Effects of a Synthetic Allosteric Modifier of Hemoglobin Oxygen Affinity on Outcome From Global Cerebral Ischemia in the Rat

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Background and Purpose—Neuronal injury results from an insufficient supply of oxygen to the brain. This experiment examined whether a pharmacologically induced rightward shift of the partial pressure of oxygen at which 50% of hemoglobin is saturated (P50) would improve outcome from either incomplete and/or near-complete forebrain ischemia–induced hypoxia in the rat.

Methods—For incomplete ischemia (attenuated electroencephalogram), fasted rats (n=17 to 19 per group) were given a synthetic allosteric modifier of hemoglobin affinity for oxygen (RSR13; 150 mg/kg IV) before or immediately after 20 minutes of bilateral carotid occlusion combined with a decrease in mean arterial pressure to 40 mm Hg. For near-complete ischemia (isoelectric electroencephalogram), rats (n=15 per group) were given RSR13 (150 mg/kg) at onset of reperfusion after 10 minutes of bilateral carotid occlusion combined with a decrease in mean arterial pressure to 30 mm Hg. In both experiments, control rats were given vehicle (0.9% NaCl IV) only. Outcome (defined as percent dead hippocampal CA1 neurons) was determined at 5 days after ischemia.

Results—RSR13 (150 mg/kg) produced a 68% rightward shift of P50 (34±6 to 57±8 mm Hg). RSR13 reduced CA1 damage resulting from incomplete ischemia by 28% (P=0.02), but only when administered at the onset of reperfusion. RSR13 had no effect on outcome from near-complete ischemia.

Conclusions—A postischemic pharmacologically induced increase in P50 may improve outcome from incomplete global cerebral ischemia. More severe (near-complete) ischemia negates this benefit. (Stroke. 1998;29:1650-1655.)

Key Words: cerebral ischemia ■ hemoglobin, allosteric modification ■ rats

Central nervous system ischemia constitutes a reduction of oxygen delivery. If sustained, tissue viability may be lost. A variety of approaches to augment oxygen delivery have been investigated in laboratory models of ischemia, including manipulations of perfusion pressure,1 blood rheology,2 and blood oxygen-carrying capacity.3–5 An alternative approach is to increase oxygen release from hemoglobin.

The synthetic allosteric modifier of hemoglobin, RSR13 (2-[4-[[3,5-dimethylanilino]carbonyl[methyl]phenoxy]-2-methylpropionic acid), causes decreased hemoglobin-oxygen affinity.6 As a result, the partial pressure of oxygen at which 50% of hemoglobin is saturated (P50) is shifted rightward. This could potentially increase tissue oxygen tension in marginally perfused tissue if arterial hemoglobin oxygen saturation is maintained normal with an adequate FIO2.7,8

Recent work performed in a feline model of focal cerebral ischemia examined the efficacy of RSR13 in reducing tissue injury.9 Mean cerebral infarct size, measured immediately after 5 hours of permanent middle cerebral artery occlusion, was reduced by 36% in cats administered RSR13 before and during ischemia. Tissue Po2 in the ischemic penumbra was increased by 22%.

Global forebrain ischemia may occur with different grades of severity. Work in our laboratory has defined a state of cerebral hypoperfusion (combined bilateral carotid occlusion and systemic hypotension in the rat) that allows attenuated but persistent EEG activity.10 If persistent for 20 minutes, this insult causes delayed neuronal necrosis in the CA1 sector of the hippocampus. Under such conditions, where marginal flow exists, it can be postulated that augmentation of oxygen delivery would be beneficial. We hypothesized that a rightward P50 shift would alter histological outcome from an incomplete global cerebral ischemic insult. The benefit observed with RSR13 treatment in the incomplete global ischemia model suggested that RSR13 be further examined in a more severe model of global ischemia.
Materials and Methods

This study was approved by the Duke University Animal Care and Use Committee.

Dose Determination Study

Male Wistar rats (age, 8 to 10 weeks; weight, 300 to 350 g; Harlan, Indianapolis, Ind) were studied to determine the effects of RSR13 on SaO2 and the P50 of the oxygen-hemoglobin dissociation curve. Rats were fasted from food but allowed free access to water for 12 to 16 hours before the experiment. Rats were then anesthetized with 3% halothane. The trachea was intubated, and the lungs were mechanically ventilated with 50% oxygen/balance nitrogen and halothane. Inspired O2 concentration was continuously measured with a polarographic O2 monitor (model 210; Ohio Medical Products). Ventilation was adjusted to maintain a PaCO2 of 37 to 42 mm Hg. Anesthesia was maintained with 0.5% to 2.0% halothane (inspired). By surgical incision, catheters were inserted into the right jugular vein and tail artery. The arterial catheter was used for obtaining samples for blood gas analysis, co-oximetry, and P50 measurement. The venous catheter was used for drug administration. Heparin (50 IU) was given intravenously. After a 30-minute stabilization period, rats (n = 1 to 2 per group) were allocated to receive one of five RSR13 doses (75, 100, 125, 150, or 200 mg/kg IV). RSR13 was dissolved in 0.45% NaCl (40 mg/mL). After withdrawal of a 2-mL baseline blood arterial sample, RSR13 was infused over 15 minutes followed by the immediate withdrawal of a second 2-mL sample of arterial blood. A third sample was withdrawn 90 minutes later. Blood was collected in heparinized tubes and placed on ice. Samples were analyzed immediately for SaO2 as determined by co-oximetry (Osm 3 Hemoximeter, Radiometer). Samples collected in parallel were analyzed within 18 hours for P50 by multipoint tonometry.11 Rats were then administered a lethal dose of halothane.

To confirm that the measured effects of RSR13 are not specific to the Wistar rat, six Sprague-Dawley rats underwent the same protocol at a single dose of RSR13 (150 mg/kg IV) for determination of effects on P50 and SaO2.

Experiment 1 (Incomplete Ischemia)

Male Sprague-Dawley rats (age, 8 to 10 weeks; Harlan, Indianapolis, Ind) were fasted from food but allowed free access to water for 12 to 16 hours before experimentation. Rats were then anesthetized with 3% halothane. The trachea was intubated, and the lungs were mechanically ventilated with 50% oxygen/balance nitrogen and halothane. Ventilation was adjusted to maintain a PaCO2 of 37 to 42 mm Hg. Anesthesia was maintained with 0.5% to 2.0% halothane (inspired). Pericranial temperature was monitored and servo-regulated to 38.0±0.1°C (reported normal temperature for rat brain)2 with a heat lamp and cooling fan controlled by a temperature regulation system (YSI model 524 22-gauge needle thermistor and model 73ATA indicating controller). The tail artery was cannulated for blood pressure monitoring and arterial blood gas analysis. The right jugular vein was cannulated for drug administration as well as induction of hypotension by exsanguination. Through a neck incision, both carotid arteries were encircled with suture. The rat received 50 IU heparin intravenously. EEG activity was monitored with active needles inserted pericranially below the temporalis muscle bilaterally, with a ground lead in the tail. Blood pressure, EEG, and pericranial temperature were continuously recorded during the experiment on a Macintosh 7100 PowerPC (Apple Computer Co) with a MacLab 4E analog-to-digital converter (AD Instrument Pty Ltd).

After surgical preparation (~25 minutes), local anesthetic (1% lidocaine) was instilled in the wounds, and the inspired halothane concentration was reduced to 0.5%. A stabilization interval of 20 minutes was allowed. Before the onset of ischemia, 1 mg succinylcholine was given intravenously. Rats were randomly allocated to one of three groups (n = 17 to 19 per group): (1) Vehicle: 3.75 mL/kg IV 0.9% saline over 10 minutes immediately before ischemia and again during the first 10 minutes after reperfusion; (2) RSR13 Pretreatment: 150 mg/kg IV RSR13 over 10 minutes immediately before ischemia and 3.75 mL/kg IV 0.9% saline during the first 10 minutes after reperfusion; and (3) RSR13 Postischemia: 3.75 mL/kg IV 0.9% saline over 10 minutes immediately before ischemia and 150 mg/kg IV RSR13 during the first 10 minutes after reperfusion.

Saline (0.9%) was chosen as the vehicle comparator to allow osmolarity to be similar to that present in the RSR13 solution. Investigators were blinded to group assignment until all neurological and histological analyses were completed.

Incomplete forebrain ischemia was induced by blood withdrawal from the venous catheter until the MAP was 40 mm Hg. Both common carotid arteries were temporarily occluded with cerebral aneurysm clips. Continued withdrawal of blood was performed as required to maintain MAP at 40 mm Hg. After 20 minutes, the carotid arteries were deoccluded, blood removed through the venous catheter was reinfused, and 0.4 mEq sodium bicarbonate was infused intravenously. Pilot studies were performed to ensure that this severity and duration of ischemia would allow a persistent but attenuated EEG resulting in histological damage in the hippocampal CA1 sector.

Rats were maintained anesthetized with 0.5% halothane for 120 minutes after ischemia with continued pericranial temperature servo-regulation at 38.0±0.1°C. Anesthesia was then discontinued, and the trachea was extubated. Animals were allowed to recover in an oxygen-enriched environment (FIO2=0.5) for 3 hours before being returned to cages with free access to water and food for the next 5 days.

On the fifth postoperative day, motor function tests were performed according to an established protocol including assays of prehensile traction and balance beam performance.12,13 The motor score was graded on a scale of 0 to 9 (best score=9). Rats were then anesthetized with halothane and underwent in situ brain fixation by intracardiac injection of buffered 4% formalin. After overnight stabilization, the brains were removed and stored in 4% formalin. Paraffin-embedded brain sections were cut in 6-µm sections frozen in 10 minutes, and stained with acid fuchsin/celestine blue. Injury to the CA1 sector of the hippocampus was evaluated by light microscopy. Viable and nonviable neurons were manually counted, and the percentage of nonviable neurons was calculated (percent CA1 dead). By convention, values from the hemisphere with the worst damage were used for the final analysis.

The Kruskal-Wallis H statistic was used to compare percent CA1 dead neurons among groups. Pairwise intergroup comparisons were performed with the Mann-Whitney U test when the Kruskal-Wallis test was significant. A value of P<0.05 was considered significant.

Experiment 2 (Near-Complete Ischemia)

The following experiment was performed after analysis of the data from experiment 1. Male Sprague-Dawley rats (age, 8 to 10 weeks; Harlan, Indianapolis, Ind) were anesthetized with halothane and surgically prepared for ischemia as described above. Near-complete ischemia was produced by exsanguination to MAP of 30 mm Hg combined with bilateral common carotid artery occlusion so as to produce EEG isoelectricity. Ischemia was allowed to persist for 10 minutes.

The rats were randomly allocated to one of two groups (n = 15 per group): (1) Vehicle: 3.75 mL/kg IV 0.9% saline given during the first 10 minutes of reperfusion and (2) RSR13 Postischemia: 150 mg/kg IV 0.9% saline given during the first 10 minutes of reperfusion.
TABLE 1. Effects of RSR13 on P50

<table>
<thead>
<tr>
<th>RSR13 Dose, mg/kg</th>
<th>n</th>
<th>Baseline</th>
<th>0 Min After Infusion</th>
<th>90 Min After Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>2</td>
<td>41 ± 1</td>
<td>48 ± 6 (17)</td>
<td>49 ± 1 (20)</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>42</td>
<td>52 (24)</td>
<td>47 (12)</td>
</tr>
<tr>
<td>125</td>
<td>2</td>
<td>44 ± 2</td>
<td>57 ± 7 (30)</td>
<td>59 (34)</td>
</tr>
<tr>
<td>150</td>
<td>2</td>
<td>43 ± 2</td>
<td>75 ± 7 (74)</td>
<td>60 ± 12 (60)</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>43 ± 1</td>
<td>90 ± 3 (109)</td>
<td>76 ± 3 (77)</td>
</tr>
</tbody>
</table>

Values are mean ± SD. P50 values are expressed in millimeters of mercury. Values in parentheses indicate percent rightward shift in P50 relative to preinfusion baseline. Respective doses of RSR13 were infused intravenously over a 10-minute interval.

IV RSR13 dissolved in 0.45% saline during the first 10 minutes of reperfusion.

Investigators were again blinded to group assignment until after the neurological and histological analyses were completed. The neurological examination, in situ formalin fixation, histological preparation, and analyses were identical to the incomplete ischemia study protocol.

The Mann-Whitney U statistic was used to test for between-group differences in percent CA1 dead neurons. A value of $P \leq 0.05$ was considered significant.

### Results

#### Dose Determination

Time-dependent effects of RSR13 on P50 are presented in Table 1. RSR13 caused a dose-dependent rightward shift of P50. Figure 1 depicts the relationship between P50 and SaO2 as a function of RSR13 dose. Doses of RSR13 ≤150 mg/kg allowed preservation of SaO2 at values >90%. On the basis of these observations, an RSR13 dose of 150 mg/kg was chosen for further study. Similar observations were made in the Sprague-Dawley rat. RSR13 (150 mg/kg IV) shifted the baseline P50 value of 34 ± 3 mm Hg to the right (57 ± 8 mm Hg) by 68% while retaining SaO2 at 90 ± 2%.

#### Experiment 1 (Incomplete Ischemia)

Physiological values are reported in Table 2. No important differences were present among groups. Motor function scores (mean ± SD) were not different among groups (Vehicle = 8 ± 2; RSR13 Preischemia = 6 ± 2; RSR13 Postischemia = 7 ± 2; $P = 0.17$). CA1 damage (percent dead neurons) is presented in Figure 2. RSR13 when administered at the onset of reperfusion (but not when administered before ischemia) resulted in a 28% reduction in percent CA1 dead neurons (Vehicle = 81 ± 20; RSR13 Preischemia = 78 ± 24; RSR13 Postischemia = 58 ± 32; $P = 0.02$).

#### Experiment 2 (Near-Complete Ischemia)

Physiological values are reported in Table 3. No important differences were present between groups. Neither motor function (Vehicle = 8 ± 1; RSR13 Postischemia = 8 ± 1; $P = 0.31$) nor percent dead neurons in hippocampal CA1

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**Figure 1.** The relationship between SaO2 and P50 is depicted as a function of RSR13 dose. Rectangles represent mean values of SaO2 for 1 to 2 rats exposed to an FiO2 of 0.5. The line graph (open squares) represents corresponding SaO2 values. The dose of 150 mg/kg was chosen for further study on the basis of the observation that this was the maximal dose that allowed SaO2 to be preserved at >90% in the presence of a rightward P50 shift.

**Figure 2.** Open circles depict percent dead hippocampal CA1 neurons in individual rats subjected to 20 minutes of incomplete forebrain ischemia and a 5-day recovery interval. RSR13 (150 mg/kg) was administered either 10 minutes before ischemia or immediately after reperfusion. Rectangles denote median values for each group are shown. A significant difference was observed between the vehicle and postischemia treatment groups ($P = 0.02$).
A hallmark of cerebral ischemia is diminished delivery of oxygen to neural tissue. Considerable effort has been directed toward improving oxygen delivery by therapies including manipulations of perfusion pressure, improvement of blood rheology, and enhancement of blood oxygen-carrying capacity. These therapies have been effective in reducing ischemic injury in laboratory animals. For example, Aronowski et al. observed a 53% reduction in cerebral infarct volume in rats subjected to hypervolemic hemodilution with DCLHb. Presumably, delivery of oxygen by residual collateral flow was sufficient to diminish the severity of the ischemic insult and improve outcome.

Oxygen availability can also be augmented by enhancing the release of oxygen from hemoglobin at the tissue level. 2,3-DPG is an endogenous allosteric modifier of hemoglobin-oxygen saturation. This organic phosphate binds to the reduced hemoglobin tetramer. Chemical treatment can be used to increase red blood cell 2,3-DPG content. Transfusion of such cells causes up to a 50% rightward shift in P50, which is sufficient to allow preservation of brain high-energy phosphate concentrations under conditions of sustained hypoperfusion. RSR13 similarly decreases the affinity of hemoglobin for oxygen by binding to deoxyhemoglobin in red blood cells sampled from multiple species at a site distinct from that of 2,3-DPG. Advantages of RSR13 over 2,3-DPG principally relate to pharmacological stability and ability to readily cross the erythrocyte membrane and dose-dependently cause a rapid increase in P50.

Watson et al. have examined the protective effects of RSR13 in a feline model of focal cerebral ischemia. A 36% reduction in infarct size (compared with vehicle-treated controls) was demonstrated in animals administered RSR13 before and during permanent middle cerebral artery occlusion. In addition, tissue PO2 was measured in the ischemic penumbra. A trend toward higher brain oxygen partial pressure was observed in the RSR13 group. This is consistent with observations made by others that RSR13 reduces the cerebral vasodilatory response to arterial hypoxemia, presumably by increasing oxygen unloading from hemoglobin at the tissue level.8

Because maintenance of some residual blood flow would be necessary to obtain benefit from enhanced release of oxygen from hemoglobin, we chose to initially study RSR13 during incomplete global ischemia. Pilot studies allowed us to define an MAP and duration of carotid occlusion sufficient to produce neurological injury in the presence of a persistent but attenuated EEG. This end point is consistent with work done by Gionet et al., who found graded cerebral blood flow, EEG, and histological injury responses to variations in intraschismic MAP administered in combination with bilateral carotid artery occlusion in the rat. We anticipated that an improvement in outcome would be seen in rats that were administered RSR13 immediately before ischemia by enhancing oxygen delivery during ischemia. Instead, RSR13 provided benefit only when given after ischemia.

There are plausible explanations for a lack of effect from RSR13 when administered before ischemia. Tissue acidosis rapidly develops during ischemia. Such an acidosis would also be expected to cause a rightward P50 shift, perhaps reducing benefit from RSR13. However, unpublished data (1996) indicate that the effects of pH and RSR13 on P50 are additive. Alternatively, because RSR13 causes a reduction in hemoglobin-oxygen affinity, any beneficial effect of RSR13
on off-loading of oxygen at the tissue level may have been counterbalanced by a reduction in arterial blood oxygen content due to reduced oxygen loading in the lungs. In our pilot dose determination study, $\text{SaO}_2$ was approximately 90% in rats administered RSR13 150 mg/kg at an $\text{FiO}_2$ of 0.5. Increasing $\text{FiO}_2$ to 1.0 would be expected to increase $\text{SaO}_2$ and potentially increase oxygen content and delivery during the ischemic insult. Further investigation of global ischemic outcome in rats administered RSR13 at an $\text{FiO}_2$ of 1.0 may allow examination of maximal potential benefit from decreasing the affinity of hemoglobin for oxygen.

Another potential factor counterbalancing any benefit from RSR13 is increased production of reactive oxygen species during reperfusion. Augmentation of oxygen delivery has been postulated to increase substrate for free radical production. Arguments can be made against this hypothesis on the basis of existing data. First, hydroxyl radical production has been shown to be sustained for at least several hours after reperfusion from an ischemic insult. Because this is the interval when administration of RSR13 was found to be beneficial, it seems unlikely that enhanced production of oxygen radicals is caused by RSR13. Second, direct examination of the effect of different fractions of inspired oxygen administered during reperfusion from global ischemia in the rat has failed to demonstrate an effect on either hydrogen peroxide production or histological outcome. Although direct examination of the effects of RSR13 on reperfusion-mediated production of reactive oxygen species would provide the strongest evidence, available data suggest that this mechanism is unlikely to be important.

We speculated that postischemic administration of RSR13 improved outcome by improving oxygen delivery during delayed postischemic hypoperfusion. Delayed hypoperfusion can result in a reduction in cerebral blood flow for several hours after reperfusion. It is during this interval that RSR13 may have limited secondary injury by an enhanced ability of hemoglobin to release oxygen at the tissue level.

To test this hypothesis and better define the therapeutic limits of postischemic RSR13 administration, rats were given a more profound global ischemic insult (near-complete ischemia), which potentially worsened the delayed hypoperfusion. If RSR13 improved outcome from incomplete ischemia by enhancing oxygen delivery during postischemic hypoperfusion, it could also be predicted that RSR13 would reduce injury when administered after this more severe global ischemic insult. However, we were unable to demonstrate any benefit from postischemic RSR13 administration in the near-complete model. Near-complete ischemia is a more severe insult to brain. It is possible that the injury was too severe to be attenuated by augmenting oxygen delivery during reperfusion with neurons already irreparably damaged from the ischemic insult.

In summary, we found a neuroprotective effect of 150 mg/kg RSR13 when administered during reperfusion after incomplete forebrain ischemia in the rat. Further investigation of postischemic administration of RSR13 in a more severe model of global ischemia did not show a postischemic benefit. These data, combined with preliminary reports of reduced focal ischemic brain damage in cats administered RSR13, suggest that allosteric modification of the affinity of hemoglobin for oxygen may be a valuable mechanism to exploit for acute treatment of some forms of ischemic brain injury.

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References


**Editorial Comment**

A number of possible therapeutic approaches have been or are now being investigated for the treatment of acute ischemia by increasing oxygen delivery/availability to ischemic tissue. One of the novel approaches is the use of synthetic allosteric modifiers of hemoglobin that function as catalysts in unloading oxygen from hemoglobin in low PO₂ environments. Among this group of compounds, RSR13 appears to be most promising. Its efficacy has already been tested under a variety of pathophysiological conditions. In this article, Grocott and coworkers examined the effect of RSR13 in a rat model of cerebral ischemia. Although the title of this article refers to global cerebral ischemia, this model actually represents incomplete cerebral ischemia.

Grocott and coworkers found that RSR13 had no effect in animals subjected to near-complete forebrain ischemia. However, their findings on less severe ischemia models (incomplete forebrain ischemia induced by bilateral carotid occlusion plus hypotension) were unexpected yet interesting. Given the fact that there was still some brain blood flow during occlusion in these animals, one would expect ischemic neural tissue that was pretreated with RSR13 should reap the most benefit from the enhanced oxygen delivery. As it turned out, the neuroprotective effect of RSR13 prevailed only in animals if RSR13 were administered during the reperfusion period. While the mechanisms for this negative result with RSR13 pretreatment remain speculative, the positive result with posttreatment of the same compound opens up a number of possibilities. Unlike pretreatment, the benefit of posttreatment RSR13 from this study suggests a potentially workable therapeutic application in alleviating some forms of ischemic or hypoxic injuries in clinical settings. In addition, future experiments should examine (1) whether repeated posttreatment of RSR13 would have an additive or prolonged neuroprotective effect and (2) whether RSR13 at 150 mg/kg was an ideal dose. Perhaps a lower concentration could work just as effectively for posttreatment.

In view of the fact that RSR13 shifts the P₅₀ of the oxygen dissociation curve to the right, oxygen will not be properly loaded at pulmonary level by inhalation of room air. In the present study, animals were ventilated with 50% oxygen, even hours after treatment, to keep arterial oxygen saturation at values more than 90%. This may translate into an inconvenience to patients undergoing such treatment.

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