Effects of the Spin Trap Agent Disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-Oxide (Generic NXY-059) on Intracerebral Hemorrhage in a Rabbit Large Clot Embolic Stroke Model

Combination Studies With Tissue Plasminogen Activator

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Background and Purpose—It has been proposed that the novel spin trap agent disodium-[(tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (NXY-059) may be useful in the treatment of ischemia and stroke. To date, there is little information concerning the safety of NXY-059 when administered in combination with the only Food and Drug Administration–approved pharmacological agent for the treatment of stroke, the thrombolytic tissue plasminogen activator (tPA). Thus, we determined the effects of NXY-059G, a generic form of NXY-059, on hemorrhage and infarct rate and volume when administered alone or in combination with tPA. In addition, we determined whether NXY-059G affected 2 physiological variables, blood glucose levels and body temperature.

Methods—Male New Zealand White rabbits were embolized by injecting a large blood clot into the middle cerebral artery via a catheter. Five minutes after embolization, NXY-059G (100 mg/kg) was infused intravenously; control rabbits received infusions of saline, the vehicle required to solubilize NXY-059G. In tPA studies, the thrombolytic was administered intravenously starting 60 minutes after embolization (20% bolus injection/80% infusion over 30 minutes). Body temperature and blood glucose levels were measured throughout the study. Postmortem analysis included assessment of hemorrhage and infarct rate, size, and location.

Results—In the vehicle control group, the hemorrhage rate after a thromboembolic stroke was 52% (n=23), and this was increased by 67% if tPA was administered (n=15). The rabbits treated with NXY-059G in the absence of tPA had a 79% incidence of hemorrhage (n=19), an increase of 52% over the control group. In the combination drug–treated groups, the NXY-059G/tPA group had a 47% incidence of hemorrhage (n=15). There was a decrease of hemorrhage volume in the NXY-059G/tPA group compared with the other 3 groups included in the study. There was no significant effect of NXY-059G either alone or in combination with tPA on infarct rate or volume. NXY-059G did not significantly alter the physiological variables that were measured.

Conclusions—This study suggests that NXY-059G may affect the integrity of the cerebral vasculature when administered immediately after an embolic stroke, as evidenced by an increase in hemorrhage rate. However, when NXY-059G is administered in combination with tPA, it may improve the safety of tPA by reducing the incidence of tPA-induced hemorrhage. The mechanism(s) involved in the NXY-059G–induced increase in hemorrhage rate and reduction of tPA-induced hemorrhage rate remains to be elucidated. (Stroke. 2002;33:1665-1670.)

Key Words: indoles ■ ischemia ■ neuroprotection ■ oxygen radical ■ reactive oxygen species ■ reperfusion ■ rabbits

Tissue plasminogen activator (tPA) is the only treatment for stroke approved by the Food and Drug Administration because it has been shown to be beneficial.1 tPA rapidly restores cerebral perfusion;2 however, reperfusion is commonly associated with neuronal and endothelial cell damage and an increased risk of intracerebral hemorrhage (ICH).3-6 In tPA clinical trials, there was an approximately 6% incidence of symptomatic ICH, with a mortality rate near 50%.1,7,8 Currently there are no Food and Drug Administration–approved treatments to guard against tPA-induced hemorrhage or to be used as adjunct therapy with tPA to promote neuronal survival.

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1665
We reported that the spin class family of compounds that includes α-phenyl-N-t-butyl nitrene (PBN) may reduce the hemorrhage after tPA administration. However, paradoxically, we found that in the absence of tPA, PBN significantly increases hemorrhage rate. The diverse effects of PBN may be associated with several pharmacological activities, including the ability of PBN to trap a variety of radicals (alkoxy, superoxide, hydroxyl) and regulate the activity of enzymes, voltage-gated channels, and transcription factors. One or more of the actions may be involved in the pharmacological activities of PBN in vivo. PBN has been shown to be neuroprotective after middle cerebral artery occlusion and to decrease ICH in rodent models.

Recent developments in the structure–activity relationships of PBN have led to the synthesis of disodium-[tert-butylamino)methyl]-benzene-1,3-disulfonate N-oxide, which is being referred to as NXY-059. NXY-059 is neuroprotective in a rodent focal ischemia model and in a primate permanent occlusion model. We recently showed that generic NXY-059 (NXY-059G) is neuroprotective in a rabbit thromboembolic stroke model and also that NXY-059G may increase the therapeutic window for tPA. In that study, NXY-059G reduced embolism-induced behavioral deficits if administered 5 minutes after small clot embolization. On the basis of the neuroprotective activities of NXY-059 in various preclinical models, it appears that NXY-059 is being considered as a candidate for a neuroprotection clinical trial in acute stroke patients.

However, because NXY-059G is structurally related to PBN and PBN has previously been shown to have numerous pharmacological activities and side effects, including the ability to increase hemorrhage rate after an embolic stroke, studies aimed at determining the safety profile of NXY-059G are warranted and essential. Therefore, we determined the pharmacological effects of administration of NXY-059G on hemorrhage rate and volume in the presence or absence of tPA treatment in a rabbit large clot embolism model (RLCEM).

Materials and Methods

All procedures followed were within institutional guidelines. Male New Zealand White rabbits weighing 2 to 3 kg were used for the study. The common carotid artery was catheterized as described previously. Emboli were prepared, and the rabbits were embolized according to the procedure of Lapchak et al. If the animal did not react behaviorally (nystagmus, hemiparesis) to the embolization, a second blood clot was injected in the same way 3 minutes after the first embolization. If there was no behavioral reaction to either embolization, no additional blood clots were administered. Animals that had no behavioral reaction after administration of 2 clots were treated in the same manner as animals responding to emboli. Inclusion or exclusion of animals was based on the criteria described below. After the embolization process was completed, the catheter was ligated close to the neck, and the rest of the catheter and injection port was cut off.

Animals that died before they were euthanatized were included in the study, and the brains were fixed and sectioned as below. The surviving animals were euthanatized 48 hours after embolization. The brains were removed and immersion fixed in 4% paraformaldehyde for at least 1 week and then examined by a naive observer. The right middle cerebral artery of each brain was examined for the presence of emboli. The surface blood vessels were then stripped from the right hemisphere of each brain. The cerebellum was also removed from the brain stem. Hemispheres and brain stem were cut into seven 5-mm-thick coronal slices, each having 2 faces, for a total of 14. We noted the presence, location, size, and type of each hemorrhage and infarct. We recorded the size of hemorrhage and/or infarct as the number of section faces showing hemorrhage or infarct. Infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. Three major types of hemorrhage were identified with the use of the grading system we have used in previous studies. Hemorrhagic infarction necrotic was red speckling of an area, usually surrounded by soft infarcted tissue; punctate hemorrhage was isolated small red marks within the tissue that did not extend through the tissue as a blood vessel would; parenchymatous ICH was a large homogeneous mass of blood within the tissue. After evaluation for hemorrhage and infarcts, the total radioactivity in the brains was measured by placing the slices into a gamma counter. The surface vessels from the right hemisphere were placed in a separate container and counted. Then the cerebellum, each hemisphere, and brain stem were counted in separate tubes. The amount of radiolabel present in the brain (including the right hemisphere vessels) was compared with that contained in the labeled blood clot at embolization. If fewer than 10% of the counts were found in the brain and vessels, it was assumed that the labeled blood clot had not reached the brain, and the data from these animals were excluded from further analyses.

Drug Administration

We randomly allocated the rabbits into 4 different treatment groups before the embolization procedure. The randomization sequence was generated with the use of randomization tables, and concealment of the randomization was achieved by use of an independent third party. The randomization sequence was not revealed until all postmortem analyses were complete. Sample size was based on power analysis with α=0.05 and β=0.90, a coefficient of variation of 15%, and a difference between means of 20%. It was determined that a sample size of approximately 12 to 14 animals per group was required. The spin trap agent disodium-[tert-butylamino)methyl]-benzene-1,3-disulfonate N-oxide (NXY-059G [generic NXY-059]) was custom synthesized in our laboratories, as described previously by Lapchak et al., according to the scheme of Hinton and Janzen and Carney. NXY-059G was dissolved in normal saline and administered at a dose of 100 mg/kg by intravenous infusion over 30 minutes, starting 5 minutes after embolization. Vehicle was administered 5 minutes after embolization. This dose and treatment regimen were previously shown to be neuroprotective in a rabbit small clot embolism model (RSCEM) when NXY-059G was administered 5 minutes after embolization. In the remaining groups of rabbits, we then administered tPA or vehicle 1 hour after embolization. The tPA regimen used in this study is as follows: 3.3 mg/kg tPA, 20% as a bolus injection given over 1 minute, followed by the remainder infused over 30 minutes. Genentech, Inc (South San Francisco, Calif) supplied tPA and its vehicle. The tPA was reconstituted with sterile water, at a concentration of 1 mg/mL.

Physiological Measurements

Throughout the studies, blood glucose levels withdrawn from the left ear vein were measured with a Bayer Glucometer Elite XL 3901B, and body temperature (left ear) was measured with a Braun Thermoscan Type 6013 digital thermometer.

Statistical Analysis

We analyzed the data using the χ² test corrected for multiple comparisons, using the Bonferroni technique and ANOVA when relevant. The Fisher’s exact test was used as the post hoc test after ANOVA.

Results

Stroke Success Rate

Of 91 embolized rabbits included, we found that 72 (79%) had >10% recovered radioactivity in the brain postmortem.
The majority of embolized rabbits responded with behavioral manifestations including nystagmus, pupil dilatation, hemiparesis, or brief uncoordinated jerking movements. Rabbits that did not have behavioral manifestations of embolization but did have >10% recovered in the brain postmortem were included in the analyses. The remaining 21% of the subjects had ≤10% of the label present in the brain postmortem, indicating that the injected blood clot did not reach the brain. In the present study, 91% of rabbits included in the final analyses had behavioral manifestations of stroke after embolization. The breakdown for behavioral manifestations after embolization is as follows: vehicle, 91%; tPA, 87%; NXY-059G, 95%; and NXY-059G/tPA, 93%. There were no imbalances among the 4 treatment groups. Rabbits that did not reach criteria were excluded from the study, and the data were not used in the final analysis. The cerebral embolism success rate is similar to that described in previous studies.9,30,31,36

Physiological Variables
Blood glucose level and body temperature were measured to determine whether pharmacological treatments affected either parameter after a thromboembolic stroke. Table 1 presents the results of the blood glucose measurements. Within 5 minutes of embolization, there was an increase in blood glucose levels that was maintained for the first 2 hours (P<0.05). Blood glucose levels returned to control levels by 24 hours regardless of the extent of stroke-induced behavioral deficits. Drug administration did not significantly affect glucose levels at any time (P>0.05). Neither embolization nor drug treatment significantly affected body temperature in any group of rabbits.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Baseline Before Embolization</th>
<th>5 min After Embolization</th>
<th>60 min After Embolization</th>
<th>120 min After Embolization</th>
<th>24 h After Embolization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>101.2±3.2</td>
<td>122.1±8.8*</td>
<td>141.6±13.7*</td>
<td>174.7±33.9*</td>
<td>101.1±3.1</td>
</tr>
<tr>
<td>tPA</td>
<td>101.4±2.1</td>
<td>127.4±10.6*</td>
<td>162.8±17.4*</td>
<td>150.4±11.5*</td>
<td>114.0±15.0</td>
</tr>
<tr>
<td>NXY-059G</td>
<td>102.8±3.1</td>
<td>120.2±5.6*</td>
<td>146.5±11.0*</td>
<td>143.6±20.2*</td>
<td>93.2±12.1</td>
</tr>
<tr>
<td>NXY-059G+tPA</td>
<td>100.3±2.5</td>
<td>118.2±4.9*</td>
<td>143.1±8.2*</td>
<td>137.0±8.0*</td>
<td>118.1±3.7</td>
</tr>
</tbody>
</table>

*P<0.05.

Hemorrhage Rate, Volume, and Type
Table 2 shows the hemorrhage rate for the 4 groups of rabbits included in this study. The percentages of rabbits with brain hemorrhages in the 4 groups were as follows: 52% in the vehicle-treated group (n=23), 87% in the tPA-treated group (P=0.0154; n=15), 79% in the group treated with NXY-059G (P=0.0645; n=19), and 47% in the group treated with the combination of NXY-059G+tPA (P=0.1441; n=15).

Overall, there was an increase in hemorrhage rates (Table 2) caused by tPA (67%) and by NXY-059G (52%). The combination of NXY-059G+tPA showed a decreased hemorrhage rate to vehicle control levels. Table 2 also shows the number of faces with observed hemorrhage, which is a measure of hemorrhage volume. For each animal in the study, the maximum number of faces observed was 14. The vehicle-treated group had 2.91±0.82 faces with hemorrhage present. When tPA or NXY-059G was administered, the number of faces was 3.00±0.50 and 2.88±0.46, respectively. Even though tPA and NXY-059G increased the hemorrhage rate (see above), neither treatment significantly altered the number of faces with hemorrhage. However, there was a significant difference between the groups treated with tPA and with NXY-059G+tPA (P=0.0019). The drug combination reduced the number of faces with hemorrhage.

Table 3 shows the types of hemorrhage present in each of the experimental groups. Most of the hemorrhages seen were hemorrhagic infarction necrotic, but punctate hemorrhages and parenchymatous ICHs were seen in some groups. Some of the animals included in the study had more than 1 type of hemorrhage present in the brain. For quantitative purposes, we treated each individual hemorrhage observed as a separate entity. Hemorrhages occurred throughout the brain and included the following structures: caudate putamen; thalamus; hippocampus; frontal, parietal, and occipital cortex; hypothalamus; suprachiasmatic area; cerebellum; pons; and midbrain.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Hemorrhagic Infarction, Necrotic</th>
<th>Punctate Hemorrhage</th>
<th>Parenchymatous ICH</th>
<th>Total No. of Hemorrhages (All Types)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>tPA</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>NXY-059G</td>
<td>14</td>
<td>8</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>NXY-059G+tPA</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>
There were no apparent differences among the groups in the distribution of types or locations of hemorrhages, although the total number of hemorrhages measured in the group treated with NXY-059G was elevated.

### Infarct Rate and Volume

In the experimental groups used in this study, we also determined whether the spin trap agent NXY-059G affected the rate or size of the ischemic infarct produced by embolism. In the vehicle- and tPA-treated groups, infarcts were found in 70% and 87% of the rabbits, respectively (Table 4). Neither tPA, NXY-059G, nor NXY-059G+tPA administration significantly affected the infarct rate or volume (Table 4).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Infarct Rate, %</th>
<th>Infarct Volume, No. of Faces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>70 (16/23)</td>
<td>6.80±0.84</td>
</tr>
<tr>
<td>tPA</td>
<td>87 (13/15)</td>
<td>3.93±0.60</td>
</tr>
<tr>
<td>NXY-059G</td>
<td>79 (15/19)</td>
<td>6.43±1.02</td>
</tr>
<tr>
<td>NXY-059G+tPA</td>
<td>80 (12/15)</td>
<td>5.60±1.17</td>
</tr>
</tbody>
</table>

Table 4. Effect of Pharmacological Treatments on Infarct Rate and Volume After Thromboembolic Stroke

Note: tPA but not NXY-059G administration slightly reduced infarct volume (P=0.11 compared with vehicle). All other statistical comparisons are not statistically different (P>0.05).

### Discussion

In the present study we found that the spin trap agent NXY-059G had differential effects on hemorrhage rate depending on whether or not the drug was coadministered with tPA. NXY-059G administration 5 minutes after embolization increased hemorrhage rate by 52% but not hemorrhage volume. tPA administration also significantly increased hemorrhage rate by 67%. NXY-059G administration after embolization attenuated the tPA-induced increase in hemorrhage rate and volume.

The spin trap agent NXY-059G did not significantly affect embolism-induced infarct rate or volume. This result is in contrast to previous studies in which a more artificial means of middle cerebral artery occlusion was used.19,24 First, the rodent study by Kuroda et al19 used a fixed 2-hour occlusion with various times of reperfusion and beginning of NXY-059 administration. The authors suggest that NXY-059 could be neuroprotective if administered up to 6 hours after reperfusion, if given as a loading dose (bolus) followed by long-term infusion. Marshall et al24 showed that administration of a bolus loading dose of NXY-059 beginning 5 minutes after occlusion, followed by chronic infusion via a minipump for 48 hours, resulted in significant behavioral improvements and neuronal sparing. Our study, which used a 30-minute infusion of NXY-059G beginning 5 minutes after embolization, was not neuroprotective if a large blood clot was injected into the brain via the carotid artery. However, we have found that NXY-059G is neuroprotective and increases behavioral performance when administered 5 minutes after the injection of small blood clots into the brain.27 The differences between our 2 studies, the RLCEM (present study) and RSCEM,27 may be related to a variety of factors, such as the extent of ischemia induced by large clots versus that induced by small clots. In the RLCEM there is an abundance of ICH caused by a large clot embolus, which may alter the progression of the ischemic stroke, whereas the hemorrhage rate in the RSCEM is low. Additionally, the method of infarct quantification we used in the present study is relatively insensitive, and we may have failed to detect a significant treatment effect (type II error). Nevertheless, there were no significant effects of the drug/drug combinations on infarct rate or volume in the present study.

However, NXY-059G did have pharmacological activity in our RLCEM. When we measured the incidence of hemorrhage after NXY-059G administration, we found that NXY-059G, like PBN,9 increased the hemorrhage rate above the baseline hemorrhage rate measured in the vehicle-treated group. It is extremely important to note that tPA administration increased the incidence of parenchymatous ICH, whereas NXY-059G treatment increased punctate hemorrhage incidence. From a clinical perspective, the parenchymatous ICH data are most relevant because parenchymatous ICHs are most often considered to be life-threatening,37,38 whereas punctate hemorrhages are not. The observation that tPA increased parenchymatous ICH incidence is consistent with the National Institute of Neurological Disorders and Stroke rt-PA Stroke Study.1 Additionally, NXY-059G, when administered in combination with tPA, reduced the hemorrhage rate to control levels. This is also reminiscent of the effects of PBN in the same model.9 Moreover, Asahi et al40 reported that PBN effectively decreased tPA-induced hemorrhage in a rat embolic focal cerebral ischemia model. Hu et al41 also showed that MDL 101,002, a conformationally constrained cyclic analogue of PBN, reduced hemorrhage rate in rabbits after an embolism.

The reason for an NXY-059G-induced increase in hemorrhage rate after an embolic stroke is not clear but may be related to the hypothesis of Kuroda et al,19 which suggested that the pharmacological effects of NXY-059 are exerted at processes occurring at the blood–endothelial cell interface. Nevertheless, the consistent finding is that PBN and its analogue NXY-059G increase hemorrhage rate in the thromboembolic stroke model. The results suggest that the base nitrore molecule has properties that can result in hemorrhage, properties not inherent in the chemical structure of 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO),9 since TEMPO did not increase hemorrhage rate in our previous study.9 It is entirely possible that PBN and even NXY-059G may differentially affect 1 or more of the radicals, receptors, enzymes, or proteins that have previously been shown to be affected by PBN,10,12–16 resulting in decreased vascular integrity and hemorrhage. NXY-059G and PBN have recently been shown to be effective at trapping free radical agents, with PBN being more effective than NXY-059.29 From the recent work of Kuroda et al,19 it appears that NXY-059, in contrast to PBN, cannot readily cross the blood-brain barrier, suggesting that endothelial cells may be a target for the actions of NXY-059G. Since endothelial cells have a high density of mitochondria, it is possible that NXY-059G may induce hemorrhage by inhibiting mitochondrial complex I function.13 Further studies are required to understand the mechanism of...
NYX-059–induced changes in vascular integrity and hemorrhage.

Our result, showing that NYX-059G reduces tPA-induced hemorrhage, suggests that NYX-059G effectively scavenges free radicals produced during and after an ischemic stroke. The beneficial effect of NYX-059G in reducing hemorrhage in tPA-treated rabbits may be due to mechanisms that are based on the known pharmacology of PBN since both drugs have similar structures. PBN is reported to scavenge free radicals at the blood–endothelial cell interface. The spin trap agent may exert its effects by reducing microvascular dysfunction indirectly caused by polymorphonuclear neutrophil leukocytes that give rise to free radicals. It is conceivable that free radicals are directly responsible for endothelial weakening and damage, resulting in increased hemorrhage.

In the present study we have tested NYX-059G at a dose (100 mg/kg) that is equivalent to an equimolar dose of approximately 0.55 mol of PBN to determine whether NYX-059G was pharmacologically active against hemorrhage or infarcts in our thromboembolic stroke model. In our study, after a dose of 100 mg/kg NYX-059G, we did not observe any changes in the 2 independent physiological variables that were measured. A recent clinical study with NYX-059 indicated that the spin trap was well tolerated in patients with acute stroke. Our finding that NYX-059G increases hemorrhage rate in the absence of thrombolytic therapy suggests that, under certain conditions, spin trap compounds can produce adverse effects. Additional preclinical safety studies with NYX-059 are warranted before initiation of a clinical trial.

In conclusion, we have shown that effective combination drug treatments can be developed as novel treatments for stroke. Administration of the spin trap agent NYX-059G before tPA significantly reduced tPA-induced hemorrhage rate and volume. Overall, our study suggests that NYX-059G may improve the safety of tPA by reducing thrombolyis-induced hemorrhage.

Acknowledgments
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