Postischemic (S)-Emopamil Therapy Ameliorates Focal Ischemic Brain Injury in Rats

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(S)-Emopamil is a calcium channel blocker of the phenylalkylamine class, having potent serotonin S2 antagonistic properties and high blood–brain barrier penetrability. Previous studies have documented cerebroprotective effects in animal models of both focal and global ischemia. The present study was undertaken to define the postischemic “window” of therapeutic efficacy for this agent. Sprague-Dawley rats were subjected to permanent proximal middle cerebral artery occlusion, combined with an initial 30-minute period of halothane-induced hypotension (50 mm Hg). (S)-Emopamil (20 mg/kg) was administered intraperitoneally either 20–30 minutes prior to middle cerebral artery occlusion or 1 hour, 2 hours, or 3 hours following occlusion. Treated groups received a second similar dose 2.5 hours later and twice daily for 2 days thereafter. Brains were perfusion-fixed on the third day. Planimetric analysis of hematoxylin and eosin-stained coronal brain sections documented a cortical infarct averaging 72.9±33.3 mm3 (mean±SD) in untreated rats. Cortical infarct volume was reduced by 48% (to 37.6±27.6 mm3) when therapy was initiated 1 hour postischemia (<0.05). When treatment was deferred to 2 hours postischemia, mean cortical infarct volume was reduced by 34%, but this difference did not attain statistical significance. Infarct volume in rats with treatment initiated at 3 hours postischemia was indistinguishable from that in controls. Striatal infarct volume was similar in all groups. These results document a postischemic therapeutic window of cerebroprotection for (S)-emopamil lying between 1 and 2 hours after middle cerebral artery occlusion. (Stroke 1991;22:355–360)

Proximal middle cerebral artery (MCA) occlusion of the rat, originally described by Tamura et al, yields a focal cerebral infarct, the size of which has been shown to vary according to a variety of factors, including the supplier and the strain of rats, the site and length of the MCA segment that is coagulated, and the perioperative values of blood glucose and arterial blood pressure. By standardizing these known factors, one may better be able to assess putative cerebral protective agents in the setting of focal cerebral ischemia.

Our laboratory has previously shown that (S)-emopamil, a calcium channel blocker of the phenylalkylamine class with superior blood–brain barrier permeability and potent serotonin S2 antagonist activity, reduces infarct volume in a rat model of MCA occlusion even when treatment is commenced 1 hour following the onset of ischemia. In the clinical setting, however, initiation of treatment is often delayed beyond this time point. Therefore, the present study was undertaken to examine the protective effect of (S)-emopamil treatment initiated up to 3 hours following an ischemic insult.

Materials and Methods

Normally fed male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, Mass.) weighing between 310 and 440 g were used. After induction of anesthesia with 4% halothane, the rats were intubated with PE-240 polyethylene tubing and mechanically ventilated with 2% halothane, 70% nitrous oxide, and a balance of oxygen. A polyethylene catheter was inserted into the right femoral artery for blood pressure measurement and arterial blood sam...
pling. Arterial blood gases were measured at intervals, and ventilatory adjustments were made as needed to ensure normocarbia. Rectal temperature was maintained between 36.5° and 37.5°C by means of a heating pad beneath the animal.

Rats were mounted on a frame, and the right proximal MCA was exposed, electrocoagulated, and transected as described previously. Briefly, a skin incision was made between the eye and ear; the zygomatic arch was removed; and the temporal muscle flap was retracted downward together with the intact mandibular bone, thus exposing the inferotemporal fossa. A craniectomy was then made with a saline-cooled dental drill rostral to the foramen ovale. The dura mater was incised with a 26-gauge needle. The segment of the MCA electrocoagulated was approximately 2.5 mm in length, lying medial to the lateral edge of the olfactory tract and containing the origin of the branch to the lateral olfactory tract. Electrocoagulation was carried out with a fine bipolar forceps. The coagulated MCA segment was then divided to ensure complete MCA occlusion. Rats having excessive arterial bleeding during the intracranial procedure were discarded.

In pilot (unpublished) studies in these intubated, normocarbic rats, Morikawa obtained smaller infarcts than had the previous operator (Nakayama et al9) in spontaneously breathing, mildly hypercapnic animals. In order to produce larger cortical infarcts comparable to those in the earlier study, we added an initial 30-minute period of halothane-induced hypotension—a procedure previously demonstrated to increase infarct volume substantially. Just prior to MCA occlusion, the inspired halothane concentration was increased to 4%, as a result of which the mean arterial blood pressure was reduced to 50 mm Hg. The mean blood pressure was then maintained between 40 and 60 mm Hg for the first 30 minutes following MCA occlusion by appropriate small adjustments in the inspired halothane concentration. During this period, the cranial wound was closed. At the end of this 30-minute period, halothane was discontinued and the blood pressure was allowed to return to normotensive levels. The rats were then extubated and returned to their cages with free access to food and water.

Six milligrams of (S)-emopamil were dissolved in 1 ml of distilled water. Treated rats received (S)-emopamil in a dose of 20 mg/kg body wt i.p. (The dose was doubled from the previous study in the hope of increasing the therapeutic effect.) Fifty-four rats were divided into five groups according to the time of initiation of drug therapy. In group 1, the pretreatment group (n=9), (S)-emopamil was injected 20–30 minutes prior to MCA occlusion. In group 2 (n=13), group 3 (n=12), and group 4 (n=8), (S)-emopamil therapy was initiated 1 hour, 2 hours, and 3 hours, respectively, following MCA occlusion. In each of these groups, following the first dose of (S)-emopamil, a second dose similar to the first was administered 2.5 hours later, and additional 20 mg/kg doses were injected twice daily over the following 2 days, until the day before sacrifice. Group 5 (n=12) consisted of controls that received intraperitoneal injections of vehicle twice daily, beginning 20–30 minutes before (n=9) or 1 hour after (n=3) MCA occlusion; the volume injected was similar to the volume of drug injected in the (S)-emopamil-treated groups.

Three days after MCA occlusion, the rats were tracheostomized under 2% halothane anesthesia, immobilized with 5 mg i.p. d-tubocurarine, and mechanically ventilated. The brain was perfusion-fixed by transcardiac perfusion with physiologic saline followed by a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 by volume), which was delivered at 110 mm Hg for 20 minutes. Coronal brain blocks were embedded in paraffin. Brain sections 10 μm thick were prepared at 200-μm intervals. These sections were stained with hematoxylin and eosin. For morphometric study, eight coronal levels having readily identifiable anatomic landmarks were selected as previously described. Each such section was viewed at low power (×10), and the infarcted area was traced onto paper using a camera lucida microscope attachment. Each drawing was then re-traced onto a digitizing tablet interfaced to a computer, which calculated infarcted areas at each coronal level. Infarct volume was calculated by numeric integration of sequential infarct areas.

The analytical strategy was to determine first whether there was any effect of (S)-emopamil therapy on infarct size compared with control and, if so, what was the effect of the timing of treatment on infarct size. A one-way analysis of variance (ANOVA) of all five treatment means followed by Dunnett's test comparing the (S)-emopamil means with the control mean was used to determine the presence of an effect. This was followed by a regression analysis of the timed measurements to determine the shape of the (S)-emopamil response over time and to compare among time points. For these latter comparisons, the Tukey and Dunn tests were used.

Results

In the initial studies of this series, animals tended to lose weight (estimated as approximately 15%12) during the 3 days between MCA occlusion and sacrifice. This could be alleviated, however, simply by providing pellet food on the floor of the cage, that is, within closer reach. After completion of MCA occlusion and allocation to the respective treatment group, six rats died prior to histopathologic analysis and hence were excluded from data analysis: these consisted of two rats in the pretreatment group, one in the 1-hour posttreatment group, one in the 2-hour posttreatment group, and two in the control group. All six deaths occurred in the first half of the series, when pellet food was not accessible on the floor of the cage.

Table 1 summarizes the physiological variables. No significant intergroup differences were noted in arte-
In contrast to the (S)-emopamil-associated reduction of cortical infarct volume, treatment failed to influence the striatal infarct volume in any group.

Figure 2 shows representative histological appearances of the cerebral infarct in the five animal groups. The reduction in the size of the cortical infarct associated with (S)-emopamil therapy was chiefly evident in the dorsolateral cortical region.

**Discussion**

The results of this study confirm a pronounced therapeutic effect of (S)-emopamil in reducing infarct volume of the cerebral neocortex (mean reduction, 48%) when administered in the early (1 hour) postischemic period. A tendency toward reduced cortical infarct volume (mean reduction, 34%) was also apparent when (S)-emopamil was first administered 2 hours following MCA occlusion. In contrast, there was no suggestion of benefit when treatment was initiated 3 hours following the ischemic insult. These data, therefore, strongly suggest that the upper limit of the postischemic “therapeutic window” for (S)-emopamil lies between 1 and 2 hours.
FIGURE 2. Representative paraffin-embedded brain sections, taken at coronal level of maximal infarct area, from rats subjected to permanent middle cerebral artery (MCA) occlusion 3 days earlier. Sections have been stained with hematoxylin and eosin. Arrowheads indicate dorsolateral border of cortical infarct. Right hemisphere is shown on left. a: Untreated control. b: (S)-Emopamil treatment prior to MCA occlusion. c: Treatment 1 hour postocclusion. d: Treatment 2 hours postocclusion. e: Treatment 3 hours postocclusion.

In contrast to the previous study, no effect was observed with (S)-emopamil pretreatment. The higher (S)-emopamil dose used in this study, which appeared to decrease mean arterial blood pressure by an additional 5 mm Hg during the period of halothane-induced hypotension in the pretreatment group (Table 1), may have contributed to this negative result. It is also possible that the deep halothane anesthesia used to induce transient hypotension might have maximally dilated the cerebral blood vessels and thus have provided no chance for blood flow to increase further in the (S)-emopamil pretreated animals. Other possibly contributory factors include hypercapnia, which was present in the previous study but absent here. We cannot be certain as to which of these factors was primarily responsible for the loss of the response to (S)-emopamil pretreatment.

(S)-Emopamil has been found by our laboratory as well as by others to reduce ischemic neuronal death in rat models of transient high-grade forebrain ischemia and to improve postischemic hypoperfusion. Indeed, recent reviews of calcium channel blockers suggest that the anti-ischemic efficacy of these agents, at least in certain settings, may derive primarily from their cerebrovascular action rather than from the prevention of cellular calcium uptake or from the diminution of calcium-mediated metabolic dysfunction. Like many other calcium channel blockers, (S)-emopamil is a potent cerebral vasodilator, capable of increasing cortical blood flow by 50% or more without affecting local cerebral glucose utilization. Results observed with emopamil in an isolated rat brain preparation suggest that the agent is readily delivered to the brain...
vasculature and is capable of enhancing postischemic perfusion; its salutary effects on postischemic energy metabolism appear to be attributable to a primary vascular influence.\textsuperscript{21}

(S)-Emopamil also has a strong serotonin S\textsubscript{2}-receptor blocking ability.\textsuperscript{22} Serotonergic neurons are located in the raphe nuclei complex of the midbrain, and serotonergic terminals innervate cortical neurons\textsuperscript{23} and are associated with cerebral vessels.\textsuperscript{24} It has been proposed that serotonin release after ischemia may aggravate the ischemic insult by constricting cortical arteries.\textsuperscript{25} The serotonin S\textsubscript{2} antagonist ketanserin was shown to reduce the remote hemodynamic consequences of thrombotic infarction.\textsuperscript{26} Thus, it is possible that the anti-ischemic effect of (S)-emopamil may be related, at least in part, to its S\textsubscript{2}-receptor blocking ability.

(S)-Emopamil possesses an extremely high brain uptake index (110 relative to water), and its cerebral availability is 18 times that of its congener verapamil following parenteral administration.\textsuperscript{20} The superior blood-to-brain permeability of this agent may be another key to its anti-ischemic efficacy, even when treatment is initiated following an ischemic insult.

The addition of halothane-induced hypotension following MCA occlusion in the rat, previously assessed by Osborne et al.,\textsuperscript{4} was shown to result in a significantly larger infarct than occurred in rats without hypotension. Halothane dilates the peripheral vasculature and decreases blood pressure in a dose-related fashion, which facilitates adjustment of blood pressure. The effect of halothane on cerebral metabolism and the cerebral vasculature, however, should be considered. In a qualitative deoxyglucose study, deep halothane anesthesia was said to decrease glucose metabolism in the gray matter homogeneously, although some subcortical structures showed relatively higher activity.\textsuperscript{27} In another study, MCA occlusion in rats with transient halothane-induced hypotension led to increased cerebrovascular permeability.\textsuperscript{28}

Alternative methods of increasing infarct size in this model of proximal MCA occlusion consist of induction of transient hemorrhagic hypotension or temporary occlusion of the cervical carotid arteries,\textsuperscript{29} both of which are aimed at further reducing cerebral perfusion pressure in the early postocclusion period. Distal MCA occlusion in combination with permanent ipsilateral and transient contralateral common carotid artery occlusion was shown to be technically easier and less invasive.\textsuperscript{30} This model, however, leaves open occasional posteriorly directed cortical branches from the proximal segment of the MCA, which were shown to be present in 16\% of Sprague-Dawley rats.\textsuperscript{31} Nonetheless, Brint et al\textsuperscript{3} recently showed, in spontaneously hypertensive rats, that distal MCA occlusion plus ipsilateral common carotid artery occlusion yields a reproducible cortical infarct. Although rats may suffer more severe neurologic deficits (grades 2 or 3 of Bederson et al\textsuperscript{4}) and more chewing difficulty after proximal MCA occlusion procedures than after distal MCA occlusion, we observed no postoperative mortality in the second half of the present series, when pellet food was made more easily accessible to the animals.

To summarize, the results of this study confirm a substantial therapeutic effect of (S)-emopamil in reducing cortical infarct volume following permanent MCA occlusion when therapy is initiated 1 hour following the onset of ischemia. A therapeutic trend was evident, as well, in rats treated beginning 2 hours postischemia, but animals commencing (S)-emopamil therapy 3 hours postischemia had infarct volumes indistinguishable from those of vehicle-treated controls. These data thus define an upper limit of the therapeutic window of postischemic efficacy, lying between 1 and 2 hours following MCA occlusion—a time frame that makes this agent potentially suitable for very early clinical application following ischemic stroke.

In this study, permanent MCA occlusion was employed. As some degree of reperfusion is common in human ischemic stroke and serves to alter the resulting histopathologic picture,\textsuperscript{32} it will be important in future studies to assess the therapeutic efficacy of (S)-emopamil in the latter setting as well. This is particularly relevant in that therapeutic thrombolysis is currently under active clinical scrutiny in the setting of ischemic stroke.\textsuperscript{33}

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