Early Changes in Blood Brain Barrier Permeability To Small Molecules After Transient Cerebral Ischemia

J. I. Sage, M.D.,* R. L. Van Uitert, M.D.,† and T. E. Duffy, Ph.D.

SUMMARY Brain unidirectional extraction and flux of leucine were measured simultaneously with cerebral blood flow (CBF) at various times after transient global cerebral ischemia in the rat. The results permit an evaluation of blood-brain barrier permeability in the postischemic period independent of alterations in CBF at the time of measurement. Leucine extraction was higher (p < 0.001) than that of CBF-matched controls at 15 min and 6 hr after 30 min of global cerebral ischemia, but was not different from control at 30 min and 1 h after ischemia. Leucine flux into brain was increased only at 15 min after reperfusion of the brain.

Cerebral edema occurs 15–30 min after reperfusion in this ischemia model, but the permeability of the blood-brain barrier to large molecules is unaltered during this period (Petito et al: J Neuropath Exp Neurol 41: 423–436, 1982). Increased barrier permeability to small molecules such as leucine may contribute to the production of this early postischemic edema.

ISCHEMIA may compromise the normal function of the blood-brain barrier to selectively limit the entry of substances into brain. Studies which measure the quantity of substances accumulating in the brain over time suggest that when ischemia progresses to infarction, both large molecules [e.g. Evans blue-albumin and horseradish peroxidase4,5] and small molecules [e.g. sucrose6,7] readily penetrate the barrier. In contrast, present evidence indicates that in settings of ischemia without infarction the blood-brain barrier remains competent to prevent large molecules from accumulating in brain; little information is available regarding barrier permeability to small molecules under such conditions.

Traditionally, blood-brain barrier permeability changes have been assessed by measuring the accumulation of tracers in brain. Accumulation measurements, however, must occur over relatively long periods of time and cannot differentiate increased entrance of substances into brain due to disruption of the barrier from decreased utilization or removal of these substances from brain. Possible disruption of blood-brain barrier functional integrity within minutes of an ischemic insult therefore cannot be detected by such procedures. In order to assess barrier function after ischemia, unidirectional extraction by brain and flux of materials into brain must be measured.

The rapid shifts in cerebral blood flow (CBF) that occur after cerebral ischemia alter the availability of materials to brain, and thus complicate studies designed to assess changes in blood-brain barrier permeability due to ischemia alone. Extraction of substances from blood by brain is not constant even in the normal animal, but varies as a complex function of CBF8. The most frequently used measure of barrier function, the brain uptake index technique,7,8 does not measure CBF but relies upon constant blood flow for accuracy; therefore, it cannot be employed appropriately after cerebral ischemia. In contrast, the technique of Sage et al.6 permits the simultaneous measurement of brain extraction and CBF, and thus allows data derived from previously ischemic animals to be compared to control animals with similar cerebral blood flows. The present study employs this method to measure the extraction of leucine by brain and its unidirectional flux into brain at various times after global cerebral ischemia in rats.

Methods

Transient bilateral cerebral hemispheric ischemia was achieved according to the method of Pulsinelli and Brierley.9 One day prior to the experiment, male Wistar rats weighing between 250 and 300 gm were anesthetized with diethyl ether and both common carotid arteries were isolated through a ventral, midline cervical incision. An atrumatic arterial clasp10 was placed loosely around each common carotid artery without interrupting carotid blood flow, and the incision was closed with metal clips. A second incision, 1 cm in length, was made behind the occipital bone overlying the first two cervical vertebrae, and with the aid of an operating microscope the right and left alar foramina of the first cervical vertebra were exposed. A 0.5 mm diameter electrocautery needle (Bovie Monopolar Electrocautery, Cincinnati, OH) was inserted through each alar foramen and both vertebral arteries were permanently occluded by electrocautery. Tail artery and jugular vein cannulae (PE-50) were inserted, and the animals recovered without sequelae. The rats were fasted overnight but were allowed free access to water.

The next day, awake rats were hand held and loosely

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restrained, the ventral neck clips were removed, and both carotid clamps were tightened to produce vascular occlusion. Carotid clamps were removed after 30 min of ischemia and restoration of carotid flow was verified by direct observation. The behavior of the animals was observed during the period of occlusion, and only those animals that did not exhibit a righting response to manually applied pressure on the tail were accepted for further experimentation. Body temperature during and following the period of carotid occlusion was maintained close to 38°C with a rectal thermometer (Yellow Springs Instrument Co., Yellow Springs, OH) coupled to a heating lamp.

CBF and leucine extraction were measured simultaneously by a method described previously at 15 min, 30 min, 1 h, and 6 h after reperfusion had begun. For these determinations, each rat was placed in a fenestrated acrylic plastic restrainer with its upper incisors positioned over a metal bar and its head secured by a nose brace. A guillotine was placed across the restrainer so that the rat could be decapitated in place. The tail artery cannula was connected via PE-90 tubing to a syringe fitted in a Harvard pump that was calibrated to withdraw blood at a constant rate of 1.57 ml/min. The pump was started and 0.2 ml of a solution containing 5 μCi of n-[1,14C]butanol (1.71 mCi/mmol, New England Nuclear, Boston, Mass.), 7.5 μCi of L-[4,5-3H]-leucine (105 Ci/mmol, Amersham Corp., Arlington Heights, Ill.), 150 mM non-radioactive L-leucine, and 10 mM HEPES-Ringer buffer, pH 7.45, was rapidly injected through the jugular venous cannula. At a time between 7 and 12 s after injection that was predetermined by the anticipated CBF rate, the animal was decapitated and the cannula was simultaneously detached from the tail.

The brain was rapidly removed from the skull and four brain regions (right and left frontal and parieto-occipital cortices) were separated within 1 min of decapitation. Each section was placed into a tared scintillation vial containing 2 ml of Protosol (New England Nuclear). The vials were reweighed and 10 ml of toluene-Omnifluor (New England Nuclear) scintillation mixture were added. The vials were sealed and heated at 50°C for 12 hours to solubilize the tissue. The arterial blood in the cannula was expelled into a scintillation vial and processed in the same manner as the brain samples. Radioactivity in all samples was assayed in a liquid scintillation spectrometer (Searle Mark III).

The relationship between CBF and extraction in non-ischemic rats is E = CBF−2 and has been described in a previous publication. Briefly the rats were prepared with jugular vein and tail artery cannulae in a similar fashion as experimental animals, and were allowed to recover from surgery overnight. The next day each animal was placed inside a clear plastic bag that was continuously flushed with a gas mixture of oxygen, nitrogen, and carbon dioxide at a rate of 4.1 to 8.1 l/min. In order to increase CBF, the CO2 content of the inspiratory gas mixture was altered to produce PaCO2 values ranging from 34 to 84 mm Hg. Some animals spontaneously hyperventilated and consequently lowered PaCO2 to 24–34 mm Hg; CBF did not fall below 80 ml/100 g/min in such animals. In order to decrease CBF below this rate, rats were injected with pentobarbital (55 mg/kg I.P.) 10 min prior to the experiment. Arterial blood gases, pH and blood pressure were monitored, and rectal temperature was maintained close to 38°C. When stable blood gases were achieved, CBF and leucine extraction were measured simultaneously. In this way, a CBF versus extraction curve for non-ischemic control animals was obtained for comparison with leucine extraction in postischemic animals. Cerebral blood flow was calculated according to the method of Van Uttert and Levy, using n-[1-14C]butanol as a reference:

\[
\frac{F_b}{M_b} = \frac{Q_s(T) \cdot F_s}{Q_s(T) \cdot M_s}
\]

where, \( F_b \) = blood flow to the brain; \( F_s \) = rate of withdrawal of blood into syringe; \( M_b \) = mass of brain tissue in grams; \( Q_s(T) \) = quantity of indicator present in brain at time, \( T \); \( Q_s(T) \) = quantity of indicator present in syringe at time, \( T \). This calculated flow (\( F_b \)) was then converted to the actual CBF by correcting for the known butanol extraction at that CBF.

The cerebral extraction of leucine was calculated as described by Sage et al.:

\[
E = \frac{Q_s(T) \cdot F_s}{Q_s(T) \cdot CBF}
\]

where: \( E \) = cerebral extraction of leucine; \( CBF \) = actual cerebral blood flow.

Unidirectional flux of leucine into brain, \( J \), was calculated according to the equation: \( J = E \cdot CBF \cdot C \), where \( C \) = arterial blood concentration of leucine. \( C \) was calculated from the specific activity of injected leucine and the amount of radioactivity accumulated in the syringe.

Blood pressure was monitored continuously from the tail artery cannula by a Statham transducer connected to a Beckman recorder. The pH (Radiometer, Copenhagen, Denmark), PaCO2, and PaO2 (L. Eschweiler and Co., Kiel, West Germany) were determined with microelectrodes on 0.1 ml samples of arterial blood.

Results

Brain extraction of leucine and CBF were measured in 27 rats after subjecting the animals to 30 min of global cerebral ischemia; 7 animals were studied 15 min after carotid clamp release. 8 After 30 min, 5 after 1 h, and 7 after 6 h. In order to evaluate the effects of ischemia on extraction while controlling for changing CBF, each experimental group was compared to the control population by an analysis of covariance (Biomed Package, Program P2V, Analysis of Variance and Covariance Including Repeated Measures). Both covariate and dependent measures were trans-
formed logarithmically prior to analysis in order to reduce non-normality of data.

Five control rats with PaCO₂ values between 35 and 40 mm Hg had cerebral blood flows of 142 ± 4 (S.E.M.) ml/100 g/min (table 1). CBF was significantly elevated at 15 min after ischemia but then fell below the normocapnic control value at 30 min, 1 h, and 6 h after ischemia (table 1).

Leucine extraction in the normocapnic control group with a mean CBF of 142 ml/100 g/min was 0.08 ± 0.01 (S.E.M.) (Table 1), but varied in the other control animals as a function of CBF. Mean leucine extraction by brain at 15 min after ischemia was 0.09, a value that was significantly higher than control when adjusted for differences in CBF between the experimental and control animals (f(1,58) = 15.32; p < 0.001) (table 1). At 6 h after ischemia, mean leucine extraction was 0.22; this value was also higher than control when adjusted for differences in CBF between the experimental and control populations (table 1).

Leucine flux into brain in awake non-ischemic normocapnic control rats (CBF = 142 ± 4) was 0.11 ± 0.02 µmol/g/min. In the experimental animals tested 15 min after ischemia, influx was significantly increased to 0.18 ± 0.04, (p < 0.001 compared to the normocapnic controls; table 1). Subsequent leucine influx at 30 min, 1 h, and 6 h after the ischemic episode tended to be less than normocapnic control, though the difference was significant only at 1 h (p < 0.05).

In restrained animals freely breathing room air, arterial pH and PaO₂ were 7.40 ± 0.05 (S.E.M.) and 83 ± 6, respectively, and PaCO₂ ranged from 24-33 mm Hg. Mean arterial blood pressure was 132 ± 10 mm Hg; mean arterial blood pressure was 130 ± 8 mm Hg. In postischemic animals, arterial pH and PaO₂ were 7.44 ± 0.05 and 88 ± 8, respectively. PaCO₂ was 28 ± 4 mm Hg and arterial blood pressure was 132 ± 10 mm Hg.

### Table 1: Cerebral Blood Flow, Extraction of Leucine by Brain, and Unidirectional Flux of Leucine into Brain after Bilateral Hemispheric Ischemia in Rats

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Measured CBF (± SEM)</th>
<th>Measured extraction (± SEM)</th>
<th>Calculated means of extraction adjusted for differences in CBF (± SEM)</th>
<th>Influx (µmol/g/min) (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-ischemic (n = 5) Normocapnic</td>
<td>142 ± 4</td>
<td>0.08 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Ischemia for 30 min + reperfusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min (n = 7)</td>
<td>200 ± 36*</td>
<td>0.09 ± 0.04</td>
<td>0.09 f(1,58) = 15.32*</td>
<td>0.18 ± 0.04†</td>
</tr>
<tr>
<td>30 min (n = 8)</td>
<td>50 ± 6*</td>
<td>0.18 ± 0.06</td>
<td>0.06 f(1,59) = 4.22</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>1 h (n = 5)</td>
<td>38 ± 2*</td>
<td>0.22 ± 0.02</td>
<td>0.08 f(1,56) = 0.87</td>
<td>0.08 ± 0.01‡</td>
</tr>
<tr>
<td>6 h (n = 7)</td>
<td>50 ± 7*</td>
<td>0.21 ± 0.02</td>
<td>0.11 f(1,58) = 36.24*</td>
<td>0.10 ± 0.03</td>
</tr>
</tbody>
</table>

Cerebral ischemia was produced by permanent occlusion of the vertebral arteries and reversible occlusion of the common carotid arteries. Animals were studied after restoration of carotid blood flow for the time indicated.

*The difference from normocapnic nonischemic animals is significant with p < 0.01 by the Students t-test.

†p < 0.001; ‡p < 0.05.

§Calculated means of extraction are compared to a control value of 0.07 which is the CBF adjusted mean of extraction in hypercapnic and pentobarbital-treated, non-ischemic, controls. *p < 0.001.

### Discussion

Little information exists concerning blood-brain barrier function after ischemia without infarction. The model of bilateral carotid and vertebral artery occlusion in the rat provides an opportunity for such studies. Blood flow to the cerebral cortex during the period of occlusion falls below 3% of control 14 and consistently produces neuropathological evidence of cortical ischemia. 9 However, cerebral cortical infarction has been shown to occur in less than 12% of animals. 5, 14 Post-ischemic cortical blood flow follows a time course similar to that found in other models of ischemia; 16, 17 i.e., transient hyperemia immediately after release of the carotid clamps is followed by a prolonged period of postischemic hypoperfusion that gradually returns toward normal.

The present work demonstrates a biphasic increase of leucine extraction by brain after a 30-min ischemic insult (table 1). Extraction at 15 min and 6 h after ischemia was greater than in control rats with similar cerebral blood flows. The data suggest an early impairment of blood-brain barrier integrity followed by a period of normal barrier function, which is then followed by a second compromise of the barrier's ability to restrict the diffusional movement of small molecules into brain. The work of Dienel et al., using the same model of ischemia, is consistent with our own results. 18 They found that accumulation of TCA-soluble [14C]valine was enhanced in the cortex after 30 min of ischemia and that the increase persisted for 12 h. Spatz et al., 4 previously demonstrated an increased brain uptake index for small molecules (glucose analogues) 5 min after cerebral reperfusion in gerbils that were subjected to unilateral common carotid artery occlusion. The results of Spatz et al, are difficult to interpret, however, since CBF was not measured.

Although calculated leucine extraction increased both at 15 min and 6 h following ischemia, leucine influx into the brain increased only at the earlier time period. The dependence of leucine influx on CBF as well as on...
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extraction accounts for this difference. Fifteen minutes after reperfusion had begun, both increased extraction and increased blood flow contributed to increased leucine influx. Six hours after ischemia, however, CBF fell to a greater degree than extraction rose and thus limited the amount of leucine available in blood circulating to the brain.

The transient increases in CBF and extraction early after ischemia may be linked pathophysiological. Autoregulation of the cerebral vasculature fails after brain ischemia. The period ofposts ischemic hyperperfusion may result from the unopposed effects of blood pressure on a system lacking autoregulatory capacity. Normotension in the posts ischemic period may act on the brain vessels in a fashion analogous to acute hypertension in the non-isch emotic animal and cause a similar disruption of blood-brain barrier integrity. An increase in flux of small molecules into brain may then result, as it does during hypertension, but subsequently resolve once the mechanisms causing hypoperfusion of the brain supervene.

Edema occurs early after an ischemic episode. The concept that this edema may be cytotoxic in origin has evolved from data demonstrating increased brain water content and the concomitantly inability of large molecules to pass from intravascular to extravascular space. In the present model, a significant increase in brain water content has been demonstrated 15–30 min after 30 min of ischemia, a time when the blood-brain barrier is still intact to Evans blue-albumin. At 15 min after the ischemic period, however, barrier permeability to leucine was increased and the flux of leucine into brain was also enhanced (table 1). The entry into brain of small molecules, such as leucine, provides a higher osmotic gradient to water than does the entry of large proteins. Increased barrier permeability to leucine and other small molecules that normally have limited access to brain may thus contribute to the early posts ischemic edema observed in this model. The finding that leucine influx into brain was not different from control at 6 h after the ischemic period is consistent with the absence of cerebral edema at this time.

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The present findings suggest that the "cytotoxic" and "vasogenic" components of brain edema secondary to ischemia may not be readily separable. Small, physiologically-important molecules that enter brain in increased amounts early in the posts ischemic period, such as leucine, are likely to be taken up by cells and thereby contribute to the cytoplasmic swelling seen in posts ischemic edema. Early posts ischemic edema may therefore have both cytotoxic and vasogenic components or may even be largely vasogenic, i.e. secondary to blood-brain barrier incompetence in limiting the movement of small molecules into brain.

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References

Flow Patterns in the Human Carotid Artery Bifurcation

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SUMMARY To elucidate the connection between blood flow and the localized genesis and development of atherosclerosis and thrombosis at the human carotid artery bifurcation, detailed studies of the flow patterns and distributions of fluid velocity and wall shear rate in this region were carried out using a transparent segment of the carotid artery, prepared from a human subject postmortem, and cinemicrographic techniques.

It was found that a recirculation zone which consisted of a pair of complex spiral secondary flows, symmetrical about the common median plane of the bifurcation, was formed in the carotid sinus over wide ranges of inflow Reynolds numbers, $Re_0$, and flow rate ratios, $Q_1/Q_0$ (internal/common). The formation and the size of the recirculation zone were largely dependent on $Q_1/Q_0$, as well as on $Re_0$. The size of the recirculation zone increased from $-4 \text{ mm at } Re_0 = 300$ to a maximum of $-9 \text{ mm at } Re_0 > 800$. The results suggest that, under physiological conditions ($Re_0 \sim 600$, $Q_1/Q_0 \sim 0.7$), a standing recirculation zone exists in the carotid sinus, thereby affecting local mass transfer and interactions of blood cells with the vessel wall, which may lead to the incidence of atherosclerosis and thrombosis in this region.

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THERE IS CONSIDERABLE EVIDENCE that both early atherosclerotic changes of vessel walls and the deposition of platelet thrombi occur preferentially at the entrances of branching arteries where, from a fluid mechanical point of view, flow is likely to be disturbed and separation of streamlines from the vessel wall and formation of eddies may occur. Hence, to elucidate the possible connection between blood flow and the localized genesis and development of atherosclerosis and thrombosis, a considerable amount of work, both theoretical and experimental, has been carried out by many investigators.

Among the theories and hypotheses proposed to account for the localization of atherosclerosis, the causative effects of wall shear stress on atherogenesis have received much attention and are still being fervently debated. Based on his experimental findings in dogs, Fry suggested that atherosclerotic changes occur preferentially at the arterial wall experiencing high shear stress because of the resulting mechanical damage to the arterial endothelium and because of an enhanced transport of lipids, including cholesterol, from the blood to the endothelium. In contrast to this, Caro et al in their post-mortem studies, found that early atherosclerotic lesions in human arteries develop more readily in regions where the wall shear rate is expected to be low, such as at the inner walls of curved vessels and at the hips of bifurcations. Therefore, they suggested that the local wall shear rate exercises a control on atheroma formation through flow dependent diffusion of lipids (synthesized within the arterial wall) away from the vessel wall, leading to the accumulation of lipids in the vessel wall in areas of low wall shear rate.

Because of these contradictory hypotheses on atherogenesis, it became important to obtain exact and precise flow patterns and distributions of shear rate (or shear stress) existing in various branching arteries and to correlate these results with the clinical findings of atherosclerosis and thrombosis.

In the early stage of our investigation into this problem, we described the detailed flow patterns in various glass models of stenoses and T-junctions. More recently, we have developed a new technique to prepare isolated transparent natural blood vessels from animals and humans post-mortem. This has, for the first time, enabled us to study the exact flow patterns and distributions of fluid velocity and shear rate existing in var-
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