

## Short Communication

# Agmatine inhibits hypoxia-induced TNF- $\alpha$ release from cultured retinal ganglion cells

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**Key words:** retinal ganglion cell, tumor necrosis factor- $\alpha$ , hypoxia, agmatine.

**ABSTRACT:** The effect of hypoxia on the release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in transformed rat retinal ganglion cells (RGCs) and the effect of agmatine on the hypoxia-induced production of TNF- $\alpha$  in RGCs were evaluated. RGCs were cultured under hypoxic conditions with 5% oxygen, with or without 100  $\mu$ M agmatine. The expression levels of TNF- $\alpha$  and its receptor-1 (TNF-R1) were investigated by Western blot analysis. After 6 hours of hypoxia, we noted an increase in TNF- $\alpha$  production in RGCs. Agmatine significantly reduced TNF- $\alpha$  level after 12 hours of hypoxic treatment. The expression of TNF-R1 was not affected by the hypoxia or agmatine treatment. Our results show that agmatine inhibits the TNF- $\alpha$  production of RGCs in hypoxic condition. These results demonstrate a possible neuroprotective mechanism for agmatine against hypoxic damage in RGCs.

## Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a cytokine that may be involved in the pathogenesis of glaucoma; it is upregulated in glial cells of glaucomatous eyes (Yan *et al.*, 2000; Yuan and Neufeld, 2000, 2001; Tezel *et al.*, 2001), and its production is increased by ischemia or elevated hydrostatic pressure in glial cells (Tezel and Wax, 2000). *In vivo* intravitreal injection of TNF- $\alpha$  induces the axonal degeneration and delayed loss of retinal ganglion cell (RGC) cell bodies (Kitaoka *et al.*, 2006). Even though there is no the direct evidence that

TNF- $\alpha$  contributes to RGC death, previous reports suggest that TNF- $\alpha$  from glial cells may play a critical role in the death of RGCs in glaucomatous eyes. However, we could not find any reports in the literature about the production of TNF- $\alpha$  in RGCs.

Agmatine, 1-amino-4-guanidobutane, is an endogenous polyamine formed by the decarboxylation of L-arginine (Tabor and Tabor, 1984; Reis and Regunathan, 2000; Grillo and Colombatto, 2004; Moinard *et al.*, 2005). Many reports have demonstrated that agmatine protects neuronal cells from various injuries, including ischemia (Gilad *et al.*, 1996; Olmos *et al.*, 1999; Gilad and Gilad, 2000; Yu *et al.*, 2000, 2003; Zhu *et al.*, 2003, 2006; Kim *et al.*, 2004, 2006; Gilad *et al.*, 2005; Kotil *et al.*, 2006; Wang *et al.*, 2006). Although there is a report showing that agmatine does not inhibit the increase of TNF- $\alpha$  after reoxygenation (Feng and LeBlanc, 2003), the effect of agmatine on production of TNF- $\alpha$

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has not been well established. Some studies have shown that aminoguanidine can attenuate the production of TNF- $\alpha$  and its cytotoxic effects (Jo *et al.*, 1995; Marzocco *et al.*, 2004; Lin *et al.*, 2006; Yilmaz *et al.*, 2006).

The purpose of this study was to investigate the effect of hypoxic injury on the release of TNF- $\alpha$  from rat RGCs and the effect of agmatine on this hypoxia-induced production of TNF- $\alpha$ . We observed that agmatine lowers the hypoxia-induced levels of TNF- $\alpha$  in RGCs. Our data suggest that this effect of agmatine may be a neuroprotective mechanism against hypoxic damage in RGCs.

## Materials and Methods

### Cell culture

The RGC-5 cell line, a retinal ganglion cell line developed from post-natal Sprague-Dawley rats, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL of penicillin and 100  $\mu$ g/mL of streptomycin. The cells were passaged every 2 to 3 days and incubated at 37°C in 5% CO<sub>2</sub> and air. During cultivation, the cells exhibited the same morphology. For all experiments, cells were used at 80% confluence.

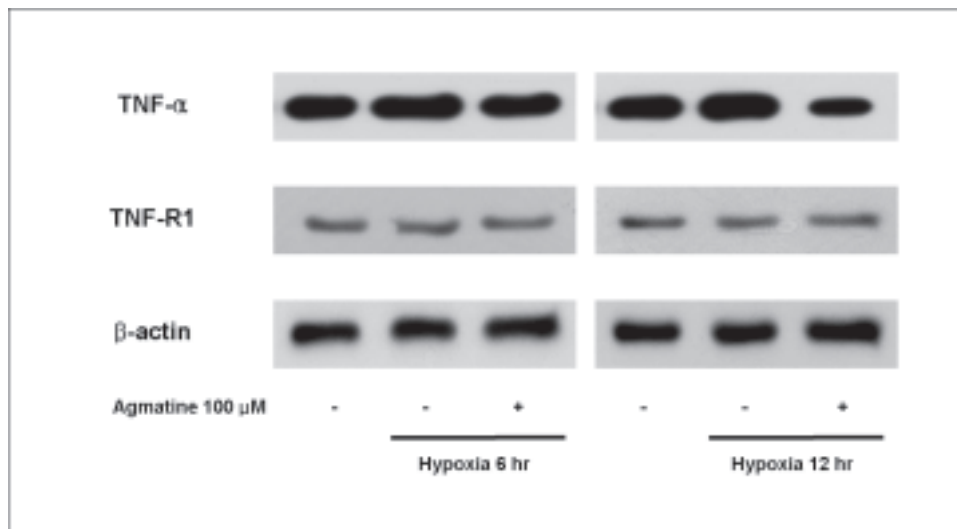
### Hypoxic injury

Cultures were transferred into a closed hypoxic chamber (Forma Scientific Co. Seoul, Republic of Korea) with controlled oxygen level (5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub>) and temperature (37°C). After washing twice with deoxygenated serum-free DMEM, cells were maintained in the hypoxic chamber. Control cells were not exposed to hypoxia. An amount of 100  $\mu$ M agmatine (Sigma, St. Louis, MO) was added to the culture medium at the start of hypoxia treatment as indicated.

### Western blot analysis

For extraction of whole cellular proteins, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with cell lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 1 mM PMSF, 1  $\mu$ g/mL aprotinin, 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL pepstatin) on ice for 30 minutes. Lysates were sonicated and the cell homogenates were centrifuged at 15,000g for 10 minutes (4°C).

The protein concentrations in the resultant supernatants were determined with the Bradford reagent, and equal amounts of protein (40  $\mu$ g) were boiled in Laemmli sample buffer and resolved by 10 or 15% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes and incubated overnight with antibod-



**FIGURE 1.** Representative Western blot analysis showing the effect of agmatine (100  $\mu$ M) on TNF- $\alpha$  and TNF-R1 levels after 6 and 12 hours hypoxic injury. Hypoxia –induced TNF- $\alpha$  production in RGCs was decreased by the presence of agmatine. All procedures repeated triplicate and this one is representative.

ies against TNF- $\alpha$  (Cell Signaling Technology, Danvers, MA), TNF-R1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti- $\beta$ -actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as indicated (diluted 1:1000). The immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence. Bands were analyzed with ImageJ program (National Institutes of Health, Bethesda, MD). All procedures were repeated in triplicate.

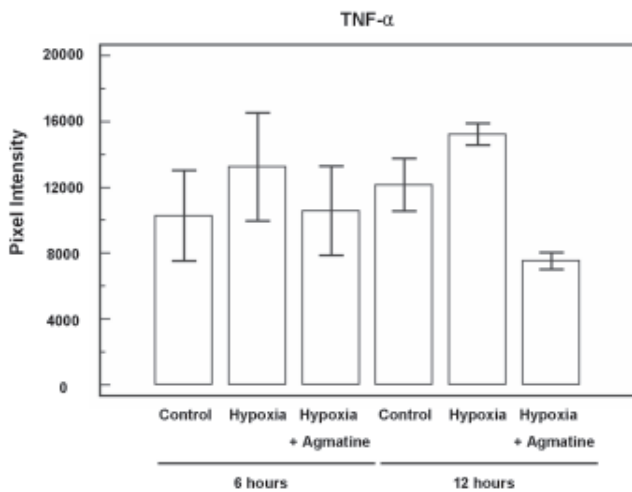
**Results**

Representative Western blots for levels of TNF- $\alpha$ , TNF-R1, and  $\beta$ -actin in hypoxia-treated RGCs are

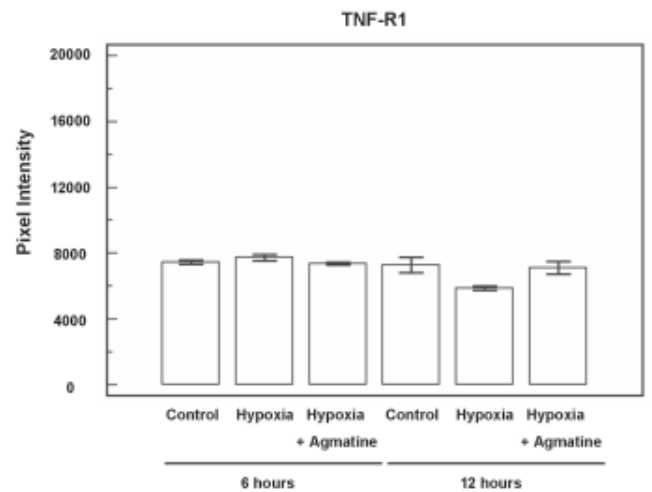
shown in figure 1. Mean intensity of bands are demonstrated in figure 2. TNF- $\alpha$  was upregulated after hypoxia, but TNF-R1 production was not apparently influenced by hypoxic injury. Agmatine suppressed the hypoxia-induced TNF- $\alpha$  production in RGCs. This tendency was more significant at 12 hours rather than 6 hours. The  $\beta$ -actin was used as an internal control.

The antibody against TNF- $\alpha$  detected a single band at 17 kDa. An increase in TNF- $\alpha$  in hypoxic RGCs became evident after 6 hours of hypoxic injury and remained elevated for 6 hours after treatment. Treatment with agmatine significantly suppressed this increase at 12 hours after hypoxic injury, even though its effect was present at 6 hours (Fig. 2A).

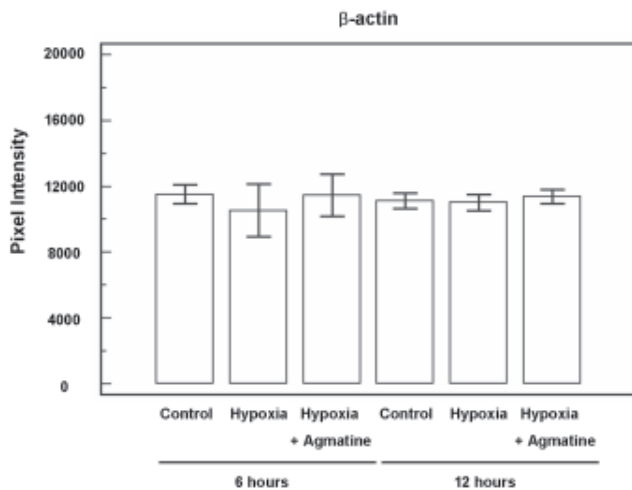
The antibody against the TNF-R1 also detected a single band at 55 kDa. After 12 hours hypoxia, expres-



**A**



**B**



**C**

**FIGURE 2.** Mean intensity of bands on Western immunoblots. (A) TNF- $\alpha$  was upregulated after hypoxia; but (B) TNF-R1 production was not apparently influenced by hypoxic injury. Agmatine (100  $\mu$ M) suppressed the hypoxia-induced TNF- $\alpha$  production in RGCs. This tendency was more significant at 12 hours rather than 6 hours. (C) The  $\beta$ -actin was used as an internal control. All procedures repeated triplicate. Intensity of each band was calculated with the ImageJ program and a mean value with 95% confidence interval was presented.

sion of TNF-R1 decreased, but the expression of TNF-R1 was not apparently changed by hypoxic injury or agmatine treatment (Fig. 2B).

The  $\beta$ -actin bands were kept nearly at a constant level (Fig. 2C). It acted as an internal control.

## Discussion

Many reports suggest that the TNF- $\alpha$  from glial cells might cause damage to RGCs in glaucomatous eyes (Yan *et al.*, 2000; Yuan and Neufeld, 2000, 2001; Tezel *et al.*, 2001). In the present study, we show that TNF- $\alpha$  production in RGCs is increased by hypoxic injury and that agmatine treatment lowers hypoxia-induced TNF- $\alpha$  levels.

The selective loss of RGCs is a distinctive feature of glaucoma (Osborne *et al.* 1999; Kaushik *et al.*, 2003; Kuehn *et al.*, 2005). Previous studies have focused on lowering the intraocular pressure to prevent the death of RGCs, but recently there has been a shift towards identifying neuroprotective mechanisms to prevent the apoptosis of RGCs (Kuehn *et al.*, 2005).

Agmatine is an aminoguanidine that has a neuroprotective effect on neuronal death resulting from various injuries including ischemia and ischemic-reperfusion injury (Gilad *et al.*, 1996; Olmos *et al.*, 1999; Gilad and Gilad 2000; Yu *et al.*, 2000, 2003; Zhu *et al.*, 2003, 2006; Kim *et al.*, 2004, 2006; Gilad *et al.*, 2005; Kotil *et al.*, 2006; Wang *et al.*, 2006). Even though agmatine is known as an inhibitor of the N-methyl-D-aspartate (NMDA) receptor and an agonist for the  $\alpha$ 2-adrenergic and imidazoline receptors, the precise mechanisms of its neuroprotective effect have not been established (Li *et al.*, 1994; Olmos *et al.*, 1999; Yang and Reis 1999; Head and Mayorov, 2006; Wang *et al.*, 2006; Zhu *et al.*, 2006). In addition, the effect of agmatine on TNF- $\alpha$  production has not yet been reported.

The upregulation of TNF-R1 in RGCs of glaucomatous eyes has been reported (Tezel *et al.*, 2001). In this study, the expression of TNF-R1 was not influenced by hypoxic injury. However, this discrepancy may be due to the fact that several mechanisms may contribute to the development of glaucoma.

In conclusion, the present study demonstrates that TNF- $\alpha$  production increases in RGCs after hypoxic injury, and that agmatine inhibits hypoxia-induced TNF- $\alpha$  levels. Further studies are needed to elucidate the neuroprotective mechanism of agmatine for the prevention of RGC death.

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