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# Damage to lens fiber cells causes TRPV4-dependent Src family kinase activation in the epithelium

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ABSTRACT

The bulk of the lens consists of tightly packed fiber cells. Because mature lens fibers lack mitochondria and other organelles, lens homeostasis relies on a monolayer of epithelial cells at the anterior surface. The detection of various signaling pathways in lens epithelial cells suggests they respond to stimuli that influence lens function. Focusing on Src Family Kinases (SFKs) and Transient Receptor Potential Vanilloid 4 (TRPV4), we tested whether the epithelium can sense and respond to an event that occurs in fiber mass. The pig lens was subjected to localized freeze-thaw (FT) damage to fibers at posterior pole then the lens was incubated for 1–10 min in Krebs solution at 37 °C. Transient SFK activation in the epithelium was detectable at 1 min. Using a western blot approach, the ion channel TRPV4 was detected in the epithelium but was sparse or absent in fiber cells. Even though TRPV4 expression appears low at the actual site of FT damage to the fibers, SFK activation in the epithelium was suppressed in lenses subjected to FT damage then incubated with the TRPV4 antagonist HC067047 (10 μM). Na,K-ATPase activity was examined because previous studies report changes of Na,K-ATPase activity associated with SFK activation. Na,K-ATPase activity doubled in the epithelium removed from FT-damaged lenses and the response was prevented by HC067047 or the SFK inhibitor PP2 (10 μM). Similar changes were observed in response to fiber damage caused by injection of 5 μl hyperosmotic NaCl or mannitol solution beneath the surface of the posterior pole. The findings point to a TRPV4-dependent mechanism that enables the epithelial cells to detect remote damage in the fiber mass and respond within minutes by activating SFK and increasing Na,K-ATPase activity. Because TRPV4 channels are mechanosensitive, we speculate they may be stimulated by swelling of the lens structure caused by damage to the fibers. Increased Na,K-ATPase activity gives the lens greater capacity to control ion concentrations in the fiber mass and the Na,K-ATPase response may reflect the critical contribution of the epithelium to lens ion homeostasis.
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## 1. Introduction

The bulk of the mammalian lens consists of tightly packed fiber cells carefully arranged in a hexagonal array (Bassnett et al., 2011). The anterior surface of the fiber cell mass is covered by an epithelial monolayer and new fiber cells are formed by mitosis and elongation of cells at the periphery of the epithelial sheet. Fully differentiated mature lens fibers lack mitochondria, endoplasmic reticulum and nuclei. These unusual cellular specializations contribute to a low degree of light scattering but as a result of such differentiation, lens fibers are unable to function in isolation and their homeostasis relies on the anterior monolayer of epithelial cells. Metabolic substrates such as glucose and amino acids enter the lens and lactate is exported via transporters in the epithelium. The epithelium also plays a critical role in active sodium–potassium transport. Lens epithelial cells have a high Na,K-ATPase activity (Tamiya et al., 2003). In contrast, mature fiber cells that account for the bulk of the lens structure have negligible Na,K-ATPase activity (Delamere and Dean, 1993). Sodium and potassium homeostasis of the fiber mass, and thus water homeostasis, is supported to a large extent by Na,K-ATPase activity in epithelial cells at the anterior-equatorial lens surface (Gao et al., 2000).

The detection of ERK, Src, TGFβ, Wnt/β-catenin, Smad3 and other signaling pathways in lens epithelial cells points to the potential for these cells to respond to cues that influence lens function (Dawes et al., 2013; Leonard et al., 2013). External stimuli may take the form of growth factors, hormones, peptides such as endothelin-1 or muscarinic and purinergic agonists in the aqueous humor

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(Duncan and Collison, 2002; Sanderson et al., 2014). Here we consider the possibility that epithelial cells also are able to sense and respond to events that occur in lens fibers.

Src tyrosine kinase and related Src family kinases (SFKs) have been detected in the lens epithelium of several species (Tamiya and Delamere, 2005; Zhou et al., 2007). SFKs are expressed in most cells serve as points of control in a wide variety of signaling pathways (Ingley, 2008). In chick lens there is convincing evidence that c-Src promotes epithelial cell proliferation and the maintenance of an epithelial phenotype while Fyn, a different SFK, promotes lens fiber morphogenesis (Leonard et al., 2013). In porcine lens, SFK activation has been shown to increase Na,K-ATPase activity in the epithelium of lenses that are exposed to purinergic agonists (Tamiya et al., 2007) or hyposmotic solution (Shahidullah et al., 2012a). The present studies show evidence for rapid SFK activation and an increase of Na,K-ATPase activity in the epithelium in response to damage at a remote region of the lens fiber mass. Our data suggests that SFK response mechanism in the epithelium is dependent on activation of the TRPV4 ion channel. Studies by other investigators suggest TRPV4 activation is mechanosensitive (Luo et al., 2014). In a previous study TRPV4 was shown to be involved in the response of the lens to osmotic swelling (Shahidullah et al., 2012b).

## 2. Materials and methods

### 2.1. Materials

Ouabain, HC067047, PP2, alamethicin and DMSO were purchased from Sigma, St. Louis, MO, USA. Other chemicals including those for preparing the Krebs solution were purchased from Sigma St. Louis, MO, USA. Rabbit anti TRPV4 polyclonal antibody was purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-phospho-Src-family (Tyr 416) kinase antibody and rabbit polyclonal anti-β-actin antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-GAPDH monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz. CA, USA). Goat anti-rabbit secondary antibody conjugated with IR Dye 680 and goat anti-mouse secondary antibody conjugated with IR Dye 800 were obtained from LICOR Biosciences (Lincoln, NE, USA).

### 2.2. Lenses

Porcine eyes were obtained from the University of Arizona meat Science Laboratory or from West Valley Processing Meat processors, Buckeye AZ or Hatfield Quality Meats (Philadelphia, PA). The use of porcine eye tissues was approved by the University of Arizona Institutional Animal Care and Use Committee and conformed to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. The posterior of the eye was dissected open and the zonules were cut, allowing the intact lens to be removed and transferred to Krebs solution that contained (in mM) 119 NaCl, 4.7 KCl, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 5.5 glucose. Prior to use the Krebs solution was equilibrated with 5% $CO_2$ for 30–40 min and adjusted to pH 7.4. Lenses were allowed to equilibrate in Krebs solution for 3 h at 37 °C and 5% $CO_2$ in a humidified incubator prior to use.

### 2.3. Damage to the posterior surface of the lens

The freeze-thaw maneuver was carried out on lenses placed in a 24 mm culture well and partially immersed in Krebs solution such that the posterior surface protruded above the solution surface. The posterior pole of the lens was touched for 10 s with a 2.5 mm diameter stainless steel probe that had been cooled in liquid nitrogen, and then a Pasteur pipette was used to flush 37 °C Krebs solution onto the probe to thaw, release and cover the lens with solution. In specified experiments, damage to a localized area of posterior fiber mass was achieved by injecting 5 μl of hyperosmotic (2400 mOsm) NaCl solution approximately 1.0 mm beneath the posterior capsule using a 30 gauge Hamilton syringe needle. Following the damage maneuver, the intact lens was incubated 1–10 min in Krebs solution. Then, the lens was removed from the Krebs solution and the epithelium was isolated from the fiber mass using fine forceps as described earlier (Shahidullah et al., 2012b). The epithelium samples were immediately snap frozen and stored at −80 °C until further use.

### 2.4. Na,K-ATPase activity

The frozen epithelium-capsule from each lens was homogenized in 350 μl of an ice-cold double-strength ATPase assay buffer that contained (mM): L-Histidine, 80; NaCl 200; KCl, 10; $MgCl_2$, 6.0; EGTA, 2.0 (pH 7.4) plus 10 μl/ml of a Protease Inhibitors Cocktail (Thermo Scientific, Rockford, IL). The homogenization protocol, which was carried out on ice, was 4 cycles of 15 s at 5 s intervals using a Misonix S3000 sonicator at a 6 Watt power setting (Misonix, New York, USA). The homogenate was centrifuged at 13,000 g for 30 min at 4 °C to remove nuclei, larger mitochondria and unbroken debris. Then the supernatant was used to measure Na,K-ATPase activity. Protein in the supernatant was measured by the bicinchoninic acid (BCA) assay (Smith et al., 1985) (Pierce Biotechnology, Rockford, IL, USA), using bovine serum albumin as a standard.

Na,K-ATPase activity was measured using a modification of a method described earlier (Shahidullah and Delamere, 2006). In brief, 150 μl of the supernatant was placed in duplicate tubes containing 50 μl of double-strength ice-cold Na,K-ATPase buffer. To ensure access of ions and ATP to membrane vesicles, alamethicin (5 μl) was added to give a final approximate concentration of 0.1 mg alamethicin per mg of protein (Xie et al., 1989). Ouabain, a selective inhibitor of Na,K-ATPase (Wallick and Schwartz, 1988), was added to half the tubes (final concentration 300 μM). Previous studies (data not shown) indicate 300 μM ouabain causes maximal inhibition of porcine lens Na,K-ATPase. Remainder of the tubes received an equivalent volume of distilled water. An additional 150 μl of distilled water was added to each tube. The tubes were pre-incubated at 37 °C for 5 min then concentrated ATP solution (40 μl) was added to each tube (final ATP concentration of 2.0 mM), bringing the total volume of the assay mixture to 400 μl and the concentration of the double-strength Na,K-ATPase buffer to single strength. The assay mixture was incubated in the dark at 37 °C for 30 min with slow shaking. At the end of the incubation period, the ATP hydrolysis reaction was stopped by the addition of 150 μl of 15% ice-cold trichloroacetic acid and the tubes were placed on ice for 30 min with occasional mixing by shaking to encourage protein precipitation.

ATP hydrolysis was determined by measuring the amount of inorganic phosphate released in each reaction tube. The tubes were placed in a centrifuge at 3000 rpm for 15 min at 4 °C then 400 μl of the supernatant was removed and mixed with 400 μl of 4.0% $FeSO_4$ solution in ammonium molybdate (1.25 g of ammonium molybdate in 100 ml of 2.5 N sulfuric acid). Standard solutions, which contained $NaH_2PO_4$ and were equivalent to 0, 62.5, 125, 250 and 500 nmoles of $PO_4^{-3}$, were treated similarly. The mixture was allowed to sit for 5 min at room temperature, and then 200 μl of each standard or sample was transferred to a 96-well plate and absorbance was measured at 750 nm using a Perkin Elmer plate reader (Victor V1420-040, Perkin Elmer, CT, USA). Na,K-ATPase activity was calculated as the difference between ATP hydrolysis in the presence