Rapid, Transient Drop in Brain Glucose After Intravenous Phloretin or 3-0-Methyl-D-Glucose

WILLIAM H. OLDENDORF, M.D.,*† PAUL D. CRANE, PH.D.,*† PABLO M. LAWNER, M.D.,*‡ AND LEON D. BRAUN, B.A.*†

SUMMARY Rats were injected intravenously with either phloretin (100 mg/kg) or 3-0-methyl glucose (2 g/kg) to reduce the carrier-mediated flux of glucose into brain. Plasma glucose and brain free glucose (BFG), lactate, and glycogen were measured over a 16 min time course. Injection of these substances caused a rapid drop in BFG to 60% of control at one minute and a minimum (50% of control values) at 4 min., followed by a gradual rise to control levels at 16 min. While plasma glucose fell, and then increased after injection, brain lactate and glycogen content was unaffected. Repeated injections of phloretin eventually caused a drop in brain glycogen; but with either competitor, BFG never fell below 50% of normal values. The i.v. injection of the glucose analog, 3-0-methyl glucose (the less toxic of the two drugs) is proposed as a possible means of cutting off the potentially hazardous supply of blood glucose to the postischemic brain.

Methods

Materials
Radioisotopes were purchased from New England Nuclear Corp., Boston, MA. The specific activities were [3H] H2O, 5 mCi/g, and D-[2-14C]-glucose, 57 uCi/μmol. The isotopes were greater than 98% pure as determined by radioscanning after thin layer chromatography, using solvent systems recommended by the manufacturer. Phloretin and 3-OMG were obtained from Sigma Chemical Co., St. Louis, MO. Soluene-100 tissue solubilizer and Instagel liquid scintillation fluid were purchased from Packard Instrument Co., Downers Grove, IL.

Intravenous Injection
Male Wistar rats (Mission Laboratory Supply, Inc., Rosemead, CA), 200-300 g, were given free access to Purina rat chow and water and maintained on a 12 hour light-dark cycle (lights on, 7 a.m.–7 p.m.). The rats were prepared surgically on the afternoon before the experiment. The animals were anesthetized with diethyl ether and a polyethylene catheter (PE-10) was inserted into a tail vein. The catheter was filled with 10% (v/v) heparin (1000 U/ml) in Ringers solution, heat sealed, and the tail covered with an aluminum sheath to prevent catheter dislocation. The next morning the animals were weighed and the sheaths removed immediately prior to injection.
The animals were placed in plastic holders and injected intravenously with Ringers solution (4 ml/kg) containing either phloretin (adjusted to pH 9.5 with NaOH in order to dissolve the phloretin), 3-OMG (2 g/kg), or Ringers solution (4 ml/kg) alone. Another group of animals was not injected. The Ringers solution injected into the controls compared with the phloretin-treated was pH adjusted to 9.5 with NaOH. At various times after administration of the intravenous bolus, the animals were killed by high intensity microwave fixation (2.1 s exposure, 3.8 kW; Metabostat 4094, Gerling Moore, Palo Alto, CA). The heart was exposed within 30 s after brain fixation; and cardiac blood was withdrawn into a heparinized syringe. After immediate centrifugation (600 g, 5 min), 0.025 ml of plasma were removed, mixed with 2 ml of cold 0.05 M Ba(OH)₂, and incubated at room temperature for 10 min. Two ml of cold 0.05 M KH₂PO₄ were added, and after 10 min, the precipitate was removed by centrifugation. The supernatants were stored at −20°C for subsequent glucose analysis. The microwaved head was excised, wrapped in latex and cooled in an ice bath for about 30 minutes. The brain was then removed and a hemisphere weighed and homogenized in 10 ml of distilled water in a glass homogenizer (Ten-Broeck, 15 ml). The homogenate was centrifuged at 10,000 g for 30 min at 4°C in polycarbonate tubes. The supernatant was stored at −20°C for further analysis.

Carotid Injection

In some animals, serum from a decapitated, similarly treated rat was used as the vehicle for carotid injection. Serum was prepared for a carotid bolus vehicle in the following manner. Rats were injected intravenously with Ringers solution (4 ml/kg, pH 9.5) with or without phloretin (100 mg/kg). Two min after injection, the rats were decapitated and trunk blood was collected. The blood was centrifuged (600 g, 5 min), and the serum used for bolus mixture. A 0.2 ml aliquot of either Ringers solution or serum was mixed with 0.5 uCi of [2,14C]-D-glucose and 4 uCi of [3H]-H₂O₂, and rapidly (<1 sec) injected into the right common carotid artery of a sodium pentobarbital anesthetized rat (50 mg/kg, i.p.). The rat has been pretreated intravenously with either 100 mg/kg phloretin or 4 ml/kg of Ringers solution two min before the carotid injection. Five sec. later the rat was decapitated, the brain removed, and the right hemisphere was solubilized in 1.5 ml of Soluene-100 at 60°C for 2 hr. Ten ml of Instagel were added and the samples were counted for 3H and 14C in a Packard Tricarb Model 3390 Liquid Scintillation Spectrometer. The brain uptake index (BUI) was calculated:

\[
\text{BUI} = \left( \frac{14\text{C}}{3\text{H}} \text{ dpm in brain} \right) \times 100,
\]

Biochemical Analysis

Plasma glucose and BFG and lactate levels were determined by enzymatic (fluorometric) analysis. Since brain glycogen was found to be distributed in both the 10,000 g supernatant and precipitate fractions, it was necessary to assay both fractions for glycogen content. Five ml of cold, 95% ethanol were added to the 10,000 g precipitate; and, after vortexing for 30 sec., a second supernatant was obtained by centrifugation at 3000 g for 15 min. at 4°C. This supernatant was discarded; and the glycogen extracted as glucose from the precipitate by incubation at 100°C for 30 min. in 1M HCl. The hydrolysate was adjusted to pH 8 with 1M Tris base and assayed for glucose content as above. One ml of the 10,000 g supernatant was mixed with one ml of 2 M HCl and incubated at 100°C for 30 min. The hydrolysate was adjusted to pH 8 with 1M Tris base and assayed for glucose as above.

Statistical Analysis

Statistical significance at the p < 0.05 level was assessed by Student's t test. Multiple comparisons were tested with Dunnett's test for multiple comparisons with a control.

Results

A time course of brain and plasma glucose content, determined after intravenous administration of either phloretin (100 mg/kg), or Ringers solution (4 ml/kg, pH 9.5), and in noninjected animals, is shown in figures 1 & 2. Phloretin injection caused an immediate drop in plasma glucose, followed by a rapid rise (fig. 1). Significant increases in plasma glucose were seen from 8-16 min. after phloretin injection. Rats given Ringers solution (pH 9.5) experienced the same early
A rapid fall and rise in plasma glucose; but at later times, in contrast to the phloretin-treated rats, they encountered a mild hypoglycemia. The content of glucose in brain also fell rapidly after phloretin injection, reaching a minimum of 1 μmol/g at 4 min., figure 2. The effect was relatively short-lived, as normal BFG levels were achieved at 16 min. Ringers (pH 9.5) treated rats had a transient (< 1 min.) fall in BFG. However, at later times the levels did not differ from non-injected rats until 16 min. post-injection. No significant changes were observed in brain glycogen or lactate content throughout the 16 min time course, figure 3.

The dose dependency of alterations in brain and plasma glucose content at 4 min after phloretin injection is given in table 1. While no significant changes were observed in plasma glucose, BFG content dropped at doses above 50 mg/kg, leading to a 50% decrease in the volume of distribution of glucose between brain and plasma. Increasing the phloretin dose from 100 to 200 mg/kg produced no further drop in BFG.

This threshold of BFG content was maintained even after more prolonged exposure to phloretin, table 2. Rats injected with three doses of phloretin, with a total exposure time of 20–24 min., had no further reduction in BFG than the minimum (approx. 1 μmol/g) encountered after a single injection. While no alterations were seen in either plasma glucose or brain lactate, a significant drop in brain glycogen content appeared after three injections.

The glucose analog, 3-OMG, had a similar effect on brain and plasma glucose content, figure 4. A rapid fall in BFG occurred, with a minimum near 1 μmol/g. Control levels were regained by 16 min. Plasma glucose rose after 4 min. and a hyperglycemic condition persisted for the remainder of the experimental period.

The BUI's for 14C glucose in control and phloretin-treated rats are given in table 3. Inclusion of 0.1 mM phloretin in the carotid bolus resulted in a 38% reduction in brain uptake. A similar reduction in glucose uptake was effected by intravenous injection of 100 mg/kg of phloretin 2 min before measurement of glucose transport. In order to maintain the bolus phloretin level at the same concentration as the systemic level, serum, obtained from a rat 2 min. after 100 mg/kg phloretin administration, was used as the vehicle. Control rats, injected with serum from a rat pretreated with Ringers solution (adjusted to pH 9.5 to account for the alkaline solution used to solubilize the phloretin) had significantly lower BUI's than non-serum controls. This was expected, as plasma glucose was 5–7 mM and the Km for glucose transport across the BBB is 9 mM. Phloretin-treated animals had a further re-

**Table 1. Dose Response of Glucose Content in the Rat 4 min after Phloretin Administration**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Brain glucose (μmol/g)</th>
<th>Plasma glucose (μmol/g)</th>
<th>V_D* (ml/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.24 ± 0.18</td>
<td>7.90 ± 1.06</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>50</td>
<td>1.90 ± 0.11</td>
<td>7.94 ± 1.13</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>100</td>
<td>1.04 ± 0.28</td>
<td>9.90 ± 0.50</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>200</td>
<td>1.03 ± 0.31</td>
<td>6.78 ± 1.15</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
</table>

*V_D* is the volume of distribution of glucose between brain and plasma, i.e., brain glucose/plasma glucose. The data is expressed as mean ± S.D.

*p < 0.01, using Dunnett's method for making multiple comparisons with a control.

*Number of animals in each group.
TABLE 2  Plasma and Brain Glucose, Lactate, and Glycogen in the Rat After Repeated Intravenous Injections of Phloretin*  

<table>
<thead>
<tr>
<th># of Injections</th>
<th>Injection† times (min)</th>
<th>Microwave† time (min)</th>
<th>Plasma glucose (μmol/ml)</th>
<th>Brain glucose (μmol/g)</th>
<th>Brain lactate (μmol/g)</th>
<th>Brain glycogen (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.76 ± 1.71</td>
<td>1.86 ± 0.20</td>
<td>1.45 ± 0.28</td>
<td>2.99 ± 0.56</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>16</td>
<td>12.08 ± 1.73</td>
<td>2.10 ± 0.18</td>
<td>0.89 ± 0.27</td>
<td>2.47 ± 0.62</td>
</tr>
<tr>
<td>2</td>
<td>0,8</td>
<td>12</td>
<td>8.22 ± 0.04</td>
<td>1.49 ± 0.43</td>
<td>1.20 ± 0.18</td>
<td>2.59 ± 0.75</td>
</tr>
<tr>
<td>3</td>
<td>0,8,16</td>
<td>20</td>
<td>7.37 ± 0.93</td>
<td>1.09 ± 0.24</td>
<td>1.25 ± 0.31</td>
<td>1.99 ± 0.57</td>
</tr>
<tr>
<td>3</td>
<td>0,8,16</td>
<td>24</td>
<td>7.71 ± 0.23</td>
<td>0.93 § ± 0.24</td>
<td>1.36 ± 0.64</td>
<td>1.37 § ± 0.08</td>
</tr>
</tbody>
</table>

*Fifteen rats are either controls or injected intravenously 1, 2, or 3 times with phloretin (100 mg/kg) in Ringers solution, pH 9.5. Data are the means ± SD of three rats per entry.  
†Elapsed times are given as minutes after the first injection.  
‡p < 0.05.  
§p < 0.01.  

Discussion  
The large flux of glucose from blood to brain is dependent upon carrier mediated BBB transport. That glucose has a trivial diffusible component is suggested by the lack of transport of L-glucose. Our expectation was that flooding the BBB with substances inhibiting BBB glucose transport could measurably impair the flux of glucose into brain and this would result in a reduction of BFG as the brain used up this substrate in the presence of its reduced flux from blood. The two substances chosen here were a highly competitive non-hexose, phloretin and the non-metabolized hexose, 3-OMG.  
Phloretin is the aglycone of the glycoside phlorizin found in the bark and stems of apple and other fruit trees. While both phlorizin and phloretin have high affinities for both passive and sodium-dependent glucose transport, phlorizin has a higher affinity for sodium dependent transport and phloretin has a greater affinity for passive glucose transport. Since the BBB transport of glucose appears to be passive (bidirectionally symmetrical and non-sodium dependent) it might be expected that phloretin would have an especially high affinity for the BBB hexose carrier. Previous studies in this laboratory indicate that phlorizin has about 25 times the affinity for the hexose carrier as does glucose and that phloretin has about 150 times the glucose affinity. We have also measured the affinity of a number of hexoses for the hexose carrier and among these was 3-OMG which has about 0.9 times the affinity for the carrier as does glucose. Since 3-OMG has no affinity for hexokinase (nor does it have a known metabolic pathway in brain) it was chosen as an alternative competitor.  
Both inhibitors caused similar drops in BFG. However, the BFG could not be depressed below about one micromole per gram brain. Doubling the dose of either competitor did not depress the BFG further. The BFG supply could not have been maintained by glycogenesis, since glycogen levels were unchanged, Fig. 3. The glycogen present in brain is inadequate to be the sole source of the residual one micromole/gram BFG for even these few minutes of impaired BBB flux without showing a measurable reduction of brain glycogen. An alternative explanation is that the BBB transport is not entirely carrier mediated and that there is a substantial diffusible component. We have discarded this notion in the past because L-glucose has no measurable unidirectional flux into brain after carotid injection and we have presumed this would be representative of any diffusible component in glucose transport. When
saturating \(^{14}\)C-D-glucose uptake by adding cold D-glucose to the arterial injectate in a single pass clearance model, we have been unable to reduce the brain uptake of \(^{14}\)C-D glucose below about 5% in the presence of even 80 mM glucose.\(^2\), \(^3\) The Km of the BBB hexose carrier is 8–9 nM in the rat, and it is assumed that the failure to further inhibit glucose entry represents this very large capacity of the carrier and perhaps some dilution of the arterial bolus by arterial blood. Although non-carrier mediated transport of glucose remains an unattractive hypothesis to explain the failure of large doses of competitors to more completely depress BFG it remains a possibility. We can offer no satisfactory explanation for the failure of large doses of competitor to more completely depress BFG.

The early plasma hypoglycemia seen after the phloretin and Ringers injections (figure 1) can be explained as a dilutional effect to the injection of a large volume of glucose-free solution. In future studies this can be prevented by adding 8 mM glucose to the injectate.

The impairment of brain recovery after incomplete ischemia has been correlated with a high level of tissue lactic acidosis.\(^12\)-\(^14\) Recovery is worsened by glucose treatment either before the ischemic episode\(^12\)-\(^18\) or during the recirculation period.\(^19\) The source of the lactic acid is ultimately BFG as brain carbohydrate reserves are converted nearly quantitatively to lactic acid during severe ischemia.\(^20\) The condition may be further aggravated by hypermetabolism during the recovery period.\(^21\), \(^22\) Since total ischemia results in a lower content of tissue lactic acid and a better recovery of metabolic\(^23\) and electrophysiological\(^24\) function than does incomplete ischemia, many investigators, although not all,\(^25\) have suggested that the postischemic supply of blood glucose may represent a hazard to the recovering brain.

The basic motivation behind the studies presented here was to demonstrate whether or not systemic flooding of the BBB hexose carrier by non-toxic substances could measurably reduce the BFG. The speed with which this reduction of BFG occurs suggests it could usefully be studied in an animal stroke model. The rapid onset of the reduced BFG and its brief duration suggest it would have considerable clinical flexibility (by repeated doses) in manipulating BFG in the presence of ischemia.

To be considered for clinical application requires that potential competitive substances be non-toxic. Although phloretin has been found noncompetitively to inhibit glucose transport,\(^10\) it is also associated with increased phosphatase activity and decreased ATP levels.\(^26\) We have also found (unpublished observation) that phloretin has a measurable effect on the BBB transport of monocarboxylic and amino acids. Phloretin is not very water-soluble unless the pH is raised above 9. Our initial studies were carried out using propylene glycol as the diluent. This caused hematuria which is due to the propylene glycol, since the studies reported here used alkalinization to solubilize the phloretin and no hematuria was noted. We are unaware of any literature concerning the toxicity of 3-OMG. We have injected, as a single dose, 4 grams/kg in 3 ml of Ringers solution intraperitoneally in one Wistar rat with no evident immediate toxic effect or any observable influence on subsequent behavior or weight gain followed for one month. If there is any toxicity, it is likely to be very low. Of the two inhibitors reported here, 3-OMG appears to be the better choice for subsequent trials in an animal stroke model due to its lower toxicity and greater specificity.

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References


TABLE 3 Brain Uptake Indices For \(^{14}\)C Glucose in Control and Phloretin-Treated Rats

<table>
<thead>
<tr>
<th>Intravenous injection*</th>
<th>Bolus vehicle for carotid injection</th>
<th>BUI† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninjected</td>
<td>Ringers solution, 15 mM Hepes, pH 7.4</td>
<td>33.0±1.9</td>
</tr>
<tr>
<td>Noninjected</td>
<td>Ringers solution, 0.1 mM Phloretin, pH 9.5</td>
<td>12.5±3.4</td>
</tr>
<tr>
<td>Ringers solution, pH 9.5, 4 ml/kg</td>
<td>Serum from a rat injected i.v. with Ringers solution (pH 9.5) 2 min before sampling</td>
<td>21.3±0.9</td>
</tr>
<tr>
<td>Phloretin, 100 mg/kg, in Ringers solution, pH 9.5</td>
<td>Serum from a rat injected i.v. with phloretin, 100 mg/kg, in Ringers solution, pH 9.5, 2 min before sampling</td>
<td>12.9±0.4</td>
</tr>
</tbody>
</table>

* Rats were injected with the indicated dosage via an indwelling catheter implanted in a tail vein.
† Values are the means ± SD of three rats.
Cerebral Artery Mass in the Rabbit is Reduced by Chronic Sympathetic Denervation

ROSEMARY D. BEVAN, M.D., HIROMICHI TSURU, M.D., AND JOHN A. BEVAN, M.D.

SUMMARY  Weights of matching right and left middle or posterior cerebral arteries and their main branches from the same animal were compared 8–10 weeks after unilateral denervation by superior cervical ganglionectomy. When compared in pairs, the denervated arterial systems weighed significantly less (mean 85%) than their innervated counterparts. This suggests that the sympathetic innervation exerts a trophic influence on extracerebral arteries.

THERE IS NOW CONSIDERABLE EVIDENCE that the sympathetic nervous system influences not only the tone of vascular smooth muscle1 and its sensitivity to vasoactive agents,2 but also the structure and constituents of the blood vessel wall.3,4 It may be of particular importance in influencing the structure and reactivity of the vasculature during its development.1,7 A number of studies based on experiments in which the effects of pre- or postganglionic interruption of the sympathetic supply to the rabbit ear artery have been analyzed, indicate a complex age-dependent effect on the blood vessel wall which weighs less, is thinner and stiffer, and because of a diminution in muscle mass is less capable of developing tone. The sympathetic nervous system modulates the metabolic activity of cells and therefore might be expected to affect the balance of synthesis and degradation not only of smooth muscle constituents but the elaboration of extracellular collagen, elastin and glucosaminoglycans.

Diseases of cerebral blood vessels in man are a common cause of morbidity and mortality. For this reason it was considered of interest to determine if the sympathetic innervation influenced cerebral blood vessels in a manner similar to that established in peripheral vessels. Because of the extreme variability of the pial circulation and the difficulty in demonstrating the small differences that result from denervation, measurements were restricted to weights of comparable innervated and denervated vascular segments. Our measurements show that the net weights of two cerebral arteries and their main branches are significantly reduced by this procedure, a finding that suggests that cerebral arterial structure is influenced in a trophic manner by the sympathetic nervous system.

A preliminary report has been previously published.4

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From the Department of Pharmacology, School of Medicine and Brain Research Institute, University of California, Los Angeles, California 90024.

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Address correspondence to: Rosemary D. Bevan, M.D., Department of Pharmacology, School of Medicine, University of California, Los Angeles, California 90024.

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