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Regulation of TRPC6 ion channels in podocytes – Implications for focal segmental glomerulosclerosis and acquired forms of proteinuric diseases (Review)

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The glomerular filtration barrier is a highly specialized tri-layer structure with unique functional properties. Podocyte dysfunction and cytoskeletal disorganization leads to disruption of the slit diaphragma, and proteinuria. Inflammatory diseases involving the kidney as well as inherited podocytopathies or diabetic nephropathy cause injury of the podocyte network. Focal segmental glomerulosclerosis (FSGS) is a pathologic entity that is a common cause of nephrotic syndrome with severe proteinuria in both adults and children. Several causative genes have been identified in the pathogenesis of FSGS. Mutations of the transient receptor potential canonical-6 (TRPC6), a non-selective cation channel that is directly activated by diacylglycerol (DAG), cause a particularly aggressive form of FSGS. Angiotensin II, acting through its AT1 receptor, plays a critical role in generation of proteinuria and progression of kidney injury in a number of kidney diseases, including FSGS. Mounting evidence suggest the central role of TRPC6 and perhaps other TRPC channels in the pathogenesis of FSGS as well as of acquired forms of proteinuria such as diabetic nephropathy or hypertension. Identification of signaling pathways downstream of TRPC6 may provide novel targets for the treatment of proteinuria and prevent progression of podocyte injury.

Keywords: proteinuric disease, focal segmental glomerulosclerosis (FSGS), TRPC6, diabetic nephropathy, angiotensin II, podocyte

Filtration barrier, podocytopathy and proteinuria

Glomerular visceral epithelial cells, also known as podocytes, are highly specialized epithelial cells that cover the outer layer of the glomerular basement membrane. They form the final barrier to protein loss, which explains why podocyte injury is typically associated with marked proteinuria syndromes. The glomerular filtration barrier is a highly specialized tri-layer structure with unique functional properties. The slit diaphragm which, structurally, is a cell-cell junction between adjacent podocyte foot processes is made up of a sophisticated multi-protein complex which dynamically controls foot process architecture via signaling to the actin cytoskeleton. Podocyte dysfunction and cytoskeletal disorganization leads to foot process effacement, disruption of the slit diaphragma, and proteinuria, and is often an ignition point for progressive kidney diseases (20, 65). The degree of proteinuria correlates well with the progression of glomerulosclerosis. Inflammatory diseases involving the kidney as well as

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inherited podocytopathies or diabetic nephropathy cause injury of the podocyte network by disrupting the fine intercellular connections (foot processes and slit membrane) and resulting in progressive proteinuric state and eventually glomerulosclerosis (45, 48, 62). More recently, biopsy studies in humans with diabetic kidney disease have provided strong evidence that podocytes are injured very early in the course of nephropathy. This podocytopathy, which is characterized by a decreased podocyte number and density correlates closely with the development and progression of proteinuria (35, 57, 59, 66, 72). Components of the diabetic milieu such as high glucose, hyperinsulinemia, insulin resistance as well as altered angiotensin II (Ang II) signaling appears to be a major cause in the pathogenesis of proteinuria (16, 35). Inhibition of the Ang II pathway is the gold standard in preventing the progression of glomerular sclerosis in proteinuria; however these drugs (i.e. Ang II convertase inhibitors [ACE-I] and type I Ang II receptor blokers [ARB)] has only limited efficacy in halting the progression of the glomerulosclerosis to end-stage renal disease (ESRD).

Focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is a pathologic entity that is a common cause of nephrotic syndrome both in adults and children. FSGS establishes a substantial risk for progression to ESRD. Over the past two decades, the incidence of FSGS has been increasing (26, 33, 62). The increased incidence of idiopathic FSGS suggests that both genetic and environmental factors may play an important role in the pathogenesis of this disease. The clinical hallmarks of FSGS include proteinuria, nephrotic syndrome, and quite frequently, the progressive loss of renal function. The primary treatment for FSGS is corticosteroid therapy; however, the response rates are inconsistent. Immunomodulators and cytostatic drugs can be used in steroid-resistant forms. Regardless of the treatment, approximately 50% of patients with persistent nephrotic range proteinuria reach ESRD after 10 years of follow-up evaluation. The phenotypic differences show the heterogeneity of this disease process, and support the existence of varying biological mechanisms causing the final histopathologic end-point (26, 33, 62).

Several causative genes have been identified in the pathogenesis of FSGS (3, 13, 49, 68). Mutations of the *NPHS1* gene that encodes nephrin on the chromosome 19q13.1 has been found to be responsible for an autosomal recessive form of congenital nephrosis syndrome also termed as Finnish nephropathy (13, 29, 56, 63). In addition, mutations in the gene *NPHS2* (podocin) have been associated with steroid-resistant idiopathic nephrosis syndrome. Podocin mutations have been reported in both familial autosomal-recessive disease and in individuals with sporadic adult-onset FSGS (28, 29, 38). Autosomal-dominant FSGS is typically a disease of adults, with variable age of onset, severity, and progression to ESRD. The first reported locus for inherited autosomal-dominant FSGS mapped to chromosome 19q13, where the *ACTN4* gene has been identified (3, 71)

TRPC6 in the kidney

The most recently reported disease-causing mutation for hereditary FSGS has provided striking new insight into the genetic heterogeneity and pathogenesis of nephrotic syndrome. Mutations of gene encoding transient receptor potential canonical-6 (TRPC6) cause a particularly aggressive form of FSGS (25, 61, 73).

TRPC6 is part of the large transient receptor potential (TRP) ion channel superfamily which plays crucial roles in a plethora of cellular functions. Up-to-date, 28 mammalian members have been identified within the TRP family which can be further classified into the

subfamilies of the canonical (or classical, TRPC), the vanilloid (TRPV), the melastatin (TRPM), the mucolipin (TRPML), the polycystin (TRPP), and the ankyrin (TRPA) groups (9). TRP ion channels function as broadly expressed polymodal "cellular sensors" and "cellular effectors" of cell and tissue homeostasis (10, 54). Their activities can be equally modulated by e.g. changes in temperature, pH, osmolarity, membrane potential or ionic concentrations; a plethora of endogenous mediators and their exogenous counterparts, etc. (12, 52, 58, 69). Moreover, they are involved in the regulation of practically all cellular processes, e.g. sensation, metabolism, cell growth, and survival (reviewed in 52).

Human TRPC6, which belongs to the canonical TRP subfamily, is a non-selective cation channel that is directly activated by diacylglycerol (DAG) in a membrane-delimited fashion, independently of the protein kinase C (PKC) system, the "classical receptor" for DAG. Based on the described activation characteristics, TRPC6 represents a new member of the second-messenger-operated cation channels, which are activated by DAG (31). Immunofluorescence staining revealed TRPC6 expression throughout the kidney in glomeruli and tubules (61, 73). All three cell types within the glomerulus (endothelial cells, mesangial cells, and podocytes), cells of the tubulo-interstitial compartment as well as the microcirculatory elements express TRPC6 in association with several other TRPC members of which some may represent potential binding partners (14). The expression of TRPC6 in glomeruli is particularly noteworthy, because abnormal podocyte function appears to be a final common pathway in a variety of proteinuric kidney diseases (35, 45, 48, 62, 72, 73). A missense mutation (proline-to-glutamine substitution) in the *TRPC6* gene has been carried in a large family with hereditary FSGS (61, 73). Functional analysis of the disease-causing mutation revealed enhanced TRPC6-mediated calcium signals in response to agonists such as Ang II. The P112Q mutation in *TRPC6* causes gain of function; in turn, the Ca^{2+} entry is enhanced and is particularly exaggerated in response to G-protein agonists such as Ang II (73). Enhanced cell surface expression of $TRPC6^{P112Q}$ protein suggests a mechanism of exaggerated calcium signaling and flux. Based on *in vitro* electrophysiologic analysis of different mutants of TRPC6 it was hypothesized that calcium-dose effects mediated by TRPC6 might play a role in determining the onset and severity of disease (17, 61).

Ang II, acting through its AT1 receptor, plays a critical role in the generation of proteinuria and in the progression of kidney injury in a number of kidney diseases including FSGS (16, 30). AT1 receptors are coupled to G-proteins and activate phospholipase C-b (PLC-β) isoforms that hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2). This triggers the production of inositol 1,4,5-trisphosphate ($InsP_3$) and DAG which releases internal calcium stores and activates Ca^{2+} entry, and activates the PKC system, respectively. Regulation of TRPC6 activity by PKC was revealed in smooth muscle cells (5). Indeed, in human and mouse cultured podocytes, we observed that activation of PKC by phorbol 12-myristate 13-acetate (PMA) almost completely abolished agonist-induced TRPC6 activation (Ambrus et al. unpublished data). Furthermore, apparently PKCs also regulate the expression level of TRPC6 in podocytes but molecular events involved remain to be clarified (unpublished data). It has been shown that tyrosine phosphorylation of TRPC6 induces a complex formation with PLC-γ1, which is a pre-requisite for TRPC6 surface expression. Furthermore, nephrin binds to phosphorylated TRPC6 via its cytoplasmic domain, competitively inhibiting TRPC6-PLC-γ1 complex formation, TRPC6 surface localization, and TRPC6 activation. Importantly, FSGS-associated mutations render the mutated TRPC6 insensitive to nephrin suppression, thereby promoting their surface expression and channel activation (37).

Induced expression of wild-type TRPC6 seems to be a common feature of human proteinuric kidney diseases as well (48, 62). It has been shown that TRPC6 protein is functionally connected to the podocyte actin cytoskeleton, which is rearranged upon overexpression of TRPC6 (20, 48). Previous studies suggested the involvement of TRPC6 in the pathology of nongenetic forms of proteinuric disease (16, 35, 48, 51). Under pathological conditions, activation of TRPC6 may cause an increase in intracellular calcium and affect critical interactions with podocyte structural proteins, leading to abnormalities in the slit diaphragm and/or podocyte foot processes. Likewise, TRPC6 may also amplify injurious signals mediated by Ang II resulting in apoptosis of podocytes. As detailed below, functional link between TRPC6 action and expression and Ang II pathway activation has been proposed (51).

Interaction of TRPC6 with other SD proteins of the filtration barrier

Several gene products have been described to be a major component of the filtration barrier where podocytes are apparently the most important players. Genes like nephrin, podocin, α-4 actinin or TRPC6 are located in close proximity in the podocyte foot processes suggesting that not only structural but functional interactions exist (61). As detailed above, lately TRPC6 has attracted special attention. It has been shown that albumin overload, a common feature of proteinuric states, may induce ER stress and subsequent apoptosis in podocytes via TRPC6-mediated Ca^{2+} entry (8). Chen et al. (8) has shown that long-term albumin exposure resulted in an up-regulation of TRPC6 expression in podocytes, which was inhibited by TRPC6-specific siRNA. Additionally, the inhibition of TRPC6 prevented the F-actin cytoskeleton disruption that is induced by albumin overload. Moreover, albumin overload induced expression of the endoplasmic reticulum (ER) stress protein GRP78, led to caspase-12 activation and ultimately podocyte apoptosis, all of which were abolished by the knockdown of TRPC6 using TRPC6 siRNA. In case of TRPC6 overexpression, an increase of intracellular Ca^{2+} down-regulates the expression of two important molecules, nephrin on slit diaphragm and synaptopodin in cytoskeleton, and stimulates RhoA activity. Consequently, F-actin derangement and a decrease of podocyte foot processes occur (2, 36) Taken together, over-expression of TRPC6 in podocytes may be one of the fundamental changes relating to the dysfunction of the slit diaphragm and proteinuria.

Ang II-TRPC6 "crosstalk"

Podocytes are constantly exposed to mechanical stress, generated by blood pressure in the glomerular capillaries, as well as shear stress resulting from fluid flow through the slit diaphragma and over the apical membrane of the podocyte. Ca^{2+}-dependent remodeling of the actin cytoskeleton in response to mechanical load is essential to counteract these forces (18, 19, 39). In the context of proteinuric kidney disease, angiotensin 1 receptor-mediated (AT1R) signaling is of particular importance. In an elegant study by Hoffmann and colleagues, direct evidence was presented in transgenic rats when podocyte-specific over-expression of AT1R was sufficient to cause proteinuria with FSGS like histological changes (30). Importantly, Ang II evokes a nonselective cationic current in podocytes (23, 53). At a cellular level, AT1R signaling is upstream of a number of pathways that may be important to TRPC6 signaling as well. One is the AT1R-dependent activation of calcineurin (40). Furthermore, AT1R signaling causes transactivation of the EGF receptor (EGFR) in podocytes (22). AT1R-EGFR interactions also activate downstream serine/threonine kinases such as the MAPK

pathway in a process that is known to require an increase in cytosolic [Ca^{2+}] (34). Ang II induces membrane ruffling and loss of stress fibers, thereby phenocopying the depletion of synaptopodin or TRPC6 (8, 34, 47, 77).

Ang II has been recognized as an apoptosis inducer in podocytes but the mechanism of apoptosis is unclear. The protein level of TRPC6 was found to be markedly increased in response to Ang II stimulation, in parallel with the induced elevation of intracellular Ca^{2+} concentration. By transfection with TRPC6 siRNA, Ang II-induced podocyte apoptosis and the transient Ca^{2+} influx were inhibited (77). The activation of ERK pathway and subsequent translocation of NF-kappaB also seem to b necessary for the up-regulation TRPC6 induced by Ang II (77). Likewise, Nijenhuis and colleagues demonstrated that Ang II regulates TRPC6 mRNA and protein levels in cultured podocytes and that Ang II infusion enhances glomerular TRPC6 expression *in vivo*. Moreover, TRPC6 expression correlated with glomerular damage markers and glomerulosclerosis. The regulation of TRPC6 expression by Ang II required TRPC6-mediated Ca^{2+} influx and the activation of calcineurin and its substrate NFAT (51). Accordingly, calcineurin inhibition by cyclosporine decreased TRPC6 expression and reduced proteinuria, whereas podocyte-specific inducible expression of a constitutively active NFAT mutant increased TRPC6 expression and induced severe proteinuria. These important findings demonstrated that the deleterious effects of Ang II on podocytes and its pathogenic role in glomerular diseases most probably involve an enhanced TRPC6 expression via a calcineurin/NFAT positive feedback signaling pathway (51).

Potential role of Ang II/TRPC6 signaling in diabetic nephropathy

Microalbuminuria is the earliest clinical manifestation of diabetic nephropathy (DN), although we lack sufficient available evidence to distinguish whether insulin resistence or hyperinsulinemia is the significant risk factor for the development of microalbuminuria (47). Importantly, insulin can influence glomerular permeability to albumin in patients with type 2 diabetes but not in healthy individuals (7), suggesting that disruption of the insulin signaling cascade is required for the insulin-induced microalbuminemia. Loss of podocyte structure and function has been reported as one of the earliest features of DN in both types of diabetes (57, 59, 66, 72). It is assumed that the pathophysiological mechanism of podocyte malfunction is multiple, and mounting evidence suggests a pivotal role for both Ang II-mediated signaling and deranged glucose metabolism. Evidence also suggests that podocytes are direct targets of Ang II in diabetes-related podocyte injury. Correspondingly, pharmacological inhibition of Ang II restores nephrin levels (41) and reverses foot process effacement (46). Furthermore, it was demonstrated that in nondiabetic proteinuric diseases podocytes are capable of synthesizing Ang II, which is able to mediate cell injury such as apoptosis in an autocrine fashion (15). Recent studies have showed that high glucose levels also increase production of Ang II by podocytes (74, 75).

Experimental data on immortalized podocytes identified increased Ang II production triggered by high glucose, which effect was independent of ACE activity (16). High glucose also elevates AT1R density on the cell surface as well as renin levels in immortalized podocyte cultures (16). AT1 receptor is known to be responsible for mediating several deleterious nonhemodynamic effects of Ang II on kidney injury. High glucose induces apoptosis and reduces the viability of differentiated podocytes. It also causes time-dependent up-regulation of TRPC6 and activation of the canonical Wnt signaling pathway in mouse podocytes, indicating that Wnt/β-catenin signaling pathway may potentially be active in pathogenesis of TRPC6-mediated diabetic podocyte injury (44).

In summary, the aforementioned experimental data indicate that high glucose leads to activation of a local angiotensin system in podocytes by increasing Ang II generation and renin level and activity as well as by augmenting AT1R density in the plasma membrane. In agreement with these facts glomerular prorenin receptor levels were reported to be increased in an experimental model of DN (32, 50). Blockade of Ang II remained a cornerstone in the management of DN and affords renal protection beyond blood pressure lowering. One additional possibility is that insulin *per se,* particularly in the setting of compromised insulin receptor signaling, may signal through alternative pathways and cause podocyte injury. Insulin may directly affect the activity of TRPC6 in a PI3K/PIP3-dependent manner (67), and TRPC6 ion channels expressed in podocytes may serve as an "amplifier" of Ca^{2+}-mediated signals downstream of AT1R. The fact that Ang II infusion was shown to increase the expression of TRPC6 in an animal model, and that TRPC6 activation was reported to increase the expression of its own receptor further support that notion (51). Based on available scientific data we assume that there is a complex network with multiple interactions in the pathomechanism of proteinuria of DN.

TRPC6 and downstream signaling pathways

An important link between intracellular Ca^{2+} increase and podocyte injury is the activation of the Ca^{2+}-dependent phosphatase calcineurin. NFATc transcription factors are extensively studied calcineurin substrates and are major regulators of transcription in response to Ca^{2+}/calcineurin signals (11, 43). Upon activation by increased intracellular Ca^{2+}, calcineurin dephosphorylates NFATc proteins leading to cytoplasm-to-nucleus translocation. In the nucleus, NFATc proteins form NFAT transcription complexes with their nuclear partners to control the transcription of target genes. Upregulation of Wnt6 and Fzd9 was shown in TRPC6-mutant glomeruli before the onset of significant proteinuria, suggesting a potential role of Wnt signaling in the pathogenesis of NFAT-induced podocyte injury and FSGS (70).

The activation of calcineurin in podocytes was found to be sufficient to cause degradation of synaptopodin and to induce proteinuria (21). Corresponding to that, cyclosporin A (CsA), a calcineurin inhibitor, prevents synaptopodin degradation *in vitro*, and mice resistant to cathepsin-mediated synaptopodin degredation are protected from proteinuria *in vivo* (21). These important finding revealed a T-cell and NFAT independent mechanism for the antiproteinuric effect of CsA. FSGS-causing TRPC6 mutations, but not the wild-type TRPC6, induce constitutive activation of calcineurin-NFAT-dependent gene transcription (64). TRPC channel-mediated intracellular events, especially calcineurin activation and related downstream effectors were found to be important in podocytes (64). One possible explanation is a positive feedback loop, whereby the calcineurin-NFAT-mediated increase in transcription leads to elevated TRPC6 channel density in the plasma membrane, thus enhancing calcium influx and related podocyte injury. Recent studies performed on cardiac myocytes suggest that this pathway is not exclusive for TRPC6; other TRPC channels, for example TRPC4, may also be involved and AT1R activation may be an important initial signaling event (40, 55). These findings strongly suggest the existence of a podocyte loop linking TRPC channels to NFAT activation and subsequent TRPC transcriptional upregulation (24, 70).

Clinical implications: TRPC6 as a potential pharmacological target

Proteinuria is a cardinal sign and a prognostic marker of kidney diseases, and also an independent risk factor for cardiovascular morbidity and mortality (1). For decades,

proteinuric kidney diseases, such as hypertensive and diabetic nephropathy, have been treated with essentially the same agents, targeting the renin-angiotensin system (6). The Ang II–mediated regulation of podocyte actin dynamics involves several signaling levels, each of which can receive additional inputs from multiple other signaling pathways, forming a complex network (40). The discovery that Ang II acts through TRPC6, and possibly other TRPCs, as well as other downstream effectors, offers a new approach to the treatment of proteinuria beyond the conventional ACE inhibitor and ARB drugs (42).

Targeting TRPC6 could be a promising new approach since both hyperactivity of TRPC6 channels in genetic FSGS and induced overexpression of TRPC6 in acquired proteinuric states including membranous nephropathy, diabetic nephropathy or hypertension related proteinuria, may have benefit of it. For example, in membranous glomerulopathy, which is the most common cause of idiopathic proteinuric kidney diseases, multiple experimental hypotheses can be defined such as e.g. modifying TRPC6 expression or specifically blocking TRPC6 channels expressed by podocytes (60). Indeed, TRPC6 siRNA coupled with a podocyte-specific delivery system has already been shown to significantly decrease TRPC6 expression in podocytes, and presumably extensive research is currently being done to discover highly specific antagonists for TRPC6 (27). The difficulty in such pharmacological approaches is the specific delivery of the drug to podocytes. Calcineurin inhibitors have been routinely used in the treatment of nephrotic syndrome. Refining current therapeutic protocols to target TRPC6-mediated downstream signals would make sense. In fact, a non-T-cell inhibitory dose of CsA is presumably sufficient to affect podocytes, sparing many of the side effects of regular dose, is of interest. CsA has been reported to decrease proteinuria even in genetically inherited forms of FSGS, where the beneficial effect of specific T-cell mediated immunosuppression can hardly be expected (4, 76). In a model, where proteinuria arises from dysfunctions in a multilevel signaling cascade (bringing together upstream receptor pathways, TRPCs, synaptopodin, NFAT, Rho GTPases, and downstream targets such as the actin cytoskeleton or the mineralocorticoid receptor), multiple therapeutic targets may be identified. This model may provide a clue why ACE inhibitors and ARBs have their limits in efficacy as antiproteinurics. Targeting different downstream mechanisms may promise more effective treatment approach to control proteinuria. Obviously, gaining deeper insight into the pathomechanism of different proteinuric states would surely help to identify more specific targets at molecular or transcriptional levels. If one can employ a “drug combination” with combined or synergistic effect of specific agents targeting to different levels within the signaling cascade (similar to oncologic treatments) may ultimately be the best approach to improve the efficacy of antiproteinuric treatment. All in all, the detailed understanding of podocyte-specific signaling further validates the podocyte as the target of choice for the treatment of proteinuria.

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