Effects of High Glucose-Induced Cx43 Downregulation on Occludin and ZO-1 Expression and Tight Junction Barrier Function in Retinal Endothelial Cells

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Purpose. To investigate whether high glucose (HG)-induced downregulation of connexin 43 (Cx43), a gap junction protein, alters ZO-1 and occludin expression and cell monolayer permeability.

Methods. Rat retinal endothelial cells (RRECs) were grown in normal (N; 5 mM) medium, high glucose (HG; 30 mM) medium, N medium transfected with Cx43 siRNA, or N medium transfected with scrambled siRNA. To determine Cx43, occludin, and ZO-1 protein expression, Western blot (WB) analysis and immunostaining were performed. Gap junction intercellular communication (GJIC) was determined using scrape load dye transfer (SLDT) assay. In parallel, cell monolayer permeability was assessed in the four groups of cells, and in cells transfected with Cx43 plasmid or dominant negative Cx43 plasmid.

Results. Connexin 43 protein expression was significantly reduced in cells grown in HG (67 ± 15% of control), and a significant reduction in Cx43 was achieved when cells grown in N medium were transfected with Cx43 siRNA (76 ± 12% of control), with concomitant decrease in GJIC activity. Cells grown in HG showed significant reduction in occludin (77 ± 9% of control) and ZO-1 (80 ± 11% of control) protein level compared with cells grown in N media. Importantly, cells transfected with Cx43 siRNA and grown in N medium showed significant downregulation in occludin (78 ± 8% of control) and ZO-1 (81 ± 6% of control) expression, and exhibited increased cell monolayer permeability. Furthermore, Cx43 upregulation protected cells against HG-induced excess cell monolayer permeability.

Conclusions. Our findings indicate that HG-induced downregulation of Cx43 expression and GJIC may contribute to the breakdown of endothelial barrier tight junctions associated with diabetic retinopathy.

Keywords: connexins, gap junctions, tight junctions

Tight junction abnormalities are closely associated with the progression of retinal pathology in diabetic retinopathy (DR). In particular, breakdown of the blood–retinal barrier (BRB), a prominent pathophysiological event in DR, is linked to reduced expression of tight junction proteins, occluding,[1] ZO-1,[2] and ZO-2.[3] Studies have identified compromised tight junctions in endothelial cells exposed to high-glucose (HG),[1,2,4] retinas of diabetic rats,[1,3,4] and those of diabetic patients.[5] While altered tight junctions are well-established contributors to BRB breakdown, recent studies suggest that decreased level of connexin 43 (Cx43), a gap junction protein, is required to maintain endothelial barrier function.[6,7] Studies have indicated that endothelial Cx43 regulates injury-based permeability[6] and plays a critical role in the maintenance of the blood–testis barrier.[8] We have previously shown that HG or diabetes reduces Cx43 expression and gap junction intercellular communication (GJIC), and promotes vascular damage associated with DR.[9–12] It is unknown, however, whether altered Cx43 level influences excess vascular permeability associated with DR.

Direct association between Cx43 and specific tight junction proteins ZO-1 and occludin is necessary for regulation of functional interdependences between tight junctions and gap junctions. Although HG and diabetes alter the expression of Cx43 and tight junction genes, the molecular interaction and functional relationship between tight and gap junctions remains unclear. The binding of the carboxyl tail of Cx43 to the second PSD95, Dlg1, ZO-1 (PDZ) domain of ZO-1 has been shown to regulate gap junction plaque size in various cell types including cardiomyocytes and neurons.[13–15] Moreover, active binding between ZO-1 and connexins contributes to the dynamic remodeling of gap junctions.[16,17] Recent studies have revealed that specific domains of Cx43, such as the ser(9) and ser(10) at the C-terminal serve as binding sites for interaction with various proteins including ZO-1.[18] Studies have shown that Cx43 colocalizes and coprecipitates with tight-junction molecules occludin and ZO-1, and that ZO-1 may regulate gap junction formation by mediating Cx43 delivery from lipid rafts to gap junction plaques.[19] Taken together, these studies underscore the functional dependence between tight junction and gap junction protein interactions. However, it is currently unknown whether or not HG influences the interactions between the tight junction and gap junction proteins, and if Cx43 influences tight junction assembly in retinal endothelial cells.

While gap junctions have traditionally been known to facilitate cell–cell communication, recent studies have shed light on the adhesive properties resulting from docking of hexameric connexons between two adjacent cells. The adhesive strength conferred by docking of Cx43 hemichannels facilitates aggregation of cells, independent of the cell–cell coupling properties ascribed to the gap junction.[20] Studies have shown that Cx43 expression alone enabled adhesion between these cells,[21] and that docked hemichannels exhibited significant resistance to pulling forces.[22] Currently, it is unknown whether the gap junctions contribute to tight junction barrier characteristics through its adhesive properties.

Although the role of Cx43 in cell–cell communication is well established, its role in facilitating tight junction assembly and maintenance of BRB characteristics in DR is not completely understood. In this study, we have investigated whether HG-induced Cx43 downregulation alters tight junction protein expression and contributes to increased permeability, and whether overexpression of Cx43 could prevent HG-induced excess permeability.

Materials and Methods

Cell Culture

Endothelial cells derived from rat retinas and ascertained positive for von Willebrand (vWF) protein as previously described[23] were used in this study. Rat retinal endothelial cells (RRECs) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), antibiotics, and antimycotics. All experiments were repeated at least four times. To examine the effect of HG-induced Cx43 downregulation on ZO-1 and occludin expression, RRECs were grown in normal (N) medium (5 mM glucose) or HG medium (30 mM glucose) for seven days, and a subset of cells grown in N medium was transfected with Cx43 small interfering RNA (siRNA) after five days in culture. In parallel, normal cells were also grown in mannitol (30 mM) as osmotic control. At confluency, cells were subjected to protein isolation for Western blot (WB) analysis, immunostaining, or scrape-load dye transfer.

Transfection With Cx43 Small Interfering RNA

Rat retinal endothelial cells were transfected with Cx43 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) or nonspecific siRNA (scrambled siRNA, 5′-aauauuggcguuaagauucua-3′; Ambion, Austin, TX) in the presence of 8 μM Lipofectin (Invitrogen, Grand Island, NY) prepared in Opti-MEM (Invitrogen). The Cx43 siRNA contained a pool of three siRNA duplexes (5′-CUGGGUCCUUCAGAUCAUAtt-3′, 5′-CUGAGAA CUACAUCAUCAtt-3′, and 5′-CUCUCGCUUUGAACAUCAUtt-3′) targeted against the rat Cx43 transcript.

Western Blot Analysis

Total protein was isolated from RRECs grown in N, HG, N transfected with Cx43 siRNA, and N transfected with scrambled siRNA. Briefly, to extract total protein, cells were lysed in buffer containing 1xPBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 3M urea, pH 7.4. Cells were centrifuged at 72*g* for 20 minutes at 4°C. Protein concentration in each sample was determined by the bicinchoninic acid protein assay reagents (bicinchoninic acid protein assay; Pierce, Rockford, IL). Western blot analysis was performed with samples containing equal amounts of protein (20 μg) in a 6% or 10% SDS–PAGE. The separated proteins in the gel were then transferred onto a PVDF membrane. Nonspecific binding sites were blocked by incubating the polyvinylidene difluoride (PVDF) membrane in Tris-buffered saline containing 0.1% Tween-20 (TTBS) with 5% nonfat dry milk. Membranes were then incubated overnight at 4°C with rabbit Cx43 (Cell Signaling, Danvers, MA), rabbit ZO-1 (Invitrogen), and rabbit occludin (Invitrogen) antibodies, washed with TTBS three times each for 10 minutes, and then incubated with the anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:3000) (Cell Signaling). Experiments presented here were repeated at least four times. After washing with TTBS, Immuno-Star Chemiluminescent Protein Detection System (BioRad, Hercules, CA) was used to detect protein levels of Cx43, ZO-1, and occludin. Molecular weights were determined by comparison with prestained protein molecular weight standards (ProsieveQuadcolor Protein Markers; Lonza, Allendale, NJ). Densitometric analysis of the chemiluminescent signal was performed at nonsaturating exposures using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Co-Immunoprecipitation Assays

Protein was isolated from RRECs grown in N medium, HG medium, or N medium transfected with Cx43 siRNA, or scrambled siRNA using lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton X-100). One milligram of protein extract from each sample was incubated with 5 uL rabbit anti-Cx43 polyclonal antibody (Cell Signaling) overnight at 4°C. Protein A agarose (Sigma-Aldrich) beads were added and incubated for 2 hours at 4°C. The beads were washed three times with lysis buffer. The retained proteins were eluted with 2× loading buffer and subjected to WB with anti–ZO-1 or antioccludin antibody.

Immunostaining of Cx43, ZO-1, and Occludin

To examine the effect of HG and Cx43 downregulation by siRNA on the localization and distribution of Cx43, ZO-1, and occludin in RRECs, immunostaining for Cx43, ZO-1, and occludin was performed in cells plated on coverslips. Briefly, the cells were fixed with methanol, blocked with 2% BSA in PBS for 30 minutes, and incubated overnight in a moist chamber with mouse Cx43 (Millipore, Danvers, MA), rabbit ZO-1 (Invitrogen), and rabbit occludin (Invitrogen) antibodies in a PBS-BSA antibody solution (1:600, 1:200, and 1:200, respectively). Cells were then washed in PBS and incubated with goat anti-rabbit IgG or anti-mouse IgG secondary antibody conjugated with rhodamine or FITC (Jackson Immunoresearch Labs, West Grove, PA) for 1 hour at 37°C in a dark chamber. The cells were then washed three times in PBS, mounted in Slow-Fade (Invitrogen, Carlsbad, CA), and examined. Negative control samples were processed in the same manner, except that the primary antibody was omitted. The cells were viewed and photographed with a Nikon Diaphot fluorescence microscope and a Nikon F1 digital camera at 800 ms exposure (Nikon Instruments, Inc., Melville, NY). The punctuate Cx43 plaques were assessed at the site of contract between adjacent cells.

In Vitro Permeability as a Function of Cx43 Expression

To examine the effect of HG-induced Cx43 downregulation on cell monolayer permeability, RRECs were grown on cell culture inserts (0.4-μm pore size; Falcon, Paramus, NJ) of transwell plates in N or HG medium for 7 days. Rat retinal endothelial cells grown in parallel were transfected at subconfluency with

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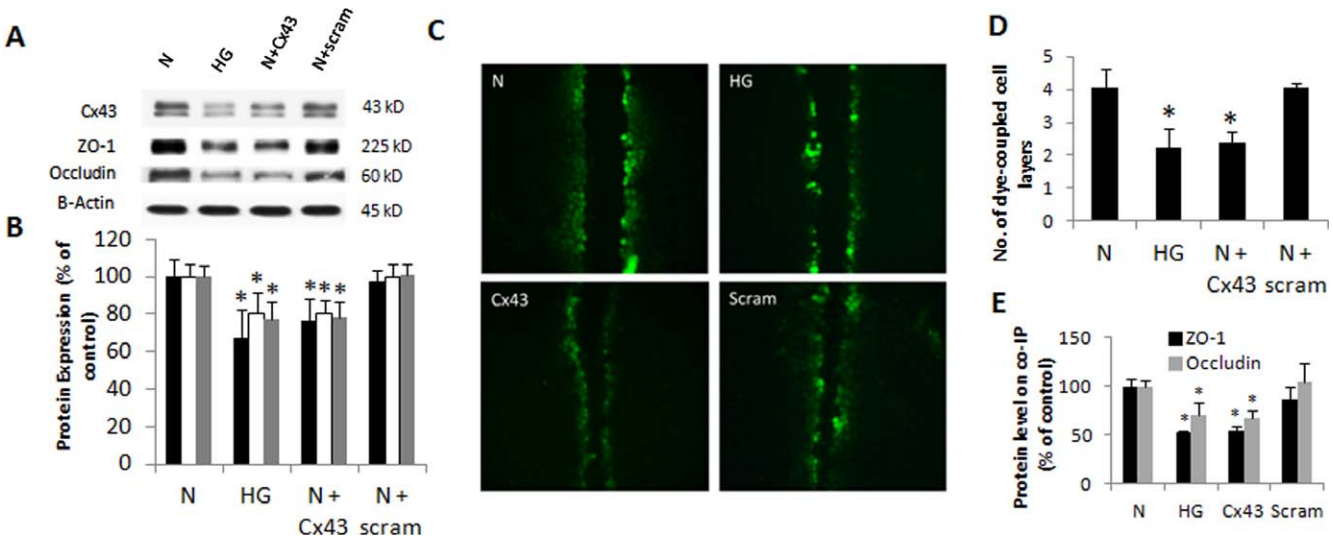

Figure 1. Western blot analysis of Cx43, ZO-1, and occludin protein levels and gap junction intercellular communication activity in RRECs grown in N, HG, N transfected with Cx43 siRNA, and N transfected with scrambled siRNA. (**A**) Cells grown in HG medium showed decreased Cx43, ZO-1, and occludin expression compared with those grown in N medium. Cells grown in N medium and transfected with Cx43 siRNA also showed reduced Cx43, ZO-1, and occludin expression compared to cells grown in N medium. (**B**) A *bar graph* shows cumulative data indicating significant reduction in ZO-1 and occludin expression in cells grown in HG medium or cells transfected with Cx43 siRNA, *bar graph*, *black*: Cx43, *white*: ZO-1, *gray*: occludin. (**C**) Scrape load dye transfer assay after scrape loading indicated reduced transfer of Lucifer yellow into contiguous cells in RRECs grown in HG or transfected with Cx43 siRNA compared with cells grown in N medium or cells transfected with scrambled siRNA. Fluorescent dye was detected in two to three layers in either side of the scrape line in cells transfected with Cx43 siRNA compared with four to five layers in cells grown in N medium or cells transfected with scrambled siRNA, indicating reduced GJIC activity in cells transfected with Cx43 siRNA. Magnification ×100. (**D**) A *bar graph* shows significant reduction in the number of dye-coupled cell layers in cells grown in HG condition or cells transfected with Cx43 siRNA. (**E**) A *bar graph* shows cumulative data from co-immunoprecipitation assays indicating significant reduction in ZO-1 and occludin expression in cells grown in HG medium or cells transfected with Cx43: Cx43 siRNA; scram: scrambled siRNA. Data are presented as mean ± SD, $^*P < 0.05$; $n = 3$ for co-immunoprecipitation assays; all other experiments $n = 4$.

Cx43 siRNA. Cell monolayer permeability was assessed by measuring the diffusion of FITC-dextran (molecular weight [MW] 43 kD; Sigma-Aldrich) from the upper to the lower chamber. The in vitro permeability (IVP) assay was performed as reported earlier.[24–26] Briefly, after cells were allowed to reach full confluence, media from both the upper and lower chamber of all groups was replaced with fresh phenol red-free DMEM. Media in the upper chamber of all groups was replaced with FITC-dextran solution (0.5 mg/mL) and cells were allowed to incubate at 37°C. At the 2-hour time point, 200 μL samples from the lower chamber of all transwells were collected and measured at 492 nm using a spectrophotometric microplate reader (SpectraMax Gemini Vmax; Molecular Devices, Sunnyvale, CA). All experiments were performed in triplicate, and the solute permeability was calculated based on the following formula: (lower chamber fluorescence/input fluorescence) × 100%.

Cell–Cell Communication Assay

We used the scrape-loading dye transfer (SLDT) technique[27,28] to assess GJIC activity. Briefly, cells were grown to confluency and rinsed three times with PBS containing 0.01% Ca^{2+} and 0.01% Mg^{2+} (Ca^{2+}, Mg^{2+}-PBS). An aliquot of 1.5 mL PBS containing 0.05% Lucifer yellow (Molecular Probes, Eugene, OR) was added to the cells, and several scrapes (cuts) were made on the cell monolayer using a surgical scalpel. The cells were incubated for 3 minutes at room temperature in the dye solution and rinsed three times with Ca^{2+}-, Mg^{2+}-PBS to remove any background fluorescence. The cells were then mounted in SlowFade (Invitrogen) and examined. Cells were photographed using a Nikon Diaphot fluorescence microscope and a Nikon F1 digital camera. The dye-coupled cells on either side of the scrape line were counted in random areas to evaluate the GJIC activity in RRECs grown in N or HG medium and in RRECs transfected with Cx43 siRNA or scrambled siRNA.

Plasmid Transfection pNUTHE916

Rat retinal endothelial cells were transfected with plasmid pNUTHE916 containing full length Cx43 cDNA (provided by Nalin Kumar, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago) or pNUTHE916 containing a dominant negative Cx43 (provided by Dr Nalin Kumar) using Lipofectamine 2000 transfection reagent (Invitrogen) at a ratio of 1 μL Lipofectamine 2000 for every 1 μg plasmid DNA in Opti-Mem medium (Invitrogen). Cells were transfected overnight and subsequently grown in the presence of MTX (Sigma-Aldrich) at 25 nM in DMEM with 10% FBS, antibiotics, and antimycotics until stably transfected cells colonies formed (7–21 days). After transfection, protein was isolated and WB analysis was performed to determine Cx43, ZO-1, and occludin protein levels in the transfected cells. Cells transfected with Cx43 plasmid were also subjected to in vitro permeability analysis as described above and SLDT for assessment of GJIC activity.

The dominant negative construct was created in the pNUT vector, the same vector in which the wild-type Cx43 construct was created[29]; the dominant negative construct contained only 1 to 235 amino acids out of the 382 amino acids of the rat a1Cx43 sequence, which prevented its trafficking to the cell surface.

Statistical Analysis

All data are reported as mean ± SD; one-way ANOVA followed by a Student's *t*-test was used to analyze all data.

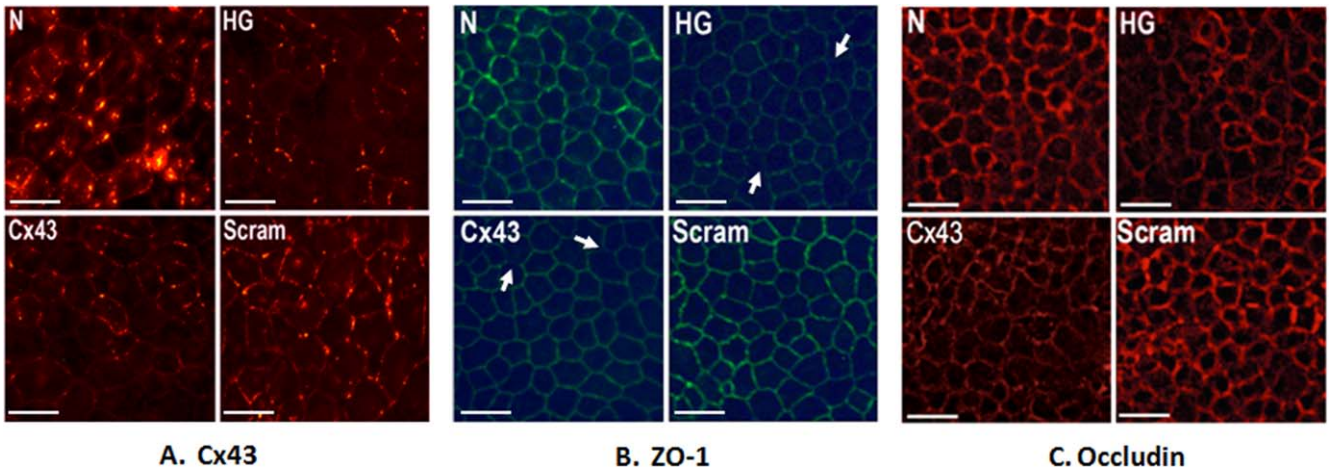

FIGURE 2. Effect of high glucose and Cx43 siRNA on Cx43, ZO-1, and occludin immunoreactivity in RREC. (**A**) Representative images of Cx43 immunostaining show decrease in Cx43 plaques in cells grown in high-glucose medium and cells transfected with Cx43 siRNA compared with cells grown in N medium or cells transfected with scrambled siRNA and grown in N medium. (**B**) Cells grown in high-glucose medium or cells transfected with Cx43 siRNA show reduced ZO-1 immunostaining and increase number of breaks (*arrows*) in ZO-1 at tight junctions as compared with cells grown in N medium or cells transfected with scrambled siRNA and grown in N medium. (**C**) Cells grown in high-glucose medium and cells transfected with Cx43 siRNA show reduced occludin immunostaining as compared with cells grown in N medium or cells transfected with scrambled siRNA and grown in N medium. Images were captured at 800 ms exposure. *Scale bar*: 25 μm.

Data with values of P less than 0.05 were considered significant.

RESULTS

Downregulation of Cx43 Reduces ZO-1 and Occludin Expression in RRECs

To determine whether Cx43 downregulation alters ZO-1 and occludin expression, WB analysis was performed with total protein isolated from RRECs grown in N medium, HG medium, cells transfected with Cx43 siRNA and grown in N medium, and cells transfected with scrambled siRNA and grown in N medium. A significant decrease in Cx43, occludin, and ZO-1 protein levels were observed in cells grown in HG medium compared with those of RRECs grown in N medium (67 ± 15% of control, $P < 0.005$, n = 4; 77 ± 9% of control, $P < 0.01$, $n = 4$; 80 ± 11% of control, $P < 0.05$, $n = 4$, respectively). Importantly, transfection with Cx43 siRNA not only reduced Cx43 protein expression (76 ± 12% of control, $P < 0.01$, $n = 4$), but also significantly reduced occludin, and ZO-1 protein expressions compared with those of cells grown in N medium (78 ± 8% of control, $P < 0.01$, $n = 4$; 81 ± 6% of control, $P < 0.005$, $n = 4$, respectively), or cells transfected with scrambled siRNA (101 ± 6% of control, $P < 0.05$, $n = 4$; 100 ± 7% of control, $P < 0.05$, $n = 4$, respectively). Mannitol used as osmotic control had no significant effect on Cx43, ZO-1, or occludin expression (data not shown). Beta-actin level used as an internal control showed no change in the transfected cells (Figs. 1A, 1B). Additionally, co-immunoprecipitation assay confirmed our initial finding that when Cx43 is downregulated, it negatively affects both ZO-1 and occludin levels in retinal endothelial cells (55 ± 5% of control, $P < 0.05$, $n = 3$; 66 ± 9% of control, $P < 0.05$, $n = 3$) (Fig. 1E). The effect of Cx43 downregulation was also verified at the functional level using SLDT assay. Cells from each of the four groups were analyzed for cell–cell communication. The SLDT results confirmed that downregulation of Cx43 expression in the Cx43 siRNA transfected cells resulted in reduced GJIC activity (Figs. 1C, 1D).

Effect of HG and Cx43 Downregulation on ZO-1 and Occludin Distribution and Localization in RRECs

To determine whether Cx43 downregulation contributes to changes in localization and distribution of tight junction proteins, immunostaining for Cx43, occludin, and ZO-1 was performed in cells grown in N medium, HG medium, cells transfected with Cx43 siRNA and grown in N medium, and cells transfected with scrambled siRNA and grown in N medium. Cells grown in HG medium showed reduced Cx43 immunostaining, as expected, and cells transfected with Cx43 siRNA exhibited significantly decreased distribution of occludin and ZO-1 (Fig. 2) without change in the localization of these proteins. The effect of Cx43 expression on ZO-1 and occludin was not significantly influenced by the state of confluence.

Effect of Altered Cx43 Expression on GJIC Activity in RRECs

To understand the association between reduced Cx43 expression and GJIC activity in RRECs, we measured cell-dye coupling by assessing transfer of Lucifer yellow through gap junctions using the SLDT technique. The total number of dye-coupled cells on either side of the scrape line was significantly reduced in HG condition compared with N condition (2.3 ± 0.6 vs. 4.3 ± 0.5; $P < 0.05$, $n = 4$). Rat retinal endothelial cells grown in N media and transfected with Cx43 siRNA showed a significant reduction in the number of dye-coupled cell layers compared with those transfected with scrambled siRNA (2.4 ± 0.3 vs. 4.1 ± 0.1; $P < 0.05$, $n = 4$) (Figs. 1C, 1D).

Cx43 Downregulation Alone Increases Cell Monolayer Permeability

To determine whether Cx43 downregulation alone alters cell barrier characteristics, we assessed cell monolayer permeability in RRECs grown in N medium, HG medium, cells transfected with Cx43 siRNA and grown in N medium, and cells transfected with scrambled siRNA and grown in N medium. As expected,[30] RRECs grown in HG medium showed

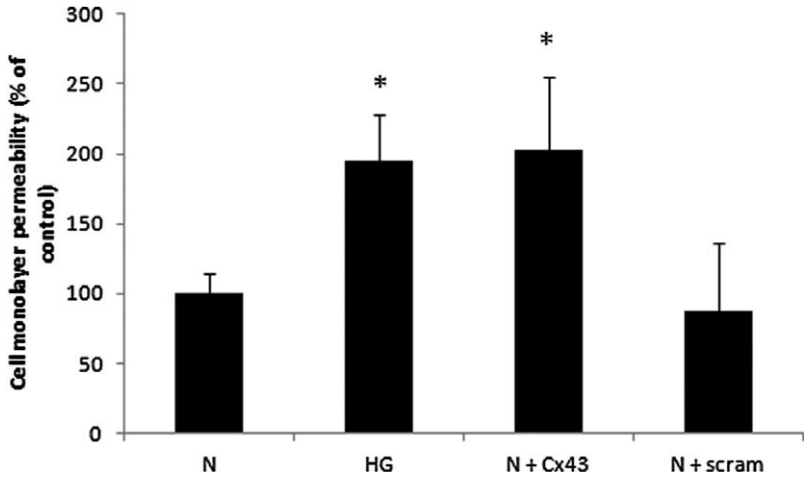

FIGURE 3. Cx43 downregulation alone increases cell monolayer permeability in RRECs. Cells grown in HG medium showed increased monolayer permeability compared with cells grown in N medium. Importantly, cells grown in N medium and transfected with Cx43 siRNA showed increased monolayer permeability compared with cells grown in N medium only. No difference in monolayer permeability was observed in cells grown in N medium compared with those transfected with scram siRNA. Data are presented as mean ± SD, $^*P < 0.05$, $n = 4$.

increased cell monolayer permeability compared with cells grown in N medium (195 ± 34% of control, $P < 0.01$, $n = 4$). Interestingly, cells transfected with Cx43 siRNA showing reduced Cx43 protein expression, exhibited significantly increased cell monolayer permeability compared with those of cells transfected with scrambled siRNA (203 ± 52% of control vs. 88 ± 49% of control, $P < 0.05$, $n = 4$) (Fig. 3).

Cx43 Upregulation Protects Cells Against HG-Induced Increase in Cell Monolayer Permeability and Changes in Tight Junction Protein Expression

To determine whether Cx43 upregulation protects RRECs against HG-induced excess cell monolayer permeability, cells grown in HG were transfected with plasmid pEGFPN1 containing full-length Cx43 cDNA to upregulate Cx43 expression, and as control, cells were transfected with Cx43 dominant negative cDNA. Western blot analysis confirmed Cx43 upregulation in cells transfected with plasmid pEGFPN1 (152 ± 9% of control, $P < 0.05$, $n = 4$) and Cx43 downregulation in cells transfected with Cx43 dominant negative cDNA (59 ± 9% of control, $P < 0.05$, $n = 4$) (Figs. 4A, 4B). In parallel, a significant increase in the number of dye-coupled cell layers was observed in cells transfected with pEGFPN1 and conversely, a reduction in the number of dye-coupled cell layers was observed following Cx43 DN transfection (Figs. 4C, 4D). Interestingly, Cx43 upregulation led to a concomitant increase in both occludin (126 ± 3% of control, $P < 0.05$, $n = 4$) and ZO-1 expression (127 ± 7% of control, $P < 0.05$, $n = 4$). In HG condition, Cx43 plasmid transfection restored Cx43 level to normal (112 ± 10% of control, $P < 0.05$, $n = 4$), and protected cells from HG induced decreases in both occludin (96 ± 4% of control, $P < 0.05$, $n = 4$) and ZO-1 expression (97 ± 6% of control, $P < 0.05$, $n = 4$) (Figs. 4A, 4B). Importantly, Cx43 upregulation inhibited HG-induced increased cell monolayer permeability compared with those of HG cells alone (118 ± 29% of control vs. 195 ± 34% of control, $P < 0.05$, $n = 6$) (Fig. 4E). Cells transfected with dominant negative Cx43 cDNA increased cell monolayer permeability when grown in either N (204 ± 55% of control, $P < 0.05$, $n = 6$) or HG condition (240 ± 16% of control, $P < 0.05$, $n = 6$). Mannitol used as osmotic control had no significant effect on cell monolayer permeability or protein expression. The results demonstrate that Cx43 upregulation could be an effective approach to inhibit HG-induced changes in tight junction protein expression and excess cell monolayer permeability.

DISCUSSION

In the present study, we demonstrated that HG condition significantly reduces Cx43 expression and GJIC activity, and concomitantly decreases ZO-1 and occludin expression in retinal endothelial cells. Furthermore, we observed that downregulation of Cx43 expression, using siRNA in cells grown in N medium, decreases ZO-1 and occludin expression, and increases cell monolayer permeability. Importantly when cells grown in HG condition were transfected with Cx43 plasmid, which abrogated HG-induced Cx43 downregulation and elevated Cx43 expression to near normal levels, a significant decrease in cell monolayer permeability was observed. Taken together these results suggest that Cx43 levels influence cell monolayer permeability, at least in part, by reducing tight junction protein expression.

The possibility that gap junctions may participate in barrier functions of tight junctions has been investigated in brain and lung endothelial cells.[7] The study indicated that Cx43 formed complexes with occludin and ZO-1 in these endothelial cells involved with the blood–brain barrier and that Cx43-based gap junctions contributed to the maintenance of endothelial barrier function. Several studies examining other cell types indicated that change in Cx43 level influences ZO-1 and occludin protein expression and regulates tight junction function. In Sertoli cells, Cx43 not only played a role in cell–cell communication, but also in the regulation of junctional proteins associated with the blood–testis barrier, including occludin[31,32] and ZO-1.[33] In human airway epithelial cells, Cx26 upregulation prevented ouabain-induced disruption in tight junction barrier function.[34] It is currently unclear whether HG-induced downregulation of Cx43 impacts tight junction function in RRECs. The importance of Cx43 in barrier function was underscored in a recent study, which reported increased mortality due to a defect of the epidermal permeability barrier in mice lacking the C-terminal region of Cx43.[35] Further studies are necessary to determine whether HG alters the C-terminal of Cx43 and compromises Cx-mediated adhesion complexes.

Studies have shown that HG decreases the expression of both tight junction and gap junction proteins and compromis-

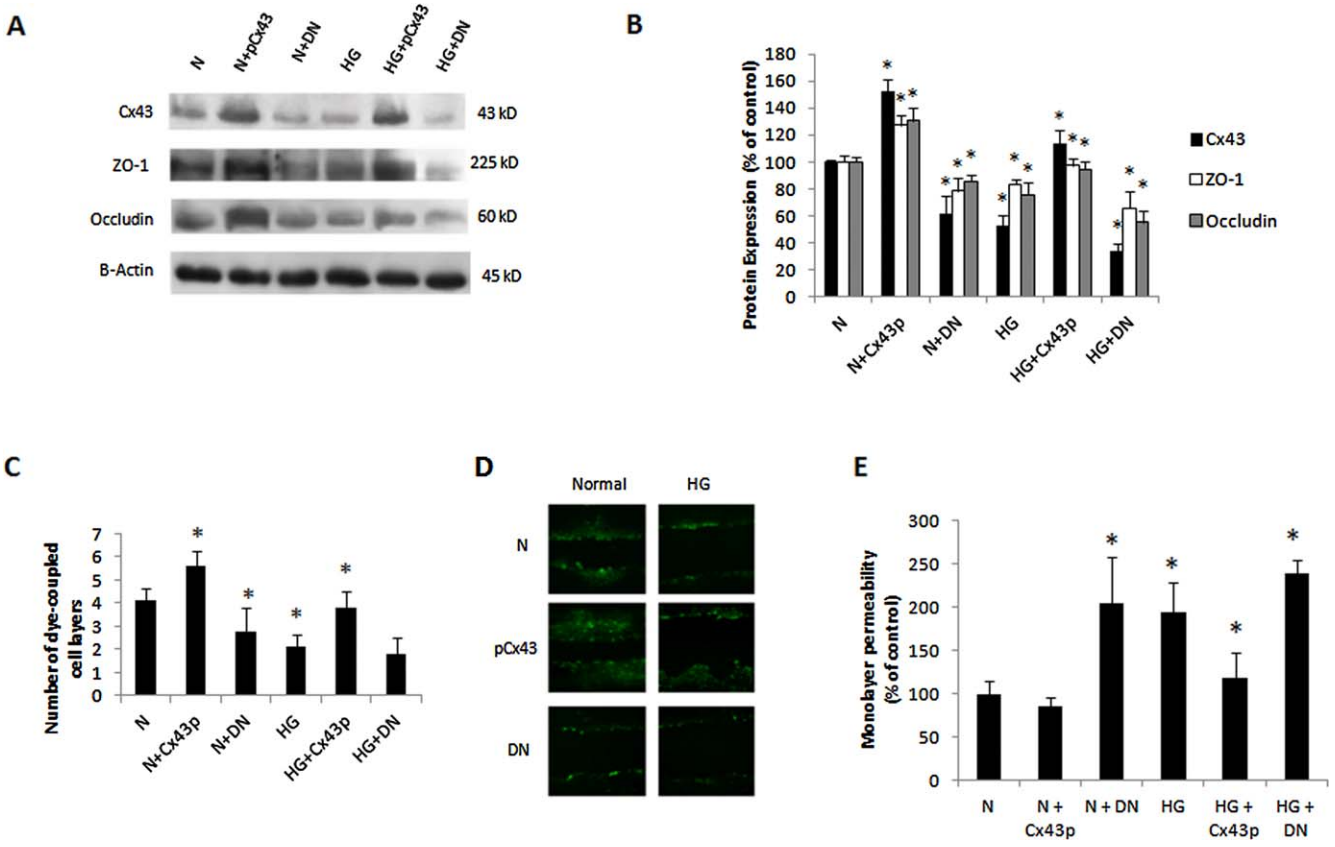

Figure 4. Transfection with Cx43 plasmid upregulated Cx43 level and reduced HG-induced increase in cell monolayer permeability. (**A**) Cells transfected with the pEGFPN1 (Cx43p) showed increased expression of Cx43 in normal condition as well as in high-glucose condition. This increase in Cx43 expression through plasmid transfection resulted in increased ZO-1 expression and occludin expression in both normal- and high-glucose condition. Transfection with dominant negative Cx43 (DN) was sufficient to decrease Cx43 expression, resulting in a concomitant decrease in both ZO-1 and occludin expression. (**B**) A *bar graph* shows cumulative data indicating that cells transfected with Cx43 cDNA showed a significant increase in Cx43, and those transfected with DN Cx43 exhibited a decrease in Cx43 expression. Both ZO-1 and occludin expression increased and decreased relative to Cx43 expression. Data are presented as mean ± SD, $^*P < 0.05$; $n = 4$. (**C**) Cells grown in HG medium exhibited increased monolayer permeability compared with cells grown in N medium. Importantly, transfection with Cx43 plasmid protected cells against HG-induced excess cell monolayer permeability. Additionally, transfection with DN Cx43 cDNA increased cell monolayer permeability in N medium and exacerbated monolayer permeability in cells grown in HG medium, $n = 6$. (**D**) Representative images showing GJIC is increased following Cx43p transfection and reduced following Cx43 DN transfection. (**E**) A *bar graph* shows cumulative data from four experiments. A significant increase in the number of dye-coupled cell layers was observed in cells transfected with Cx43p and conversely, a reduction in the number of dye-coupled cell layers was observed following Cx43 DN transfection. Data are presented as mean ± SD, $^*P < 0.05$. Cx43, Cx43 plasmid.

es their functions. However, it is unclear whether decreased expression of one affects the expression of the other, or whether they are independently affected by HG. While an association between tight junction protein expression and gap junction protein expression has been reported in Sertoli cells,[31,33] this is the first study showing an association between tight junction and gap junction protein expression in RRECs. Interestingly, studies have identified a physical connection between the last 19 amino acid residues of the C-terminus of Cx43 and the second PDZ domain of ZO-1.[36] While both ZO-1 and occludin are known to coprecipitate with Cx43, the exact interaction between Cx43 and occludin remains unclear.[7,37] A few studies have begun to examine the association between Cx43 and ZO-1 and the functional consequences of this interaction. In rat osteoblasts, ZO-1 has been shown to regulate Cx43-mediated GJIC activity and alter membrane localization of gap junctions.[19] Furthermore, absence of ZO-1 has been shown to result in abnormally large gap junction plaques with inactive channels.[38–40] Conversely, Cx43 has been shown to be involved in the maintenance of the tight junctions and play a critical role in the reassembly of the blood–testis barrier.[8] Findings from the current study indicate that HG-induced downregulation of Cx43 inhibits tight junction protein expression. Since there is a physical association between Cx43 and tight junction proteins, it is possible that downregulation of Cx43 levels inhibits tight junction protein expression. Currently, it is unknown whether changes in tight junction protein expression impacts Cx43 expression.

Although the principle role of the gap junction is to allow intercellular communication between two adjacent cells, it has been thought that docking between two hemichannels could provide adhesive strength between these cells. Only recently have studies on cellular adhesive properties reported this possibility. In particular, hemichannels have been shown to initiate adhesive interactions between cells without forming functional gap junctions[21] and promote tissue assembly.[20] Similarly, another study reported that adhesion complexes involving gap junction channels are capable of resisting significant pulling forces similar to those supported by integrins.[22]

Currently the mechanism(s) underlying how Cx43 changes ZO-1 and occludin levels is unknown. However, studies have identified that a relationship exists between the expression levels of these proteins.[41] Changes in connexin expression

have been shown to influence the expression of tight junction proteins. In mouse hepatic cells and human airway epithelial cells, connexin (Cx32 or Cx26) overexpression induced tight junction protein expression and improved barrier function.[34,42] In hepatic cells, for instance, transfection with Cx32 was able to induce the expression of various tight junction proteins including ZO-1 and occluding.[43]

While Cx43 deficiency has been identified as a critical mediator in several diseases, such as arrhythmias,[44] congestive heart failure,[45] and oculodentodigital dysplasia,[46] among others, these diseases are primarily due to functional defects arising from altered cell–cell communication. To the best of our knowledge this is the first study that suggests Cx43 downregulation may contribute to the development of vascular permeability by compromising tight junction protein expression. Aberrant behavior of this nonconventional function of connexin, which is independent of cell–cell communication, may contribute to retinal vascular permeability in DR. Further studies are necessary to determine if improvement in Cx43 levels could be useful as a novel therapeutic intervention in early stage DR.

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