Verapamil: Failure of Metabolic Amelioration Following Global Forebrain Ischemia in the Rat

Joseph R. Berger, M.D., Raúl Busto, B.S., and Myron D. Ginsberg, M.D.

Summary The potential efficacy of the calcium channel blocker verapamil in modifying ischemic brain injury was evaluated in anesthetized rats subjected to 60 or 90 min of diffuse forebrain ischemia produced by bilateral occlusion of the carotid and vertebral arteries. Treated animals received verapamil, 2 mg/kg intravenously, prior to ischemia. Four hours of posts ischemic recirculation was permitted by reversing the carotid occlusions. Intermittent high-voltage slow-wave activity was noted on electroencephalograms shortly after verapamil infusion, prior to ischemia. The ischemic insult induced an isoelectric EEG, which tended to persist during recirculation in both treated and untreated animals. Similarly, verapamil pretreatment failed to influence brain water content or cerebral energy metabolites (phosphocreatine, ATP, ADP, AMP) or cerebral energy charge when assayed after four hours of recirculation. Thus, verapamil failed to confer a protective effect on brain electrical activity, water content, or energy metabolites following ischemia in this model.

CONSIDERABLE RECENT ATTENTION has been devoted to the role of perturbed calcium ion homeostasis in contributing to hypoxic-ischemic cellular injury. Normally, intracellular calcium ion activity is regulated at a level several orders of magnitude less than that of the extracellular space. During cerebral ischemia, intracellular calcium ion activity has been shown to decline at cerebral blood flows below 6–9 ml/100 g/min, concomitant with membrane depolarization as signalled by rising extracellular potassium ion activity, suggesting that the opening of voltage-dependent calcium ion channels within the plasma membrane has permitted the intracellular influx of this ion. In addition, the energy failure of ischemia results in a release of calcium from its normal ATP-dependent sites of sequestration in the endoplasmic reticulum. Finally, ischemia impairs the ability of mitochondria to accumulate calcium. The resulting increase in free cytoplasmic calcium activity disrupts a variety of cellular functions and, in particular, may be responsible in part for the breakdown of membrane phospholipids and massive release of free fatty acids due to stimulation of phospholipases. The latter events, in turn, have been implicated in contributing to irreversible cellular injury.

A deleterious effect of the intracellular influx of calcium is strongly suggested by recent studies in extracerebral tissue. Schanne et al reported that hepatocytes cultured in a calcium-depleted medium were able to withstand exposure to a variety of otherwise noxious membrane-active toxins. Farber and colleagues demonstrated that survival of heart and liver cells following ischemia, verapamil decreased intracellular calcium accumulation in association with the preservation of high energy phosphate compounds and myocardial function following reperfusion. Thus, it is possible that calcium channel blockers might exert a protective effect in brain ischemia as well. Preliminary studies of calcium antagonists in brain ischemia have demonstrated improved cerebral blood flow in treated animals following the ischemic insult. The present study was designed to assess the potential efficacy of a calcium-channel entry blocker, verapamil, in mitigating the consequences of transient cerebral ischemia in the rat. For this purpose, we utilized a well-characterized model of brain ischemia in the rat and assessed recovery by means of the electroencephalogram, water content, and energy metabolites. Since ischemia of 30-min duration in this species is generally followed by high-grade recovery of energy metabolism and function, we have instead investigated longer periods of ischemia (60 and 90 min), — periods after which brain energy metabolism in the untreated animal characteristically remains somewhat depressed.

Methods Male Wistar rats weighing 280–300 g were subjected to periods of diffuse forebrain ischemia by the method of Pulsinelli and Brierley as modified in our laboratory. In brief, the animals were prepared as follows: The vertebral arteries were electrocoagulated under halothane anesthesia. One day later, anesthesia was induced with diethyl ether. D-tubocurarine, 5 mg, was injected intraperitoneally, a rapid tracheostomy was performed, and animals were respired with a constant-volume rodent respirator on 1–1.5% halothane, 69% nitrous oxide, and 30% oxygen. The rectal temperature was maintained at 37.0 ± 0.5 degrees C, and arterial blood pressure recordings, bipolar electroencephalogram, and intermittent measurements of arterial PO2, PCO2, and pH were obtained as described previously.

After initial preparation, halothane was discontinued and at least 20 minutes was allowed to elapse to ensure a steady-state prior to initiation of the ischemic insult. Bilateral forebrain ischemia was produced by occlusion of both common carotid arteries with poly-
ethylenediaminetetraacetic acid (EDTA) as described previously. Since preliminary studies had shown that forebrain energy metabolites were measured by the direct enzymatic-fluorometric technique of Lowry and their levels of high-energy phosphate compounds during ischemia varied considerably among animals and tended to correlate with the degree of EEG suppression during ischemia and mean arterial blood pressure levels during recirculation, the following criteria were utilized in the present study to ensure uniform severity of ischemia: 1) total abolition of EEG activity within 1 min from the onset of carotid occlusion; 2) isoelectric EEG throughout the occlusion; and 3) maintenance of mean arterial blood pressure above 90 mm Hg during the recirculation period. To ensure uniform, severe ischemia, the blood pressure was reduced to 100–110 mm Hg by controlled arterial hemorrhage into a heparinized plastic syringe, beginning 10 min prior to the onset of carotid occlusion (total amount 4–5 ml). (In some animals, it was necessary to reinfuse a portion of this blood during the ischemic insult in an effort to prevent hypotension.)

Two durations of ischemia were studied: 60 min and 90 min. Verapamil-treated and non-treated animal groups were compared. Treated animals received verapamil, 2 mg/kg, by a 5-min intravenous infusion delivered 30 min before initiating carotid occlusion; this dose was the highest shown in preliminary studies to be tolerated without inducing hypotension or significant bradycardia. Non-treated control animals received an identical amount of saline adjusted to the same pH as that of the verapamil-containing vehicle. Additional verapamil (1 mg/kg) or saline placebo, respectively, was administered slowly at the time of the ischemic insult. Total numbers of successful animals are given in table 3a. An additional 5 animals failed to meet the physiological criteria presented above; these were discarded.

Post-ischemic recirculation was initiated by removal of the carotid ligatures and reinfusion of the shed blood. After four hours of recovery, brains were frozen in situ. Right and left supratentorial brain structures from each animal were combined and ground under liquid nitrogen. Brain water content was assayed by the wet weight/dry weight method as previously described. Energy metabolites were measured by the direct enzymatic-fluorometric technique of Lowry and Passonneau. A portion of the tissue powder was weighed in a Cahn balance at ~30 degrees C and extracted with 0.1 N hydrochloric acid in 99% methanol. The sample was then deproteinized with perchloric acid and the solution neutralized to pH 6.0. The precipitated salt solution was centrifuged and the final supernatant used for assays of phosphocreatine, ATP, ADP, AMP, pyruvate, lactate and glucose. Energy charge was calculated according to Atkinson as:

\[
\text{ATP + 0.5 x ADP} = \text{ATP + ADP + AMP}
\]

Statistical Analysis

Comparison of corresponding data in non-treated vs. treated animal groups was performed by means of Student's t-test. A two-way analysis of variance was used to analyze the physiological variables of table 1.

Physiological Variables

These are shown in table 1. A slight (statistically insignificant) degree of acidosis was present during the ischemic period in both the non-treated and verapamil-treated animals and increased somewhat following four hours of recirculation. No differences were noted, however, between non-treated and treated animals. Arterial PCO2 in the treated 60-min-ischemia group declined somewhat during the ischemic insult. Otherwise, arterial blood gases were maintained in the normal range. Mean arterial blood pressure during ischemia was lower in the verapamil-treated animals than in the non-treated groups, but these differences did not attain statistical significance (Student t-test). This blood-pressure difference possibly represents an effect of the drug itself.

Table 1: Physiological Measurements in Non-treated and Treated Animals

<table>
<thead>
<tr>
<th></th>
<th>Non-treated</th>
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<th>Treated</th>
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<tbody>
<tr>
<td></td>
<td>60 and 90 min ischemia (n = 6)</td>
<td>60 min ischemia (n = 5)</td>
<td>90 min ischemia (n = 3)</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>During</td>
<td>After 4 hour recirculation</td>
</tr>
<tr>
<td>PCO2 (mm Hg)</td>
<td>37.7</td>
<td>±1.4</td>
<td>38.1</td>
</tr>
<tr>
<td>PO2 (mm Hg)</td>
<td>143.9</td>
<td>±5.0</td>
<td>118.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.438</td>
<td>±0.013</td>
<td>7.349</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>124.1</td>
<td>±5.0</td>
<td>100.8</td>
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</table>

Values are mean ± SEM. Significant difference compared to corresponding value before ischemia: *p < 0.05, †p < 0.01.

MAP = mean arterial pressure.
mals, transient intermittent high-voltage slow-wave activity was noted within one minute of verapamil administration (fig. 1). During ischemia, the EEG remained isoelectric in all animals. Occasional slow-wave activity emerged during the early recirculation period in some animals, but by four hours of recirculation the EEG was virtually isoelectric in all cases, and no difference was noted in the EEGs of non-treated vs. verapamil-treated animals.

Brain Water Content

The results are shown in Table 2. In the non-treated animal groups, values obtained in the 90- and 60-min subgroups did not differ from each other and hence were pooled. As is evident from table 2, brain water content of verapamil-treated animals with 60 or 90 min of prior ischemia did not differ from the pooled non-treated ischemic controls.

Cerebral Metabolites

Table 3 presents the metabolite data of this study. Following 4 hours of postischemic recirculation, no significant differences in energy metabolite levels were apparent between non-treated and verapamil-treated animals in either the 60- or 90-min ischemia subgroups. Similarly, there were no significant differences in energy charge potential, in the sum of ATP, ADP, and AMP, or in the ATP/ADP ratio (table 3b).

Discussion

Verapamil was selected as the calcium channel blocker in these experiments because it is a nonpolar compound of low molecular weight (— 491) and hence expected to cross the blood-brain barrier. The transient blocker in these experiments because it is a nonpolar compound of low molecular weight (— 491) and hence expected to cross the blood-brain barrier.

Table 2: Brain Water Content

<table>
<thead>
<tr>
<th></th>
<th>Normal 60- and 90-min ischemia</th>
<th>60-min ischemia</th>
<th>90-min ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 6)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>No ischemia</td>
<td>77.4 ± 0.1</td>
<td>78.6 ± 0.1</td>
<td>78.3 ± 0.2</td>
</tr>
<tr>
<td>4-hour recirculation</td>
<td></td>
<td>79.5 ± 0.5</td>
<td></td>
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</table>

Values are means ± SEM.

Brain Water Content

The results are shown in Table 2. In the non-treated animal groups, values obtained in the 90- and 60-min subgroups did not differ from each other and hence were pooled. As is evident from table 2, brain water content of verapamil-treated animals with 60 or 90 min of prior ischemia did not differ from the pooled non-treated ischemic controls.

Cerebral Metabolites

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Discussion

Verapamil was selected as the calcium channel blocker in these experiments because it is a nonpolar compound of low molecular weight (— 491) and hence expected to cross the blood-brain barrier. The transient high-voltage slowing of the electroencephalogram occurring within one minute of its intravenous administration in this study is consistent with its entry into brain.

We were unable to demonstrate any change in EEG recovery, brain water content or energy metabolites with verapamil treatment after either 60 or 90 minutes of ischemia. (The somewhat lower intra-ischemic blood pressure in verapamil-treated animals (table 1) was not statistically significant and is unlikely to have influenced the degree of ischemia since, even without induced hypotension, ATP levels are virtually depleted in neocortex, striatum, and hippocampus by 30 min of ischemia. Thus, verapamil did not confer any protective effect on the brain following ischemia. These results are consistent with its lack of protection of rat brains subjected to hypoxic injury in the experiments of Karasawa and colleagues. Calcium channel blockers, including verapamil, have also failed to protect rat brains subjected to hypoxic injury in the experiments of Karasawa and colleagues. Calcium channel blockers, including verapamil, have also failed to protect rat brains subjected to hypoxic injury in the experiments of Karasawa and colleagues. In a recent study carried out in a global ischemia model in the rat, Smith et al. were unable to demonstrate an effect of nimodipine pretreat-

Table 3A: Metabolite Levels in Normals and Following Four Hours of Recirculation

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>PCr</th>
<th>Glu</th>
<th>La</th>
<th>Py</th>
<th>La/Py</th>
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<tr>
<td>Normals*</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>(n = 5)</td>
<td>3.01</td>
<td>0.36</td>
<td>0.03</td>
<td>5.21</td>
<td>1.97</td>
<td>1.06</td>
<td>0.11</td>
<td>9.63</td>
</tr>
<tr>
<td>± 0.09 ± 0.03 ± 0.005 ± 0.20 ± 0.17 ± 0.08 ± 0.01 ± 1.14</td>
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<tr>
<td>60 min ischemia</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>1.97</td>
<td>0.19</td>
<td>0.02</td>
<td>5.39</td>
<td>4.48</td>
<td>1.58</td>
<td>0.15</td>
<td>10.86</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>± 0.07 ± 0.01 ± 0.003 ± 0.15 ± 0.47 ± 0.17 ± 0.01 ± 0.60</td>
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<tr>
<td>Treated</td>
<td>1.96</td>
<td>0.20</td>
<td>0.03</td>
<td>4.74</td>
<td>5.55</td>
<td>2.30</td>
<td>0.15</td>
<td>15.66</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>± 0.03 ± 0.01 ± 0.002 ± 0.16 ± 0.64 ± 0.63 ± 0.02 ± 4.31</td>
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<tr>
<td>90 min ischemia</td>
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<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>1.66</td>
<td>0.13</td>
<td>0.07</td>
<td>4.63</td>
<td>6.64</td>
<td>3.31</td>
<td>0.18</td>
<td>20.89</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>± 0.16 ± 0.01 ± 0.04 ± 0.37 ± 0.50 ± 0.81 ± 0.02 ± 8.00</td>
<td></td>
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</tr>
<tr>
<td>Treated</td>
<td>1.58</td>
<td>0.16</td>
<td>0.03</td>
<td>4.57</td>
<td>4.84</td>
<td>6.33</td>
<td>0.23</td>
<td>25.37</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>± 0.19 ± 0.01 ± 0.01 ± 0.54 ± 0.61 ± 3.15 ± 0.04 ± 9.35</td>
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</tbody>
</table>
Values are mean ± SEM (μmoles/g of wet weight.)
* Nitrous oxide anesthetized rats; values taken from Yoshida et al.24
ment on postischemic electrophysiological function, despite evidence of a patchy improvement of delayed postischemic hyperperfusion in treated animals.

Major differences between brain and non-neural tissues may exist with respect to their responses to ischemia as well as with regard to the ability of calcium channel blockers to modify the outcome of ischemia. In cerebral tissue, the dominant phospholipase, phospholipase A₂, has been shown recently to be calcium-independent; this contrasts with the calcium-sensitivity of phospholipase A₁ in non-neural tissues. Large decreases in phospholipid content have been demonstrated in ischemic liver and myocardium, whereas cerebral phospholipids decrease only slightly after ischemia. Several explanations are possible for the resistance of ischemic brain to verapamil treatment: a) the blood-brain barrier may limit the entry of the agent into brain parenchyma; b) release of sequestered intracellular calcium may be more important in contributing to ischemic injury than the influx of extracellular calcium into the cells; and c) a rise in intracellular calcium may not be the primary mediator of metabolic injury in this model of forebrain ischemia.

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References

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