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Protective mechanism of agmatine pretreatment on RGC-5 cells injured by oxidative stress

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Abstract

Agmatine has neuroprotective effects on retinal ganglion cells (RGCs) as well as cortical and spinal neurons. It protects RGCs from oxidative stress even when it is not present at the time of injury. As agmatine has high affinity for various cellular receptors, we assessed protective mechanisms of agmatine using transformed RGCs (RGC-5 cell line). Differentiated RGC-5 cells were pretreated with 100 μ M agmatine and consecutively exposed to 1.0 mM hydrogen peroxide (H_2O_2). Cell viability was determined by measuring lactate dehydrogenase (LDH), and the effects of selective alpha 2-adrenergic receptor antagonist yohimbine (0-500 nM) and N-methyl-D-aspartic acid (NMDA) receptor agonist NMDA (0-100 μ M) were evaluated. Agmatine's protective effect was compared to a selective NMDA receptor antagonist MK-801. After a 16-h exposure to H_2O_2 , the LDH assay showed cell loss greater than 50%, which was reduced to about 30% when agmatine was pretreated before injury. Yohimbine almost completely inhibited agmatine's protective effect, but NMDA did not. In addition, MK-801 (0-100 μ M) did not significantly attenuate the H_2O_2 -induced cytotoxicity. Our results suggest that neuroprotective effects of agmatine on RGCs under oxidative stress may be mainly attributed to the alpha 2-adrenergic receptor signaling pathway.

Key words: Agmatine; Neuroprotection; Oxidative stress; Retinal ganglion cell

Introduction

Glaucoma is the leading cause of irreversible blindness worldwide. Although elevated intraocular pressure has been known as a major risk factor for the development and progression of glaucoma, oxidative stress is also associated with glaucomatous visual field deterioration (1-3). Retinal ganglion cells (RGCs) are susceptible to oxidative stress (3-5). As glaucomatous damages are mostly irreversible, many glaucoma researchers are searching for a neuroprotective strategy for RGCs.

Agmatine is a primary amine, which is formed by decarboxylation of L-arginine in the mammalian brain. Agmatine has high affinity for various cellular receptors. It is an agonist for alpha 2-adrenergic and imidazoline receptors and an antagonist for N-methyl-D-aspartic acid (NMDA) receptors (6-8). Agmatine has neuroprotective effects against cortical and spinal neuronal injuries (9-11). It also protects RGCs

from hypoxic damage (12). Even when agmatine is not present at the time of injury, it seems to protect RGCs by later blocking cellular death induced by oxidative stress (13). However, the precise protective mechanisms of agmatine pretreatment have not been well established.

We investigated the mechanisms of action of agmatine pretreatment on RGCs under oxidative stress using transformed RGCs (RGC-5 cell line).

Material and Methods

Cell culture and agmatine pretreatment

RGC-5 cells, which belong to a transformed RGC line developed from postnatal Sprague-Dawley rats, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) plus 10% heat-inactivated fetal bovine serum

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(FBS; Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). For differentiation, the cells were incubated with 1.0 µM staurosporine (Sigma-Aldrich, USA) for 6 h; and then they were cultured in 10% FBS-DMEM for another 3 days (13).

After differentiation, the cells were pretreated with 100 µM agmatine (Sigma-Aldrich) in serum-free DMEM for 2 h (13), and then rinsed with phosphate-buffered saline (PBS; Gibco). Control cells were incubated in serum-free DMEM.

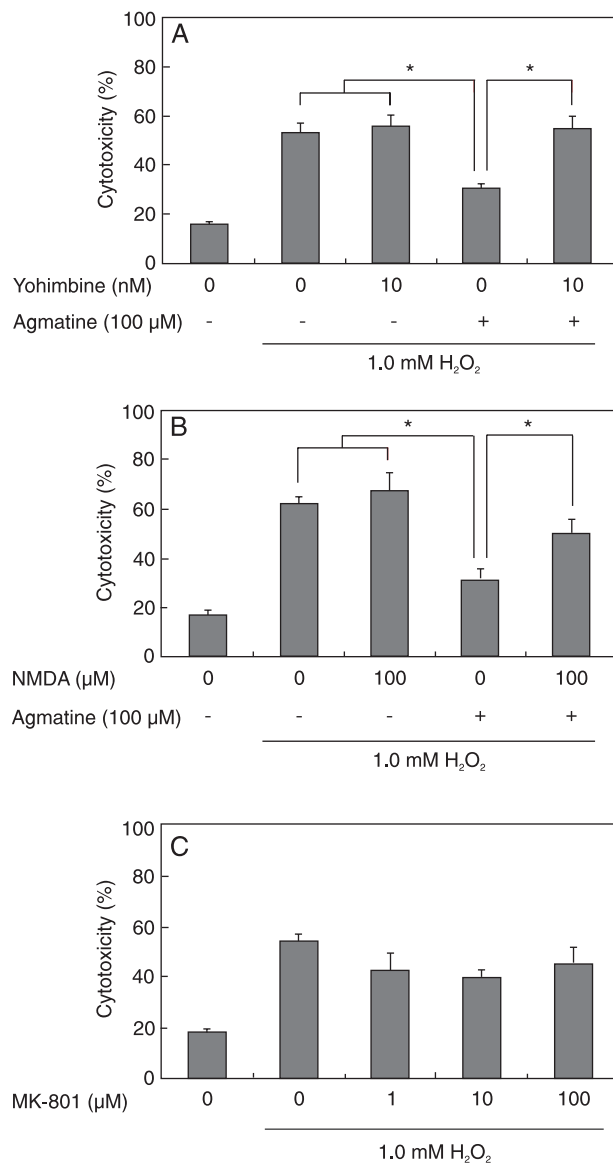


Figure 1. Determination of H₂O₂ cytotoxicity by the lactate dehydrogenase assay. Differentiated RGC-5 cells were pretreated with 100 µM agmatine and then exposed to oxidative stress with yohimbine (A) or N-methyl-D-aspartic acid (NMDA) (B). The cells were also treated with MK-801 (C). *Panels A and B*, *P < 0.05 (one-way ANOVA followed by *post hoc* comparisons).

Oxidative stress with agonist/antagonist

After agmatine (100 µM) pretreatment for 2 h, the cells were exposed to 1.0 mM hydrogen peroxide (H₂O₂; Sigma-Aldrich) for 16 h (13). The mechanisms of action of agmatine pretreatment were investigated using the specific alpha 2-adrenergic receptor antagonist yohimbine (0-500 nM; Sigma-Aldrich), and NMDA receptor agonist NMDA (0-100 µM; Calbiochem, USA). In addition, the effect of agmatine was compared to that of MK-801 (0-100 µM; Sigma-Aldrich), a selective NMDA receptor antagonist.

Cytotoxicity assay

Cell viability was determined by measuring lactate dehydrogenase (LDH; CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit; Promega, USA) according to manufacturer instruction. Briefly, the LDH released from injured cells into the culture medium was reacted with tetrazolium salt. The red formazan product was measured at 490 nm using an ELISA plate reader (Vmax; Molecular Device, USA). Cytotoxicity was calculated as the proportion of injured cells to the total cell population (14). Data are reported as means ± SEM.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by *post hoc* comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences (SPSS) program for Windows, version 12.0 (SPSS Inc., USA). P values below 0.05 were considered to be significant.

Results

Differentiated RGC-5 cells were pretreated with 100 µM agmatine for 2 h, and then exposed to 1.0 mM H₂O₂ for 16 h for induction of oxidative stress. After agmatine pretreatment, the cytotoxicity of H₂O₂ declined from above 50 to approximately 30% (Figure 1A,B).

Neither yohimbine nor NMDA, at any concentration, had any significant effect on oxidative stress-induced cell death (data not shown); 10 nM yohimbine and 100 µM NMDA were used for further experiments. While yohimbine almost completely blocked the protective effects of agmatine pretreatment (Figure 1A), NMDA did not (Figure 1B).

To evaluate the contribution of NMDA receptor signaling, the protective effect of agmatine was compared to that of MK-801. MK-801 did not significantly affect the H₂O₂-induced cytotoxicity of RGC-5 cells (Figure 1C).

Discussion

Agmatine is known to be an agonist for alpha 2-adrenergic and imidazoline receptors as well as an antagonist of NMDA receptors (6-8). Wheeler and WoldeMussie (15) and Kalapesi et al. (16) studied the alpha 2-adrenergic receptors of human ganglion cells and the RGC-5 cell line. They dem-

onstrated the presence of alpha 2A-adrenergic receptors on both undifferentiated and succinyl concanavalin-A differentiated RGC-5s. They also showed a higher expression of alpha 2A-adrenergic receptors on 7-day differentiated RGC-5 cells compared to the undifferentiated and early differentiated cells. In addition, RGCs also expressed NMDA glutamate receptors.

In the present study, the protective effects of agmatine pretreatment on RGC-5s under oxidative stress were completely abolished by 10 nM yohimbine but only partially decreased by 100 μ M NMDA. Even the powerful NMDA antagonist MK-801 did not reduce the RGC-5 cell death induced by H₂O₂. These results suggest that agmatine pretreatment may rescue RGCs from oxidative stress mainly through alpha 2-adrenergic signaling rather than through the NMDA receptor pathway.

We have demonstrated that agmatine pretreatment has anti-apoptotic effects on differentiated RGC-5s under

oxidative stress (13). In the present study, its protective mechanisms were investigated. Considering the many biological functions of agmatine, this study is not sufficient since its focus was limited to alpha 2-adrenergic and NMDA receptor pathways. Further experiments are needed to fully understand the neuroprotective mechanisms of agmatine pretreatment.

The neuroprotective mechanisms of agmatine pretreatment on RGCs under oxidative stress *in vitro* may be mainly associated with the alpha 2-adrenergic receptor signaling pathway.

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