Biphasic Opening of the Blood-Brain Barrier Following Transient Focal Ischemia: Effects of Hypothermia

Z. Gao Huang, Dong Xue, Edward Preston, Hasneen Karbalai, Alastair M. Buchan

ABSTRACT: Objective: Tracer constants (K_i) for blood-to-brain diffusion of sucrose were measured in the rat to profile the time course of blood-brain barrier injury after temporary focal ischemia, and to determine the influence of post-ischemic hypothermia.

Methods: Spontaneously hypertensive rats were subjected to transient (2 hours) clip occlusion of the right middle cerebral artery. Reperfusion times ranged from 1.5 min to 46 hours, and i.v. 3H-sucrose was circulated for 30 min prior to each time point (1h, 4h, 22h, and 46h; n=5-7 per time point). K_i was calculated from the ratio of parenchymal tracer uptake and the time-integrated plasma concentration. Additional groups of rats (n=7-8) were maintained either normothermic (37.5°C) or hypothermic (32.5°C or 28.5°C) for the first 6 hours of reperfusion, and K_i was measured at 46 hours. Results: Rats injected after 1.5 – 2 min exhibited a 10-fold increase in K_i for cortical regions supplied by the right middle cerebral artery (p<0.01). This barrier opening had closed within 1 to 4 hours post-reperfusion. By 22 hours, the blood-brain barrier had re-opened, with further opening 22 and 46 hours (p<0.01), resulting in edema. Whole body hypothermia (28°C-29°C) during the first six hours of reperfusion prevented opening, reducing K_i by over 50% (p<0.05).

Conclusions: Transient middle cerebral artery occlusion evokes a marked biphasic opening of the cortical blood-brain barrier, the second phase of which causes vasogenic edema. Hypothermic treatment reduced infarct volume and the late opening of the blood-brain barrier. This opening of the blood-brain barrier may enhance delivery of low permeability neuroprotective agents.

RÉSUMÉ: Ouverture biphasique de la barrière hémato-encéphalique suite à une ischémie focale transitoire: effets de l’hypothermie. Objectif: Nous avons mesuré les constantes d’un traceur (K_i) de la diffusion de sucre du sang vers le cerveau chez le rat afin d’observer l’évolution des dommages subis par la barrière hémato-encéphalique après une ischémie focale temporaire et pour déterminer les effets d’une hypothermie post-ischémique. Méthodes: Des rats spontanément hypertendus ont été soumis à une occlusion de deux heures de l’artère cérébrale moyenne par un clip. Le temps de reperfusion variait de 1.5 minute à 46 heures et une perfusion intraveineuse de 3H-sucrose a été administrée pendant 30 minutes avant chaque évaluation ponctuelle (1h, 4h, 22h, et 46h; n=5-7 par évaluation ponctuelle). La constante K_i a été calculée à partir de l’indice de captation du traceur par le parenchyme et de la concentration plasmatique en fonction du temps. Des groupes additionnels de rats (n=7-8) ont été maintenus soit à la température normale (37.5°C) ou en hypothermie (32.5°C ou 28.5°C) pendant les 6 premières heures de la reperfusion et K_i a été mesurée à 46 heures. Results: Les rats qui ont reçu l’injection après 1.5 – 2 minutes présentaient une augmentation de K_i de dix fois supérieure dans les régions corticales irriguées par l’artère cérébrale moyenne (p<0.01). Cette ouverture de la barrière s’était refermée 1 à 4 heures post-reperfusion. À 22 heures, la barrière hémato-encéphalique s’était réouverte, davantage à 22 et à 46 heures (p<0.01), ce qui a donné lieu à de l’œdème. L’hypothermie généralisée (28°C-29°C) pendant les 6 premières heures de la reperfusion a empêché son ouverture, diminuant ainsi la constante K_i de plus de 50% (p<0.05). Conclusions: L’ouverture transitoire de l’artère cérébrale moyenne provoque une ouverture biphasique importante de la barrière hémato-encéphalique corticale dont la deuxième phase cause de l’œdème. L’hypothermie a diminué la taille de l’infarctus cérébral et l’ouverture tardive de la barrière hémato-encéphalique. Cette ouverture de la barrière hémato-encéphalique peut accroître la distribution d’agents neuroprotecteurs à basse perméabilité.


Brain capillary walls are distinguished by an endothelial cell layer replete with tight junctions and a scarcity of fenestrae. This blood-brain barrier (BBB) is a specialization which maintains homeostasis of the neuronal micro-environment, limiting blood-to-brain diffusion of hydrophilic molecules. Penetration is largely restricted to lipophilic substances capable of directly traversing endothelial membranes, and to hydrophilic substances such as amino acids and glucose, for which specific membrane carriers exist.1,2 BBB drainage, following brain ischemia, leads to the extravascular leakage of plasma proteins and other solutes, resulting in an imbalance with osmotic forces drawing excess water into the tissue (i.e. vasogenic edema).3 Tissue swelling ensues within the rigid confines of the skull, elevating intracranial pressure with secondary ischemia due to compression of microvasculature, and, ultimately, brain herniation.4

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A reproducible rodent model used to study focal brain ischemia involves temporary (e.g., 0.5 to 4 hours, or permanent occlusion of a middle cerebral artery [MCAO model]) with tandem occlusion of the ipsilateral common carotid artery. Temporary MCAO enables the study of both positive and negative aspects of post-ischemic reperfusion, which occurs clinically, both spontaneously and therapeutically, with the advent of rt-PA, newly licensed as a thrombolytic agent for ischemic stroke in the first three hours. Restoration of blood flow, if early enough, may offer the advantage of reducing neuronal damage and limiting infarct extension and enhances potentially cytoprotective drug delivery. These benefits may be undermined by reperfusion injury to the microvasculature, compromising BBB function, exacerbating edema formation and the inflammatory developments that follow. Dynamic changes in BBB permeability, which follow temporary MCAO, are therefore of critical importance.

The objective of this study was to delineate the time course and intensity of the BBB opening after reversible MCAO ischemia in the rat using a method involving a removable,atraumatic clip. Post ischemic changes in the BBB were studied using a modification of a radiotracer methodology, which quantifies even minor degrees of BBB opening with a high degree of sensitivity. The radiotracer method used in this study is based on a 2-compartment (plasma/brain) simple diffusion model, which assumes that the amount of tracer crosses the inferior cerebral vein in the rhinal fissure. The MCA was then cut and retracted. A #1 micro-clip (Codman) was placed on the MCA at a site proximal to the point where it crosses the inferior cerebral vein in the rhinal fissure. The incisions were then closed with wound clips.

Animals were subjected to two hours of ischemia, during which time the anesthesia was discontinued and the animals allowed to regain consciousness. At the end of ischemia, the rats were briefly re-anesthetized with halothane, the MCA clip removed, and blood re-flow through the MCA visually verified. The wound was sutured closed, and the animal permitted to regain consciousness. Sham groups of animals were treated in the same manner, except the micro-clip was placed on the MCA and then removed immediately. Animals were maintained at a rectal temperature of 37°C - 38°C for all procedures, except where indicated otherwise in the hypothermic experiments.

Regional transfer constants (K_r) for BBB permeation of 3H-sucrose were measured by a previously published method. Measurements were made at different time points after reperfusion of the right MCA. The rats were anesthetized with pentobarbital (65 mg/kg i.p.) and, after cannulation of a femoral artery and vein, 3H-sucrose (NET-341) was injected intravenously (20 µCi/100g, in 0.5 ml saline). Immediately upon tracer injection, syringe-pump sampling of femoral arterial blood was begun at a constant rate (0.039 ml/min⁻¹) and continued for 30 min. At this point, sampling was stopped and the brain was immediately cleared of intravascular tracer by perfusing 25 ml saline at 100 – 130 mm Hg pressure through a cannula inserted a few minutes beforehand into the right carotid artery. The rat was decapitated, the brain removed and dissected bilaterally into the cortex (about 180 mm⁻³, representing the complete MCA supply territory), striatum, and hippocampus. Brain samples (weighed) and measured volumes of plasma from the arterial sample were placed in the scintillation vials and solubilized overnight at 37.5°C in 1.3 ml Soluene 350 (Packard Instr.). 10 ml of fluor (HionicFluor) was added to all vials and the samples were counted by liquid scintillation to determine the tracer level in the brain parenchyma (C paren, dpm.g⁻¹) and the time integral of the plasma tracer level (αdiff[C paren, dpm.g⁻¹] dt, dpm.s.ml⁻¹). The integral was obtained by multiplying the plasma concentration (C plasma, dpm.ml⁻¹) by the circulation time (1800s). The transfer constant (K_r, mL.g⁻¹.s⁻¹) was calculated from the relationship: K_r = C paren, dpm.g⁻¹ / (αdiff[C paren, dpm.g⁻¹] dt, dpm.s.ml⁻¹) 

In Experiment 1, radiotracer studies were carried out at 1, 4, 22, and 46 hours after reperfusion (or 3, 6, 24, and 48 hours after the onset of ischemia) with five animals in each group. In an additional group (n = 5), the radiotracer experiments were initiated within 1.5 – 2 min after reperfusion. In this case only, pentobarbital rather than halothane, anesthesia was induced 15 min before reperfusion for canulations and clip removal. A group of sham-operated rats (n = 7 total) was studied at 1, 2, 3, 5, and 24 hours after sham occlusion, with one or two rats at each time point. No sham-operated rats were studied at 1.5 - 2 minutes.

In Experiment 2, rats underwent mild or moderate hypothermia during the first 6 hours of reperfusion. Ten minutes before the end of 2 hours of MCA occlusion, the rats were lightly halothane anesthetized and were surrounded by bags of crushed ice. This caused body temperature to drop to 32°C - 33°C at the time that the MCA clip was removed. Rectal temperature was maintained for 6 hours at 32°C - 33°C for the mild hypothermic group (n=7) or 28°C - 29°C for the moderate hypothermic group (n=7). Control animals, maintained at 37°C - 38°C, were concurrently studied with their hypothermic peers, such that two normothermic groups were formed (n = 8 for the mild hypothermia controls, and n = 7 for the moderate hypothermia controls). During the six hours, halothane was continued at 0.5 – 0.7% for the hypothermia groups only, as the control group did...
RESULTS

Experiment 1

Mean regional transfer constants for the six groups of normothermic rats are summarized in Table 1. In 7 sham-stroked rats, radiotracer measurements were initiated at 1, 2, 3, 5, or 24 hours after the sham procedure. Each time period group contained 1 animal, except for the 3 and 24 hour groups, which contained 2 animals each. There were no significant differences between the regional Ki values for cerebral tissues on the right side of the brain (which had undergone a complete surgical procedure, except for MCA occlusion), and those of corresponding territories on the left side. In the 5 experimental groups of rats which underwent 2 hours of MCA occlusion (each group: n = 5), the largest blood-brain barrier openings and increases in Ki took place in the right neocortical tissue supplied by the occluded right MCA (Table 1). When the 30 min tracer circulation period began 1.5 – 2 min after reperfusion, there was evidence of an early increase in mean Ki to greater than 10-fold of the baseline value. This was followed by a partial recovery, with subsequent Ki measurements and 1 and 4 hours post-reperfusion, which were significantly lower than the acute (1.5 – 2 min) group values, although still elevated above baseline and above values for the contralateral, non-ischemic side. A late opening in the BBB was then demonstrated 4 and 22 hours post-reperfusion. This was most pronounced between 22 and 46 hours after reperfusion and was more pronounced on the left side of the brain, as compared to the right side of the brain (Figure 1).

Striatum removed from the right hemisphere of stroked rats exhibited a slight but significant elevation in Ki, 1.3 – 2 min post-reperfusion (Table 1). This change was no longer present at 1 or 4 hours post-reperfusion, however, a significant increase in Ki was demonstrated between 22 and 46 hours after reperfusion. The dorsal hippocampus ipsilateral to the MCA occlusion showed little change in Ki, except at the 46 hour time point, when a slight elevation was present. In the contralateral, non-ischemic

![Table 1: Regional Transfer Constant (Kᵣ) in MCA Model (Experiment 1)](https://www.cambridge.org/core/core)
hemisphere, all regions exhibited mean Ki values slightly higher than the baseline, sham stroke values. However, in no instance was this statistically significant.

Experiment 2
Table 2 shows the effect of mild or moderate hypothermia for 6 hours post-reperfusion on BBB opening caused by MCAO. Mean Ki measured 46 hours post-reperfusion was lower in rats that underwent hypothermia (32°C - 33°C) compared to that of normothermic controls. With moderate hypothermia of 28°C - 29°C, the reduction in BBB opening was more striking.

Experiment 3
Table 3 reports edema measurements based on the wet to dry weight difference as a percentage of water per cerebral hemisphere (ml.g⁻¹ x 100). In 5 rats sacrificed 4 hours post-reperfusion, the values (mean ± SD) were 80.4 ± 0.3% and 78.5 ± 0.2% for the right and left sides, respectively (p<0.001). Furthermore, the mean % H₂O measured in the stroked hemisphere at 22 hours post-reperfusion was significantly higher than that at 4 hours (p< 0.01), whereas, in the non-ischemic hemisphere there was no significant difference between these two time points.

Table 2: Effect of Mild and Moderate Post-Ischemic Hypothermia on Transfer Constant (Ki) (Experiment 2)

<table>
<thead>
<tr>
<th>Variability Group (n)</th>
<th>Right (Mean ± SD) (mL.g⁻¹.s⁻¹ x 10⁶)</th>
<th>Left (Mean ± SD) (mL.g⁻¹.s⁻¹ x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variability Group (n)</td>
<td>Right (Mean ± SD) (mL.g⁻¹.s⁻¹ x 10⁶)</td>
</tr>
<tr>
<td></td>
<td>Variability Group (n)</td>
<td>Cortex</td>
</tr>
<tr>
<td>Normothermia (8)</td>
<td></td>
<td>30.6 ± 9.1</td>
</tr>
<tr>
<td>Hypothermia (7) 32-33°C</td>
<td></td>
<td>23.1 ± 9.1</td>
</tr>
<tr>
<td>Normothermia (7)</td>
<td></td>
<td>34.7 ± 10.9</td>
</tr>
<tr>
<td>Hypothermia (7) 28-29°C</td>
<td></td>
<td>15.4 ± 8.8**</td>
</tr>
</tbody>
</table>

** p<0.01, student t-test.
Post-ischemic hypothermia (28°C - 29°C) for the first 6 hours had no ameliorative effect upon the edema measured in the cortex 46 hours after reperfusion. The mean (± SD) percentage water (ml.g⁻¹ x 100) for the normothermic group was 86.1 ± 0.7% for the post-ischemic right cortex and 79.8 ± 0.7% for the contralateral left cortex, versus 85.7 ± 1.4% (right) and 79.6 ± 0.3% (left) for that of the hypothermic group.

Experiment 4

Despite the failure of moderate post-ischemic hypothermia to reduce the accumulation of edema in hypothermia (28°C - 29°C) for the first 6 hours of the 46 hour reperfusion period, this resulted in a mean cortical infarct volume of 122 ± 57 mm³ (n = 6) (Figure 2), which was significantly lower than that of the normothermic control group (175 ± 22 mm³, n = 6) (p < 0.05) (Table 4). Animals receiving 6 hours of mild hypothermic reperfusion (32°C - 33°C) had a total infarct volume of 149 ± 39 mm³ (n = 6) at 46 hours, which was less than that of the control group, but not significantly so. The reduction in the size of injury relates to smaller volumes of infarction rather than differences in the amount of swelling or edema, confirming Experiment 3.

DISCUSSION

In these transient ischemic experiments, the widest BBB openings and increases in $K_i$ were seen in the post-ischemic right cerebral cortex, which was dissected to include both the core and edge of the region perfused by the MCA. The opening was clearly biphasic, characterized by an initial 10-fold augmentation in $K_i$ during the first half hour of reperfusion, followed by partial closing, and then a delayed, but progressive, opening between 22 and 46 hours post-reperfusion. Moderate hypothermia during ischemia dramatically reduced infarction and edema, as well as preventing BBB opening, but also had partial effects on infarct size when instituted during the post-ischemic period following normothermic ischemia. In these studies, we have demonstrated that postischemic moderate hypothermia affects not only the size of the infarct, it does so in tandem with reductions in the opening of the BBB, not by simply reducing the amount of vasogenic edema, but possibly by interfering with the post-ischemic inflammatory response.

This profile of BBB injury shows similarities to findings based on the assessment of Evan’s Blue dye extravasation in the cat. Following one hour of temporary MCA occlusion, dye injected i.v. early in reperfusion caused staining of brain parenchyma. This was followed by a refractory period, and then a delayed opening, which was visible in cats sacrificed five hours or three days post-stroke. The initial acute opening has been described as a ‘hemodynamic’ BBB opening. Because of acidosis, loss of autoregulation, and vasodilation of the cerebral vasculature, reperfusion results in excessive blood flow or ‘luxury perfusion’. High intraluminal blood pressure in the cerebral microvasculature has been shown to induce abnormal pinocytotic transport across endothelial cells, and opening of interendothelial tight junctions. A significant role of arterial pressure in the degree of post-reperfusion opening after

**Table 3: Percentage of Water in MCA Model (Experiment 3)**

<table>
<thead>
<tr>
<th>Variability</th>
<th>Right Cortex</th>
<th>Left Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (n)</td>
<td>% of Water (Mean ± SD)</td>
<td>% of Water (Mean ± SD)</td>
</tr>
<tr>
<td>Normothermia</td>
<td>4 hours post-reperfusion (5)</td>
<td>80.4 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>22 hours post-reperfusion (5)</td>
<td>83.7 ± 0.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>46 hours post-reperfusion (6)</td>
<td>86.1 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moderate Hypothermia (6)</td>
<td>85.7 ± 1.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>79.6 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>p < 0.01 for ANOVA plus Tukey’s test.</sup>

<sup>a – right side values different from corresponding left side values; b – different from 4 hr post-reperfusion value.</sup>

**Figure 2:** Volume of neocortical infarction for each rat following 2 hours of normothermic transient MCA occlusion and 46 hours of reperfusion, in the first 6 hours of which animals were maintained at either 37°C-38°C (normothermia), 32°C-33°C (mild hypothermia), or 28°C-29°C (moderate hypothermia). The mean edema and infarct sizes are displayed ±SD (the asterisk denotes significantly less injury (p<0.05). The n value for each group is indicated in brackets.
MCAO has been demonstrated. After 3 hours of MCAO and 30 min re-circulation, BBB opening to Evan’s Blue dye was greatly augmented in rats that had been rendered hypertensive with phenylephrine during the reperfusion period. The magnitude of acute hyperemic BBB opening, and of modulating factors, such as blood pressure, would appear to be an important consideration in utilizing and interpreting the MCAO model. For example, factors such as hypertension, which augment post-reperfusion opening, and the ensuing homeostatic changes might thereby indirectly influence subsequent neuropathological events. On the other hand, one might anticipate that the degree of early post-ischemic BBB opening could have a positive impact on the efficacy of experimental drugs when the chemical nature of these compounds limits their ability to cross the normal BBB. For instance, with the competitive AMPA antagonist NBQX, which penetrates the blood-brain barrier, effective concentrations of the drug are achieved after 3 to 4 hours, with an even more dramatic upward increment taking place between 22 and 46 hours. Accounting for this time delay would seem important in any proposal on the cause(s) of BBB opening, which presumably differs from that underlying the acute post-ischemic opening. In fact, the delayed BBB opening to $^3$H-sucrose is consistent with the published observations that between 24 and 48 hours after transient MCAO, there occurs a rapid evolution of delayed edema, which peaks within this time period, and that infiltration of polymorphonuclear cells follows a similar time course. Vascular endothelial leakiness was proposed to result from the release of lipid inflammatory mediators through the interaction of injured tissue with infiltrating leukocytes and aggregating platelets. Among the possible mediators of BBB dysfunction and formation of vasogenic edema, proteases, bradykinin, histamine, and eicosanoid products of arachidonic acid metabolism and free radicals have been strongly implicated.

It is well documented that neuronal injury is reduced by induction of hypothermia during ischemia, or even by its induction during the post-ischemic reperfusion period. The experiments in the present study quantitate for the first time a protective effect of post-ischemic hypothermia on the delayed BBB injury that follows temporary MCAO. Mean $K_i$ values after 46 hours of reperfusion were more than 50% lower in rats that underwent cooling to 29°C for 6 hours. Although a light degree of halothane anesthesia in the cooled rats may have contributed a protective effect, efficacy of lowered brain temperature per se was suggested by the fact that 28°C - 29°C was more protective than 32°C - 33°C. Separate experiments showed, however, that the 6 hours of hypothermic treatment at 28°C - 29°C did not reduce the amount of edema present at 46 hours post-reperfusion, even though this treatment appeared to favourably affect both BBB damage and infarct volume.

Post-ischemic hypothermic protective mechanisms may be related to a slight attenuation in the reperfusion hyperemia. During reperfusion, hypothermia may reduce leukotrienes, improve glucose utilization and blood flow, and slow free radical reactions and the propagation of lipid peroxidation cascades. This could prevent the leakage of proteins and the accumulation of extracellular fluid and inhibit the biosynthesis, release and uptake of neurotransmitters, such as glutamate and dopamine. There are two massive glutamate release points in the brain damage. Therefore, any reduction of glutamate concentration in the extracellular space by delayed hypothermia may protect against brain damage.

In conclusion, following transient focal ischemia, measurements of $K_i$ for BBB permeation of $^3$H-sucrose have demonstrated both an acute opening, likely hemodynamic in nature, and a delayed opening of the ipsilateral cortex MCAO. The quantal release of glutamate has been recently correlated with the size of neocortical infarction in focal ischemia and may result in endothelial cell damage in the BBB. The blockade on the non-NMDA glutamate receptors attenuates brain damage. Therefore, any reduction of glutamate concentration in the extracellular space by delayed hypothermia may protect against brain damage.

In this study, partial recovery from the acute opening of the BBB was evidenced by the fact that $K_i$ values at one or four hours post-reperfusion were significantly lower than those measured acutely, but were higher than baseline values, or values for contralateral non-ischemic cortex. The second part of the biphasic opening was then indicated by the significant elevation for contralateral non-ischemic cortex. The second part of the opening, which presumably differs from that underlying the acute post-ischemic opening, is likely hemodynamic in nature, and a delayed opening of the ipsilateral cortex MCAO. Clearly, hypothermia has potent effects on BBB opening and does reduce infarct size, but through mechanisms other than reducing edema. The quantitative power of $K_i$ measurements should facilitate the exploration of drug or other treatments to offset the disadvantages of reperfusion therapy (BBB opening and edema) and facilitate its benefits. The understanding of the interplay between microvascular damage, edema, inflammation, and neuronal death hours after thromboembolic stroke is critical to the development of successful stroke therapies.
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