Toxicology and Applied Pharmacology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Evidence for a role of claudin 2 as a proximal tubular stress responsive paracellular water channel

Anja Wilmes *, Lydia Aschauer, Alice Limonciel, Walter Pfaller, Paul Jennings

Division of Physiology, Department of Physiology and Medical Physics, Innsbruck Medical University, Innsbruck, Austria

ARTICLE INFO

Article history:
Received 10 January 2014
Revised 16 May 2014
Accepted 27 May 2014
Available online xxxx

Keywords:
Claudin 2
Tight junction
Water permeability
Proximal tubule
Cyclosporine A
Trans-epithelial electrical resistance

ABSTRACT

Claudins are the major proteins of the tight junctions and the composition of claudin subtypes is decisive for the selective permeability of the paracellular route and thus tissue specific function. Their regulation is complex and subject to interference by several factors, including oxidative stress. Here we show that exposure of cultured human proximal tubule cells (RPTEC/TERT1) to the immunosuppressive drug cyclosporine A (CsA) induces an increase in transepithelial electrical resistance (TEER), a decrease in dome formation (on solid growth supports) and a decrease in water transport (on microporous growth supports). In addition, CsA induced a dramatic decrease in the mRNA for the pore forming claudins -2 and -10, and the main subunits of the Na^+/K^+ ATPase. Knock down of claudin 2 by shRNA had no discernable effect on TEER or dome formation but severely attenuated apical to basolateral water reabsorption when cultured on microporous filters. Generation of an osmotic gradient in the basolateral compartment rescued water transport in claudin 2 knock down cells. Inhibition of Na^+/K^+ ATPase with ouabain inhibited dome formation in both cell types. Taken together these results provide strong evidence that dome formation is primarily due to transcellular water transport following a solute driven osmotic gradient. However, in RPTEC/TERT1 cells cultured on filters under iso-osmotic conditions, water transport is primarily paracellular, most likely due to local increases in osmolarity in the intercellular space. In conclusion, this study provides strong evidence that claudin 2 is involved in paracellular water transport and that claudin 2 expression is sensitive to compound induced cellular stress.

Introduction

The immunosuppressive drug cyclosporine A (CsA) was approved for clinical use in the 1980s and revolutionized transplant medicine. Today, CsA is still one of the leading immunosuppressive agents despite the fact that CsA is a nephrotoxin, causing disturbances to the renal vasculature, the glomerulus and the proximal tubule (Burdmann et al., 2003; Olbricht et al., 1994; Pfaller et al., 1986). As a consequence, patients that undergo kidney transplantations have excellent short term survival, but long term survival is challenged by CsA-mediated toxicity and has not been improved since CsA's first introduction to the clinics (Casey and Meier-Kriesche, 2011). CsA belongs to the calcineurin inhibitors and it exhibits its pharmaceutical effect by binding to cyclophilin. This complex inhibits the phosphatase activity of calcineurin, which in turn prevents the activation of nuclear factor of activated T-cells (NFAT) and thereby inhibits IL-2 transcription and T-cell activation (Casey and Meier-Kriesche, 2011). While calcineurin inhibition has been suggested to be directly involved in CsA toxicity (Grinyo and Cruzado, 2004; Naesens et al., 2009), we have recently shown compelling evidence for a calcineurin independent toxicity mechanism (Wilmes et al., 2013). We could show that a supratherapeutic concentration of CsA (15 μM) caused a release of cyclophilin B from the cells, induced mitochondrial injury and ER stress and strongly activated the Nrf2 mediated oxidative stress response and all three branches of the unfolded protein response in RPTEC/TERT1 cells. However, at lower concentrations that still induce full cyclophilin B secretion, no cellular stress was observed (Wilmes et al., 2013). We further demonstrated that CsA increased transepithelial electrical resistance (TEER), a highly sensitive endpoint of chemical induced epithelial dysfunction and toxicity (Mathieu et al., 2005; Rotoli et al., 2002).

Toxic concentrations of compounds will typically decrease TEER due to a loss of monolayer integrity. However, sub toxic concentrations of compounds that do not result in cell death can also increase TEER, although the molecular basis of compound-induced TEER has not yet been elucidated. TEER in intact monolayers is governed by tight junction protein expression. Tight junction proteins include membrane spanning proteins (occludin and the claudin family), and intracellular binding proteins, including zonula occludens 1-3 (ZO1-3). They are responsible for the precise control of paracellular transport of ions and water across epithelial barriers and are additionally involved in regulation of polarity and the control of cell proliferation and differentiation (Aschauer et al., 2013; Findley and Koval, 2009; Koval et al., 2010). Specific claudin

* Corresponding author at: Innsbruck Medical University Department of Physiology and Medical Physics, Fritz-Pregl-Strasse 3, 6020 Innsbruck, Austria. Fax: +43 512 900373800.
E-mail address: Anja.Wilmes@i-med.ac.at (A. Wilmes).

http://dx.doi.org/10.1016/j.taap.2014.05.013
0041-008X/

members can either seal (CLDN 1, 3, 4, 5, 6, 7, 8, 9, 11 and 19) or form selective pores in the paracellular space. The pore forming claudins can be further divided into cation (CLDN 2, 10b, 15, and 16) and anion channels (10a and 17) (Hou et al., 2008; Hou et al., 2012; Krug et al., 2012). Epithelial tightness is therefore governed by the expression of specific claudin subtypes (Hou et al., 2012). Claudin subtype expression varies in different types of tissues and also within specific compartments within the tissue (Turksen and Troy, 2011) and is thus tailored to the physiological role of the tissue. This is best illustrated in the nephron, where different combinations of claudins are expressed within each segment. The proximal tubular region, which constitutively exhibits high rates of transport, expresses the pore forming claudin -2 and -10, whereas distal tubular segments are characterized by high expression levels of sealing claudins, including claudin -3, -4 and -8 and are devoid of pore forming claudins (Kirk et al., 2010; Turksen and Troy, 2011).

The aims of the current study were to further investigate the observed increase in TEER in response to CsA in the human renal proximal tubular cell line RPTEC/TERT1. CsA at 15 μM induced an increase in TEER and a decrease in water transport when cultured on microporous growth supports. CsA also inhibited dome formation when cells were cultured on solid growth supports and decreased mRNA levels of the pore forming claudins 2 and 10 and the Na^+/K^+ ATPase subunits ATP1A1, ATP1B1, FXYD2 and NKAIN4. Thus it is likely that CsA affects both, paracellular and transcellular transport. These processes were further delineated utilizing claudin-2 knock down (KD) cells and inhibition of Na^+/K^+ ATPase with ouabain. We describe for the first time a difference in mechanisms of water transport when cells are cultured on solid growth supports compared to cells cultured on microporous growth supports.

Material and methods

Materials were purchased from Sigma (Vienna, Austria) unless otherwise stated.

Cell culture. RPTEC/TERT1 cells (Wieser et al., 2008) were obtained from Evercyte GmbH (Vienna, Austria) and were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mix (50:50 solution Invitrogen, cat. No. 11966 and No. 21765, respectively) supplemented with 2 mM glutamax (Invitrogen, Vienna, Austria), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite, 100 U/ml penicillin and 100 μg/ml streptomycin, 10 ng/ml epithelial growth factor and 36 ng/ml hydrocortisone as previously described (Limonciel et al., 2012). Before each of the following experiments cells were cultured for at least 10 days to allow full contact inhibition. Medium was refreshed every 2nd or 3rd day during the stabilization period.

Cell treatment with CsA. For microarray analysis, RPTEC/TERT1 cells were cultured on 0.2 μm pore size, 25 mm diameter aluminum oxide filters (Nunc, Thermo Scientific, Vienna, Austria), with 1 ml apical and 2 ml basolateral medium. Cells were treated with 15 μM CsA (Calbiochem, Darmstadt Germany) or 0.1% DMSO as a vehicle control daily for up to 14 days and were harvested at days 1, 3 and 14 as previously described (Wilmes et al., 2013). All other CsA experiments were carried out for a maximum of 7 days, with treatment of 15 μM CsA every 48 h. For western blot and dome formation experiments RPTEC/TERT1 cells were grown in 6-well plates, for immunofluorescence microscopy cells were grown on 18 mm glass coverslips and for TEER and water transport studies cells were grown on 1.0 μm pore size, 24 mm diameter wide PET filters (Millipore, Darmstadt, Germany), with 1.5 ml apical and 2 ml basolateral medium.

Microarray. RNA was extracted on days 1, 3 and 14 using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Microarray analysis was conducted with Illumina ® HT 12 v3 BeadChip arrays (~47,000 transcripts) as previously described (Wilmes et al., 2013). Significance of gene deregulation was calculated using the Benjamini–Hochberg (BH) equation to correct for false discovery rate with a threshold of 0.05 (Benjamini and Hochberg, 1995). Probes which exhibited a BH corrected of p-value <0.05 and fold-change >1.5 were considered significant.

Western blots. Cells were washed in PBS twice and lysed in RIPA buffer supplemented with 1% protease inhibitor cocktail. Cell lysates (20 μg) were separated by 1-DE on a 4–12% gradient NuPage Bis-Tris gel (Invitrogen, Vienna, Austria) in MES buffer for 35 min at 200 V and were transferred to low fluorescence PVDF membranes (Millipore, Darmstadt, Germany) in NuPage transfer buffer with 20% methanol for 0.5 h at 20 V (semi-dry transfer, Invitrogen, Vienna, Austria). Blocking was performed with 5% milk powder in TBS-0.1% Tween (TBS-T) for 1 h. Primary antibody incubation was carried out in TBS-T at 4 °C for 18 h. Secondary antibody was incubated for 1 h at RT. The following dilutions of antibodies were used: rabbit anti-CLDN1 1:500 (Zymed 71-7800), mouse anti-CLDN2 1:500 (Zymed 32-5600), rabbit anti-CLDN3 1:500 (Sigma SAB4500435), rabbit anti-CLDN10 1:500 (abcam ab52234), mouse monoclonal anti-beta-actin 1:40,000 (Sigma A2228), mouse monoclonal anti-aquaporin 1 1:500 (abcam ab9566), Cy5 anti-rabbit 1:5000 (GE Healthcare PA45011), Cy3 anti-mouse 1:5000 (GE Healthcare PA43009). Membranes were scanned using a LAS4000 fluorescent imager (GE-Healthcare, Munich, Germany).

Immunofluorescence. Cells were analyzed by immunofluorescence microscopy as previously described (Limonciel et al., 2012). Briefly, cells were fixed in 100% methanol, blocked in 5% BSA/1% Triton X-100 for 30 min and stained with primary and secondary antibody at RT for 1 h and 35 min, respectively. Slides were mounted with *p*-phenylene-diamine glycerol and images were obtained using a Zeiss Axiophot fluorescent microscope equipped with a cooled CCD camera (Spot Diagnostics, MI, USA) using METAVUE image processing software (Universal Imaging, PA, USA). Primary antibodies are described above and were used at concentrations of 1:300. Secondary antibodies were labeled with Alexa488 (1:800 Invitrogen A21206 and A21059).

Phase contrast microscopy. Phase contrast images were acquired using a Zeiss inverted microscope (Axiovert 100) equipped with a Spot RT3™ camera. RPTECT/TERT1 cells and claudin 2 KD clone 3 were grown on 6-well plastic dishes for at least 10 days after confluence to allow for dome formation. Cells were then treated with either 15 μM CsA or 10–100 nM ouabain.

Functional readouts: Transepithelial electrical resistance (TEER) and water transport studies. TEER was measured using the Endohm and EVOM systems from World Precision Instruments.

Initial experiments for water transport and phenol red absorbance were carried out using iso-osmotic and osmotic apical and basolateral gradients. An osmotic gradient was induced by applying 50 or 100 mM mannitol to either the basolateral or the apical compartment. In addition the Na^+/K^+ ATPase was inhibited with 10 nM ouabain applied basolaterally for 48 h. For the rest of the experiments with either compound treated RPTEC/TERT1 cells or claudin 2 knock down clones, iso-osmotic conditions were used. Water transport and phenol red concentration were analyzed after 48 h. Statistical analysis was performed using a one-way ANOVA with a Dunnett's multiple comparisons post test ($n = 3$).

Total amount of water transport from apical to basolateral was calculated by weighting out total volumes of apical and basolateral media after 48 h. Phenol red absorbance was measured on a Tecan infinitive M200 Pro plate reader A560 after allowing the supernatant sample (100 μl) to equilibrate to ambient CO_2 at RT in ambient air for 1 h (in order to remove the major acid component). Phenol red has been previously used to analyze paracellular permeability in Caco2

Please cite this article as: Wilmes, A., et al., Evidence for a role of claudin 2 as a proximal tubular stress responsive paracellular water channel, Toxicol. Appl. Pharmacol. (2014), http://dx.doi.org/10.1016/j.taap.2014.05.013