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Acute and chronic exposure to high levels of glucose modulates tight junction-associated epithelial barrier function in a renal tubular cell line

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ABSTRACT

Aims: Type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases worldwide. Diabetic nephropathy (DN) is a complication of diabetes and the mechanisms underlying onset and progression of this disease are not fully understood. It has been shown that hyperglycemia is an independent factor to predict the development of DN in individuals with T2DM, however, a link between high plasma glucose levels and renal tubular injuries in DN remains unknown. In this study, we investigated the effect of high levels of glucose (i.e. 180 or 360 mg/dL) for up to 24 h (acute) or over 72 h (chronic) upon tight junction (TJ)-mediated epithelial barrier integrity of the kidney tubular cell line, MDCK.

Methods/key findings: High levels of glucose (180 and 360 mg/dL) induced a decrease in transepithelial electrical resistance associated with an increase in TJ cation selectivity at 24 h or in TJ permeability to a paracellular marker, Lucifer Yellow, at 72 h-exposure when compared to control group (exposed to 100 mg/dL glucose). Immunofluorescence analyses showed that glucose treatment induced a significant decrease in the tight junctional content of claudins-1 and -3 as well as a significant increase in claudin-2 (particularly at 24 h-exposure) and a time-dependent change in occludin/ZO-1 junctional content. The analyses of total cell content of these junctional proteins by Western blot did not reveal significant changes, except in claudin-2 expression.

Significance: Our data suggest that high levels of glucose induce time-dependence changes in TJ structure in MDCK monolayers, suggesting a possible link between hyperglycemia-induced tubular epithelial barrier disruption and diabetic nephropathy.

1. Introduction

Type 2 diabetes mellitus (T2DM) is one of the most prevalent metabolic disorders that are directly associated with obesity and a modern lifestyle [1,2]. The T2DM is initially characterized by a moderate hyperglycemia associated with insulin resistance, which is partially compensated by beta cell hyperplasia/hypertrophy [3]. In later stages, there is a progressive decrease in beta-cell secretory function and mass, leading to a permanent state of dependence on exogenous insulin administration [4–7]. It is known that hyperglycemia can induce various disorders such as cardiac dysfunction, neuropathy, glaucoma, and renal dysfunction [8–10].

Diabetic nephropathy (DN) is a complication of diabetes and the mechanisms underlying the onset and progression of this disease is not fully understood [11–12]. It has been shown that hyperglycemia is an independent factor that predicts the development of DN in individuals with T2DM [11,13,14]. However, the mechanisms involved in kidney injuries associated with high plasma glucose levels remain unknown. In the kidney, acute hyperglycemia is linked to the development of microalbuminuria which occurs within the first five years of T2DM onset, prior to diabetic nephropathy structural changes [13,15]. These latter changes include glomerular hypertrophy and renal fibrosis associated with proteinuria and decreased glomerular filtration rate [12,16–18].

Several studies on DN have shown alterations in the glomerular filtration barrier [11,19]; however, the impact of impairment of the structure and function of renal tubules has been less investigated. In addition, in vitro studies with high glucose concentrations on renal tubular barrier mediated by tight junction (TJ) are lacking. The renal tubular epithelium is well adapted for efficient transport of water, ions, and molecules through the transcellular and paracellular routes, resulting in adjustment of the composition of the glomerular filtrate. This function is vital for the homeostasis of body fluid composition and volume [20]. TJs, the most apically-located of the junctional complex, play a central role in transporting epithelia, such as that lining the renal tubules. While maintaining the apical-basal domains of the plasma membrane, this intercellular junction favors the energy-dependent,

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directional transcellular transport mediated by asymmetrically distributed membrane transporters, carriers, and pumps [21,22]. In addition, TJ provides the paracellular permeability seal through four-pass membrane proteins such as occludin and claudins, which are anchored to the actin cytoskeleton via scaffolding complexes of PDZ-containing proteins (i.e. ZO-1,-2,-3) [23–26]. Differential expression of distinct subtypes of claudins and other junctional proteins has been associated with differences in the functional properties of TJ in epithelia from different organs, including the kidney [20–23]. Yet, alteration to the biochemical organization of TJ, involving cellular redistribution and down- or overexpression of junctional proteins, has been related to the modulation of paracellular epithelial permeability in vitro and in vivo conditions [20,22–30].

In the present study, we investigated whether the glucose at relatively high concentrations may interfere with the TJ-mediated paracellular barrier in the renal tubular epithelium, in order to shed light on a possible repercussion of this mechanism in the development of DN. Therefore, we have examined the acute (up to 24 h) and chronic (at 72 h) effects of two different glucose levels (180 and 360 mg/dL), which represent different severity degrees of hyperglycemia in the diabetic state [5,31,32]. For that, we analyzed the effect of these glucose concentrations on the epithelial barrier function mediated by TJs in MDCK cells, used as an in vitro model of the tubular epithelium [27,28,30,33].

2. Materials and methods

2.1. Cell culture and treatment

MDCK renal epithelial cells were obtained from the Adolfo Lutz Institute (São Paulo, SP, Brazil). MDCK cells were cultured in plastic flasks (Corning, USA) with Minimum Essential Medium (MEM) mixed with 10% fetal bovine serum and 100 IU of penicillin/mL and 100 μg of streptomycin/mL (Cultilab – Campinas, Brazil) at 37 °C in a 5% CO_2 humidified atmosphere (Incusafe Sanyo MCO-17A, Sanyo Electric Ltd., Japan) [27]. For experimental purposes, MDCK cells were seeded at the density of 0.5×10^6 cells/cm^2 on 12 mm or 30 mm (diameter) permeable inorganic membrane inserts (Millicell, Ireland) coated with collagen extracted from Wistar rats tails and placed in 6-well plates (Nest Biotech Co. Ltd., China) containing supplemented MEM. The culture medium was changed every 2 days, and cells passaged weekly. When fully confluent (after 3 to 4 days of seeding), MDCK cells were incubated with different glucose concentrations (100, 180 and 360 mg/dL) for up to 24 h (acute) or 72 h (chronic treatment) on both apical and basal surfaces. MDCK monolayers grown on 12 mm diameter inserts were used for the measurement of transepithelial electrical resistance and then processed for immunocytochemistry. Those monolayers grown on 30 mm diameter inserts were employed to assess the dilution potential difference or paracellular transepithelial flux and then collected for Western blotting. The experiments for each procedure were done in duplicate or triplicate per experimental group and repeated at least three times.

2.2. Measurement of the transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was determined using two Ag/AgCl chopstick electrodes coupled to a voltmeter (EVOM, World Precision Instruments, UK) up to 72 h [27]. For that, monolayers grown on 12 mm Millicell inserts were exposed acutely to Krebs-bicarbonate buffer (composition in mM: NaCl 115, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, $NaHCO_3$ 10, HEPES 15; pH = 7.4) containing 100, 180 or 360 mg/dL glucose and the TEER was measured at 2, 4, 6, 8 and 24 h. In the chronic experiments, the MDCK monolayers were kept in sterile unsupplemented MEM containing the same tested concentrations of glucose for up to 72 h; in this case, the media were changed every 24 h just after the measurement of TEER.

2.3. Measurement of dilution potential difference

The cation selectivity of the paracellular pathway was assessed by measuring the mannitol dilution potential difference (MPD) across MDCK monolayers [28]. For that, MDCK cells, grown on 30 mm permeable tissue culture supports, were first exposed to Krebs-bicarbonate buffer or unsupplemented MEM containing 100, 180 or 360 mg/dL glucose for 24 or 72 h, respectively. After that, the initial potential difference (PD) (mV) was measured and, after the NaCl from the basolateral Krebs solution was isosmotically replaced by mannitol (concentrations in mM: mannitol 229, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, $NaHCO_3$ 10, HEPES 15; pH = 7.4), the final potential difference was determined. The MPD values were obtained by subtracting the initial PD from final PD value and expressed as mV.

2.4. Paracellular transepithelial flux

In order to evaluate the paracellular transepithelial permeability to molecules, we employed the extracellular marker, Lucifer Yellow (LY) (457 kDa) (Life Tech, USA) as previously described [29]. In the case of acute treatment, MDCK monolayers grown on 30 mm permeable supports were transferred to a new plate containing Krebs-bicarbonate buffer with 100, 180 or 360 mg/dL glucose, whereas the apical medium was replaced with Krebs plus glucose containing LY at concentration of 100 μM. As for the chronic treatment, MDCK monolayers were treated with different glucose concentrations added to unsupplemented MEM for 48 h then exposed to Krebs-bicarbonate for 24 h as described above. After the incubation period, triplicates of 0.2 mL samples were taken from the apical and basolateral surfaces and read at 428 nm of excitation wavelength and 535 emission wavelength using a microplate reader (Synergy H1, Biotek Instruments, USA). To calculate the transepithelial flux of LY, apical solution absorbance was expressed as the percentage in relation to the sum of basal + apical solution absorbance (taken as 100% marker concentration).

2.5. Immunocytochemistry

MDCK cell monolayers grown on 12 mm Millicell inserts were fixed and kept in − 20 °C methanol until the immunocytochemistry reaction [30]. For occludin and ZO-1 immunoreactions, cells were firstly permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing with phosphate buffered saline (PBS − 0.05 M; pH = 7.4), the monolayers were incubated with 3% fetal bovine serum (FBS) in PBS for 30 min at room temperature (RT) and then with the primary antibody to claudin-1 (Abcam cat number ab15098, dilution 1:50), claudin-2 (Invitrogen cat number 32-5600, dilution 1:50), claudin-3 (Invitrogen cat number 34-1700, dilution 1:50), occludin (Invitrogen cat number 33-1500, dilution 1:50) or ZO-1 (Invitrogen cat number 61-7300, dilution 1:50) diluted in 3% FBS-containing PBS overnight at 4 °C. Subsequently, monolayers were incubated for 1 h with FITC-conjugated specific secondary antibody (Sigma) and DAPI (Sigma) (in 1% FBS-containing PBS) at RT. Monolayers were then washed several times with PBS, mounted with Vectashield (Vector Laboratories; USA) and analyzed at the same microscopic session using identical illumination/contrast settings in order to compare fluorescence between treated and control groups using an inverted fluorescence microscope (Leica TCS SP5 II or Observer.Z1, Zeiss, Germany). The fluorescence degree was measured using the free software Image J (http://rsbweb.nih.gov/ij/) in 5–6 random areas of each monolayer analyzed. Using the multi-point tool, 60 points were randomly selected at the intercellular contact region in every area sampled and expressed as arbitrary fluorescence units given a total of 1020–2100 point values per experimental group.

2.6. Western blot

MDCK monolayers were scrapped from 30 mm Millicell inserts and

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