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Retinal protective effects of topically administered agmatine on ischemic ocular injury caused by transient occlusion of the ophthalmic artery

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Abstract

Agmatine, an endogenous polyamine and putative neuromodulator, is known to have neuroprotective effects on various neurons in the central nervous system. We determined whether or not topically administered agmatine could reduce ischemic retinal injury. Transient ocular ischemia was achieved by intraluminal occlusion of the middle cerebral artery of ddY mice (30-35 g) for 2 h, which is known to also induce occlusion of the ophthalmic artery. In the agmatine group (N = 6), a 1.0 mM agmatine-containing ophthalmic solution was administered four times daily for 2 weeks before occlusion. In the control group (N = 6), a 0.1% hyaluronic acid ophthalmic solution was instilled at the same times. At 22 h after reperfusion, the eyeballs were enucleated and the retinal sections were stained by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Transient ocular ischemia induced apoptosis of retinal cells in the entire retinal layer, and topically administered agmatine can significantly reduce this ischemic retinal injury. The proportion of apoptotic cells was definitely decreased ($P < 0.001$; Kruskal-Wallis test). Overall, we determined that topical agmatine application effectively decreases retinal damage in an *in vivo* ocular ischemic injury model. This implies that agmatine is a good candidate as a direct neuroprotective agent for eyes with ocular ischemic diseases.

Key words: Agmatine; Apoptosis; Ischemia; Neuroprotection; Retina

Introduction

Agmatine is an endogenous aminoguanidine compound formed by the decarboxylation of L-arginine, and has been reported to be a putative neuromodulator in neurons of the central nervous system (CNS) (1,2). Although agmatine was initially described as a ligand for imidazoline receptors (3), it is now recognized to act as an agonist on imidazoline I and α_2 -adrenergic receptors, as an antagonist on N-methyl-D-aspartic acid (NMDA) receptors, and as an inhibitor of neuronal/inducible nitric oxide synthases (NOSs) (1,2).

A large body of experimental evidence has demonstrated the neuroprotective effects of agmatine on various noxious neuronal injuries (4-6). Given the suggestions that agmatine can reduce neuronal loss after cerebral/spinal cord ischemia (5,6), we confirmed its protective effects on hypoxia-induced apoptosis of RGC-5 cells *in vitro* (7). Although we also revealed that topically administered

agmatine rescues retinal ganglion cells (RGCs) in the eyes of chronic ocular hypertensive rats (8), the *in vivo* data providing evidence for the neuroprotective effects of agmatine are not yet sufficient.

Theoretically, agmatine is a good neuroprotective drug candidate because of its high binding affinity for α_2 -adrenergic and NMDA receptors. Although various neuroprotective agents, including α_2 -adrenergic receptor agonists and NMDA receptor antagonists, have been widely studied, a novel candidate drug is not yet available (9). If we can prove the neuroprotective effects of agmatine and identify its mechanisms of action, agmatine might provide new therapeutic strategies as a neuroprotective drug.

In the present report, we determined whether or not topically administered agmatine could reduce retinal injury induced by transient ocular ischemia using intraluminal occlusion of the ophthalmic artery.

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Material and Methods

Animals and topical agmatine administration

A total of 12 adult male ddY mice (7 weeks old, 30 to 35 g) were used for this study. The animals were maintained under controlled conditions with a 12:12-h light/dark cycle and standard food and water provided *ad libitum*. They were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research after obtaining permission of the Institutional Animal Care and Use Committee. Every effort was made to minimize the number of animals sacrificed and their suffering throughout our experiments.

An agmatine-containing ophthalmic solution (1.0 mM; Sigma-Aldrich, USA) was formulated as previously described (8). Mice in the agmatine group (N = 6) received agmatine eye drops in both eyes four times daily for 2 consecutive weeks. Mice in the control group (N = 6) received a 0.1% hyaluronic acid ophthalmic solution (Santen Pharmaceutical Co., Japan) in both eyes at the same times.

Transient ocular ischemia caused by ophthalmic artery occlusion

Intraluminal occlusion of the middle cerebral artery (MCA) was induced as previously described (10,11) using a silicone rubber-coated monofilament (Doccol Co., USA). Briefly, after the mice were anesthetized with an intraperitoneal injection of a mixture of zolazepam/tiletamine (80 mg/kg; Zoletil 50®, Virbac, France) and xylazine (20 mg/kg; Rompun®, Bayer HealthCare, Germany), the filament

was introduced into the left internal carotid artery through an arteriotomy in the left common carotid artery. The filament was advanced up to the origin of the anterior cerebral artery and caused occlusion of the ophthalmic artery as well as the MCA (12).

After 2 h of occlusion, the animals were re-anesthetized and the filament was withdrawn to permit restoration of blood flow. After an additional 22 h, the mice were perfused with 4% paraformaldehyde and their eyeballs were enucleated.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

The enucleated eyeballs were immersed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin; 4- μ m sections were cut parallel to the maximum circumference of the eyeball through the optic disc. Apoptotic cells were identified using TUNEL (DeadEnd™ Fluorometric TUNEL System; Promega, USA), and fluorescein isothiocyanate (FITC)-12-dUTP-labeled fragmented DNA was directly visualized using a fluorescence microscope. Nuclear counterstaining was performed using 4',6-diamidino-2-phenylindole (DAPI). Four sections were selected from each eyeball and areas within a field between 375 and 625 μ m from the optic disc were evaluated.

Results

Representative retinal sections of the TUNEL are shown in Figure 1. Mice in the control group were treated with a 0.1% hyaluronic acid ophthalmic solution (vehicle) in

both eyes four times daily for 2 consecutive weeks before occlusion of their left MCA. Their right eyes were defined as the 'no treatment control' group (Figure 1A,B) and their left eyes were defined as the 'ocular ischemic injury only' group (Figure 1C,D). Mice in the agmatine group received agmatine eye drops in both eyes four times daily for 2 consecutive weeks before occlusion of their left ophthalmic artery. Their right eyes were defined as the 'agmatine treatment only' group (Figure 1E,F) and their left

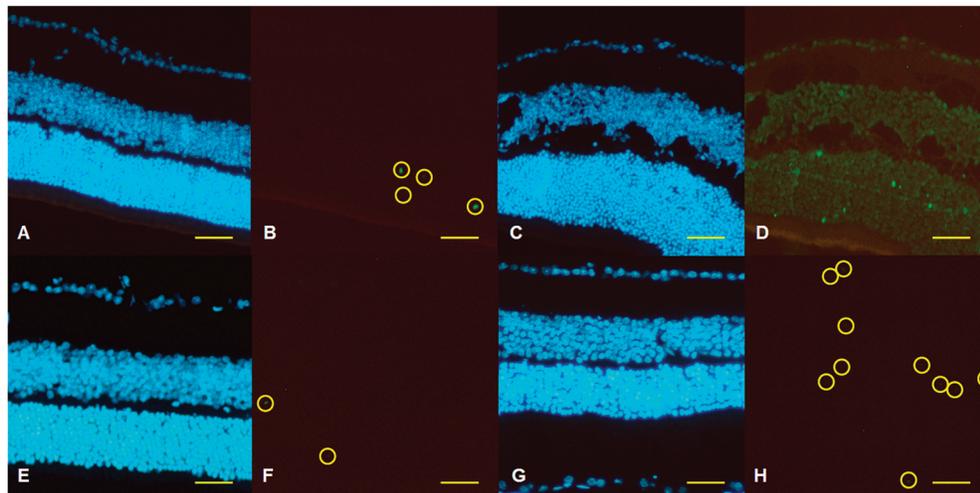


Figure 1. Representative retinal sections of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), in which the fluorescein isothiocyanate (FITC)-12-dUTP-labeled fragmented DNA and nuclear counterstaining were performed using 4',6-diamidino-2-phenylindole (DAPI). A, B, No treatment control; C, D, ocular ischemic injury only; E, F, agmatine treatment only; G, H, ocular ischemic injury after agmatine treatment. A, C, E, G, Blue fluorescence indicates DAPI; B, D, F, H, green fluorescence indicates FITC. The TUNEL-positive cells are emphasized as yellow circles in Panels B, F, and H, but all cells are TUNEL-positive in Panel D. Scale bar = 100 μ m.

eyes were defined as the 'ocular ischemic injury after agmatine treatment' group (Figure 1G,H).

In the retinas of the control group, a few TUNEL-positive apoptotic cells were noticed in the outer nuclear layer (Figure 1B). Transient ocular ischemia definitely induced extensive retinal damage (Figure 1D); every single cell in the whole retinal layer was TUNEL-positive and large vacuolations were found in the inner nuclear and plexiform layers. However, topically administered agmatine dramatically reduced the proportion of apoptotic cells ($P < 0.001$; Kruskal-Wallis test) and improved the retinal integrity (Figure 1H).

Discussion

In this investigation, we demonstrated that topically administered agmatine effectively rescues retinal cells from ischemic damage. Although many researchers have used an acute ischemia-reperfusion injury model caused by a transient elevation in intraocular pressure (13-15), this animal model has the mixed pathophysiology of pressure-dependent and pressure-independent mechanisms. Retrobulbar/intravitreal injection of endothelin-1 has also been reported to completely obstruct retinal vessels (16,17); however, this is a very expensive method and it is hard to control the duration of occlusion, especially in small rodents (18). Intraluminal MCA occlusion also causes transient/permanent occlusion of the ophthalmic artery (12). While intraluminal MCA occlusion requires a skilled technician, it can precisely control the time of occlusion. Thus, transient intraluminal occlusion of the MCA can serve as a useful experimental model that mimics ocular ischemia-reperfusion injury.

Agmatine is a putative neuromodulator in CNS neurons

(1,2) and is known to act as an agonist on imidazoline I and α_2 -adrenergic receptors, as an antagonist on NMDA receptors, and as an inhibitor of neuronal/inducible NOSs (1,2). Recently, agmatine has received considerable attention due to its neuroprotective effects on various neuronal injuries (4-6). Regarding ocular tissue, we demonstrated that agmatine protects damaged hypoxic RGC-5 cells (7) and that topically administered agmatine rescues RGCs in chronic ocular hypertensive rat eyes (8). In the present report, we investigated whether topically administered agmatine could reduce ischemic retinal injury. In a previous investigation, high concentrations of agmatine (0.1 M) were presumed to have caused corneal toxicity, while 1.0 mM agmatine exhibited good ocular hypotensive effects (8). As such, in this study we also used 1.0 mM agmatine eye drops. In addition, since we have some evidence of the neuroprotective effects of agmatine pretreatment (19,20), we decided to start agmatine treatment before inducing ischemic injury.

Our results confirm that topically administered agmatine can significantly reduce ischemia-reperfusion retinal injury in a mouse model of transient ocular ischemia. Although the precise mechanisms of agmatine in this retinal protective process have not been well established, agmatine is a good potential candidate as a direct neuroprotective agent for eyes with ocular ischemic diseases, including glaucoma.

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