Cerebral ischemia can result from a wide range of disturbances and is associated with several intracellular and molecular events leading to neuronal death. Despite many investigations into the biochemical mechanisms of ischemia-induced neurodegeneration, the precise molecular factors leading to cell death remain unclear. Several metabolic and neurochemical changes have been demonstrated in models of ischemia. Necrotic cells contain high levels of intracellular calcium while extracellular calcium concentrations decrease during ischemia, suggesting an important role for calcium-dependent biochemical processes associated with ischemia.

An important consequence of the ischemia-induced rise in calcium concentration is the massive release of neurotransmitters and neuropeptides. Recent evidence suggests that in postischemic neuronal degeneration there is an overactivity of excitatory amino acid (viz. glutamate and aspartate) transmitter systems. The excessive activation of glutamatergic pathways causes prolonged depolarization at postsynaptic receptors, accompanied by alterations in membrane permeability to calcium ions. These mechanisms may also contribute to the vulnerability of other neurons, such as those affected by neuropeptides, to ischemia.

It has been proposed that endogenous opioids play a pathophysiological role in brain ischemia. Numerous groups have examined the potential therapeutic effects of naloxone, an opiate antagonist, in different stroke models, with mixed results. Differences in methodology, animals, and clinical outcome measures may account for some of the discrepancies. Moreover, high doses of naloxone are required, suggesting that the therapeutic effects of this drug may result from actions that are not mediated by μ-opioid receptors. Such conclusions are consistent with recent findings in which selective κ-opioid antagonists improved hemodynamic function and behavioral/neurologic deficits. Since dynorphin has selectivity for the κ-opioid receptor, attempts have been made to identify the specific opioid and receptor that mediate the pathologic effects of cerebral ischemia.

We examined changes in the number ($B_{max}$) and dissociation constant ($K_d$) of κ-opioid receptors in a...
FIGURE 1. Sample of electroencephalogram under baseline conditions (left) and 1 hour after embolization (right) in rabbit. MC, motor cortex; SC, sensorimotor cortex; VC, visual cortex; r, right; l, left; ECG, electrocardiogram.

previously described model of massive cerebral ischemia induced in rabbits.21

Materials and Methods

Using 25 New Zealand white rabbits weighing 3.0–3.5 kg, we isolated and then cannulated the right common carotid artery after occlusion of all its external branches. Embolization through a catheter inserted in the internal carotid artery was carried out in 11 rabbits by means of 10 microspheres, 0.3–0.35 mm in diameter, suspended in 1 ml of a sterile solution containing physiological saline plus 1% xanthan gum and perfused by a constant perfusion pump for 60 seconds (flow rate of 62 ml/hr). The five sham-operated rabbits received carotid injections of 1 ml of the vehicle. The nine control rabbits received no carotid injections. We performed all surgical procedures with the rabbits under anesthesia (10-15 minutes duration) with 4% halothane and used 2% xylocaine locally to ensure their pain-free conditions.

We prepared another group of 17 rabbits (six sham-operated and 11 embolized) for electroencephalography (EEG) according to the stereotactic method of Monnier and Gangloff22 to study the motor, sensorimotor, and visual cortices of both hemispheres. All electrophysiological variables were evaluated for 30 minutes at baseline conditions and 1 hour after surgical preparation and carotid injection. The quantified EEG analysis was calculated on-line using a Nova 4/c, 16-bit minicomputer of the Data General series (Westboro, Massachusetts) equipped with a 12.5-megabyte hard disk, a 512-kilobyte floppy disk, hardware multiply-divide, and an analog-to-digital conversion board and then evaluated by means of a minicomputer, according to the method standardized in our laboratory.21 We considered the power density spectrum and four frequency bands (0.15–3.7, 3.7–7.2, 7.2–12.2, and 12.2–20.2 Hz) for both the right and left cortices. Somatosensory evoked potentials (SEPs) were obtained by unilateral stimulation of the right and left median nerves. The data were evaluated by two-factor analysis of variance23 to examine the factors treatment, times within a treatment, rabbit, and the treatment × rabbit interaction. All the in vivo experiments were carried out in strict accordance with the “Guiding Principles in the Care and Use of Laboratory Animals” of the American Physiological Society.

The binding studies were conducted 1 hour after surgical preparation or carotid injection to avoid the presence of necrotic tissue to a significant extent even if it cannot be excluded that pathologic processes were developing. The 25 rabbits were decapitated and their brains were homogenized as described by Quirion and Pilapil.24 For 16 rabbits (five controls, five sham-operated, and six embolized) 1.0 ml homogenized right frontoparietal cortex (12 mg/ml) was increased to 2.0 ml with solutions of the unlabelled ligand dynorphin A (1–8) plus [3H]dynorphin A (1–8) (specific activity 25.5 Ci/mmol at 1, 3, 5, 7, 10, and 15 nM). Binding was assayed in the remaining nine rabbits (four controls and five embolized) in the presence of a saturating concentration of U 69593 (10−8 M), a specific κ-opioid receptor ligand.25 Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 10−6 M unlabeled dynorphin A. The mixture was incubated for 1 hour at 0°C, filtered, and washed three times with cold buffer. The protein content was measured according to the method of Lowry et al.26 Kd and Bmax were calculated using the methods of Munson and Rodbard.27 The control, sham-operated, and embolized groups were compared using analysis of variance as modified by Scheffé.23

For histologic examination, the brains of all 17 rabbits were fixed in 12% formalin solution. Five coronal sections were prepared and stained with hematoxylin and eosin and Luxol fast blue for myelin.

Results

Neither the control nor the sham-operated rabbits showed any changes in the parameters considered. All 11 embolized rabbits developed massive bilateral edema. At the time of the binding assay no ischemic lesions were evident; they were detectable histologically only later.
The EEG recordings of the 11 embolized rabbits were characterized by continuous delta activity of very low voltage on the right side and delta activity of voltage higher than normal on the left (Figure 1). Quantified EEG showed that the relative power in the 0.15-3.7-Hz frequency band increased bilaterally. A significant decrease (p<0.05) in the power density spectrum on the right and a significant increase (p<0.05) on the left were observed (Figure 2). SEP recordings indicated bilateral loss of all cortical components. In all the 11 embolized rabbits the subcortical components (N1 and P1) were delayed (Figure 3).

Histologic results showed that the 11 embolized rabbits developed massive bilateral brain edema without histologic evidence of ischemic lesions. According to histologic localization of the ischemic sites previously described, 21 samples of the right frontoparietal cortex submitted to biochemical determinations showed that our experimental stroke significantly increased Bmax without changing Kd (Table 1). In the experiments using 10^-8 M U 69593, no changes were observed in the kinetic properties of κ-opioid receptors in embolized rabbits compared with controls (Table 2).

**Table 1. Kinetic Variables of κ-Opioid Receptors in Rabbit Brain**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Bmax (fmol/mg protein)</th>
<th>Kd (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>181.66±12.61</td>
<td>3.42±0.18</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>5</td>
<td>193.77±16.06</td>
<td>4.22±0.32</td>
</tr>
<tr>
<td>Embolized</td>
<td>6</td>
<td>247.90±15.54*</td>
<td>3.77±0.26</td>
</tr>
</tbody>
</table>

Data are mean±SEM.

*p<0.005 different from control and sham-operated according to Munson and Rodbard. 27

**FIGURE 2.** Bar graphs of quantified electroencephalogram analysis in rabbits: power density spectrum (PDS) and relative power in four frequency bands. MC, motor cortex; SC, sensorimotor cortex; shaded bars, baseline conditions; cross-hatched bars, 1 hour after embolization. *p<0.05 different from baseline by analysis of variance.

**FIGURE 3.** Typical somatosensory evoked potentials of rabbit under baseline condition (Bas) (top) and 1 hour after embolization (1 h) (bottom). R, right hemisphere (stimulation of left median nerve); L, left hemisphere (stimulation of right median nerve); N1 and P1, subcortical components; N2 and P2, N3 and P3, N4 and P4, cortical components.

**Discussion**

Experimental intravascular embolization in rabbits seems to be a valuable model of stroke that reproduces a pattern of massive cerebral edema commonly found during the course of ischemic insult to the brain. Previously we standardized a different model of stroke by injecting different amounts of microspheres into the internal carotid artery. 21 The pathologic picture ranged from a single infarct, ipsilateral to the injection and localized in the frontoparietal area to massive brain edema. In that study, cerebral blood flow was monitored by the clearance curve of xenon-133. Rabbits submitted to massive embolization demonstrated a critical decrease (>80%) in gray matter blood flow. These neurophysiological results...
indicate the existence of brain damage since the increase in delta activity is typical of this pathology. In particular, SEP recordings showed a loss of cortical components, indicating a primary cortical lesion.

Recent evidence suggests a role for endogenous opioid peptides in the pathogenesis of cerebral ischemia and shock. These findings are based mainly on the demonstration that the opiate antagonist naloxone improves neurologic recovery. However, these results have not been confirmed by other investigators.17,18 Even if there are no clear reasons to explain the apparently contradictory findings with naloxone, some hypotheses can be made. Naloxone is effective only at very high doses, reflecting actions of non-μ-opioid receptors. Beneficial doses of naloxone are nearly three times those used to reverse morphine effects in animals. For these reasons, it has been suggested that other opiate receptors, such as δ- and κ-opioid receptors, could be involved. Studies using opiates acting at κ sites showed beneficial effects in a rabbit model of spinal stroke.19,20 Moreover, it has been demonstrated that dynorphin concentrations increase following traumatic thoracic spinal cord injury in rats.21 The increase in dynorphin concentrations is localized to the ischemic site and is closely correlated with the degree of damage and neurologic deficits. Dynorphin is considered to be the endogenous neuropeptide with selectivity for κ-opioid receptors.22 Because of these findings, it has been suggested that dynorphin is the pathologic opioid involved in cerebral ischemia. McIntosh and coworkers23 showed that κ-opioid agonists exacerbate hypoperfusion after brain injury, but no data are available regarding changes in κ-opioid receptor binding after cerebral ischemia.

Our results indicate that massive cerebral ischemia in rabbits significantly increases the number of κ-opioid receptors without changing the dissociation constant. The increase in Bmax is localized at the ischemic site in the frontoparietal cortex since this brain area is the most affected by embolization. Even if [H]dynorphin A (1–8) also binds to μ- and δ-opioid receptors, our experiments using saturating concentrations of the specific κ-opioid agonist U 69593 clearly demonstrate that only κ-opioid receptors are modified during brain ischemia. In fact, no changes in the number of [H] dynorphin binding sites were detected in the ischemic tissue under these experimental conditions.

In conclusion, our results suggest that κ-opioid receptors and dynorphin-related peptides appear to be involved in this model of cerebral ischemia. Therefore, specific κ-opioid antagonists might possess greater therapeutic efficacy than nonselective opiate antagonists such as naloxone in the treatment of cerebral ischemia.

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References


KEY WORDS • cerebral ischemia • endorphins • rabbits
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