Neuroprotection by the stable nitroxide Tempol during reperfusion in a rat model of transient focal ischemia

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Object. The use of thrombolytic agents in the treatment of stroke has yielded surprisingly modest success, possibly because of reperfusion injury mediated by reactive oxygen species (ROS). Therefore, scavenging ROS may be of therapeutic value in the treatment of stroke. Nitroxides are low-weight superoxide dismutase mimics, which allows them to act as cell-permeable antioxidants. In this study the nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) is investigated to determine its ability to reduce reperfusion injury.

Methods. Male Sprague–Dawley rats weighing between 280 g and 350 g underwent middle cerebral artery occlusion with an intraluminal suture for 60 minutes. Regional cerebral blood flow, blood pressure, cerebral temperature, and rectal temperature were monitored during the procedure. After reperfusion, the animals were randomized to groups receiving blinded intravenous administration of either Tempol (10 mg/kg; eight animals) or vehicle (eight animals) over the first 20 minutes of reperfusion (Study I). In a second study to determine dose dependency, animals were randomized to groups receiving Tempol (20 mg/kg; eight animals), low-dose Tempol (5 mg/kg; eight animals), or vehicle (eight animals; Study II). The rats were killed after 4 hours of reperfusion, and brain sections were stained with 2,3,5 triphenyltetrazolium chloride. Infarct volumes were measured using digital imaging.

Animals receiving Tempol had significantly reduced infarct volumes at doses of 20 mg/kg and 10 mg/kg compared with controls (49.01 ± 18.22% reduction [p = 0.003] and 47.47 ± 34.57 [p = 0.02], respectively). No significant differences in the physiological variables measured were observed between groups.

Conclusions. Tempol provides significant neuroprotection after reperfusion in a rat model of transient focal ischemia. These results support the importance of ROS in reperfusion injury and encourage further study of this molecule as a therapeutic agent following thrombolysis.

Keywords • free radical • Tempol • transient focal ischemia • reperfusion injury • neuroprotection • stroke

ISCHEMIC stroke continues to be a major health problem, for which a promising treatment has been the use of thrombolytic agents to reopen cerebral arteries, re-establishing blood flow to ischemic parts of the brain. In particular, recombinant tissue plasminogen activator has been demonstrated to be effective in improving clinical outcome but only if treatment is begun within the first 3 hours of the onset of ischemia.25 The lack of a more robust effect and the short time window of efficacy are thought to be at least in part a result of reperfusion injury, a series of damaging biochemical processes that may paradoxically counteract the beneficial effects of reperfusion. Because ROS may play a role in this process, scavenging these oxygen-derived radicals may be of therapeutic value in decreasing reperfusion injury and increasing the effectiveness of current thrombolytic treatments.

Although it has long been suggested that ROS play a role in cerebral ischemia,2,10,11,19,20,21 the pathophysiological effect of these free radicals has not been completely elucidated in vivo. It has been shown experimentally that ROS are produced after transient focal ischemia4,15 (Pluta, et al., unpublished data) and that the administration of free radical scavengers15,23 may have protective effects in rat models of cerebral vessel occlusion. However, administration of the body’s natural ROS scavenging enzymes, such as superoxide dismutase and catalase, has been ineffective; therefore their role as endogenous protectants after ischemia could not be confirmed.22 Intravenous administration of these agents does not ensure adequate delivery to the tissue, in part because of the BBB.4

Nitroxides are a unique group of compounds that mimic...
Tempol and focal ischemia

...the enzyme SOD. They have been used for a variety of applications, including as biophysical probes for monitoring cellular pH and membrane stability, and also contrast agents in nuclear magnetic resonance imaging. These molecules are low-molecular-weight stable free radicals that are highly cell permeable and may easily cross the BBB, properties that should permit the molecule to enter the cell and scavenge both intra- and extracellular deleterious ROS radicals.

Two steps characterize the main reaction of nitroxides (Fig. 1). The stable nitroxide is reduced by the superoxide radical, forming a hydroxylamine intermediate. This intermediate is then oxidized to reform as the initial nitroxide. Thus the reaction is catalytic, allowing the molecule to act as a self-replenishing antioxidant. Not only do these molecules catalytically quench extra- and intracellular ROS radicals, they have also been hypothesized to oxidize transition metal ions, reducing production of hydroxyl radicals17(Fig. 1). Nitroxides have also been shown to prevent cytotoxicity caused by H2O2 in vitro14 and to be potent antioxidants in such in vivo models as cardiac reperfusion injury1 and closed head injury.

In this study we focus on the nitroxide, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), which is a nonelectrolyte, and can freely cross the BBB. We investigated the ability of Tempol to reduce brain damage after ischemia and reperfusion in rats.

Materials and Methods

Study Protocol

All animal procedures were approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee and met National Institutes of Health guidelines for animal care. Male Sprague–Dawley rats, each weighing between 280 g and 350 g, were used. The animals were housed under light conditions and provided with unlimited access to food and water.

The effects of Tempol during brain reperfusion after ischemia were investigated in two separate studies. The first was designed to determine the effect of Tempol on infarct size after focal ischemia and reperfusion in rats. A dose of 10 mg/kg was selected because, in unpublished pilot studies, it has been shown to be the highest dose tolerated without causing hypotension. In Study I, animals were randomized to either Tempol (10 mg/kg; eight rats) or vehicle groups (0.9% saline; eight rats). Study II was conducted to determine a dose–response relationship between Tempol and infarct size. Animals were randomized to one of three groups: Tempol (20 mg/kg; eight rats), low-dose Tempol (5 mg/kg; eight rats), or vehicle (eight rats).

General Preparation

A state of general anesthesia was induced in the rats by using 5% isoflurane in a gas chamber. After tracheal intubation, ventilation was provided by a servomotor at a rate of 25 breaths/minute, and anesthesia was maintained with 2% isoflurane. The rats’ rectal temperature was monitored and normothermia (36.5˚C and 37.5˚C) was maintained with a thermostatically regulated heating pad. A PE-50 catheter was introduced into the caudal artery of the animal’s tail for continuous monitoring of blood pressure. Physiological variables were recorded at 10-minute intervals and tabulated at seven representative time points throughout the experiment. For drug infusion, the right femoral vein was catheterized with a PE-10 catheter connected to an infusion pump.

The calvaria was exposed, and a laser Doppler perfusion probe was placed onto the cortex in the left MCA distribution via a small twist drill hole and affixed rigidly to the skull. On the contralateral side, a thread-sized thermistor probe was passed under the calvaria for measurement of brain temperature.

Occlusion of the MCA

Transient MCA occlusion was performed in all groups by using an intraluminal thread technique. The neck area was exposed with a ventral midline skin incision. With the aid of an operating microscope, the right CCA, ECA, and ICA and their ramifications were exposed. The pterygopalatine artery was ligated at the ICA bifurcation, and the ECA was divided distally and used as a conduit to access the ICA. After placing a microvascular clip across the right CCA, a 25-mm length of No. 3-0 monofilament, silicon-coated nylon suture was introduced into the ECA lumen. The suture was then advanced slowly and gently until the rCBF dropped to at least 25% of the baseline value, as measured using a laser Doppler perfusion monitor. After a 60-minute occlusion, the thread was withdrawn, and reperfusion was confirmed with the laser Doppler perfusion monitor. Failure to achieve an appropriate drop in CBF and/or failure to achieve reperfusion were exclusion criteria.

Drug Administration

In all groups, the investigators were blinded to whether drug or vehicle had been administered. In Study I, animals received 10 mg/kg Tempol or vehicle (volume calculated based on a Tempol dose of 10 mg/kg). In Study II, animals received 5 mg/kg Tempol, 20 mg/kg Tempol, or vehicle. Solutions administered in all groups were infused over 20 minutes at a rate of 50 μl/minute.

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pressed as the mean control rats. * p < 0.03 by unpaired two-tailed t-test. Values are expressed as the mean ± SD.

**Determination of Infarct Size**

After 4 hours of reperfusion, anesthesia was again induced with a lethal dose of pentobarbital, and the rats were decapitated. Their brains were removed and placed in a brain matrix for tissue slicing. Two-millimeter coronal sections were cut and stained in 2% 2,3,5-triphenyltetrazolium chloride for 20 minutes at 37°C, and the slices were then fixed and stored in 10% formalin. The slices were photographed using a digital camera, and images were analyzed using commercially available image analysis software. The infarct volume from each coronal section was mathematically reconstructed to give the total infarct volume in cubic millimeters.

**Statistical Analysis**

Infarct volumes and physiological variables were compared among groups in the experiments by using unpaired, two-tailed t-tests. A probability value of less than 0.05 was considered significant.

**Sources of Supplies and Equipment**

The rats were obtained from MCI-DCT, Frederick, MD. The servoventilator was acquired from Harvard Apparatus, Millis, MA. The rectal thermometers were purchased from Yellow Springs Instrument Co., Inc., Yellow Springs, OH, and the heating pads were provided by American Hamilton Co., Cincinnati, OH. The laser Doppler perfusion and thermistor probes and the laser Doppler perfusion monitor were obtained from Perimed, Inc., Smithtown, NY.

The 2,3,5-triphenyltetrazolium chloride was purchased from Sigma Chemical Co., St. Louis, MO. The digital camera was acquired from DAGE-MTI, Inc., Michigan City, IN, and the image analysis software (Adobe Photoshop) was purchased from Adobe Systems, Inc., Seattle, WA. The statistical software (Statview) was obtained from SAS Institute, Inc., Cary, NC.

**Results**

**Study I**

Infarct size was significantly smaller in rats receiving Tempol than in untreated animals. Infarct size, expressed as mean infarct volume ± SD, was 101.2 ± 65.5 mm$^3$ in Tempol-treated rats compared with 194.3 ± 78.3 mm$^3$ in controls (47% reduction, p = 0.02; Fig 2). Comparison of physiological variables (MABP, rectal and cerebral temperature, weight) between the two groups at preocclusion, occlusion, and reperfusion timepoints showed no significant differences (p > 0.05; Table 1). There was no significant difference in rCBF between the two groups during.

**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Preocclusion</th>
<th>Occlusion</th>
<th>Occlusion 2</th>
<th>Reperfusion</th>
<th>Infusion</th>
<th>Infusion 2</th>
<th>Postinfusion</th>
</tr>
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<tbody>
<tr>
<td>MABP (mm Hg) vehicle</td>
<td>129.2 ± 5.2</td>
<td>132.5 ± 4.6</td>
<td>131.5 ± 5.8</td>
<td>134.2 ± 10.6</td>
<td>133.8 ± 11.6</td>
<td>131.0 ± 12.5</td>
<td>131.2 ± 6.8</td>
</tr>
<tr>
<td>10 mg/kg Tempol vehicle</td>
<td>131.7 ± 5.3</td>
<td>134.2 ± 7.0</td>
<td>133.0 ± 5.2</td>
<td>130.1 ± 6.4</td>
<td>131.5 ± 4.5</td>
<td>130.8 ± 5.8</td>
<td>131.6 ± 8.9</td>
</tr>
<tr>
<td>rectal temp (°C) vehicle</td>
<td>36.8 ± 0.1</td>
<td>37.0 ± 0.1</td>
<td>37.0 ± 0.2</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.1</td>
<td>37.1 ± 0.2</td>
<td>37.1 ± 0.4</td>
</tr>
<tr>
<td>10 mg/kg Tempol vehicle</td>
<td>37.0 ± 0.2</td>
<td>37.1 ± 0.1</td>
<td>37.0 ± 0.2</td>
<td>37.0 ± 0.2</td>
<td>37.0 ± 0.2</td>
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<td>37.0 ± 0.2</td>
</tr>
<tr>
<td>cranial temp (°C) vehicle</td>
<td>36.9 ± 0.2</td>
<td>37.1 ± 0.1</td>
<td>37.0 ± 0.2</td>
<td>37.3 ± 0.3</td>
<td>37.2 ± 0.2</td>
<td>37.2 ± 0.3</td>
<td>37.1 ± 0.2</td>
</tr>
<tr>
<td>10 mg/kg Tempol vehicle</td>
<td>36.9 ± 0.2</td>
<td>36.9 ± 0.4</td>
<td>36.9 ± 0.3</td>
<td>36.9 ± 0.4</td>
<td>36.9 ± 0.4</td>
<td>36.9 ± 0.4</td>
<td>37.0 ± 0.2</td>
</tr>
<tr>
<td>rCBF (% of baseline) vehicle</td>
<td>100 ± 10.7</td>
<td>11.4 ± 8.6</td>
<td>110.0 ± 12.2</td>
<td>111.0 ± 13.5</td>
<td>108.0 ± 16.2</td>
<td>106.0 ± 12.2</td>
<td>104.0 ± 16.4</td>
</tr>
<tr>
<td>10 mg/kg Tempol vehicle</td>
<td>100 ± 14.0</td>
<td>13.5 ± 10.2</td>
<td>108.0 ± 15.8</td>
<td>105.0 ± 12.8</td>
<td>107.0 ± 13.8</td>
<td>107.0 ± 13.8</td>
<td>109.0 ± 16.4</td>
</tr>
</tbody>
</table>

* The seven time points are defined as follows: preocclusion = baseline reading; occlusion = beginning of occlusion; occlusion 2 = 30 minutes into occlusion; reperfusion = beginning of reperfusion; infusion = 1 minute after beginning of infusion; infusion 2 = 10 minutes after infusion; postinfusion = after infusion.
ischemia and reperfusion, and Tempol did not affect the rCBF during reperfusion.

**Study II**

Infarct size again was significantly smaller in Tempol-treated animals as opposed to controls. At doses of 20 mg/kg and 5 mg/kg the infarct size, expressed as mean infarct volume ± SD, was 91.7 ± 32.8 mm³ and 131.9 ± 70.5 mm³, respectively, compared with 179.8 ± 59.9 mm³ in controls (48% and 23% reduction, respectively; Fig 3). The difference in infarct size between animals receiving the 20-mg/kg dose and controls was statistically significant (p = 0.003), whereas the difference between those receiving the 5-mg/kg dose and controls did not reach statistical significance (p = 0.12). Comparison of physiological variables between Tempol-treated animals and controls again yielded no significant differences, and there were no significant differences in rCBF (p > 0.05; Table 2).

**Dose Response**

When results from Studies I and II were normalized to the percentage reduction of infarct size ± SD, a dose–response relationship compared with the control group could be found for the three dose levels: 5 mg/kg, 10 mg/kg, and 20 mg/kg. The mean percentage reduction compared with the corresponding control ± SD was 23.6 ± 39.1%, 47.5 ± 34.6%, and 49 ± 18.2% for doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively (Fig. 4).

**Discussion**

These data indicate that the nitroxide Tempol has a substantial, reproducible neuroprotective effect when given during reperfusion in a rat model of transient focal ischemia. At doses of 10 mg/kg and 20 mg/kg, the infarct size was significantly decreased (approximately 50% reduction) compared with controls. Also, the administration of Tempol did not affect any of the physiological variables measured. This study is the first study in which the use of nitroxides for treatment of stroke has been investigated.

Nitroxides are cell-permeable molecules that cross the BBB and are able to scavenge both inter- and intracellular ROS. These substances should therefore provide protection against ROS-mediated damage after cerebral ischemia and reperfusion. This assumption is supported by the fact that there have been numerous experimental studies in which a protective effect for antioxidants and spin-trapping agents after ischemia–reperfusion has been demonstrated. The goal of this study was to use this novel, potent antioxidant in vivo in a clinically relevant scenario of postischemic administration after reperfusion, and to time the administration to correlate with treatment timing that could be applied in humans (that is, after thrombolysis).

Our positive results in Study I prompted us to reproduce these data and to investigate a possible dose–response re-

![Bar graph showing dose–response relationship of Tempol on infarct reduction. * p < 0.03, and ** p < 0.005 by unpaired two-tailed t-test. Values are expressed as the percentage reduction in infarct volume compared with control ± SD.](image-url)
relationship. In Study II we found that Tempol provided almost the same protection at doses of 10 mg/kg and 20 mg/kg, but it was ineffective at a dose of 5 mg/kg. Although Tempol provided relatively the same protection at the two higher doses, there was less variability in the infarct size (SD = ± 32.8 mm³ compared with ± 65.5 mm³ at doses of 20 mg/kg and 10 mg/kg, respectively) at the 20-mg/kg dose compared with the 10-mg/kg dose, indicating that the highest dose is most effective. Of interest is the observation that the highest dose tested (20 mg/kg) did not decrease infarct size more than in the 10-mg/kg group, but it was more consistent (that is, there was less variability in lesion size). This indicates that we may have reached the maximum protective effect of the molecule at a dose of 20 mg/kg. There is most likely a limit to the amount of tissue that postischemic therapy can salvage, because there is a core infarct area that cannot be saved. Our results indicate that with the 60-minute occlusion used in this model, the possible infarct reduction is limited to 50%.

Possible mechanisms of action of these molecules include the catalytic removal of inter- and intracellular peroxide ions, the oxidation of transition metals thereby preventing the Fenton reaction, the quenching of damaging semiquinone radicals, reduction of hypervalent metal complexes such as ferryl ions, and termination of radical-chain reactions. Nitroxides have also been found to increase nitric oxide levels in endothelial cells in vitro, but it is still unclear how this affects infarction. Our analysis of the physiological variables measured indicates that, at the concentrations used in these experiments, Tempol does not work by affecting blood pressure, cerebral temperature, or rCBF.

Questions about the role of free radicals following reperfusion remain. Perhaps the most important question is whether free radical scavengers can increase the window of opportunity for administration of thrombolytic agents. If this window could be increased, treatment would be available to many more stroke victims. The limitations of our current model, as it relates to treating stroke in humans, are great. Important questions about the treatment window, patient survival, long-term histological damage, and behavioral protection and recovery have yet to be answered and are the focus of ongoing studies.

With the emergence of thrombolytic agents, interventions to prevent damage caused by reperfusion have become increasingly important. Currently, no therapy is undertaken after thrombolysis in the clinical treatment of stroke. The significant protective effect of Tempol after reperfusion indicates that this drug may be an effective complement after administration of a thrombolytic agent. Certain characteristics of this compound make its clinical use advantageous. Tempol can be administered intravenously, thus entailing little technical difficulty in its administration and allowing its infusion immediately after thrombolysis. The small size of the molecule also allows it to cross the BBB easily and penetrate cells to scavenge inter- and intracellular radicals in the brain.

Nitroxides are currently being used for a wide variety of clinical applications, and they have also been used as antioxidants in a wide variety of experimental models. Nitroxides are also radioprotectors and are currently being assessed for clinical use for radiation-induced alopecia and to provide radioprotection. This compound is presently being assessed for clinical use, which may expedite its approval in the treatment of stroke.

Conclusions

The nitroxide Tempol provides significant neuroprotection after reperfusion in a rat model of transient focal ischemia. These findings offer further evidence of the deleterious role of ROS in ischemia and reperfusion and indicate that Tempol may have clinical application as a brain-protecting agent after thrombolyis.

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References


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