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Full paper

Toll-like receptor 4 inhibitor protects against retinal ganglion cell damage induced by optic nerve crush in mice

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ABSTRACT

Toll-like receptor 4 (TLR4) plays key roles in innate immune responses and inflammatory reactions. TAK-242 (resatorvid) is a small-molecule cyclohexene derivative that selectively inhibits TLR4 signaling pathways and suppresses inflammatory reactions. Here we investigated the protective effects of TAK-242 against optic nerve crush (ONC) which induces axonal injury like glaucoma in mice. TAK-242 was injected intravitreally immediately after ONC. The effect of TAK-242 was evaluated by measuring the number of fluorogold-labeled retinal ganglion cells (RGCs) at 10 days after ONC. Furthermore, the expression levels of phosphorylated-nuclear factor-kappa B (p-NF-κB) and phosphorylated-p38 (p-p38) were measured by Western blotting. In addition, we examined activated astrocytes by immunostaining. TAK-242 significantly abrogated the loss of RGCs associated with ONC. Moreover, the expression levels of p-NF-κB and p-p38 were significantly reduced by TAK-242 treatment. Furthermore, TAK-242 and C34, a TLR4 inhibitor, significantly reduced astrocyte activation in the ganglion cell and inner plexiform layers, compared with vehicle treatment. These findings indicate that TAK-242 inhibits not only the TLR4 signaling pathway but also astrocyte activation downstream of this pathway, suggesting that the inhibition of TLR4 signaling is a promising candidate for the treatment of glaucoma.

1. Introduction

Glaucoma is a main cause of blindness worldwide (1). This neurodegenerative disease is characterized by the progressive death of retinal ganglion cells (RGCs) and axonal degeneration, along with visual field losses. Although an elevated intraocular pressure (IOP) is considered a main risk factor for glaucoma, treatment to reduce the IOP does not completely suppress the progression of visual field defects in some glaucoma patients. Therefore, novel glaucoma therapies that extend beyond IOP reduction are needed.

Currently, the optic nerve crush (ONC) model is used as an experimental model of glaucoma. In this model, RGCs death and axonal degeneration are induced *via* direct optic nerve compression (2). In addition, the expression levels of chemokines and cytokines involved in inflammatory and immune responses are upregulated. These mediators secondarily induce RGCs death *via* the exogenous apoptosis pathway (3, 4). This animal model is useful for the clarification of pathologic mechanisms that underlie optic neuropathy.

The pattern recognition receptor Toll-like receptor 4 (TLR4) is thought to play an important role in innate immune responses and inflammatory reactions. Furthermore, within the central nervous system, TLR4 is a key player in the response to injury-induced endogenous ligands which is termed damage-associated molecular patterns (DAMPs) (5). In addition, TLR4 is expressed in the retina and it is associated with high levels of IOP-induced retinal damage and retinal ischemia/reperfusion injury (6). Notably, in the Japanese population, normal-tension glaucoma has been associated with TLR4 gene polymorphisms (7).

TAK-242 {ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1- carboxylate}, or resatorvid, a small-molecule cyclohexene derivative, selectively inhibits TLR4 signaling (8). TAK-242 obstructs the interactions of TLR4 with adaptor molecules by binding Cys747 in the intracellular domain of TLR4 (9). Therefore, TAK-242 suppresses inflammatory responses and thus inhibits the production of nitric oxide (NO) and cytokines, which are factors downstream of TLR4 signaling (10). Previously, we demonstrated that

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TAK-242 reduced middle cerebral artery occlusion-induced infarct volume by inhibiting TLR4 signaling in mice (11). Furthermore, TLR4 knockout mice were recently found to be more resistant to ONC damage, compared with wild-type mice (12). However, it is not clear whether TLR4 inhibition represents a pharmacologically protective effect in an ONC model. Therefore, the present study aimed to investigate the effects of the TLR4 inhibitor TAK-242 in the ONC model.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Eight week-old male ddY albino mice (body weight, 30–40 g; Japan SLC Ltd., Hamamatsu) were purchased for this study. Animals were housed in an air-conditioned room maintained at 22 ± 2 °C, under controlled lighting conditions (12 h:12 h; light:dark) with free access to a standard diet (CLEA Japan, Inc., Tokyo) and tap water.

2.2. Optic nerve crush

ONC was induced as described previously (4, 13). Mice were anesthetized intraperitoneally with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo) and xylazine (6 mg/kg; Bayer Health Care, Tokyo). A small incision was made in the superior and lateral conjunctiva and then, a gentle dissection was made using fine forceps (#5/45; Dumont, Jura, Switzerland) to expose the optic nerve. The optic nerve was crushed for 10 s using self-closing forceps (#N7; Dumont) at a site approximately 1–2 mm posterior to the globe. After surgery, ophthalmic ointment (Daiichi-Sankyo) was applied to both eyes to ensure that the corneas did not dry out and to protect against infection.

2.3. Drug treatments

TAK-242, {ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1- carboxylate} was synthesized in the laboratory of Pharmaceutical and Medicinal Chemistry, Department of Organic and Medicinal Chemistry, Gifu Pharmaceutical University, using a procedure that was based on a previous report (14). The compound was verified using ^{1}H-NMR and mass spectra. C34, Isopropyl 2-(acetylamino)-2-deoxy α-D-glucopyranoside 3,4,6-triacetate, was purchased from Sigma–Aldrich (St. Louis, MO, USA). TAK-242 and C34 were dissolved in 1% dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto); 1% DMSO in saline was used as a vehicle. Immediately after the ONC procedure, TAK-242 (0.02 μg/2 μl/eye) and C34 (0.2 μg/2 μl/eye) were injected into the vitreous body of the left eye using a 10-μL Hamilton glass syringe (701N; Hamilton Co., Reno, NV, USA) fitted with a sterile 34-gauge needle (Terumo, Tokyo). Previously, we reported that TAK-242 (0.01 μg/μl, but not 0.001 or 0.003 μg/μl) which was administrated intracerebroventricularly at 1 μl showed not only protective effect against neuronal damage induced by cerebral ischemia but also suppression of the phosphorylated NF-κB and p38 *via* TLR4 signaling seen after ischemia and reperfusion (11). Therefore, in the present study, TAK-242 was used at 0.01 μg/μl, which was intravitreally injected at 2 μl.

2.4. Retrograde labeling of retinal ganglion cells with fluorogold

Three days before the ONC procedure, mice were anesthetized intraperitoneally with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo) and xylazine (6 mg/kg; Bayer Health Care). Mice were then fixed on a stereotaxic apparatus; after hair was shaved, followed by disinfection, the skin was removed along the parietal midline. A small drill was used to make a hole in the skull through which 1 μL of 4% fluorogold (Biotium, Hayward, CA) was injected using a Hamilton syringe; this was completed over a 2-min at a depth of 2 mm from the brain surface. The location of the hole was 2.92 mm behind the bregma along the anteroposterior axis, and 0.5 mm lateral to the midline.

2.5. Assessment of retinal ganglion cell survival in mice

Ten days after the ONC procedure, mice were euthanized by cervical dislocation under deep anesthesia, and each eye was enucleated. Eyes used for histological analysis were immersed in a fixative solution containing 4% paraformaldehyde (PFA) at 4 °C for 6–8 h. The enucleated eyes were placed under a microscope, and the retinas were dissected. Retinas were quartered and flat-mounted on glass slides. Coverslips were mounted using Fluoromount aqueous mounting medium for fluorescent staining (Diagnostic BioSystems, Pleasanton, CA, USA). Fluorescently labeled RGCs were viewed using a BX50 fluorescence microscope (Olympus, Tokyo). Fluorescent micrographs were captured with a high resolution (16-bit) charge-coupled device camera (DP30BP, Olympus) at 1360 × 1024 pixels, using MetaMorph software. RGCs were counted at approximately the same distance [0.5 μm (central), 1.5 μm (middle), peripheral] from the optic nerve head, and the average of RGCs per 12 identically sized microscopic fields was recorded.

2.6. Western blot analysis

Western blot analysis was performed as previously reported (15). Mice were euthanized by cervical dislocation, and the eyeballs were quickly removed. The retinae were carefully separated from the eyeballs and rapidly frozen on dry ice. To extract proteins, tissues were homogenized in cell-lysis buffer (RIPA buffer (Tris–HCl 50 mM, NaCl 150 mM, 0.5% Sodium Deoxycholate, 0.1% SDS and 1% NP-40) and 1% protease inhibitor cocktail (Sigma–Aldrich), 1% phosphatase inhibitor cocktails 2 and 3 (Sigma–Aldrich)) using a Physcotron homogenizer (Microtec Co. Ltd., Chiba). The lysate was centrifuged at 12,000 × g for 20 min. The protein concentration of the supernatant was determined prior to use in subsequent experiments. Protein concentrations were measured by a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA) using bovine serum albumin as standard. A mixture of equal parts of protein lysate and sample buffer containing 10% 2-mercaptoethanol was subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA, USA). The membranes were blocked for 30 min at room temperature with 5% Blocking One-P (Nacalai Tesque Inc.) in 10 mM Tris-buffered saline containing 0.05% Tween 20, and were subsequently incubated overnight at 4 °C with the primary antibody. For immunoblotting, the following primary antibodies were used: rabbit anti-phospho-nuclear factor kappaB p65 (Ser536) polyclonal antibody (1:1000 dilution; Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182) polyclonal antibody (1:1000; Cell Signaling), mouse anti-phospho-neurofilament heavy (p-NFH) monoclonal antibody (1:1000; Millipore), mouse anti-TLR4 monoclonal antibody (1:1000; IMGENEX, San Diego, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000; Cosmo Bio Co., Ltd., Tokyo), rabbit anti-p38 polyclonal antibody (1:1000; Cell Signaling), rabbit anti-NF-κB polyclonal antibody (1:1000; Cell Signaling), and mouse anti-β-actin monoclonal antibody (1:1000;

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