Connexin 43 (Cx43) Uptregulation Protects Retinal Endothelial Cells Against High Glucose Insult

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Abstract

Background and Objectives: Apoptotic cell death of retinal endothelial cells, including endothelial cells, is a prominent characteristic of early diabetic retinopathy (DR). In this study, we investigated whether or not high glucose induced downregulation of Connexin 43 (Cx43) could be protected against via plasmid transfection of full length Cx43 cDNA and studied its effects on Gap junction Intercellular Communication (GJIC), apoptosis, and permeability of rat retinal endothelial cells (RRECs).

Methods: To determine whether or not Cx43 expression could be protected against in high glucose condition, RRECs were grown in normal (N) or high glucose (HG) medium for 7 days and transfected with a plasmid containing full length Cx43 cDNA isolated from these cells, as well as non-transfected cells was subjected to Western Blot Analysis to determine Cx43 expression. Additionally, cells were assessed for changes in GJIC using a scrape loading dye transfer assay (SLDT). To identify cells undergoing apoptosis, differential staining with acridine orange/ethidium bromide was performed, and an in vitro permeability assay (IVP) was used to assess changes in cell monolayer permeability.

Results: Western blot analysis confirmed a significant increase in Cx43 expression in cells transfected with Cx43 cDNA compared to non-transfected cells grown in N condition, and the negative effect of HG on Cx43 expression was protected against in these cells. Similarly, SLDT analysis indicated that HG-induced decrease in GJIC was returned to near normal levels in transfected cells. Furthermore, Cx43 transfection protected 50% of the HG cells from apoptosis compared to cells that were not transfected, and reduced cell monolayer permeability in these cells to a similar extent.

Conclusions: Findings from this study indicate that upregulation of Cx43 protects cells from HG-induced apoptosis by improving GJIC, and reduces cell monolayer permeability in RRECs grown in high glucose condition.

Introduction

Intercellular communication is an essential process that allows for the coordination of cellular activity via gap junction channels. These low-resistance channels and hemi-channels are formed by proteins called connexins, and interruption of connexin-mediated intercellular communication disrupt vascular homeostasis. In the retina, the predominant gap junction protein, Connexin43 (Cx43) is abundantly expressed, suggesting an essential role for Cx43 in the maintenance of retinal vascular homeostasis. Decreased expression of Cx43 has been shown to promote apoptosis of endothelial cells in vitro, and lead to the development of vascular lesions in vivo.

Diabetic retinopathy (DR), is the leading cause of blindness in the working age population, and hyperglycemia, the prominent characteristic of diabetes, is known to play a significant role in the development of DR. Early DR is characterized by vascular cell apoptosis and increased permeability. High glucose has been shown to decrease the expression of Cx43 in retinal pericytes and endothelial cells, leading to a decrease in gap junction activity. The suggested importance of Cx43 in the development of diabetic retinopathy is underscored by the fact that streptozotocin-induced diabetic mice and Cx43 heterozygous knock out mice (Cx43−/−) contained similar vascular lesions on microscopic examination of their retinal vascular networks. It is unknown however, whether or not these hallmark changes in diabetic retinopathy can be reversed by increasing Cx43 expression.

To the best of our knowledge, no study has attempted to increase Cx43 expression in retinal endothelial cells to protect them from high glucose insult. In this study, we attempt to rescue rat retinal endothelial cells from high glucose-induced apoptosis, decreased gap junction intercellular communication, and increased vascular permeability by upregulating Cx43 through plasmid transfection.

Methods

Cell Culture

RRECs were grown in normal (5 mM glucose; N) or high glucose (30 mM; HG) medium with 10% FBS and antibiotics for 7 days at 37°C.

Plasmid Transfection

RRECs were grown to 70% confluency in N and HG medium and transfected with plasmid pNPUTHE16 (generously donated by Nalin Kummar, San Diego, CA). This plasmid contained full length Cx43 cDNA using Lipofectamine 2000 transfection reagent (Invitrogen; Grand Island, NY), at a ratio of 1 μL Lipofectamine to 2 μg of DNA. After 48 hr, cells were transfected overnight and subsequently grown in the presence of G418 (Invitrogen) at 500 μg/mL until stable colonies formed (7-14 days).

Western Blot

RRECs from all experimental groups were homogenized and protein samples were isolated. Western blot analysis was carried out on samples containing equal amounts of protein (20 μg) as determined by a bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL) on a 10% SDS-polyacrylamide gel. Western blot procedure was carried out as described and rabbit anti-Cx43 (Cell Signaling; Daves, MA) was used as the primary antibody with an alkaline-phosphatase conjugated anti-rabbit antibody (Cell Signaling) as the secondary. A rabbit anti-β-actin antibody (Invitrogen) was used after membrane stripping to ensure equal amounts of protein were loaded.

Differential Apoptosis Staining

To study apoptosis in transfected and non-transfected cells, a phosphatase buffered saline solution (PBS) containing ethidium bromide and acridine orange at 25 μg/mL each was added for 5 minutes. These cells were then stained with 4% paraformaldehyde (PFA) and visualized under a fluorescence microscope with a DAPI filter. Apoptotic cells have orange nuclei and healthy cells remain mostly green.

Scrape Loading Dye Transfer (SLDT)

SLDT was used to determine GJIC activity. RRECs were grown to confluence on cover slips and random cuts were made in the monolayer using a razor blade. PBS with 0.05% Lucifer Yellow CH (Molecular Probes) was added to the cells and equilibrated for 5 minutes. Cells were then fixed with 4% paraformaldehyde (PFA) and visualized under a fluorescence microscope using a FITC filter. The number of dye-coupled cell layers was counted in ten random fields and averaged.

In Vitro Permeability (IVP)

The permeability of RRECs monolayers was determined by measuring the movement of fluorescein isothiocyanate (FITC) dextran (40 kDa) (Sigma, Saint Louis, Missouri). RRECs were grown on transwell inserts (BD Falcon; San Jose, CA) to confluence and FITC-dextran was added to the upper chambers of the transwell apparatus at 100 μg/mL. After a 2 hour incubation, a 200 μL sample was obtained from each bottom chamber and absorbance at 492 nm was measured.

Results

Figure 1: Western blot data demonstrated ability to control Cx43 expression through plasmid transfection. (A) Cumulative data showed a substantial increase in Cx43 level when transfected with the plasmid containing full length Cx43 cDNA (pCx43) compared to normal. Similarly, RRECs transfected with the plasmid containing dominant negative Cx43 (CX43 Dom Neg) showed a significant decrease in Cx43 expression. Furthermore, when grown in high glucose condition, Cx43 plasmid transfection restored Cx43 expression to a normal level. Data presented as mean ± SD, p<0.05, n=6 (B) Representative WB image demonstrating ability to control of Cx43 expression through plasmid transfection.

Figure 2: Differential staining apoptosis assay. (A) Cumulative apoptosis data demonstrated a significant increase in percentage of apoptosis in normal HG conditions compared to normal and HG conditions. A significant decrease in percentage of apoptosis was a result of Cx43 plasmid transfection. (B) Normal HG conditions. Data presented as mean ± SD, p<0.05, n=6 (B) Representative images demonstrating differences in apoptosis seen in the various growth conditions.

Figure 3: Scrape loading dye transfer assay. (A) As expected, cumulative data indicated a significant increase in GJIC in Cx43 cells transfected with Cx43 compared to normal. Interestingly, GJIC was returned to near normal levels in HG transfection cells grown in high glucose condition. RRECs transfected with dominant negative Cx43 exhibited a consistently decreased level of GJIC activity, as gap junction function was inhibited. Data presented as mean ± SD, p<0.05, n=6 (B) Representative images showing number of dye-coupled cell layers penetrated by Lucifer Yellow in various growth conditions.

Figure 4: In vitro permeability assay. Cumulative data revealed a significant increase in monolayer permeability when non-transfected RRECs were grown in HG condition. Interestingly, when RRECs were transfected with the plasmid containing Cx43 cDNA, this increase in permeability was substantially reduced. RRECs transfected with dominant negative Cx43 demonstrated a consistently increased level of permeability. Data represented as mean ±SD, p<0.05, n=6.

Summary

High glucose growth condition resulted in a significantly reduced level of Cx43, increased apoptosis, decreased GJIC, and increased monolayer permeability. The importance of Cx43 was highlighted by the fact that transfection with dominant negative Cx43 resulted in these same changes, regardless of growth condition. Importantly, in high glucose condition, transfection with Cx43 cDNA restored Cx43 levels to normal, decreased apoptosis, increased GJIC, and suppressed cell monolayer permeability. Cx43 Cx43 cDNA transfection protected RRECs from high glucose insult.

Conclusion

Cx43 cDNA plasmid transfection has a protective effect on RRECs subjected to high glucose insult. Transfection protects against changes in apoptosis, GJIC, and monolayer permeability. Our findings suggest that upregulation of Cx43 may be a useful tool in preventing the vascular cell changes that are characteristic of diabetic retinopathy.

References

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