LENS PKC-γ AND PKC-ε HAVE OPPOSING INTERACTIONS WITH Cx43.

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Abstract

Purpose:

Regulation of intercellular communications (ICC) via gap junction coupling (GJC) is extremely important for maintaining cell growth and surviving under stress conditions such as hypoxia and oxidative stress. Such combination of growth and stress conditions is typical for the lens epithelial cells (LEC). Previously, we have shown that insulin-like growth factor-1 (IGF-1) and stress factors such as phorbol-12-myristate-13-acetate (TPA) or oxidative stress (H_2O_2) inhibited ICC in LEC via direct activation of PKC-γ. Activated PKC-γ interacts with and phosphorylates on Serine-368 the main gap junction protein Cx43 that results in the disassembly of gap junction plaques and the loss of ICC. Besides γ, there is the ε isoform of PKC in LEC. Previously it has been shown that PKC-ε plays a key role in cardiac protection against hypoxia. In the heart, PKC-ε activated by growth factors or TPA or hypoxia, interacts with and phosphorylates Cx43 that also has been shown to be involved in the reduction of the size of the infarct area during ischemic preconditioning. The functional role of PKC-ε in the LEC is still unclear. We believe that PKC-ε might serve the same protective function in the LEC. The purpose of this study is to investigate interactions between PKC-ε and Cx43 in the LEC and show how stress factors like TPA, oxidation and growth factors, which are activators of PKC-γ can regulate PKC-ε.

Working Model

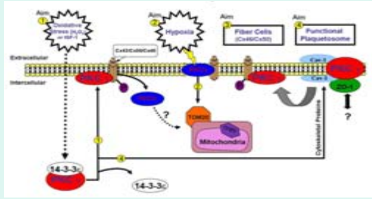

Figure 1 – PKC-γ and PKC-ε are Stress Protectors in Lens –

Aim#1 – Oxidative stress activates PKC-γ which then leaves 14-3-3 and binds to Cx43 (Cx50 and Cx46) and phosphorylates Connexins at the C-terminus. PKC-ε is inhibited and is removed from the Connexins.

Aim#2 – As the lens matures the inner lens is more hypoxic and this activates PKC-ε which translocates to the mitochondria and activates cytIV.

Aim#3 – PKC-γ remains bound to 14-3-3 to be activated by oxidative stress to control fiber cell Cx50 and Cx46.

Aim #4 – The process of PKC delivery to the Cx43 occurs via lens cytoskeletal proteins to create a functional "plaquetosome."

Methods

Tissue Culture : N/N 1003A Rabbit Lens Epithelial Cells or Human Lens Epithelial Cells were grown in DMEM with 10% fetal bovine serum until 90% confluent under normoxic condition in incubator (ambient air plus 5% CO_2).

Cells were treated at 37 °C for 30 minutes with stress factors H_2O_2 (100 μM), TPA (200 nM) and growth factors FGF-2 and IGF-1 (both at 25 ng/mL). After that lens cells were collected and homogenized by sonication and the whole cell homogenate was used for immunoprecipitation (IP) with antibodies against PKC-γ, PKC-ε and Cx43 followed by Western blotting (WB) to analyze protein composition in IP.

Hypoxia : Hypoxic conditions (5% O_2, 5% CO2, 37 ⁰C, 90% humidity) were created with the help of Proox C21 hypoxic chamber (BioSpherix, NY) using nitrogen and CO_2 as a displacement gases. Petri dishes were used for cell culture to ensure appropriate and fast ventilation of cell cultures in the chamber. No changes of pH of cell media were registered during hypoxia incubation up to 4 days.

Western Blot (WB): Proteins were electophoretically transferred from gels to nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in phosphate-buffered saline and probed with PKC-γ, PKC-ε, and Cx43 antibodies overnight at 2 μg/mL. Species specific secondary antibodies, conjugated with horse radish peroxidase followed by x-ray to detect chemoluminescence were used to visualize specific bands.

Confocal Scanning Fluorescent Microscopy: To visualize the specific location of PKC-γ, -ε and Cx43 in the cells before and after abovementioned treatments Confocal Scanning Fluorescent Microscopy with image analyses were used. Lens cells were grown on cover slips, treated as mentioned above and then fixed in 4% paraformaldehyde, permeabilized, blocked and treated with primary antibodies against PKC-γ, -ε and Cx43 at a 5μg/ml concentration. To visualize the specific location of primary antibodies, secondary fluorescent Alexa Fluor antibodies were used.

PKC Enzyme Activity: PKC activity was determined by use of a PepTag Assay Kit (Promega). Each PKC was immunoprecipitated from whole cell homogenate as mentioned above. The immunoprecipitate–agarose bead complexes were used to measure the activity of each PKC isoform.

Immunoprecipitation Analysis (IP): Lens cells were collected from flasks and homogenized by sonication and centrifuged for 30 minutes at 4 °C. The whole cell homogenate was immunoprecipitated with antibodies against Cx43 followed by agarose beads-protein-A immunoprecipitation. The immunoprecipitate–agarose bead complexes were resolved by SDS-PAGE and visualized by Western blot with antisera to PKC-γ, PKC-ε and Cx43.

TPA, IGF-1 and Oxidation activate PKC-γ and inhibit PKC-ε

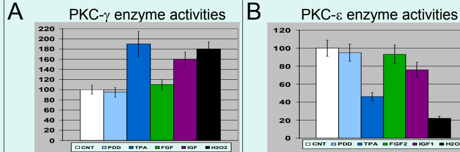

Figure 2 – (A) – PKC-γ and (B) – PKC-ε enzyme activities in the cell homogenates. CNT – control. Cells were treated with PDD (200 nM), TPA (200 nM), FGF-2 (25 ng/mL), IGF-1 (25 ng/mL) and H_2O_2 (100 μM) for 30 min at 37 ⁰C under normoxic condition. Each PKC was immunoprecipitated and enzyme activity was assayed as described in Methods.

Hypoxia activates PKC-ε but not PKC-γ

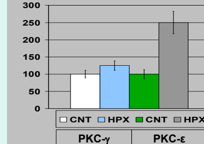

Figure 3 – The effect of hypoxia (5% O_2, 5% CO_2, 37 °C, 24 hours) on enzymatic activity of PKC-γ and PKC-ε in cell homogenates: (CNT) - control, normoxic. (HPX) - hypoxic (5% O_2, 5% CO_2) conditions. Each PKC was immunoprecipitated and enzyme activity was assayed as described in Methods.

TPA activation disrupts the interaction of PKC-ε with Cx43 and promotes interaction with PKC-γ

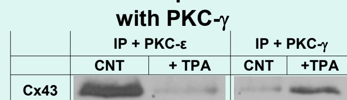

Figure 4 – Western blot analyses of Cx43 in immunoprecipitates with anti-PKC-ε- or PKC-γ antibodies. (CNT) – Control. (+TPA) - TPA treatment (200 nM, 30 min).

Cx43 colocalizes with PKC-ε

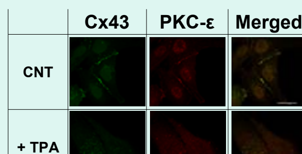

Figure 5 - Confocal images of immunolabeled Cx-43 (Green), PKC-ε (Red) and merged images (yellow). Typical punctate patterns of junctional Cx43 plaques can be seen in control cells. Merged picture demonstrates colocalization of PKC-ε with Cx43 in control normoxic conditions. PKC-γ activators such as TPA (200 nM) induces disappearance of the junctional Cx43 and its colocalization with PKC-ε.

Hypoxia does not induce degradation of Cx43, PKC-γ or PKC-ε

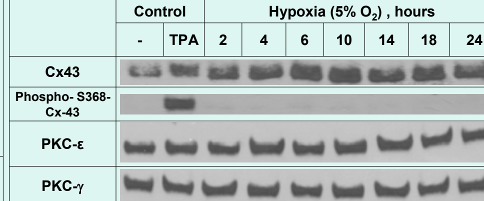

Figure 6 – Western blot analyses of Cx43 in cell homogenates under normoxic (CNT) conditions and under hypoxia. TPA treatment (200 nM, 30 min) induces strong phosphorylation of Cx43 on Ser368. Hypoxia does not induce phosphorylation of Cx43 on Ser368.

Growth and Stress Factors Regulates Cx43 phosphorylation.

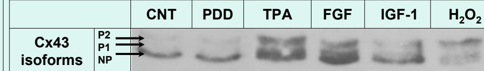

Figure 7 – Western blot analyses of Cx43 in cell homogenates under normoxic conditions. (CNT) – control. TPA and PDD at 200 nM, (30 min). FGF-2 and IGF-1 at 25 ng/mL (30 min). Oxidation by H2O2 at 100 μM (30 min). NP – nonphosphorylated Cx43 isoform. P1 and P2 – phosphoisoforms of Cx43. S368-phosphoisoform of Cx43 migrates together with NP isoform after TPA treatment.

Cellular localization of Cx43, PKC-γ and PKC-ε

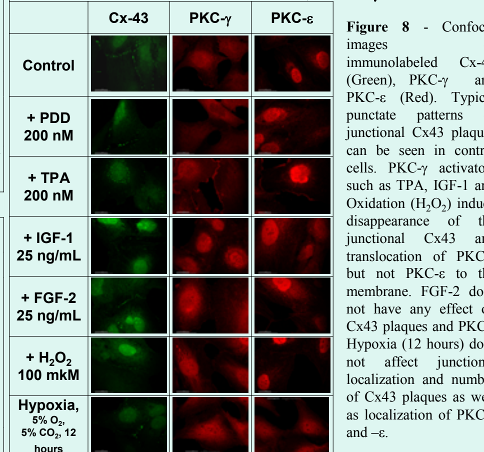

Figure 8 - Confocal images of immunolabeled Cx-43 (Green), PKC-γ and PKC-ε (Red). Typical punctate patterns of junctional Cx43 plaques can be seen in control cells. PKC-γ activators such as TPA, IGF-1 and Oxidation (H_2O_2) induce disappearance of the junctional Cx43 and translocation of PKC-γ but not PKC-ε to the membrane. FGF-2 does not have any effect on Cx43 plaques and PKCs. Hypoxia (12 hours) does not affect junctional localization and number of Cx43 plaques as well as localization of PKC-γ and –ε.

Hypoxia does not reduce the number of Cx43 junctional plaques

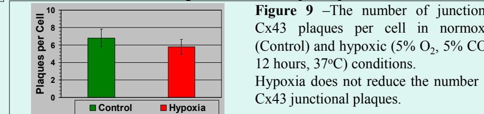

Figure 9 –The number of junctional Cx43 plaques per cell in normoxic (Control) and hypoxic (5% O_2, 5% CO_2, 12 hours, 37°C) conditions.

Hypoxia does not reduce the number of Cx43 junctional plaques.

PKC-ε Knockout Mouse Lenses Show Abnormal Morphology

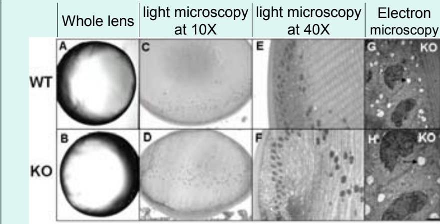

Figure 10 – PKC-ε Knockout Mouse Lenses (KO) Compared to wild type Controls (WT). Lenses from PKC-ε knockout mice (B, D, F, G, and H) were compared to WT mouse lenses (A, C, and E).

(A) and (B) - Whole lens,

(C) and (D) - light microscopy images of stained sections at 10X,

(E) and (F) - light microscopy images of stained sections at 40X.

Electron microscopy images of PKC-ε knockout lenses were taken at 4000X (G) or 7000X (H). Numerous large vacuoles are the main features (left to right, arrows). Lenses are from 6 week old mice.

The PKC-ε knockout lenses shown, from 6-week-old mice, are not altered in size when compared to control lenses (A and B), but have large vacuoles in the bow region and this region is disorganized (E and F). Electron microscopy (G and H), indicates that there are numerous vacuoles in lenses from the PKC-ε knockout mice. Like the PKC-γ knockout, the PKC-ε knockout lenses have nuclei which may reflect a delay in maturation (C and D).

Summary

1) Stress factors such as phorbol ester (TPA), oxidation (H_2O_2) and hypoxia and IGF-1 have opposite effects on enzyme activity in the lens epithelial cells (Figure 2).
2) PKC-γ was activated and PKC-ε was inhibited by phorbol ester (TPA), oxidation, and IGF-1 (Figure 2).
3) Hypoxia activates PKC-ε (Figure 3).
4) Phorbol ester (TPA) induces a decreased association of Cx43 with PKC-ε and increased association of PKC-γ with Cx43 (Figure 4).
5) Cx43 colocalizes with PKC-ε (Figure 5).
6) Stress factors such as phorbol ester (TPA), oxidation (H_2O_2) but not Hypoxia cause the disassembly of junctional Cx43 plaques (Figure 5 and 8).
7) PKC-ε activation by Hypoxia (Figure 3) does not induce the phosphorylation of Cx43 (Figure 6).
8) PKC-γ activation by phorbol ester (TPA), oxidation (H2O2) or growth factor (IGF-1) induces the phosphorylation of Cx43 and produces several phosphoisoform of Cx43 (Figure 7).
9) Hypoxia does not induce the disassembly of junctional Cx43 plaques (Figure 8 and 9), does not induce the phosphorylation or degradation of Cx43, PKC-ε or -γ (Figure 6).
10) Lenses from PKC-ε knockout mice are smaller and have an elongated shape and exhibit abnormal morphology of the bow region (Figure 10).

Conclusion

PKC-γ and PKC-ε have opposing effects on lens Cx43. PKC-γ is activated by stress factors or growth factors and this increases its interaction with Cx43 and displaces PKC-ε from Cx43. Similar treatments cause inhibition of PKC-ε activity and a decreased association of PKC-ε with Cx43. On the contrary, hypoxia, another stress factor, does not affect PKC-γ activity but activates PKC-ε. Results of PKC-ε knockouts mouse lenses suggest that PKC-ε is required for normal lens morphology.

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