Originales

EFECTO DE LA HIPERGLUCEMIA EN LA EXPRESIÓN DE LAS PROTEÍNAS DE LAS *TIGHT JUNCTIONS* EN CÉLULAS DE EPITELIO PIGMENTARIO DE LA RETINA HUMANA

Introducción: Una de las primeras consecuencias de la retinopatía diabética es la rotura de la barrera hematorretiniana (BHR) causada por la interrupción de las tight junctions. Mientras que la alteración de las proteínas implicadas en la interrupción de las tight junctions de la BHR interna ha sido estudiada extensamente, la información sobre este proceso en la barrera hematorretiniana externa (constituida por el epitelio pigmentario de la retina) es escasa. El objetivo de este trabajo es estudiar el cambio de expresión (ARNm y proteína) de ocludina, zonula occludens-1 y claudina-1 en células de epitelio pigmentario de retina (EPR) humanas con dos concentraciones diferentes de glucosa.

Materiales y métodos: Se utilizó una línea de células de EPR humano (ARPE-19), cultivada durante 3 semanas en un medio suplementado con un 10% de suero bovino fetal y con una concentración de 5,5 mmol de D-glucosa (simulando condiciones fisiológicas) o 25 mmol de D-glucosa (simulando la hiperglucemia que ocurre en pacientes diabéticos). Las proteínas de las *tight junctions* ocludina, zonula occludens-1 y claudina-1 se estudiaron por *Western blot* y PCR real time. Todas las determinaciones se hicieron a los 14 y 21 días.

Resultados: La expresión de ARNm y proteína de ocludina y ZO-1 fue similar en cultivos mantenidos a 5,5 y 25 mmol de D-glucosa. Por el contrario, en condiciones de hiperglucemia (25 mmol) se produjo un claro aumento en la expresión de ARNm de la claudina-1 y en el contenido de esta proteína a los 21 días (concentraciones de ARNm, 1,03 frente a 2,29; proteína, 0,92 frente a 1,14).

Conclusiones: La elevada concentración de glucosa produce una expresión diferencial de las proteínas de las *tight junctions* en células del epitelio pigmentario de la retina humana. Además, nuestros resultados sugieren que la hiperexpresión de la claudina-1 mediada por glucosa participaría en la función de sellado de las *tight junctions.* Las consecuencias funcionales y la traducción clínica de estos hallazgos serán motivo de futuras investigaciones.

Palabras clave: Barrera hematorretiniana. Cultivo celular. Epitelio pigmentario de la retina. Tight junction.

High glucose concentration leads to differential expression of tight junction proteins in human retinal pigment epithelial cells

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Introduction: One of the early features of diabetic retinopathy is the breakdown of the blood-retinal barrier (BRB) due to disruption of the tight junctions. Whereas impairment of the proteins involved in the disruption of the tight junctions of the internal BRB has been extensively studied, there is no information on the direct effect of high glucose concentration on the barrier function of the outer blood-retinal barrier (formed by the retinal pigment epithelium [RPE]). The aim of this study was to explore the effect of high glucose concentration on the expression of tight junction proteins (occludin, zonula occludens-1 [ZO-1] and claudin-1) in a human RPE line under two distinct glucose concentrations.

Materials and methods: An RPE cell line (ARPE-19) were cultured for 3 weeks in a medium supplemented with 10% fetal calf serum containing 5.5 mmol D-glucose (mimicking physiological conditions) or 25 mmol D-glucose (mimicking the hyperglycemia that occurs in diabetic patients). Occludin, ZO-1 and claudin-1 were studied by real-time polymerase chain reaction and Western blot at 14 and 21 days.

Results: Occludin and ZO-1 mRNA levels and protein content were similar in cultures maintained at 5.5 mmol and 25 mmol of D-glucose. In contrast, high glucose concentration (25 mmol) induced a clear upregulation in claudin-1 mRNA expression and protein content at 21 days (mRNA level: 1.03 vs 2.29; protein content: 0.92 vs 1.14).

Conclusions: High glucose concentration leads to differential expression of tight junction proteins in ARPE-19 cells. In addition, our results suggest that the upregulation of claudin-1 by glucose is involved in the increase of tight junction sealing function. The functional consequences and clinical applicability of these findings require further investigation.

Key words: Blood-retinal barrier. Cell culture. Retinal pigment epithelium. Tight junction.

INTRODUCTION

Diabetic retinopathy remains the leading cause of blindness among the working age population, and macular edema is one of the primary causes of poor visual acuity in patients with diabetic retinopathy[1,2]. The breakdown of the blood retinal barrier (BRB) due to the disruption of the tight junctions is the main factor accounting for diabetic macular edema[3]. While extensive work has been carried out to identify the factors involved in the disruption of the tight junctions of the inner BRB, the mechanisms implicated in the outer BRB regulation have been poorly explored.

The retinal pigment epithelium (RPE) is a highly specialized epithelium that serves as a multifunctional and indispensable component of the vertebrate eye. Through the expression and activity of specific proteins, RPE regulates the transport of nutrients and waste products to and from the retina, contributes to outer segment renewal by ingesting and degrading the membranous disks shed by the photoreceptor outer segments, protects the outer retina from excessive high-energy light and light-generated oxygen reactive species and maintains retinal homeostasis through the release of diffusible factors[4]. In addition, RPE forms the outer BRB, thus controlling the flow of solutes and fluid from the choroidal vasculature into the outer retina[4,5]. The inner BRB is constituted by the blood vessels of the retina and directly controls the flux into the inner retina[4,5]. The strict control of fluid and solutes that cross the BRB is achieved through well-developed tight junctions. Over 40 proteins have been found to be associated with tight junctions, including transmembrane, scaffolding, and signaling proteins[6]. Zonula occludens-1 (ZO-1), claudins and occludin are the most studied of these proteins, especially concerning the aspects on how they are related to the BRB.

Treatment of RPE cells with either serum, interferon gamma, tumor necrosis factor alpha, hepatocyte growth factor (HGF), interleukin (IL)-1β or placental growth factor-1 (PLGF-1) decreased transepithelial electrical resistance (TER), increased permeability and altered the expression or content of tight junction molecules[7-11]. However, to the best of our knowledge, the direct effect of high glucose concentrations has never been reported.

The aim of the study was to explore the effect of 5.5 mmol D-glucose (mimicking physiological conditions) and 25 mmol D-glucose (mimicking hyperglycemia that occurs in diabetic patients) on expression (mRNA and protein) of occludin, ZO-1 and claudin-1 in a spontaneously immortalized human RPE line (ARPE-19).

METHODS

Human RPE cell cultures

The immortalized human RPE cell line ARPE-19 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in tissue culture flasks in DMEM/F12 1:1 (Gibco; Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 5.5 mmol D-glucose in a humidified incubator at 37 °C in 5% CO_2. The medium was changed every 3-4 days. The cells used in these experiments were between passages 16 and 19. In order to rule out a potential bias by an osmotic effect the experiment was also performed using manitol as an osmotic control agent.

We preferred to maintain ARPE-19 cultures in base medium with 10% FBS throughout the experiment because it more closely resembled the physiological conditions. In addition, it should be noted that serum deprivation produces a depletion of nutrients that could lead to a non-specific reduction in protein biosynthesis/secretion.

Real-time PCR

RNA was extracted with the Rneasy Mini kit with DNAase digestion. RT-PCR specific primers were used (TaqMan assays): OCLN Hs00170162_m1; TJP1 (ZO-1) Hs00268480_m1; CLN1 Hs00221623_m1. Automatic relative quantification data was obtained with ABI Prism 7000 SDS software (Applied Biosystems, Foster City, CA, USA) using β-actin as endogenous control gene. The measurements were performed at 14 and 21 days.

Western blot analysis

ARPE-19 cells were cultured at confluence in Petri dishes during 14 and 21 days in DMEM/F12 medium containing 10% fetal bovine serum and 5.5 mmol D-glucose or 25 mmol D-glucose. Protein was extracted using lysis buffer (10 mmol TRIS, 50 mmol NaCl, 2 mmol EDTA, 1 mmol $MgCl_2$, pH 7.5, 1% SDS, phenylmethylsulfonyl fluoride and complete protease inhibitors) and then homogenized by sonication. The protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 20 μg protein was resolved by 10% SDS-PAGE (for claudin-1 and occludin) and 7.5% SDS-PAGE (for ZO-1) and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with rabbit primary antibody against claudin-1, rabbit primary antibody against occludin and mouse primary antibody against ZO-1, all diluted 1:1000 (Zymed Lab Gibco; Invitrogen, San Diego, CA, USA), and further incubated with goat anti rabbit or mouse horseradish peroxidase-conjugated secondary antibody (Pierce; Thermo Scintific, Rockford, IL, USA). Proteins were visualized using the enhanced chemiluminescence detection system (Supersignal CL-HRP Substrate System; Pierce; Thermo Scientific, Rockford, IL, USA). The same blot was stripped and reblotted with a mouse primary antibody specific to β-actin (Calbiochem; EMD, Nottingham, UK) to normalize the protein levels. Densitometric analysis of the autoradiographs was performed with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with Quantity One 4.6.2 software (Bio-Rad Laboratories, Hercules, CA, USA). The measurements were perfomed at 14 and 21 days.

Immunohistochemistry

Inmmunohistochemistry was performed in cells grown in 24-well plates containing one circle cover slip of glass (12 mm of diameter) (Thermo scientific, Menzel-Gläser;

Braunschweig, GE) inside each well. The initial concentration was 20,000 cells/ml and cells were grown at confluence for 21 days in DMEM/F12 medium containing 10% FBS under the conditions previously described. Cells were washed with PBS and fixed with methanol for 10 minutes, washed again with PBS two times and blocked with PBS BSA 2% 0.05% Tween overnight at 4 °C. Rabbit anti-claudin1 or occludin, and mouse anti-ZO1 (Zymed Lab Gibco; Invitrogen, San Diego, CA, USA), all diluted 1/200 were incubated for 1 hour at room temperature. After washing two times with PBS, cells were further incubated with Alexa 488 goat anti-rabbit and Alexa 594 donkey anti-mouse secondary antibodies (Invitrogen; San Diego, CA, USA) for 1 hour at room temperature. After washing two more times with PBS the slides were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories; Burlingame, CA, USA) and were observed with a fluorescent microscope (BX61; Olympus, Hamburg, Germany). Images were acquired with a confocal laser scanning microscope (FV1000; Olympus, Hamburg, Germany). Each image was saved at a resolution of 2,048 × 2,048 pixel image size.

Cell counting

Nuclei from seven fields of each condition were counted to determine the total number of cells and cells in division per field. Images were acquired at 20× with the Olympus fluorescence microscope BX61 (equivalent to an area of 0.57 mm^2).

Cytotoxicity

Lactate dehydrogenase (LDH) was measured as an indicator of cell death by using a cytotoxicity detection kit (Roche; Applied Science, Barcelona, Spain). LDH activity was measured in a 96-well plate with two replicates for each condition at an absorbance of 490 nm. Results are expressed as percentage of cells showing cytotoxicity ± SD. Percent cytotoxicity = (exp value – low control) / (high control-low control) × 100.

Statistical analysis

The Kolmogorov-Smirnov test was employed to confirm the assumption of the normality of the variables. Student's t test was used to compare continuous variables that were expressed as mean ± SD. Levels of statistical significance were set at $p < 0.05$. Unless otherwise specified, the results are expressed in arbitrary units.

RESULTS

Real-time PCR

The results of real-time PCR for occludin, ZO-1 and claudin-1 mRNA obtained at 14 and 21 days of culture are shown in figure 1. Occludin and ZO-1 mRNA levels were similar in cultures maintained in 5.5 mmol and 25 mmol of D-glucose at 14 and 21 days. By contrast, high glucose concentration produced a clear upregulation of claudin-1 mRNA expression at 21 days ($1.03 ± 0.48$ vs $2.29 ± 0.7$; $p = 0.039$).
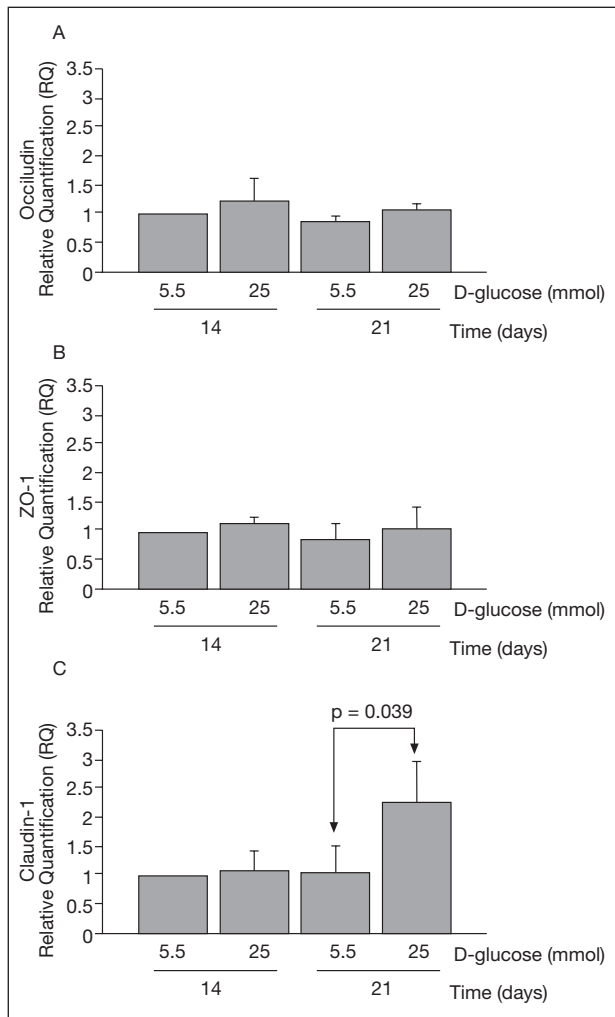

Fig. 1. Results of real-time PCR. A: results of occludin. B: zo-1. C: claudin-1. The vertical axis is the relative expression level of each gene against the 5.5 mmol D-glucose medium at 14 days. Gene expression levels were calculated after normalizing with β-actin. Bars represent the mean ± SD of the values obtained.

Western blot analysis

The results of Western blot analysis are displayed in figures 2-4. Bands at approximately 60 kDa were found for occludin, but there were no significant differences between both glucose conditions in the measurements performed at 14 days ($0.52 ± 0.22$ vs $0.66 ± 0.28$; $p = 0.42$) and at 21 days ($0.44 ± 0.10$ vs $0.38 ± 0.19$; $p = 0.56$) (fig. 2). For ZO-1, a low protein content was observed in samples grown at 25 mmol of D-glucose at 14 ($0.53 ± 0.15$ vs $0.27 ± 0.06$; $p = 0.09$) and 21 days ($0.28 ± 0.09$ vs $0.09 ± 0.07$; $p = 0.1$), but these differences were not significant (fig. 3). By contrast, we found significant differences in claudin-1 expression between the two glucose conditions. At 14 days, we observed a significantly higher claudin-1 protein content in cells cultured under 25 mmol of D-glucose ($0.14 ± 0.08$ vs $0.28 ± 0.11$; $p = 0.04$). This difference was
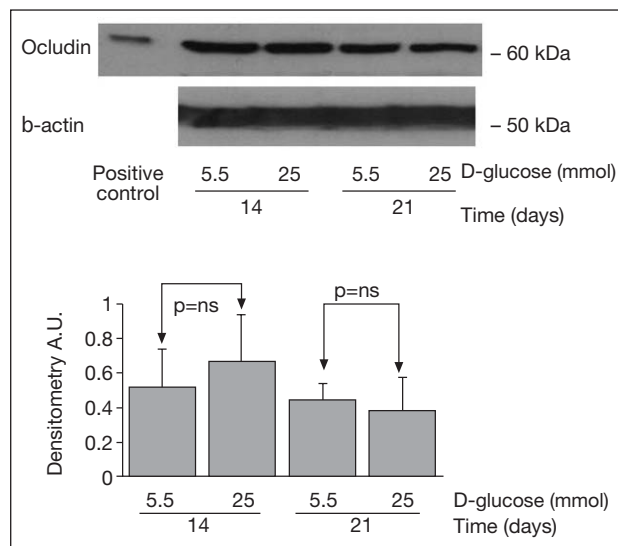
Fig. 2. Results of Western blot analysis of occludin. Protein levels are expressed in arbitrary units after correction for β-actin. Bars represent the mean ± SD. NS: no significant.
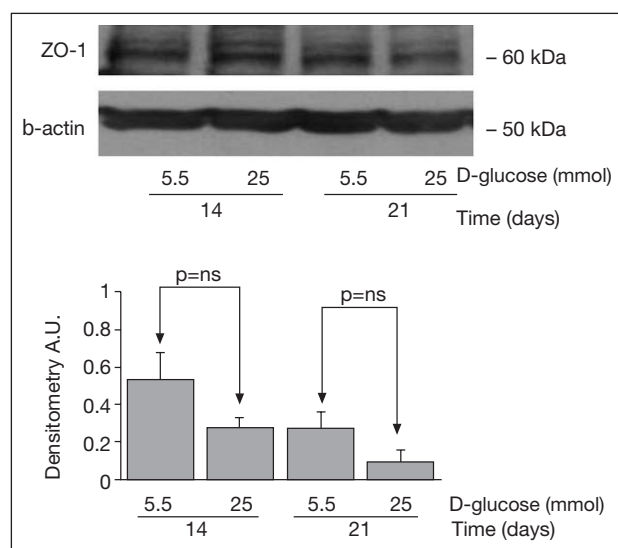

Fig. 3. Results of Western blot analysis of ZO-1. Protein levels are expressed in arbitrary units after correction for β-actin. Bars represent the mean ± SD.

even more evident at 21 days (0.92 ± 0.12 vs 1.14 ± 0.28; p = 0.03) (fig. 4).

Immunohistochemistry

The results of the immunohistochemistry performed at 21 days of the experiment are shown in figure 5. Immunofluorescence confocal micrographs demonstrate that in both glucose conditions the cells were grown forming a monolayer. When cells were cultured at 25 mmol of D-glucose, claudin-1 staining (fig. 5D) appeared to be

stronger than when cultured at 5.5 mmol (fig. 5A). Claudin-1 was observed to colocalize with ZO-1 in junctional complexes (fig 5C, F).

Cell counting and cytotoxicity detection

In order to rule out a potential bias in the results due to changes in cell proliferation, the total number of cells and cells in division were counted. No significant differences were found in the total cell number be-tween 5.5 mmol of D-glucose and 25 mmol (146 ± 27.37 vs. 141.29 ± 20.01; p = NS). The number of cells in division was very similar between both glucose concentrations (16.14 ± 4.95 vs 19.57 ± 7; p = NS). In addition, we did not observe any significant differences regarding cytotoxicity as measured by LDH assay (5.43% ± 0.56 vs 6.72% ± 0.46; p = NS).

Controlling by osmotic effect

The results of RT-PCR and Western blot of tight junction proteins were very similar to the above mentioned when the experiment was controlled by the osmotic effect using manitol (data not shown).

DISCUSSION

The effect of the RPE on the properties of the neighboring cells is well documented but the effects of neighboring environments on RPE are less well studied[12,13]. Intercellular junction integrity of RPE can be impaired by several proinflammatory cytokines, such as HGF and PLGF-1[8-11]. However, the specific
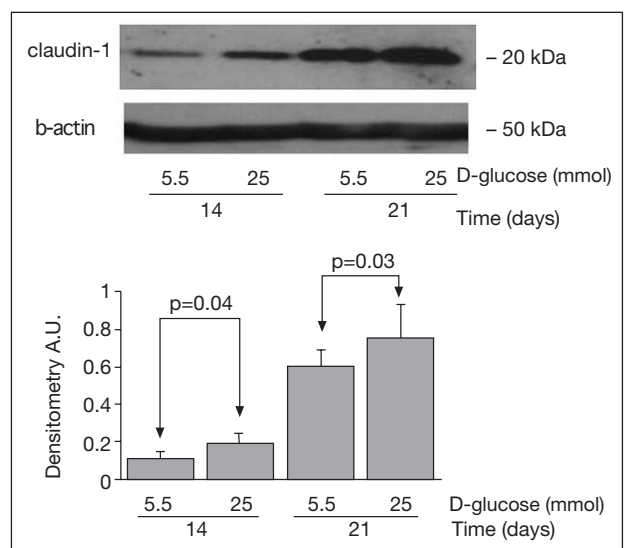

Fig. 4. Results of Western blot analysis of claudin-1. Protein levels are expressed in arbitrary units after correction for β-actin. Bars represent the mean ± SD.
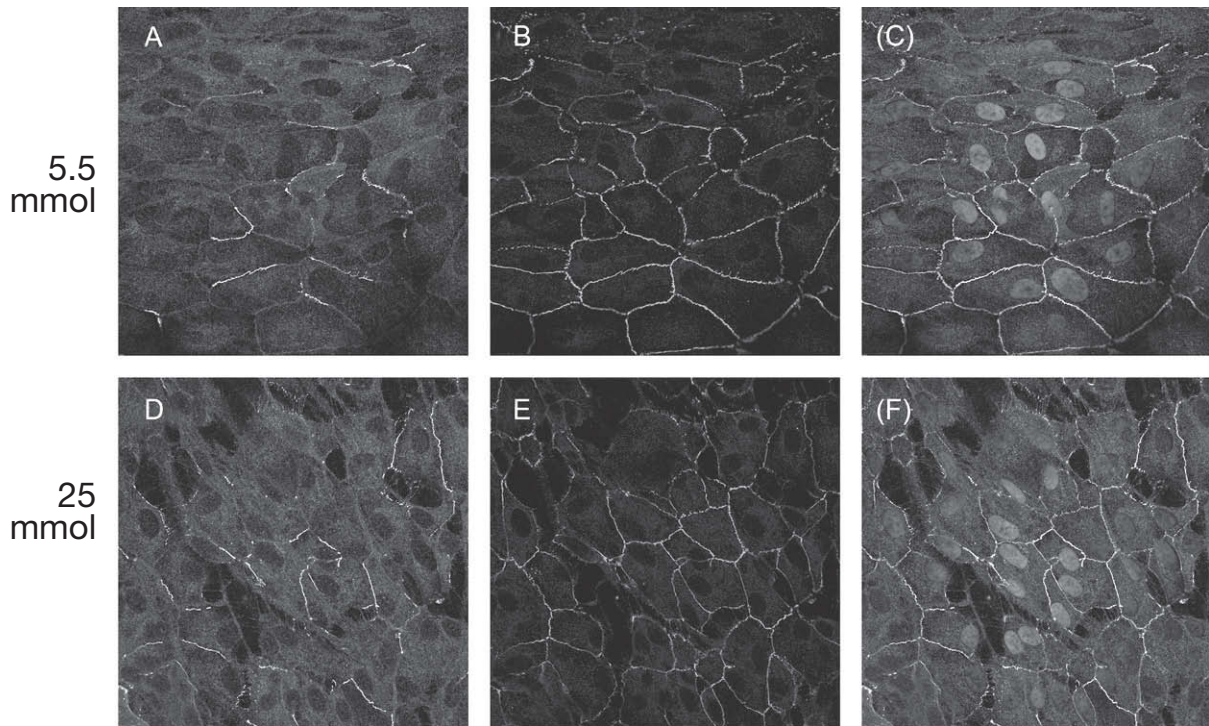
Fig. 5. Results of immunohistochemistry for claudin-1 and ZO-1. Expression of claudin-1 (A, D) and zo-1 (B, E) in ARPE-19 cultured cells. C, F: merged image showing colocalization of claudin-1 and ZO-1.

effects of high glucose concentrations on the function and molecular constituents of RPE cell tight junctions have never been reported. In the present study, we found that glucose at a concentration mimicking severe hyperglycemia (25 mmol) significantly increases the expression and content of claudin-1. Therefore, it seems that high glucose concentrations strengthen rather than weaken the tight junction properties of ARPE-19 cells. These findings have important implications in both the design and the interpretation of the results of in vitro experimental studies using ARPE-19 cultured cells.

There is little information regarding the effect of the pathogenic factors involved in diabetic retinopathy on ARPE-19 tight junction proteins. Abe et al[10] reported that IL-1β impaired the barrier function in ARPE-19 cells and this was accompanied by an aberrant expression of the tight junction molecules (downregulation of occludin and upregulation of claudin-1). Ghassemifar et al[14] demonstrated that VEGF significantly upregulates ZO-1α+ and ZO-1α− transcripts and proteins. In the current study, we found that high glucose concentrations lead to a differential expression of tight junction proteins in ARPE-19 cells. Whereas occludin expression (mRNA and protein levels) was unaffected, a low but not significant protein content of ZO-1 was detected at 14 and 21 days. By contrast, a significant upregulation of claudin expression (mRNA and protein levels) was found at day 14 and was even higher at day 21. This increase of claudin-1 protein content in cultu-

res treated with glucose 25 mmol as compared with 5.5 mmol suggests that glucose exerts its effects on the barrier function by a process involving a specific increase in claudin-1 expression. The complexity of the tight junction complex is just beginning to be understood in epithelial model systems and the relative contribution of the various junctional proteins to BRB properties and the changes in permeability in disease states will be critical areas for future studies. In fact, claudins are thought to constitute the backbone of tight junction strands[15].

The results of the present study can not be easily transferred to clinical practice because the diabetic milieu is something more than high blood glucose levels, and other elements such as cytokines, growth factors, reactive oxygen species and advanced glycation end-products could be involved in the tight junction dysfunction that occurs in diabetic retinopathy. However, our findings strongly suggest that hyperglycemia per se is not an important factor accounting for the impairment of the outer BRB in diabetic retinopathy. It is worth noting that Busik et al[16] have recently reported that in vivo diabetes-related endothelial injury in the retina may be due primarily to the release of cytokines induced by glucose but not a direct effect of high glucose. Another potential weakness of our study is that cultured cells do not perfectly fit in as a model of the tissue from which they were derived, simply because cells need to interact with their environment to maintain a native phenotype. One of the most difficulty pro-

perties to retain in epithelial cell culture is precisely the barrier function performed by tight junctions. However, ARPE-19 cell line is a spontaneously transformed line of human RPE that retains barrier function and, therefore, is a good model for studying RPE tight junctions[17].

In conclusion, high glucose concentration leads to differential expression of tight junction proteins in ARPE-19. Our results suggest that the upregulation of claudin-1 by glucose is involved in the increase of junction sealing function. However, further investigation is needed to determine the mechanisms involved in the up-regulation of claudin-1 mediated by glucose as well as its functional consequences and the clinical applicability of these findings.

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