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Tropisetron as a neuroprotective agent against glutamate-induced excitotoxicity and mechanisms of action

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ABSTRACT

The objective of this study was to determine the neuroprotective role of tropisetron on retinal ganglion cells (RGCs) as well as to explore the possible mechanisms associated with alpha7 nAChR-induced neuroprotection. Adult pig RGCs were isolated from all other retinal tissue using a two-step panning technique. Once isolated, RGCs were cultured for 3 days under control untreated conditions, in the presence of 500 μM glutamate to induce excitotoxicity, and when tropisetron was applied before glutamate to induce neuroprotection. 500 μM glutamate decreased RGC survival by an average of 62% compared to control conditions. However, RGCs pretreated with 100 nM tropisetron before glutamate increased cell survival to an average of 105% compared to controls. Inhibition studies using the alpha7 nAChR antagonist, MLA (10 nM), support the hypothesis that tropisetron is an effective neuroprotective agent against glutamate-induced excitotoxicity; mediated by α7 nAChR activation. ELISA studies were performed to determine if signaling cascades normally associated with excitotoxicity and neuroprotection were up- or down-regulated after tropisetron treatment. Tropisetron had no discernible effects on pAkt levels but significantly decreased p38 MAPK levels associated with excitotoxicity from an average of 15 ng/ml to 6 ng/ml. Another mechanism shown to be associated with neuroprotection involves internalization of NMDA receptors. Double-labeled immunocytochemistry and electrophysiology studies provided further evidence that tropisetron caused internalization of NMDA receptor subunits. The findings of this study suggest that tropisetron could be an effective therapeutic agent for the treatment of degenerative disorders of the central nervous system that involves excitotoxicity.

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1. Introduction

Excitotoxicity has been implicated in the pathology of a number of neurodegenerative disorders of the brain, such as, Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis (Romano et al., 1998; Mattson, 2003). Neurodegenerative diseases of the eye, including glaucoma, retinal ischemia, and diabetic retinopathy, also have pathologies linked to excitotoxicity (Lipton, 2001; Kim et al., 2007; Schmidt et al., 2008). While not all neurodegenerative diseases occur with elevated concentrations of glutamate, alteration of glutamate receptor activity is a common key in the process of excitotoxic cell death, giving rise to the term glutamate-induced excitotoxicity (Michaelis, 1998; Mattson, 2003). Many recent studies have indicated that excitotoxicity is initiated in response to excessive Ca^{2+} influx (Sattler and Tymanski, 2000; Arundine and Tymianski, 2003; Brandt et al., 2011), which initiates signaling cascades to activate caspases that ultimately destroy the cells (Li et al., 1997; Tenneti et al., 1998; Tenneti and Lipton, 2000). Recent studies have progressed in the direction of determining the specific intracellular signaling pathways that are involved in glutamate-induced excitotoxicity. Several studies have demonstrated that apoptosis associated with excitotoxicity is regulated through the p38 MAP kinase pathway (Dineley et al., 2001; Pearson et al., 2001; Manabe and Lipton, 2003; Zarubin and Han, 2005; Wang et al., 2007).

Abbreviations: ACh, acetylcholine; AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; α7 nAChR, alpha7 nicotinic acetylcholine receptor; ANOVA, analysis-of-variance; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GluRs, glutamate receptors; 5-HT_3, serotonin; NGF, nerve growth factor; CNS, central nervous system; IgG, immunoglobulin G; IgM, immunoglobulin M; MAPK, mitogen activated protein kinase; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; RGC, retinal ganglion cell; SDS, sodium dodecyl sulfate; TEA, tetraethylammonium chloride; Thy, glycoprotein originally identified in thymus gland; tropisetron, (1*R*,5*S*)-8-methyl-8-azabicyclo[3.2.1] octan-3-yl 1methyl-indole-3-carboxylate.

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Asomugha and Linn (2010) found chronically stimulated glutamate receptors activates the MAPKKK > MAPKK > p38 MAP kinase intracellular signaling pathway and leads to apoptosis. Using ELISA techniques, they reported that when adult pig RGCs are cultured with 500 μM glutamate for 12 h, there was an average resulting cell death of 40% from untreated control conditions and an increase in phosphorylation of p38 MAP kinase by an average of 72% over control. When the p38 MAP kinase inhibitor, SB 203580, was applied before excessive glutamate, RGC death and phosphorylation of p38 MAP kinase were significantly decreased. This strengthens the notion that the p38 MAP kinase pathway activation is key in glutamate-induced excitotoxicity in cultured pig RGCs.

With excitotoxicity playing a key role in some of the most widely suffered neurodegenerative disorders, the clinical implications involved with protecting cells from excitotoxicity are immense. A recent line of research has shown that, in various neural tissues, when cells are pre-treated with substances to stimulate nicotinic acetylcholine receptors (nAChR), the toxic effects of excessive glutamate can be prevented (Akaike et al., 1994; Kaneko et al., 1997; Dajas-Bailador et al., 2000; Wehrwein et al., 2004; Thompson et al., 2006). However, the neuroprotective mechanisms by which excitotoxicity is prevented by nAChR activation are not fully understood. Several studies have suggested that Ca influx through activated nAChRs affects phosphorylation level of the p38 MAPK and Akt intracellular signaling pathways resulting in neuroprotection (Asomugha and Linn, 2010; Brandt et al., 2011).

Another mechanism that has been proposed for neuroprotection from glutamate-induced excitotoxicity is reduction of Ca^{2+} influx through internalization of calcium channels or through internalization of glutamate receptors. Studies by Cristofanilli and Akopian (2006) found that treatment with actin destabilizing agents caused internalization of Ca_v 1.3 L-type calcium channels and protected dissociated RGCs from excitotoxicity induced by activation of iGluRs, suggesting a possible mechanism for the regulation of the Ca^{2+} current and neuroprotection (Cristofanilli et al., 2007). These same studies have linked nAChR activation to internalization of receptor proteins. Shen et al. (2010) found that activation of nAChRs in fetal rat cortical neurons by treatment with nicotine and donepezil, an acetylcholinesterase inhibitor, caused internalization of glutamate receptors, resulting in attenuation of the glutamate induced Ca^{2+} influx, reduction in caspase-3 activation, and protection of cells from glutamate-induced excitotoxicity. This tie between nAChR mediated neuroprotection and internalization of glutamate receptors led us to explore glutamate receptor internalization as a possible mechanism of neuroprotection.

In the current study, we investigate the neuroprotective properties of tropisetron, ((1*R*,5*S*)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl 1methyl-indole-3-carboxylate), against glutamate-induced excitotoxicity in isolated and cultured adult pig retinal ganglion cells. Tropisetron has long been used as an anti-emetic to help alleviate symptoms associated with chemotherapy and post-operative condition. However, it has been discovered that tropisetron is a unique substance in that it also has a partial α7 nAChR agonist action as well (Papke et al., 2005; Macor et al., 2001). It is this action that has been analyzed in this study. In previous studies, activation of α7 nAChRs has been shown to protect cells from glutamate-induced excitotoxicity in a number of model systems (Kawamata and Shimohama, 2011; Yu et al., 2011; Liu et al., 2012). Previous studies from this lab using pharmacological and immunocytochemical techniques have identified a number of specific nAChR subunits that are involved in neuroprotection against glutamate-induced excitotoxicity in isolated cultured adult pig RGCs (Thompson et al., 2006). In this previous study, it was demonstrated that nAChRs composed of α4 and β2 subunits are only found on small pig RGCs and selective agonists for α4 nAChRs produced significant neuroprotection against excitotoxicity. α7 nAChRs were found only on large pig RGCs and ACh or nicotine provided significant neuroprotection against a glutamate insult (Wehrwein et al., 2004; Thompson et al., 2006). As tropisetron also exhibits an agonist action at α7 nAChRs, it may prove to be useful as a neuroprotective agent on RGCs.

As a result, pharmacology studies were designed using isolated adult pig RGCs in culture to investigate the efficacy of tropisetron as a neuroprotective agent. Further studies using competitive agonists and antagonists were performed to determine which receptors elicit tropisetron's neuroprotective effect. Two different approaches were used to further investigate the mechanisms involved in tropisetron's ability to protect adult pig RGCs from glutamate-induced excitotoxicity. ELISA studies were used to look at the role of p38 MAPK and Akt signaling proteins in tropisetron treatments against glutamate. Internalization of NMDA GluRs in response to tropisetron treatment was also investigated using fluorescent immunocytochemistry and electrophysiology techniques. It was hypothesized that if tropisetron protects cells from excitotoxicity, then it may occur through internalization of NMDA receptors. Understanding the mechanisms involved in ACh-induced neuroprotection in the pig retina could ultimately lead to therapeutic treatment for any central nervous system (CNS) disease that involves excitotoxicity.

2. Experimental procedures

2.1. Retinal ganglion cell isolation

In order to obtain pure retinal ganglion cells (RGCs) for *in vitro* studies, adult pig eyes were removed from animals at a local slaughterhouse (Pease Slaughterhouse, Scotts, MI) and transported on ice to the laboratory for removal of retinas and isolation of RGCs. To isolate the RGCs, we used a modified two-step panning procedure described in Wehrwein et al. (2004). The retinas were removed from eyes according to the methods described by Wehrwein et al. (2004). Isolated retinas were then placed in a modified CO_2-independent medium (Gibco, Carlsbad, CA) kept at 37 °C, containing 4 mM glutamine, 10% fetal bovine serum (FBS), 5% antibiotic/antimycotic, and 4 mM HEPES and enzymatically dissociated using papain (27 u/mg) for 20 min at 37 °C. After 20 min in papain, tissue was rinsed with fresh CO_2-independent medium to stop the papain action and 1 mg/ml DNase. Complete dissociation of the retina was obtained using an unpolished Pasteur pipette to gently triturate the tissue.

RGCs were isolated from all other retinal tissue using a two-step panning technique according to methods previously described (Wehrwein et al., 2004; Thompson et al., 2006; Brandt et al., 2011). The first step in this process plated dissociated retinal tissue onto dishes, coated with goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA; 0.5 mg in 10 ml of 20 mM Tris buffer) to eliminate nonspecific binding. After 1 h of incubation on the IgG plates, cells from each dish were transferred onto Petri dishes coated with mouse anti-rat Thy 1.1 antibody (BD Biosciences, San Diego, CA; 12.5 μg in 10 ml PBS containing no magnesium chloride and no calcium chloride) bound to goat anti-mouse IgM (Jackson ImmunoResearch; 0.36 mg in 10 ml of 20 mM Tris buffer) for 1 h at 37 °C. This represented the second panning step in the process. After 1 h, the culture medium was replaced with fresh CO_2-independent medium including supplemental factors consisting of NGF, transferrin and insulin (Wehrwein et al., 2004). Each 4 ml of culture medium contained 50 μl of 15 μg/ml nerve growth factor (NGF), 48 μl of 500 μg/ml transferrin, and 12 μl of 10 mg/ml insulin.

2.2. Pharmacology studies

In pharmacology studies, isolated RGCs were evenly distributed into dishes at a density of 1×10^5 cells/ml. Each dish contained isolated RGCs that were cultured under six different conditions. The first dishes in each experiment always contained isolated RGCs that were untreated. The second condition consisted of dishes containing isolated RGCs treated with 500 μM glutamate to induce excitotoxicity. The remaining four conditions consisted of dishes containing cultured RGCs that were treated with appropriate concentrations of agonists and/or antagonists. In dose–response studies, conditions 3–6 were treated with various concentrations of tropisetron for 1 h prior to a 500 μM glutamate insult. Glutamate was obtained from Sigma (St. Louis, MO). Tropisetron was obtained from RBI (Natic, MA). In inhibition studies, the α7 nAChR antagonist, methyllycaconitine (MLA), obtained from Tocris (Bristol, UK) was applied to conditions 3–6 for 1 h before tropisetron application to allow the antagonist time to bind to receptors. Since tropisetron has both α7 nAChR agonist and 5-HT_3 antagonist properties, control experiments using the 5-HT_3

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