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

MARTA VILLARROEL[a], MARTA GARCÍA-RAMÍREZ[a,b], LIDIA CORRALIZA[a,b], CRISTINA HERNÁNDEZ[a,b] AND RAFAEL SIMÓ[a,b]

[a]Diabetes and Metabolism Research Unit. Institut de Recerca Hospital Vall d'Hebron. Universitat Autònoma de Barcelona (UAB). Barcelona. Spain.
[b]CIBER of Diabetes and Associated Metabolic Diseases (CIBERDEM). Barcelona. Spain.

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-1by 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.

This study was supported by grants from, Novo Nordisk Pharma SA, Fundación para la Diabetes, Academia de Ciències Médiques de Catalunya i Balears, Instituto de Salud Carlos III (CIBERDEM) and Ministerio de Ciencia y Tecnología (SAF2006-05284).

Corresponding autor: R. Simó, MD, PhD.
Head of Diabetes and Metabolism Research Unit. Institut de Recerca Hospital Vall d'Hebron.
Pg. Vall d'Hebron, 119-129. 08035 Barcelona. Spain.
e-mail: rsimo@ir.vhebron.net

Manuscrito recibido el 17-11-2008 y aceptado para su publicación el 9-1-2009.

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;