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Role of TRPC6 and heat-sensitive TRPV channels in regulation of human podocytes

by Lídia Kardosné Ambrus

Supervisor: Tamás Bíró, MD, PhD, DSc

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By Lídia Kardosné Ambrus molecular biologist (MSc degree)

Supervisor: Tamás Bíró, MD, PhD, DSc

Doctoral School of Molecular Medicine, University of Debrecen

Head of the Examination Committee:	János Szöllősi, PhD, DSc, MHAS
Members of the Examination Committee:	Zoltán Papp, MD, PhD, DSc
	Éva Szőke, PhD

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Head of the Defense Committee:	János Szöllősi, PhD, DSc, MHAS
Reviewers:	Attila Tóth, PhD, DSc
	Péter Sántha, MD, PhD
Members of the Defense Committee:	Zoltán Papp, MD, PhD, DSc
	Éva Szőke, PhD

The PhD Defense takes place at the Lecture Hall of Department of Dermatology, Faculty of Medicine, University of Debrecen
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INTRODUCTION

The kidney and the filtration barrier

The kidneys are two bean-shaped organs found in vertebrates. They are located on the left and right in the retroperitoneal space. The nephron is the morphological and funcional unit of the kidney, which consists of glomerulus and tubules, that drain into collecting duct.

The glomerular capillary wall (GCW) consists of three distinct but closly interacting layers. The inner layer is formed by the fenestrated endothelium while the outer layer is made up of podocytes. Between these cells there is the glomerular basement membrane (GBM), which is the third member of the GCW. The three layers of this GCW acts as filtration barrier. Ultrafiltration through this barrier depends on the charge and size of the molecules in the plasma, and also the net filtration pressure in the glomerulus. At physiological conditions, the net filtration pressure is around 15 mmHg from the capillary to the Bowmn-capsule, so in the direction of filtraion.

The elements of the filtration barrier contribute to the filtration to varying degrees and with different properties. Glomerular capillaries are different from other capillaries in the body. The glomerular capillary wall is not surrouned by smooth muscles, and is highly perforated since the endothel cell layer is not continuous, with 60-100 nm wide fenestrae found between the cells. The endothelium, due to the large gaps and lacking diaphragm, does not act as a real barrier to the molecules in the plasma. Their main role lies in charge filtration, because the endothelial cells are covered with glycocalyx from the luminal side, which is heavily negatively charged, which inhibits the movement of other negatively charged molecules.

The GBM is not amorphous, but is rather a highly organized labyrinth of interconnected polygonal fibrils, which form heterogenous pores. These pores,

ranging from 4 to 10 nm, allow the GMB to act as a physical filter and hold back the large molecules (<10 nm).

The GBM has several components with negative charges, thereby also acting as a charge barrier, but this filtration is not as effective. The stucture and localization of the GBM is especially important, because it physically links the endothelium with podocytes, stabilizing the glomerular filtration membrane. If the molecular structure of GBM is altered, it can lead severe kidney diseases with associated proteinuria.

Podocytes form the outer layer of the GCW. Their structural integrity is key to the development and maintenance of the glomerular filtration barrier. Podocytes play important roles in other ways, and can therefore be referred to as multifunctional cells, as they produce proangiogenic factors that contribute to the development, proliferation, and survival of endothelial cells, the most important being the vascular endothelial growth factor-A (VEGFA). Podocytes together with endothelial cells, produce the constituents of the GBM: endothelium produces cα1α1α2 (IV) collagen and α3α4α5 (IV)-es collagen is created by the podocytes.

The integrity of all three layers are essential for physiological filtration, and a deficit either layer leads to proteinuria.

Podocytes and development of podocytes associated diseases

Proteinuria is the first hallmark of most of kidney injuries, which is an early consequence of podocytopathy. Podocytes are terminally differentiated epithelial cells, which have unique and complex organisation. Differenciated podocytes have an arboriezed body shape, and they form projections like major processes, are reinforced by microtubules and intermedier filaments; and actin-rich foot processes, which are associated with GBM by focal adhesions. The neighboring foot processes are interdigitated by a special adhesion complex,

called the slit diaphram (SD), thereby forming the final barrier for the primer filtrate.

The SD provides a tight fit between neighboring cells, similar to tight junctions, however it lacks E-cadherin and is structuraly porous. Research in the past couple of decades has proven that mutations or malfunctions in the proteins that form the complex, as well as proteins which are in the same signaling pathway with SD proteins play essential roles in the development of proteinuric states in the kidney.

As a result of persistent stressors, podocytes undergo pathological transformation, the central mechanism of which is the so-called foot process effacement (FPE). During this process, the foot processes lose their structural complexity. The surface of the podocytes become smoother and enlarged, foot processes will be shorthened, therefore the filtration slits will rarely develop properly. This mechanism leads to the injury of barrier functions, which leads to the development of proteinuria. Several studies revealed the relationship between podocyte injury and glomerulosclerosis associated with proteinuria. Focal segmental glomerulosclerosis (FSGS) is a pathological entity which is produced by podocytopathia and caracterized with proteinuria, nephrosis syndrome which can lead to loss of kidney functions. There are three forms of FSGS: dysregulated, inflammatory and degenerative.

Over the last two decades, the incidence of idiopathic FSGS has been increasing. Both genetic and enviromental factors can play a role in the development of the disease. Genetic studies revealed that mutations of several proteins which are involved in the podocyte functions can play causative roles in the development of FSGS. Gain-of function mutations of TRPC6 channel, which is a member of TRP superfamily and belongs to the SD complex, can cause a particularly agressive form of autosomal-dominant FSGS.

TRP ionchannels in the kidney and TRPC6

The transient receptor potential protein superfamily consists of 50 proteins, of which 28 members are expressed in mammals. Mammalian TRPs were classified according to sequence homology into 6 subfamilies: canonical/classical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP) and mucolipid (TRPML). TRPC channels showed the highest sequence homology with the Drosophila trp gene, hence their name.

All TRP channels consist of four six-transmembrane domains that assemble as tetramers to form a functional ion channel. The kation permeable pore is formed by the loop between S5 and S6 of all four subunits. The cytosolic N- and C-termini comprise several regulatory domains which contol the activity of the functional channel.

Most of the TRP channels are modulated by Ca^{2+}, therefore they can be considered as the modulators of intracellular Ca^{2+}-signaling. They are expressed in several excitable and non-excitable cells, where they are involved in a wide range of cellular mechanisms, such as homeostasis, sensory and motor functions, muscle contraction, vasomotor regulation. The kidney forms an interesting microcosm on the plasticity of TRP channels, since many members of the family are expressed along the nephron, where they are essential for physiological functions of the kidney. There is more and more evidence that the mutations of TRP channels play crucial roles in genetic and aquired kidney diseases. It is also known that the gain-of-function mutation of TRPC6 leads to FSGS, malfunction of TRPP2 is linked to autosomal-dominantpolycystic kidney disease, mutation of TRPM6 results in hypomagnesemia with secondary hypocalcemia, and dysfunction of TRPV1 is implicated in renal hypertension. TRPV5, TRPV6 and TRPM6 are essential for Ca^{2+} and Mg^{2+} reabsorption.

TRPC6 in the kidney is expressed in all of three cell types of the glomerulus (podocytes, endothelial and mesangial cells), and is also found in the collecting duct. In the podocytes' membrane, TRPC6 is involved in the

formation of the filtration barrier by interacting with SD proteins. TRPC6 creates a complex with podocin, nephrin, α-aktinin-4 and other SD proteins, which is essential in podocyte functions. Nephrin and TRPC6 association is very important in the translocation of TRPC6, because nephrin binds to phosphorilated TRPC6 and inhibits TRPC6 translocation to the plasma membrane.

Elevated TRPC6 expression is a common feature of kidney diseases with proteinuria. TRPC6 functionally associates to the actin cytoskeleton, which rearranges as a result of TRPC6 overexpression. All this suggests that TRPC6 plays a role in the development of pathological conditions in the kidney. Its activation results in an increase of $[Ca^{2+}]_{IC}$, and this effect leads to the abnormalities of SD and/or degradation of foot processes. These pathological processes are amplified by TRPC6 activation.

TRPC6 belongs to the canonical/classical group of TRP superfamily. TRPC channels act as a non-selective cation channel, permeable for Na^{+} and Ca^{2+}. Human TRPC6 is directly activated by diacylglycerol (DAG) and its derivates, independently of the protein kinase C (PKC) system. Therefore TRPC6 can be considered as the classical receptor for DAG. Other $[Ca^{2+}]_{IC}$ signaling pathways also can regulate the function of TRPC6. In the cell, the increased $[Ca^{2+}]_{IC}$ along with DAG activates PKC enzymes, which regulates activity of several proteins in Ca^{2+}-signaling, including TRPC6.

Protein kinase C (PKC) system

The PKC isoenzymes are lipid-activated serine/threonine kinases, which are involved in several signaling pathways and are implicated in regulation of a wide rage of cellular processes. The PKCs are activated by receptors that stimulate the PLC enzyme, which hydrolyzes phosphatydilinositol 4,5-bisphosphate (PIP_2) to DAG and IP_3. These molecules initiate other signaling pathways: DAG activates PKC enzymes, which phosphorylate several proteins and IP_3 mobilizes intracellular Ca^{2+}.

The PKC family is comprised of 10 members, that are divided into three subfamilies, based on their structural differences. The first group is the classical PKCs (cPKC), namely PKCα, -β_1, -β_2 and –γ. For their activation, they require DAG or its analogs, like phorbol 12-myristate 13-acetate, and Ca^{2+}. PKCδ, -ε, -η and –θ belong to the novel PKC subfamily (nPKC). These isoforms are insensitive to Ca^{2+}-, only activated by DAG. The members of the third, so-called atypical PKC subfamily (aPKC), PKCλ/ι és -ζ, are very different from the other PKC isoforms, being insensitive to both DAG, and Ca^{2+}.

In 2011 it was described for the first time, that the PKC system negatively regulates TRPC6 channel activity. While PKC enzymes do not activate TRPC6 channel, they can decrease the channel activity via phosphoriltion. Serine 488, a non-canonical phosphorilation site of the TRPC6, is a target of PKCδ, thereby this enzyme negatively regulates TRPC6. To the best of our knowledge, we do not have information about the regulation of TRPC6 by PKC activity on human podocytes.

Heat-sensitive TRPV channels in the kidney

TRPV1 and TRPV4 of the heat-sensitive TRPV channels, play a crutial role in kidney functions. TRPV1 is involved in mediating renal filtration, and Na^+ and water homeostasis. In chronic kidney diseases and in the inflammatory processes of the kidney, induction of TRPV1 activation might play a beneficial role and could be renoprotective in acute kidney injury.

TRPV4 is present in the water impermeable segments of the nephron. On the renal epithelial cells TRPV4 acts as an osmosensor, but its expression was also described on renal epithelium and smooth muscle cells.

However, we still lack data about the expression of heat-sensitive TRPV channels on the glomerular podocytes and their function in the physiological and pathological processes of podocytes.

AIMS

In our study, we aimed to analyse the regulatory effect of the PKC system on human podocytes expressing TRPC6. In our experiments we used a differentiated conditionally immortalized human podocyte cell line.

We were looking for the answers to the following questions:

1. Is TRPC6 expressed on human podocytes and if so, is it functionally active?
2. Which PKC isoforms are expressed on human podocytes?
3. Which PKC isoenzymes regulate the funcional activity of TRPC6?

In the second section of our experiments we examined the heat-sensitive TRPV1-V4 channels on human podocytes.

Our quetions:

1. Which heat-sensitive TRPV channel is expressed on human podocytes?
2. Do human podocytes react to heat stimulation?
3. Are the TRPV1-V4 channels functionaly active on human podocytes?

MATERIALS AND METHODS

Materials

In our experiments we have prepared stock solutions with 1000-fold concentration compared to the final working solutions. Before treatments the stocks were diluted in culture medium or working buffer to keep the solvent concentration at 0.1 %.

Cell culturing

The conditionally immortalised human podocyte cell line was derived from a nephrectomy specimen. The isolated podocytes were transfected with a temperature-sensitive tsA58 T antigen using a retroviral vector. Cells were able to proliferate at the 'permissive' conditions (33°C), and went through differentiation after transfering to the 'nonpermissive' temperature (37°C). During the differenciation process, cells were converted from cobblestone into arborized cells, and continuously expressed podocyte-specific markers (WT-1, nephrin, podocin). These processes were accompanied by the onset of synaptopodin synthesis, which protein we used as a differentiation marker.

The human podocyte cell line was cultured in 'permissive conditions' in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml fungizone and insulin-transferrin-selenium (1:100) at 33 °C to maintain proliferation. Differenciation was induced by transferring cells to 37 °C and kept in culture for 7 days ('non-permissive' conditions).

Human embrionic kidney cells (HEK293) were cultured in DMEM medium with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml fungizone and 2 mM L-glutamine. The culture medium in case of HEK293T cells was supplemented with non-essential aminacids instead of L-glutamine and cells were kept at 37 °C. Both cell lines were maintained in a humidified, 5% CO_2 containing atmosphere.

RNA isolation, reverse transcription, RT-PCR

Total RNA was isolated using TRIzol reagent. 1 μg of isolated total RNA after DNase treatment were reverse-transcribed (RT) into cDNA with „High Capacity cDNA Kit" and RNasin ribonuclease inhibitor. cDNA samples were amplified on a GeneAmp® PCR System 2400 DNA Thermal Cycler by using specific primers and probes. PCR products were separated with EZ-Vision DNA Dye on 1.5% agarose gel and visualized under UV.

Quantitative real-time PCR (Q-PCR)

Total RNA of human podocyte sample was isolated using TRIzol and reverse-transcribed as described before. PCR amplification was performed on an ABI Prism 7000 sequence detection system according to the 5' nuclease assay by using specific primers and probes and TaqMan Universal PCR Master Mix.

Immunocytochemistry

Human podocytes were seeded and cultured on sterile coverslips under non-permissive conditions for 7 days. On the 7th day the coverslips with differentiated podocytes were fixed and permeabilized. After incubation with blocking solution, cells were probed with primary antibodies overnight at 4°C. Following washing steps in PBS, coverslips were treated with Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 1 hour at room temperature. Nuclei were stained with propidium-iodide or DAPI (4′,6-diamidino-2-phenylindole), then visualisation of the proteins was performed by using Zeiss LSM 510 Meta Confocal Microscope.

Western blot

Cells were seeded and cultured on Petri-dishes and harvested in protease inhibitor cocktail containing detergent mixture. Samples were homogenized using a sonicator on ice. Protein concentrations were determined by BCA reagent and set to 1 mg/ml concentration with destillated water and sodium-dodecyl-sulfate. Protein samples were subjected to SDS-PAGE and transferred

to nitrocellulose membranes. After blocking the free binding sites, membranes were probed with the primary antibodies overnight at 4°C. As secondary antibodies, horseradish peroxidase-conjugated IgGs (1:1000) were employed and the immunoreactive bands were visualized by a SuperSignal West Pico Substrate-Enhanced Chemiluminescence kit using LAS-3000 Intelligent Dark Box Gel Logic 1500 Imaging System. Densitometric analysis was performed using KODAK Molecular Imaging Systems.

Fluorescent Ca^{2+} imaging in single-cell format

Podocytes were seeded to coverslips, and after differentiation cells were loaded with Fura-2 AM fluorescent dye. The culture medium was supplemented with neostigmin to inhibit acethylcholinesterase. Coverslips with the Fura-2 loaded cells were then placed on the stage of an inverted fluorescent microscope. Cells were continuously washed with NTY performed with a background perfusion system. Test solutions were directly applied onto the cells through a perfusion capillary tube at a 0.35 ml/min rate, performed with a local perfusion system. Excitation wavelength was alternated between 340 and 380 nm by a dual wavelength monochromator, while the emission was monitored at 510 nm performed with a photomultiplier.

Fluorescent Ca^{2+} imaging in multi-well format

Human podocytes were cultured and differentiated in 96-well plates. On the 7th day, cells were loaded 1 μM Fluo-4 AM fluorescent dye containing Hank's solution for 30 min. The plates were then placed into a FlexStation 3 fluorescent microplate reader and cytoplasmic Ca^{2+} concentration (reflected by fluorescence; λEX: 494 nm, λEM: 516 nm) was monitored during application of compounds in various concentrations. Data are presented as F1/F0, where F0 is the average fluorescence of the baseline (before compound application) and F1 is the actual fluorescence. When applying antagonists, cells were pretreated for

30 min and the measurements were carried out in the continuous presence of fixed concentration of the applied antagonist.

Patch-clamp measurements

Human podocytes cultured in Petri dishes and differentiation were induced at ’non-permissive’ conditions. Whole-cell patch clamp measurements were made by using an Axopatch 1.D amplifier and Clampex 10.2 software. We recorded GSK1016790A-evoked transmembrane currents. During the experiments, the holding potential was 0 mV, and cells were ramped every 2 s from -120 to +100 mV over the course of 400 ms.

Measuring effect of heat stimulation

Differentiated podocytes were loaded with 1 μM Fluo-4 AM fluorescent dye, and than using Olympus IX83 inverted fluorescent, cells were imaged with constant settings in every 9 s in autofocus mode between each capturing. During measurement, heated solution (50°C) was pipetted into the Petri dishes, wich was cooled down to 40-45°C near the cells. As control, we were used buffer at room temperature.

Gene silencing by RNA interference (RNAi)

Podocytes were seeded in Petri dishes or 96-well plate for fluorescent measurments, and cultured for 7 days under ’non-permissive’ conditions. After podocyte differentiation, cells were transfected with siRNA oligonucleotides targeting human TRPV3 using Lipofectamine^{TM} RNAiMAX transfection reagent and serum-free Optimem medium. For controls, siRNA Negative Control Duplexes (scrambled RNA) were employed. 48 hours after transfection, cells in Petri dishes were harvested to quantitatively evaluate the efficacy of siRNA-driven silencing by Q-PCR and fluorescent Ca^{2+}-measurments were performed on transfected cells in 96-well plates.

Stabile plasmid DNA transfection

HEK293T cells were cultured on 35 mm Petri dishes and at 70-80% confluence were transfected with TRPC6 overexpressing vector (EX-U0193-M09) and Lipofectamin™ 2000 transfection reagent according to the manufaturer's protocol. After 3 and a half hours, medium was changed to selection medium, which was culture medium supplemented with 750 μg/ml geneticin.

Selected cells were cultured in 500 μg/ml geneticin containig culture medium, after which the cells were harvested for RNA and protein samples for RT-PCR and Western blot experiments as described above.

Transient plasmid DNA transfection

We transiently overexpressed human recombinant TRPV1–4 proteins in HEK293T cells. As DNA constructs, sequence of human TRPV1 was cloned in the pCAGGSM2-IRES-GFP-R1R2 vector, and sequences of human TRPV2, TRPV3 and TRPV4 isoforms were cloned in the pCINeoIRES-GFP vector.

HEK293T cells were cultured in Petri dishes and transfected at 50-60% confluence using a mixture of TransIT-293 transfection reagent and plasmid DNA costruct. 48 hours after transfection, cells were harvested and analysed by western blot.

Statistical analyses

Our data were processed using OriginPro 8.6 software. Standard deviations were calculated with OriginPro 8.6 and IBM SPSS Statistics 23. Software. We used Student's *t*-test for comparing two groups or one-way analyses of variance (ANOVA) followed by Bonferroni and Dunnett *post-hoc* tests in case of more than two groups. A value of $p<0.05$ was used to determine statistical significance. Data were presented as mean ± SEM.

RESULTS

In vitro differentiation of human podocytes

In our experiments we used differentiated human podocytes. Conditionally immortalized human podocytes were cultured at 33°C (’permissive’ conditions) where cells could proliferate. Differentiation was induced by transferring cells to 37°C (‘non-permissive’ conditions) and kept in culture for 7 days. Differentiated podocytes express podocyte-specific podocin and actin-bound synaptopodin at high levels. In our culturing system we assessed the process of differentiation using Western blot and immmunocytochemistry by determining the expression of the podocyte-specific markers.

TRPC6 are functionally expressed on differentiated human podocytes

Proliferating and differentiated human podocytes, both expressed TRPC6. As revealed by RT-PCR as well as by Western blotting and immunocytochemistry, TRPC6 channels are expressed by differentiated human podocytes, both at the mRNA and protein levels.

Confirming of specificity of the TRPC6 antibody, we established TRPC6-overexpressing HEK293 cell culture (HEK293-TRPC6). Using RT-PCR and Western blotting, we demonstrated that HEK293-TRPC6 cells expressed TRPC6.

In the next step we analysed the functionallity of the TRPC6 channel by employing Fluo 4-AM-based Ca^{2+}-imaging. In our measurments we used 1-oleoyl-2-acetyl-sn-glycerol (OAG) as a TRPC6 channel activator. OAG markedly increased intracellular Ca^{2+}-level in a dose-dependent manner in human podocytes. Repeating the experiment in low Ca^{2+}-containing buffer, this effect was almost completely abolished suggesting that the OAG induced Ca^{2+}-influx, most probably via TRPC6.

Expression pattern of PKC enzymes on human podocytes – podocytes express multiple PKCs

Since we aimed to uncover the potential regulatory role of the PKC system on TRPC6, we than analysed the expression profile of different PKC izoenzymes on human podocytes. Using Q-PCR, we demonstrated that human podocytes express PKCα and -γ of classical PKC group, PKCδ, -ε, -η and -θ, which are members of novel PKCs and finally the atypical PKCζ at mRNA levels. We confirmed these results of by western blotting: all PKC isoforms detected on the mRNA level were found to be expressed on human podocytes.

Endogenous activity of the PKC system exerts a tonic inhibitory effect on TRPC6

We next assessed whether the modulation of the endogenous activities of these PKC isoforms (especially of those that showed high expression levels) affect the function of TRPC6. In our experiments we used three inhibitors: Gö6976 for inhibition of classical PKCs; GF109203X, which is classical and novel PKC inhibitor and we analysed the effect of rottlerin, as PKCδ blocker. In the Ca^{2+}-measurements differentiated human podocytes were pretreated with one of the mentioned PKC inhibitors for 30 min, than TRPC6 activity was induced by OAG. All inhibitors markedly elevated the Ca^{2+}-signal induced by OAG. These data suggest that, in differentiated human podocytes, the endogenous activities of classical and novel PKCs exert a constitutively present, 'tonic' inhibition on TRPC6.

We also examined the opposite of these effects: by elevating the activity of the PKC system using an exogenous agonist. In the experiments we used the general PKC activator PMA. Following pretreatment of the cells with PMA for 30 min, the OAG induced Ca^{2+}-influx was significantly abrogated, which findings further argue for the inhibitory effects of certain PKC isoforms on TRPC6 function.

It is generally accepted, that the down-regulation of PKC isoforms indicates their activity. We next investigated the putative effects of PMA treatment on expression of PKC isoforms. Using Western blotting we demonstrated that the PMA pretreatment down-regulated the levels of PKCα, -β_1, -β_2 and -η.

All these data suggested that PKCα, -β_1, -β_2 and -η exerted inhibitory effect on TRPC6 channel activity.

The PKC system down-regulated TRPC6 expression on differentiated human podocytes

Previous studies showed that PMA suppressed expression of TRPC6 on glomerular mesangial cells. Therefore, on human podocytes we also examined the effect of PMA treatment on the protein level of TRPC6 using western blotting. PMA treatment markedly suppressed TRPC6 expression in a time-dependent fashion similar to what was seen in the cases of PKC isoform down-regulation.

Human podocytes express heat-sensitive TRPV1, TRPV2, TRPV3 and TRPV4 channels

At the start of our experiments we did not have any data about the expression of heat-sensitive TRPV channels in the podocytes. Therefore we aimed at investigating the molecular expression and functionality of heat sensitive TRPV1–4 channels in human podocytes. First, we investigated the molecular expression pattern of these channels. By Q-PCR we found that all four channels were present on human podocytes. mRNA expression of TRPV1 and TRPV4 was high, but TRPV2 and TRPV3 were expressed at relatively low levels. These data were supported by immunocytochemistry and western blotting: TRPV1 staining was weaker compared to TRPV2-4 channels, which showed intense signals. Specificity of the applied antibodies was checked on TRPV channel-overexpressing HEK293T cells.

Podocytes can be activated by heat pulse

In the next step after confirming the molecular expression of heat-sensitive TRPV channels on human podocytes, we investigated their functionality. Differentiated podocytes were loaded with Fluo 4-AM Ca^{2+}-sensitive fluorescent dye, and than were stimulated with warmed (approximately 45-50 °C) buffer. The majority of the cells reacted to the heat pulse by elevating the intracellular Ca^{2+}-concentration. This result indicated the functional presence of heat-sensitive TRPV channels on podocytes.

TRPV1 channel is not active in human podocytes

To analyse the function of individual TRPV isoforms, we used various pharmacological tools to activate these channels and assessed their specificity by applying specific antagonists, where they were available.

Capsaicin is a potent and specific TRPV1 activator. During the fluorescent Ca^{2+}-measurements, capsaicin was not abble to induce any alteration in the intracellular Ca^{2+}-concentration of the podocytes up to 1 mM. The specific TRPV1 inhibitor capsazenipe and AMG9810 had no effect on the lack of channel activity. Parallel with these experiments we examined the ultrapotent TRPV1 agonist RTX. Similarly, RTX was also ineffective in evoking any Ca^{2+}-signal in podocytes.

These results suggested that TRPV1, in spite of the relatively high expression of the mRNA transcripts, does not form a functional channel in the cultures of differentiated human podocytes.

TRPV2 and TRPV4 were showned functional activity on human podocytes

For activating TRPV2 we applied the phytocannabinoid cannabidiol (CBD). Application of 10 μM CBD moderately elevated the intracellular Ca^{2+}-level in podocytes. In low Ca^{2+} conditions this effect was eliminated, suggesting that CBD activated Ca^{2+}-permeable ion channels in the plasma membrane. The presence of tranilast, a potent TRPV2 inhibitor, effectively abolished the Ca^{2+}-

signal induced by CBD. Our lab, among others, showed that CBD is also a weak agonist of TRPV4. Therefore, we investigated the involvement of TRPV4 in the CBD-induced Ca^{2+}elevations, repeating the experiments in the presence of HC067047, a potent and selective blocker of TRPV4. We demonstrated that HC067047 inhibited the CBD-induced Ca^{2+}-responses approximately as effectively, as tranilast did. These data supported our previous results, that CBD can activate TRPV4 channels and suggested the presence of functional TRPV4 channels in human podocytes.

To further investigate the functionality of TRPV4 channels, we used the hyper-potent TRPV4 channel agonist GSK1016790A. We found that in nM concentrations, GSK1016790A markedly increased the Ca^{2+}-level of human podocytes in a dose-dependent fashion. Analysis of the data showed that after agonist treatment, not only the peak of the transient was higher, but the slope as well. In the presence of HC067047, robust elevation of intracellular Ca^{2+}-concentration by GSK1016790A was strongly inhibited. In the absence of extracellular Ca^{2+}, GSK1016790A elevated the intracellular Ca^{2+}, although it was less potent and slower than in normal buffer, suggesting that functional TRPV4 channels are expressed on the intracellular Ca^{2+} stores, as well. However the fact that in the presence of extracellular Ca^{2+} the rising phase of transients were much steeper, suggested that the majority of TRPV4 channels are activated by the agonist in the plasma membrane.

This was also supported by whole-cell patch clamp experiments in which we detected strong GSK1016790A-induced transmembrane currents whose biophysical characteristics corresponded to TRPV4 currents. GSK1016790A-induced transmembrane currents were also inhibited by HC067047.

4α-PDD is the classical TRPV4 channel agonist. In our experiments we also checked the effect of 4α-PDD on podocytes. Although 4α-PDD was less effective than GSK1016790A, it similarly raised the intracellular Ca^{2+}-concentration, and its effect was also inhibited by HC067047.

TRPV3 agonists increased the intracellular Ca^{2+}-level in human podocytes partly independently of TRPV3

Examing the activity of the TRPV3 channel is a significant challenge, because there are no commercially available specific agonists and antagonists. In the literature, there are some herbal compounds like eugenol, thymol, cavacrol, which are communly used as TRPV3 activators. We tested these compounds on differentiated human podocytes, and they demonstrated marked activation in the concentration range reported for effective activation of TRPV3 channels. However, the dose-response relationships were not saturated and did not show sigmoid shape, suggesting that these compounds can activate other targets, as well.

In contrast, using the synthetic and non-specific TRPV3 agonist 2-APB, a similarly and significantly elevated cytoplasmic Ca^{2+}-concentration was observed from the extracellular space. The dose-response relationship of 2-APB was sigmoidal.

Although we lack specific TRPV3 inhibitors, we analysed the effect of the general TRP inhibitor ruthenium red. Selected concentrations of carvacrol, thymol and 2-APB were only partially inhibited by ruthenium red. We checked the effect of endogenous TRPV3 antagonist IPP, which only partially inhibited the Ca^{2+}-influx induced by selected concentrations of carvacrol, thymol and 2-APB.

Using gene silencing by RNA interference, we further investigated the role of TRPV3. Transfection of the podocytes with siRNA targeting TRPV3 channels resulted in a marked decrease in the expression of the channel. Although the TRPV3 silencing was found effective as verified with Q-PCR, it did not influence the Ca^{2+}-responses evoked by the agonists. These results indicate that TRPV3 channel activators were effective in increasing cytoplasmic Ca^{2+}-concentration of differentiated human podocytes, their application is likely

to evoke several off-target effects and the contribution of TRPV3 channels to these Ca^{2+}-responses is minimal.

DISCUSSION

Focal segmental glomerulosclerosis (FSGS) is a pathological codition, which is a general cause of nephrotic syndrome both in adults and children. It is the most common primary disorder causing end-stage kidney disease. At the early onset of the disease, glomerulosclerosis is either focal (only some of the glomeruli are involved) or segmental (a part of the glomeruli are affected), but during progression of the disease, sclerosis become more widespread and global in the kidney.

Electronmicroscopy revealed that FSGS generally is a disease primary caused by the injury of podocytes, therefore it is considered as a podocytopathy. It is indicated by a characteristic damage of the foot processes termed foot process effacement. Transformation of foot processes requires rearrangement of the actin cytoskeleton, resulting in changes in the shape of podocytes and their withdrawal. Therefore neighboring podocytes can not connect to each other, and integrity of the glomerular filtration barrier is damaged.

The exact cause of FSGS is still unknown. Genetic studies of the last few decades revealed that several proteins of podocytes are involved in the development of glomerulosclerosis. Several causative mutaions in genes coding SD proteins have been identified in the pathogenesis of FSGS, like nephrin, podocin, TRPC6 and other structural proteins like α-actinin 4.

Mutations of the gene encoding TRPC6 channel cause a particularly aggressive form of FSGS. Interestingly, both down- and up-regulation of the channel activity may lead to development of glomerulosclerosis. Since altered TRPC6 signaling was identified in the pathogenesis of genetic and aquired FSGS as well, in the current study we aimed to analyse the putative regulatory effect of the PKC system on TRPC6 channel activity in human podocytes. In the second part of our experiments, we examined the expression of the heat-sensitive TRPV1-4 on human podocytes and we investigated the putative regulatory role of these channels in the podocytes cellular functions.

PKC system exerts tonic inhibitory effect of TRPC6 activity

According to our knowledge, we are the first in the literature to demonstrate that the PKC isoforms play a crutial role in the regulation of TRPC6 in human podocytes. PKC isoforms exert a constitutive inhibition on TRPC6 function. Inhibition of endogenous PKC activities by GÖ6976 (inhibitor of classical PKCs), GF109203X (blocker of classical and novel PKCs) and rottlerin (PKCδ antagonist) pretreatments, significantly elevated the Ca^{2+}-response induced by OAG. In our study we tried to defined, which PKC isoforms are involved in these mechanisms. Using western blotting we showed that after PMA treatment expression level of PKCα, -β1, - β2 and -η were down-regulated. So the PKC inhibitory effect on TRPC6 channel activity and expression are probably due to these PKCs.

TRPC6 and the PKC system have been implicated in numerous physiological and pathological processes of the kidney and its podocytes. On mesangial cells, the relationship between these two systems was previously described. In that study, PMA was shown to suppress TRPC6 levels. In good accordance, in our work we presented the inhibitory effect of PKC on TRPC6 expression on human podocytes. Moreover the major message of our study is that the inhibitory action of PKCs on TRPC6 is not only realized at the molecular expression level but also on the funcional level. We have also identified PKC isoenzymes that are involved in mediating the tonic inhibiton of TRPC6.

To confirm our research results, very similar results have been described in other systems. The noradrenergic neurons of the dorsal spinal cord express TRPC6 at high levels, also regulated by the PKC systems. Similar mechanisms were reported in smooth muscle cells: in TRPC6 expressing cells the Ca^{2+}-signal induced by OAG was augmented after GF109203X treatment and PMA also reduced the effect of OAG. This inhibitory action was suggested to be mediated

by the selective involvement of PKCδ by TRPC6 phosphorilation on the serine 488 residue.

In our work we also analysed the effect of PKCδ using rottlerin as PKCδ antagonist. Pretreatment with rottlerin markedly increased the Ca^{2+}-response induced by OAG, but PMA treatment had no effect on expression of PKCδ protein. Since rottlerin was shown to inhibit activities of numerous other kinase systems (MAP kinase-activated protein kinase-1β, or Rsk-2, and p70 S6 kinase), we cannot exclude the possibility that the effect of rottlerin to increase the TRPC6-mediated Ca^{2+}-signal is due to its effect on other target(s) than PKCδ.

Our findings might have clinical implications. As detailed above, the malfunction of TRPC6 expression and/or activity in a wide variety of genetic and acquired kidney disorders is associated with proteinuria. The pathologically altered TRPC6 signaling leads to pathological intracellular Ca^{2+}-concentration in podocytes, and plays a crucial role in the development of podocytopathies. Interestingly, in many case these phenomena are associated with altered PKC activity. Moreover, in glomerulonephritis up-regulation of PKCβ2 was found, and in membranous glomerulonephritis the expression of PKCα and -β was increased as well. However these studies did not clearly demonstrate that the elevated PKC levels were also accompanied by increased PKC activities.

Our results confirmed that the activation of PKCα, -β_1, -β_2 and -η play essential role in TRPC6 expression and also in the regulation of its activity. It is proposed that pathological *in vivo* conditions altering the expression and/or activation patterns of podocyte-expressed PKCs may influence TRPC6 activity and hence podocyte functions. Therefore, further pre-clinical and clinical studies are now invited to uncover whether the targeted manipulation of activities of certain PKC isoforms might be beneficial in the therapeutic management of given proteinuric kidney diseases with altered TRPC6 functions.

In pathological conditions, levels of several mediators which can activate TRPC6 signaling, are elevated for example angiotensin II (Ang II). Ang II is key

regulator in the development of proteinuria and the progression of kidney injury in kidney deseases, like in FSGS. Ang II binds to its own receptor (AT1R) on the plasma membrane in podocytes and activates PLCβ which generates IP_3 and DAG. DAG activates the PKC system, therefore Ang II by activating the PKC system can inhibit TRPC6 activity. Additionally, DAG is a direct activator of TRPC6. In this way, the signaling pathway connecting with the AT1R can counter-regulate the activity of TRPC6. Due to TRPC6 activation, the elevated intracellular Ca^{2+}-level induces the Ca^{2+}-dependent phosphatase calcineurin, which dephosphorilates NFATc proteins. NFAT translocates to the nuclei and creates an NFAT transcriptional complex with other nuclear elements and this complex regulates the transcription of target genes. TRPC6 gene is also regulated by the NFAT complex, which leads to elevated TRPC6 expression in the plasma membrane, enhancing Ca^{2+}-infux and related podocyte injury. These processes justified the application of AT1R blockers (ARB) and the inhibitor of angiotensin convertase enzyme in the therapy of proteinuria. The discovery that Ang II acts through TRPC6, offers a new approach to the treatment of proteinuria beyond the conventional ACE inhibitor and ARB drugs.

TRPC6 as a therapeutic target could be a promising new approach, since it is revealed that both hyperactivity of TRPC6 channels in genetic FSGS and induced overexpression of TRPC6 in acquired proteinuric states play a causative role in the development of kidney injuries. Research is already under way to change TRPC6 expression and to find a specific TRPC6 inhibitor. In 2010, it was already shown that TRPC6 siRNA coupled with a podocyte-specific delivery system significantly decreased TRPC6 expression in podocytes. A paper in 2018 reported that reasearchers identified (2-(benzo[d][1,3]dioxol-5-ylamino)thiazol-4-yl)((3 S,5 R)-3,5-dimethylpiperidin-1-yl)methanone as a specific and high affinity TRPC6 inhibitor.

It is clear that there is a very complex and multilevel signaling cascade that causes development of proteinuria in podocytes. Therefore, identification of

multiple terapeutic targets would be the best approch. As this is achieved by the most accurate mapping of the signaling cascade, we hope that our research has contributed to this by revealing the regulation of the TRPC6 channel.

Human podocytes express heat-sensitive TRPV1-4 channels

In the second part of our study we provided the first evidence for the functional expression of the heat-sensitive members of the vanilloid subfamily of TRP channels in human podocytes. Quantitative analysis of the mRNA transcripts revealed that TRPV1 and TRPV4 are the dominantly expressed TRPV channels but TRPV2 and TRPV3 were also detected at lower levels. These results are highly consistent with the findings of other research groups.

We examined the functionality of TRPV channels, first by analysing the activity of TRPV1. Interestingly, neither the specific agonist capsaicin nor the ultrapotent RTX were able to induce Ca^{2+}-influx in podocytes suggesting loss of capsaicin sensitivity or an impaired functionality of TRPV1 in the investigated cell line. A capsaicin insensitive splice variant TRPV1b was identified and its expression was reported in trigeminal and dorsal root ganglia and in keratinocytes as well. If TRPV1b is coexressed with TRPV1 it behaves as a dominant negative subunit disrupting capsaicin/vanilloid sensitivity of the channel. In our case, both mutations which can cause capsaicin insensitivity and the presence of the dominant negative subunit TRPV1b can be an explanation for the finding that in spite of the molecular expression of TRPV1, we did not find any functional effect of capsaicin.

In case of TRPV2, we detected the molecular expression and the functional activation of the channels in human podocytes as well. In our measurements, CBD was used as an TRPV2 agonist, which induced a moderate calcium influx from the extracellular space which was inhibited by tranilast, a suggested antagonist of TRPV2. Parallel with these experiments, the TRPV4 antagonist HC067047 also decreased CBD induced Ca^{2+}-entry. Therefore, we

concluded that functional TRPV4 channels can be involved in the effect of CBD. This is in good accordance with previously presented data showing that CBD can activate TRPV4 channels as well.

Further investigating TRPV4, the classical agonist 4α-PDD and the hyperpotent activator GSK1016790A generated rapid and robust Ca^{2+}-response in podocytes, which was strongly inhibited in the presence of the antagonist HC067047. Voltage-dependent characterization of TRPV4 also was showed marked transmembrane currents induced by GSK1016790A which were similarly inhibited by HC067047. This result suggested that in accordance with the molecular expression data, TRPV4 channels are the dominantly expressed thermosensitive TRPV channels in human podocytes.

In contrast with TRPV2 and TRPV4, functional expression of TRPV3 in podocytes is not clear. Molecular analyses showed the presence of TRPV3 mRNA and protein in human podocytes at lower levels compaired to TRPV4. Unfortunately, there are no available commercially specific TRPV3 agonists and antagonists to this day. In our work we used some botanical compounds, namely carvacrol, eugenol, thymol, which are generally accepted in the litreature as TRPV3 agonists. These drugs are potent but not specific activators of TRPV3. The same is true in case of the sythetic TRPV3 activator 2-APB. In our experiments, all of these agonists induced marked Ca^{2+}-elevation in podocytes derived from the extracellular space (at concentrations ≤1 mM), but only the effect of 2-APB reached a maximum over the concentration range applied, making possible the correct fitting of a sigmoidal dose–response curve. The experimentally determined EC50 (~500 μM) was higher than found earlier in electrophysiological studies on recombinant TRPV3 channels (~42 μM at physiological membrane potential). In addition, the general TRP channel blocker ruthenium red only partially blocked the effect of the compounds and the endogenous TRPV3 inhibitor IPP was partially effective blocking only 2-APB evoked Ca^{2+} signals. In contrast, RNAi mediated silencing of the molecular

expression of TRPV3 did not influence the effect of the agonists. All this results suggest that the TRPV3 agonists may have several off-target effects which can include the activation of several other TRP channels, and numerous other targets involved in cellular Ca^{2+}-handling. According to our pharmacological results we can conclude that TRPV3 channel functionality on human podocytes remains unclear, and requires further investigation.

The expression of heat and mechanosensitive TRPV channels has already been investigated in the lower urinary tract and in the kidney. Although the role of TRPV1 in the regulation of urinary tract functions, its expression in the urothelial cells is still under debate. It is known that TRPV4 is expressed on the non-neuronal cells of the urinary tract. In the kidney, TRPV4 is present in the water impermeable segments of the nephron. In addition, both TRPV1 and TRPV4 were described to form functionally active channels in the endothelium of the renal vasculature.

Although, the heat-sensitive TRPVs are present in the kidney, we know only little about their functionality on tubular epithelial cells, and we especially lack any data regarding podocytes.

TRPV1 and TRPV2, as well as TRPV4 were shown to be sensitive not only to thermal, but also for mechanical stimulation and osmotic challenges. Their osmosensory function is not restricted for channels expressed in neural structures but it is described in non-neuronal cells, such as in the urogenital tract. TRPV1 is required for bladder stretch detection by mediating the effect of hypotonicity in urothelial cells resulting in ATP release. However, TRPV1 is reportedly involved in the transmission of not only hypotonic, but hyperosmotic stimuli, as well. In the renal tubular system, an important role in transmitting the effect of tubular flow and osmolarity is attributed to TRPV4. TRPV4 mediated flow-induced increase in intracellular Ca^{2+}-concentration in medullary thick ascending limbs and its role was revealed in hypotonic stimuli induced Ca^{2+}-entry needed for regulatory volume decrease in renal cortical collecting duct

cells. All the above data let us conclude an intriguing new hypothesis: TRPV channels might transmit osmotic stimuli toward podocytes and influence the properties of the filtration barrier depending on osmotic challenges.

TRPV4 has an outstanding role in the formation of epithelial barrier in several tissues. In airway epithelium, activation of TRPV4 and voltage gated L-type Ca^{2+} channels by shear stress enhanced epithelial barrier function. In epidermal keratinocytes of the skin, functionally active TRPV4 channels interacts with adherent junction proteins and actin cytoskeleton, enhancing cell-cell junction and tight barrier formation. Moreover, TRPV4 was described in bladder urothelium and kidney collecting duct epithelium associating with α-catenin, an intracellular adherent junction protein. All these data further suggest a putative contribution of TRPV4 to the fine orchestration of podocytes Ca^{2+} homeostasis regulating the filtration barrier.

All things considered, further investigations are needed to reveal the putative role of TRPV channels in the physiological and pathological processes in human podocytes. Pharmacological targeting of these channels might contribute to the development of future therapies of primary and secondary podocytopathies and related kidney diseases.

SUMMARY

In our study, we investigated the expression, functionality and regulation of some TRP channels on human podocytes. We demonstrated that the endogenous activity of the PKC system blocked TRPC6. In our experiments, the PKC agonist PMA markedly suppressed the Ca^{2+}-elevation which was induced by the TRPC6 activator OAG. Long-term treatment with PMA also decreased the expression of TRPC6. In addition, exogenous inhibition of the PKC system with blockers of PKC subfamilies enhanced TRPC6 acivity. We also showed that expression level of PKCα, -β_1, -β_2 and -η proteins were down-regulated by PMA treatment, so presumably, these isoforms can regulate TRPC6.

In the second part of our study, we provided the first evidence for the functional expression of the heat-sensitive TRPV1-4 on human podocytes. TRPV1 and TRPV4 are highly expressed TRPV channels but TRPV2 and TRPV3 were also detected at lower levels. TRPV1 did not show activity after treatment with the specific TRPV1 activators capsaicin or RTX. In contrast, CBD induced moderate calcium influx which was inhibited by the TRPV2 antagonist tranilast, and the TRPV4 antagonist HC067047. Furthermore, investigating TRPV4, the classical agonist 4α-PDD and the hyperpotent activator GSK1016790A generated rapid and robust Ca^{2+}-response. Therefore, TRPV4 can be the dominantly expressed thermosensitive TRPV in human podocytes. In case of TRPV3, the botanical compounds and also 2-APB induced marked Ca^{2+}-elevation. This effect with hardly influenced by the TRPV3 antagonist IPP or only partially blocked by the general TRPV blocker ruthenium red. In contrast, RNAi mediated silencing of TRPV3 did not influence the effect of the agonists. These results suggest that TRPV3 agonists may have several off-target effects.

Our findings confirmed that TRP channels and its regulatory systems play a crucial role in the regulation of human podocytes functions.

New scientific results presented in the dissertation:

- TRPC6 ion channels expressed in human podocytes are negatively regulated by the PKC system
- PKCα, -β1, -β2 and -η isoenzymes may participate in the inhibition of the TRPC6 channel
- Activation of a PKC system results in down-regulation of the TRPC6 protein
- Heat-sensitive TRPV1-4 channels are expressed on human podocytes
- TRPV1 does not form a functional ion channel on human podocytes
- TRPV2 and TRPV4 form functional ion channels
- Contribution of TRPV3 channels to the Ca^{2+} responses of the TRPV3 agonists is minimal

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List of publications related to the dissertation

1. **Ambrus, L.**, Kelemen, B., Szabó, T., Bíró, T., Tóth, I. B.: Human podocytes express functional thermosensitive transient receptor potential vanilloid (TRPV) channels.
   Br. J. Pharmacol. 174 (23), 4493-4507, 2017.
   DOI: http://dx.doi.org/10.1111/bph.14052.
   IF: 6.81
2. **Ambrus, L.**, Oláh, A., Oláh, T., Balla, G., Saleem, M. A., Orosz, P., Zsuga, J., Bíró, K., Csernoch, L., Bíró, T., Szabó, T.: Inhibition of TRPC6 by protein kinase C isoforms in cultured human podocytes.
   J. Cell. Mol. Med. 19 (12), 2771-2779, 2015.
   DOI: http://dx.doi.org/10.1111/jcmm.12660
   IF: 4.938

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List of other publications

3. Páyer, E., Szabó-Papp, J., **Ambrus, L.**, Szöllősi, A. G., Andrási, M., Dikstein, S., Kemény, L., Juhász, I., Szegedi, A., Bíró, T., Oláh, A.: Beyond the physico-chemical barrier: glycerol and xylitol markedly yet differentially alter gene expression profiles and modify signalling pathways in human epidermal keratinocytes.
Exp. Dermatol. 27 (3), 280-284, 2018.
DOI: http://dx.doi.org/10.1111/exd.13493
IF: 2.608 (2017)

4. Oláh, A., **Ambrus, L.**, Nicolussi, S., Gertsch, J., Tubak, V., Kemény, L., Soeberdt, M., Abels, C., Bíró, T.: Inhibition of fatty acid amide hydrolase exerts cutaneous anti-inflammatory effects both in vitro and in vivo.
Exp. Dermatol. 25 (4), 328-330, 2016.
IF: 2.532

5. Szabó, T., **Ambrus, L.**, Zákány, N., Balla, G., Bíró, T.: Regulation of TRPC6 ion channels in podocytes: implications for focal segmental glomerulosclerosis and acquired forms of proteinuric diseases.
Acta Physiol. Hung. 102 (3), 241-251, 2015.
DOI: http://dx.doi.org/10.1556/036.102.2015.3.2
IF: 0.814

6. Oláh, A., Tóth, I. B., Borbíró, I., Sugawara, K., Szöllősi, A. G., Czifra, G., Pál, B., **Ambrus, L.**, Kloepper, J. E., Camera, E., Ludovici, M., Picardo, M., Voets, T., Zouboulis, C. C., Paus, R., Bíró, T.: Cannabidiol exerts sebostatic and antiinflammatory effects on human sebocytes.
J. Clin. Invest. 124 (9), 3713-3724, 2014.
DOI: http://dx.doi.org/10.1172/JCI64628
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