Transient Middle Cerebral Artery Occlusion Influence on Systemic Oxygen Homeostasis and Erythropoiesis in Wistar Rats

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Background and Purpose—Systemic hypoxia is a common complication in stroke patients and may exacerbate ischemic brain damage. Expression of the hypoxia-inducible cytokine erythropoietin (Epo) is upregulated in the brain in both stroke patients and in animal stroke models and exerts local neuroprotective effects in the ischemic brain. Epo is also well known to stimulate red blood cell (RBC) production. The purpose of the present study was to evaluate whether poststroke systemic hypoxia is present in the rat model and whether it is associated with increased peripheral Epo and RBC production.

Methods—Wistar rats underwent 1-hour transient middle cerebral artery occlusion (MCAO) under mechanical ventilation, followed by reperfusion without further ventilation. Groups of MCAO and sham-operated animals were evaluated at extended times after reperfusion for assessment of arterial blood gases, plasma Epo, and complete blood count.

Results—Arterial oxygen saturation was significantly lower in the infarct group between 6 and 24 hours after reperfusion (P=0.0005), and plasma Epo levels were increased 6 hours after reperfusion (P<0.05). RBC counts and hematocrit were transiently increased 2 to 7 days after reperfusion in animals with MCAO compared with sham. Maximal increases were seen at day 7 (22% and 16% increases of RBC count and hematocrit, respectively; P<0.001). In contrast, the white blood cell counts in animals with MCAO decreased by >30% in the same time period.

Conclusions—Plasma Epo levels, RBC counts, and hematocrit are all increased in response to systemic hypoxia aftercerebral ischemia in rats. (Stroke. 2004;35:000-000.)

Key Words: hypoxia ■ hematocrit ■ stroke

Clinical observations indicate that hypoxia is a common complication of stroke in the acute phase and for hours to days after stroke onset.1,2 Even in stroke patients who appear normoxic during the day, 25% develop hypoxia at night.3 Studies in rats using intraluminal filament transient middle cerebral artery occlusion (MCAO) and graded levels of hypoxia have shown that hypoxia exacerbates ischemic brain damage.4 It is not known whether animals with MCAO spontaneously develop systemic hypoxia after a stroke, but this might be expected.

Erythropoietin (Epo) is a hypoxia-inducible cytokine, originally identified as a kidney-derived stimulator of erythroid progenitor cell proliferation and differentiation.5,6 Increased synthesis of Epo is known to stimulate the production of red blood cells (RBCs), thereby increasing oxygen delivery to tissues as a mechanism of physiological adaptation to hypoxia.7,8 Recent studies have shown that Epo and its receptor (EpoR) are expressed in rodent and human brain tissues.9,10 Epo system expression is upregulated in the ischemic brain after MCAO in rodents11,12 and in human autopsy brains with ischemic infarcts or general hypoxic damage.13 Neutralization of endogenous brain Epo by administration of soluble EpoR potentiates ischemic brain injury in a rodent MCAO stroke model,14,15 suggesting a role for the brain Epo system in neuroprotection against ischemic damage. Little is known about potential upregulation of the levels of peripheral Epo and its erythropoietic activity in response to systemic hypoxia aftercerebral ischemia. In MCAO mice, a transient increase of blood hematocrit was observed at the end of occlusion, with normalization 1 hour after reperfusion.16 A similar study showed increases in hematocrit 24 hours after reperfusion.17 Potential changes in blood hematocrit at later reperfusion time points and their relationship with changes in blood gases and plasma Epo are not known. The purpose of the present

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study was to evaluate the changes in plasma Epo, RBC numbers, and hematocrit from days 0 to 28 after transient MCAO in Wistar rats and to examine whether these changes are associated with systemic hypoxia.

Materials and Methods

Animals
All procedures were performed in accordance with current guidelines of the Canadian Council on Animal Care. Adult male Wistar rats (Charles River Breeding Center, St Constant, Quebec, Canada) were housed in groups of 3 to 5 and maintained under a natural 14/10-hour light/dark cycle, with food and water available ad libitum. Before surgery, animals were fasted for 2 hours and then weighed once before surgery and again at the moment of euthanization. Animal weights were uniform at the beginning of the experiments and ranged between 350 and 400 g.

Transient MCAO
The transient MCAO technique was modified from Zhu and Auer, as described previously. Anesthesia was induced with 5% isoflurane inhalation and maintained with 1% to 2% isoflurane in nitrous oxide/oxygen (70%/30%). Animals were intubated and ventilated (model 683 rodent ventilator; Harvard). Rectal temperature was maintained throughout surgery at 37°C with a heating blanket (homeothermic blanket control unit; Harvard). The tail artery was cannulated for blood sample collection and blood pressure monitoring (Gould 13-6615-50 preamplifier and 6600 amplifier, Mark8 recorder; Graphtec). Blood gases and glucose level before and immediately after ischemia were obtained. The total amount of blood removed never exceeded 400 mL and was replaced with an equal volume of saline. Arterial blood gases (PaO$_2$, PaCO$_2$, SaO$_2$, pH) were measured using a blood gas analyzer (ABL520; Radiometer). Animal ventilatory rate was adjusted to maintain normal levels of oxygen and carbon dioxide. MCAO was induced with a 3-0 monofilament coated with poly-L-lysine at a mean arterial blood pressure (MABP) of 60 mm Hg by regulating the percentage of isoflurane for a period of 60 minutes. The filament was then withdrawn to allow reperfusion and discontinuation of the isoflurane restored blood pressure. All wounds were closed, and the animals were allowed to recover in a room maintained at 21.6°C to 21.8°C. Sham-operated animals underwent the same surgical treatment without suture insertion or MABP reduction. In some experiments, a separate sham hypotensive group was also studied. This sham hypotensive group was identical to the normotensive one except for MABP, which was reduced to 60 mm Hg for 60 minutes after isolation of the internal carotid. All other physiological parameters were similar in MCAO, sham normotensive, and sham hypotensive rats, both before and immediately after surgery. Manipulation of the carotid body was identical in all sham and ischemic groups. When follow-up periods exceeded 24 hours, a subcutaneous bolus of 10 mL 0.9% NaCl was given immediately after surgery and daily for 2 days to prevent dehydration. Neurological assessment was performed in all rats before surgery and at the moment of euthanization, using clinical scales described previously. There was a 30% to 40% decrease in sensory and motor activity in rats undergoing MCAO. No neurological abnormalities were observed in sham and sham hypotensive groups.

Experiment 1: Blood Gases, Plasma Epo, and Hematological Changes After Reperfusion
A total of 4 to 5 animals in left sham-operated groups and 5 to 6 animals in left MCAO groups were killed at 8 different time intervals...
(0, 1, 2, 4, 6, 18, and 24 hours) after reperfusion. The animals were anesthetized and blood from the tail artery was used for measurement of blood gases, followed by cardiac puncture for measurement of Epo levels and complete blood count (CBC). Plasma Epo levels were measured by competitive radioimmunoassay using the Epo-Trac 125I RIA kit (DiaSorin). 20 CBC was performed with a counter analyzer (Coulter), and white blood cell (WBC) differential count was measured by light microscopy on peripheral blood smears stained with Wright–Giemsa.

Experiment 2: Hematological Changes 0 to 28 Days After Reperfusion
MCAO was induced in the left or right middle cerebral artery. Changes in hematological parameters were measured at 0, 2, 7, 14, and 28 days after reperfusion. A total of 10 to 15 animals in each of the left or right sham-operated groups and 5 to 9 animals in each of the left or right MCAO groups were killed at each time point. Blood was harvested by intracardiac puncture, and CBC and WBC differential count were performed as described above.

Statistical Analysis
After data validation and normality checks, the effects of the main factors were assessed by means of a mixed ANOVA model with a first-order autoregressive covariance structure. Main treatment effects were calculated and plotted for each of the factors. Comparisons at specific time points were analyzed using simple ANOVA for independent groups, and post hoc analysis was done by the Wilcoxon rank–sum test. Spearman coefficients (r) were used to assess the degree of correlation between the main factors. Statistical significance was reached if P<0.05.

Results
Effect of MCAO on Blood Gases After Reperfusion
There was a precipitous drop in SaO2 immediately after surgery in both MCAO and sham groups because the animals were no longer oxygenated and ventilated (Figure 1). SaO2 stabilized at ~85% in sham animals 6 hours after reperfusion but continued to drop to 75% to 80% in MCAO animals, with a significant difference between these groups at 24 hours (P=0.03). The average SaO2 values for the period 0 to 4 hours after reperfusion were virtually identical in the 2 groups (Figure 2A; 94.3±1.2% versus 93.7±1.8% in sham and MCAO rats, respectively). Between 6 and 24 hours after reperfusion, average SaO2 values were significantly lower in MCAO rats and were in the hypoxic range (86.8±0.8% versus 78.8±1.7%; P=0.0005; Figure 2A). PaO2 changes were similar to those of SaO2, with no difference between MCAO and sham animals 0 to 4 hours after reperfusion and significantly lower PaO2 levels in MCAO rats between 6 and 24 hours after reperfusion (66.7±1.4 mm Hg versus 58.0±2.0 mm Hg; P<0.0013; Figure 2B). There was no significant difference in PaCO2 values between sham and MCAO animals, although there was a trend for PaCO2 from 6 to 24 hours after reperfusion to be higher in the MCAO group (P=0.0551; Figure 2C).

### TABLE 1. Time Course of Hematological Changes During the First 24 Hours After Reperfusion in MCAO and Sham Rats

<table>
<thead>
<tr>
<th>Reperfusion Time (Hours)</th>
<th>Group</th>
<th>n</th>
<th>RBC (10^12/L)</th>
<th>Hematocrit (L/L)</th>
<th>WBC (10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Sham</td>
<td>5</td>
<td>6.41±0.28</td>
<td>0.38±0.02</td>
<td>8.80±1.01</td>
</tr>
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<td></td>
<td>MCAO</td>
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<td>5.94±0.52</td>
<td>0.34±0.03</td>
<td>8.98±2.14</td>
</tr>
<tr>
<td>1</td>
<td>Sham</td>
<td>5</td>
<td>5.53±0.21</td>
<td>0.32±0.01</td>
<td>6.42±0.66</td>
</tr>
<tr>
<td></td>
<td>MCAO</td>
<td>5</td>
<td>5.81±0.19</td>
<td>0.33±0.01</td>
<td>6.32±0.73</td>
</tr>
<tr>
<td>2</td>
<td>Sham</td>
<td>4</td>
<td>6.31±0.13</td>
<td>0.36±0.00</td>
<td>9.50±0.80</td>
</tr>
<tr>
<td></td>
<td>MCAO</td>
<td>5</td>
<td>6.06±0.20</td>
<td>0.35±0.01</td>
<td>7.84±0.83</td>
</tr>
<tr>
<td>4</td>
<td>Sham</td>
<td>5</td>
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<td>0.35±0.01</td>
<td>12.50±2.04</td>
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<tr>
<td></td>
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<td>6.58±0.33</td>
<td>0.37±0.02</td>
<td>9.12±0.84</td>
</tr>
<tr>
<td>6</td>
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<td>4</td>
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<td>0.32±0.03</td>
<td>10.80±1.26</td>
</tr>
<tr>
<td></td>
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<td>6.48±0.46</td>
<td>0.36±0.03</td>
<td>9.72±0.62</td>
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<tr>
<td>18</td>
<td>Sham</td>
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<td>6.76±0.15</td>
<td>0.39±0.01</td>
<td>10.90±1.20</td>
</tr>
<tr>
<td></td>
<td>MCAO</td>
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<td>6.74±0.36</td>
<td>0.39±0.02</td>
<td>9.64±1.15</td>
</tr>
<tr>
<td>24</td>
<td>Sham</td>
<td>5</td>
<td>6.88±0.23</td>
<td>0.39±0.01</td>
<td>9.10±0.89</td>
</tr>
<tr>
<td></td>
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<td>6</td>
<td>7.17±0.21</td>
<td>0.40±0.01</td>
<td>6.74±0.72*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM.

*Different from sham group (P<0.05, ANOVA followed by Wilcoxon rank–sum test).
Effect of MCAO on Peripheral Epo Production and Hematological Parameters After Reperfusion

Epo concentrations were similar in both groups at time 0, and they increased gradually with time in both cases, followed by a decline toward baseline levels. Peak concentration of Epo was 20% higher in MCAO animals (89.2 ± 8.9 mU/mL versus 74.4 ± 3.1 mU/mL; P < 0.05; Figure 3) and occurred 2 hours later (6 versus 4 hours after reperfusion). As well, plasma Epo levels in MCAO rats tended to have a negative correlation with SaO2 values in these animals (r = −0.62; P = 0.056). Changes in hematological parameters during the first 24 hours after reperfusion are summarized in Table 1, with no difference between groups except a 26% decrease in WBC count at 24 hours in MCAO rats compared with sham (P < 0.05).

Effect of MCAO on Hematological Parameters 0 to 28 Days After Reperfusion

CBC was performed on all animals at 0, 2, 7, 14, and 28 days after reperfusion. Data from left- and right-sided MCAO rats were pooled because no difference was observed between the sides of occlusion. RBCs (Figure 4A) and hematocrit (Figure 4B) were unchanged in MCAO versus sham rats at the end of the surgical procedure (time 0). At day 2, the increases were significant in the stroke group: 12% (P < 0.01) and 14% (P < 0.01) for RBCs and hematocrit, respectively. Maximal changes were observed at day 7, with a 22% increase for RBCs (P < 0.001) and a 16% increase for hematocrit (P < 0.001). These parameters normalized 14 and 28 days after reperfusion. Hemoglobin concentration changes in MCAO rats were similar to those of RBC count and hematocrit, with 10% (P < 0.01) and 20% (P < 0.001) increases at days 2 and 7, respectively, and no changes at days 14 and 28, compared with sham. In contrast to the increases induced by MCAO in RBC variables, WBC count was decreased by 33% (P < 0.01), 45% (P < 0.001), and 38% (P < 0.01) at 2, 7, and 14 days, respectively (Figure 4C). These changes in WBC count reflected as much the decrease within the MCAO group (27% decrease at day 7 compared with day 0) as the increase in counts in the sham group (58% increase at day 7 compared with day 0). The WBC counts were similar in both groups by day 28 (Figure 4C). Hematological parameters were assessed at days 0, 2, and 7 after surgery in the sham hypotensive group and compared with the sham normotensive group. MABP values in the hypotensive group were 22% to 25% lower than those in corresponding normotensive animals, and there was no difference in RBC count, hematocrit, and WBC count between these groups (Table 2).

Discussion

This study shows that MCAO rats develop moderate hypoxia (SaO2 ≤ 80%; PaO2 ≤ 60%) from 6 to 24 hours after reperfusion. The SaO2 and PaO2 decreases in ischemic animals are significant not only compared with sham controls but because these values are now at levels that compromise oxygen delivery. The poststroke systemic hypoxia in MCAO rats correlates well with previous clinical observations in stroke patients. Other studies using a transient MCAO model in Wistar rats have found no difference in PaO2 up to 4 hours after reperfusion, but no measurements were made after the 4-hour time frame.

In the ischemic brain, where autoregulation is often impaired, even low or moderate hypoxia can produce detrimental effects. The causes of poststroke hypoxia may be multiple, including alterations in the central regulation of respiration, weakness of the respiratory muscles on the
hemiplegic side, and obstructive sleep apnea. Medical complications arising after stroke such as asphyxia, chest infection, and pulmonary emboli may also impair oxygenation. In our model, hypoxia is not likely caused by aspiration pneumonia or pulmonary embolism because the animals were not infected or immobile. The low PaO2 associated with slightly increased PaCO2 points to a problem of ventilation, such as respiratory muscle weakness, central respiratory dysfunction, or sleep apnea. It is also possible that MCAO animals had a greater respiratory depressant response to anesthesia because of progressive underlying brain damage. The immediate response to hypoxia involves reflex hyperventilation mediated by chemoreceptors in the carotid body. Manipulation of the carotid body during surgery was identical in the sham and MCAO groups, thus eliminating chemoreceptor damage as a possible reason for the difference in PaO2 between the 2 groups.

Hypoxia is the main stimulus of Epo production in both peripheral and brain tissues by activation of the transcription factor hypoxia-inducible factor-1 α. A prolongation of the increase over time of plasma Epo is observed in MCAO rats 6 hours after reperfusion, followed by moderate polycythemia 2 to 7 days after reperfusion. The fact that Epo levels increased only transiently in the presence of persistent hypoxia is consistent with observations in other models, and this may be explained, at least in part, by cellular adaptation to hypoxia. The early time course of RBC increase is also consistent with the known effects of hypoxemia and Epo increase on erythropoiesis in rats. The maximal increases in plasma Epo, RBC count, and hematocrit in MCAO rats are 20% to 30% over sham controls. These responses remain below the magnitude required to increase blood viscosity or contribute to cerebral infarction. As well, the amounts of brain Epo induced by MCAO are also relatively low and likely insufficient to protect against acute cerebral ischemia. However, they may play a neuroprotective role in tolerance to further stroke episodes by mechanisms involving prevention of neuronal cell death through inhibition of apoptosis. By analogy, the moderate polycythemia observed in MCAO rats might represent an attempt to precondition the brain against subsequent ischemia.

Systemic hypotension is a feature of the MCAO model but not present in the sham group. The results of the sham hypotensive group eliminate hypotension as a contributor to the observed hematological changes, and previous studies have demonstrated that controlled mild hypotension does not affect systemic oxygen delivery to tissues. Therefore, hypoxia and the changes in hematological parameters in the stroke group appear independent of hypotension.

It is possible that the increase in hematocrit is simply the result of dehydration and hemoconcentration, but this is unlikely. Hydration was assured by subcutaneous administration of saline, and if hematocrit increased as a result of dehydration, a concomitant increase in WBC count would be seen as well. In this study, the increase in RBCs and hematocrit was accompanied by a concomitant decrease in WBC, which is inconsistent with dehydration. Future studies measuring reticulocyte counts will provide additional proof of true erythropoiesis. Interestingly, a decrease in spleen white cells was observed previously in this same model, indicating that white cells may shift from both blood and the spleen into other lymphoid or nonlymphoid tissues, including the ischemic brain. Alternatively, increased cell death may play a role. To our knowledge, this is the first study relating systemic hypoxia to plasma Epo increase and erythropoiesis after MCAO. This supports the hypothesis that hypoxia occurs after cerebral ischemia and may affect outcome.

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