PKCγ Knockout Mouse Lenses Are More Susceptible to Oxidative Stress Damage

S.S. Lobell, D. Lin, Lloyd Willard*, M. Barnett, E. Gustafson, D. Madgwick, D.J. Takemoto

Department of Biochemistry and *Diagnostic Medicine, Kansas State University, Manhattan, KS 66506

Abstract

Purpose: Protein kinase C γ (PKCγ) plays critical roles in control of gap junctional communication and oxidative stress responses in the lens epithelial cells. In this report we wished to determine if the deletion of endogenous PKC γ causes lenses to be more sensitive to oxidative stress.

Methods: Animals were 6 weeks old unless otherwise indicated. Both the control (b6129pf21j100903) mice and PKC γ knockout (B6;129p-Prkcctm1St1) mice were from Jackson Laboratory (Bar Harbor, MA). For light microscopy, all eyes were removed immediately after death and treated with hydrogen peroxide for 1 hour. The concentration for 6 week old mice was 50 μM, and the concentration for the 3 day old mice was 10 μM. Lenses were then fixed in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate. Lenses were post-fixed with osmium tetroxide, dehydrated with increasing concentrations of ethanol, and embedded in epon (LX112). Sections (one μm thick) were stained with toludine blue. PKC γ enzyme activity was measured by use of the PepTag PKC assay kit. Cx 50 phosphorylation was measured by western blot with anti-phosphoserine antibodies. Whole lens gap junction activity was determined by dye transfer assay. Lucifer yellow and rhodamine dextran were microinjected and dye transfer was measured by confocal microscopy.

Results: In control mouse lenses, endogenous PKCγ enzyme was activated by application of phorbol ester (TPA 200nM) or H_2O_2 (100μM). However, no PKCγ enzyme activity was detected in PKCγ knockout mice. Cx50 phosphorylation experiments showed that activation of PKCγ by TPA or H_2O_2 caused Cx50 phosphorylation on serines in control lenses, but no decrease in dye transfer was observed in PKCγ knockout lenses. Structural studies by light microscopy demonstrated that H_2O_2 challenge resulted in extensive structural damage in knockout lenses, while the control lenses showed much less damage. Data indicates that open gap junctions causes lens to be more sensitive to oxidative stress in PKCγ knockout mice.

Conclusions: PKCγ phosphorylates connexin proteins in response to oxidative stress which, in turn, causes inhibition of gap junctions in the lens. PKCγ is required for protection of lens from oxidative damage. A failure to inhibit gap junctions in response to oxidative stress may contribute to the oxidative damage of lenses in PKCγ knockout mice.

METHODS

Animals: Male and female mice at 6-week of age were used in this study. Both the control mice (b6129pf21j100903) and PKCγ knockout mice (B6;129p-Prkcctm1St1) were from Jackson Laboratory. All experiments conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and were performed according to an institutionally approved animal protocol.

PKCγ Enzyme Activity Assay: PKCγ activity was analyzed using a PepTag Assay kit. Briefly, equal protein amounts of whole cell extracts from whole lens were immunoprecipitated with PKCγ antisera. Immunoprecipitated PKCγ/agarose bead complexes were incubated with a PKC reaction mixture, and the PKCγ reaction products (fluorescent PepTag peptides) were resolved by agarose gel electrophoresis and visualized under UV light. The phosphorylated peptide bands were excised, and their fluorescence intensities were quantified by spectrophotometry, according to the manufacturer's instructions. Results are expressed as the percentage of nontreated specific PKCγ activity and as the average of three experiments ± SEM.

Immunoprecipitation, Western blot, and Phosphorylation of Cx50 on Ser and Thr: The whole lenses were homogenized with cell lysis buffer followed by sonication. The cell lysis buffer contained 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 0.1% protease inhibitor cocktail, 5 mM NaF, 5 μM Na3VO4, and 2 mM PMSF. After centrifugation at 12,000x g for 20 min, the supernatants were used as whole cell extracts. Whole cell extracts were immunoprecipitated with anti-Cx50 at 4°C for 4 hours. The immunoprecipitate/agarose bead complexes were resolved by SDS-PAGE and visualized by Western blot with antisera to phosphoserine (pS), phosphothreonine (pT), and/or connexin 50 (Cx50). Some whole cell homogenates were blotted using antisera as shown (Fig. 2B)

Lens Gap Junction Activity Dye-Transfer Assay: The lenses of Six-week-old PKCγ knockout or control mice were removed immediately after the death of the animal and washed in PBS. Lenses were incubated with 50 μM H2O2 or 200 nM TPA for 20 minutes in 2 mL serum-free DMEM (low glucose) media. After treatments, a total 126 nL of Lucifer yellow (2.5 mg/ml in PBS) and rhodamine dextran (1%) was injected into the superficial cortical fibers (around 20 μm in depth) per injection site. After incubation in serum-free DMEM at room temperature for 30 minutes, the lenses were fixed in 2.5 % paraformaldehyde, dissected, and mounted in 3% agar. The extent of dye transfer (in millimeters; diffusion distance of rhodamine-dextran subtracted from Lucifer yellow diffusion distance) as a measure of gap junction permeability in the lens was determined by confocal microscopy. Each experimental group contained six lenses and the distance of dye transfer was determined in six areas of the bow region of each lens in coded samples. Results are expressed as mean ± SEM with $P \leq 0.05$.

Lens Light Microscopy: All lenses were removed immediately and treated with 50 μM H_2O_2 for 1 hour. Lenses were then fixed in a solution of 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate. Lenses were post-fixed with osmium tetroxide, dehydrated, and embedded in epon (LX112). Sections (one μm thick) were stained with toludine blue, and viewed and photographed under a Nikon microscope.

Figure 1

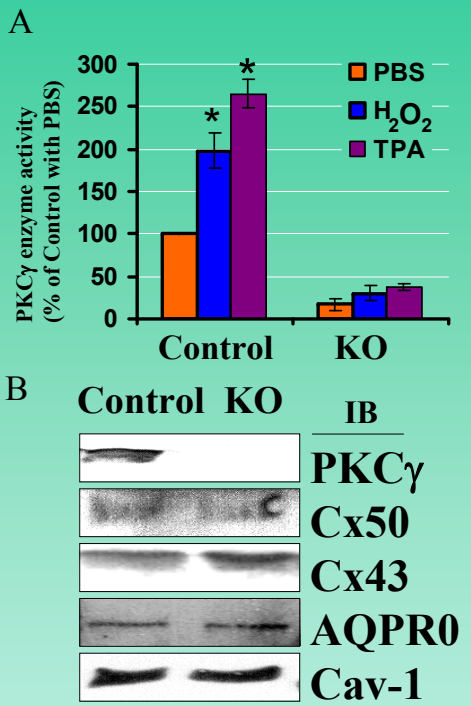

Figure 1 H_2O_2 activates endogenous PKCγ enzyme activity in lenses from the control, but not the PKCγ knockout mice

Figure 2

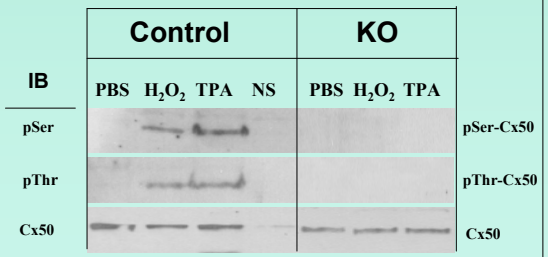

Figure 2 Phosphorylation of Cx50 on serines and threonines is stimulated by H_2O_2 or TPA in lenses from the control, but not the PKCγ knockout mice

Figure 3

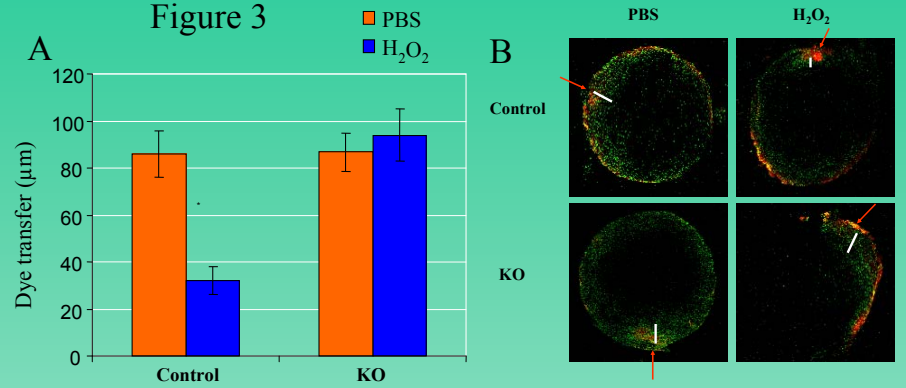

Figure 5 PKCγ knockout abolishes effects of H_2O_2 on decreases in lens dye transfer

Figure 4

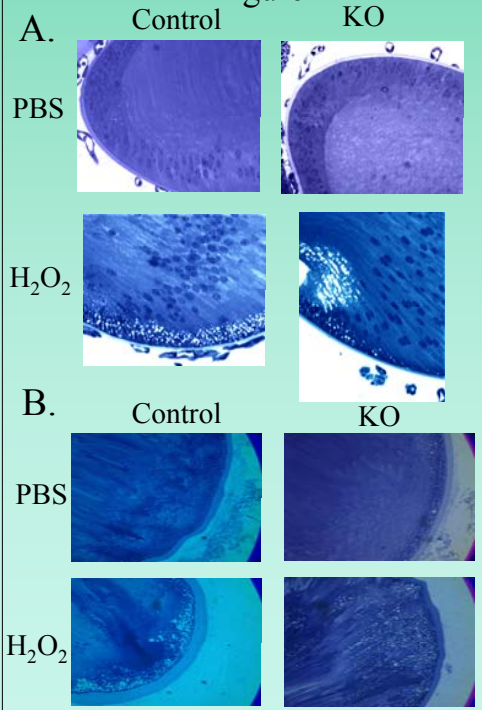

Figure 6 PKCγ knockout lenses are more sensitive to H_2O_2.
A. Two-day-old lenses treated in 10μM H_2O_2 for 1 hour.
B. Six-week-old lenses treated in 50μM H_2O_2 for 1 hour.

Summary

Since gap junctions play important roles in lens homeostasis, passage of apoptotic signals such as high Ca 2+ or ATP to an adjacent cell through open gap junctions could be linked to oxidative cell death. The process is well documented in neural cells and immune cells. Cx50, initially found in the lens, is now known to be extensively found in neural systems, for example, in the optic nerve and central nervous system projections. Cx50 knockout results in a small lens with less differentiation, suggesting that Cx50 is required for lens differentiation and cell growth as well. Cx50 is recently found to bind to calmodulin and to be regulated by Ca 2+. In this report we measured PKCγ activation in both PKCγ knockout and control mice and found that Cx50 phosphorylation and lens gap junction activity in the bow region are specifically controlled by PKCγ. We have demonstrated that the deletion of PKCγ causes lens fiber cells to have open and uncontrolled gap junctions and this subsequently results in fiber cell damage. Oxidative stress is one of the most common causative agents of disease. A life-time accumulation of sublethal oxidative stress damage through open gap junctions would be harmful. It is thus possible that regulation of gap junctions via PKCγ is essential for survival of cells not just in lenses but also in other tissues where this critical enzyme is expressed. Therefore, studies of the interplay of gap junction channels and PKCγ in control and knockout animals might provide useful information for understanding how tissues respond and deal with stress and disease.

Conclusions

- **Deletion of PKCγ alters the arrangement of cortical fibers**
- **H_2O_2 activates endogenous lens PKCγ enzyme activity**
- **Phosphorylation of Cx50 on serines and threonines was stimulated by H_2O_2 or TPA in lenses from the control, but not the PKCγ knockout mice**
- **PKCγ knockout abolishes effects of H_2O_2 on decreases in lens gap junction dye transfer**
- **PKCγ knockout lenses are more sensitive to H_2O_2 damage**

Supported by EY-13421 (D.T.) and a Howard Hughes Fellowship (S.L.)