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Agmatine pretreatment protects retinal ganglion cells (RGC-5 cell line) from oxidative stress *in vitro*

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ABSTRACT: Agmatine, 2-(4-aminobutyl)guanidine, has been reported to have neuroprotective effects against various neuronal damages. In this study it was investigated whether agmatine pretreatment rescues the retinal ganglion cells from oxidative injury *in vitro*. After differentiation of transformed rat retinal ganglion cells (RGC-5 cell line) with staurosporine, agmatine (0.0 to 100.0 μM) pretreatment was performed for 2 hours. Subsequently, they were exposed to hydrogen peroxide (0.0 to 2.5 mM) as an oxidative stress. Cell viability was monitored for up to 48 hours with the lactate dehydrogenase (LDH) assay and apoptosis was examined by the terminal deoxynucleotide transferase-mediated terminal uridine deoxynucleotidyl transferase nick end-labeling (TUNEL) method. As a result, differentiated RGC-5 cells were found to have decreased viability after addition of hydrogen peroxide in a dose-dependent manner. This hydrogen peroxide induced cytotoxicity caused apoptosis characterized by DNA fragmentation. Agmatine pretreatment not only increased cell viability but also attenuated DNA fragmentation. In conclusion, agmatine pretreatment demonstrated neuroprotective effects against oxidative stress induced by hydrogen peroxide in differentiated RGC-5 cells *in vitro*. This suggests a novel therapeutic strategy rescuing retinal ganglion cells from death caused by oxidative injury.

Introduction

Agmatine, 2-(4-Aminobutyl)guanidine, is a decarboxylation product of L-arginine and an intermediate in the polyamine metabolism (Reis and Regunathan, 2000; Grillo and Colombatto, 2004). Even though the precise mechanisms by which agmatine acts are not yet well established, it is known that agmatine binds to α_2-adrenergic and imidazoline receptors and blocks the N-methyl-D-aspartate (NMDA) receptors (Li *et al*., 1994; Yang and Reis, 1999).

There are several reports on the neuroprotective effects of agmatine in various experimental models of trauma to the central nervous system (Gilad *et al*., 1996; Gilad and Gilad, 2000; Zhu *et al*., 2003; Kim *et al*., 2004, 2006; Kotif *et al*., 2006; Wang *et al*., 2006). It can reduce areas affected by infarction and attenuates neuronal loss following cerebral and spinal cord injuries. In a glaucoma model, we have reported that agmatine shelters the retinal ganglion cells from hypoxic injury (Hong *et al*., 2007), and this protection seems to be related to the c-Jun N-terminal kinase (JNK) and the nuclear factor-kappa B (NF-κB) signaling pathways.

Since it is not possible to predict exactly when retinal ganglion cells will be injured, it would be ideal to

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protect them before injury. Thus, in the present study, we investigated whether agmatine pretreatment may offer neuroprotective abilities against oxidative stress.

Materials and Methods

Cell culture

RGC-5 cells, a transformed retinal ganglion cell line developed from post-natal Sprague-Dawley rats, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 100 U/mL penicillin and 100 ug/mL streptomycin (Gibco, Carlsbad, CA). Cells were seeded at a density of $6.0 \infty 10^4$ cells/well in 6-well plates and incubated at 37ºC in 5% CO_2 environment. They were passaged every 3 days, and all experiments were performed at a confluence of 70%.

Differentiation of RGC-5 cells

Cells from this cell line were exposed to 1.0 μM staurosporine (Sigma-Aldrich, St. Louis, MO), a broad-spectrum protein kinase inhibitor, for 6 hours. Subsequently, they were recovered in the standard culture media up to 5 days, and their morphological changes were monitored to determine the optimal recovery time. Well differentiated RGC-5 cells were defined as those having multiple long neurites and displaying contacts among them.

Oxidative stress and agmatine pretreatment

Oxidative stress was caused by addition of hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) to the culture media. Briefly, after 2 hours of serum starvation, differentiated RGC-5 cells were incubated with 0.0, 1.0, 10.0, and 100.0 μM agmatine (Sigma-Aldrich, St. Louis, MO) for 2 hours. Then, cells were injured by

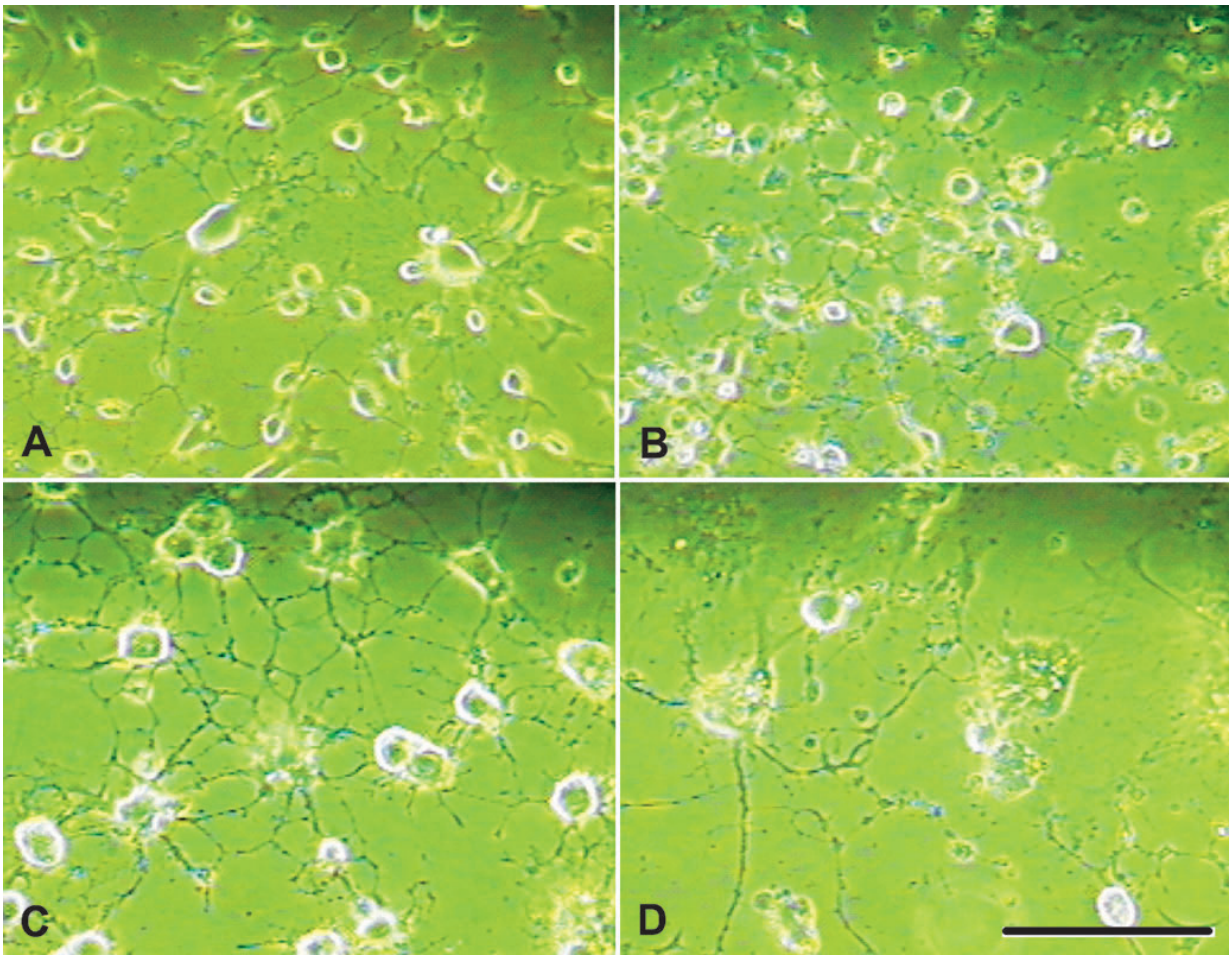

FIGURE 1. Differentiation of RGC-5 cells (bright field microscopy). RGC-5 cells were exposed to 1.0 mM staurosporine for 6 hours and let to recover for up to 5 days. (A) No recovery, (B) day 1, (C) day 3, and (D) day 5 of recovery. Scale bar represents 20 mm.

several concentrations of hydrogen peroxide (0.0, 0.5, 0.8, 1.0, and 2.5 mM) during 48 hours.

Lactate dehydrogenase (LDH) assay

Cell viability was quantified by measurement of LDH released to culture media from damaged cells according to the manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit; Promega Corporation, Madison, WI), and the proportion of injured cells in the total cell population was calculated.

Terminal deoxynucleotide transferase-mediated terminal uridine deoxynucleotidyl transferase nick end-labeling (TUNEL) assay

Using the Apo-BrdU *In Situ* DNA Fragmentation Assay Kit (BioVision, Inc., Mountain View, CA), apoptotic features of damaged cells were assessed. Transferred bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) to the free 3'-OH of cleaved DNA by terminal deoxynucleotide transferase (TdT) were detected by the anti-BrdU-FITC antibody under fluorescence microscopy. Propidium iodide was used for counter-staining.

Statistical analysis

LDH assay data were analyzed by one-way ANOVA, followed by post hoc comparisons (Student-Newman-Keuls) using the SPSS program for Windows, version 12.0 (SPSS Inc., Chicago, IL). Results are expressed as means ± *SD*.

Results

Differentiation of RGC-5 cells by staurosporine

After 6 hours incubation with 1.0 μM staurosporine, RGC-5 cells were recovered at day 1, 3 or 5 for observation of their morphological features (Figs. 1 and 2). Their somas became rounder and their neurites gradually increased. After 3 days of recovery, RGC-5 cells sufficiently contacted each other with multiple long neurites and were thus presumed to be well differentiated. Then, day 3 was selected as the optimal recovery time, and further experiments were performed under this condition.

Oxidative stress induced cytotoxicity on differentiated RGC-5 cells

After recovery, differentiated RGC-5 cells were damaged by exposure to either 0.0, 0.5, 0.8, 1.0, or 2.5 mM hydrogen peroxide for either 16, 24, or 48 hours. Cytotoxicity was quantified by measurement of released LDH to culture media from injured cells (Fig. 3). Compared to control, 0.5 and 0.8 mM hydrogen peroxide exposure did not make any apparent difference (both $p>0.05$) but 1.0 and 2.5 mM hydrogen peroxide signifi-

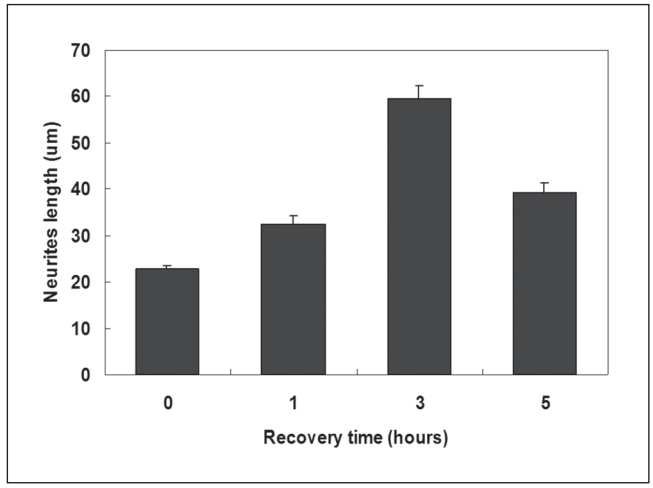

FIGURE 2. Neurites length of differentiated RGC-5 cells according to the recovery time after staurosporine treatment. Data are expressed as means ± SD.

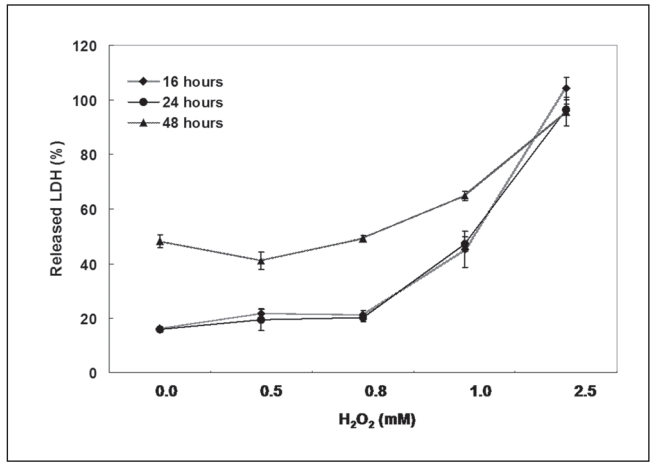

FIGURE 3. Hydrogen peroxide (H_2O_2) induced cytotoxicity in differentiated RGC-5 cells.

cantly induced LDH leakage (both p<0.001). Because hydrogen peroxide increased LDH release in a dose-dependent manner between 0.8 and 2.5 mM, and because 16 and 24 hours exposure to hydrogen peroxide showed similarly effective cytotoxic insults, we selected the 16 hours exposure to 1.0 mM hydrogen peroxide as the oxidative stress condition for further experiments.

Protective effect of agmatine pretreatment

Agmatine pretreatment attenuated hydrogen peroxide-induced death of differentiated RGC-5 cells (Fig. 4). As the concentration of agmatine was increased, the protective effect was also increased. However, even though maximal protection was apparent with 100.0 μM, the protective effect of 10.0 μM agmatine was not statistically different with that of 10.0 μM agmatine. LDH leakage markedly increased with addition of 1.0 mM hydrogen peroxide (40.94 ± 3.53%) when compared to control (13.72 ± 1.40%; $P < 0.001$). However, 100.0 μM agmatine pretreatment attenuated these cytotoxic effects (27.35 ± 2.08%).

Anti-apoptotic effects of agmatine pretreatment

In Figure 5, TUNEL assay for broken DNA from apoptotic cells revealed FITC (green fluorescence) positive signal. Propidium iodide counter-staining demonstrates living cells (orange fluorescence). Pretreatment of agmatine markedly reduced the loss of RGC-5 cells possibly through a decrease in apoptosis.

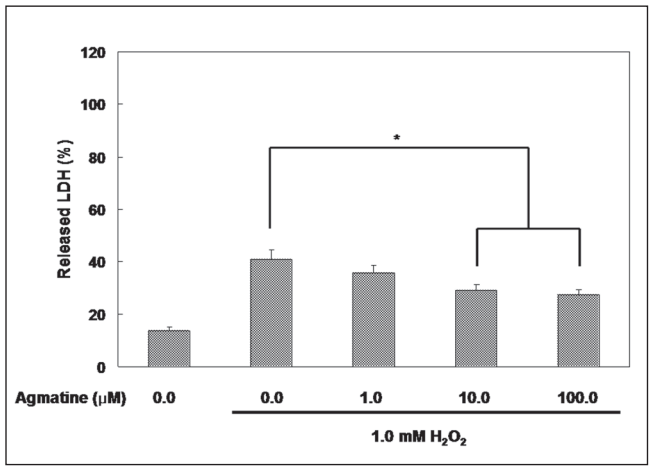

FIGURE 4. Protective effects of agmatine pretreatment. Release of lactate dehydrogenase (LDH) was significantly decreased when the cells were pretreated with agmatine before injury. Data are expressed as means ± *SD*. Asterisk indicates a statistically significant difference.

Discussion

An important feature of our study is that we have used differentiated RGC-5 cells in culture. In fact, it is generally thought that experiments on primary cultured cells are better on immortalized cells. However, because there are many difficulties and limits in dealing with primary cultured retinal ganglion cells, transformed cells have been preferred in the current experiments. Although RGC-5 cells express neuronal markers characteristic of primary cultured retinal ganglion cells and they are presumed to be biologically similar to primary cells, they are mitotically active and exhibit morphological differences to primary cultured retinal ganglion cells. Previous reports have demonstrated that when RGC-5 cells are differentiated, they show no mitoses, and they sufficiently express neurites by exposure to staurosporine (Frassetto *et al*., 2006; Harvey and Chintala, 2007; Lievin *et al*., 2007). In our study, RGC-5 cells were differentiated by incubation in 1.0 μM staurosporine for 6 hours and let to recover for 3 days before we started our major experiments.

In the present study, pretreatment with agmatine rescued the differentiated RGC-5 cells from apoptosis caused by oxidative stress in a statistically significant and dose-dependent manner. Kalapesi *et al*. (2005) have reported that the expression of α_2-adrenergic receptors increased on fully differentiated RGC-5 cells compared to undifferentiated RGC-5 cells. As mentioned above, agmatine acts as an agonist for the α_2-adrenergic receptors. Taken together, there is a possibility that the effects of agmatine revealed on differentiated RGC-5 cells may be related in some way to the expression of the α_2-adrenergic receptors.

Agmatine has been mainly studied as a neuroprotective agent for neuronal ischemic injuries of the central nervous system. We have already applied this concept to a glaucoma model and successfully demonstrated the neuroprotective effects of agmatine against hypoxia-induced apoptotic death of retinal ganglion cells *in vitro* (Hong *et al*., 2007). In the same direction, this study investigated agmatine's neuroprotective effects on retinal ganglion cells when exerted in advance, i.e., before cells are exposed to damage.

Even though further studies should be performed to elucidate the precise neuroprotective mechanisms of

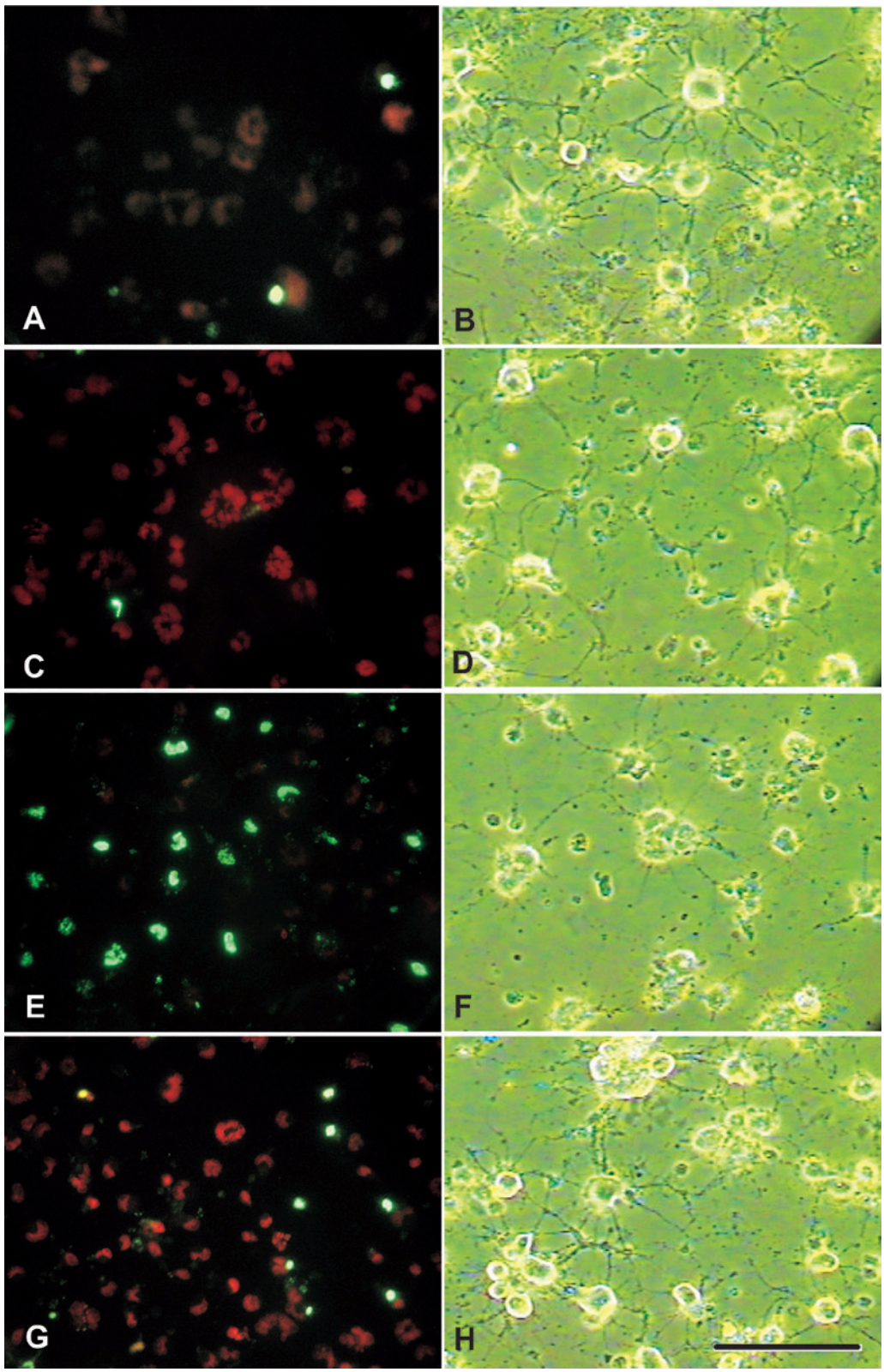

FIGURE 5. Anti-apoptotic effects of agmatine pretreatment. After pre-incubation with agmatine, differentiated RGC-5 cells became more resistant against the hydrogen peroxide injury. Green fluorescence indicates apoptotic cells while orange fluorescence, indicates living cells. (A, B) No treatment, (C, D) 100.0 μM agmatine only, (E, F) 1.0 μM hydrogen peroxide only, and (G, H) 1.0 μM hydrogen peroxide after 100.0 μM agmatine. Panels A, C, E and G are fluorescence micrographs, while panels B, D, F and H are bright field micrographs. Scale bar represents 20 mm.

agmatine, the present results encourage us to consider it as a potential protective agent, with the ability to rescue retinal ganglion cells from apoptotic death.

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