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Effects of chemical inhibition of N-WASP, a critical regulator of actin polymerization on aqueous humor outflow through the conventional pathway

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ABSTRACT

The integrity of actin cytoskeletal organization in aqueous humor outflow pathway is thought to play a critical role in modulation of aqueous humor outflow through the trabecular meshwork. Our understanding of the regulation of actin cytoskeletal dynamics in outflow pathway, however, is very limited. To explore the potential importance of Neural Wiskott–Aldrich syndrome protein (N-WASP), a critical regulator of actin polymerization/nucleation in aqueous humor outflow pathway, the effects of Wiskostatin, a selective pharmacological inhibitor of N-WASP, on aqueous humor outflow facility were evaluated using enucleated porcine eyes and a constant pressure perfusion system. Further, drug induced effects on actin cytoskeletal organization, cell adhesions, myosin II phosphorylation, matrix metalloproteinase (MMP) activity, and cytoskeletal protein profile in porcine trabecular meshwork (TM) cells were determined by immunofluorescence, zymography, and mass spectrometry. Aqueous humor outflow facility was increased significantly and progressively in the Wiskostatin perfused porcine eyes. The Wiskostatin perfused eyes appear to exhibit increased giant vacuoles in the inner wall of aqueous plexi and deformation of aqueous plexi. The Wiskostatin treated TM cells demonstrated extensive vacuoles in their cytosol, and both actin stress fibers and focal adhesions were decreased in a reversible manner. The drug-treated TM cells also revealed decreased myosin II and actin in the cytoskeletal enriched triton insoluble fraction but did not affect myosin II phosphorylation or MMP-2 activity. These data demonstrate that the chemical inhibition of N-WASP increases aqueous humor outflow facility in association with decreased actomyosin interaction and cell adhesive interactions revealing the importance of N-WASP in homeostasis of aqueous humor outflow.

1. Introduction

Glaucoma, characterized by optic nerve degeneration and retinal ganglion cell death, is a leading cause of blindness worldwide. In primary open angle glaucoma (POAG), the most prevalent type of this disease, intraocular pressure (IOP) is commonly elevated as a result of increased resistance of the conventional aqueous humor outflow process (Quigley, 1993; Weinreb and Khaw, 2004). In humans this pathway is a predominant route of aqueous humor outflow (Tan et al., 2006), and the increased resistance in POAG is thought to be related to functional and structural abnormalities in the juxtacanalicular region (JCT) of the trabecular meshwork (TM) and/or Schlemm's canal (SC). Although retinal ganglion cell survival represents a primary goal for the treatment of this disease, lowering IOP is the only option available to treat glaucoma, at the present time (Weinreb and Khaw, 2004). Hence, understanding the molecular mechanism(s) of both normal and perturbed aqueous humor outflow in glaucoma is necessary and important.

TM, JCT and SC cells are believed to influence aqueous outflow facility by altering their morphology, cell adhesive interactions and extracellular environment, leading to changes in the geometry of the aqueous outflow pathway, and paracellular and transcellular permeability (Epstein et al., 1999; Johnson et al., 1992; Rao and Epstein, 2007; Tian et al., 2009, 2000). The TM cells have smooth muscle-like contractile and relaxation properties regulated by actomyosin interaction (Coroneo et al., 1991; Rao and Epstein, 2007). The involvement of the actin cytoskeleton in glaucoma pathobiology was suggested by observing disordered actin fibers in glaucomatous outflow tissue specimens (Read et al., 2006). The influence of actin cytoskeletal integrity on aqueous humor outflow facility has also been well documented in both in vivo and ex-vivo studies (Rao and Epstein, 2007; Tian et al., 2009, 2000). Moreover, inhibitors of

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various kinases including Rho kinase (ROCK), protein kinase C (PKC), and myosin light chain kinase (MLCK), which regulate myosin light chain phosphorylation and actomyosin interaction, have been demonstrated to increase aqueous outflow facility and lower IOP (Honjo et al., 2001; Khurana et al., 2003; Rao et al., 2001; Tanihara et al., 2008; Tian et al., 2009). Although cellular relaxation, altered cell morphology, and loss of cell adhesive interactions are commonly observed in outflow pathway cells treated with actin depolymerizing agents and kinase inhibitors (Rao and Epstein, 2007; Tian et al., 2009, 2000), the cellular and molecular basis for the potential involvement of the actin cytoskeleton and the regulatory proteins of actin cytoskeleton in the homeostasis of aqueous humor outflow function have not been well characterized.

Neural Wiskott–Aldrich syndrome protein (N-WASP), a member of the WASP family of proteins, is a key regulator of actin polymerization and nucleation; its activity is regulated by phosphatidylinositol bisphosphate (PIP_2) and the small GTPase Cdc42, which induces a conformational switch that initiates actin polymerization (Bompard and Caron, 2004; Takenawa and Suetsugu, 2007). N-WASP in its active confirmation interacts with actin-related protein-2/3 (ARP2/3) and activates ARP2/3 complex to induce actin nucleation and polymerization (Bompard and Caron, 2004; Takenawa and Suetsugu, 2007). Actin nucleation and polymerization in turn plays a crucial role in various cellular functions, such as membrane protrusion, migration, endocytosis, vesicle and organelle trafficking, podosome formation, transcription, and exocytosis (Goley and Welch, 2006; Oikawa and Takenawa, 2009; Takenawa and Suetsugu, 2007; Wu et al., 2006). Recently, Wiskostatin, a small molecular chemical compound, has been identified as a selective and reversible inhibitor of N-WASP (Peterson et al., 2004), and this compound keeps N-WASP in its autoinhibited form and thereby prevents N-WASP membrane association and ultimately actin polymerization.

In this study, we determined the effects of Wiskostatin on aqueous humor outflow facility and its cellular responses in cultured TM cells to explore the potential role of N-WASP in aqueous humor outflow function. Enucleated porcine eyes perfused with Wiskostatin demonstrated increased aqueous outflow facility, which was associated with structural and morphological changes in aqueous humor outflow pathway. These data reveal that N-WASP may be involved in modulation of aqueous humor outflow facility via trabecular meshwork pathway, and provide novel insights into the participation of different cytoskeletal regulatory proteins in aqueous humor outflow homeostasis.

2. Materials and methods

2.1. Materials

Wiskostatin was purchased from Biomol International, LP (Plymouth Meeting, PA). N-WASP polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Plus detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). Cell culture media and fetal bovine serum were obtained from Gibco-BRL (Gaithersburg, MD). Tetrarhodamine isothiocyanate (TRITC) –phalloidin, monoclonal antibody against β-actin, and monoclonal antibody against vinculin were purchased from Sigma–Aldrich (St. Louis, MO). Phospho-MLC2 polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Myosin IIA and IIB polyclonal antibodies were from Covance (Princeton, NJ).

2.2. Perfusion and histological examination

Freshly obtained enucleated porcine eyes from a local abattoir were perfused with 50 μM Wiskostatin (initially dissolved in DMSO) in perfusion medium containing Dulbeco phosphate-buffered saline (DPBS) and 5.5 mM D-glucose at 25 °C using a Grant constant pressure perfusion system as described previously (Rao et al., 2005). After establishing the initial baseline outflow measurements at 15 mmHg and 25 °C, the anterior chambers of the eyes were exchanged with drug and perfused with drug continuously for 5 h. Outflow measurements were recorded at hourly intervals. Drug effects were expressed as percentage change in outflow facility (compared to baseline values) over 5 h, in drug treated versus sham treated (DMSO) paired controls (contra lateral eyes). Data were analyzed by a paired two-tailed Student's *t*-test to determine significance. At the end of a 5 h perfusion period, sham control and drug-treated fellow eyes were fixed and subjected to light and electron microscopic analysis as described in our previous study (Rao et al., 2001).

2.3. Cell culture and viability assay

Porcine TM (PTM) cells were isolated as we described earlier from pig eyes obtained from a local abattoir (Rao et al., 2001). PTM cells were used between passages 3 and 5. All experiments were conducted using confluent cultures and cells were cultured at 37 °C under 5% CO_2, in Dulbecco's modified Eagle Medium (DMEM) containing 10% FBS and Penicillin–Streptomycin–Glutamine. For cell viability assays, drug-treated PTM cells were incubated with fluorescein diacetate and propidium iodide as described previously and cell viability and toxicity was assessed by following the fluorescein diacetate green fluorescence and propidium iodide red staining, respectively (Rao et al., 2001).

2.4. Immunofluorescence staining

Immunofluorescence staining was performed as we described previously (Rao et al., 2001). Briefly, the drug-treated PTM cells (cultured on gelatin-coated glass coverslips) were fixed with 3.7% formaldehyde for 12 min after morphologic examination by phase-contrast microscopy (IM35; Carl Zeiss, Thornwood, NY). Cells were then washed with cytoskeletal buffer (10 mM 2-[N-morpholino] ethane sulfonic acid (MES), 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM glucose pH 6.1), permeabilized with 0.5% Triton X-100, and blocked with 10% serum buffer. For F-actin staining, cells were incubated with TRITC-phalloidin (1:500) for 20 min. For detection of focal adhesions and myosin II, cells were immunostained with mouse anti-vinculin (1:200) and rabbit anti-myosin IIA antibody (1:500) for 2 h respectively, followed by incubation with secondary antibody conjugated with Alexa 488, and counterstaining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI) for 30 min.

2.5. MLC phosphorylation

MLC phosphorylation status of PTM cells was determined as described by Garcia et al.(Garcia et al., 1995). Briefly, confluent cell cultures of both control and drug treated were extracted with cold 10% trichloroacedic acid and 0.5M dithiothreitol, and cell precipitates obtained after centrifugation at 13,000 rpm were dissolved in 8 M urea buffer using a sonicator, separated on glycerol slab gels, and transferred onto nitrocellulose filters. The filters were then subjected to immunoblot analysis using a rabbit anti-phospho-MLC2 antibody (1:1000) followed by development with peroxidase-conjugated goat anti-rabbit IgG and an ECL plus detection system.

2.6. Gelatin zymography

Matrix metalloproteinase-2 (MMP-2) activity was evaluated by gelatin zymography as described previously (Sanka et al., 2007).