Reperfusion-Induced Injury to the Blood-Brain Barrier After Middle Cerebral Artery Occlusion in Rats

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Background and Purpose  The integrity of the blood-brain barrier may play an important pathophysiological role during postischemic reperfusion. To determine the factors that lead to exacerbation of brain injury by reperfusion, we investigated changes in cerebral blood flow, blood-brain barrier permeability, edema formation, and infarction in permanent or temporary middle cerebral artery occlusion in rats and studied the relation between focal cerebral blood flow and blood-brain barrier disruption.

Methods  Middle cerebral artery occlusion was performed with the rat suture model, allowing either permanent (6 hours) or temporary occlusion (3 hours of occlusion and 3 hours of reperfusion). We measured brain water, ion contents, and infarct volumes and determined cerebral blood flow using laser Doppler flowmetry and blood-brain barrier permeability with [3H]a-aminoisobutyric acid.

Results  During occlusion, cerebral blood flow was reduced to 7% to 15% (permanent) and 10% to 17% (temporary) of the baseline. During 3 hours of reperfusion, it returned to 47% to 80% (lateral cortex) and 78% to 98% (medial cortex) of the baseline. Compared with the contralateral hemisphere, the water content in the ischemic area increased in both permanent and temporary groups (P<.05, P<.01). Both infarct volume and blood-brain barrier disruption were greater in the reperfusion group compared with the permanent occlusion group (P<.05). Blood-brain barrier disruption correlated with cerebral blood flow during reperfusion (P<.05).

Conclusions  These findings demonstrate that brain infarct and blood-brain barrier disruption are exacerbated after reperfusion in this model of focal ischemia. Blood-brain barrier disruption may relate to the degree of cerebral blood flow recovery. Thus, although early reperfusion in focal ischemia may preserve penumbra tissue, late reperfusion may increase the tissue injury. (Stroke. 1994;25:1658-1665.)

Key Words  • blood-brain barrier • brain edema • cerebral blood flow • reperfusion • rats

The effects of postischemic reperfusion have been widely studied in rats, rabbits, cats, and monkeys. Several investigators have reported that 30 minutes of global ischemia plus 1 to 3 hours of reperfusion results in blood-brain barrier (BBB) disruption and brain edema in a four-vessel occlusion model in rats. The integrity of the BBB could play an important role in the subsequent pathophysiology, since BBB disruption may result in significant brain edema formation and infarction.

BBB permeability after reperfusion has been less well studied in focal ischemia. In focal cerebral ischemia, there is an ischemic penumbra area that is free of the ischemic insult for a considerable period, and reperfusion after temporary middle cerebral artery occlusion (tMCAO) is still of benefit to this zone for up to 90 minutes. However, blood flow restoration may exacerbate brain edema in temporary ischemia. Thus, reperfusion presents a dilemma: on the one hand, reperfusion may reduce the infarct area and attenuate edema formation. On the other hand, it may disrupt the BBB and exacerbate edema formation. The relation between cerebral blood flow (CBF) and BBB permeability after postischemic reperfusion is still not clear. To identify factors that lead to exacerbation of brain injury after postischemic reperfusion, we compared changes in CBF, BBB permeability, and edema formation in permanent and temporary MCAO in rats. The relation between local CBF and BBB disruption is discussed.

Materials and Methods

Experimental Protocols

Rats were subjected to either 6 hours of permanent MCAO (pMCAO) or 3 hours of occlusion followed by 3 hours of reperfusion (tMCAO). In each group of animals, three sets of experiments were performed: (1) measurement of brain edema and tissue ion content, (2) measurement of infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC) staining, and (3) measurement of BBB permeability using [3H]a-aminoisobutyric acid ([3H]AIB) as a passive permeability marker. During all experiments, physiological parameters were monitored and controlled and CBF was measured by laser Doppler flowmetry (LDF).

Rat MCAO Suture Model

Adult male Sprague-Dawley rats (Charles River) weighing 250 to 300 g were induced with 5% isoflurane, intubated (16G, 2-in Angiocath), and ventilated with 1.5% isoflurane in N2O/O2 (70%/30%). A PE-50 catheter was introduced into the femoral artery for continuous monitoring of arterial blood pressure and sampling for analysis of blood gases and blood pH. The temperatures of the temporalis muscle and rectum were monitored and controlled.
values were averaged over 5-second intervals and recorded around the probe during the experiment to prevent desiccation was stereotactically advanced to gently touch the intact dura mater. To get a clearer optical medium between the LDF leakage. Large blood vessels were avoided under microscopic cortex. The dura was left intact to prevent cerebral spinal fluid sites represented the ischemic core and perifocal areas, re-

FIG 1. Illustration of the location of the cerebral blood flow measurements and brain tissue sampling. A, Laser Doppler flowmetry was performed at three cortical sites: point A was 6 mm lateral and point B 1.5 mm lateral in the ipsilateral ischemic hemisphere; point C was 6.0 mm lateral in the contralateral hemisphere. All three points were 1.0 mm posterior to the bregma. B, Sampling of brain tissue for the water, ion, and blood-brain barrier permeability determination. Coronal sections 4 mm thick were cut through the middle cerebral artery distribution. The sections were divided into six areas: A, lateral cortex; B, medial cortex; and C, basal ganglia from the ipsilateral and contralateral sides. The tissue samples were processed for water, Na⁺, K⁺, and Cl⁻ measurements and PS product determination.

were measured with thermocouples (E type, Omega Engineering, Inc), and brain temperature was regulated with a heating lamp and heating pad to maintain normothermia. Arterial blood pressure was maintained at >100 mm Hg by adjustment of the isoflurane concentration. Blood gases were analyzed one time during the experiments to maintain an arterial PaO₂ level of ≥100 mm Hg.

MCAo occlusion was produced as described previously. Briefly, all branches of the left common carotid artery were isolated and coagulated, including the terminal lingual maxil-

Cortical Blood Flow (CBF) Measurement

For the CBF measurement, we used an LDF monitor (model BPM² system, Vasamedics Inc) equipped with a small-caliber probe of 0.7-mm diameter (P-433, Vasamedics). The scalp was incised in the midline and reflected. Flow was measured at three points on the surface of cortex (Fig 1A): point A was placed 6 mm lateral and point B 1.5 mm lateral to the midline of the ipsilateral hemisphere. Point C was placed 6.0 mm from the midline in the contralateral hemisphere. All three points were 1.0 mm posterior to the bregma. In preliminary studies, we found that after MCAo, the A and B sites represented the ischemic core and perifocal areas, respectively. At each point, a 2-mm hole was drilled in the skull and the bone was carefully removed to prevent damage to the cortex. The dura was left intact to prevent cerebral spinal fluid leakage. Large blood vessels were avoided under microscopic guidance. The LDF probe was held in a micromanipulator and was stereotactically advanced to gently touch the intact dura mater. To get a clearer optical medium between the LDF probe and the dura, warmed 0.9% saline was slowly rinsed around the probe during the experiment to prevent desiccation of the exposed brain. Stable baseline LDF readings were obtained for at least 20 minutes from all three sites of the MCA before occlusion. Continuous digital display of LDF values were averaged over 5-second intervals and recorded every 20 minutes during the MCAo. The CBF values were then calculated and expressed as percentage of the baseline values.

Water, Na⁺, K⁺, and Cl⁻ Content

At the end of the experiment, the brains were removed, slices 4 mm thick were taken through the MCA distribution, and the brain tissue was divided into lateral cortex, medial cortex, and basal ganglia regions (Fig 1B). These samples were weighed on an electronic analytical balance (Mettler AF100, Mettler Instrument Co) with 0.0001-ng precision to obtain wet weight (W). The tissue was then dried at 95°C for 24 hours and reweighed to obtain dry weight (D). The water content was expressed as percent wet weight, calculated as (W–D)/W×100. The dehydrated section was digested in 1 mL of 1N nitric acid for 1 week. Then a 0.2-mL aliquot was removed and diluted to 2 mL with deionized water and 3 mmol/L CsCl solution, and the Na⁺ and K⁺ contents were measured in this solution by atomic emission spectroscopy (IL943 Automatic Flame Photometer, Instrumentation Laboratory Inc). Cl⁻ was measured with a digital chloridometer (model 442-5000, Haake Buchler Inc).

Morphometric Measurement of Infarct Volume

The area of cerebral infarction was quantified by TTC staining. The brains were removed, and six coronal slices at 2, 4, 6, 8, 10, and 12 mm distal from the frontal pole were dissected with a brain slicer (Activational System Co, Inc). All slices were incubated in 2% TTC (Sigma Chemical Co) solution for 30 minutes at 37°C. Then the slices were fixed in 10% formaldehyde solution. The area of infarction in each section was determined by measuring the area of normally staining brain in the hemispheres ipsilateral and contralateral to the MCAo. The area of infarction was then calculated as (normal volume contralateral minus normal volume ipsilateral). The total volume of infarction was calculated by summing the infarct area in each section and multiplying by the distance between sections.

BBB Permeability and Brain Plasma Volume

The permeability–surface area (PS) product of the BBB to [³H]AIB was determined by a modification of the method of Ohno et al. [³H]AIB (35 μCi) was injected through a femoral vein 10 minutes before the end of the experiment, while [¹⁴C]inulin (20 μCi), a plasma volume marker, was given as a second injection 2 minutes before the end of experiment (both from Du Pont–New England Nuclear). A peristaltic pump withdrew arterial blood continuously throughout the course of the experiment. At the end of the experiment, a terminal plasma sample was taken, and the rat was killed by decapitation. Blood samples were digested in methylenbenzenethionium hydroxide (Sigma), bleached with H₂O₂, and counted in an aqueous-based liquid scintillation cocktail. Brain tissue samples were obtained as described in Fig 1B, digested in methylenbenzenethionium hydroxide, and counted in a Beckman 3801 two-channel liquid scintillation counter. The PS product was calculated from the formula

$$PS=(C_p-P_xC_v)/(C_p \cdot dt)$$

where $C_p$ is the counts per gram of brain, $P$ is the plasma volume of the brain determined from the inulin space, $C_v$ is the terminal plasma concentration of [³H] AIB, and $C_p \cdot dt$ is the integral of the [³H] AIB plasma concentration ($C_p$) for the experiment. The latter was calculated from the radioisotope content of the continuously withdrawn arterial blood sample. The transfer constant ($K_t$) is a function of the effective volume fraction (V) of the blood in which the substance resides, the CBF, the permeability (P) of the substance at the BBB, and the surface area (S) of the BBB. The following
TABLE 1. Physiological Parameters of Rats During the Experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>MABP, mm Hg</th>
<th>pH</th>
<th>PCO₂, mm Hg</th>
<th>PO₂, mm Hg</th>
<th>Hematocrit, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMCAO</td>
<td>101 ± 3</td>
<td>7.51 ± 0.05</td>
<td>30 ± 4</td>
<td>112 ± 14</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>tMCAO</td>
<td>104 ± 8</td>
<td>7.48 ± 0.06</td>
<td>31 ± 6</td>
<td>112 ± 16</td>
<td>39 ± 1</td>
</tr>
</tbody>
</table>

MABP indicates mean arterial blood pressure; pMCAO, permanent middle cerebral artery occlusion; and tMCAO, temporary MCAO. Values are mean ± SD for 21 rats in each group.

equation describes the relation between \( K_t \), CBF, \( V_f \), and PS product:

\[
PS = -CBF \times V_f \times \ln[1-(K_t/\text{CBF} \times V_f)]
\]

When the ratio \( K_t/\text{CBF} \times V_f \) is ≤ 0.2, then \( K_t \) will underestimate the PS product by no more than 10%. The ratio \( K_t/\text{CBF} \times V_f \) in our previous studies for AIB was never greater than 2%, indicating \( K_t \approx PS \).

Statistical Analysis

The data are expressed as the mean ± standard deviation of the mean (SD). Statistical differences between the pMCAO and tMCAO groups were evaluated by a two-tailed Student's \( t \) test for unpaired samples. Within a group of animals, results from the left and right hemispheres were compared by a two-tailed Student's \( t \) test for paired samples. \( P < .05 \) was considered to represent significance.

Results

Cardiovascular and respiratory data from pMCAO and tMCAO are given in Table 1. The values were measured during the occlusion, and they are all in the normal range. The pattern of mean arterial blood pressure during ischemia and in the reperfusion phases is shown in Fig 2. When the suture was inserted or withdrawn, there was usually a slight fall in blood pressure, but it recovered quickly. The brain temperature, based on the temporals muscle reading, was regulated at 37°C to 37.5°C, and the body temperature averaged 37.5°C during experiments in both pMCAO and tMCAO groups.

Cerebral Blood Flow

Introduction of the suture to block the blood supply to the MCA produced an equal fall in the relative surface blood flow in the lateral and medial cortex areas in the pMCAO and tMCAO groups (Fig 3). Mean percentage of baseline CBF in the contralateral hemisphere was comparable, remaining around 100% in both groups. During the MCAO, the level of CBF was reduced to 7% to 15% (lateral cortex) and 16% to 30% (medial cortex) of the baseline in the pMCAO group. CBF was reduced to 10% to 17% (lateral cortex) and 28% to 40% (medial cortex) of the baseline in the tMCAO group. During the 3 hours of reperfusion, CBF recovered to 47% to 80% (lateral cortex) and 78% to 98% (medial cortex) of the baseline. It should be mentioned that immediately after reperfusion, there was no consistent pattern of hyperemia. Hyperperfusion after 3 hours of MCAO was found in only three rats, in which the blood flow in the ipsilateral hemisphere was > 100% of baseline after 1 to 2 hours of reperfusion.
Changes in H₂O and Ion Contents

The changes in water content in the lateral cortex between pMCAO and tMCAO at 6 hours are displayed in Fig 4A. Water in the MCA core territory increased from a value of 79.4±1.3% to 83.5±1.7% in the tMCAO group (P<.001). The increase in the pMCAO group (82.0±1.3%) was less, but statistical analysis showed no significant difference between the two groups (P>.05, Table 2). The water gain of the tissue was accompanied by shifts in Na⁺, K⁺, and Cl⁻. The Na⁺ content increased from 207 to 343 μEq/g dry wt in the tMCAO and from 191 to 292 μEq/g dry wt in the pMCAO group, whereas the K⁺ content decreased from 491 to 359 μEq/g dry wt in the tMCAO and from 464 to 372 μEq/g dry wt in the pMCAO group (Fig 4B and 4C). The changes in Cl⁻ content paralleled changes in Na⁺ content in both groups (Fig 4D). The differences in water and ions between the contralateral and ipsilateral hemispheres for all regions are shown in Table 2. There were no significant differences in ion content between pMCAO and tMCAO groups.

Infarct Volume

The method for inducing focal cerebral ischemia described above yielded reproducible infarction in the MCA territory of the rat’s brain as measured by mitochondrial dehydrogenase staining with TTC. As shown in Fig 5, the infarct volumes were significantly smaller in the pMCAO than in the tMCAO group. In the pMCAO group, the infarct volume was 103±39 mm³, whereas in the tMCAO group, it was 203±38 mm³ (P<.01). When the infarct volumes were divided into cortical and subcortical components, the cortical infarct volumes in the pMCAO group were still smaller than in the tMCAO group (44±23 versus 122±27 mm³, P<.01); however, the subcortical infarct volumes in both groups were not statistically different (57±20 versus 80±14 mm³). The infarct volumes in the subcortical regions were not affected by intervention with reperfusion, which may be because of a poor collateral blood supply in these areas. The ratios of left to right hemispheric areas, which represent swelling, were 1.060±0.006 in the pMCAO and 1.073±0.011 in the tMCAO groups, respectively, and were not significantly different between the two groups.

BBB Permeability

The BBB permeability was measured as the PS products for [3H]AIB. Since AIB enters the brain by...
simple diffusion, the PS product for AIB is a measure of the passive permeability. PS products in the contralateral hemispheres were comparable in both pMCAO and tMCAO groups, and they were all in the normal range (Fig 6). In the pMCAO group, a breakdown of the BBB in the ischemic hemisphere was not observed (lateral cortex, 1.25 ± 0.43; medial cortex, 1.58 ± 0.52; and basal ganglia, 1.41 ± 0.39 μL · g⁻¹ · min⁻¹, respectively). However, the PS product was significantly increased in the tMCAO group (lateral cortex, 3.69 ± 1.36, P < .001; medial cortex, 2.74 ± 0.71, P < .01; and basal ganglia, 2.60 ± 0.69, P < .01, respectively). These results indicate that the integrity of the BBB was maintained during the first 6 hours of pMCAO but that the BBB is damaged by 3 hours of reperfusion after 3 hours of ischemia.

**Correlation Between the Degree of Reperfusion and BBB Disruption**

The correlation between the degree of reperfusion and BBB disruption is shown in Fig 7. In the pMCAO group, the final CBF in the lateral cortex was 15 ± 7% of baseline, and the PS product was 1.25 ± 0.43 μL · g⁻¹ · min⁻¹. During 3 hours of reperfusion, the pattern of restoration was variable; therefore, the rats were divided into two groups according to the degree of reperfusion. Three rats exhibited hypoperfusion with average CBF in the lateral cortex 35 ± 6% of baseline. Six rats showed good reperfusion, with average CBF in the lateral cortex 93 ± 12% of baseline. The PS product in the poorly reperfused rats was 2.6 ± 0.72 compared with 4.2 ± 1.35 μL · g⁻¹ · min⁻¹ in rats with good reperfusion. The BBB disruption was significantly greater in the rats with good reperfusion (P < .05).

**Discussion**

The postulated mechanisms underlying reperfusion injury are multifactorial and intricately interconnected. They are governed by positive and negative feedback control, cooperativity, and maintenance of homeostatic balance by means of counterpoised antagonists.²³ To study the mechanisms, we developed a suture MCAO model for both permanent and temporary ischemia in the MCA territory without craniotomy. Recirculation is relatively complete because vascular spasm and direct mechanical damage by clips or ligation are avoided. In addition, decompression of intracranial pressure, as occurs with craniotomy, can be avoided. We did not determine whether the ultimate extent of injury was greater after tMCAO compared with pMCAO. It is possible that injury was simply accelerated in tMCAO but that the eventual extent of injury was the same. However, the reason for this study was to develop a model of reperfusion injury.

LDF measurements were used to continuously monitor the changes of local CBF during the MCAO throughout our experiments. The LDF provides instantaneous, non-directional, continuous, and noninvasive measurements of microcirculatory blood flow in a tissue sample of ~1 mm². Other authors have shown good correlation between LDF and other techniques of monitoring blood flow in the brain microcirculation.²⁴,²⁵ Although the results show only relative rates of cortical blood flow, it is essential to verify both occlusion and
reperfusion when studying ischemia-reperfusion. The results demonstrate that failure to establish reperfusion will produce injury. The average CBF in the ischemic lateral cortex of both groups was around 7% to 17% of baseline. The threshold for infarction is similar to that for energy failure/loss of membrane homeostasis.26-29 However, the threshold for infarction varies with the duration of the insult.12 In the rat MCAO model, the ischemic lesion does not improve if reperfusion is delayed beyond the first 90 minutes. The reason is probably a high cerebral metabolic rate and a relatively poor blood supply in rats.2-30 During the 3 hours of reperfusion, the blood flow was above the baseline CBF in only a few rats. Thus, there was no reactive hyperemia in most animals in our experiment. Other investigators revealed that in the MCAO model, hyperemia occurs after 1 to 2 hours of occlusion but not after 3 hours of occlusion.31,32

The degree of BBB disruption was measured quantitatively in the present study by determination of the permeability and surface area product for $[^{3}H]$AIB.33 The results indicate that after 6 hours of pMCAO, the PS product was still in the normal range.34 This suggests that the BBB permeability to serum proteins remains intact for at least several hours after pMCAO.35,36 In the tMCAO group, however, the BBB disruption was clearly observed. Since the PS product was two times as high as that in the pMCAO group, our results demonstrate that 3 hours of occlusion followed by 3 hours of reperfusion can disrupt the BBB permeability in all regions, including the cortical, subcortical, and basal ganglia in this model. The BBB opening may be one important factor that aggravates brain infarction.

A close association between increased CBF values and BBB opening has been shown in various pathological processes such as epilepsy,37 hypercapnia and hypoxia,32 and hypertension.38,39 Kuroiwa and colleagues reported that postischemic reactive hyperemia is also associated with BBB opening and brain edema. However, they could not quantitatively determine the BBB permeability by Evans blue staining. Our data show that in the tMCAO rats, BBB disruption is related to the degree of reperfusion. BBB opening was observed only in the well-reperfused rats in which CBF was >50% of baseline. Because BBB disruption after reperfusion could aggravate brain edema and infarction, it may be an important limiting factor in using reperfusion to improve ischemic brain injury.

Our results show that the water content is significantly increased in the ipsilateral hemisphere in both the pMCAO and tMCAO groups, although the edema formation is not significantly different between these two groups. The volume of infarction, however, in the tMCAO group is greater than in the pMCAO group ($P<.05$). Reperfusion may aggravate brain injury by restoring the supply of water and osmotic equivalents that may then exacerbate edema.42 A second possible mechanism of reperfusion injury is the resupply of oxygen after the period of ischemia. Oxygen free radicals may cause peroxidation of lipids in the cell membranes, resulting in the failure of membrane ATPase, changes in ion homeostasis, and elevation of the concentration of free fatty acids.43 Although we did not identify the sources of free radicals, formation of these toxic mediators may result from arachidonic acid metabolism, catecholamine oxidation, neutrophil activity, nitric oxide synthesis, xanthine oxidase activity, or reduced metabolite accumulation.44,45 A third possible mechanism of reperfusion injury is the release of a variety of biochemical mediators associated with edema and infarction. Many of these agents may be released directly from neurons or from endothelial cells. These mediators include excitatory amino acids, bradykinin, histamine, and cations. Considering the documented importance of oxygen free radicals in reperfusion injury in other tissues, the role of these destructive molecules in brain injury seems very probable.

In summary, our experiments, using the rat suture MCAO model, demonstrate that BBB permeability is increased in the rats after tMCAO but not after 6 hours of pMCAO. In addition, the infarct volume in the tMCAO group is greater than in the pMCAO group. Therefore, reperfusion after 3 hours of MCAO may be of little or no benefit in reducing infarct volume and edema and may also cause an earlier BBB disruption.

Acknowledgments

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References

Editorial Comment

A potentially deleterious consequence of reperfusion in ischemic tissue is reoxyegenation-induced excessive formation of oxygen free radicals.\(^1\)\(^2\) Indirect evidence supports the contention that free radicals, including superoxide dismutase and hydroxyl radicals, are formed during reperfusion after cerebral ischemia.\(^3\)\(^4\) A number of studies showing the favorable effects of endogenous or exogenous free radical scavenging enzymes\(^5\)\(^6\) also support a pathogenetic role of oxygen free radicals in ischemic brain injury. However, there is little or no evidence in animal studies that has clearly shown that reperfusion after temporary arterial occlusion accentu-
Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats.
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