Ischemia-Related Changes in Galanin Expression in the Dentate Hilar Region after Transient Forebrain Ischemia in Gerbils

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Abstract: Although galanin (GAL) protects hippocampal neurons from ischemic damage, no study has examined ischemia-related changes in endogenous GAL in the hippocampal dentate gyrus. We investigated the chronological changes of GAL, well-known as the potassium channel opener, expression in the dentate gyrus at various times after 5 min of transient forebrain ischemia in gerbils. A few GAL-immunoreactive (IR) neurons were found in the polymorphic layer of the sham-operated group. Three hours after ischemia-reperfusion, the pattern of GAL immunoreactivity was similar to that of the sham-operated group and the number of GAL-IR neurons and immunoreactivity were highest 12 h after ischemic insult. At this time, GAL-IR neurons in the polymorphic layer showed strong GAL immunoreactivity. Thereafter, GAL-IR neurons and immunoreactivity significantly decreased in the dentate hilar region. Four days after ischemic insult, GAL-IR neurons were not detectable. In addition, the results of a Western blot study showed a pattern of GAL expression similar to the immunohistochemical changes. GAL protein content also was highest 12 h after ischemia. In conclusion, the increased expression of endogenous GAL in the dentate gyrus after ischemia is related to response to the ischemic damage.

Key words: dentate gyrus, galanin, gerbil, polymorphic cells, potassium channel opener, transient forebrain ischemia

Introduction

Transient forebrain ischemia, produced by prolonged deprivation of blood flow to the brain, results in the

insidious delayed degeneration of specific vulnerable neurons within the brain [19, 24]. The hippocampus is one of the most vulnerable brain regions in terms of ischemic injury [12, 13, 30].

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Transient bilateral occlusion of common carotid arteries in the Mongolian gerbil (*Meriones unguiculatus*) is widely used as a model for transient forebrain ischemia. Cerebral infarction can be produced in the gerbil by ligating common carotid arteries and is due to a peculiarity of the circle of Willis in which collateral blood flow between the internal carotid and basilar circulation is absent [4, 17].

In 1982, Kirino reported that 3 different types of ischemic damage were observed in the hilar region of the dentate gyrus, the hippocampal CA2 region and the hippocampal CA1 region after 5 min of transient forebrain ischemia in gerbils. In the hilar region of the dentate gyrus, neuronal changes were rapid and corresponded to ischemic cell change. Alterations in the CA2 region were relatively slow and identical to what has been called reactive change. On the contrary, changes in the CA1 pyramidal cells were even slower, and this process was described as delayed neuronal death [19].

Glutamate is one of the most important neurotransmitters in the development of cerebral ischemia-induced cell death [8, 9, 31]. Treatment with glutamate release inhibitors and receptor antagonists decreases neuronal death after transient forebrain ischemia [14, 16, 28]. One possible type of cellular protection after ischemic injury is reduced glutamate release from glutamatergic neurons by activated potassium channels, leading to the hyperpolarization of nerve endings [23]. Potassium channel openers such as galanin (GAL) and RP 52891 protect hippocampal neurons from ischemic damage [32]. GAL, a 29 amino acid neuropeptide, is an inhibitory neuromodulator in a variety of central systems. It is related to memory function, the regulation of secretion of hypothalamopituitary hormones and the regulation of seizure activity [2, 15, 20].

Mazarati *et al.* [20] examined the role of hippocampal GAL in an animal model of status epilepticus. Control rats showed abundant GAL-immunoreactive fibers in the dentate hilar region, whereas no GAL-immunoreactive neurons were observed. They suggested that hippocampal GAL acts as an endogenous anticonvulsant via GAL receptors. No studies have examined the normal distribution of GAL in the dentate hilar region, furthermore, Kirino [19] reported that neuronal death in the dentate hilar region was more rapid than that in the hippocampal CA1 region. Until

now, no studies have reported the ischemia-related changes of endogenous GAL expression in the dentate gyrus after transient forebrain ischemia in the Mongolian gerbil. Therefore, in the present study, we examined the chronological expression and alterations of GAL in the dentate gyrus after 5 min of transient forebrain ischemia using the gerbil ischemia model.

Materials and Methods

Experimental animals

This study used the progeny of Mongolian gerbils (*Meriones unguiculatus*) obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with a fixed 12 h light/dark cycle and free access to food and water. Procedures involving animals and their care conformed to institutional guidelines which are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996).

Induction of transient forebrain ischemia

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 66–75 g were placed under general anesthesia with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck and both common carotid arteries were isolated, freed of nerve fibers, and occluded using non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central arteries in eyeballs using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. Restoration of blood flow (reperfusion) was directly observed under the ophthalmoscope. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded. Body temperature was monitored and maintained at $37 \pm 0.5^\circ\text{C}$ during the surgery and during the immediate post-operative period until the animals had fully recovered from the anesthesia. At designated reperfusion times, the sham-operated and operated animals were sacrificed. The animals were anesthetized with pentobarbital sodium, and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by

4% paraformaldehyde (in 0.1 M PB, pH 7.4) at 30 min, 3 h, 12 h, 24 h, 2 days and 4 days ($n=8$ at each time point) after the induction of ischemia. The sham-operated animals served as controls ($n=8$).

Immunohistochemistry for GAL

Five animals in each group were used for the immunohistochemical study. Their brains were removed and post-fixed in the same fixative for 6 h and the brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the tissues were frozen and sectioned with a cryostat at 30 μ m and consecutive sections were collected in six-well plates containing PBS.

The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were next incubated with diluted rabbit anti-GAL antibody (1:10,000, Chemicon, USA) overnight at room temperature and were subsequently incubated with biotinylated horse anti-rabbit IgG and streptavidin peroxidase complex (Vector, USA). Then, the sections were visualized with 3,3'-diaminobenzidine in 0.1 M Tris buffer and mounted by Polymount (Polyscience, USA). In order to establish the specificity of the immunostaining, a negative control test was carried out by substituting normal rabbit serum for primary antibody or by omitting the primary antibody. The negative control showed the absence of immunoreactivity in any structures.

Western blot study

Three animals in each group were used for the immunoblot study. After sacrificing them and removing the dentate gyrus, the tissues were homogenized in 50 mM Tris containing 50 mM HEPES (pH 7.4), EGTA (pH 8.0), 0.2% NP-40, 10 mM EDTA (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After centrifugation, the protein concentration was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, USA). Aliquots containing 20 μ g of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Then, each aliquot

was loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, USA). To reduced background staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min followed by incubation with rabbit anti-GAL antiserum (1:5,000), with peroxidase conjugated horse anti-rabbit IgG (Sigma, USA), and then with ECL kit (Amersham, USA).

Quantification of data and statistical analysis

Twenty sections per animal were viewed through a microscope connected via CCD camera to a PC monitor. At a magnification of 25–50 $\times$, the regions were outlined on the monitor and their areas measured. Each studied field for each tissue was selected within the dentate gyrus containing all layers. Images of GAL immunoreactivity in the dentate gyrus of each animal were captured with an Applescanner. The brightness and contrast of each image file was calibrated by Adobe Photoshop version 2.4.1, and analyzed using NIH Image 1.59 software. Values of the background staining were obtained and subtracted from the immunoreactive intensity. The intensity of GAL immunoreactivity was evaluated by means of a relative optical density (ROD) value which value was obtained after the transformation of mean gray values into ROD using a formula. Also the results of the Western blot study were scanned and a ROD value was obtained using Scion Image software (NIH). All obtained quantitative data were analyzed using one-way ANOVA to determine statistical significance and Bonferroni's test was used for post-hoc comparisons. A P -value below 0.05 or 0.01 was considered to be statistically significant.

Results

Changes in GAL immunoreactivity

A few GAL-immunoreactive neurons were detectable in the polymorphic layer (PL) of the sham-operated group (Fig. 1A). The pattern of GAL immunoreactivity 3 h after ischemic insult was similar to that of the sham-operated group (Fig. 2). Twelve hours after ischemic insult, GAL-immunoreactive neurons and immunoreactivity were significantly increased in the dentate gyrus (Figs. 1B and 2). At this time, a few GAL-immunoreactive neurons were found in the mo-

Fig. 1. Immunohistochemical staining for GAL in the dentate gyrus of the sham-operated group (A), 12 h (B), 2 days (C) and 4 days (D) after ischemia-reperfusion. In the sham-operated group (A), a few GAL-immunoreactive neurons (arrows) are detectable in the polymorphic layer (PL). GAL-immunoreactive neurons are increased 12 h after ischemic insult (B). GAL immunoreactivity decreases 2 days after ischemic insult (C) and GAL immunoreactivity is not detectable 4 days after ischemia-reperfusion (D). GCL, granule cell layer; ML, molecular layer. Bar=100 μ m.

lecular layer, and GAL-immunoreactive neurons in the polymorphic layer showed strong GAL immunoreactivity. In addition, the granule cell layer showed higher GAL immunoreactivity than that of the sham-operated group. Thereafter, the number of GAL-immunoreactive neurons and the immunoreactivity decreased in the dentate gyrus (Fig. 2).

Two days after ischemia-reperfusion, GAL-immunoreactive neurons and immunoreactivity were significantly decreased in the dentate gyrus as compared with the 12 h post-ischemic group (Figs. 1C and 2). At this time, a few GAL-immunoreactive neurons were detectable in the polymorphic layer, but four days after ischemic insult, GAL-immunoreactive neurons were not detectable (Figs. 1D and 2). Also, GAL immunoreactivity was very weak in the dentate gyrus.

Changes in GAL protein content

The results of the Western blot study showed a pattern of GAL expression similar to the immunohistochemical changes that occurred after 5 min of transient

Fig. 2. The relative optical density of GAL immunoreactivity in the dentate gyrus after ischemia-reperfusion. The results of the quantitative data obtained using image analysis are consistent with the immunohistochemical study (* $P < 0.05$, ** $P < 0.01$, significant differences from the adjacent group). The bars indicate the means $\pm$ S.E.

forebrain ischemia (Fig. 3). The GAL protein content was highest 12 h after ischemia-reperfusion, and thereafter decreased until 4 days after ischemia.

Fig. 3. Western blot analysis of GAL in the gerbil dentate gyrus derived from the sham-operated and operated groups. The relative optical density of the immunoblot band is also represented. Two asterisks indicate significant differences ($P < 0.01$) from the adjacent group. The bars indicate the means $\pm$ S.E.

Discussion

Some experimental studies have suggested that neurotoxicity via excitatory amino acids such as glutamate plays a role in the pathogenesis of ischemic damage in the hippocampus [1, 8, 9, 18]. In addition, tissue culture studies have found that the excitotoxic action of glutamate occurs in the presence of calcium ions, and that it disrupts cellular homeostasis and leads to neuronal damage [6, 22, 25].

Ionic current behavior under conditions of acute ischemia is a consequence of elevated $[K_o]$ and acidosis [3]. It results in the enhancement of the inward rectifier potassium current and activation of ATP-sensitive K^+ channels [10, 21, 26, 27].

In the present study, we found that ischemic insult evoked changes in GAL in the dentate gyrus in gerbils. GAL expression was significantly increased in the dentate hilar region 12 h after ischemia-reperfusion and at this time, many hilar cells showed strong GAL immunoreactivity. This enhanced GAL expression might imply a neuroprotective effect from the excitotoxic event and this result is supported by a previous study

that found that the release of glutamate evoked by a 6- or 8-min period of ischemia is reduced by 25–40% in the presence of GAL (0.3 μ M). The opening of ATP-sensitive K^+ channels reduces ischemia-induced necrotic cell death in the heart [11] and brain [7, 29].

The efflux of potassium ions inhibits the excitatory post-synaptic potential (EPSP) of the CA1 pyramidal cells in the hippocampus [5]. Thus, GAL is induced after ischemia-reperfusion to open the potassium channels, which cause pyramidal cells in the CA1 region to become hyperpolarized. The hyperpolarized pyramidal cells prevent the glutamatergic CA1 pyramidal cells from releasing glutamate which suggests that GAL as a potassium channel opener might attenuate the accumulation of glutamate and reduce ischemic damage.

Galanin expression plays a role in memory and cognitive function [15]. GAL reduces a suite of potassium currents, including calcium-activated potassium (IC), the delayed rectifier (IK), and transient outward potassium (IA) conductances, but not calcium or sodium currents [15].

In conclusion, our results suggest that the increased expression of endogenous GAL in the dentate gyrus 12

h after ischemia-reperfusion may be associated with the response to ischemic damage.

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