Effects of Calcium Channel Blockers on Neurologic Outcome After Focal Ischemia in Rabbits

P.D. Lyden, MD, J.A. Zivin, MD, PhD, A. Kochhar, PhD, and V. Mazzarella, BS

To determine the efficacy of calcium channel blockers in preserving neurologic function after central nervous system ischemia, we studied three such agents in two animal models. We treated rabbits after inducing ischemia in the brain with intra-arterial microspheres and in the spinal cord using a removable aortic occluding device. We found no benefit, in terms of neurologic functional outcome, from lidoflazine, nimodipine, or nicardipine. All three agents elevated regional blood flow in the spinal cord. We conclude that calcium antagonists are not likely to prove beneficial if used alone in the treatment of focal central nervous system ischemia. (Stroke 1988;19:1020–1026)

Calcium channel blockers increase cerebral blood flow in humans and experimental animals.1–8 When used after ischemic insult, these agents have been shown to ameliorate changes in concentrations of energy metabolism intermediates in the brain, to reduce histopathologic evidence of cerebral infarction, and to restore neuronal electrophysiologic function in some,9–14 but not all,15–18 previous reports. After ischemia, the effect of calcium antagonists on integrated neurologic function, such as behavior, is unclear. In some studies of global cerebral ischemia, calcium antagonists improved neurologic functional outcome when used in conjunction with intensive resuscitation.3,19–24 In other studies, calcium antagonists did not augment postischemic recovery of function.25–27 To clarify the effect of calcium antagonists on recovery of neurologic function, we studied three such agents in new, highly reproducible animal models of central nervous system (CNS) ischemia.

Nicardipine and nimodipine are dihydropyridine calcium channel blockers thought to be selective for cerebral vessels.2,5,9 Lidoflazine is a diphenylalkylamine also thought to have preferential activity in cerebral versus coronary and peripheral vascular territories.19,22 We attempted to establish the efficacy of this class of agents for cerebral ischemia by testing structurally different compounds that are thought to have similar pharmacologic properties.

Materials and Methods

Animal Preparation

The rabbit spinal cord ischemia model (RSCIM) of reversible CNS ischemia has been described in detail.28 Under halothane anesthesia, New Zealand White rabbits (2.5–3.0 kg) have a snare ligature occluding device placed around the abdominal aorta below the renal arteries. The end of the occluder is left accessible through the skin. The rabbit is allowed to recover from anesthesia for a minimum of 2 hours. To induce ischemia, the occluder is tightened and clamped. The rabbit is examined for paraparesis 60 seconds after tightening, and the occluder is tightened again if necessary. All rabbits are completely paralyzed by 120 seconds. At the end of a predetermined occlusion period, the device is unclamped and removed and the skin is closed with surgical staples. A range of occlusion durations are used to generate an ischemia dose–response curve.

To induce irreversible CNS ischemia we have devised a multiple cerebral emboli model (MCEM) using microspheres29 in New Zealand White rabbits (2.5–3.0 kg). The rabbit is anesthetized with halothane, and through a lateral neck incision a 3-cm-long 20-gauge catheter is implanted anterograde in the common carotid artery after the external carotid artery is ligated. The catheter is exposed externally as the incision is closed and is filled with 0.25 ml heparin in normal saline (100 units/ml solution) through an injection cap. The rabbit is allowed to

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recover from the effects of anesthesia for a minimum of 3 hours before embolization.

Unlabeled and iodine-125-labeled tracer microspheres (50 μm, 3M Co., St. Paul, Minnesota) are carefully mixed, suspended in acetone, sonicated vigorously, and dried. From this stock mixture we daily prepare a set of microsphere doses. For each rabbit an appropriate weight of dried microspheres is added to 100 μl of 0.05% Tween-80 in normal saline. The amount of radioactivity present is measured in a gamma counter, and the specific activity of each dose of microspheres is calculated. The suspension is agitated at full power in a sonicator (Branson Model 200 Sonifer Cell Disruptor, Danbury, Connecticut) for 5 minutes, diluted to 0.5 ml total volume with normal saline, and quantitatively transferred to a 0.5 ml gas-tight syringe (Hamilton Co., Reno, Nevada). The rabbit is restrained, and the injection cap is removed to clear the heparin solution from the catheter by allowing backfill with the rabbit’s own blood. The cap is replaced, and the microsphere suspension is quickly injected while the rabbit is awake. The syringe and catheter are flushed with 1.5 ml normal saline to ensure that nearly all the microspheres are delivered into the artery. After injection the rabbit is removed from restraint and examined for evidence of cerebral insult.

**Neurologic Function Ratings**

In RSCIM, each rabbit undergoes aortic occlusion for a predetermined time. In previous work we have determined the general range of durations of ischemia that will produce all grades of function, from completely normal to totally paraplegic. Two and 18 hours after the insult, each rabbit is graded as normal or paraplegic by an examiner blinded to the treatment. Rabbits with questionably detectable motor activity (decreased feeding, grooming, exploration, or obtundation) were graded as abnormal. Rabbits are allowed to live for 4 days and are then killed. Any rabbit that died early is excluded from all data analysis.

In MCEM, rabbits are observed 18 hours after injection of a predetermined weight of microspheres by two observers blinded to treatment. Rabbits are scored as normal, abnormal, or dead. Unequivocal abnormal findings include generalized weakness, markedly reduced motor activity (decreased feeding, grooming, exploration), or obtundation. In our study, the two observers agreed on >95% of the scorings. Equivocal rabbits were examined and scored by a third independent observer and considered to be abnormal if any question remained. Eighteen hours after injection, the rabbits were asphyxiated, the brains were removed and sectioned, and the radioactivity present was measured in a gamma counter. From the specific gravity of the injected mixture of microspheres and the total radioactivity measured in each brain, the total weight of microspheres delivered to each brain was calculated.

**Physiologic Measurements**

To assess the effect of the agents compared with 5 ml saline on blood pressure, heart rate, and blood flow, we prepared two separate groups of rabbits. In one group, mean arterial blood pressure (MABP) and heart rate (HR) were recorded through a 20-gauge catheter placed retrograde in the common carotid artery. Data was monitored continuously using a Transpac disposable transducer (Sorenson Research Co., Salt Lake City, Utah) and a Keithly 500 Data Acquisition System (Boston, Massachusetts) attached to a personal computer. The maximum blood pressure depression was noted, and MABP, HR, and elapsed time at that point were recorded.

For blood flow measurements in the second group, we used a method we have previously reported. Under halothane anesthesia, rabbits had a loop of fishing line placed around the abdominal aorta through a midline incision. After recovery from anesthesia (≥3 hours), the rabbits were restrained and an intravenous line was placed in one ear. We injected 1.5 ml verapamil (5 mg/ml in water:ethanol 10:1) subcutaneously around the central ear artery and then removed a strip of skin to expose the artery. Thirty minutes after each agent was administered, we incised the ear artery and began collecting arterial blood samples onto Whatman #1 filter paper disks (Clifton, New Jersey). Any rabbit that did not bleed briskly from the incision was not used. Twenty-five microcuries of [14C]iodoantipyrine was infused intravenously by mechanical pump over 60 seconds into the marginal vein of the contralateral ear. At the exact end of the infusion, the fishing line was pulled, thus ending blood flow to the spinal cord instantly. The spinal cord was rapidly removed, frozen in dry ice, and sectioned into 1.0-mm segments. Representative segments were digested in Protosol for 24 hours, mixed with Biofluor (New England Nuclear, Boston, Massachusetts), and then counted in a scintillation counter. The filter paper blood samples were likewise digested and counted. Regional blood flow was then calculated for each spinal cord segment.

**Experimental Design**

Lidoflazine, nimodipine, and nicardipine were obtained as gifts from Pharmacia (Piscataway, New Jersey), Miles Laboratories (West Haven, Connecticut), and Syntex (Palo Alto, California), respectively. Lidoflazine was tested in the RSCIM by administering an intravenous bolus, 1.0 mg/kg over 60 seconds, 10 minutes before the onset of ischemia. We also administered lidoflazine to rabbits using the MCEM, 1.0 mg/kg, 5 minutes after embolization. Control rabbits were studied using saline infused exactly as described above.

Nimodipine was dissolved in 100 μl polyethylene glycol 200 (PEG) and diluted in saline. The solution was prepared 1–2 hours before use and stored in
TABLE 1. Efficacy of Calcium Channel Blockers in Rabbit Spinal Cord Ischemia Model

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>n</th>
<th>ET50 (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Control</td>
<td>18</td>
<td>27.2 ± 1.5</td>
</tr>
<tr>
<td>Lidoflazine</td>
<td>1 mg/kg</td>
<td>15</td>
<td>28.0 ± 2.2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Control</td>
<td>16</td>
<td>28.1 ± 4.8</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>5 µg/kg</td>
<td>17</td>
<td>21.9 ± 9.1</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>50 µg/kg</td>
<td>13</td>
<td>22.3 ± 1.6</td>
</tr>
</tbody>
</table>

ET50, duration of ischemia in minutes that produces paraplegia in 50% of rabbits.

opaque containers. We studied nimodipine in the RSCIM using two dosages (5 and 50 µg/kg) by injecting the drug over 120 seconds beginning 5 minutes before the onset of ischemia. We studied nimodipine in the MCEM using two dosages (5 and 50 µg/kg) administered over 120 seconds beginning 5 minutes after injection of the microspheres. All control rabbits received an equivalent volume of vehicle (PEG in saline) exactly as described for treatment groups.

Nicardipine was studied only in the MCEM because of its pharmacologic similarity to nimodipine. Nicardipine was dissolved in saline and administered intravenously at either 0.2 or 1.0 mg/kg over 60 seconds, beginning 5 minutes after the onset of ischemia. Control rabbits received saline.

Data Analysis

For both the RSCIM and MCEM we constructed quantal dose–response curves from the neurologic function ratings at 18 hours. The derivation of this type of analysis has been described in detail,31 and its practical utility was demonstrated.27-29,32 In brief, the construction of a quantal dose–response curve is similar to the construction of a life table. At each point on the abscissa the percentage of abnormal (including dead) animals is calculated and plotted as the ordinate. The total increases from 0% abnormal or dead at the lowest duration of occlusion or weight of microspheres to 100% at the highest duration or weight. The resulting sigmoid curve represents the effect of a range of durations or weights on the neurologic outcome of a group of animals. From the data, the point at which 50% of the animals are abnormal is calculated; ET50 is the duration of ischemia that produces paraplegia in 50% of the animals, and ES50 is the weight of microspheres trapped in the brains of animals that produces unequivocal signs of encephalopathy in 50%. As we have shown, an agent that is effective in reducing neurologic damage will shift the quantal dose–response curve to the right, with a significant increase in ET50 or ES50.29,32 Lesser grades of neurologic damage can be used, but in our experience these other endpoints yield increased error and reduced sensitivity of the test. To determine significance, t tests were performed using the Bonferroni correction (p<0.05).

Results

The results of the efficacy studies are presented in Tables 1 and 2 and Figures 1–3. Lidoflazine did not
alter outcome after reversible or irreversible CNS ischemia, that is, ET_{50} for lidoflazine was nearly identical to saline-treated control values in the RSCIM (Table 1) as was ES_{50} in the MCEM (Table 2, Figure 1). Nimodipine did not alter ET_{50} in the RSCIM (Table 1). In the MCEM, 5 µg/kg nimodipine increased ES_{50} from 380 µg in vehicle-treated controls to 482 µg in treated rabbits, but this increase was not significant (Table 2). Figure 2 summarizes the data for nimodipine. As shown in Table 2, ES_{50} for 1 mg/kg nicardipine was 250 µg compared with 314 µg in saline-treated controls. At 0.2 mg/kg, ES_{50} for nicardipine was 219 µg (Figure 3). These values were not significantly different from control.

The effects of each agent on MABP and HR are shown in Table 3. Of the agents studied, nicardipine significantly lowered MABP at both 0.2 and 1.0 mg/kg. There were no significant changes in MABP and HR attributable to lidoflazine or nimodipine at any dose studied.

All agents studied increased spinal cord blood flow (SCBF) significantly. Control SCBF in the lumbar cord was 23.5 ± 2.1 ml/100 g/min (mean ± SEM; n = 9). SCBF was increased by 1 mg/kg lidoflazine, 5 µg/kg nimodipine, 50 µg/kg nimodipine, and 0.2 mg/kg nicardipine to 84.2 ± 2.4, 51.7 ± 6.5, 43.9 ± 8.0, 51.8 ± 4.5 ml/100 g/min, respectively (n = 3 for each agent); these changes were significant for all agents (p < 0.05, analysis of variance). Even though 0.2 mg/kg nicardipine lowered MABP 30% from baseline, this dose increased SCBF 120% from control.

Discussion

We studied three cerebral-selective calcium channel blockers in two animal models of CNS ischemia and were unable to show an unequivocal preservation of neurologic function. Although nimodipine
exhibited a tendency to improve neurologic function during cerebral ischemia, this trend was not significant. All three agents raised SCBF significantly, even though nicardipine significantly lowered MABP. Lidoflazine, structurally very different from the other two agents, was no more efficacious.

We studied three agents with similar properties in different models, and the lack of apparent benefit suggests that we have not overlooked a subtle treatment effect. Calcium channel blockers have been shown by others to raise cerebral blood flow in normal and ischemic brain at the doses we chose.1,2,8,10,12,19 Using these methods, we have shown previously that tissue plasminogen activator, serotonin antagonists, and glutamate antagonists preserve neurologic outcome.29,32,33 Thus, we believe that the probability of a Type II error that explains the results we observed has been minimized.

It is important to clarify the differences between our methods and those of previous authors. We studied focal CNS ischemia in awake rabbits not receiving any other drugs concurrently. Thus, we reduced or eliminated possible interaction between the agents under study and anesthetics, anticonvulsants, or sedatives. We studied neurologic outcome using clinically meaningful and reproducible end points in two different models. The end points chosen represent severe grades of neurologic impairment and are unequivocal, and thus error due to observer bias or interpretation are minimized. Rating schemes based on more subtle grades of dysfunction are not as sensitive in detecting treatment benefit because there is more interobserver disagreement in measurements of subtle behavioral differences, which increases variance.

Calcium channel blockers produce a variety of physiologic responses, presumably mediated by selective inhibition of calcium transport. The three agents we studied cause relatively more vasodilation of CNS blood vessels than of peripheral blood vessels.34-36 Cerebral-selective calcium antagonists have been shown to preserve neurologic function in experimental global ischemia, such as cardiac arrest.9-14,19,24 In those studies the calcium antagonist was used as part of intensive postischemic resuscitation that included artificial ventilation, arrhythmics, pressors, anesthetics, and anticonvulsants. The benefit shown in those studies may have been due to a synergistic interaction of several agents rather than to a direct effect of the calcium channel blocker.

Calcium antagonists have been studied in experimental focal cerebral ischemia in awake and anesthetized animals with mixed results.15-17,27 Although some studies have shown a positive effect on metabolic recovery, electrophysiologic parameters, or blood flow, clear beneficial effect of these agents on neurologic function has not been observed after focal ischemia in awake animals.

We demonstrated that calcium channel blockers increase SCBF, and others have shown a similar effect on cerebral blood flow.1-3,12,17,19 However, the preservation of ischemic neurons may depend on factors other than blood flow alone.37-39 Our results suggest that the restoration of blood flow to levels well above baseline does not ameliorate ischemic damage after reversible interruption of SCBF. In addition to effects on blood flow, calcium antagonists may prevent neuronal accumulation of calcium after ischemic cellular insult.40-43 Our results suggest that calcium blockade may not preserve neurologic function. Our negative finding suggests either that the doses we chose were insufficient to block calcium entry into ischemic cells or that calcium channel blockade per se may not protect ischemic tissue. We favor the latter interpretation for several reasons. Although calcium clearly accumulates in dying neurons, there is no unequivocal evidence that calcium enters the ischemic cell through voltage-dependent calcium channels; rather, calcium may enter the dying cell directly through the damaged cell membrane or may be released from sequestration in intracytoplasmic organelles.42 It has not yet been shown that elevated levels of free intracellular calcium occur before cells have been irreversibly damaged.36 That is, intraneuronal calcium concentrations may increase after or concurrent with some other irreversible metabolic step that triggers cell death. Finally, first-pass brain extraction of nicardipine is probably sufficient to

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>n</th>
<th>Time (min)</th>
<th>Baseline (torr)</th>
<th>Minimum (torr)</th>
<th>% change</th>
<th>Baseline (beats/min)</th>
<th>Minimum or maximum (beats/min)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>5 ml</td>
<td>3</td>
<td>—</td>
<td>85±2</td>
<td>81±2</td>
<td>—</td>
<td>250±11</td>
<td>244±7</td>
<td>—</td>
</tr>
<tr>
<td>Lidoflazine</td>
<td>1 mg/kg</td>
<td>4</td>
<td>2</td>
<td>85±6</td>
<td>78±9</td>
<td>-8</td>
<td>254±19</td>
<td>238±19</td>
<td>-6</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>5 µg/kg</td>
<td>4</td>
<td>5</td>
<td>82±3</td>
<td>88±6</td>
<td>7</td>
<td>242±11</td>
<td>255±14</td>
<td>-7</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>50 µg/kg</td>
<td>4</td>
<td>5</td>
<td>82±3</td>
<td>72±4</td>
<td>-12</td>
<td>242±11</td>
<td>233±13</td>
<td>-4</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>0.2 mg/kg</td>
<td>3</td>
<td>3</td>
<td>78±3</td>
<td>55±5*</td>
<td>-29</td>
<td>234±14</td>
<td>226±33</td>
<td>-3</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>1.0 mg/kg</td>
<td>3</td>
<td>1</td>
<td>92±4</td>
<td>37±63*</td>
<td>-60</td>
<td>227±8</td>
<td>219±15</td>
<td>-5</td>
</tr>
</tbody>
</table>

Values are mean±standard deviation. Time to maximal change from baseline.

*Significantly different from control by one-way ANOVA and Neuman-Keuls test (p<0.05).
provide biologically active amounts of the agent to brain calcium channels.43

We did not study the effect of calcium antagonists on histopathologic measures such as infarct volume. In previous work on the RSCIM, we have demonstrated a close correlation between the observed functional outcome and pathologic findings.28 Thus, in the RSCIM it is probable that the calcium blockers did not alter the neuropathologic pattern of damage. Our MCEM is intended to be a rapid, sensitive screening pharmacologic tool; the quantification of histopathologic damage would add little to our conclusions.

We conclude that cerebral-selective calcium blockers used alone are probably not of benefit in the treatment of experimental focal cerebral ischemia in awake rabbits. Further work may elucidate situations in which, combined with other agents, calcium channel blockers may be useful.

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**KEY WORDS** • rabbits • cerebral ischemia • calcium channel blockers
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