Flunarizine, a Calcium Entry Blocker, Ameliorates Ischemic Brain Damage in the Rat

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The effects of flunarizine, a calcium entry blocker, were evaluated in a long-term survival model of ischemia in rats. One group of animals received the drug orally at 24 and 4 h prior to the insult (40 mg·kg⁻¹·dose⁻¹). Another group was given flunarizine following the insult, intravenously at 5 min (0.1 mg·kg⁻¹), and orally at 8 and 24 h (40 mg·kg⁻¹·dose⁻¹). A third group received the solvent for the oral suspension on the same schedule as the pretreated group. Six animals from each group were subjected to 9 min ischemia and recovery of 7 days, at which time the brains were harvested for histologic study. In another six animals from each group, cortical metabolites and fatty acids were determined during early recirculation. Local cerebral blood flow was measured at 60 min recirculation in a third set of animals. Flunarizine significantly improved histological outcome (fewer irreversibly damaged cells) in both treatment groups. This amelioration was not related to improvement of cerebral blood flow during the period of delayed hypoperfusion, nor the postischemic levels of high-energy phosphates or free fatty acids. (Key words: Brain: blood flow; ischemia. Ions: calcium, blocker. Pharmacology: flunarizine.)

CEREBRAL ISCHEMIA comprises the common insult in a myriad of clinical conditions. Cardiac arrest, near drowning, stroke, and some forms of head trauma are among many situations encountered that subject the brain to varying degrees of ischemia. The pathophysiology is composed of a severely compromised blood flow and substrate delivery inadequate to maintain normal energy production. This triggers a host of events that occur either during the ischemic period or soon after blood flow has been reestablished. One of these events is the influx of Ca²⁺ into the cell, which may trigger several detrimental reactions, such as proteolysis, lipolysis, protein phosphorylation, and enhancement of transmitter release. If unabated, these processes can lead to cell death.¹²

Following resumption of cerebral blood flow after ischemia, a secondary reduction in flow or so-called delayed hypoperfusion, which may contribute further to ischemia-related cerebral damage, occurs.⁹ This delayed hypoperfusion is caused by an increase in cerebrovascular resistance and may be mediated or exacerbated by the calcium flux in ischemia. Based on the hypothesis that a decrease in the calcium flux will reduce or eliminate ischemic damage,¹ various calcium channel blockers have been evaluated in the treatment of ischemia. They seem to be effective in ameliorating ischemic myocadial damage⁴ and may be beneficial in cerebral ischemia.⁵⁻⁹

However previous studies have used a variety of ischemic models that entail the use of multiple operations,⁸ pharmacological agents for cardiovascular support, and/or techniques such as prolonged ventilation, thoracotomy, and intensive care.⁶,⁷,⁹

Recently Smith et al. reported a long-term recovery model of ischemia in rats.¹⁰ Clamping of both common carotid arteries and hypotension to a MABP of 50 mmHg, induced by trimethaphan and maintained by phlebotomy, comprise the ischemic insult. This results in ischemia of the forebrain, which can be titrated to the desired interval, allowing long-term survival of the rats and permitting quantification of histologic damage.¹¹ Using this model, we showed in a previous report that flunarizine, a new calcium channel blocker,¹² improves ischemic damage in hippocampus.¹³ The purpose of this study was to determine the efficacy of flunarizine treatment in reducing damage in other susceptible areas of the brain. In addition, we explored the possibility that this reduction in damage may be through an effect on high-energy metabolites, free fatty acids, or cerebral blood flow in the postischemic period.

Materials and Methods

Male Wistar-SPF rats (300–400 g) were obtained from Møllegaards Avslaboratorium, Copenhagen, Denmark. Flunarizine was a generous gift of Janssen Pharmaceutica, Belgium. For oral administration, flunarizine was suspended in vegetable oil (Matoja®, Nordfalks AB, Mölndal, Sweden) for a final concentration of 28 mg·ml⁻¹; this allowed the desired dose (40 mg·kg⁻¹·dose⁻¹) to be administered in a small volume (0.5 ml). The intravenous preparation contained flunarizine dissolved in Ringer's solution (0.1 mg·ml⁻¹). Other drugs and materials were obtained from commercial sources.
EXPERIMENTAL GROUPS

There were three experimental groups. Each consisted of 15–18 animals that underwent the operative procedures, ischemia, and recovery described in the following section. The nontreated ischemia group (I) was fed solvent (0.5 ml/dose) via orogastric tube at approximately 24 h and 4 h prior to the ischemic insult. The postischemia treatment (II) group received intravenous flunarizine (approximately 0.1 mg·kg⁻¹) at 5 min postischemia. In addition, the animals were administered 0.5 ml of the oral preparation via orogastric tube at 8 and 24 h postischemia. The pretreatment group (III) was fed the flunarizine suspension at 24 and 4 h prior to ischemia. Six animals from each group were used for the histological evaluation; cortical high energy metabolites and free fatty acids were determined in another six from each group; cerebral blood flow was measured in five animals from groups I and II, and three from group III. Control groups (6 animals each for histology, biochemical assays, and measurement of CBF) underwent the operative procedures, but received neither medications nor an ischemic insult.

OPERATIVE PROCEDURES

The animals were fasted overnight preceding the operation, but were allowed water ad libitum. On the day of operation, anesthesia was induced with 3.5% halothane and 2:1 N₂O in O₂. The trachea was intubated with a polyethylene tube (Intramedic PE 240®, Clay Adams, Parsippany, New Jersey) with the help of transillumination of the neck by a high intensity fiberoptic lamp. Through a midline neck incision, the jugular veins were exposed. Muscle relaxation was achieved with suxamethonium (Celocurin®, Vitrum AB, Stockholm, Sweden; approximately 4 mg·kg⁻¹) injected in the left jugular vein. This facilitated the mechanical ventilation and provided immobilization during the steady-state period. Anesthesia was maintained with 0.7% halothane and 2:1 N₂O in O₂. A silicon catheter (Dow Corning Silastic®, id 0.03 mm, od 0.065 mm) was inserted in the exposed right internal jugular vein for administration of drugs and for withdrawal of blood during the hypotensive period.

Both common carotid arteries were exposed by gentle dissection and loose suture placed around each for subsequent manipulation. The tail artery was exposed through a ventral incision and cannulated (Portex PE50®, Hythe, Kent, UK) to allow continuous blood pressure recording and intermittent sampling of arterial blood gases. Needle electrodes were placed bilaterally through small skin incisions into the temporalis muscle to monitor the interhemispheric electroencephalogram (EEG) during the ischemic period. Following these procedures, the halothane was discontinued, and the rats were allowed a 30-min period to reach steady state. Mechanical ventilation with 2:1 N₂O:O₂ was continued throughout. The PaO₂ was maintained above 80 mmHg, PaCO₂ between 30–40 mmHg, and the rectal temperature near 37° C. Heparin (50 IU or 150 IU·kg⁻¹) was administered intravenously for anticoagulation. The initial blood gases and pH were sampled 15 min following completion of the operative procedure and periodically thereafter. Samples were analyzed on Combi-Analyzer® (E. Scheweiler u. Co., Kiel, FRG) and Radiometer® pH-meter (Copenhagen, Denmark). Additional suxamethonium was administered as needed prior to the onset of ischemia.

The previously mentioned technique was modified for animals prepared for metabolite and free fatty acid determination. Briefly, the dorsal skull was exposed and a plastic funnel placed for later use. The animals were subjected to ischemia and a 15-min recirculation period. At this time, the brains were frozen in situ, removed, and stored at −80° C, as described by Pontén et al.14 The control group underwent the operative procedures and was allowed a similar period to reach steady state, but was not subjected to ischemia.

Modifications in the method also were made for the animals in which cerebral blood flow was to be measured. The operative procedures for the three groups (I, II, and III) were similar to those previously described. In addition, one femoral vein and femoral artery catheter were placed for the infusion of isotope and blood sampling, respectively. The animals were subjected to ischemia and a 15-min recirculation period. Another six animals underwent similar preparation, but did not undergo ischemia, and served as controls.

INDUCTION OF REVERSIBLE FOREBRAIN ISCHEMIA

After undergoing the operative procedure previously described, hypotension was induced with trimethaphan (Arfonad®, Hoffmann-La Roche, Basel, Switzerland; 4–5 mg·kg⁻¹) and withdrawal of blood. When the mean arterial pressure (MAP) reached 50 mmHg, vascular clamps were placed on both common carotid arteries. The EEG was recorded continuously before and 1 min after the clamping. Subsequent EEG tracings were obtained intermittently during ischemia and recirculation. At the end of ischemia, the withdrawn blood was reinfused, vascular clamps removed, and NaHCO₃ (approximately 1 mEq·kg⁻¹) administered. The duration of ischemia was 9 min; zero time was taken as the point at which the EEG became isoelectric. Following the ischemia, the jugular catheter was removed and the neck incision closed with
sutures. Nitrous oxide was discontinued at 15–20 min postischemia, the animals were extubated when they resumed adequate spontaneous ventilation. They were then placed in cages and allowed free access to water and food.

ASSessment of Neuronal Necrosis

Following a 7-day postischemia recovery, six animals in each group were reanesthetized with halothane and tracheostomized. While being mechanically ventilated with halothane, 0.7% in 2:1 N₂O:O₂, they were perfusion-fixed via the ascending aorta with 4% formaldehyde buffered to pH 7.35, following a 30 s rinse with saline. Both solutions were prewarmed to 37°C. The brains were removed the following day and stored in cold fixative. The preparation and celestin blue-acid fuchsins staining of the brains for histological study have been described in detail elsewhere. The dual staining has been used to highlight irreversibly damaged neurons in 8 μm sections. Because of their acidophilic nature, these cells appear bright red on examination under light microscopy and are readily distinguished from other neurons.

SCORING CEREBRAL DAMAGE

The extent of damage in representative areas of vulnerability was evaluated by observing the number of necrotic, acidophilic cells and the number of normal cells in each area. A direct count of these damaged cells and normal neurons was performed in representative sections of the caudate nuclei and neocortex at 400×. The observations were performed in a blinded fashion. A percentage damage score was calculated for the caudate. Because of the large number of cells present in the cortex, a crude damage index was used for comparison of the different groups. For determination of damage in the caudate nuclei, the cell counts were performed in one dorsal region and one lateral region (fig. 1). Damage in the neocortex was classified according to a crude damage index (CDI) as follows: no damage—0; rare to occasional acidophilic cells per field (approximately 10% damage)—1; moderate number of acidophilic cells per field (approximately 10–50% damage)—2; frequent acidophilic cells per field (greater than 50% damage)—3.

DETERMINATION OF HIGH-ENERGY METABOLITES AND FREE FATTY ACIDS

Samples of parietotemporal cortex (approximately 100 mg per animal) were dissected from previously obtained brains at −22°C and assayed for metabolites and free fatty acids (FFA) according to previously described methods. Cortical levels of ATP, ADP, AMP, PCr, glucose, glycogen, lactate, pyruvate, and free fatty acids were determined in six animals from each group (I, II, and III). Energy charge (EC) was calculated according to the formula:

\[ EC = \frac{(ATP + 0.5 ADP)}{(ATP + ADP + AMP)} \]

MEASUREMENT OF LOCAL CEREBRAL BLOOD FLOW

Local cerebral blood flow (LCBF) was measured according to Sakurada et al. using 14C-iodoantipyrine as tracer. For details of the methods in our laboratory, see Abdul-Rahman et al. In the experimental groups, CBF was determined at 1 h postischemia. CBF in the control group was measured at 90–120 min after completion of the operative procedure in order to approximate the postoperative time at which CBF measurements were performed in the ischemic groups. Isotope (35 μCi, approximately 100 μCi · kg⁻¹) was infused over 45 s, at which time the animal was decapitated, the brain removed and placed in isopentane at −50 to −70°C, and then stored at −80°C for subsequent preparation of autoradiographs. Blood samples were collected at 5-s intervals during the infusion. Sample activity was measured in a Beckman Liquid Scintillation Counter (LS 2800, Beckman Instruments, Fullerton, CA). Densitometric readings from the autoradiographs were performed on a Macbeth TD511 Densitometer (Kollmorgen Corp., New York, NY).
### TABLE 1A. Physiologic Variables for Groups in the Histology Study (Mean ± SEM)

<table>
<thead>
<tr>
<th>Groups (no. animals)</th>
<th>Weight* (g)</th>
<th>Glucose* (mMol·l⁻¹)</th>
<th>Temp* (°C)</th>
<th>pH*</th>
<th>PaO₂ * (mmHg)</th>
<th>PaCO₂ * (mmHg)</th>
<th>MABP (mmHg)</th>
<th>MABP 2 PI (mmHg)</th>
<th>MABP 5 PI (mmHg)</th>
<th>Ext (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (6)</td>
<td>336 ± 4</td>
<td>7.3 ± 0.8</td>
<td>37.4 ± 0.2</td>
<td>7.45 ± 0.03</td>
<td>89 ± 6</td>
<td>35 ± 2</td>
<td>119 ± 6</td>
<td>112 ± 8</td>
<td>141 ± 6</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>II (6)</td>
<td>327 ± 9</td>
<td>9.1 ± 0.7</td>
<td>37.1 ± 0.2</td>
<td>7.44 ± 0.02</td>
<td>99 ± 6</td>
<td>35 ± 1</td>
<td>137 ± 10</td>
<td>107 ± 5</td>
<td>129 ± 5</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>III (6)</td>
<td>339 ± 5</td>
<td>7.6 ± 1.8</td>
<td>37.3 ± 0.2</td>
<td>7.41 ± 0.03</td>
<td>98 ± 5</td>
<td>35 ± 2</td>
<td>113 ± 6</td>
<td>112 ± 6</td>
<td>144 ± 7</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>Control (6)</td>
<td>325 ± 5</td>
<td>6.8 ± 0.3</td>
<td>37.2 ± 0.1</td>
<td>7.36 ± 0.01</td>
<td>108 ± 5</td>
<td>37 ± 1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Groups: Control = operated, nonischemic control; I = nontreated; II = postischemia flunarizine treatment; III = preischemia flunarizine treatment.

Abbreviations: MABP, mean arterial blood pressure; preischemia, preischemia treatment.

EXT', time, in minutes, from release of carotid clamps to extubation; control animals extubated within 2–3 min after discontinuation of N₂O following steady-state period. NA, not applicable.

* Values obtained prior to the onset of ischemia.

### ANALYSIS OF DATA

Results were analyzed using analysis of variance (ANOVA). A post hoc comparison was performed among groups I, II, and III using either Dunnett’s or Neumann-Keul’s test for multiple comparisons and the Mann-Whitney U test. A P value < 0.05 was considered a significant difference.

### Results

The physiological variables (body weight, plasma glucose, pH, PaO₂, PaCO₂, and rectal temperature) were similar in all three groups (I, II, and III) and controls for each part of the study (tables 1A and 1B). EEG isoelectricity was observed in all animals shortly after carotid occlusion (10.7 ± 4.6, 8.2 ± 1.4, and 12 ± 1.3 s in groups I, II, and III, respectively). At the termination of ischemia, all animals had MABP over 90 mmHg, which had stabilized by 5 min (table 1). Although groups I and II exhibited blood pressures lower than preischemic values immediately following the insult, there was no intergroup difference. The intravenous administration of flunarizine resulted in a transient decrease in MABP of approximately 10 mmHg, which normalized within 5 min. In animals assigned to the histology study, there was no difference in the time of extubation among the groups (53 ± 4, 46 ± 5, and 43 ± 5 min recirculation for groups I, II, and III, respectively).

### NEUROHISTOLOGIC EVALUATION

The areas of selective vulnerability in this model and the locations at which neuronal damage was evaluated are depicted in figure 1.

In sections from the operated, nonischemic control group, no acidophilic (necrotic) cells were observed in the caudate or the neocortex. Extensive neuronal necrosis was observed in the caudate nuclei of the nontreated group. The mean damage was 40% and included two animals with large infarcts. Both treatment groups (II and III) exhibited significantly fewer acidophilic cells (P < 0.01) in all sections evaluated. The damage averaged 0.3–3% in the caudate nuclei of both the pre- and postischemia treated animals. There was one animal in the pretreatment group that had a small unilateral infarct.

Observations of the necrotic areas yielded results similar to those in the caudate. The damage involved the middle cortical layers in all three groups. The nontreated group (I) exhibited widespread damage throughout layers III–V (CDI 3). These layers of the parietal and entorhinal cortex consistently displayed the highest density of aci-

### TABLE 1B. Physiologic Variables for Groups in the Metabolism and CBF Studies (Mean ± SEM)

<table>
<thead>
<tr>
<th>Groups* (no. animals)</th>
<th>Weight† (g)</th>
<th>Glucose‡ (mMol·l⁻¹)</th>
<th>Temp‡ (°C)</th>
<th>pH‡</th>
<th>P_O₂ § (mmHg)</th>
<th>P_CO₂ § (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, M (6)</td>
<td>331 ± 5</td>
<td>7.5 ± 0.3</td>
<td>37.1 ± 0.1</td>
<td>7.38 ± 0.01</td>
<td>116 ± 4</td>
<td>34 ± 0.15</td>
</tr>
<tr>
<td>II, M (6)</td>
<td>322 ± 6</td>
<td>8.6 ± 0.2</td>
<td>37.1 ± 0.1</td>
<td>7.37 ± 0.01</td>
<td>106 ± 5</td>
<td>35 ± 0.4</td>
</tr>
<tr>
<td>III, M (6)</td>
<td>328 ± 5</td>
<td>8.7 ± 0.5</td>
<td>37.3 ± 0.2</td>
<td>7.40 ± 0.02</td>
<td>102 ± 4</td>
<td>36 ± 0.9</td>
</tr>
<tr>
<td>Control, M (6)</td>
<td>326 ± 6</td>
<td>4.5 ± 0.5%</td>
<td>37.1 ± 0.1</td>
<td>7.38 ± 0.02</td>
<td>102 ± 5</td>
<td>37 ± 0.6</td>
</tr>
<tr>
<td>I, F (5)</td>
<td>305 ± 7</td>
<td>6.7 ± 0.2</td>
<td>37.4 ± 0.1</td>
<td>7.38 ± 0.01</td>
<td>104 ± 5</td>
<td>39 ± 1.2</td>
</tr>
<tr>
<td>II, F (5)</td>
<td>307 ± 7</td>
<td>5.9 ± 1.5</td>
<td>37.3 ± 0.1</td>
<td>7.41 ± 0.01</td>
<td>103 ± 2</td>
<td>37 ± 0.1</td>
</tr>
<tr>
<td>III, F (5)</td>
<td>293 ± 12</td>
<td>7.1 ± 0.7</td>
<td>37.1 ± 0.2</td>
<td>7.43 ± 0.01</td>
<td>101 ± 3</td>
<td>37 ± 0.2</td>
</tr>
<tr>
<td>Control, F (6)</td>
<td>321 ± 8</td>
<td>7.9 ± 0.4</td>
<td>37.2 ± 0.1</td>
<td>7.38 ± 0.01</td>
<td>108 ± 5</td>
<td>38 ± 0.4</td>
</tr>
</tbody>
</table>

* Groups: Control = nonischemic control; I = nontreated; II = postischemia flunarizine treatment; III = preischemia flunarizine treatment.

† Values obtained prior to the onset of ischemia in groups I, II and III.

‡ Values obtained immediately prior to CBF measurement.

§ P < 0.05.

¶ Values may be low because of gross hemolysis of stored samples.
Acidophilic neurons. Damage in the treatment groups also involved layers III–V. However, sections from both groups showed few to no acidophilic cells per field (CDI 0–1). Comparison of these results is depicted schematically in figure 2.

**ENERGY METABOLITES AND FREE FATTY ACIDS FOLLOWING 9-MIN ISCHEMIA AND 15-MIN RECOVERY**

The concentrations of high-energy phosphates diminish rapidly to negligible levels after the onset of ischemia. Following resumption of cerebral blood flow, the levels increase steadily and reach near-normal concentrations by 30 min. In this study, the levels of metabolites were determined at 15 min recirculation, at which time recovery toward normal values is still incomplete. In all groups, the concentrations of ATP and total adenylates showed a significant recovery toward control. The differences among groups were small, but they were statistically significant as compared to the nontreated animals (I). The postischemia-treated animals (II) had lower values of ATP (2.14 ± 0.02 vs. 2.25 ± 0.02, in μmol·g⁻¹) and total adenylates (2.47 ± 0.02 vs. 2.56 ± 0.02; P < 0.05); whereas, the pretreatment group (III) had higher concentrations of both (2.35 ± 0.04 and 2.67 ± 0.05, for ATP and total adenylates, respectively; P < 0.05). The

**TABLE 2. Cerebral Cortical Metabolites after 9-Min Ischemia and 15-Min Recirculation**

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>TOT</th>
<th>PCr</th>
<th>EC</th>
<th>GLU</th>
<th>GLY</th>
<th>LAC</th>
<th>PYR</th>
<th>L/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>2.91</td>
<td>0.257</td>
<td>0.040</td>
<td>3.20</td>
<td>4.24</td>
<td>0.948</td>
<td>2.17</td>
<td>3.57</td>
<td>1.47</td>
<td>0.105</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.005)</td>
<td>(0.002)</td>
<td>(0.01)</td>
<td>(0.07)</td>
<td>(0.001)</td>
<td>(0.30)</td>
<td>(0.24)</td>
<td>(0.08)</td>
<td>(0.01)</td>
<td>(0.7)</td>
</tr>
<tr>
<td>I</td>
<td>2.25</td>
<td>0.259</td>
<td>0.050</td>
<td>2.56</td>
<td>5.08</td>
<td>0.930</td>
<td>6.59</td>
<td>0.37</td>
<td>4.65</td>
<td>0.557</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.008)</td>
<td>(0.004)</td>
<td>(0.02)</td>
<td>(0.12)</td>
<td>(0.002)</td>
<td>(0.22)</td>
<td>(0.07)</td>
<td>(0.80)</td>
<td>(0.04)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>II</td>
<td>2.14§</td>
<td>0.264</td>
<td>0.060</td>
<td>2.47§</td>
<td>4.88</td>
<td>0.922§</td>
<td>7.03</td>
<td>0.37</td>
<td>6.07</td>
<td>0.396</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.011)</td>
<td>(0.002)</td>
<td>(0.02)</td>
<td>(0.02)</td>
<td>(0.002)</td>
<td>(0.22)</td>
<td>(0.04)</td>
<td>(0.60)</td>
<td>(0.02)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>III</td>
<td>2.35§</td>
<td>0.270</td>
<td>0.053</td>
<td>2.67§</td>
<td>5.17§</td>
<td>0.930</td>
<td>6.24</td>
<td>0.44</td>
<td>4.25</td>
<td>0.318</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>(0.040)</td>
<td>(0.012)</td>
<td>(0.004)</td>
<td>(0.05)</td>
<td>(0.13)</td>
<td>(0.002)</td>
<td>(1.11)</td>
<td>(0.08)</td>
<td>(0.56)</td>
<td>(0.04)</td>
<td>(0.6)</td>
</tr>
</tbody>
</table>

* Groups: Cont = nonischemic control; I = nontreated; II = postischemia flunarizine treatment; III = preischemia flunarizine treatment.
Abbreviations: Ad = total adenylates; PCr = phosphocreatine; GLU = glucose; GLY = glycogen; LAC = lactate; PYR = pyruvate. Values are given as means with standard error (SEM) in parentheses.
† Values expressed as μmol·g⁻¹.
‡ Energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP).
§ P < 0.05, compared to group I.
TABLE 3. Cortical Free Fatty Acids after 9 Min Ischemia and 15-Min Recirculation

<table>
<thead>
<tr>
<th>Groups</th>
<th>16:0†</th>
<th>18:0</th>
<th>18:1</th>
<th>20:4</th>
<th>22:6</th>
<th>Total†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont (6)</td>
<td>164 (18)</td>
<td>72 (7)</td>
<td>64 (18)</td>
<td>4 (1)</td>
<td>ND</td>
<td>304 (40)</td>
</tr>
<tr>
<td>I (6)</td>
<td>206 (25)</td>
<td>227 (18)</td>
<td>63 (6)</td>
<td>13 (3)</td>
<td>6 (4)</td>
<td>515 (49)</td>
</tr>
<tr>
<td>II (6)</td>
<td>211 (21)</td>
<td>235 (14)</td>
<td>80 (17)</td>
<td>17‡ (4)</td>
<td>2 (2)</td>
<td>555‡ (50)</td>
</tr>
<tr>
<td>III (6)</td>
<td>212 (20)</td>
<td>181 (10)</td>
<td>59 (7)</td>
<td>8§ (1)</td>
<td>1 (1)</td>
<td>473§ (31)</td>
</tr>
</tbody>
</table>

* Groups: Cont = nonischemic control; I = nontreated; II = postischemia flunarizine treatment; III = preischemia flunarizine treatment. Values expressed as mean nmol·g⁻¹ tissue (SEM).
† (16:0)-palmitic acid, (18:0)-stearic acid, (18:1)-linoleic acid, (20:4)-arachidonic acid, (22:6)-docosahexaenoic acid; Total = total free fatty acids; ND = nondetectable.
‡ P < 0.05, compared to group I.
§ P < 0.05, compared to group II.

postischemic treatment group (II) had a significantly lower EC than group I or III (P < 0.05). Tissue lactate and pyruvate levels varied among the groups. Although Group II had higher concentrations than the nontreatment group, and the concentrations in group III were lower, these differences did not reach statistical significance. The results are summarized in table 2.

Free fatty acid concentrations surge to micromolar range with the onset of ischemia. Arachidonic acid (20:4) and total FFA may reach 500–1000 μmol·kg⁻¹. Resumption of CBF is associated with a progressive decrease in FFA toward normal levels by about 30 min. However, this recovery is still incomplete at 15 min recirculation. The findings in this study were consistent with the postischemia recirculation time. In all three groups, there was an apparent return toward normal levels, although the concentrations of free fatty acids, including (20:4) and FFA, were elevated. As compared to the nontreated group (I) (13.0 ± 2.9 nmol·g⁻¹), the postischemic treatment group (II) had higher concentrations of arachidonic acid (17.5 ± 3.6 nmol·g⁻¹; P < 0.05); the pretreatment group (III) had lower values (7.9 ± 0.8 nmol·g⁻¹), which were not significantly different from group I. The total free fatty acids in group (II) (554.7 ± 50.0 nmol·g⁻¹) were higher as compared to the nontreated group (I) (514.7 ± 49.0 nmol·g⁻¹; P < 0.05) and lower in group (III) (473.1 ± 31.2 nmol·g⁻¹; P < 0.05). These results are summarized in table 3.

LCBF AT 60-MIN RECIRCULATION FOLLOWING 9-MIN ISCHEMIA

Values for each of the three groups are presented as per cent of control in figure 3. In all three ischemic groups (I, II, and III), the LCBF was reduced to 25–50% of control values (P < 0.01) in the areas of primary focus of this study: hippocampus, caudoputamen, and cortex. There was no significant difference between the LCBF in nontreated animals (group I) and either of the flunarizine-treated groups (II and III).

Discussion

The model used in this report11 claims several advantages for its use in the study of ischemia. It requires minimal preparation time (15–20 min) for the operative

![FIG. 3. Cerebral blood flow (LCBF) in various structures after 9-min ischemia and 60-min recirculation. Clear bar = nontreated group (I, n = 5); speckled bar = flunarizine postischemia treatment (II, n = 5); black bar = flunarizine pretreatment (III, n = 5). Control values are given in parenthesis; results are expressed as per cent of control +SD.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931406/)
procedures. The ischemic insult can be applied in a square-wave manner for the desired duration. The animals require minimal postoperative and postischemic care. Extensive neuronal damage of certain highly vulnerable regions occurs (fig. 1) and still allows long-term survival of the animals. The histological methods employed facilitate quantification of neuronal damage by light microscopy and subsequent comparisons between groups.

Accurate evaluation of neurological function in the rat is more difficult. Smith and co-workers described several behavioral alterations in some of the animals following 10-min ischemia, such as hyperextension of the hindlimbs, hyperexcitability, and seizure activity possibly evoking to death. In this study, all animals allowed 7 days' survival demonstrated variable degrees of hindlimb hyperextension and hyperexcitability to loud noises. No seizures or deaths occurred in these groups. Correlation of changes in higher brain function to histological damage observed in rats requires a more severe insult than one needed to produce significant histological damage. Volpe et al. recently presented changes in behavioral performance in rats subjected to 30-min ischemia induced by occlusion of the carotid and vertebral arteries bilaterally. These animals exhibited severe histopathological damage (2.6-3 on a 0-3 scale) in the striatum and hippocampus. Despite this, the rats showed only a reduction in working performance, while no sensorimotor impairment was reported. Thus, methods more sophisticated than gross neurological assessment seem to be required to accurately detect neurobehavioral changes following ischemia in the rat. On the other hand, the histopathological assessment of neuronal necrosis in the susceptible areas is highly reproducible and can be quantified readily for comparison. This study was designed to evaluate the effects of flunarizine on histopathological changes following cerebral ischemia.

Preischemic treatment with calcium channel blockers has been shown to reduce cerebral damage, and our findings with flunarizine concur. This pretreatment is of clinical interest primarily in a variety of operative cases where the brain may be subject to hypoperfusion, such as those requiring deliberate hypotension, those involving certain neurosurgical procedures, and possibly those requiring cardiopulmonary bypass. The effectiveness of treatment with calcium channel blockers following the insult is of greater interest because, in a majority of clinical situations, patients present after experiencing the ischemic insult.

HISTOLOGIC FINDINGS

The extent of neuronal necrosis observed in the caudate nuclei was significantly less in the treatment groups as compared to the nontreated animals. In the neocortex, the nontreated group showed greater damage than the treated groups. In a previous study, we found that pre- and postischemic treatment with flunarizine significantly reduced damage in the hippocampus. Briefly, animals that received flunarizine showed 45 and 52% damage in this region (in the postischemia-treated and the pretreated groups, respectively), while the nontreated group had 91% damage in the dorsal hippocampus ($P < 0.01$). This reduction in damage concurs with the findings of Van Reempts et al., who reported that flunarizine (0.1 mg·kg$^{-1}$) reduced damage in the hippocampus from 30% in controls to 7% in treated animals following 20-min ischemia using the four-vessel occlusion model in the rat.

Opposite conclusions recently were reported by Newberg and co-workers, who evaluated the effects of postischemic administration of flunarizine in dogs subjected to 10-min ischemia by occlusion of the ascending aorta and both venae cavae. The authors reported that flunarizine in a dose of 6 µg·kg$^{-1}$ administered 10-min postischemia did not improve neurological function as observed during a 48 h period. There are several differences between their study and ours. The flunarizine dosage used by Newberg was a single dose of 6 µg·kg$^{-1}$, whereas, in our study, the agent was administered in a higher dose and over a 24-h period (0.1 mg·kg$^{-1}$ iv at 5 min recirculation and 40 mg·kg$^{-1}$ orally at 8 and 24 h). Although the lower dose has been reported to affect cerebral blood flow, in the studies reporting benefit of flunarizine in ischemia, higher doses of flunarizine were employed. In addition, many of the treated animals in the Newberg study died of pulmonary edema of uncertain etiology. Finally, no histopathological assessment is reported. However, of the two treated animals that survived to 48 h, one was judged to have no damage (grade 1), while none of the nontreated animals achieved higher than grade 2 (moderate damage).

Other authors have shown that postischemic treatment with calcium channel blockers may reduce cerebral damage following ischemia. Vaagenes and colleagues found that in dogs subjected to cardiac arrest induced by apnea and clamping of the tracheal tube, animals treated with lidoflazine following the insult tended to exhibit a better histologic score than the nontreated ones, although a statistical significance could not be demonstrated. It has also been reported recently that postischemic nimodipine administration reduced histological damage in monkeys subjected to 17-min global brain ischemia induced by neck cuff.

Thus, postischemic intervention with flunarizine and other calcium channel blockers seems to be effective in reducing ischemic cerebral damage. This implies that the ameliorative effect of treatment would be on physiological
events in the recirculation period. One such mechanism may be the restoration of a normal energy state. Another mechanism could be an effect on postischemic CBF.

**Effects on Metabolism**

Following the onset of ischemia, energy depletion occurs rapidly in the brain. The concentrations of ATP and other high-energy phosphates decrease from normal to negligible levels. Resumption of blood flow provides vital substrates to the brain and removal of wastes. This is accompanied by a recovery of energy stores to near-normal by 30 min. An ameliorative effect of any postischemic intervention may hasten the recovery of the energy state to normal.

Fifteen minutes after resumption of CBF, all three experimental groups showed a marked restoration of the energy stores (ATP, total adenylates, and EC). The postischemic treatment group (II) showed lower values than the nontreated one (I), whereas the values in the pretreatment group (III) were the same or greater. However, the absolute values of these differences are quite small and, therefore, their importance to pathophysiology is probably minimal. Similar evidence was obtained by Hosman et al., who could not show an improvement in ATP fluorescence in flunarizine-treated cats over controls after an ischemic insult.

Free fatty acids (FFA) have been implicated in the detrimental reactions following ischemia. During the ischemic period, the concentrations of FFA steadily increase several hundred-fold to micromolar range. These could, in turn, participate in reactions harmful to the cell, such as production of vasoactive prostaglandins and free radical formation. With the onset of cerebral recirculation, the FFA levels decrease rapidly toward normal by 30 min. A beneficial effect of postischemic treatment with flunarizine on free fatty acid metabolism may be reflected in a more rapid decrease in FFA in the treated group. In our study, with samples obtained at 15-min recirculation, the FFA concentrations in all animals were in the nanomolar range and reflect recovery toward normal. As compared to the nontreated group (I), the postischemic treatment group (II) had a significantly greater concentration of arachidonic acid and total FFA. On the other hand, group III yielded lower concentrations of both. Thus, the effect of postischemic treatment with flunarizine in reducing neuronal damage seems unrelated to the restoration of normal energy stores or to the levels of FFA.

**Local Cerebral Blood Flow**

Following resumption of flow after ischemia, there occurs an early phase of excess perfusion or hyperemia. Subsequently there is a secondary decrease in cerebral blood flow that may persist for several hours. Although the mechanism of this phenomenon is undetermined, excess influx of Ca\(^{2+}\) may cause vasoconstriction and the increased cerebrovascular resistance (CVR) present at this time. This delayed hypoperfusion has been suggested to impart a secondary insult to the postischemic brain and may be important in the pathogenesis of ischemic damage. Therefore, improved perfusion during this period may result in improved outcome after ischemia. The decreased cerebral blood flow is manifest within 30–45 min recirculation. An ameliorative effect of an agent on CBF should be evident subsequent to this period.

Our measurements at 60-min recirculation demonstrate that the LCBF in all three of the ischemic groups was universally depressed as compared to controls. More importantly, there was no difference between the nontreated (I) and postischemia (II) treatment groups. Flunarizine has been reported to have vasodilatory effects that are prominent on the cerebral vasculature, and has been shown to normalize postischemic CBF in a dog model. However, Newberg and colleagues found that flunarizine had no effect on CBF in dogs following ischemia.

Variable results also have been obtained with other calcium channel blockers. White et al. reported that lidoflazine improved CBF after an ischemic insult in dogs. However, Dean and colleagues could not substantiate that lidoflazine had any effect on delayed hypoperfusion. On the other hand, nimodipine, a calcium channel blocker of different pharmacological properties, consistently seems to increase postischemic CBF.

Our findings that postischemic administration of flunarizine reduced neuronal necrosis and the conflicting results on the effect of calcium channel blockers on delayed hypoperfusion suggest that postischemic hypoperfusion probably plays a minor role in the pathogenesis of ischemic cerebral damage.

**Other Mechanisms of Action**

Other properties of flunarizine may explain its beneficial effects in ischemia. Flunarizine is known to improve erythrocyte deformability and blood viscosity. This may allow for improved microcirculation following ischemia, even during the period of increased CVR. In turn, delivery of substrates to areas at risk would be improved.

It has been shown that calcium accumulates in damaged neurons following ischemia. Flunarizine may reduce this accumulation and related detrimental reactions. However, there seems to be no in vivo effect of flunarizine on total calcium following ischemia. The increase in total intracellular calcium may reflect the terminal event rather than
the etiology of neuronal death. Findings of a recent study indicate that total calcium increases only after 24–72 h. This accumulation is preceded by an increased calcium turnover in the cell, implying that initially the cell can maintain normal calcium homeostasis, consuming cellular energy, but it eventually succumbs. Mela-Riker and colleagues demonstrated that flunarizine reduced KCI-induced Ca$^{2+}$ influx by 50–100% in in vitro preparations of synaptosomes. In addition, the agent enhanced the ability of mitochondria to store calcium. A reduction in calcium influx and/or an increase in the ability of the mitochondria to tolerate increased calcium loads that are present following an ischemic insult may contribute to the decrease in cell damage.

Flunarizine may exert its effect through a different mechanism. The ischemic event is associated with release of large amounts of neurotransmitters in the brain. An imbalance of excitatory and inhibitory transmitter receptor stimulation has been suggested to play a role in producing ischemic neuronal damage. If so, a modification of the release of these agents may result in reduced damage. Flunarizine recently has been shown to affect the release of neurotransmitters and, thus, may act via this mechanism.

CONCLUSION

We have found that flunarizine significantly ameliorates ischemic cerebral damage, even when administered post-ischemia. This improvement does not correlate with the early recovery of cortical energy and FFA metabolism, nor an improvement of postischemic delayed hyperperfusion. Further studies are needed to elucidate this agent’s mechanisms of action.

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