Müller Cells in the Preconditioned Retinal Ischemic Injury Rat

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NISHIYAMA, T., NISHIKAWA, S., TOMITA, H. and TAMAI, M. Müller Cells in the Preconditioned Retinal Ischemic Injury Rat. Tohoku J. Exp. Med., 2000, 191 (4), 221–232 —— The role of Müller cells in the preconditioned retinal ischemic injury rat was investigated. In anesthetized Sprague Dawley rats, retinal ischemia for 5 minutes constituted the preconditioning stimulus for the left eye. After 24 hours, both eyes were clamped for 60 minutes. In 30, 60, 90, and 120, minutes and 1 day, 3 days, and 7 days after ischemia, electroretinograms were recorded, and the eyeballs were enucleated. After fixation with 4% paraformaldehyde, the avidin-biotin-peroxidase technique was applied to show glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP). Furthermore, for the solubilized retinas, Western blot analysis and enzyme-linked immunosorbent assay were performed to detect GS and GFAP in the extracts. Preconditioning performed 24 hours before ischemia significantly improved the recovery of the a- and b-waves 1 day after 60 minute ischemia. In the 30, 60, 90, and 120 minutes after ischemia, the recovery of the a-wave only was observed. There was a nonsignificant trend toward greater recovery in the first 120 minutes after 60 minute ischemia, especially in the b-wave. GS immunoreactivity had no significant difference between non-preconditioned and preconditioned groups 30, 60, 90, and 120 minutes after ischemia. In 1 day after ischemia, GS immunoreactivity decreased in both groups. In 3 and 7 days after ischemia, GS immunoreactivity recovered only in the preconditioned group. The retinas at 3 and 7 days after 1 hour of ischemia showed increased GFAP immunoreactivity in the non-preconditioned group. In the preconditioned group, only slight GFAP immunoreactivity was observed. These results suggested that the mechanism of preconditioned retinal ischemia may be related to Müller cells in the retina. ——— glial cell; glutamate; Müller cell; preconditioning; retinal ischemia © 2000 Tohoku University Medical Press

The basis for the phenomenon of ischemic preconditioning is that short periods of ischemic stress do not lead to irreversible cell injury (in contrast to prolonged ischemic stress) but paradoxically to an adaptive mechanism that results in resistance to a subsequent ischemic stress.

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Cerebral ischemia is a potent modulator of gene expression. Gene regulation, including immediate early genes, is required for programmed neuronal death after trophic factor deprivation and is predicted to be involved in apoptosis triggered by cerebral ischemia (Atkins et al. 1996). Hypoxia induces upregulation of gene products (Takagi et al. 1996). It is reported that the tolerance to ischemia is induced by the incompletely defined protective factors in hippocampal neurons (Kirino et al. 1991). In myocardium (Parratt and Szekeres 1995; Martin and Walter 1996) and in the brain (Kogure and Kato 1993; Simon et al. 1993), the phenomenon of ischemic tolerance has been shown. But once ischemia has begun, preconditioning by a previous, brief nondamaging period of ischemia cannot protect the retina. It is significant in the search for effective methods to prevent or treat ischemic injury in the central nervous system because preconditioning utilizes the endogenous protective potential of tissue.

It has been demonstrated that preconditioning provided complete protection against retinal ischemic injury in rats (Roth et al. 1998), although the mechanism was unclear. It was thought that Müller cells play a central role in the homeostatic regulation of the retina (Newman and Reichenbach 1996). In this study, we investigated glutamine synthetase (GS), which is the marker enzyme of Müller cells, and glial fibrillary acidic protein (GFAP), which is expressed in unhealthy Müller cells, in the preconditioned retina.

**Materials and Methods**

This experiment conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

**Animals**

Sprague Dawley rats on postnatal day 90 were anesthetized with intramuscular injections of xylazine (13 mg/kg) and ketamine (87 mg/kg). To induce ischemia, we used aneurysm clips (large aneurysm clip for permanent occlusion, Sugita, Tokyo). We incised the bulbar conjunctiva around the limbus, and then clamped the left eye for 5 minutes as the preconditioned eye. After 24 hours, both eyes were clamped for 60 minutes. In 30, 60, 90, and 120, minutes and 1 day, 3 days, and 7 days after ischemia, electroretinograms (ERGs) were recorded, and the eyeballs were enucleated.

**ERG Recordings**

Rats were dark-adapted overnight and then, in dim red light, were anesthetized with intramuscular injections of xylazine (13 mg/kg) and ketamine (87 mg/kg). Full-field scotopic ERGs were elicited with 10-μsecond flashes of white light and responses were recorded using a UTAS-E 3000 Visual Electrodiagnostic System (LKC Technologies, Gaithersburg, MD, USA). The corneas of the rats were anesthetized with a drop of 0.5% propacaine hydrochloride, and the pupils
were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses with gold wire loops were placed on both corneas with a drop of 2.5% methylcellulose to maintain corneal hydration. A silver wire reference electrode was placed subcutaneously in the hind leg. Stimuli were presented at intensities of 1.9 log candela (cd) m² at 1-minute intervals. Responses were amplified at a gain of 4000, filtered between 0.3 to 500 Hz, and digitized at a rate of 2000 Hz on two channels. Three responses were averaged. The a-waves were measured from the baseline to the peak in the cornea-negative direction, and b-waves were measured from the cornea-negative peak to the major cornea-positive peak. (Preconditioned: \( n = 10 \) per group, non-preconditioned: \( n = 4 \) per group).

**Immunohistochemical staining**

Eyeballs were fixed overnight with periodate-lysine paraformaldehyde fixative. The 3-mm thick sections were treated with 0.3% hydrogen peroxide-methanol for 15 minutes at room temperature. Next, they were incubated overnight at 4°C with 1 \( \mu \)g/ml anti-GS and GFAP antibody, with biotin-conjugated goat anti-mouse IgG, 1:200 dilution overnight at 4°C, and with avidin-biotin-peroxidase complex for 1 hour at 37°C. Antibodies were dissolved in phosphate buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin (BSA), and sections were rinsed with PBS containing 0.05% Tween 20 between incubations. Color was developed by incubation in 50 mM Tris-HCl, pH 7.6, containing 0.02% 3-3'-diaminobenzidine-HCl, and 0.05% hydrogen peroxide for 10 minutes at room temperature. The sections were then dehydrated and mounted.

**Immunoblots of extracts of retinas with GS and GFAP**

After the retinas were carefully removed from uvea and sclera, they were solubilized with 1% sodium dodecyl sulfate. Protein concentrations were measured according to the method of Lowry et al. (1953), using BSA as the standard. Western blot analysis was performed according to Towbin et al. (1979) using each 5 mg protein sample. After proteins were transferred to nitrocellulose membrane by electrophoresis, the membrane was treated with 3% gelatin for 1 hour at 37°C. We used 1 \( \mu \)g/ml mouse monoclonal anti-GS antibody (Chemicon International INC., Temecula, CA, USA) and mouse monoclonal anti-GFAP antibody (ICN Biomedicals, Inc., Aurora, OH, USA). Next, 1:5000 diluted anti-rabbit IgG alkaline phosphatase was used as the second antibody. The antibodies were diluted with PBS (0.14 M NaCl and 10 mM phosphate buffer, pH 7.4) containing 1% BSA and 0.05% Tween 20. Incubation time was 1 hour at room temperature. Washing was performed after each step with PBS containing 0.05% Tween 20. Color development was carried out with 5 ml of dye solution (100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris-HCl buffer, pH 9.5) containing 33 \( \mu \)l nitroblue tetra-
zolium solution (50 μg/ml nitroblue tetrazolium in 70% dimethylformamide) and 16.5 μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (50 μg/ml BCIP, p-toluidine salt in dimethyl-formamide).

Assay of relative contents of GS and GFAP contents by ELISA

Relative GS and GFAP contents were measured by enzyme-linked immunosorbent assay (ELISA). After the retinas were carefully removed from uvea and sclera, they were solubilized with 1% sodium dodecyl sulfate. Protein concentrations were measured, using BSA as the standard. Next, 50 μl of diluted samples of retinas (5 μg/ml) was applied in each well. The binding of this antigen to microtiter wells was performed overnight at 4°C. The diluted anti-GS and GFAP antibodies were applied to the wells in 0.05 ml aliquots. After 2 hours of incubation at room temperature, the plate was washed with PBS containing 0.1% Triton X-100 (Tx-PBS) and PBS four times each. Peroxidase-labeled goat anti-rabbit IgG at a dilution of 1:1000 (0.05 ml) was added to the wells. The plates were incubated at room temperature for 2 hours and then washed with Tx-PBS and PBS. Fifty μl of 3.7 mM o-phenylenediamine and 0.006% H₂O₂ in citrate-phosphate buffer (pH 5.0) was added to each well. After another 2 hour incubation, the same amount of 2.4 M H₂SO₄ was added to each well. The absorbance at 492 nm was read on a two-wavelength microplate photometer. Preischemic: n = 4, others: n = 3 per group.

Statistical analysis

In all instances, data are shown as mean ± s.d.; p < 0.05 was considered statistically significant (t-test).

Results

ERG

Fig. 1 shows the representative electroretinogram at 3 days after 60 minute ischemia with and without preconditioning. Preconditioning performed 24 hours before ischemia significantly improved the recovery of the a-, and b-waves 1 day after 60 minute ischemia (p < 0.01). In the 30, 60, 90, and 120 minutes after ischemia, the recovery of the a-wave only was observed (p < 0.05). There was a nonsignificant trend toward greater recovery in the first 120 minutes after 60 minutes of ischemia, especially in the b-wave (Figs. 2a and b).

Immunohistochemical staining

GS immunoreactivity had no significant difference between non-preconditioned and preconditioned groups pre and 60 minutes after ischemia. At 1 day after ischemia, GS immunoreactivity decreased in both groups. At 3 days after ischemia, GS immunoreactivity recovered only in the preconditioned group (Fig. 3a).
The retinas at 3 days after 60 minute of ischemia showed increased GFAP immunoreactivity in the non-preconditioned group. In the preconditioned group, only slight GFAP immunoreactivity was observed (Fig. 3b).

**Immunoblots of extracts of retinas with GS and GFAP**

Extracts from the retinas 30 and 90 minutes after ischemia gave a single band at the same position, which corresponded to 44 kDa for GS. In the non-preconditioned group extracts at 1, 3 and 7 days after ischemia yielded a slightly positive band. At 3 and 7 days after ischemia, the GS band had recovered in the preconditioned group only (Fig. 4a).

The retinas 1, 3 and 7 days hours after 60 minutes of ischemia demonstrated an increased GFAP band, which corresponded to 50 kDa for GFAP in the non-preconditioned group. In the preconditioned group, a band was scarcely observed (Fig. 4b).

**Assay of relative GS and GFAP contents measured by ELISA**

The changes of GS and GFAP concentrations in the retina were shown in Figs. 5a and b. High GS concentration was observed in the extracts from the retinas 30, 60, 90, and 120 minutes after ischemia. In the non-preconditioned group, the extracts from 1, 3, 7 days after ischemia yielded low concentrations of
Fig. 2. ERG summary (a, a-wave; b, b-wave). Preconditioning performed 24 hours before ischemia significantly improved recovery of the a- and b-waves 1, 3 and 7 days after 60 minute ischemia. There was a nonsignificant trend toward greater recovery in the first 120 minutes after ischemia, especially in the b-wave. ■, preconditioned; □, non-preconditioned; pre, 24 hours after preconditioning. All values are mean±s.d. (preconditioned: n = 10 per group; non-preconditioned: n = 4 per group).

GS. At 3 and 7 days after ischemia, GS had recovered only in the preconditioned group (p < 0.05) (Fig. 5a).

The retinas at 1, 3 and 7 days after 60 minutes of ischemia showed increased GFAP in the non-preconditioned group. However, the preconditioned group had a low concentration of GFAP (p < 0.05) (Fig. 5b). The results obtained from ELISA agreed with those obtained from Western blot analysis.
Fig. 3. Immunohistochemistry (Fig 3a, GS; Fig 3b, GFAP).
GS immunoreactivity showed no significant difference between non-preconditioned and preconditioned groups pre and 60 minutes after ischemia. At 1 day after ischemia, GS immunoreactivity decreased in both groups. At 3 days after ischemia, GS immunoreactivity recovered only in the preconditioned group (Fig. 3a).

The retinas 3 days after 60 minute ischemia had increased GFAP immunoreactivity in the non-preconditioned group. In the preconditioned group, only slight GFAP immunoreactivity was found. Nuclei were stained by methyl green (Fig. 3b).

**Discussion**

The possible mechanisms involved in the protection of the heart that results from classical ischemic preconditioning, that is the ability of short periods of ischemia to protect the heart against a subsequent, more prolonged, period of ischemic stress initiated several minutes later have been discussed recently.
Fig. 4. Immunoblots of extracts of retinas with GS (Fig. 4a) and GFAP (Fig. 4b). Extracts from retinas 30 and 90 minutes after ischemia gave a single band at the same position, which corresponded to 44 kDa for GS. In the non-preconditioned group, extracts at 1, 3 and 7 days after ischemia yielded a slightly positive band. At 3 and 7 days after ischemia, the GS band was recovered only in the preconditioned group (a). +, preconditioned; −, non-preconditioned.

The retinas at 1, 3 and 7 days after 60 minute ischemia had increased the GFAP band, which corresponded to 50 kDa for GFAP in the non-preconditioned group. In the preconditioned group, a band was scarcely observed (b).

(Parratt 1994). In retinal ganglion and Müller cells in culture, sublethal hypoxia induced tolerance to glutamate and anoxia and increased heat shock protein (HSP)-70 expression. The protective effect of sublethal hypoxia in these cultured cells was abolished by the HSP-70 synthesis inhibitor quercetin (Caplioli et al. 1996). Another potentially protective protein whose expression was increased after ischemic preconditioning in the brain was the free-radical scavenger superoxide dismutase (Kato et al. 1995). Li and Roth (1999) demonstrated that adenosine, acting through the A1 and A2a receptors, is a critical component in the induction of ischemic tolerance after preconditioning in the retina. On the other hand, it was thought that Müller cells play a central role in the homeostatic regulation of the retina. In this study we investigated the role of the Müller cells in the process of preconditioning rescue.

We chose 5 minute ischemia to serve as a brief ischemic stimulus for precon-
Fig. 5. Assay of relative contents of GS and GFAP contents measured by ELISA. The changes of GS and GFAP concentrations in the retina were shown in Figs. 5a and 5b. High GS concentration was observed in the extracts from retinas 30, 60, 90, and 120 minutes after ischemia. In the non-preconditioned group the extracts at 1, 3 and 7 days after ischemia yielded low concentrations of GS. At 3 and 7 days after ischemia, GS had recovered in the preconditioned group only ($p < 0.05$) (Fig. 5a). The retinas at 1, 3 and 7 days after 60 minute ischemia showed increased GFAP in the non-preconditioned group. However, in the preconditioned group, they had low concentration of GFAP ($p < 0.05$) (Fig. 5b). ■, preconditioned; □, non-preconditioned; pre, 24 hours after preconditioning. All values are mean ± s.d.; preischemic: $n = 4$, others: $n = 3$ per group.
ditioning because the electroretinogram exhibited a nearly complete recovery 1 day after ischemia. Studies we designed to test the ability of preconditioning to preserve retinal function after 60 minute ischemia. This duration of ischemia was chosen because it induced an approximately 10% recovery of the baseline electroretinogram a- and b-wave amplitudes 7 days after ischemia, and because it caused significant histologic alterations.

Müller cells are giant cells that occupy full-thickness retina from the internal to the external limiting membrane and beyond the external limiting membrane as villous processes. They are recognized by their increased cytoplasmic density, as compared to adjacent neurons. These cells are important because they furnish glucose to the nerve cells, and they are able to synthesize and store glycogen (Cogan and Kuwabara 1967). Their extensions enclose most of the neurons and insulate nerve fibers. Müller cells also can be considered space-occupying cells, since their extensions fill all retinal areas not occupied by neurons, except in the inner retina, where astrocytes also participate in this process.

Müller cells play a central role in the homeostatic regulation of the retina (Newman and Reichenbach 1996). Normal neuronal and vascular function relies on interactions with glia. Glial cells are implicated in the maintenance of the blood-retinal barrier at the endothelial lining of retinal microvessels (Gardner et al 1997), as has also been shown for the blood-brain barrier (Rubin et al. 1991). The extracellular ionic environment is optimized by glia for proper electrophysiological function of neurons. Also, glia maintains low synaptic levels of neurotransmitters. The major excitatory neurotransmitter in the retina is glutamate, an amino acid that is toxic to retinal neurons when present in high abundance (Romano et al. 1995). Thus, the regulation of retinal glutamate by glia (Riepe and Norenburg 1977) is essential for normal vision.

According to Riepe and Norenburg (1977), Müller cells take up neuronally released glutamate, thereby inactivating and conserving amino acids, which are subsequently converted to glutamine by the action of GS. Glutamine, which is freely diffusible, then leaves the Müller cell and is available to neurons for the formation of glutamate. The localization of GS to Müller cells thus lends firm support for a crucial role of these cells in glutamate metabolism. It is proposed that the Müller cell plays a central role in ameliorating glutamate toxicity under normal conditions and that the mechanism by which it accomplishes this is disrupted by an ischemic insult that ultimately results in delayed neuronal cell degeneration (Perlman et al. 1996). It is in this way, we think that the process of preconditioning rescue is related to Müller cells.

Together with astrocytes, Müller cells form the macroglia of the retina. GFAP is normally expressed in astrocytes. On the other hand, GFAP is not expressed by normal, healthy Müller cells, but it is expressed in an unhealthy state (Distler and Dreher 1996). That is, GFAP is thought to be expressed due to gliosis, and hypertrophy of macroglial cells.
In this way, we regarded the GS as the enzyme that serve functional glioneuronal interactions, and the GFAP as the pathological marker.

Petito et al. (1992) showed that at 3 and 24 hours after ischemia, GS immunoreactivity increased in the rat hippocampus. Tanaka et al. (1992) demonstrated that GS immunoreactivity increased transiently on day 2 after ischemia and decreased to the initial level on day 5 in the gerbil hippocampus. On day 2 after ischemia, and more prominently on day 3, reactive astrocytes were intensely stained for GFAP in the hippocampal formation. In this study, we observed GS immunoreactivity showed no significant difference between non-preconditioned and preconditioned groups in 30, 60, 90 and 120 minutes after ischemia. In 1 day after ischemia, GS immunoreactivity decreased in both groups. In 3 and 7 days after ischemia, GS immunoreactivity was recovered only in the preconditioned group. These results showed that Müller cells were disrupted by an ischemic insult after 1 day, but the functions recovered after 3 and 7 days only in the preconditioned group.

The retinas 1, 3 and 7 days after 60 minute ischemia had increased GFAP immunoreactivity in the non-preconditioned group. But in the preconditioned group, they exhibited only slight GFAP immunoreactivity. These results showed that Müller cells were damaged after 1, 3 and 7 days in the non-preconditioned group, but they were rescued in the preconditioned group.

In summary, our results suggested that the mechanism of preconditioned retinal ischemia might relate to Müller cells in the retina.

References


