Research Report

Taurine protects transformed rat retinal ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction

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ABSTRACT

Hypoxia-induced apoptosis of retinal ganglion cells (RGCs) is the major cause of progressive vision loss in numerous retinal diseases, including glaucoma and diabetic retinopathy. Taurine is a naturally occurring free amino acid that has been shown to have neurotrophic and neuroprotective properties in the retina. We investigated the specific potential for taurine to be protective for immortalized rat retinal ganglion cells (RGC-5) exposed to hypoxia (5% O_2). Pretreatment of RGC-5 cells with 0.1 mM taurine significantly reduced the extent of apoptosis detected by DAPI staining, MTT, and Annexin V-FITC/PI assays. To further study the mechanism underlying the beneficial effect of taurine, interactions between taurine and the process of mitochondria-mediated apoptosis were examined. Taurine treatment of RGC-5 cells suppressed the induction of the mitochondrial permeability transition (mPT) by reducing intracellular calcium levels and inhibiting the opening of mitochondrial permeability transition pores (mPTPs). Moreover, the loss of mitochondrial membrane potential, a decline in cellular ATP levels, a reduction in the amount of cytochrome *c* translocated to the cytoplasm and caspase-3 activation were observed in taurine-treated cultures. These results demonstrate the potential for taurine to protect RGCs against hypoxic damage *in vivo* by preventing mitochondrial dysfunction.

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1. Introduction

Retinal ganglion cells (RGCs) are solely responsible for the relay of visual signals from the eye to the brain, and therefore are of critical importance to the visual system. When RGCs are exposed to hypoxic conditions, which can occur in various ocular diseases including glaucoma, diabetic retinopathy, and central retinal artery occlusion, the lack of oxygen can impair or even induce apoptosis of RGCs (Levin, 2003; Sennlaub et al., 2002). Thus, it is urgent to develop neuroprotective strategies for RGCs. Protective agents of RGCs have been identified that include calcium channel blockers (Yamada et al., 2006), nitric oxide (NO) synthase inhibitors (Hong et al., 2007), and NMDA receptor antagonists (Hare et al., 2004). However, these agents are also associated with negative side effects and compliance problems. Therefore the potential neuroprotective functions of endogenous molecules, such as erythropoietin (Yamasaki et al., 2005), growth factors (Johnson et al., 1986), and taurine

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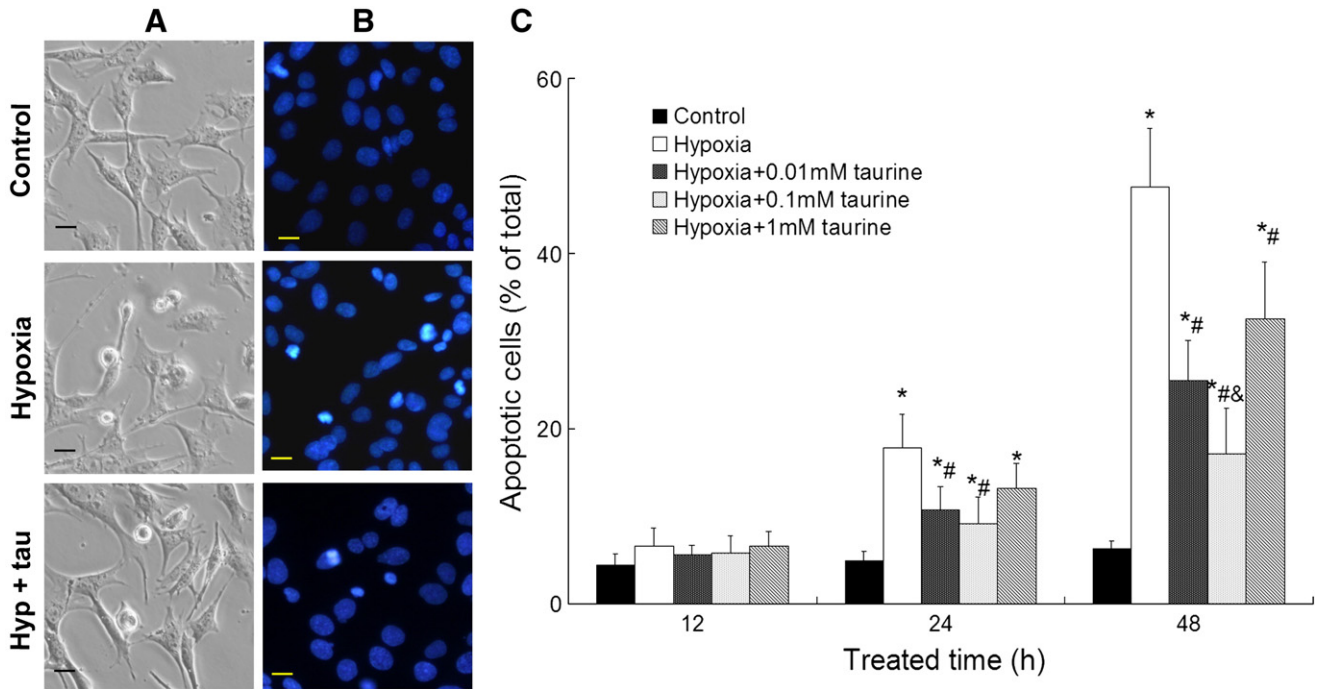 E-mail address: mimt2005@sina.com (M. Mi).

Fig. 1 – Taurine reduces hypoxia-induced apoptosis of RGC-5 cells. (A) Phase-contrast micrographs of RGC-5 cells exposed to 24 h hypoxia with and without taurine (0.1 mM) administration. Scale bar 10 μm. (B) Photomicrographs of nuclear DAPI staining. RGC-5 cells were cultured with and without hypoxia for 24 h in the presence or absence of taurine (0.1 mM). Scale bar 10 μm. (C) Percentage of apoptotic cells detected by Annexin V-FITC/PI staining by flow cytometry. Apoptotic cells included Annexin V(+)/PI(−) and Annexin V(+)/PI(+) cells. Results are expressed as the means ± SD of 5 independent experiments. (*$p < 0.05$ as compared to the control group for each time point; #$p < 0.05$ as compared to the hypoxia group for each time point; and $p < 0.05$ as compared to the hypoxia group pretreated with 0.01 mM or 1 mM taurine.)

(2-aminoethanesulfonic acid) are being considered. In the present study, the neuroprotective effects of taurine on RGCs was investigated.

Taurine is a sulfur-containing amino acid that is enriched in many excitable tissues including the retina, brain, skeletal and cardiac muscles. Physiological actions of taurine are widely involved in membrane stabilization, osmoregulation, neuro-modulation, regulation of calcium homeostasis, and antioxida-tion (Bouckenooghe et al., 2006). Since Hayes et al. reviewed in 1975 that retinal degeneration of cats was associated with taurine deficiency (Hayes et al., 1975), increasing evidence has accumulated to indicate the pivotal role of taurine in the development and survival of retinal neurons (Militante and Lombardini, 2002). In addition to the protective effects of taurine demonstrated in diabetic retinopathy (Yu et al., 2008) and photochemical stress (Yu et al., 2007), taurine has also been shown to render neurons (El Idrissi, 2008; Sun and Xu, 2008) and cardiomyocytes (Takatani et al., 2004a,b) resistant to an array of detrimental stimuli such as hypoxia and ischemia. We there-fore hypothesize that taurine has neuroprotective effects on RGCs under hypoxia.

Although the exact mechanisms of hypoxia-induced apop-tosis have not been completely elucidated, mitochondria have been shown to play an important role (Wang, 2001; Kristian, 2004). During hypoxia, intracellular Ca^{2+} levels are elevated and the presence of excessive free radicals, acidosis, and low adenine nucleotide levels all trigger the opening of mitochon-drial permeability transition pores (mPTPs), which initiates the

mitochondrial permeability transition (mPT) (Kristian, 2004; Kristian and Siesjo, 1998).In spite of the complexity of mito-chondria-mediated apoptosis, the mPT is commonly regarded as an irreversible event leading to mitochondrial failure and even cell death (Galluzzi et al., 2008). Initiation of the mPT can lead to the functional impairment of mitochondria through
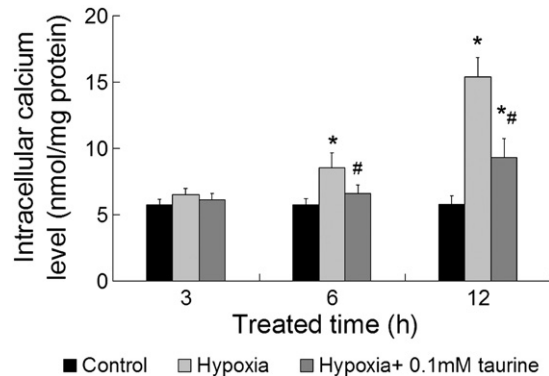

Fig. 2 – The effect of taurine on the intracellular calcium level in RGC-5 cells exposed to hypoxia. [Ca^{2+}]$_i$ was measured using Fura-2/AM in cultures exposed to normoxia or hypoxia for 3, 6, and 12 h in the presence or absence of taurine (0.1 mM). Results are expressed as the means ± SD of 5 independent experiments, with 5 parallel samples in each experiment. (*$p < 0.05$ compared to the control group for each time point; #$p < 0.05$ compared to the hypoxia group for each time point.)
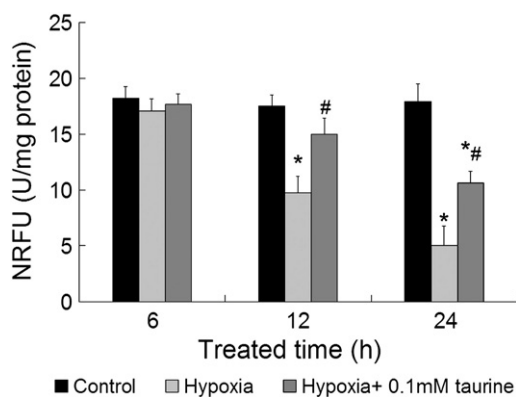
Fig. 3 – The influence of taurine on the opening of mPTPs in RGC-5 cells exposed to hypoxia. Cells pretreated with or without taurine (0.1 mM) and exposed to normoxia or hypoxia for 6, 12, and 24 h were used to measure the normalized relative fluorescence units (NRFU) of calcein. Results are expressed as the means±SD of 5 independent experiments, with 5 parallel samples in each experiment. (*$p < 0.05$ compared to the control group for each time point; #$p < 0.05$ compared to the hypoxia group for each time point.)

depolarization of mitochondrial membrane potential ($\Delta\psi m$) and a reversal of ATP synthase function to accelerate cellular energy depletion (Foster et al., 2006). Induction of the mPT can also release proteins normally localized to mitochondria into the cytosol, including cytochrome c (cyt c) and apoptosis-inducing factor (AIF) which can activate caspase and caspase-independent effectors of apoptosis (Galluzzi et al., 2008). Taurine appears to be able to block mitochondria-mediated apoptosis through its ability to regulate intracellular Ca^{2+} homeostasis both in the cytoplasm and in mitochondria (Foos and Wu, 2002; El Idrissi, 2008) to affect the process of antioxidation, and to provide a pH buffer in the mitochondrial matrix (Hansen et al., 2006). However, previous studies have not unequivocally confirmed the effects of taurine on the mPT (El Idrissi, 2008; Palmi et al., 2006; Takatani et al., 2004a,b), resulting in the need to further clarify the actions of taurine on mitochondria of RGCs exposed to hypoxia.

In this study, the immortalized RGC line, RGC-5 (Krishnamoorthy et al., 2001), was used instead of primary RGCs due to the difficulties of culturing primary cell lines and the similarities that RGC-5 cells share with primary RGCs. The effect of taurine on the survival of RGC-5 cells exposed to hypoxia was evaluated, particularly the neuroprotective role of taurine in the modulation of Ca^{2+}-dependent mPT and regulation of mitochondrial function as effectors of the mitochondrial apoptotic pathway.

2. Results

2.1. Taurine reduces hypoxia-induced apoptosis of RGC-5 cells

To verify the protective effects of taurine on RGC-5 cells exposed to hypoxia, serial concentrations of taurine (i.e. 0.001,

0.01, 0.1, 1, and 10 mM) were administered to cultures of RGC-5 cells prior to induction of hypoxia and morphological changes were examined. Control, untreated RGC-5 cells exhibited an epithelial-like phenotype with short extensions. With prolonged exposure to hypoxia (5% O_2, 5% CO_2, and 90% N_2), an increasing number of cells rounded up (Fig. 1A). To quantitate cell viability, MTT assays were performed. While cell viability of untreated RGC-5 cells dropped to 48.7±4.5% after 48 hours (h) of hypoxic exposure, RGC-5 cells pretreated with 0.01 mM, 0.1 mM and 1 mM of taurine exhibited elevated levels of cell viability to 66.6±3.7%, 73.5±4.1%, and 62.9±3.9% respectively (all $p < 0.05$ vs. Hypoxia group; $n = 5$), but no significant improvement was detected with 0.001 mM or 10 mM of taurine administration. In addition, there were no significant differences in cell viability between RGC-5 cells with and without taurine under normoxia conditions. Diamidino-phenyl-indole (DAPI)-staining was also used to characterize hypoxic-induced cell death of RGC-5 cells. Hypoxic exposure resulted in clear changes in chromatin morphology such as condensation and fragmentation, and the changes were significantly attenuated by taurine (Fig. 1B).
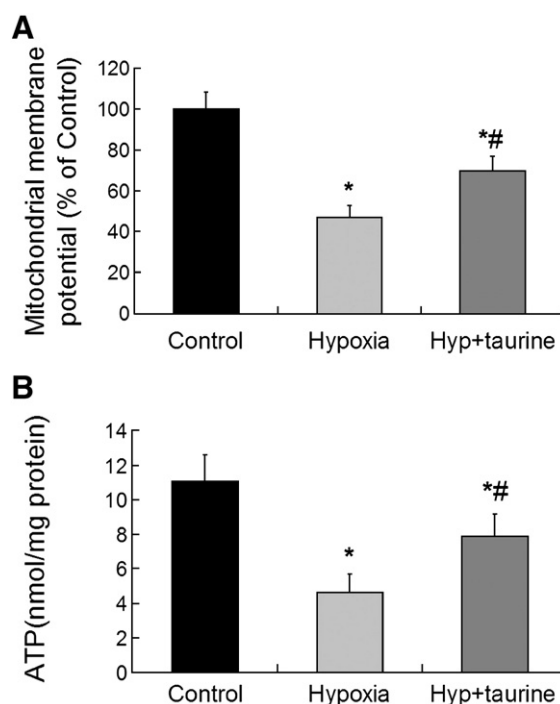

Fig. 4 – Effects of taurine on mitochondrial function in RGC-5 cells exposed to hypoxia. (A) Mitochondrial membrane potential of RGC-5 cells exposed to normoxia or hypoxia for 24 h in the absence or presence of taurine (0.1 mM) were determined using JC-1. Results are expressed as the means±SD from 5 independent experiments, with 5 parallel samples in each experiment. (*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the hypoxia group.) (B) Cellular ATP concentrations were detected in RGC-5 cells exposed to normoxia or hypoxia (24 h) with or without taurine (0.1 mM). Results are expressed as the means±SD from 5 independent experiments, with 5 parallel samples in each experiment. (*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the hypoxia group.)

The anti-apoptotic effects of taurine were further studied using an Annexin V-FITC/PI assay (Fig. 1C). In agreement with previous data from Hong et al. (2007), hypoxia-induced apoptosis of RGC-5 cells was found to be time-dependent. Although the initial 12 h of hypoxic exposure did not change the percentage of apoptotic cells relative to the control culture, there were marked increases in the percentage of apoptotic cells after 24 h (17.75 ± 3.95 %; $p < 0.05$) and 48 h (47.59 ± 6.70%; $p < 0.05$). However, taurine suppressed hypoxia-induced damage to RGC-5 cells in a dose-dependent manner. All the taurine doses between 0.01 mM and 1 mM effectively reduced the percentage of apoptotic cells, but taurine at 0.001 mM and 10 mM only has tendency to reduce hypoxic damage. As shown in Fig. 1C, when concentrations of 0.01, 0.1, and 1 mM taurine were used to pretreat the RGC-5 cells prior to the induction of hypoxia, the percentage of apoptotic cells at 48 h detected decreased to 25.50 ± 4.51%, 17.14 ± 5.14%, and 32.52 ± 6.42%, respectively (all $p < 0.05$). Since the lowest percentage of apoptotic cells was detected in the presence of 0.1 mM taurine ($p < 0.05$), this concentration was used in subsequent experiments.

2.2. Taurine modulates intracellular calcium homeostasis

Hypoxic injury of neurons is hypothesized to be triggered by an accumulation of intracellular Ca^{2+} (i.e. $[Ca^{2+}]_i$) (Yamada et al., 2006). To determine whether taurine affected the $[Ca^{2+}]_i$ of RGC-5 cells, the Fura-2/AM fluorescence method was used. As shown in Fig. 2, a significant increase in the $[Ca^{2+}]_i$ in taurine-untreated RGC-5 cells was observed as early as 6 h after the induction of hypoxia. In contrast, no significant elevation in the $[Ca^{2+}]_i$ was detected in taurine-treated RGC-5 cells until 12 h after hypoxic exposure when a relatively lower $[Ca^{2+}]_i$ was detected (i.e. a 1.5-fold increase vs. 2.7-fold increase in taurine-untreated cells) (Fig. 2). These findings support a role for taurine in modulating the intracellular calcium homeostasis of RGC-5 cells in response to hypoxia.

2.3. Taurine inhibits the opening of mPTPs

The opening of mPTPs can be triggered by increases in Ca^{2+} levels during hypoxia, which can also initiate the mPT and further lead to mitochondrial dysfunction (Zoratti et al., 2005).
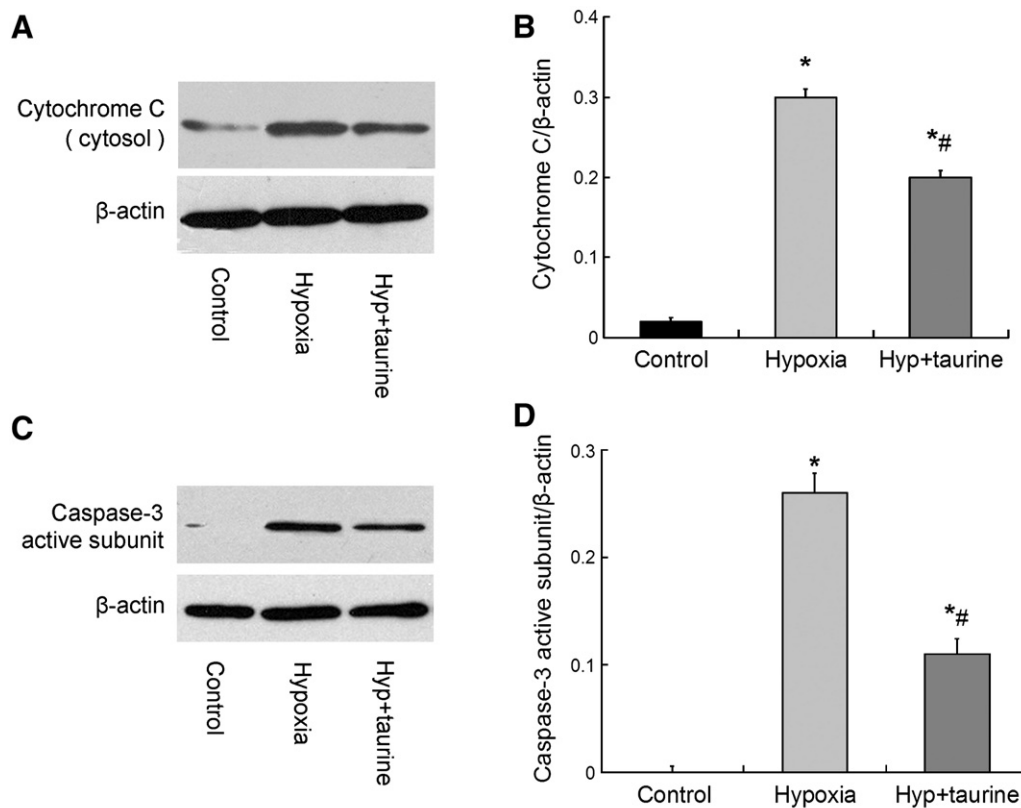

Fig. 5 – Influences of taurine on the cytochrome c/caspase-3 apoptosis pathway. (A) Western blot analysis of cytochrome c (14 kDa) and β-actin (42 kDa) in total cytosolic protein fractions from cells exposed to normoxia or hypoxia for 24 h with or without taurine (0.1 mM). Representative results of 3 independent experiments are shown. (B) Quantitative analysis of cytosolic cytochrome c levels normalized to the internal control, β-actin. Results are expressed as the means ± SD from 3 independent experiments. (*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the hypoxia group.) (C) Western blot analysis of the active subunit of caspase-3 (17 kDa) and β-actin (42 kDa). Protein samples derived from cells exposed to normoxia or hypoxia for 24 h with or without the addition of taurine (0.1 mM). Representative results of 3 independent experiments are shown. (D) Quantitative analysis of the active subunit of caspase-3 normalized to the internal control, β-actin. Results are expressed as the means ± SD from 3 independent experiments. (*$p < 0.05$ compared to the control group; #$p < 0.05$ compared to the hypoxia group.)

Therefore, the influence of taurine on mPTPs was detected using the calcein–cobalt method to monitor the distribution of green fluorescence emitted from calcein as a readout of intact mPTPs. A marked decrease in the fluorescence intensity of taurine-untreated RGC-5 cells was observed after more than 12 h of hypoxic exposure (Fig. 3). However, in taurine-treated cultures, quantitative analysis of the normalized relative fluorescence units (NRFU) detected revealed no significant decrease up to 12 h after induction of hypoxia (Fig. 3). After 24 h of hypoxia, comparatively higher NRFU (10.62 ± 1.08 U/mg protein; $p < 0.05$) were observed in cultures pretreated with taurine compared to untreated cells (5.08 ± 1.67 U/mg protein). Taken together our results demonstrate the ability of taurine to reduce the extent of opened mPTPs in response to hypoxia.

2.4. Taurine prevents mitochondrial dysfunction

To further investigate the effects of taurine on mitochondrial function, indicators of mitochondrial activity such as membrane potential ($\Delta\psi$m) and levels of cellular ATP were determined. After 24 h of hypoxic exposure, the $\Delta\psi$m for taurine-treated and untreated cells were reduced (Fig. 4A), although the untreated cells did exhibit lower membrane potential values ($p < 0.05$). Cellular ATP content was also a sensitive readout of mitochondrial function. For example, Fig. 4B shows that, after 24 h of hypoxia, a drastic decrease in cellular ATP levels from 11.07 ± 1.52 to 4.64 ± 1.03 nmol/mg protein was detected. In contrast, treatment of cultures with taurine resulted in cellular ATP levels of 7.89 ± 1.25 nmol/mg protein ($p < 0.05$). These findings provide direct evidence that taurine protects mitochondrial function during hypoxia.

2.5. Taurine blocks the mitochondrial apoptotic pathway

Mitochondrial dysfunction may initiate apoptosis by releasing pro-apoptotic factors, such as cyt c, from the mitochondrial intermembrane space into the cytoplasm to trigger apoptosis via a caspase 3-dependent pathway (Li et al., 1997; Takatani et al., 2004a). Given the protective effects of taurine on mitochondrial function demonstrated in this study, levels of cytosolic cyt c and the active subunit of caspase 3 were assessed to identify specific mechanisms of taurine's potential anti-apoptotic signaling. After 24 h of hypoxia, RGC-5 cells showed a substantial increase in levels of cytosolic cyt c, while the 17-kDa fragment of caspase-3 was detected in RGC-5 cells with and without taurine pretreatment (Figs. 5A, C). Quantitative analysis of the pro-apoptotic protein bands detected revealed that taurine partially attenuated the release of cyt c and blocked the activation of caspase 3 (Figs. 5B, D).

3. Discussion

Using the RGC-5 cell line, we demonstrate that taurine can suppress hypoxia-induced apoptosis in RGCs. Although the cytoprotective actions of taurine have previously been observed in brain neurons and cardiomyocytes in vivo and in vitro under hypoxic/ischemic conditions (El Idrissi, 2008; Sun and Xu, 2008; Takatani et al., 2004a,b), to our knowledge, this is the first report to elucidate the neuroprotective action of taurine on RGCs in response to hypoxia. It is well known that taurine is abundant in the retina, even amounted to mM range. However, the decrease in intracellular concentration of taurine and the loss of intracellular:extracellular concentration ratio due to the release of taurine under various cell-damaging conditions such as hypoxia, may result in neuronal damage (Sun and Xu, 2008). In our in vitro hypoxia model, taurine administration did not completely block cellular damage resulting from hypoxic injury, although treatment of RGC-5 cells with 0.1 mM taurine reduced the total number of apoptotic cells by $\sim 64\%$, thereby providing a neuroprotective effect. Furthermore, the combination of data from the Annexin V-FITC/PI assays, which represent a reliable method to identify apoptotic cells in both the early and late stages of apoptosis, the MTT assays, and the DAPI staining studies, all provide strong evidence in support of our hypothesis that taurine provides neuroprotective effects for RGCs.

While various functions of taurine have been previously reported, the precise cellular mechanisms involving taurine had not been well established. Previous studies suggested that the cytoprotective action of taurine during hypoxia was related to its inhibition of intracellular calcium levels, antioxidation, and excitotoxicity (Saransaari and Oja, 2000). In the present study, the possible correlation between taurine and mitochondria function were evaluated. It is well known that mitochondria are key components to the function of RGCs and that mitochondrial dysfunction is the primary cause of hypoxia-induced apoptosis of RGCs. A critical factor mediating mitochondrial dysfunction is the mitochondrial permeability transition (mPT). When induced, functional breakdown and morphological disintegration of mitochondria occurs and initiates cell death (Kristian, 2004). In this study, we further confirmed that the anti-apoptotic action of taurine was mediated by suppressing the Ca^{2+}-dependent mPT to prevent mitochondrial dysfunction and block activation of the cyt c/caspase-3 apoptotic pathway.

It has been shown that an increase in intracellular free Ca^{2+} predisposes cells to damage (Choi, 1988; Sasaki and Kaneko, 2007) and involves numerous signaling pathways. Initiation of Ca^{2+}-dependent mPT has specifically been implicated in hypoxia/ischemia-induced cell death (Ichas and Mazat, 1998). During hypoxia, the lack of oxygen rapidly depletes ATP levels below the threshold for maintaining cellular ionic gradients and the subsequent neuronal depolarization provokes an excessive Ca^{2+} influx. The efficacy of taurine in modulating $[Ca^{2+}]_i$ has been well characterized in neurons and cardiomyocytes (Saransaari and Oja, 2000), and consistent with these studies, we also found that taurine attenuates the elevation in $[Ca^{2+}]_i$ in RGC-5 cells during hypoxia. While this attenuation may involve effects on Ca^{2+} influx (Lehmann et al., 1985) or the sequestration of Ca^{2+} in mitochondria (El Idrissi, 2008), we can only conclude from our data that taurine can alleviate hypoxia-induced damages by suppressing Ca^{2+}-dependent mPT.

The mPT is based on the opening of non-specific pores (mPTPs) that are located at the junction between the internal and outer membranes of mitochondria. The diameter of a mPTP allows molecules up to ~ 1500 Da to cross the mitochondrial membrane. During hypoxia, excessive amounts of Ca^{2+}, combined with elevated cellular ROS levels and adenine nucleotide depletion, triggers the opening of mPTPs (Zoratti et al., 2005).

Given the ability of taurine to counteract calcium overload and antioxidation, we hypothesize a role for taurine in the regulation of mPTPs. Consistent with this hypothesis, we observed that taurine was able to partially inhibit the opening of mPTPs and decrease $[Ca^{2+}]_i$ levels in RGC-5 cells. In contrast, however, is data from a study on the effects of taurine on mPTPs isolated from a rat liver exposed to high levels of Ca^{2+} and phosphate (Palmi et al., 2000). This divergence may be correlated with the different regulations of the mPTPs opening in neurons and in other cells like liver or heart (Kristian, 2004). Moreover, it is reasonable to presume that the ability of taurine in controlling pore-opening only under milder physiological mPT-inducing conditions (Palmi et al., 2000; Palmi et al., 2006). However, the data from our experiments support the inhibitory effects of taurine on the mPT.

The activation of the mPT can lead to a bioenergetic, biosynthetic, and redox crisis in a cell which can directly threaten cell survival (Armstrong, 2006). When the mPT is induced, the mitochondrial inner membrane becomes permeable to protons which leads to uncoupling of the electron respiratory chain and the collapse of membrane potential, which in turn leads to a cessation of ATP generation in mitochondria (Armstrong, 2006; Galluzzi et al., 2008). By pretreating RGC-5 cells with taurine, the hypoxia-induced loss of mitochondrial membrane potential and decline in ATP levels were alleviated, supporting the notion that neuroprotection is, in part, due to prevention of mitochondrial dysfunction. These results are consistent with recent studies that demonstrate the ability of taurine to enhance the mitochondrial activity of cerebella granual cells exposed to glutamate (El Idrissi, 2008).

Another threatening consequence of altered mitochondrial permeability is the release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol (Armstrong, 2006; Kristian, 2004; Galluzzi et al., 2008). Cyt c is associated with the inner mitochondrial membrane and serves as an essential component of the electron transfer chain. With induction of the mPT and translocation of cyt c into the cytosol, mitochondrial function is compromised. In the current study, the relatively lower cytosolic cyt c levels detected in taurine-treated cultures demonstrated that a correlation exists between taurine and the preservation of mitochondrial function. Moreover, once cyt c enters the cytosol, it associates with apoptotic peptidase activating factor 1 (Apaf-1) and dATP to form an apoptosome. Apoptosomes recruit and activate pro-caspase-9, which in turn activates other downstream caspases to initiate apoptosis (Galluzzi et al., 2008). Caspase-3 has classically been considered as an executioner caspase. In this experiment, taurine suppressed the activation of caspase-3 and was associated with lower cytosolic levels of cyt c. In addition, recent studies have revealed that taurine participates in the intrinsic apoptosis process through inhibition of Apaf-1/caspase-9 apoptosome assembly, or alternatively through the Akt/caspase-9 signaling pathway (Takatani et al., 2004a,b). Although the underlying mechanisms require further study, it is clear that taurine has an effect on inhibiting intrinsic signaling pathways of apoptosis.

In conclusion, our experiments with RGC-5 cells provide evidence that taurine can prevent hypoxia-induced mitochondrial dysfunction and inhibit apoptosis. Although RGC-5 cells represent an *in vitro* model, mechanistic insights derived from these cells have the potential to partially address how RGCs react to hypoxia during the course of glaucoma, diabetic retinopathy, or other related retinal diseases. Further *in vivo* studies are needed to confirm the therapeutic efficacy of taurine in protecting RGCs function, and given that taurine is an authenticated nutritional additive, taurine represents a highly attractive candidate for application in the clinic for ophthalmic, long-term oral treatment of hypoxic conditions resulting from retinal diseases.

4. Experimental procedures

4.1. Cell culture

The transformed retinal ganglion cell line, RGC-5 (PTA6600, ATCC), developed from post-natal Sprague–Dawley rats, was grown in DMEM (Gibco, China) containing 5 mM glucose, 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (TBD, China). RGC-5 cells were cultured in a CO_2 incubator (Shel Lab 2300, USA) with 5% CO_2 at 37 °C. Doubling time of these cells was ~20 hours (h) and cells were passaged every 2–3 days at a ratio of 1:6. RGC-5 cells of passages 10–20 were used in our experiments, and cells were used at 80% confluence for all experiments.

4.2. Hypoxic injury to RGC-5 cells

Hypoxia was induced according to Hong et al. (2007). Briefly, cultures were transferred into an automatically-controlled Multi Gas Incubator (YCP-50S,Hua-xi,Changsha,China) in which oxygen level (5% O_2,5% CO_2, and 90% N_2) and temperature (37 °C) were maintained. After cells were washed twice with deoxygenated serum-free DMEM, cells remained in the hypoxic incubator for various incubation periods. Various concentrations of taurine were added into the culture medium 4 h prior to the induction of hypoxia and again at the start of hypoxic injury. Control cells were not exposed to hypoxia.

4.3. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used as an indicator of cell viability based on its mitochondrial-dependent reduction to formazone (Hansen et al., 1989). Cells were seeded at a density of 5×10^3 cells/well in 96-well plates and cultured with various concentrations of taurine (0.001, 0.01, 0.1, 1, and 10 mM) or PBS. After 4 h, hypoxia was induced and maintained for 48 h. MTT (5 mg/mL) was added after 44 h, and at 48 h DMSO was added to dissolve the formazan generated. Absorbance at 490 nm was detected using a monochromator microplate reader (Safire II, Tecan, Switzerland). Cell viability was expressed as the percentage of viable cells in the hypoxic samples relative to cell viability detected in the control cells.

4.4. DAPI staining

To visualize fragmented nuclei, RGC-5 cells were stained with DAPI (Beyotime, China). After 24 h of hypoxia, cells were fixed

with methanol-acetone (7:3) for 10 min at room temperature then incubated with DAPI for 5 min at room temperature. RGC-5 cells were viewed using an FV1000 Olympus microscope (Olympus, Tokyo, Japan).

4.5. Annexin V-FITC/PI assay

Apoptosis was detected using an Annexin V-FITC/PI detection kit (Bipec Biopharma Corp., USA) according to the manufacturer's directions. Briefly, cells were harvested and resuspended in binding buffer (10^6 cells/mL). Aliquots of 10^5 cells were mixed with 5 μL of Annexin V-FITC and 10 μL of PI. After 15 min at room temperature in the dark, fluorescence was detected by flow cytometry (FACS-400, USA). The per-centage of apoptosis was determined from the number of Annexin V(+)/PI(–) cells relative to the number of Annexin V(+)/PI(+) cells.

4.6. Determination of free intracellular [Ca^{2+}]

The free intracellular [Ca^{2+}] in RGC-5 cells was measured using the fluorescence Ca^{2+} indicator, fura-2/AM, as previously reported, with some modifications (Das et al., 2006; Yamada et al., 2006). Briefly, RGC-5 cells were seeded in 24-well plates (3×10^5/per well). After 4 h, cells with and without taurine (0.1 mM) were exposed to hypoxia for 3, 6, or 12 h. Fura-2 AM (5 μg/mL) was then added to the cells. After 45 min cells were rinsed twice with 2% BSA/PBS and incubated another 10 min at 37 °C before fluorescence intensities at 340 nm (F^{340}) or 380 nm (F^{380}) were detected simultaneously by a monochromator microplate reader (Safire II, Tecan, Switzerland). The [Ca^{2+}]$_i$ was calculated as follows (Das et al., 2006): [Ca^{2+}]$_i = K_d \beta$ $(R - R_{min})/(R_{max} - R)$, where β is the ratio of F^{380}_{max} (the fluorescence emission at 380 nm at a zero level of free Ca^{2+}) to F^{380}_{min} (the fluorescence intensity at a saturating level of free Ca^{2+}) and R represents the fluorescence ratio of F^{340} and F^{380}. The maximal fluorescence ratio (R_{max}) was determined after adding Triton X-100 (0.1%) and the minimal fluorescence ratio (R_{min}) was determined by adding EGTA (5 mM; Sigma). The value of K_d, a cell-specific constant, was determined to be 0.387 μM for the RGC-5 cells. After determining [Ca^{2+}]$_i$, cells were lysed in 20 μL of 0.1 M NaOH and total cellular protein concentrations were measured using the Bradford Protein Assay method. [Ca^{2+}]$_i$ was normalized to total cellular protein and presented as nmol/mg protein.

4.7. Detection of opened mPTPs

Opened mPTPs of RGC-5 cells were detected using calcein–cobalt with a mPTP assay kit (Genmed Scientifcs Inc., USA) according to the manufacturer's directions. Briefly, RGC-5 cells were seeded in 24-well plates (3×10^5 /per well). After 4 h, cells treated with or without taurine (0.1 mM) were exposed to hypoxia for 6, 12, or 24 h. Cells were then washed with Reagent A, incubated with Reagents B and C (1:50) at 37 °C for 20 min, then washed twice with Reagent A again. Fluorescence intensity was measured using a monochromator microplate reader (Safire II, Tecan, Switzerland; λex 488 nm, λem 505 nm). Cells were subsequently lysed in 20 μL of 0.1 M NaOH and protein concentrations were measured using the Bradford Protein assay. Results were presented as NRFU (U/mg protein).

4.8. Measurement of mitochondrial membrane potential

The fluorescent, lipophilic and cationic probe, JC-1 (Beyotime, China), was employed to measure the mitochondrial membrane potential (Δψm) of RGC-5 cells according to the manufacturer's directions. Briefly, after indicated treatments, cells were cultured in 24-well plates and incubated with JC-1 staining solution (5 μg/mL) for 20 min at 37 °C. Cells were then rinsed twice with JC-1 staining buffer and fluorescence intensity of both mitochondrial JC-1 monomers (λex 514 nm, λem 529 nm) and aggregates (λex 585 nm, λem 590 nm) were detected using a monochromator microplate reader (Safire II, Tecan, Switzerland). The Δψm of RGC-5 cells in each treatment group were calculated as the fluorescence ratio of red (i.e. aggregates) to green (i.e. monomers).

4.9. Detection of cellular ATP levels

Cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime, China) according to the manufacturer's instructions. Briefly, after 24 h of hypoxia, RGC-5 cells pretreated with or without taurine (0.1 mM) were schizolysised and centrifuged at 12,000 ×g for 5 min. In 24-well plates, 100 μL of each supernatant was mixed with 100 μL ATP detection working dilution. Luminance (RLU) was measured by a monochromator microplate reader (Safire II, Tecan, Switzerland). Standard curves were also generated and the protein concentration of each treatment group was determined using the Bradford Protein assay. Total ATP levels were expressed as nmol/mg protein.

4.10. Preparation of cytosolic fractions and total cell lysates

Preparation of cytosolic fractions was achieved using a commercially available cytosol/mitochondria fractionation kit according to the manufacturer's protocol (Beyotime, China). Cellular proteins were extracted with T-PER (Pierce Biotechnology, Inc., Rockford, IL) and centrifuged at 12,000 rpm for 20 min. Protein concentrations were determined using the Bradford protein assay with bovine serum albumin (BSA) as a standard. Protein samples were stored at –80 °C.

4.11. Western blot analysis

Protein samples (40 μg) were separated by 15% SDS-PAGE and transferred to PVDF membranes (Millipore, USA) blocked with 2% BSA/Tween 20/TBS at room temperature for 1 h. Immunoblots were incubated at 4 °C overnight with primary antibodies specific for cytochrome c (Boaoshen, China) and caspase-3 (CST, USA). After washing, membranes were incubated with horseradish peroxidase conjugated secondary antibodies at 37 °C for 1 h. Antibody binding was visualized using enhanced chemilumines-cence (ECL; Pierce, USA). Detection of β-actin (Boaoshen, China) was used as a loading control. Quantitative analysis of immuno-blotted bands was performed using Quality One software.

4.12. Statistical analysis

Statistical significance was determined using the ANOVA method followed by Tukey's post-hoc test as appropriate. Each

value was expressed as a mean±standard deviation (SD). Differences were considered significant when the calculated p value was <0.05.

Acknowledgments

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