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# Administration of Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Insulin-Like Growth Factor-II Protects Phosphate-Activated Glutaminase in the Ischemic and Reperfused Rat Retinas

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Tomita, H., Ishiguro, S., Abe, T. and Tamai, M. *Administration of Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Insulin-Like Growth Factor-II Protects Phosphate-Activated Glutaminase in the Ischemic and Reperfused Rat Retinas.* Tohoku J. Exp. Med., 1999, **187** (3), 227-236 —— Phosphate-activated glutaminase (PAG) activity decreases markedly in the early period of ischemia. The decrease of the enzyme activity is reversible if the ischemic period is relatively short, but it becomes irreversible after 90 minutes of ischemia. The deterioration is a functional damage of the retinas caused by ischemia. We studied effects of growth factors and neurotrophic factors on protection of PAG in the ischemic and reperfused rat retinas. Before ischemia, 1 $\mu$l of growth factors or neurotrophic factors (0.1 $\mu$g/$\mu$l for insulin-like growth factor-I [IGF-I], insulin-like growth factor-II [IGF-II], brain-derived neurotrophic factor [BDNF], nerve growth factor [NGF]; 1 $\mu$g/$\mu$l for basic fibroblast growth factor [bFGF]) were injected into the vitreous cavity of the left eyes of anesthetized Sprague Dawley rats. As a control, phosphate buffered saline was injected to the right eyes. To induce ischemia, we clamped left eyes for 90 minutes after bulbar conjunctival incision all around limbus. The rat retinas were homogenized with distilled water 1 day after reperfusion and used for PAG assay. Retinal ammonia concentration was also determined as a ischemic marker. About 80% decrease of retinal PAG activity and 50% increase of retinal ammonia concentration were observed after 90 minutes of ischemia and 1 day of reperfusion as compared with unoperated normal eyes. IGF-II, BDNF and NGF had protective effects on the retinal PAG activity, whereas IGF-I, bFGF, stable bFGF were less effective. In addition, IGF-II and BDNF suppressed elevation of retinal ammonia concentration. BDNF, NGF and

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IGF-II have marked effect on the protection of PAG activity in the ischemic and reperfused rat retinas, whereas bFGF, which is very effective for the protection of ischemic cell death, shows moderate effect. ———— ischemia; phosphate-activated glutaminase; ammonia; growth factors 

Glutamate is a leading candidate of excitatory transmitter in the retina (Ehinger 1974; Slaughter and Miller 1983; Ishida et al. 1984; Bloomfield and Dowling 1985; Ehinger et al. 1988; Massey and Miller 1988) and has potent neurotoxic activity (Olney 1982). In the mechanisms of ischemic injury, it is generally reported that the excess glutamate released from retinal cells binds to its receptor and the binding induces a calcium overload of the cells (Benveniste 1991). Subsequently the influx of calcium leads to a cellular destruction through increasing the activities of calcium-dependent proteases and free radicals (Farooqui and Horrocks 1994).

Although the role of glutamate in the ischemia has been studied extensively, change of glutamate metabolism is not well understood. Recently, Watanabe et al. (1995) reported that phosphate-activated glutaminase (PAG) decreased in the rat retinas immediately after ischemia, whereas the other enzymes, such as glutamate dehydrogenase and glutamine synthetase, were not affected. PAG catalyzes hydrolytic cleavage of glutamine to yield glutamate and ammonia, and is suggested to be a key enzyme in glutamate-glutamine cycle. PAG is rich in neuronal cells, especially photoreceptor cells (Ross et al. 1987), whereas glutamine synthetase for glutamine formation mainly exists in Müller cells in the retina (Riepe and Norenberg 1978; Hartig et al. 1995). Decrease of PAG activity in the retina immediately after ischemia means suppression of toxic glutamate and ammonia. Therefore, the decrease of the enzyme activity seems to be adaptive response to the ischemic injury.

In retinal ischemia, several cytokines protect the retinal function and retinal cell death. Siliprandi et al. (1993) reported that nerve growth factor (NGF) promotes functional recovery of retinal ganglion cells after ischemia. Unoki and Lavail (1994) demonstrated that brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF) transiently protect the retina from pressure-induced ischemic injury. In this study, we evaluated the effects of some growth factors and neurotrophic factors on the PAG activity and ammonia concentration in the ischemic rat retinas. Preliminary reports of some of these studies have been made (Ishiguro et al. 1996).

## Materials and Methods

### *Animals*

All procedures involving rats adhered to the ARVO Resolution on the Use of Animals in Research and the guidelines of the UCSF Committee on Animal

Research. Sprague Dawley (SD) rats weighing 250 g to 350 g were used for all experiments. Rats were anesthetized with an intramuscular injection of ketamine hydrochloride (66 mg/kg) and xylazine hydrochloride (3.3 mg/kg) mixture.

*Preparation of retinal ischemia*

To induce ischemia, we used the aneurysm clips (large aneurysm clip for permanent occlusion, Sugita, Tokyo). We incised the bulbar conjunctiva all around limbus, and then clamped left eye. Sham operation was performed on right eye. Ocular ischemia was sustained for 30, 60 or 90 minutes. Three animals were used for each experiment. Interruption of blood flow was confirmed ophthalmoscopically, and the a- and b-waves of single flash electroretinograms disappeared within 3 minutes of ischemia. One day after repurfusion, rats were sacrificed and PAG activity and ammonia concentration were measured.

*Intravitreous injection*

Growth factors used in this study were NGF from mouse submaxillary gland (0.1 $\mu$l/$\mu$g, Beohringer Mannheim, Detroit, MI, USA), human recombinant bFGF (1 $\mu$l/$\mu$g, Beohringer Mannheim), stable bFGF (1 $\mu$l/$\mu$g, Takeda, Osaka) (Seno et al. 1988), recombinant human BDNF (0.1 $\mu$l/$\mu$g, Pepro Tech Inc, England), IGF-I (0.1 $\mu$l/$\mu$g, Beohringer Mannheim) and IGF-II (0.1 $\mu$l/$\mu$g, Beohringer Mannheim). For experiments, these growth factors were dissolved in phosphate buffered saline (PBS). The concentration of each growth factors referred to LaVail et al. (1992). Before ischemic insults, we injected intravitreously one of the growth factors or PBS to left eye. PBS was injected into right eye of each rats as control. We used a microsyringe with 33-gauge needle for the injection. Ocular ischemia was sustained for 90 minutes. One day after repurfusion, rats were sacrificed and rats eyes were enucleated. The rat retinas were homogenized in distilled water, and used for measuring PAG activity and ammonia concentration.

*PAG assay*

For PAG assay, homogenized solution was dialyzed against distilled water for 90 minutes. Decrease of PAG activity is observed specifically in the membrane-bound form, but not in the soluble form after ischemic injury (Watanabe et al. 1995). However, as more than 85% of the PAG activity is of membrane-bound form (Hans et al. 1987), we measured the activity without the separation.

PAG activity was determined by the assay described by Hans et al. (1987). The activity was assayed by ammonia formation after 30 minutes incubation. In detail, this assay was the use of the *o*-phthaldialdehyde/mercaptoethanol reagent for estimating ammonia released from glutamine. The substrate solution consists of 100 mM potassium phosphate, 171 mM glutamine, 1.5 mM ammonium chloride

and 10% ethyleneglycol, pH 8.0. The dialyzed samples of 25 ml were added to 35 $\mu$l of substrate. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 10 $\mu$l of 7% trichroloacetic acid (TCA), kept on ice for 15 minutes, and centrifuged at 15 000 rpm for 3 minutes. Fifty microliters of supernatant were mixed with 900 ml of the *o*-phthaldialdehyde/mercaptoethanol reagent (10 ml of 0.2 M potassium phosphate, pH 7.4, 0.56 ml of 72 mM mercaptoethanol in ethanol and 0.56 ml of 186 mM *o*-phthaldialdehyde in ethanol). They were kept on dark at room temperature for 45 minutes, and measured at 410 nm with a spectrophotometer. The optical density were converted to ammonia concentration from the $NH_4Cl$ standard curve. Each data was shown as ammonia concentration.

*Assay of ammonia concentration*

Ten microliter of 7% TCA was immediately added to 60 $\mu$l of homogenized solution of the retina. After standing at 4°C for 15 minutes, the samples were centrifuged at 10 000 rpm for 3 minutes at 4°C. Seventy microliters of distilled water was added to 30 $\mu$l of the supernatants. The ammonia concentration was measured by adding 2.9 ml of *o*-phthaldialdehyde/mercaptoethanol reagent. They were kept on dark at room temperature for 45 minutes, and measured with a fluorophotometer (Exitation: 410 nm, Emission: 470 nm) (Taylor et al. 1974). The optical density were converted to ammonia concentration from the $NH_4Cl$ standard curve. Each data was shown as ammonia concentration.

## Results

We applied 30, 60 and 90 minutes of ischemia on the rat retinas and reper-

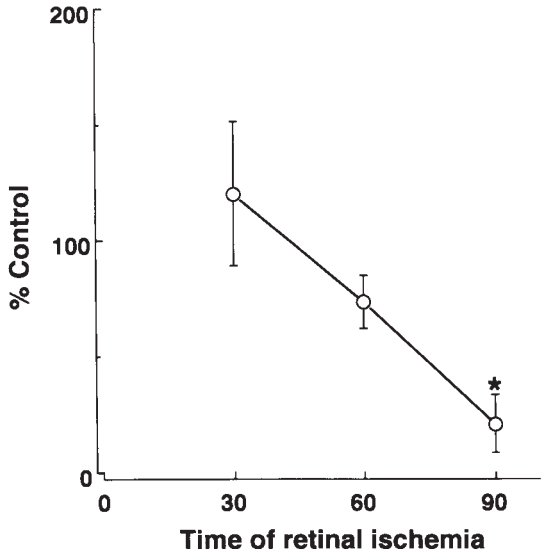


Fig. 1. PAG activity in the ischemic rat retinas 1 day after reperfusion. Percentage of PAG activity as compared with sham-operated eyes is shown as mean ± S.D. ($n=3$). Note that the values for statistical significance by paired $t$-test. ($^*p<0.05$).

fused for one day. Time dependent decrease of PAG activity was observed (Fig. 1), but no significant decrease of PAG activity was present in 30 (control: 51.86±13.86, ischemia: 56.32±7.48 nmol/mg protein) and 60 minutes of ischemia (control: 69.7±6.67 nmol/mg protein, ischemia: 50.15±6.76 nmol/mg protein) except 90 minutes of ischemia (control: 51.19±11.31 nmol/mg protein, ischemia: 9.46±5.21 nmol/mg protein). Watanabe et al. (1995) has shown that about 50% decrease of PAG activity occures mostly in the membrane-bound form even after 30 minutes of ischemia. Therefore, the retinal damage of 30 minute and 60 minute-ischemia seemed to be recovered until one day after reperfusion. On the other hand, 90 minutes of ischmia made irreversible damage on the retinas. Similar irreversible response obtained by the experiments of ammonia concentration support the above results (30 minute-ischemia; control: 6.13±0.55, ischemia: 6.53±0.22 nmol/mg protein, 60 minute-ischemia; control: 6.01±0.35, ischemia: 8.31±1.94 nmol/mg protein, 90 minute-ischemia; control: 6.79±0.32, ischemia: 10.30±0.71 nmol/mg protein). Only 90 minutes of ischemia showed significant ammonia elevation in the reperfused retinas (Fig. 2).

One day after reperfusion, the response is reversible if the ischemia is relatively short period. The enzyme activity recovers the normal level. In the retina given long period of ischemia, such as 90 minutes of ischemia, however, recovery of PAG activity is not observed at 1 day after reperfusion. Irreversible decrease of PAG activity shown in the present study is thought to be a functional damage caused by ischemia. We used PAG activity as a biochemical marker to evaluate the protective effect of some cytokines on ischemic injury.

As shown in Fig.3, PAG activity of IGF-II-, BDNF- and NGF-injected rat

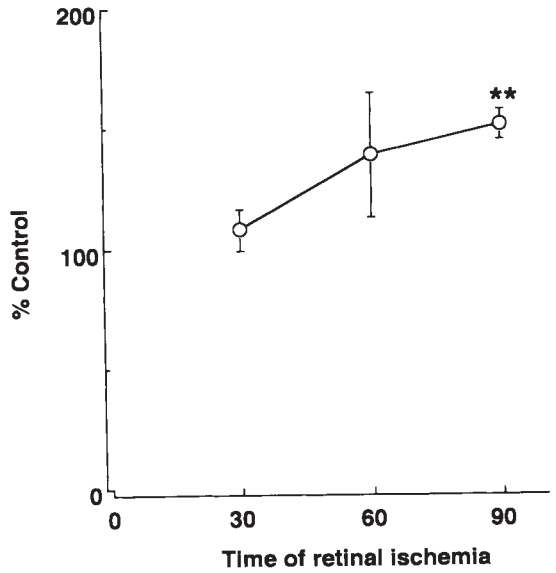


Fig. 2. Ammonia concentration in the ischemic rat retinas 1 day after reperfusion. Percentage of ammonia concentration as compared with sham-operated eyes is shown as mean±S.D. ($n=3$). Note that the values for statistical significance by paired $t$-test. ($^{**}p<0.01$).

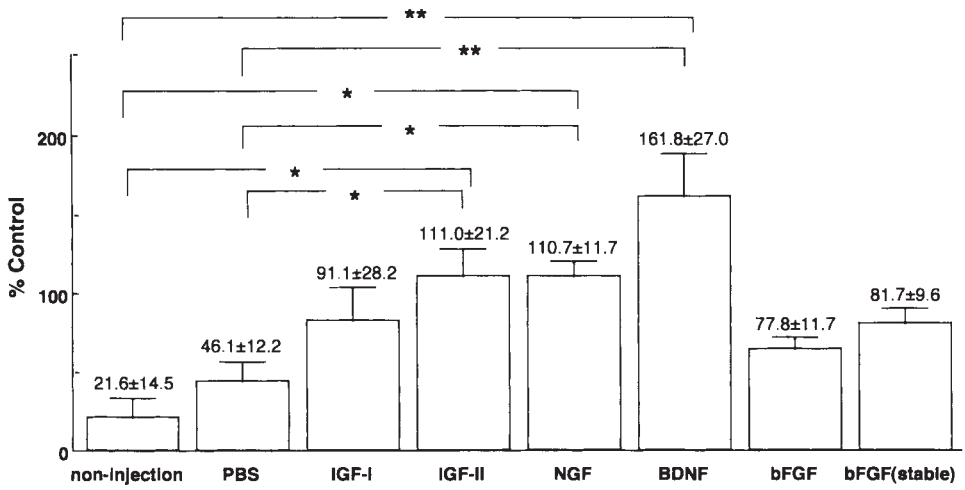


Fig. 3. Effects of pre-administration of growth factors and neurotrophic factors on PAG activity in the ischemic rat retinas 1 day after reperfusion. Percentage of PAG activity as compared with sham-operated contralateral eyes is shown as mean $\pm$ S.D. ($n=3$). Note that the values for statistical significance by dunnet test ($^*p<0.05$).

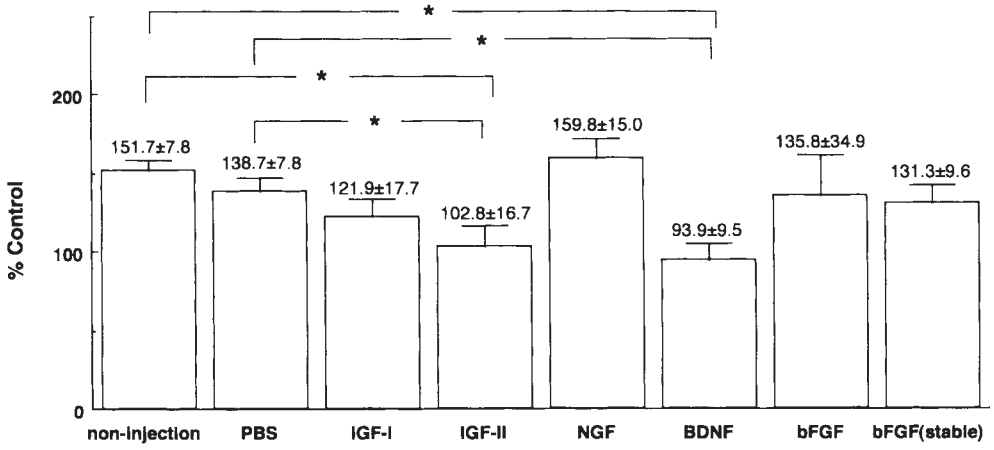


Fig. 4. Effects of pre-administration of growth factors and neurotrophic factors on the ammonia concentration in the ischemic rat retinas 1 day after reperfusion. Percentage of ammonia concentration as compared with sham-operated contralateral eyes is shown as mean $\pm$ S.D. ($n=3$). Note that the values for statistical significance by Dunnet test ($^*p<0.05$).

retinas was protected as compared with PBS-injected eyes. IGF-I and bFGF were less effective. As bFGF is unstable, we repeated the same experiments using modified and stable bFGF, but only moderate effect on the protection was obtained. On the basis of ammonia concentration, IGF-II and BDNF were effective (Fig. 4).

## Discussion

We investigated the effects of NGF, BDNF, IGF-I, IGF-II and bFGF on PAG activity in the ischemic and reperfused rat retinas. We expected the protective effect of bFGF on PAG activity, because bFGF receptor exists in

photoreceptor cells (Gao and Hollyfield 1992). However, the effects of bFGF on PAG activity was moderate as compared with other cytokines in the present study. As bFGF is not stable, we re-estimated the effects of bFGF using the stable bFGF (Seno et al. 1988), in which two out of three cysteine residues were exchanged to serine. The effects of the stable bFGF was similar with normal bFGF. Unexpectedly, the stable bFGF also showed moderate protective effect on the PAG activity. Mattson et al. (1993) indicated that NGF and IGF-II were effective in preventing loss of mitochondrial function when added up to 4 hours following the onset of hypoglycemia, whereas the protective effect of bFGF required pretreatment. This difference suggest that a different mechanism of action exists between bFGF and the other two growth factors. The reason why bFGF was less effective to PAG decrease in the rat retinal ischemia is unknown.

The enzyme PAG can exist in soluble form, located in the mitochondrial inner matrix, and in membrane-bound form, associated with the inner mitochondrial membrane. Watanabe et al. (1995) reported that PAG, especially the membrane bound PAG which is located in mitochondrial inner membrane, decreased during ischemia. Mattson et al. (1993) indicated that depletion of ATP under conditions that prevented an elevation of intracellular free calcium by incubation in calcium free medium did not result in loss of mitochondrial trans membrane potential in hippocampal neurons. These results demonstrated that elevation of intracellular free calcium can cause damage on mitochondria in hippocampal neurons. Pre-treatment of NGF, IGF-II and bFGF could prevent the loss of mitochondrial function caused by hypoglycemia.

Cheng and Mattson (1992) have investigated the involvement of calcium channels in the mechanism of hypoglycemic damage and growth factor protection. They suggested that hypoglycemic damage of cultured hippocampal and septal neuron occurred by the loss of calcium homeostasis, which was induced by calcium influx triggered by activation of N-methyl-D-aspartate (NMDA) receptors. They have reported that NGF, bFGF, IGF-I and IGF-II stabilized calcium homeostasis in ischemia (Cheng and Mattson 1991, 1992, 1993). However, the protective effects of these cytokines on PAG activity are different in the present study. Probably, these results may reflect the distribution and concentration of those receptors in the retinal photoreceptor cells, where the most PAG activity of the rat retina is present (Ross et al. 1987).

Calcium-dependent intracellular protease such as calpain is involved in PAG protein degradation. In vivo and in vitro the treatment with leupeptin and/or calpain inhibitors I benefits the protection against the neuronal degeneration caused by cytotoxic hypoxia or transient global cerebral (Rami and Krieglstein 1993) NGF causes a decrease in the activity of calpain in PC12 cells by causing an increase in the activity of calpastatin (Oshima et al. 1989). In the rabbit retina, calpain and calpastatin are localized in inner segments, outer segments, the outer and inner plexiform layer and ganglion cells (Persson et al. 1993). These

localization coincide with localization of PAG in the retina. It is likely that NGF protects the decrease of PAG by inhibiting calpain activity.

The mechanism of BDNF for inhibiting the decrease of PAG activity is not clear, although it is well-known that the levels of neurotrophic agents such as NGF and BDNF increase markedly after transient forebrain ischemia (Lindvall et al. 1992). It is possible that these agents have a protective effect from neuronal injury. Our result may be clue to clarify the mechanism of BDNF for ischemic injury.

In this study, we demonstrated that these growth factors have a protective effect on the functional damage was indicated by the decrease of enzyme activity was caused by transient ischemia. Many experiments for the protection of retinal ischemia have been investigated over the last decade. These neurotrophic factors are well-known cytokines for protecting the ischemic cell injury. Estimation, however, has been made by morphological or electrophysiological methods. We used PAG activity as a biochemical marker to evaluate the protective effect of some cytokines on ischemic injury. Our results coincided with other research has been made by morphological or electrophysiological methods. These results raise the possibility that analysis using the PAG activity is useful method for evaluating the protective effects on the functional damage caused by transient ischemia.

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