Effects of the nitric oxide donor 3-morpholinosydnonimine (SIN-1) in focal cerebral ischemia dependent on intracellular brain pH

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Object. A nitric oxide (NO) donor that has been successfully used in the treatment of myocardial infarction, 3-morpholinosydnonimine (SIN-1), may be a potential neuroprotective agent. Production of NO in brain microsomes is dependent on the pH. The purpose of this study was to determine the efficacy of SIN-1 and its dependence on pH in vivo during periods of focal cerebral ischemia.

Methods. At 0.1 or 1 mg/kg, SIN-1 was administered to 54 Wistar rats 30 minutes before a 2-hour period of focal cerebral ischemia under moderate hypo-, normo-, and hyperglycemic conditions. Measurements of brain intracellular pH (pHi); regional cortical blood flow, and the redox state of nicotinamide adenine dinucleotide were obtained in three additional animals to confirm the effects of the serum glucose manipulations. The animals were killed at 72 hours after the ischemic period to obtain infarction volumes. Administration of SIN-1 significantly reduced infarction in normoglycemic animals and, to a lesser extent, in hyperglycemic animals, indicating that SIN-1 was less effective under hyperglycemic conditions. At either dose SIN-1 had no significant effect on infarction volume in moderately hypoglycemic animals because moderate hypoglycemia in itself significantly (p < 0.005) reduced infarction volume.

Conclusions. The NO donor SIN-1 may be a useful intraoperative cerebral protective agent. Furthermore, it is hypothesized that a mechanism that could explain the published discrepancies regarding the effects of NO donors in vivo may be affected by differences in ischemic brain acidosis.

KEY WORDS • nitric oxide • nitric oxide donor • focal cerebral ischemia • intracellular brain pH • 3-morpholinosydnonimine • rat

THE role of NO in the development of ischemic brain damage is complex. Endothelial cells, which produce endothelium-derived relaxing factor NO, regulate the basal tone of cerebral vessels, platelet aggregation, neutrophil infiltration, and neuronal function. The NO donor SIN-1 is currently being used in interventional cardiology to minimize myocardial infarction. Treatment by NO donors in different animal studies has shown a trend toward neuroprotection; however, isoenzyme- and concentration-dependent dual mechanisms have been proposed to explain both protective and detrimental effects of nonselective inhibitors of NOS at different dosages. Hecker, et al., demonstrated that the activities of endothelial and NOS enzymes were dependent on pH. The “enzyme activity pH curve” was shown to be a narrow bell-shaped curve with an optimal enzyme activity at a pH of 7.6 for eNOS and at a pH of 6.7 for nNOS. Therefore, the variable effects of endogenous NO donors described in different published studies not only might be related to the ischemia model, dosage of the drug treatment, and timing, but also to the effects of intra- and perischemic pH on NO activity. Supporting this concept is the observation that the nonselective NOS inhibitor, L-NAME, was not as efficacious in situations of severe focal cerebral ischemia compared with those of moderate ischemia.

In this study we tested the efficacy of SIN-1, an NO donor, as a neuroprotectant, as well as the hypothesis that the severity of ischemic brain acidosis influences the neuroprotective effects of the NO donor. The degree of brain acidosis was manipulated by altering serum glucose concentrations. A sydnonimine, SIN-1 is considered to be an endogenous endothelium-derived relaxing factor that is produced by the endothelium and is used to mimic the intravascular actions of NO. Given the current technology, it is not possible to measure brain pH and NO simultaneously in vivo. We therefore conducted a comparative

Abbreviations used in this paper: CA = carotid artery; CCA = common CA; eNOS = constitutive nitric oxide synthase; enNOS = endothelial NOS; L-NAME = nitro-L-arginine methyl ester; MABP = mean arterial blood pressure; MCA = middle cerebral artery; NADH = reduced form of nicotinamide adenine dinucleotide; NO = nitric oxide; nNOS = neuronal NOS; pH = arterial pH; pH = intracellular pH; SIN-1 = 3-morpholinosydnonimine; SNP = sodium nitroprusside; TTC = 2,3,5-triphenyltetrazolium chloride.
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Intracellular brain pH and the NADH redox state, and regional cortical blood flow, and the NADH redox state, can be measured in vivo by using umbelliferone, a fluorescent indicator. A PE-10 catheter was placed in the right external CA, with the tip at the carotid bifurcation for retrograde injection of umbelliferone. A video-fluorescent microscope was focused on the parietal cortex to measure brain pH, regional cortical blood flow, and the NADH redox state. The umbelliferone solution (0.2 g in 200 ml of 5% glucose) was injected into the external CA catheter at 30- to 60-minute intervals before, during, and after MCA and bilateral CCA occlusion.

The pH indicator, umbelliferone, has two fluorophors, anionic and isobestic, which are excited at 370 and 340 nm, respectively, and have a common emission wavelength of 450 nm. The fluorescence of the anion varies directly with the pH, whereas the isobestic fluorescence varies with the drug concentration. Brain pH can then be calculated from the 340/370-nm ratio. The NADH fluorescent images excited at 370 nm are acquired before umbelliferone is injected to correct for background fluorescence and for analysis of mitochondrial function. The scale factor for the percentage of change in NADH fluorescence from baseline is set so that 100% represents the level of NADH fluorescence in the healthy brain, whereas an increase to 300% represents brain death. The images obtained from the 340-nm excitation were processed to compute the regional cortical blood flow by using the 1-minute initial slope index with a partition coefficient of unity for umbelliferone. All images of pH, regional cortical blood flow, and NADH redox states are stored on tape for analysis.

Regional cortical blood flow as measured using umbelliferone is defined as those areas that are relatively avascular and primarily contain arterioles and capillary beds. The imaging system allows the measurement of regional cortical blood flow by allowing the investigator to outline cortical areas of interest, which are devoid of major surface conducting vessels.

Histopathological Study

Seventy-two hours after flow restoration, the animals were again placed in a state of anesthesia, induced by pentobarbital, and then intracardially perfused using a warm (37°C) 2% TTC solution. The animals’ brains were quickly removed and immersed in the TTC solution for 5 days. Eleven serial coronal sections were cut from each brain at 1-mm intervals and photographed. Total cortical infarction volume was calculated by integrating the infarcted areas of all sections (area of infarction in square millimeters × thickness of section). The total infarction volume was multiplied by the ratio between total left hemisphere volume and total right hemisphere volume to correct for cerebral edema.

Statistical Analysis

Analysis of variance was followed by the Fisher post hoc test for multiple comparisons to test the statistical significance of differences between groups. The Student unpaired t-test was used to compare measurements between different time points within a group. A probability value lower than 0.05 was considered significant. Data are presented as the means ± standard errors of the means for all groups, with the exception of data obtained from the video images (brain pH, and regional cortical blood flow), which are presented as the means ± standard deviations. All analysis was conducted using STATVIEW statistical software (Abacus Concepts, Inc., Berkeley, CA).

Results

In Vivo Fluorescence Imaging of Brain pH, regional cortical blood flow, and the NADH Redox State

Brain pH, the NADH redox state, and regional cortical blood flow were measured in three typical animals (Fig. J. Neurosurg. / Volume 97 / October, 2002 915
1). Baseline values measured before initiation of ischemia in the normoglycemic animal (serum glucose 10.7 mmol/L, PaCO\textsubscript{2} 45.1 mm Hg, pH 7.329, and MABP 86 mm Hg) were as follows: brain pH\textsubscript{i} 7.01 ± 0.03, the NADH redox state 100%, and regional cortical blood flow 66 ± 15.5 ml/100 g/min. After 2 hours of ischemia the brain pH\textsubscript{i} had declined to 6.58 ± 0.07, the NADH redox state had increased to 44% of baseline, and the regional cortical blood flow had significantly declined to 23.7 ± 13.4 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pH\textsubscript{i} was 6.69 ± 0.03, the NADH redox state had increased by 60%, and the regional cortical blood flow had increased to 85.7 ± 13.8 ml/100 g/min.

In the moderately hypoglycemic animal (serum glucose 5.2 mmol/L, PaCO\textsubscript{2} 43 mm Hg, pH 7.397, and MABP 86 mm Hg) the baseline brain pH\textsubscript{i}, which had been 7.01 ± 0.08, had decreased to 6.79 ± 0.06 after 2 hours of ischemia, the NADH redox state had increased by 14% of baseline, and the regional cortical blood flow had declined from 79.8 ± 14.4 to 32.8 ± 10.3 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pH\textsubscript{i} was 6.89 ± 0.05, the NADH redox state had decreased by 17%, and the regional cortical blood flow had increased to 89.1 ± 20.1 ml/100 g/min.

Baseline values in the hyperglycemic animal (serum glucose 19 mmol/L, PaCO\textsubscript{2} 48 mm Hg, pH 7.436, and MABP 89 mm Hg) before initiation of ischemia were the following: brain pH\textsubscript{i} 7.01 ± 0.07, the NADH redox state 100%, and regional cortical blood flow 75.3 ± 24.4 ml/100 g/min. After 2 hours of ischemia, the brain pH\textsubscript{i} had decreased to 6.12 ± 0.05, the NADH redox state had increased to 75% of baseline, and the regional cortical blood flow declined to 16.4 ± 10.7 ml/100 g/min. Thirty minutes after restoration of blood flow, the brain pH\textsubscript{i} was 6.45 ± 0.1, NADH redox state had decreased by 60%, and the regional cortical blood flow had increased to 46.4 ± 17.4 ml/100 g/min. The difference in brain pH\textsubscript{i} during periods of focal cerebral ischemia between the normoglycemic and hyperglycemic groups was significant (p < 0.005).

**Physiological Parameters**

Treatment with SIN-1 did result in a temporary reduction in MABP directly after intravenous injection, but recovered during the 30 minutes between injection of SIN-1 and occlusion of the MCA and CCAs. Weight loss was reduced significantly (p < 0.05) in both normoglycemic and hyperglycemic SIN-1–treated animals in response to both low and high doses. The SIN-1 did not significantly affect the glucose response to insulin in the hypoglycemic groups (Table 1).

**Infarction Volume**

The decrease in serum glucose levels from 10 to 3.3 mmol/L resulted in a significant (p < 0.001) reduction in cortical infarction volume by 80%, from 95.8 ± 12 to 19.1 ± 11 mm$^3$, compared with the normoglycemic group (Fig. 2). Hyperglycemia (glucose level 22.4 mmol/L) resulted in exacerbation of cortical ischemic damage by 178%, to 170.3 ± 14 mm$^3$ (p < 0.005 compared with normoglycemic animals).

When compared with the normoglycemic controls, SIN-1 at 0.1 mg/kg significantly decreased infarction volume by 71% (p < 0.003), from 95.8 ± 12 to 27.9 ± 12 mm$^3$. Increasing the dose of SIN-1 to 1 mg/kg also caused a significant reduction in ischemic damage by 69% (p < 0.018), to 33.4 ± 10 mm$^3$, compared with the normoglycemic control group. Infarction volumes between the two normoglycemic SIN-1–treated groups were not significantly different.

In hyperglycemic animals SIN-1 treatment at 0.1 and 1 mg/kg significantly reduced cortical infarction volume by 45% (p < 0.025) and 51% (p < 0.023), from 170.3 ± 14 mm$^3$ to 87.3 ± 18.7 and 83.5 ± 17.3 mm$^3$, respectively. Increasing the SIN-1 treatment dose in hyperglycemic animals from 0.1 to 1 mg/kg did not significantly decrease cortical infarction volume.

The differences in infarction volumes between the SIN-1–treated normoglycemic groups (69–71%) and SIN–treated hyperglycemic groups (45–51%) was statistically significant (p < 0.05).
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FIG. 2. Bar graph of infarction volumes shown in cubic millimeters comparing animals treated with SIN-1 at 0.1 and 1 mg/kg with ischemic controls during moderate hypoglycemia, normoglycemia, and hyperglycemia. The percentage differences in infarction volumes between treated and nontreated animals were 45 ± 11% and 51 ± 10% in the hyperglycemic group and 71 ± 13% and 69 ± 10% in the normoglycemic group for 0.1 mg/kg and 1 mg/kg SIN-1, respectively. Therefore, SIN-1 was less effective in reducing infarction volume as brain pH became more acidic. During moderate hypoglycemia, differences between the treated and nontreated groups were 56 ± 27% and 66 ± 19% for 0.1 and 1 mg/kg SIN-1, respectively; this was not statistically significant. Data are expressed as the means ± standard errors of the means. *Statistically different from normoglycemic ischemic control values (p < 0.005). †Statistically different from respective ischemic control values (p < 0.025, analysis of variance).

In the insulin-induced moderately hypoglycemic animals, SIN-1 tended to reduce infarction volume at both dosages of 0.1 mg/kg (8.4 ± 4.9 mm³) and 1 mg/kg (6.6 ± 3.4 mm³), but this reduction did not reach statistical significance (p < 0.22 and p < 0.29, respectively).

Discussion

Three hypotheses can be supported by this study. First, SIN-1, an NO donor that has been used in the treatment of myocardial infarction may also be useful as an intraoperative neuroprotectant and as a treatment for stroke. Second, the efficacy of SIN-1 is dependent on brain pH, and may be more effective in cases of moderate ischemia than in those of severe ischemia. Third, when evaluating the potential effects of pharmaceutical agents on the treatment of central nervous system disorders, such as cerebrovascular disease and brain tumors, brain pH must be considered to be a possible influencing factor.

The NO Donor SIN-1

The NO donor SIN-1 is currently used in interventional cardiology because it produces antispasitic and vasodilatory effects without inducing tolerance.1 In human acute coronary syndromes, titration of SIN-1 to the desired antisch-
The mean systemic parameters in the study groups*

<table>
<thead>
<tr>
<th>Group</th>
<th>PaCO₂ (mm Hg)</th>
<th>PaO₂ (mm Hg)</th>
<th>pH₃</th>
<th>MABP (mm Hg)</th>
<th>Glucose (mmol/L)</th>
<th>Hematocrit (%)</th>
<th>Temperature (°C)</th>
<th>Weight Loss (%)</th>
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<tr>
<td>before occlusion</td>
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<td>ischemic control group</td>
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<td>at 1 hr ischemia</td>
<td>42.4 ± 1.5</td>
<td>193 ± 8.8</td>
<td>7.416 ± 0.016</td>
<td>89.1 ± 2.7</td>
<td>2.7 ± 0.6</td>
<td>36.0 ± 1.5</td>
<td>38.2 ± 0.4</td>
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<td>at 2 hrs ischemia</td>
<td>41.0 ± 2.3</td>
<td>181 ± 7.0</td>
<td>7.412 ± 0.021</td>
<td>89.5 ± 3.0</td>
<td>2.4 ± 0.3†</td>
<td>36.2 ± 1.2</td>
<td>38.2 ± 0.4</td>
<td>5.6 ± 2.7</td>
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<td>SIN-1 0.1 mg/kg treatment group</td>
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<td>before occlusion</td>
<td>34.8 ± 1.7</td>
<td>194 ± 7.9</td>
<td>7.454 ± 0.020</td>
<td>84.0 ± 3.9</td>
<td>4.2 ± 0.4†</td>
<td>36.2 ± 1.1</td>
<td>38.3 ± 0.4</td>
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<td>at 1 hr ischemia</td>
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<td>181 ± 7.0</td>
<td>7.412 ± 0.021</td>
<td>89.5 ± 3.0</td>
<td>2.4 ± 0.3†</td>
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<tr>
<td>before occlusion</td>
<td>39.7 ± 1.5</td>
<td>212 ± 9.7</td>
<td>7.442 ± 0.013</td>
<td>81.4 ± 2.2</td>
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<tr>
<td>at 1 hr ischemia</td>
<td>36.4 ± 1.6</td>
<td>207 ± 5.9</td>
<td>7.450 ± 0.015</td>
<td>84.4 ± 2.9</td>
<td>2.3 ± 0.2†</td>
<td>38.1 ± 1.1</td>
<td>37.0 ± 0.1</td>
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<tr>
<td>at 2 hrs ischemia</td>
<td>38.9 ± 1.5</td>
<td>191 ± 6.7</td>
<td>7.431 ± 0.016</td>
<td>84.9 ± 2.8</td>
<td>1.9 ± 0.3†</td>
<td>39.2 ± 0.5</td>
<td>37.0 ± 1.0</td>
<td>6.4 ± 2.3</td>
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*p Values are expressed as the means ± standard errors of the means.
† Statistically different from normoglycemic control values (p < 0.05).
‡ Statistically different from respective ischemic control values (p < 0.05).

(pH 7.6). Similar studies on pH sensitivity of the neuronal isofrom of cNOS revealed an optimal pH of 6.7 within a bell-shaped curve similar to endothelial cNOS. Combining their data on both cNOS enzymes, we can conclude that, within the pH range encountered (6.12–6.82), enzyme activity would vary considerably. The following supports this hypothesis: 1) increased intracellular acidosis achieved by augmenting the severity of ischemia caused a loss of the neuroprotective effect by the nonselective NOS inhibitor, l-NAME, suggesting that NOS inhibition was less effective because of inhibition of NOS activity in acidosis; 2) in a separate study (Coert, et al., in press), administration of 7-NI, a selective nNOS inhibitor, was far less effective during periods of hyperglycemia (27.5% reduction compared with approximately 48% in this study) and more effective during periods of both normoglycemia (93.5% reduction compared with approximately 70% in this study) and hypoglycemia (72.6% reduction compared with approximately 61% in this study).

Based on the data the following hypothesis can be supported. In the healthy brain, NO is produced to maintain basal tone. In the brain with cerebral ischemia, NO production increases as the brain becomes ischemic, to a pH below approximately 6.7—NO donors become more effective during periods of hyperglycemia (72.6% reduction compared with approximately 61% in this study).

**Role of NO in Cerebral Ischemia**

Direct measurements of NO production in vivo have revealed both increased and decreased NO concentration during cerebral ischemia. Using a porphyrin microsensor, Malinski, et al., reported an increase from a baseline level of lower than 10⁻⁸ M to an approximate level of...
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$10^{-6}$ M in cases of focal ischemia. Using Nafion and porphyrine-coated carbon fiber electrodes, Lin, et al., recorded NO production in rats during 40 minutes of combined MCA and bilateral CCA occlusion. Basal extracellular NO concentration increased to a mean of $18.8 \pm 3.4$ nmol/L. The differences between these values and those reported by Malinski, et al., were explained by the smaller tip of the probe, a different occlusion technique, more superficial cortical measurements, and the higher selectivity of the probe. In a cat model of focal cerebral ischemia, NO concentrations were shown to increase during the first 10 minutes in regions exhibiting depolarization. The course of NO production after this was found to be variable and heterogeneous, ranging from a continuous reduction to a sustained overproduction. It was suggested that NO production could be pH dependent. Altogether, these data suggest that outcome is determined by the individual contributions of eNOS and nNOS through their specific and local effects rather than by the absolute concentration of NO during ischemia.

A limited number of in vivo studies have been performed in which the effect of NO donors in focal cerebral ischemia was shown to be primarily protective. Differences in methodology, including animal models, anesthetic agents, occlusion techniques, duration of ischemia, and drug delivery and dosing complicate a comparison of effects in these different studies. In the study by Morikawa, et al., in which 300 mg/kg L-arginine was used as a treatment dose, a 28 to 35% reduction in ischemic damage was reported. Sodium nitroprusside was used in three studies in dosages ranging from 0.11 mg/kg/hr (total dose 0.22 mg/kg/hr) to 3 mg/kg/hr (total dose 3 mg/kg/hr), resulting in reductions in infarction volume of 67 and 27%, respectively. In a study by Solom, et al., a high dose of SNP (1.1 mg/kg/hr administered intravenously for 2 hours), which did not significantly reduce regional cortical blood flow but significantly reduced MABP, did not attenuate ischemic damage compared with a lower dose (0.11 mg/kg/hr), which improved treatment outcome. In contrast, a high dose of SNP with addition of phenylephrine (10–100 $\mu$g/hour intracarotid infusion) to prevent hypotension was protective in the study by Zhang, et al. At 3 mg/kg/hr SIN-1 was shown to attenuate ischemic damage in a permanent model of focal ischemia when administered up to 60 minutes after onset of ischemia. In a previous study by Coert, et al., intravenous administration of SIN-1 at 1 mg/kg reduced the mean cortical infarction volume, but this reduction was not statistically significant. Reducing occlusion times from 3 to 2 hours in the present study did not significantly change cortical infarction volume or variability, although a reduction in the occlusion time from 3 hours to 1 hour of ischemia in this same model did just that.

Initiating intravenous SNP (0.19 $\mu$g/kg/min) in patients with white-matter lacunar (four patients) or cortical infarcts (18 patients) at a mean of 21.3 hours (range 9.3–27 hours) after onset of stroke, Butterworth, et al., were able to improve cerebral blood flow, although MABP was reduced. A reduction in platelet function was also found in patients who were not on a regimen of aspirin prior to their ischemic strokes. Overall outcome in the SNP-treated group, however, was not different from that of the control group.

The neuroprotectiveness of NO donors can be attributed to either a parenchymal or vascular effect. It has been demonstrated that NO donors enhance regional cortical blood flow by vasodilation, which reduces neuronal damage in the area surrounding the ischemic core. These studies used either SNP or SIN-1, which was given by intracarotid infusion, whereas in three other studies these donors were given intravenously and demonstrated no significant changes in regional cortical blood flow. This may suggest that intracarotid infusion may result in a higher concentration of NO donor in the brain, thereby exerting a profound dilatory effect, or there could be a loss of vascular reactivity because of cerebral ischemia. In this study we chose the intravenous route for administration of SIN-1 because clinically it is routinely given intravenously. On the other hand, a parenchymal effect of NO could, for example, decrease neuronal death by attenuating the rise in intracellular calcium or by reducing free radical formation. Inhibition of NOS also has been demonstrated in a number of published reports to be either neuroprotective or neurotoxic. This suggests in part that NO can be neuroprotective or neurotoxic, depending on the ischemic environment, which in part may be pH dependent.

Model of Graded Focal Cerebral Ischemia

To ascertain the relationship between intracellular brain acidosis and NO, animals were subjected to moderate hypoglycemia, normoglycemia, or hyperglycemia to provide models of three graded levels of ischemia. There have been several studies in which brain pH has been measured before, during, and after global and focal cerebral ischemia. Brain pH becomes more acidic during ongoing ischemia, declining from approximately 6.7 to less than 6 in response to increasing serum glucose levels (approximately 6.5 mmol/L to > 28 mmol/L). Conversely, as serum glucose levels become more hypoglycemic (approximately 6.7–7 mmol/L to approximately 7 mmol/L), brain pH becomes less acidic (6.7–7.0). Cerebral infarction is reduced under moderate hypoglycemic conditions, whereas it becomes exacerbated under hyperglycemic conditions. In our present study, hypoglycemia (serum glucose level of approximately 3 mmol/L) reduced the cortical infarction volume by approximately 80%, from 95.8 ± 12 to 19.1 ± 10 mm$^3$ in the normoglycemic control group, whereas in the hyperglycemic animals there was a 178% increase in infarction volume (to 170.3 ± 14 mm$^3$).

Conclusions

In this experiment the protective effect of NO enhancement by SIN-1 during focal cerebral ischemia was altered by serum glucose concentrations, which in effect reflected a manipulation of brain pH. As a neuroprotectant in this study SIN-1 was significantly effective in reducing infarction volume in animals with hyperglycemia and, to a greater extent, in animals with normoglycemia. The successful use of NO donors in the treatment of myocardial ischemia makes them attractive candidates for use as neuroprotective agents. The apparent effect of pH on NO is consistent with in vitro data. We propose that brain pH is an important factor for determining NO activity and that the observed variability in effects of NO enhancement in
different models of cerebral ischemia is partly due to differences in brain pH during ischemia. The effect of pH on NO enhancement provides an additional mechanism by which acidosis contributes to ischemic brain damage. We also propose that, depending on the environment, brain pH might influence how pharmaceutical agents perform as a treatment modality. For example, brain tumors have been shown to alter pH.\textsuperscript{13-14} Further investigations will need to be undertaken to elucidate the mechanism by which the concentration of the H\textsuperscript{+} affects NO activity.

Acknowledgments
The authors are indebted to Ms. Heidi Martin for her technical assistance and to Ms. Mary Soper for preparation of the manuscript.

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J. Neurosurg. / Volume 97 / October, 2002

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Intracellular brain pH and SIN-1


Manuscript received January 18, 2002.
Accepted in final form June 10, 2002.
This work was supported by the Thoralf M. Sundt, Jr., Research Fellowship
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