Reduction in Ischemic Brain Injury in Rabbits by the Anion Transport Inhibitor L-644,711

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Background and Purpose: We studied the anion transport inhibitor L-644,711, which is known to reduce astrocyte swelling and excitotoxin release in primary astrocyte culture, in two models of thromboembolic stroke to assess its capacity to influence ischemic brain injury.

Methods: New Zealand White rabbits were used in this study. The two models include autologous clot embolized to the brain via the carotid artery, with one model using a transient period of systemic hypotension. Cerebral blood flow was determined by the hydrogen clearance method, intracranial pressure was measured with a fiberoptic transducer, and infarct size was assessed with triphenyltetrazolium chloride staining of the coronally sectioned brain. Both models received a 2-hour infusion of L-644,711 (total dose, 12 mg/kg) beginning 20 minutes before embolization.

Results: In both the normotensive (p<0.01) and the hypotensive (p<0.05) model, treatment with L-644,711 resulted in a significant reduction in infarct size and a significant improvement in regional cerebral blood flow (p<0.03, normotensive model, and p<0.05, hypotensive model). Raised intracranial pressure, unique to the hypotensive model, was abolished by the administration of L-644,711 (p<0.05). A hyperglycemic response associated with embolization, also unique to the hypotensive model, was significantly reduced by the administration of L-644,711 (p<0.05).

Conclusions: The ability of L-644,711 to limit brain injury in two related models of thromboembolic stroke suggests a potential therapeutic role for anion channel blockers in cerebral ischemia. (Stroke 1992;23:93-97)

L-644,711 \((R^+)(5,6\text{-dichloro }9a\text{-propyl} 2,3,9,9a\text{-tetrahydro }3\text{-oxo-1H fluoren}-7\text{yl})\) oxy acetic acid) is an anion transport inhibitor that has been shown to be effective in reducing mortality in a feline model of head trauma/hypoxia\(^1\)\(^2\) and a tyramine-induced model of Reyes syndrome in dogs.\(^3\) In the head injury model, L-644,711 was shown to inhibit astrocyte swelling.\(^4\) Since astrocyte swelling is also an early event in cerebral ischemia,\(^5\) we decided to examine the effects of L-644,711 in two models of thromboembolic stroke in the rabbit, one with and one without superimposed transient hypotension. The model including hypotension produces a severe ischemic insult and generates a substantial rise in intracranial pressure (ICP).\(^6\) The normotensive model produces a less extensive infarct without raised ICP.\(^7\) We present data demonstrating that pretreatment with L-644,711 prevents ischemic brain injury in both models.

Materials and Methods

The two rabbit models of thromboembolic stroke used in the present study have been previously described in detail.\(^6\)\(^7\) Briefly, New Zealand White rabbits of either sex weighing 2.7-3.2 kg were anesthetized with a solution of acepromazine (20 mg) and ketamine (50 mg/kg). An aortic catheter was placed transfemorally for mean arterial pressure (MAP), arterial blood gas (pH, PaO\(_2\), and PaCO\(_2\)), hematocrit, and glucose determinations. A platinum-iridium probe was inserted through the other femoral artery to the level of the aortic arch to monitor systemic hydrogen concentrations and four 30-gauge platinum-iridium electrodes were inserted 2 mm into the...
cortical mantle for measurement of regional cerebral blood flow (rCBF) using the initial slope of the hydrogen clearance curve (t₀, taken where systemic hydrogen concentration falls to zero, typically, 25 seconds from termination of the 7% hydrogen inhalation). Three electrodes were placed 10 mm lateral to midline and immediately posterior to the coronal suture within the embolized hemisphere. A single electrode was used to monitor rCBF in the contralateral hemisphere. The measurement of rCBF by the hydrogen clearance technique is well established and has been demonstrated to compare favorably with the techniques of stable xenon-enhanced computed tomographic scanning and thermal diffusion. A fiberoptic epidural pressure transducer (Ladd Research Industries, Burlington, Vt.) was positioned anterior to the coronal suture in midline to monitor ICP.

The rabbits were mechanically ventilated with a Baby Bird Ventilator (Bird Corporation, Palm Springs, Calif.). Adjustments in depth and rate of ventilation and inspired O₂ concentration were used to maintain arterial blood gases within physiologic range. Core temperature, as measured by an esophageal probe, was maintained to within 1°C. The common, internal, and external carotid arteries on the right side were isolated and the external carotid artery ligated. After a 30-minute equilibration period, baseline measurements of serum glucose, hematocrit, ICP, arterial blood gases, MAP, temperature, and rCBF were obtained and recorded. Each reading was repeated to ensure reproducibility; core temperature, MAP, and ICP were recorded continuously.

Twenty minutes before the unilateral embolization of an autologous clot, an intravenous infusion of L-644,711 (Merck & Dohme, prepared in 4.2% NaHCO₃, pH 6.3 at a concentration of 1 mg/kg) was initiated and continued for 2 hours, giving a total dose of 12 mg/kg in the experimental groups. The control groups received the vehicle. The investigators were blinded as to the treatment modality being instituted. A 5-cm length of autologous clot formed in polyethylene 90 tubing was then injected into the internal carotid artery via a common carotid arteriotomy. The arteriotomy was repaired and blood flow to the common and internal carotid arteries reestablished.

Two models of thromboembolic stroke differing in ischemic intensity were employed in this study. In the first model, following the embolization, all animals were subjected to controlled exsanguination to achieve an MAP of 30 mm Hg for 45 minutes. This transient hypotension is necessary to consistently produce a cerebral infarct in both the middle and posterior cerebral artery distributions. During the hypotension rCBF was reduced to <5 ml/100 g/min in all animals. At the end of this 45-minute hypotensive period, the preembolization baseline MAP (55±5 mm Hg) and hematocrit were restored by reinfusing the blood withdrawn.

In the second model, normotension was maintained throughout the entire experiment. Cerebral blood flow measurements were determined immediately following embolization. To ensure a reproducible infarct, a reduction in rCBF in the embolized hemisphere to <15 ml/100 g/min was used as the criterion for ischemic intensity in this series of experiments. The value of rCBF reduction is based on historical studies demonstrating the intensity of ischemia necessary to produce irreversible injury if no therapeutic intervention is instituted.

After the 45-minute period of hypotension after embolization in the hypotensive model and embolization alone in the normotensive model, baseline MAP was supported with homologous citrated blood. All animals were supported for a total of 4 hours from the time of the embolic event. This duration was chosen to provide sufficient time for infarct delineation. Recordings of all measurements previously described under baseline readings were performed at five time points: baseline, within 30 minutes after embolization, and at each of the final three hourly time points.

At the end of the experiments the animals were killed with sodium pentobarbital (150 mg/kg i.v.). A calvariecotomy was then performed, the brain harvested, and the location of the clot verified by visual inspection. The brain was then coronally sectioned into 2-mm slices and incubated in 1.5% buffered triphenyltetrazolium chloride (TTC) to delineate the infarct. The technique of staining with TTC to determine the region of infarct has been demonstrated to have good correlation with electron microscopic evidence of cell death at time points as early as 3 hours and with ischemic injury as demonstrated by nuclear magnetic resonance and CBF autoradiograms at time points as early as 4 hours, suggesting that this technique is an acceptable method for measuring infarct size. Infarct size, as percentage of the total hemispheric area, was determined planimetrically using an IBM Image Analyzer.

A total of 26 rabbits were used in this study. Ten animals were subjected to embolization plus hypotension, with five animals receiving L-644,711 and five animals receiving the vehicle control. Similarly, 10 animals were subjected to embolization alone, with five animals receiving L-644,711 and five animals receiving vehicle control. A total of three animals in the control groups and three animals in the L-644,711 groups failed to meet our rCBF criteria, and these experiments were terminated. Only those flow probes meeting criteria were included in the data analysis. Arterial blood gases and hematocrit were measured hourly and MAP and core temperature measured continuously in both stroke protocols. Hematocrit, arterial blood gases, serum glucose values, rCBF, and ICP were analyzed through the use of analysis of variance (ANOVA) for repeated measurements. Analysis of variance provides tests for changes over time (five recording times) and overall...
differences between control and L-644,711 treatment. When an overall treatment difference exists, either analysis of covariance adjusting for baseline differences or the Wilcoxon rank sum test where no baseline differences exist was performed to identify the time following embolization at which treatment differences occur. In this way the effect of testing at multiple time points on the probability of a type I error is minimized. Measurements of infarct size were analyzed through the use of the Wilcoxon rank sum test. All tests of hypotheses were performed at the $\alpha=0.05$ level of significance. Results are reported as the mean±SEM.

**Results**

The administration of L-644,711 did not significantly change the MAP, which was held constant at 55±5 mm Hg with the two following exceptions: 1) during the 45-minute period of exsanguination-induced hypotension in the hypotensive model, and 2) for 5 minutes immediately after embolization, during which MAP rose an average of 4.5 mm Hg in the L-644,711-treated groups and 8.5 mm Hg in the control groups. Arterial blood gases, hematocrit, and core temperature were maintained within physiological range in all groups except for a uniform transient reduction in hematocrit in both the control and L-644,711-treated groups during the 45-minute period of hypotension. No statistically significant differences, based on ANOVA, were noted for any physiological parameters.

Regional cerebral blood flow for the hypotensive (Figure 1) and the normotensive model (Figure 2) demonstrated very similar baseline values. Thirty minutes after embolization in the normotensive model, rCBF was reduced to 6.1±2.8 ml/100 g/min in the control group followed by a minimal recovery of rCBF during the course of the experimental protocol. In the L-644,711-treated group, however, there was a recovery of rCBF from the 30-minute value of 10.4±2.6 ml/100 g/min to values consistent with nonischemic flows (26–34 ml/100 g/min) by 2 hours after embolization ($p<0.03$, ANOVA group comparison of control versus L-644,711 groups). In the hypotensive model, rCBF was reduced to <5 ml/100 g/min in both treated and control animals. Cerebral blood flow in the control group remained at <10 ml/100 g/min throughout the duration of the experiment. The L-644,711 group again demonstrated a recovery of rCBF to nonischemic ranges within 2 hours of embolization (Figure 1, $p<0.05$, ANOVA group comparison of control versus L-644,711-treated groups).

In the hypotensive model, infarct size was reduced from 65.9±8.1% in the control group to 34.4±5.6% in the L-644,711–treated group ($p<0.05$, Figure 3). In the normotensive model the infarct size was reduced from 36.9±9.2% of the hemisphere in the control group to 5.4±1.9% of the hemisphere in the L-644,711 group ($p<0.01$, Figure 3). Gross examination of the harvested brain confirmed the presence of the clot in the anterior circulation on the side embolized without evidence of fragmentation or dissolution.
Mean ICP in all groups before embolization was 6–8 mm Hg. In the normotensive model no rise in ICP was observed in either the control or treated group. In the hypotensive model, ICP steadily rose in the control group to achieve a value 235±61.3% of the baseline value (Figure 4). In the L-644,711–treated group, no statistically significant rise in ICP was seen. The difference in ICP between the control and treated groups achieved statistical significance (p<0.05, ANOVA treatment comparison) at the final two time points.

The serum glucose values for all groups before embolization were very similar, ranging between 214 and 260 mg/dl. In the normotensive model there was a rise in serum glucose in both the control and L-644,711–treated groups. This rise in serum glucose concentration was not statistically significant.

In the hypotensive model, there was a marked increase in serum glucose values in the control group to 576±130.5 mg/dl; the hyperglycemic response in the L-644,711–treated group was significantly reduced to 335.2±42.4 mg/dl (p=0.05, ANOVA treatment comparison).

Discussion

We studied an anion channel blocker, L-644,711, in two models of cerebral ischemia, both involving autologous clot embolization of one internal carotid artery in the rabbit. Pretreatment with L-644,711 resulted in a statistically significant 80% and 51% decrease in infarct size in the normotensive and hypotensive models, respectively. This reduction in infarct size was associated with a statistically significant restoration of cerebral blood flow in both models. Treatment with the drug also blocked the development of raised ICP and elevated serum glucose values seen in the hypotensive model.

We suggest two possible mechanisms of action for the salutary effects of L-644,711 in our models, based on published in vitro studies and work in progress:

- Inhibition of astrocyte swelling or inhibition of neutrophil function.

Astroglial swelling is a prominent early feature of cerebral ischemia. Although the role of astroglial swelling in the pathophysiology of ischemic injury is unknown, recent studies have shown that swollen primary astrocytes cultures release glutamate, an excitotoxic neurotransmitter implicated in ischemic neuronal damage. In these in vitro experiments, L-644,711 blocked the release of glutamate from swollen astrocytes. Although not directly examined in the present study, this ability of L-644,711 to prevent the release of glutamate from swollen astrocytes may be mechanistically related to its demonstrated ability to limit ischemic brain injury. Further work is necessary to clearly establish and clarify the effect of the drug on in vivo excitotoxic release.

Another mechanism by which L-644,711 might exert its beneficial effects on cerebral ischemia is the suppression of neutrophil function. Similar to the results with other anion channel blockers, L-644,711 inhibits neutrophil function in vitro and in vivo (authors’ unpublished observations). Neutrophil activation and accumulation may play a role in ischemic injury via rheological or biochemical mechanisms. Neutrophils are known to accumulate in ischemic brain23 and may release deleterious products such as oxygen free radicals, platelet activating factor, and eicosanoids.22 The ability of L-644,711 to inhibit neutrophil function may in part explain its efficacy in cerebral ischemia. It is of interest that the depletion of neutrophils with anti-neutrophil antisera produced similar reductions in ischemic brain injury, that is, partial restoration of rCBF, amelioration of raised ICP, and reduction in infarct size in the hypotensive model.

Hyperglycemia is known to increase brain damage in models of global brain ischemia; however, the effect of hyperglycemia on focal cerebral ischemia is unclear since both beneficial26-27 and deleterious effects have been demonstrated. The fact that L-644,711 blunted the hyperglycemia encountered in our models indirectly supports the hypothesis that hyperglycemia may exacerbate ischemic brain injury. Of course, the cause-and-effect relationship of the hyperglycemic response and the degree of brain injury in the present models has yet to be established.

The pretreatment strategy used in the present study allowed us to examine the impact of L-644,711 on cerebral ischemia in what we anticipated to be a “best case scenario.” Comparison of pretreatment with posttreatment paradigms, as previously examined in incomplete global ischemia, may ultimately assist in defining the time frame and the magnitude of the various mechanisms contributing to ischemic cerebral injury.

In conclusion, pretreatment with L-644,711 reduced ischemic brain injury in two models of thromboembolic stroke in the rabbit. Two possible mechanisms for the salutary effect of the drug are the...
suppression of neutrophil function and inhibition of astrocyte swelling. Further studies are ongoing to evaluate the efficacy of L-644,711 as a posttreatment in experimental stroke paradigms.

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References


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