Indomethacin Ameliorates Ischemic Neuronal Damage in the Gerbil Hippocampal CA$_1$ Sector

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The purpose of our experiment was to examine whether the cyclooxygenase inhibitor indomethacin ameliorates neuronal injury in the gerbil hippocampal CA$_1$ sector following 5 minutes of forebrain ischemia. Thirty minutes before bilateral carotid artery occlusion, Mongolian gerbils were injected intraperitoneally with 1 (n = 10), 2 (n = 10), 5 (n = 12), or 10 (n = 7) mg/kg of indomethacin. Seven days after occlusion, the gerbils were perfusion-fixed and neuronal density in the hippocampal CA$_1$ sector was assessed. The mean ± SEM neuronal density in nine unoperated normal gerbils was 307 ± 9/mm$^2$, in 10 untreated ischemic gerbils 55 ± 21/mm$^2$, and in seven vehicle-treated ischemic gerbils 15 ± 9/mm$^2$. The mean ± SEM neuronal density in ischemic gerbils treated with 1, 2, 5, or 10 mg/kg indomethacin was 132 ± 28/mm$^2$, 154 ± 29/mm$^2$, 176 ± 30/mm$^2$, and 136 ± 39/mm$^2$, respectively. Indomethacin at any dose significantly ameliorated ischemic neuronal damage in the gerbil hippocampal CA$_1$ sector.

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Received March 22, 1988; accepted June 2, 1988.

Neurons in the hippocampal CA$_1$ sector are highly vulnerable to ischemic insult.$^{1-4}$ Brief bilateral carotid artery occlusion in gerbils has been shown to produce delayed neuronal damage in the hippocampal CA$_1$ sector.$^{2,3}$ Recent experiments suggest that neurotoxic actions of synaptically released excitatory amino acids play a crucial role in delayed neuronal damage in the hippocampus,$^{5-7}$ but the mechanism is not fully understood.

The role of prostaglandins (PGs) in the central nervous system has been the object of numerous investigations. Gaudet et al$^8$ showed that brain concentrations of PGD$_2$, PGE$_2$, PGF$_2\alpha$, thromboxane B$_2$, and 6-keto-PGF$_1\alpha$ were markedly elevated during recirculation following cerebral ischemia. To study the functional aspects of brain PGs, it seems important to clarify the regional distribution of PG synthesis in the brain. Using immunohistochemical techniques, it has been recently demonstrated that recirculation after ischemia resulted in increased PGF$_2\alpha$ concentration in the cytoplasm of neurons (especially in the hippocampus), in blood vessels, and in the interfascicular oligodendrocytes.$^9$ The binding proteins specific for PGF$_2\alpha$, PGE$_2$, and PGD$_2$ have also been found in the hippocampus.$^{10}$

Recent extensive studies have demonstrated that the PGs possess many impressive biologic activities$^{11-15}$: regulation of cerebral blood flow,$^{11}$ potentiation of the action of excitatory amino acids,$^{12}$ inhibition of noradrenaline release from cerebrocortical neurons,$^{13-15}$ etc. Considering these biologic activities, it can be assumed that PGs synthesized in the hippocampus during recirculation may affect the extent of ischemic hippocampal damage.

The purpose of our study was to examine whether delayed neuronal damage in the hippocampal CA$_1$ sector can be prevented by suppression of PG synthesis with indomethacin. To evaluate the effect of indomethacin, neuronal cell density in the CA$_1$ sector was used as an index of neuronal survival.$^{16}$

Materials and Methods

Sixty-five adult Mongolian gerbils weighing 60–70 g were anesthetized with 2% halothane in 70% N$_2$O and 30% O$_2$. Bilateral forebrain ischemia was induced by occluding the common carotid arteries with aneurysm clips for 5 minutes. Anesthesia was discontinued as soon as the clips were placed.

Thirty minutes before occlusion, 39 gerbils were injected intraperitoneally with 1 (n = 10), 2 (n = 10), 5 (n = 12), or 10 (n = 7) mg/kg of indomethacin (Sigma Chemical Co., St. Louis, Missouri) dissolved in 7% sodium bicarbonate solution (vehicle).
The volume of indomethacin solution was 0.1 ml/10 g body wt. In preliminary experiments, 10 gerbils had been injected with 10 mg/kg indomethacin 30 minutes before occlusion. Three of the 10 gerbils died a few days after clip removal; autopsy revealed gastrointestinal bleeding. In our present experiment, therefore, 1 mg/kg of an H\textsubscript{2} blocker, famotidine (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan),\textsuperscript{17} dissolved in physiological saline was also injected intraperitoneally 30 minutes before occlusion to prevent gastrointestinal bleeding. The volume of famotidine solution injected was 0.1 ml/10 g body wt. Seven vehicle-treated gerbils were injected with 7% sodium bicarbonate solution and famotidine.

In both indomethacin- and vehicle-treated gerbils, 1 mg/kg famotidine was administered 12 and 24 hours after clip removal. To examine the effects of vehicle and famotidine on ischemic neuronal damage, 10 gerbils underwent bilateral carotid artery occlusion but received no drugs. Nine unoperated gerbils served as normal controls.

After surgery, all gerbils were returned to their cages and permitted free access to food and water. One week after occlusion, the gerbils were perfusion-fixed with 3.5% formaldehyde in 0.1 M phosphate buffer (pH 7.3) under pentobarbital anesthesia by transcardiac perfusion at a pressure of 120 cm H\textsubscript{2}O. Fixed gerbils were refrigerated overnight, and the brains were dissected out the following day.

Two-millimeter-thick coronal sections were cut, dehydrated through a graded series of ethanol, soaked in xylene, and embedded in paraffin. Six-micrometer sections that contained the dorsal hippocampus (0.5–1.0 mm posterior to the most rostral tip of the hippocampus or 1.4–1.9 mm posterior to the bregma)\textsuperscript{18} were prepared and stained with hematoxylin and eosin or Luxol fast blue and cresyl violet.

| TABLE 1. Neuronal Cell Density of Gerbil Hippocampal CA\textsubscript{1} Sector After 5-Minute Bilateral Occlusion of Common Carotid Arteries |
|---------------------------------|-----------|----------|
| Group                           | No.       | Density  |
| Unoperated normal controls      | 9         | 307±9    |
| Bilateral forebrain ischemia     |           |          |
| Untreated                       | 10        | 55±21    |
| Vehicle-treated                 | 7         | 15±9     |
| Indomethacin-treated            |           |          |
| 1 mg/kg                         | 10        | 132±28\textsuperscript{*}     |
| 2 mg/kg                         | 10        | 154±29\textsuperscript{†}     |
| 5 mg/kg                         | 12        | 176±30\textsuperscript{‡}     |
| 10 mg/kg                        | 7         | 136±39\textsuperscript{†}     |

Values are mean±SEM cells per 1-mm linear length.

\textsuperscript{*}p<0.05, 0.01 compared with vehicle.
\textsuperscript{†}p<0.05, 0.01 compared with untreated.

\textsuperscript{‡}p<0.05, 0.01 compared with untreated.
The sections were examined in a blind fashion as reported by Kirino et al. The number of intact neurons in the CA₁ sector in one section from each gerbil was counted since similar neuronal changes are seen throughout the rostrocaudal extent of the dorsal hippocampus. The left and right dorsal hippocampi of each specimen were photographed using Polaroid type 667 films (Cambridge, Massachusetts) at ×30 magnification. On each photograph the total linear length of the CA₁ sector was measured by means of a digital curvimeter (Uchida Youkou Co., Tokyo, Japan). The number of intact neurons in the stratum pyramidale within the CA₁ subfield (Figure 1) was counted using an Olympus Vanox photomicroscope (Lake Success, New York) at ×400 magnification. Neurons that had shrunken cell bodies surrounded by empty spaces were excluded. Based on these data, the neuronal density of the right and left CA₁ sectors of each gerbil, that is, the number of CA₁ neurons per 1-mm linear length of the stratum pyramidale observed in each 6-μm section, was calculated and averaged; data for each group are expressed as mean ± SEM. Statistical analysis was done using Student's t test; p<0.05 was considered significant.

Results
In the nine unoperated normal control gerbils neuronal cell density of the CA₁ sector was 307 ± 9/mm (Table 1). In the 10 gerbils subjected to 5 minutes of bilateral forebrain ischemia but treated with no drugs extensive neuronal damage was observed in the CA₁ sector; the density of intact neurons was 55 ± 21/mm.

In the seven vehicle-treated gerbils extensive neuronal damage was also observed (Figure 2); neuronal density in the CA₁ sector was 15 ± 9/mm. There was no significant difference in the neuronal densities between untreated and vehicle-treated gerbils subjected to 5 minutes of bilateral forebrain ischemia.

Figure 2. Photomicrographs of pyramidal cells in gerbil hippocampal CA₁ sector after 5-minute bilateral common carotid artery occlusion. a: Unoperated normal gerbil; hematoxylin and eosin stain, ×369. b: Vehicle-treated gerbil; hematoxylin and eosin stain, ×369. c: 5 mg/kg indomethacin-treated gerbil; hematoxylin and eosin stain, ×369. CA₁ pyramidal cells were well preserved by treatment with indomethacin.
The CA1 pyramidal cells were well preserved by treatment with indomethacin (Figure 2). The neuronal density in 1, 2, 5, or 10 mg/kg indomethacintreated gerbils was 132±28/mm (n=10), 154 ±29/mm (n=10), 176±30/mm (n=12), and 136±39/mm (n=7), respectively (Table 1). These neuronal densities were all significantly higher than that in untreated and vehicle-treated gerbils subjected to 5 minutes of bilateral forebrain ischemia.

Discussion

In our present experiment, ischemic neuronal damage in the hippocampal CA1 sector of gerbils following bilateral forebrain ischemia was ameliorated by pretreatment with indomethacin. At doses of 1, 2, and 5 mg/kg, delayed neuronal damage was reduced in a dose-related manner.

Indomethacin is a well-known cyclooxygenase inhibitor. It has been reported that at the doses we used indomethacin inhibits brain PG synthesis by approximately 60–80%. Using immunohistochemical techniques, we also confirmed (unpublished data) that PG synthesis in the hippocampal area was markedly reduced by pretreatment with 5 or 10 mg/kg indomethacin. Therefore, our results suggest that cyclooxygenase metabolites synthesized during recirculation after ischemia are involved in the pathogenesis of delayed neuronal damage in the hippocampal CA1 sector.

Although the functional aspects of PGs in the central nervous system have not been clarified, some biologic actions of PGs can be postulated to affect the survival of pyramidal cells in the hippocampal CA1 sector following ischemia. The release of excitatory amino acids such as glutamate and aspartate has been strongly suggested to be a critical factor for delayed neuronal death following transient cerebral ischemia. Using intradendritic recording, PGF2α, PGE2, or PGD2 have been reported to potentiate the excitatory action of L-glutamate or L-aspartate on Purkinje cell dendrites. Although such potentiating actions of PGs on excitatory amino acids are not verified yet in hippocampal neurons, PGs synthesized in the hippocampal area may augment the cytotoxic effect of excitatory amino acids on CA1 pyramidal cells.

There are numerous reports indicating that PGs are important as modulators of adrenergic neurotransmission. PGE2 attenuated the release of 3Hnorepinephrine from synaptosomes prepared from rat hypothalamic tissue. In brain cortical slices, PGE2 markedly reduced the stimulation-evoked overflow of tritium. On the other hand, lesions of the locus coeruleus system, a principal inhibitory neuronal system, have been demonstrated to aggravate ischemic neuronal damage in the rat hippocampal CA1 region. Therefore, it might be assumed that PGs, principally PGE2, synthesized in the hippocampal area following recirculation attenuate depressant effects of noradrenergic transmission on neuronal excitability, thereby aggravating ischemic neuronal damage.

Several pharmacologic actions of indomethacin other than cyclooxygenase inhibition must also be considered in interpreting our results. Indomethacin is known to possess a calcium antagonistic action. Disruption of intracellular calcium homeostasis is thought to play an important role in the process of cell death. It has been demonstrated that calcium accumulated in the hippocampal CA1 region precedes a marked increase in neuronal cell necrosis, and treatment with a calcium antagonist has been reported to reduce ischemic neuronal damage in the hippocampus. Therefore, indomethacin might ameliorate neuronal damage in the CA1 sector by inhibiting calcium influx.

Indomethacin also inhibits phospholipase A2 activity. However, the possibility that indomethacin treatment reduced ischemic neuronal damage by blocking phospholipase A2 seems to be less likely because much higher concentrations are required for a significant inhibition of phospholipase A2 activity than that required to block cyclooxygenase. Furthermore, it has been reported that treatment with dexamethasone or corticosterone, which induces inhibition of phospholipase A2 activity, failed to ameliorate the ischemic neuronal necrosis.

Our results are in contrast with those of Koide et al who reported that 5 mg/kg indomethacin did not reduce ischemic neuronal damage. Differences in experimental conditions such as species, severity of ischemic insult, and drugs other than indomethacin might affect the experimental results, but the exact reason for the different results is not clear at present.

In conclusion, our results reveal that indomethacin ameliorated neuronal death in the hippocampal CA1 sector following 5 minutes of bilateral forebrain ischemia. Further studies should be performed to investigate the mechanism for such effects.

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KEY WORDS: cerebral ischemia • indomethacin • hippocampus • gerbils
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Stroke. 1988;19:1399-1403
doi: 10.1161/01.STR.19.11.1399

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