Imipramine protects retinal ganglion cells from oxidative stress through the tyrosine kinase receptor B signaling pathway

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Abstract
Retinal ganglion cell (RGC) degeneration is irreversible in glaucoma and tyrosine kinase receptor B (TrkB)-associated signaling pathways have been implicated in the process. In this study, we attempted to examine whether imipramine, a tricyclic antidepressant, may protect hydrogen peroxide (H$_2$O$_2$)-induced RGC degeneration through the activation of the TrkB pathway in RGC-5 cell lines. RGC-5 cell lines were pre-treated with imipramine 30 minutes before exposure to H$_2$O$_2$. Western blot assay showed that in H$_2$O$_2$-damaged RGC-5 cells, imipramine activated TrkB pathways through extracellular signal-regulated protein kinase/TrkB phosphorylation. TUNEL staining assay also demonstrated that imipramine ameliorated H$_2$O$_2$-induced apoptosis in RGC-5 cells. Finally, TrkB-IgG intervention was able to reverse the protective effect of imipramine on H$_2$O$_2$-induced RGC-5 apoptosis. Imipramine therefore protects RGCs from oxidative stress-induced apoptosis through the TrkB signaling pathway.

Key Words: nerve regeneration; retinal ganglion cell; imipramine; oxidative stress; apoptosis; tyrosine kinase receptor B; neural regeneration

Introduction
Optic nerve degeneration and retinal ganglion cell (RGC) loss are irreversible in glaucoma, and permanent vision loss can be developed in severe cases (Shahid and Salmon, 2012; Nouri-Mahdavi and Caprioli, 2015). Extensive efforts have been devoted to understand the underlying mechanism of RGC degeneration and to seek feasible treatment options to protect against RGC loss. In recent decades, a number of molecular pathways have been shown to be involved in the process of RGC degeneration and to exert some protective effects, such as insulin-like growth factor-1 (Kermer et al., 2000; Yang et al., 2013) and hepatocyte growth factor (Miura et al., 2003; Tönges et al., 2011). Among the identified molecular pathways, neurotrophin-regulated signaling pathways, including brain-derived neurotrophic factor (BDNF) and its receptor (tyrosine kinase receptor B, TrkB), are crucial for the survival, differentiation, and regeneration of many kinds of sensory neurons, including RGCs (Hyman et al., 1991; Jones et al., 1994; Chen and Weber, 2001; Tong et al., 2013).

Tricyclic antidepressants, including amitriptyline and imipramine, appear to stimulate BDNF/TrkB pathways in many neuronal systems (Siuciak et al., 1997; Xu et al., 2002; Balu et al., 2008; Réus et al., 2011). Imipramine, combined with ketamine, increases BDNF production and protein kinase C phosphorylation in the hippocampus of rats to modify locomotor activity (Réus et al., 2011). Imipramine protects cortical neural stem cells from inflammation-induced apoptosis by activating BDNF signaling pathways (Peng et al., 2008). Furthermore, the long-term use of imipramine can induce excessive production of BDNF in rat olfactory bulbs to extend brain plasticity after injury (Van Hoomissen et al., 2003); however, it is still unclear whether anti-depressants exert BDNF/TrkB neuroprotection in RGCs.
Figure 1 Effects of Imip on TrkB signaling in RGC-5 cells.
RGC-5 cells were maintained in vitro and Imip was added at concentrations of 0.5 or 5 µM. In the control group, no Imip was added to the culture. At 12 hours after culture, cell lysates were collected and western blot analysis was conducted with primary antibodies against brn3a, ERK1-2, pERK1-2, TrkB, and pTrkB. (B, C) The degrees of TrkB and ERK1-2 phosphorylation were measured by comparing the relative blotting intensities of pTrkB against TrkB (B) and pERK1-2 against ERK1-2 (C). The experiments were performed in triplicate. Data are expressed as the mean ± SEM. Comparison was conducted by two-tailed Student’s t-test. *P < 0.05, vs. control group. Imip: imipramine; RGC-5: retinal ganglion cells; (p)TrkB: (phosphorylated) tyrosine kinase receptor B; (p)ERK: (phosphorylated) extracellular signal-regulated protein kinase.

Figure 2 Effects of Imip against oxidative stress in RGC-5 cells.
RGC-5 cells were cultured in 6-well plates overnight. H$_2$O$_2$ (10 µM) was added to induce oxidative stress. In the control group, cells were not subjected to H$_2$O$_2$ treatment. In the H$_2$O$_2$ + Imip group, H$_2$O$_2$-treated RGC-5 cells, Imip (5 µM) was added 30 minutes prior to H$_2$O$_2$ treatment. At 48 hours after culture, the TUNEL assay was performed to identify apoptotic cells (blue staining) amongst the RGC-5 population (positive to Thy-1 staining; × 20). (B) Percentage of apoptotic RGC-5 cells. Data are expressed as the mean ± SEM. Comparison was conducted by a two-tailed Student’s t-test. The experiments were performed in triplicate.

Figure 3 Imip protects RGC-5 cells from oxidative stress by activating TrkB signaling.
RGC-5 cells were pre-treated with Imip (5 µM) 30 minutes prior to H$_2$O$_2$ (10 µM) to inhibit oxidative stress-induced apoptosis. At 1.5 hours after Imip treatment (1 hour after H$_2$O$_2$ treatment), TrkB-specific blocking antibody, TrkB-IgG (0.2 mg/mL), was added to the culture. A non-specific antibody, NC-IgG (2 µM), was added to the control culture. Two days later, TUNEL assay was performed to identify the apoptotic cells (blue staining) among the RGC-5 population (positive Thy-1 staining; × 20). (B) The percentages of apoptotic RGC-5 cells. Data are expressed as the mean ± SEM. A comparison was conducted using a two-tailed Student’s t-test. The experiments were performed in triplicate. *P < 0.05, vs. H$_2$O$_2$ + Imip group with NC-IgG. H$_2$O$_2$: Hydrogen peroxide; Imip: imipramine; RGC-5: retinal ganglion cells; TrkB: tyrosine kinase receptor B.
In this study we hypothesized that imipramine activates TrkB signaling pathways through the phosphorylation of TrkB/extracellular signal-regulated protein kinase (ERK) proteins in RGC-5. We also hypothesized that imipramine prevents oxidative stress-induced apoptosis in RGC-5 cells through activation of the TrkB signaling pathway.

Materials and Methods

RGC-5 in vitro culture

The RGC-5 cell line was developed by Dr. Agarwal at the University of North Texas in the USA (Agarwal, 2013). We obtained RGC-5 cells from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in 10-cm culture dishes with 5% CO₂ at 37°C. RGC-5 cells were grown to confluency, dissociated by 0.5% trypsin (Invitrogen), and subsequently passed every 2 or 3 days.

Oxidative stress, imipramine, and antibody intervention

in vitro

Oxidative stress was induced in RGC-5 cells in vitro by hydrogen peroxide (H₂O₂) treatment as previously described (Gupta et al., 2013). Briefly, RGC-5 cells were inoculated in 6-well culture plates at a density of 2 × 10⁵ cells/well. The majority of cells attached after 6 hours, and after 1 day the cells were treated with H₂O₂ (10 µM) for 48 hours to induce oxidative stress and apoptosis in RGC-5 cells.

Imipramine (Sigma-Aldrich) was initially dissolved in dimethyl sulfoxide to make a stock solution of 5 mM. The stock solution was then diluted in DMEM to make working concentrations of 5 µM or 0.5 µM. To treat RGC-5 cells, imipramine was added 30 minutes prior to H₂O₂ treatment.

TrkB-specific functional antibody (TrkB-IgG) was synthesized by Ribo-Bio (Guangzhou, Guangdong Province, China). An equivalent non-specific control antibody, NC-IgG (RiboBio) was used as a parallel control. IgG (0.2 mg/mL) was added 1.5 hours after imipramine treatment or 1 hour after H₂O₂ treatment.

Western blot assay

At the end of the designated culture, RGC-5 cells were trypsinized and centrifuged in ice-cold PBS. Cell lysates were then generated with a lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and protease inhibitor cocktail (Invitrogen). The collected proteins were then separated in a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. The primary antibodies applied were rabbit anti-brn3a polyclonal antibody (1:1,000; Sigma-Aldrich), rabbit anti-ERK1-2 polyclonal antibody (1:1,000; Sigma-Aldrich), rabbit anti-phospho-Erk1-2 (pERK1-2, 1:500; Sigma-Aldrich), rabbit anti-TrkB polyclonal antibody (1:1,000; Sigma-Aldrich), and rabbit anti-phosphorylated TrkB polyclonal antibody (1:500; Sigma-Aldrich). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (1:50,000; Bioread, Hercules, CA, USA). The optical density of blots were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA), and quantified by ImageJ software (NIH, Bethesda, MD, USA).

TUNEL assay

Apoptosis of RGC-5 cells under oxidative stress was quantified in situ using the TUNEL assay. Briefly, at the end of culture, RGC-5 cells were fixed with 10% paraformaldehyde (PFA; Invitrogen) in PBS (Invitrogen) for 10 minutes, and permeabilized with 3% Triton X-100 (Sigma-Aldrich) for another 10 minutes. An in situ Apoptosis Detection Kit (Chemicon, Billerica, MA, USA) was then applied as per the manufacturer’s instructions. In addition, a RGC-5-specific antibody (Thy-1, 1:100; Cell Signaling, Beverly, MA, USA) was applied during TUNEL staining to identify RGC-5 neurons. Visualization was carried out using an optical BX51 fluorescence microscope (Olympus, Tokyo, Japan). Apoptotic RGC-5 cells were counted by measuring the percentage of TUNEL-positive RGC-5 cells, which were identified by goat anti-Thy-1 polyclonal antibody (1:200; Sigma-Aldrich) immunostaining.

Statistical analysis

All data in the present study are presented as the mean ± SEM and were processed using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Data comparison was conducted using a two-tailed Student’s t-test. The experiments were performed in triplicate. P-values < 0.05 were considered statistically significant.

Results

Imipramine activated TrkB signaling pathways in RGC-5 cells

To determine whether imipramine activates TrkB signaling pathways in RGC-5 cells, RGC-5 cells were cultured in vitro and treated with 0.5 or 5 µM imipramine. After 12 hours, 5 µM imipramine significantly phosphorylated TrkB and ERK1-2 (P < 0.05), whereas 0.5 µM imipramine had little effect on TrkB and ERK1-2 phosphorylation (Figure 1).

Imipramine protected RGC-5 cells from oxidative stress-induced apoptosis

To determine whether imipramine inhibits oxidative stress-induced apoptosis in RGC-5 cells, a well-known in vitro retinal injury model (oxidative stress model) was used. RGC-5 cells were cultured in 6-well plates at a density of 2 × 10⁵ cells/well for 1 day. On the second day of culture, RGC-5 cells were exposed to 10 µM H₂O₂ to induce oxidative stress. After 48 hours of H₂O₂ treatment, a considerable number of TUNEL-positive RGC-5 cells were counted by measuring the percentage of TUNEL-positive RGC-5 cells, which were identified by goat anti-Thy-1 polyclonal antibody (1:200; Sigma-Aldrich) immunostaining.
Imipramine protected RGC-5 cells against oxidative stress through the TrkB signaling pathway

To determine whether imipramine inhibits apoptosis in RGC-5 cells through TrkB signaling activation, TrkB-IgG was used to block the activation of the TrkB signaling pathway and added 1.5 hours after imipramine treatment (1 hour after H2O2 treatment). At 47 hours after TrkB-IgG intervention, a larger number of TUNEL-positive RGC-5 cells were observed compared with cells treated with non-specific antibody NC-IgG (P < 0.05; Figure 3).

Discussion

Our study demonstrated imipramine-activated TrkB signaling pathways in RGC-5 cells, and illustrated that imipramine activated TrkB signaling pathways through the phosphorylation of TrkB and ERK1/2. These results are in line with previous studies showing that imipramine stimulates BDNF production after olfactory bulbectomy (Van Hoomissen et al., 2003), activates the TrkB signaling pathway to exert antidepressant-induced behavioral effects (Saarelainen et al., 2003), or regulates neural plasticity in the brain (Rantamaki et al., 2007). Thus, in RGCs, imipramine is likely to act as a TrkB agonist, a novel finding that has not been reported.

The functional assay using the TrkB blocking antibody, TrkB-IgG, demonstrated that the protective effect of imipramine on RGC-5 cells against oxidative stress-induced apoptosis was realized through the activation of the TrkB signaling pathway, thus further confirming our hypothesis that imipramine acts as a TrkB agonist in RGCs. Future studies to inhibit downstream TrkB targets or block BDNF production are necessary to completely understand the underlying molecular mechanisms of imipramine acting on TrkB pathways to inhibit retinal apoptosis or degeneration (e.g., the involvement of TrkB/ERK phosphorylation or BDNF production). Taken together, our study identifies, for the first time, that imipramine reduces oxidative stress-induced apoptosis of RGCs in a TrkB-dependent manner. The methods of targeting imipramine or other anti-depressant small molecules will undoubtedly help our understanding of the mechanisms underlying retinal injury, as well as proposing novel therapeutic interventions to prevent retinal degeneration.

Author contributions: JH wrote the paper and conducted experiments; GHL, JG and SJY conducted experiments and statistical analysis; MLH designed the study. All authors approved the final version of this paper.

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