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PROTECTIVE EFFECT OF MAGNESIUM ACETYLTAURATE AGAINST ENDOTHELIN-INDUCED RETINAL AND OPTIC NERVE INJURY

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Abstract—Vascular dysregulation has long been recognized as an important pathophysiological factor underlying the development of glaucomatous neuropathy. Endothelin-1 (ET1) has been shown to be a key player due to its potent vasoconstrictive properties that result in retinal ischemia and oxidative stress leading to retinal ganglion cell (RGC) apoptosis and optic nerve (ON) damage. In this study we investigated the protective effects of magnesium acetyltaurate (MgAT) against retinal cell apoptosis and ON damage. MgAT was administered intravitreally prior to, along with or after administration of ET1. Seven days post-injection, animals were euthanized and retinae were subjected to morphometric analysis, TUNEL and caspase-3 staining. ON sections were stained with toluidine blue and were graded for neurodegenerative effects. Oxidative stress was also estimated in isolated retinae. Pre-treatment with MgAT significantly lowered ET1-induced retinal cell apoptosis as measured by retinal morphometry and TUNEL staining. This group of animals also showed significantly lesser caspase-3 activation and significantly reduced retinal oxidative stress compared to the animals that received intravitreal injection of only ET1. Additionally, the axonal degeneration in ON was markedly reduced in MgAT pretreated animals. The animals that received MgAT co- or post-treatment with ET1 also showed improvement in all parameters; however, the effects were not as significant as observed in MgAT pretreated animals. The current study showed that the intravitreal pre-treatment with MgAT reduces caspase-3 activation and prevents retinal cell apoptosis and axon loss in ON induced by ET1. This protective effect of ET1 was associated with reduced retinal oxidative stress.

Key words: RGC apoptosis, endothelin-1, optic nerve, magnesium acetyltaurate.

INTRODUCTION

Optic neuropathies such as glaucoma are characterized by loss of retinal ganglion cells (RGC) and visual field damage. Although, elevated intraocular pressure (IOP) is the major risk factor for glaucoma, impaired microcirculation of optic nerve (ON) head is also considered a contributing or causal factor in glaucomatous optic neuropathy (Agarwal et al., 2009). The retinal hemodynamic changes in optic neuropathy have been associated with increased aqueous humor levels of endothelin-1 (ET1) (Sin et al., 2013), the most potent vasoconstrictor known. Some studies have also reported increased ET1 levels in the plasma of glaucoma patients (Emre et al., 2005; Cellini et al., 2012), while others did not find the same (Kunimatsu et al., 2006; Chen et al., 2013). Nevertheless, there is accumulating evidence that ET1 plays a significant role in the pathogenesis of glaucomatous optic neuropathy (Sugiyama et al., 1995; Tezel et al., 1997; Yorio et al., 2002; Prasanna et al., 2003).

Currently all antiglaucoma drugs act by lowering IOP. However, as the role of vasospastic mechanisms and oxidative stress in RGC apoptosis is becoming widely recognized, more targeted therapies are being investigated. In this study we investigated a substance, magnesium acetyltaurate (MgAT) synthesized from magnesium and taurine, for its protective role against ET1-induced retinal and ON damage in rats. Magnesium is a natural calcium antagonist. It increases blood flow and reduces vascular resistance in various vascular beds (Shechter et al., 2000; Touyz, 2003). It also has a direct vasorelaxant effect (Ishiguro et al., 1997). Furthermore, it improves the endothelial function (Ishiguro et al., 1997). Additionally both, magnesium and taurine reduce oxidative stress. The role of magnesium in preventing ophthalmic diseases has been reviewed extensively (Agarwal et al., 2014). In one of the studies, dietary taurine effectively prevented retinal photochemical damage in rats by reducing oxidative stress (Yu et al., 2007). Taurine may also regulate the

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Abbreviations: CAT, catalase; DAPI, 4,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; ET1, endothelin-1; GCL, ganglion cell layer; GSH, glutathione; IOP, intraocular pressure; IR, inner retina; MDA, malondialdehyde; MgAT, magnesium acetyltaurate; MPA, metaphosphoric acid; ON, optic nerve; PBS, phosphate-buffered saline; RGC, retinal ganglion cell; RIPA, radio-Immunoprecipitation assay lysis; SOD, superoxide dismutase; TBA, thiobarbituric acid; TUNEL, Terminal Transferase dUTP nick end labeling.

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calcium transport (Lombardini, 1991). In one of our previous studies we demonstrated that magnesium taurate reduces oxidative stress in the lenses of galactose-fed rats (Agarwal et al., 2013a,b). Hence, we hypothesized that the compound consisting of both the magnesium and taurine protects against ET1-induced retinal and ON damage.

In this study, we used ET1-induced rat model of RGC apoptosis. Intravitreal injection of ET1 cause impaired retinal and ON blood flow resulting in retinal and ON head ischemia, which culminates into RGC damage and pathological changes at the ON head (Sugiyama et al., 1996, 2009). Ischemia also leads to increased retinal oxidative stress, which promotes RGC apoptosis (Chen et al., 2015). Accordingly, intravitreal injection of ET1 in animals such as rabbits and monkeys results in optic neuropathy similar to ON head damage and axon loss seen in glaucoma (Orgül et al., 1996; Oku et al., 1999; Sugiyama et al., 2009). To investigate our hypothesis, we performed morphological and biochemical studies to understand the effects of intravitreal administration of MgAT on RGC apoptosis, retinal oxidative stress and ON damage induced by intravitreal ET1 in rats. In this study we used MgAT as it has more neutral pH in aqueous solution compared to magnesium taurate, which is more alkaline.

EXPERIMENTAL PROCEDURES

All experiments in this study were conducted in accordance with the Associations for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. *Sprague Dawley* rats of either sex aged 8–12 weeks and weighing 200–250 g were used. Animals were housed under standard laboratory conditions and were provided with normal pellet diet and tap water *ad libitum*. Those found normal on general and ophthalmic examination were included in the study after 1 week of acclimatization.

MgAT was synthesized using a mixture of 25.0 g (0.200 mol) of taurine and 4.1 g (0.102 mol) of magnesium oxide in 200 ml of water. The mixture was stirred at 80–90 °C for 10 min with slow addition of 25.0 ml (0.265 mol) of acetic anhydride. The mixture was further stirred at the same temperature for 30 min and then evaporated in vacuum. The residue was rubbed with 100 ml of hot ethyl alcohol and cooled in refrigerator for 24 h. The precipitate was filtered, washed successively with 50 ml of cold ethyl alcohol, 25 ml of acetone and 25 ml of diethyl ether followed by drying first at room temperature in air and then at 40–50 °C in vacuum with residual pressure of 2–3 mmHg for 1 h. 32.2 g (90.4%) anhydrous MgAT was obtained as a white powder. The salt contained Mg content of 6.68% with a molecular weight was 356,656 and the empirical formula of $C_8H_{16}MgN_2O_8S_2$.

Study design

The animals were randomly divided into five groups of 30 animals each ($n = 30$). Animals in group 1 were injected with phosphate-buffered saline (PBS) intravitreally and served as control. Animals in group 2 similarly received 2.5 nM of ET1. Group 3 and 5 were intravitreally injected with MgAT (320 nM) 24 h before and after ET1 injection (MgAT pre-treatment and MgAT post-treatment groups), respectively. In group 4, MgAT was coadministered with ET1 intravitreally (MgAT co-treatment group). The choice of the dose of ET1 and MgAT was based on our preliminary dose–response study (Data not shown). For intravitreal injection, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (65 mg/kg) and a drop of 0.5% alcaine was administered for local anesthesia. A 30-gauge needle was used to puncture the superior nasal sclera 1 mm behind the limbus. The injections were made using a Hamilton syringe through the puncture keeping at an angle of 45° from ocular surface. In all animals a total volume of 5 μl was injected slowly. As per ethical guidelines, all intravitreal injections in this study were done unilaterally. Systemic blood pressure and IOP were measured before intravitreal injection and subsequently, 24 h post-injection. For measurement of the blood pressure, non-invasive tail cuff method was used, whereas for IOP, a non-contact tonometer (Tonolab) was used as described previously (Ohashi et al., 2008; Hoorn et al., 2011).

Seven days post-injection, animals were euthanized by an intraperitoneal injection of pentobarbital (100 mg/kg) and both eyes were enucleated. For proper orientation, a suture was placed at 12 o'clock position. From the eyes that received intravitreal injection, six eyes from 6 animals were fixed in 10% formalin; were paraffin embedded and sectioned at 3-μm thickness using microtome. The sections were used for hematoxylin and eosin (H&E), TUNEL and caspase-3 staining. ON segments were dissected from 6 eyes, 1 mm from the back of the globe, fixed in 10% formalin for 24 h and embedded in paraffin. Tissue was sectioned at 0.70-μm thickness and stained with 1% toluidine blue (Jia et al., 2005) for morphological analysis. Retinae from rest of the eyes were processed for estimation of superoxide dismutase (SOD) activity ($n = 6$), catalase (CAT) activity ($n = 6$), reduced glutathione (GSH) contents ($n = 6$) and malondialdehyde (MDA) contents ($n = 6$). The eyes that did not receive intravitreal injection were also processed similar to those that received intravitreal injection.

Assessment of retinal morphology

Assessment of the effects of MgAT on ET1-induced changes in retinal morphology was done by examining H&E stained retinal sections under light microscope as described previously (Takahata et al., 2003; Razali et al., 2015, 2016).The sections were taken at 1 mm from the temporal edge of the optic disc. Three randomly selected fields of view from each section were calibrated at 20× magnifications and saved in jpg image format. Images were then evaluated using image analysis software (ImageJ 1.31, National Institutes of Health, Bethesda, MD, USA) by two masked investigators independently. Morphologically distinguishable glial cells, vascular endothelial cells or other cells with the diameter of the nucleus less than 7 mm were excluded from the cell