Effect of Betaxolol on Aspartate Aminotransferase Activity in Hypoxic Rat Retina In Vitro

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ABSTRACT—We investigated the effect of betaxolol on the decrease of mitochondrial aspartate aminotransferase (mAAT) activity in rat retinas induced by hypoxia in vitro. It is reported that mAAT decreases in ischemic or hypoxic retina and that the decrease is caused by Ca^{2+} -dependent proteases such as calpain. Betaxolol is a compound that has β_1 -adrenergic receptor blocking and voltage-dependent calcium channel blocking properties. The rat eye cups were maintained with Locke's solution saturated with 95% air–5% CO_2 . The eye cups were immersed in glucose-free Locke's solution saturated with 95% N_2 /5% CO_2 (hypoxic solution). Ninety minutes of hypoxia caused a 20% decrease in mAAT activity. The eye cups incubated with the hypoxic solution containing 1 mM EGTA, 10 μM MK-801 or 100 μM betaxolol were protected from the decrease in mAAT activity, so that the residual mAAT activity was 50%, 45% or 40%, respectively, compared to the eye cups incubated in hypoxic solution alone, which had a 100% decrease in mAAT activity. In addition, co-incubation with EGTA and betaxolol had a greater protective effect against the mAAT decrease than a single application. This additive effect of betaxolol was dose-dependent. These results suggested that betaxolol had a protective effect against the decrease of mAAT caused by hypoxia and indicated that betaxolol might inhibit the Ca^{2+} release from intracellular Ca^{2+} stores.

Keywords: Aspartate aminotransferase, Hypoxia, Betaxolol, Neuroprotection, Retina

A number of events occur in glaucoma. All events ultimately lead to the death of retinal ganglion cells (1). It is thought that the cell death in glaucoma is due to the inhibition of axonal transport (2, 3) and retinal blood circulation (4–6). β -Adrenergic antagonists are commonly used in the treatment of patients with glaucoma for reducing elevated intraocular pressure.

Recently many investigators are interested in the neuroprotective effect of anti-glaucomatous agents. The primary action of β -adrenergic antagonists is to inhibit the receptor-mediated action of noradrenaline or adrenaline. However, some β -adrenergic antagonists have also been shown to act as calcium channel antagonists (7–11). Betaxolol is a selective β_1 -adrenergic blocker, but its neuroprotective action is generally thought to be due to its calcium channel blocking properties (8, 12). Betaxolol also blunted the ischemia-induced damages, more precisely *N*-methyl-D-aspartate (NMDA) toxicity (13).

It is widely believed that intracellular calcium ions play a fundamental role in regulating numerous enzyme activities and mediating the effects of hormones and growth factors that control a wide variety of cellular processes such as metabolism, cell differentiation and secretion. Under ischemic conditions, the intracellular calcium level is elevated through voltage-dependent calcium channels and NMDA receptors. A decrease of Ca^{2+} pumping results from ATP depletion and then the Ca^{2+} release from intracellular stores (14–16). Most of the previous studies have shown elevated levels of intracellular Ca^{2+} under ischemic conditions. Loss of Ca^{2+} homeostasis may activate Ca^{2+} -dependent enzymes such as proteases, phospholipases (17) and endonucleases, leading to mitochondrial damage and cell death, resulting from energy failure.

In a previous study, we reported the change of aspartate aminotransferase (AAT) activity in ischemic and hypoxic rat retinas (18). AAT catalyzes the interconversion of glutamate, which is believed to be an excitatory amino acid neurotransmitter in the retina (19–21), and oxaloacetate with aspartate and α -ketoglutarate. AAT has the highest

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activity among the enzymes for glutamate metabolism (22–27). In our previous study (18), 90 min of ischemia or hypoxia caused a 20% decrease in mitochondrial AAT (mAAT) activity, whereas cytosolic AAT (cAAT) activity remained unchanged, and we suggested that mAAT activity may be Ca^{2+} -dependent.

In this study, we tested the effect of betaxolol on decrease of AAT activity induced by hypoxic conditions *in vitro*.

MATERIALS AND METHODS

Preparation of samples

All procedures involving rats adhered to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Normal RCS (rdy+/rdy+) rats (28) weighing 200–250 g were used. After rats were anesthetized with an intramuscular injection of ketamine hydrochloride (66 mg/kg) and xylazine hydrochloride (3.3 mg/kg) mixture, eyes were enucleated. We removed the cornea, lens and vitreous, and we obtained the eye cups. The eye cups were incubated at 37°C in Locke's solution (157 mM Na^+ , 2.3 mM Ca^{2+} , 164.2 mM Cl^- , 3.6 mM HCO_3^- , 5 mM HEPES, pH 7.2) and saturated with 95% air / 5% CO_2 for 90 min as the control condition. We used glucose-free Locke's solution saturated with 95% N_2 / 5% CO_2 for 90 min as the hypoxic condition. Following this incubation, the separated rat retina was homogenized with 500 μl of 0.25 M sucrose using a glass-Teflon homogenizer. The homogenate was dialyzed with distilled water for 60 min to remove endogenous disturbing substances. We obtained a mitochondrial fraction by centrifugation at $4,500 \times g$ for 10 min. The pellet was resuspended in 500 μl of 0.25 M sucrose and used for the measurement of mAAT activity. The supernatant was centrifuged at $100,000 \times g$ for 60 min and then the supernatant was used as the soluble fraction.

Effects of EGTA, MK-801, betaxolol on mAAT activity in hypoxic rat retinas

To examine the protective effects of EGTA (*O,O'*-bis (2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid), MK-801 (noncompetitive NMDA-receptor antagonist; Sigma, St. Louis MO, USA), and betaxolol on mAAT activity, the eye cups were incubated in glucose-free Locke's solution containing 1 mM EGTA, 10 μM MK-801 or 100 μM betaxolol and saturated with 95% N_2 / 5% CO_2 for 90 min. We measured mAAT activity. To investigate the relation between Ca^{2+} and the effect of betaxolol, we studied the dose-dependency of betaxolol in the hypoxic condition using glucose-free Locke's solution containing 1 mM EGTA.

Assay of aspartate aminotransferase activity

Activity of AAT was assayed according to the modified method of Godfrey et al. (29, 30). Twenty μl (34 μg protein) of samples was added to a 30- μl reaction mixture containing 60 mM imidazole buffer, pH 7.4, 4 mM α -ketoglutarate, 0.7 g/l bovine serum albumin, 1.4 ml/l Triton X-100 and 1.4 mg/l malate dehydrogenase. Each tube was sonicated for 1 min on the ice, and the reaction was started by adding 10 μl of substrate solution containing of 160 mM L-aspartate and 1.44 mM NADH. Samples were incubated at 37°C for 2 min, and then the reaction was stopped by adding 40 μl of 0.7 M HCl. After 20 min, required to destroy the remaining NADH in the acid solution, 1 ml indicator solution containing 95 mM Tris-HCl buffer, pH 8.5, 860 mM ethanol, 2 mM β -mercaptoethanol and 11.6 mg/l alcohol dehydrogenase, was added to each sample. The NAD^+ was converted to NADH in 30 min. The intensity of fluorescence of each sample was measured with a fluorophotometer (excitation: 340 nm; emission: 460 nm). Protein concentration was measured by the method of Lowry et al. (31).

RESULTS

In our previous study, we reported that 90 min of ischemia caused a 20% decrease in mAAT activity whereas cAAT activity remained unchanged, and a similar decrease in AAT activity was observed *in vitro* after 90 min of hypoxia (18). The protective effects of EGTA, MK-801 and betaxolol on retinal mAAT were examined. The percentage of protection against decreased mAAT activity is shown in Fig. 1. The decrease in mAAT activity was 50% prevented by the addition of 1 mM EGTA, 45% by 10 μM MK-801, 40% by 100 μM betaxolol, and 70% prevented by the addition of both 1 mM EGTA and 100 μM betaxolol. Betaxolol showed a dose-dependent protective effect under the hypoxic condition when the buffer contained EGTA (Fig. 2, post-hoc Dunnett's test, $P < 0.05$).

DISCUSSION

The present results substantiate that betaxolol acts as a neuroprotective agent against retinal hypoxia. Our previous study showed that 90 min of ischemia or hypoxia caused a 20% decrease in mAAT activity and that the mAAT degradation under hypoxic conditions was 90% prevented by the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin in Ca^{2+} -free Locke's solution with EGTA. These results indicated that the decreased mAAT activity may be Ca^{2+} -dependent (18). In this study, we observed the decrease of mAAT activity was partially blocked by MK-801. This result indicated that the decrease of mAAT was caused by Ca^{2+} entry from the extracellular space mediated through

Fig. 1. Effects of EGTA, MK-801 and betaxolol on retinal mAAT activity under hypoxic conditions in vitro. The concentrations of reagent used were 1 mM EGTA, 10 μ M MK-801, 100 μ M betaxolol. The reagents were added to Locke's solution saturated with 95% N₂ / 5% CO₂. As a control, the eyecup was incubated in Locke's solution saturated with 95% O₂ / 5% CO₂ for 90 min. Decreased mAAT activity was 70% effectively prevented by the addition of 1 mM EGTA and 100 μ M betaxolol. One hundred percent protection represented no degradation of the enzyme. Each datum point shows the mean $\pm$ S.D. (n = 4; **P<0.01, *t*-test)

Fig. 2. The effects of betaxolol on retinal mAAT activity under hypoxic conditions in vitro. The concentrations of reagent used were 10^{-8} – 10^{-2} M betaxolol. The eye cups were incubated with Locke's solution containing 1 mM EGTA and various concentrations of betaxolol saturated with 95% N₂ / 5% CO₂ for 90 min. One hundred percent protection represented no degradation of the enzyme. Each datum point shows the mean $\pm$ S.D. Significant difference vs control group (n = 4; *P<0.05, post-hoc Dunnett's test for multiple comparisons). Betaxolol had significant dose-dependent protective effects.

the NMDA receptor.

Previous reports showed that the AAT activity in retinal layers is predominantly distributed in the outer plexiform layer and photoreceptor inner segments (32, 33). Most of the previous studies showed that retinal ischemia thinned the inner retinal layers and reduced the number of cells in those layers, but not in the photoreceptor nuclear layer. However, it should be noted that the photoreceptor segments were shorter and more disorganized than normal (34). Therefore, the photoreceptor cells might be sublethally affected by the cytotoxic processes, and this effect could be due either to the photoreceptors themselves, or to their interacting Müller cells or retinal pigment cells. This suggests that a 20% decrease in mAAT activity by a 90-min ischemic insult might be induced by the damage of specific cells.

In the present study, the decreased mAAT activity was 50% prevented by the addition of 1 mM EGTA, 45% by 10 μ M MK-801, 40% by 100 μ M betaxolol, and 70% prevented by the addition of both 1 mM EGTA and 100 μ M betaxolol. Betaxolol is a selective β_1 -adrenergic blocker, but its neuroprotective action is generally thought to be due to its calcium channel blocking properties (8, 12). We can not exclude the possibility that the selective β_1 -adrenergic blocking property of betaxolol is may be related to this neuroprotective effect in this study. However, it is reported that a β -adrenergic receptor antagonist such as timolol has no neuroprotective effect on NMDA-induced retinal injury (35) and that betaxolol has a greater L-type blocking activity than other β -adrenergic receptor antagonist (36) and neuroprotective effect on the ischemia-induced retinal injury (12, 13). These results suggested that betaxolol may function as a neuroprotective agent by reducing the excessive influx of calcium into the cell in some way (see the Introduction). Under ischemic conditions, the intracellular calcium level is elevated through voltage-dependent and NMDA receptor-operated channels. The ATP depletion causes a decrease of Ca²⁺ pumping and Ca²⁺ is released from the intracellular stores (14–16). In our previous study (18), we showed the decrease of mAAT induced by hypoxia was inhibited by co-incubation of EGTA and thapsigargin but not ryanodine. In this study, we observed that betaxolol had an effect similar to that of thapsigargin. These results suggested that betaxolol may reduce the concentration of intracellular calcium ions by preventing calcium ions influx from the extracellular space and by retarding the release of calcium ions from intracellular calcium stores, under hypoxic conditions.

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