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Research article

Live cell imaging of actin dynamics in dexamethasone-treated porcine trabecular meshwork cells

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ABSTRACT

The regulation of the actin cytoskeleton in trabecular meshwork (TM) cells is important for controlling outflow of the aqueous humor. In some reports, dexamethasone (DEX) increased the aqueous humor outflow resistance and induced unusual actin structures, such as cross-linked actin networks (CLAN), in TM cells. However, the functions and dynamics of CLAN in TM cells are not completely known, partly because actin stress fibers have been observed only in fixed cells. We conducted live-cell imaging of the actin dynamics in TM cells with or without DEX treatment. An actin-green fluorescent protein (GFP) fusion construct with a modified insect virus was transfected into porcine TM cells. Time-lapse imaging of live TM cells treated with 25 μM Y-27632 and 100 nM DEX was performed using an inverted fluorescence microscope. Fluorescent images were recorded every 15 s for 30 min after Y-27632 treatment or every 30 min for 72 h after DEX treatment. The GFP-actin was expressed in 22.7 ± 10.9% of the transfected TM cells. In live TM cells, many actin stress fibers were observed before the Y-27632 treatment. Y-27632 changed the cell shape and decreased stress fibers in a time-dependent manner. In fixed cells, CLAN-like structures were seen in 26.5 ± 1.7% of the actin-GFP expressed PTM cells treated with DEX for 72 h. In live imaging, there was 28% CLAN-like structure formation at 72 h after DEX treatment, and the lifetime of CLAN-like structures increased after DEX treatment. The DEX-treated cells with CLAN-like structures showed less migration than DEX-treated cells without CLAN-like structures. Furthermore, the control cells (without DEX treatment) with CLAN-like structures also showed less migration than the control cells without CLAN-like structures. These results suggested that CLAN-like structure formation was correlated with cell migration in TM cells. Live cell imaging of the actin cytoskeleton provides valuable information on the actin dynamics in TM cells.

1. Introduction

Steroids, such as dexamethasone (DEX), are of considerable benefit in the control of inflammatory reactions, and used widely to treat various inflammatory diseases. However, steroids have diverse side effects, such as ocular hypertension. DEX-induced ocular hypertension was first reported in 1963 (Armaly, 1963a). Patients with primary open-angle glaucoma are at higher risk of steroid-induced intraocular pressure (IOP) elevation (Armaly, 1963b, 1967), and prolonged IOP elevation often results in the development of secondary open-angle glaucoma (Iwao et al., 2011).

Steroid-induced ocular hypertension is thought to result from changes in the conventional outflow pathway with steroid treatment, leading to increased resistance of the aqueous outflow; steroid treatment reportedly induces the accumulation of extracellular matrix (ECM) on the trabecular meshwork (TM) (Yun et al., 1989; Johnson et al., 1990; Steely et al., 1992; Zhou et al., 1998; Clark et al., 2001; Tane et al., 2007), and decreases the phagocytic function of TM cells (Matsumoto and Johnson, 1997; Zhang et al., 2007). Recently, we reported that DEX increased the barrier function in Schlemm's canal endothelial (SCE) cells, resulting in the increased outflow resistance (Fujimoto et al., 2012). We also revealed that this DEX-induced change in SCE cells was canceled by the Rho-associated kinase (ROCK) inhibitor Y-27632.

Clark et al. (1994) reported abnormal DEX-induced changes in the actin cytoskeleton, such as cross-linked actin networks (CLAN), in cultured TM cells. They also observed the CLAN-like structure more frequently in the TM tissue of glaucoma patients compared to normal control TM tissue (Clark et al., 2005; Hoare et al., 2009).

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Subsequently, many studies have explored the molecular mechanisms of CLAN formation, and revealed that CLAN formation was related to various molecules in intracellular signaling pathways, such as integrin, PKC, and noncanonical Wnt signaling (Filla et al., 2006, 2009, 2011, 2014; Yuan et al., 2013;). However, the functions and dynamics of CLAN in TM cells are not completely clear, partly because actin stress fibers have been observed only in fixed cells. To examine this, we conducted live-cell imaging of the actin dynamics in TM cells with or without DEX treatment and evaluated the relationship between CLAN formation and cell movement.

2. Material and methods

2.1. Cell culture

Pig eyes were obtained from a local abattoir, and porcine trabecular meshwork (PTM) cells were isolated using collagenase, as described previously (Fujimoto et al., 2012). PTM cells were cultured in Dulbecco's modified Eagle's medium (DMEM; WAKO Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL amphotericin B, at 37 °C in 5% CO_2. These cells were used after 3–5 passages. The characterization of TM cells was confirmed by dexamethasone-induced myocilin expression and phagocytic activity using real-time RT-PCR and *S. aureus* bioparticles labeled with pHrodo Red fluorescent reagent, respectively (data not shown).

2.2. Transfection of actin-GFP and talin-RFP

The actin-green fluorescent protein (GFP) fusion construct and/or the talin-red fluorescent protein (RFP) fusion construct were transfected with modified insect virus baculovirus (CellLight®, Invitrogen, Waltham, MA), following the manufacturer's instructions. The CellLight® reagent was added to the PTM cells at about 50% confluence, and incubated for 24 h at 37 °C in 5% CO_2. We confirmed actin-GFP and/or talin-RFP expression using fluorescent microscopy, and subsequently used the actin-GFP and/or talin-RFP expressing TM cells in each experiment.

2.3. F-actin staining

The normal (not transfected actin-GFP) and actin-GFP expressing PTM cells were cultured on gelatin-coated glass coverslips. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS at room temperature. To stain F-actin, the cells were incubated with phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) (Sigma–Aldrich) at room temperature for 30 min. After the cells were washed with PBS, they were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) or an all-in-one fluorescence microscope (BZ-X710; Keyence, Osaka, Japan).

2.4. Time-lapse imaging

The actin-GFP and/or talin-RFP expressing PTM cells were cultured on gelatin-coated glass-bottom 35-mm dishes. The culture medium was replaced with serum-free, phenol-red-free DMEM 24 h before the Y-27632 or DEX treatment. Time-lapse imaging of the live TM cells treated with 25 μM Y-27632 was conducted using an inverted fluorescence microscope (AS-MDW; Leica Microsystems, Wetzlar, Germany). Differential interference contrast (DIC) and fluorescent images were obtained every 15 s in actin-GFP only expressing cells, or every 30 s in actin-GFP and talin-RFP expressing cells for 30 min. Time-lapse imaging of the live TM cells treated with 100 nM DEX was conducted using an all-in-one fluorescence microscope (BioStation; Nikon, Tokyo, Japan). Phase contrast and fluorescent images were obtained every 30 min for 72 h.

2.5. Image data analysis

To quantify the cell migration of TM cells with or without DEX, we used the methods described by Kikuta et al. (2013). The amount of PTM cell migration was quantified using the cell deformation index (CDI; see Fig. 4-A) based on the Image J software (NIH, Bethesda, MD). To calculate the CDI, we used images obtained at two time points 24 h apart. We drew the cell shape at each time point, and merged the two images. The merged image was separated into three regions as follows: occupied only at the initial time point (A); occupied only at the final time point (C); and overlapped at the initial and final time points (B). CDI was calculated as the ratio $(A + C)/(A + B)$.

2.6. Statistical analysis

Data are presented as the means ± standard deviation (SD). Two groups were compared using the Student's *t*-test or Wilcoxon rank-sum test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Actin-GFP transfection in PTM

To confirm the intracellular distribution of actin-GFP fusion protein induced by baculovirus, the transfected PTM cells were fixed and stained with phalloidin-TRITC (Fig. 1). The distribution of fibriform GFP structures corresponded to that of phalloidin-labeled F-actin in TM cells. The transduction efficacy of actin-GFP was 22.7 ± 10.9% (mean ± SD, average of three independent examinations).

3.2. Live cell imaging of actin dynamics after Y-27632 treatment

Rho-associated kinase (ROCK) is involved in the rearrangement of F-actin in TM cells, and ROCK inhibitors such as Y-27632 induce actin depolymerization and cell shape change in TM cells within 30 min (Honjo et al., 2001; Rao et al., 2001; Koga et al., 2006). Therefore, we performed short-term live cell imaging of actin dynamics after the Y-27632 treatment. The F-actin and cell shape of control TM cells (without Y-27632) did not change during the 30-min observation (Fig. 2, upper panels; Supplementary video 1). On the other hand, the Y-27632 treatment changed the cell shape and decreased actin stress fibers in a time-dependent manner (Fig. 2, lower panels; Supplementary video 2). Additionally, TM cells were doubly-labeled with actin-GFP and talin-RFP, and the effects of Y-27632 on the dynamics of these molecules were observed. Talin is known to bind to F-actin and integrins, and to contribute to integrin signaling and mechanosensitivity (Ye et al., 2014; Yan et al., 2015). Although F-actin was depolymerized in a similar manner as singly labeled cells, the punctate staining of talin was less affected compared to F-actin (Supplementary videos 3 and 4).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.exer.2016.02.007.