduction in CBF after suture MCAO was not different between the control and mevastatin treatment groups. This is somewhat unexpected...
and raises the possibility that other mechanisms addressed by the authors may also be in operation.

In summary, in the accompanying article interesting results are presented to strengthen the neuroprotective role of statin drugs via mechanisms that are independent of cholesterol reduction. Clinical studies that specifically explore the neuroprotective role of statin drugs are warranted.

Chung Y. Hsu, MD, PhD
Abdullah Nassief, MD
Guest Editors
Department of Neurology
Washington University School of Medicine
St Louis, Missouri

References
Mevastatin, an HMG-CoA Reductase Inhibitor, Reduces Stroke Damage and Upregulates Endothelial Nitric Oxide Synthase in Mice

Sepideh Amin-Hanjani, Nancy E. Stagliano, Masaru Yamada, Paul L. Huang, James K. Liao and Michael A. Moskowitz

Stroke. 2001;32:980-986
doi: 10.1161/01.STR.32.4.980

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/32/4/980

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/