Mefenamate, an Agent that Fails to Attenuate Experimental Cerebral Infarction

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ABSTRACT: Background: Blockade of nonselective cation channels is a potential therapeutic approach that has not been attempted in cerebral ischemia, in spite of the ability of these channels to allow cellular calcium influx into neurons. Fenamates are a class of molecules that block these channels, and many congeners are also anti-inflammatory and free radical scavenging. These three mechanisms may contribute to brain damage in ischemia. Methods: Pretreatment or posttreatment with mefenamate (30 mg/kg) was evaluated in a temperature-controlled rat transient focal ischemia model. Quantitative histopathology on 26 coronal sections allowed determination of tissue necrosis and tissue atrophy at one week survival. Results: Neither pre- nor postischemic administration of a dose previously shown effective in preventing epileptic neuronal necrosis was found to reduce necrosis in cortex, nor in any subcortical structures. Conclusions: We conclude that nonselective cation channel blockade with mefenamate affords no neuroprotection in this model. Publication bias against negative studies exists in the literature, but we here report negative findings due to the multiple potentially positive actions of the drug. Closer examination of the effects of the molecule, however, reveals several potentially negative effects as well. We conclude there may be inherent weakness in pharmacologic monotherapy, even with molecules having protean potentially beneficial effects. This conclusion seems to have been borne out by the results of recent clinical trials.

Nonselective cation channels are ubiquitous in a variety of tissues including the central nervous system (CNS), and their activation can lead to cell death. Overactivation of these channels may play a role in pathological processes related to intracellular calcium accumulation. Ischemic cell death in stroke is believed in part to involve influx of calcium into neurons. Although blockade of agonist-gated and voltage gated L-type calcium channels has been studied in models of cerebral ischemia, with some therapeutic successes, blockade of nonselective cation channels has not been evaluated.

Mefenamic acid (N-(2-3,xylyl)-anthranilic acid) is a CNS-penetrating nonsteroidal anti-inflammatory drug possessing analgesic/antipyretic actions, that also blocks calcium-activated nonselective cation (CAN) channels. These CAN channels have the ability to maintain a depolarized state for extended periods of time, possibly because these channels do not inactivate, and are voltage independent. Experiments studying potential epileptic mechanisms in the hippocampal slice suggest that CAN channels may be linked to N and L-type calcium channels in neurons, producing a positive feedback loop which allows continued calcium influx into a cell. The resulting depolarized state would cause the voltage sensitive calcium channels to remain activated. The occurrence of spreading depression may be the result of
these mechanisms. In cerebral ischemia, spreading depression-like depolarization waves occur.\(^9\) These considerations suggest CAN channels as attractive potential therapeutic targets in cerebral ischemia.

A second possible target for intervention in the treatment of stroke is the potentially damaging inflammatory response.\(^{10,12}\) Since mefenamic acid is anti-inflammatory,\(^{13}\) this further suggested to us that this agent should have potential as a neuroprotectant in cerebral ischemia.

A third mechanism of ischemic damage that is potentially affected is free radical production, by scavenging of nitric oxide radicals by mefenamic acid.\(^{14}\) With three separate modes of potentially beneficial action, we deemed mefenamic acid worthy of examination for neuroprotective effects in a temperature-controlled ischemia model that mimicks ischemic stroke.

**MATERIALS AND METHODS**

Fed male Wistar rats (320-390 g; \(n=30\)) were subjected to focal ischemia using a modified version of the intraluminal filament model for 100 minutes, at a blood pressure of 80 mm Hg.\(^{15}\) Mefenamic acid was prepared by dissolving 1.1 mg in 1 ml of a balanced physiologic 5-ion solution containing 140 mM Na\(^+\), 4 mM K\(^+\), 2.1 mM Mg\(^{2+}\), 1.44 mM Ca\(^{2+}\), 141.3 mM Cl\(^-\) and 9.54 mM HEPES buffer. NaOH was added for dissolution of the drug and to adjust the pH to 7.8. The solution was run through a bacterial filter and stored at 4°C. Mefenamic acid (30 mg/kg) or an equal volume of saline was given by intraperitoneal injection, either 45 minutes prior to ischemia or 20 minutes after ischemia, and was continued for three days at eight hour intervals until nine intraperitoneal injections had been given. Each of the three groups (control (untreated), pretreatment, posttreatment) contained ten animals, and mortality was zero in all three groups.

To induce focal brain ischemia, rats were initially anesthetized under 4% halothane for intubation and were then ventilated on a 7:3 N\(_2\)O:O\(_2\) mixture with 1% halothane (range: 0.5 - 2\%), during which a catheter was inserted into the tail artery to measure blood pressure. A lateral neck incision was made and the right common carotid artery was dissected free, clamped at the bifurcation of the carotid, and then ligated, leaving a stump. Into this carotid artery was inserted and guided up the internal branch of the carotid artery, a 25 mm length of 3-0 suture was inserted and guided up the internal branch of the carotid artery until a feeling of faint resistance was encountered, usually at 21-22 mm. Occlusion duration was 100 minutes during which time blood pressure was regulated to 80 mmHg by varying the halothane concentration. Halothane may not affect ischemic damage in the absence of hypothermia.\(^{16,17}\)

Head temperature was monitored using a tympanic membrane probe inserted into the middle ear. Body temperature was monitored using a rectal probe. Both head and body temperatures were regulated to 37°C using a heating blanket underneath the animal and by varying the distance of an overhead lamp. Blood gases, pH, glucose and hematocrit were measured pre-ischemia, during ischemia and postischemia. Blood glucose was measured, but not regulated with insulin, in order to avoid an effect of insulin itself in ischemia.\(^{18,19}\)

After one week survival the animals were injected with pentobarbital (0.5 ml/100 g) and transthoracic perfusion fixation was carried out through the ascending aorta via the left ventricle. After a brief rinse of the cerebral circulation with normal saline, 4% phosphate buffered formaldehyde was perfused and the brain was removed the next day and placed in fixative. Coronal brain slices 3 mm thick were then cut, and dehydrated in graded ethanol. After clearing in xylol, embedding in paraffin, and 6 \(\mu\)m thick sectioning at 500 \(\mu\)m intervals, 26 equispaced sections were chosen for quantitation from bregma -2.2 to -14.7 mm. Sections were stained with hematoxylin and eosin, and damage was quantitated using a microscope connected to a computerized image analysis system. Four polygons were traced: cortical and subcortical pan-necrosis (infarction), and both ipsilateral and contralateral hemisphere. Atrophy, the difference between the two hemispheres, was calculated by subtracting ipsilateral from contralateral area. Total damage (tissue lost) was calculated by summing cortical necrosis, subcortical infarction, plus atrophy. Total damage was then graphed as percent of the opposite hemisphere, thus giving damage volumes normalized for the size of that hemisphere, obviating variation due to tissue shrinkage and different animal size. Power analysis (SigmaStat, SPSS Inc.) showed that with our sample size (\(n=10\)) and standard deviation (see Figure 1) we would be able to detect a 16% difference in infarct size at \(\alpha=0.05\) and \(B=0.80\) probability levels. Cerebral blood flow measurements have previously been made in this model and validate the degree of ischemia\(^{20}\) and the consistency of the resulting infarcts.\(^{15}\) The protocol was reviewed and approved by the local Animal Care Committee.

**RESULTS**

Physiological parameters, including animal weight, blood glucose, \(P_{O_2}\), \(P_{CO_2}\), pH, hematocrit, head temperature and body temperature, from pre, during and postischemia are displayed in the Table. No significant differences exist between the three groups in any of the parameters. However, a number (\(n=7\)) of animals in the treated groups demonstrated minor, self-limiting seizures throughout the three day period of administration of mefenamic acid. These were characterized by whisker twitching.

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**Table: Physiologic parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (grams)</th>
<th>Glucose (mM)</th>
<th>MABP (mm Hg)</th>
<th>Temp (tympanic)</th>
<th>Temp (rectal)</th>
<th>pH</th>
<th>pCO(_2)</th>
<th>pO(_2)</th>
<th>Hematocrit (%)</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>358±7</td>
<td>6.4±0.2</td>
<td>80.1±0.8</td>
<td>37.02±0.01</td>
<td>37.07±0.04</td>
<td>7.40±0.01</td>
<td>37.8±1.4</td>
<td>112.7±2.7</td>
<td>44.3±0.7</td>
</tr>
<tr>
<td>Pretreated</td>
<td>356±5</td>
<td>6.2±0.3</td>
<td>78.1±1.3</td>
<td>37.03±0.01</td>
<td>37.08±0.04</td>
<td>7.39±0.01</td>
<td>37.3±1.5</td>
<td>117.1±3.4</td>
<td>43.4±0.7</td>
</tr>
<tr>
<td>Posttreated</td>
<td>351±5</td>
<td>6.4±0.3</td>
<td>79.6±0.6</td>
<td>37.03±0.01</td>
<td>37.00±0.04</td>
<td>7.40±0.01</td>
<td>37.1±1.2</td>
<td>117.6±3.8</td>
<td>44.3±0.7</td>
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</table>
Mefenamate has been shown to lower seizure threshold, potentially protective mechanisms of action of this drug. Mefenamate is neuroprotective by directly scavenging potentially damaging NO radicals. There are a number of positive effects of mefenamate which would be expected to ameliorate ischemic brain damage. Mefenamate penetrates the CNS and blocks non-selective cation channels. Mefenamate is also a nonsteroidal anti-inflammatory agent which blocks cyclooxygenase, the enzyme which catalyzes the first reaction on the pathway of oxidative arachidonic acid metabolism to thromboxane A2 and prostacyclin. We obviated hypothermia, but anti-inflammatory drugs have been reported to be neuroprotective, when hypothermia is allowed to occur.

In addition to channel blockade and anti-inflammatory action, free radical scavenging provides a third potential mechanism of neuroprotective action. Mefenamate is neuroprotective in vitro by directly scavenging potentially damaging NO radicals.

The negative results warrant explanation in light of multiple potentially protective mechanisms of action of this drug. Mefenamate has been shown to lower seizure threshold, accounting for the minor seizures we observed in the treated animals. Seizures of a global type are detrimental in ischemia but here were very mild and self-limiting. Their occurrence in the present study parenthetically indicates CNS penetration of the drug. Although seizures might have cancelled other, neuroprotective actions of the drug, our chosen dose was near the ED$_{50}$ neuroprotective dose against neuronal necrosis from pilocarpine-induced status epilepticus. No trend toward any effect was seen, and this was deemed not to warrant dose-response studies.

Mefenamate has also been shown to cause calcium release from intracellular stores and affects calcium-activated large conductance potassium (K$_{Ca}$) channels, both of which might be detrimental in ischemia.

Following ischemia, cortical spreading depression (CSD) occurs. By itself, spreading depression does not cause neuronal injury in normal brain but it may render neurons more vulnerable in ischemic brain, and may contribute to neuronal damage in tissue surrounding the infarct, or distantly. Prevention of CSD has been deemed essential if adequate recovery of the neurons is to occur following ischemia. In this study, it is not possible to determine whether mefenamic acid had any effect on CSD, although none of the authors in the ischemic cascade. Such mechanisms may be overwhelming and could be unaffected by a single drug. These considerations make it necessary to consider multi-drug therapy, or a combination of pharmacologic and effective non-pharmacologic measures in treating brain ischemia.

REFERENCES