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Original Article

Trigonelline promotes auditory function through nerve growth factor signaling on diabetic animal models

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ABSTRACT

Background: Protection of cochlear function and reconstruction of neuronal networks in damaged auditory sensory structures is crucial for therapeutic treatment of diabetic hearing loss. Nerve growth factor (NGF) has been used as a novel therapeutic target to protect against the neurodegenerative effects of Diabetes Mellitus (DM).

Purpose: We aimed to evaluate the potential effect of trigonelline (TRG) on reducing auditory damage produced by DM using NGF as a potential marker.

Method: Docking simulations were carried out using Autodock Vina software and visualized using Discovery Studio. Morphological analysis of hair cells and neuromasts was performed on alloxan-induced diabetic zebrafish by fluorescence and scanning electron microscopy. Blockage of NGF receptor phosphorylation with K-252a was used to evaluate TRG and NGF action. Further assessment of NGF by ELISA on a primary culture of spiral ganglion cells was performed as a marker of neuronal function on the hearing system. Finally, auditory function was assessed in LepR($^{db/db}$) mice using auditory brainstem response (ABR) and transient evoked otoacoustic emission (TEOAE) during 8 weeks.

Results: Docking simulations showed that TRG binds to the active site of NGF through molecular interactions with Lysine88 (Lys88) and Tyrosine52 (Tyr52). TRG treatment significantly reduced hair cell loss and neuromast damage in diabetic zebrafish ($P < .05$). Further evaluation revealed a significant increase in the number of neuromasts after NGF administration ($P < .001$). TRG and NGF action was suppressed during blockage of NGF receptor phosphorylation. Moreover, spiral ganglion cells revealed significant elevation on NGF values after TRG treatment ($P < .05$). *In vivo* evaluation of LepR($^{db/db}$) mice revealed a significant reduction in the auditory damage produced under diabetic progression, characterized by reduced ABR hearing threshold shifts and increased signal-to-noise ratio in TEOAE ($P < .05$).

Conclusions: This study suggests that the enhanced hearing function produced by TRG may be mediated by NGF, providing a potential therapeutic strategy for diabetic hearing loss.

Introduction

Diabetes mellitus (DM) involves several pathogenic pathways in different organs and can lead to complications such as nephropathy, cardiovascular disease, neuropathy, retinopathy, and hearing loss (American Diabetes Association, 2008; Hong et al., 2013). Strong evidence suggests the progressive damage in auditory structures due to DM shares cell death mechanisms and decreased essential growth factors with other noxious stimuli in the auditory system (Du et al., 2010; Op de Beeck et al., 2011). Several growth factors that mitigate against apoptosis have been studied *in vivo* to prevent degeneration of cochlear sensory structures and neural elements (Gillespie and Shepherd, 2005). Nerve growth factor (NGF) plays an essential role in maintenance of the auditory system (Salvinelli et al., 2002). NGF administration has been proposed as a novel therapeutic strategy in cochlear protection of the auditory nerve against degeneration in ototoxic conditions (Shah et al., 1995). However, NGF has a restricted ability to cross the blood–brain barrier and the blood–cochlear barrier, limiting its application

Abbreviations: ABR, auditory brainstem response; AX, alloxan monohydrate; db/db, homozygous for leptin receptor-deficient; dbh, heterozygous for leptin receptor-deficient; DM, diabetes mellitus; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; Lys88, Lysine88; LysoPI, lysophosphatidylserine; LysoPS, lysophosphatidylinositol; K-252a, NGF receptor phosphorylation blocker; MT2, TrkA agonist; NGF, nerve growth factor; PBS, phosphate buffered saline; SNR, signal-to-noise ratio; SS, sea salt; TEOAE, transient evoked otoacoustic emission; TRG, trigonelline; TrkA, tropomyosin receptor kinase A or high affinity nerve growth factor receptor; Tyr52, tyrosine52; ZF, zebrafish

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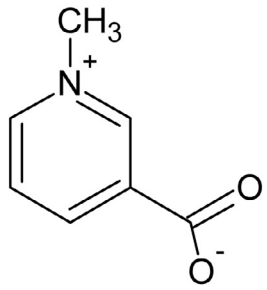

Fig. 1. Structural formula of trigonelline.

(Pardridge, 2002). These disadvantages have led to the discovery of small molecules that enhance the activity of NGF or mimic its effects.

Trigonelline (TRG, Fig. 1), a vitamin B3 precursor, is an alkaloid found in high-consumption foods and herbal drugs, such as oats, legumes, coffee, pumpkin, and fenugreek (de Zwart et al., 2003). In our previous study, TRG was found to be an active component of coffee responsible for improving auditory neuropathy (Hong et al., 2008). We recently studied the regenerative and enhanced functional effects produced by TRG on damaged β-cells produced under diabetic conditions, demonstrating increased glucose uptake and increased pancreatic islet size (Nam et al., 2015). TRG has strong clinical potential to treat DM-related complications by reducing oxidative stress, eliciting neuroprotective effects, and interacting with ototoxic signaling pathways (Tohda et al., 1999; Yoshinari et al., 2013; Zhou and Zhou, 2012). However, there are currently no strategies to effectively treat or prevent diabetic hearing damage.

The aim of this study was to evaluate whether the effects of TRG on the damage of the auditory function due to DM are mediated by NGF. TRG activity on NGF was evaluated *in silico, in vitro,* and *in vivo* using molecular docking, spiral ganglion cultures, neuromasts, and hair cells with zebrafish (ZF). The diabetic mouse strain C57BL/KsJ (db/db) was used to assess hearing function during DM progression through electrophysiological tests.

Materials and methods

Chemicals

TRG (purity > 98%), sea salt, alloxan monohydrate (AX) and tricaine were purchased from Sigma Aldrich Chem. Co. (St Louis, MO, USA). TRG was dissolved in SS for ZF experiments and in distilled water for murine experiments. AX was dissolved in SS and tricaine was dissolved in distillated water. Human NGF was purchased from R&D systems (Minneapolis, MN, USA), dissolved in phosphate buffered saline (PBS) and diluted on SS. K-252a was purchased from LC laboratories (Woburn, MA, USA), dissolved in dimethyl sulfoxide (DMSO; Daejung Chemicals Co., Ltd. (Siheung, Korea)) and diluted on SS.

In silico

AutoDock Vina (version 1.1.2) was used to predict the specific positioning of TRG (PubChem Compound Identifier 5570) in the active site of NGF. The crystal structure of NGF at 2.61 Å and of the binding motif of NGF receptor, tropomyosin receptor kinase A (TrkA), at 2.2 Å resolution was obtained from the Protein Data Bank (PDB) (PDB ID: 4XPJ and 1WWW, respectively). The first structure is a co-crystallization of NGF in a complex tested for 2 different ligands, lysophosphatidylinositol (LysoPS) and lysophosphatidylserine (LysoPI). The second structure is a co-crystallization of the TrkA ligand binding domain obtained by deletion of NGF from the structure and further reintroduction for visualization purposes. Water molecules and ligands were removed from the structures and hydrogen atoms were added. To compare and confirm the interaction, ligands that have been demonstrated to modify NGF and TrkA activity were used as controls by redocking into the structures and comparing to TRG. Docking interactions were analyzed with Discovery Studio 4.5 using the lowest binding free energy of all possible positions.

Ethical statement

All experimental procedures in animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication, No. 80-23, revised 1996). Protocols were approved by the Animal Care and Use Committee of Kyung Hee University.

ZF neuromasts and hair cells

Five days post-fertilization zebrafish larvae (AB wild-type) were harvested for evaluation of neuromasts and otic hair cells. ZF adults were maintained in glass tanks with a continuously recirculating system and 14/10 h light/dark cycle. Fish were fed commercially available fish food and newly hatched brine shrimp twice a day. Three female and three male sexually mature ZFs were mated in a breeding cage at night at 29 °C. Eggs were collected at 2 h post-fertilization and washed with SS.

Diabetic induction consisted on 72 h of exposure to freshly prepared AX (600 μM) and changed five times each day. After induction, TRG (0.1 or 1 μg/ml), NGF (25, 50 and 100 ng/ml), and K-252a (100 nM) were administered for 12 h. Validation to select an adequate concentration of K-252a (50, 100, 200, 300, 400, 500, and 600 nM) was carried out in ZF not exposed to AX prior to performing the experiments on diabetic conditions. Lateral line neuromasts and hair cells were labeled by exposing the ZF to 0.1% YO-PRO-1 fluorescent dye (Molecular Probes-Life Technologies, Eugene, OR, USA) for 30 min. Larvae were then washed twice with sea salt water and anesthetized with tricaine (0.03%). In each ZF larvae, fluorescent neuromasts and hair cells were counted under fluorescence microscopy (Olympus 1 × 70, Olympus, Shinjuku-ku, Japan). Focus Lite software was used for image analysis.

Primary cell culture

To evaluate whether TRG could be associated with NGF in the auditory system, spiral ganglion of neonatal Sprague–Dawley rats were cultured. Temporal bones of neonatal Sprague–Dawley rats were removed, and dissociated spiral ganglion cell cultures were maintained in a high-glucose culture media, as described elsewhere (Evans et al., 2007). The NGF level was determined using an enzyme-linked immunosorbent assay (ELISA) development kit (R&D System, Minneapolis, MN, USA). The supernatant of harvested media was used for the assay. TRG was applied to cells for 24 h at concentrations of 2.5 and 5 μg/μl.

Diabetic mice

Eight-week-old adult male *LepR*$^{(db/db)}$ C57BL/KsJ mice were used as a diabetic mouse model, and male *LepR*$^{(db/+)}$ C57BL/KsJ (dbh) littermates were used as non-diabetic animals. All mice were obtained from Jung-Ang Lab Animals (Seoul, Korea). The control group was matched with db/db mice that did not receive treatment. Animals were housed under a 12/12-h light/dark cycles in a room maintained at a controlled temperature (23 °C ± 2 °C) and humidity (50 ± 5%), with food and water *ad libitum*. Mice were divided into four groups (n = 10/group): non-diabetic, untreated diabetic (control), and diabetic mice treated with TRG 10 and 20 mg/kg. Experimental diabetic mice were treated orally once daily with 0.3 ml of TRG at 10 or 20 mg/kg. The dbh mice and diabetic non-treated db/db mice were treated orally with 0.3 ml of distilled water once daily for 8 weeks. Blood glucose levels were determined as described in an earlier study (Hong and Kang, 2008).