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Rho kinase inhibition in diabetic kidney disease

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Small GTPases of the Rho family and their down-stream effectors Rho associated kinases (ROCKs) are the molecules that converge a spectrum of pathophysiological signals triggered by the diabetic milieu and represent promising molecular targets for nephroprotective treatment in diabetes. The review discusses recent studies exploring the consequences of diabetes-induced Rho-ROCK activation in the kidney and the effects of ROCK inhibition (ROCKi) in experimental diabetic kidney disease (DKD). Studies in models of type 1 and type 2 diabetes have indicated blood pressure-independent nephroprotective actions of ROCKi in DKD. The underlying mechanisms include attenuation of diabetes-induced increases in renal expression of prosclerotic cytokines and extracellular matrix, anti-oxidant effects and protection of mitochondrial function, resulting in slower development of glomerulosclerosis and interstitial fibrosis. The studies have also shown antiproteinuric effects of ROCKi that could be related to reductions in permeability of the glomerular barrier and beneficial effects on podocytes. Glomerular haemodynamic mechanisms might also be involved. Despite remaining questions in this field, such as the effects in podocytes later in the course of DKD, specificity of currently available ROCKi, or the roles of individual ROCK isoforms, recent evidence in experimental diabetes suggests that ROCKi might in future broaden the spectrum of treatments available for patients with DKD. This is supported by the evidence generated in models of non-diabetic kidney disease and in clinical studies in patients with various cardiovascular disorders.

Introduction

Current treatment of diabetic kidney disease (DKD) is based on good metabolic control, control of high blood pressure and use of inhibitors of renin-angiotensin-aldosterone system (RAAS). However, despite progress in prevention and treatment, reversal and even stabilization of the progressive course of overt (proteinuric) diabetic nephropathy (DN) are still difficult to achieve, and many patients progress to end-stage renal disease. New approaches that would broaden the spectrum of available treatments for DKD are needed to improve prognosis in these patients. Over the past decade there has been increasing experimental evidence indicating pathophysiological roles for small GTPases of the Rho family and their down-stream effectors Rho associated kinases (ROCK) Here, I will briefly review this literature, discuss the impact of inhibition of this pathway in the diabetic kidney, and further focus on translational potential of this area of research.

Physiology of Rho-ROCK signalling

RhoA-ROCK signalling has been extensively and repeatedly reviewed [1–3]. In brief, Rho GTPases, members of the Ras superfamily of small GTP-binding proteins, are divided into three major classes: Rho, Rac and Cdc42. The Rho family consist of RhoA, RhoB, and RhoC. Most Rho GTPases behave as 'molecular switches' that fluctuate between inactive and active states, depending on the binding of either GDP or GTP to the GTPases. This cycle is under the direct control of three groups of regulatory proteins (Figure 1). The guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP to activate the Rho proteins. In addition, activated Rho proteins are prenylated

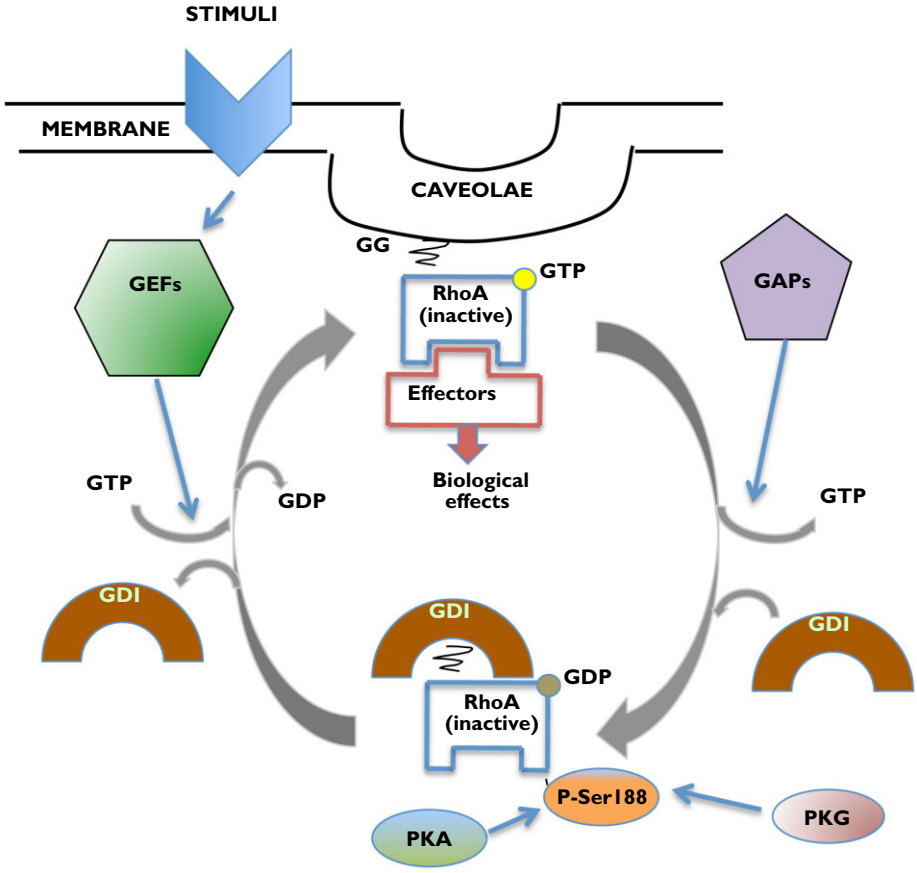

Figure 1
RhoA activation cycle

at their carboxy terminus [2]. This prenylation (farnesyl or geranyl-geranyl groups) is involved in translocation of the active GTP-bound form of Rho to plasma membrane, where it interacts with its effector proteins to generate downstream responses.

The activation of Rho proteins is then turned off by GTPase activating proteins (GAPs) that enhance the intrinsic GTPase activity of the Rho protein, leading to the hydrolysis of GTP to GDP. The third group of proteins involved in the Rho signalling cycle are guanine dissociation inhibitors (RhoGDI) that hide the isoprenyl groups of the GTPases, an action that favours the sequestration of the inactive GTPases in the cytosol. The GDIs also impede the release of GDP from the GTPase and contribute to the maintenance of the GTPases in an inactive state [4]. The dissociation of the RhoGDI from the GTPase is essential for the activation of GTPases by GEFs. In addition, Rho could be regulated by phosphorylation on Ser188, mediated by protein kinases A and G. Although the RhoA phosphorylation prevents its ubiquitination and proteozomal degradation, it also enhances association of the protein with RhoGDI and leads to attenuation of RhoA-mediated signalling. The Rho protein cycle is stimulated by agonists acting via G protein-coupled receptors (GPCR), tyrosine kinase receptors, cytokine receptor activation and mechanical stress, which essentially regulate the activity of GEFs.

In the GTP-bound state, these GTPases bind to effector molecules that, in turn, lead to the stimulation of signalling cascades that promote a myriad of cellular responses discussed below. The Rho associated kinases (ROCKs) were found to be one of the first downstream targets of RhoA [5] and remain the best studied. In mammals, ROCKs consist of two isoforms: ROCK1, which is also known as ROKβ and p160ROCK, and ROCK2, which is also known as ROKα and often referred to as Rho-kinase [5].

Inhibitors of the Rho-ROCK pathway

Several approaches have been used to inhibit Rho-ROCK signalling. Y27632 and fasudil are the most commonly used pharmacological ROCK inhibitors (ROCKi) that target

their ATP-dependent kinase domains and are equipotent in terms of inhibiting both ROCK1 and ROCK2 [5]. At higher doses these inhibitors also interfere with several isoforms of protein kinase C, A and mitogen-activated protein kinases [6]. In addition, the 3-hydroxy-3-methylglutaryl CoA reductase inhibitors (statins) prevent RhoA activation by interfering with its geranyl-geranylation and membrane tethering [7] and thus at least in part inhibit ROCK activity. This mechanism has been implicated in pleiotropic protective effects of statins in the cardiovascular system and kidney [8]. As mentioned below in more detail, some of the disadvantages of pharmacological inhibitors have been more recently overcome by genetically modified models, such as cells expressing inactive RhoA mutant [9], siRNA targeting RhoA [10] or ROCK1, respectively and ROCK2 knockout mice [11, 12].

Multiple roles of the Rho-ROCK pathway in cardiovascular and renal physiology and disease

The pleiotropic effects of the RhoA-ROCK pathway have sparkled considerable interest in most of the fields of biomedical research and detailed discussion of this literature is beyond the scope of this review. In brief, studies in the vascular system and kidney conducted in the non-diabetic context have identified physiological and pathophysiological roles for Rho-ROCK in such processes as cell contraction, proliferation, growth and production of extracellular matrix (ECM), apoptosis, migration and differentiation (reviewed in [2, 13, 14]). Rho-ROCK have also been implicated in enhanced endothelial permeability [15], angiogenesis [16], and reduced availability of endothelial nitric oxide [17]. The activity of this pathway is increased in cardiovascular system and renal cells in a wide spectrum of cardiovascular and kidney disorders, which can be, in turn, ameliorated by the treatment with ROCKi both in experimental and clinical settings [1–3, 18–23].

Activation of Rho-ROCK in diabetes

In addition to high glucose [9, 10, 24, 25], Rho-ROCK are stimulated by other components of the diabetic milieu, such as advanced glycation endproducts (AGEs) [26, 27], reactive oxygen species (ROS) [28], hexosamine pathway [24] and oxidized LDL [29, 30] in vascular and renal cells. Moreover, the Rho-ROCK pathway can be activated by or mediate the effects of hormones, cytokines, and physical forces implicated in the pathophysiology diabetic complications, such as angiotensin II (AngII) [31], aldosterone [19], vascular endothelial growth factor (VEGF) [32, 33], transforming growth factor-β (TGF-β) [34, 35] and mechanical stress [36].

It has become apparent that Rho-ROCK converge numerous pathophysiological signals triggered by the diabetic milieu in the kidney and consequently these molecules represent promising targets for nephroprotective treatment in diabetes. The evidence supporting this notion is discussed below.

RhoA-ROCK in the diabetic kidney

Similar to other cell types, the RhoA-ROCK pathway is activated in renal cells exposed to diabetic milieu. As demonstrated by initial studies, the pathway contributes to progrowth, profibrotic/prosclerotic signalling, and enhanced ECM production [9, 10, 25]. The effects of high glucose on mesangial cells (MC) were inhibited by simvastatin [25], ROCKi Y27632 or fasudil [9, 10], as well as with transfection of MC with inactive RhoA mutant [9] or siRNA targeting RhoA [10]. Other studies have implicated Rho-ROCK in MC growth and collagen production induced by VEGF [33] and AngII [31]. There is possible synergism in high glucose- and AngII-induced Rho-ROCK signalling [31].

This led to a hypothesis that ROCKi might be nephroprotective in DKD. The following studies were designed to test this hypothesis in models of DKD receiving long term treatment with ROCKi.

Initial shorter studies (4 weeks) [37] demonstrated beneficial effects of fasudil or fluvastatin in streptozotocin (STZ)-diabetic rats on renal expression of prosclerotic cytokines and NOX4 with a reduction in trace albuminuria. The following studies by Peng *et al*. [10] treated the STZ-diabetic rats with fasudil (30 mg kg^{-1} body weight) for 6.5 months and reported reductions in proteinuria, mesangial matrix accumulation and glomerular fibronectin expression, along with suppression of ROCK activity. Importantly, electron microscopic analysis showed beneficial effects of fasudil on podocyte foot process effacement. The protective effects of fasudil were comparable with those of the ACE inhibitor enalapril.

To evaluate whether ROCKi confer protective effects in a model of more advanced disease in insulinopenic diabetes, we have recently studied uninephrectomized STZ-diabetic rats that display an accelerated course of the disease. When initiated at the onset of diabetes, fasudil, administered for 18 weeks, attenuated the development of proteinuria, glomerulosclerosis and tubulointerstitial fibrosis, prevented the decrease in GFR, and ameliorated diabetes-induced increases in renal TGF-β, CTGF and ECM protein expression [38]. The treatment also resulted in a significant upregulation of nephrin mRNA and protein expression. Unlike the acute administration of ROCKi [38], chronic treatment with fasudil had no effect on blood pressure. Most of these effects were comparable with the angiotensin AT1 receptor blocker (ARB) losartan, but combination of fasudil and losartan was not more effective

than losartan alone. The late intervention with fasudil, initiated after 12 weeks of diabetes without treatment, still attenuated glomerular lesions and prevented loss of GFR, but did not significantly influence tubulointerstitial fibrosis and lacked antiproteinuric efficacy.

In non-diabetic models of kidney disease, the Rho-ROCK pathway has been shown to mediate epithelial-mesenchymal transition (EMT) [39, 40], a process closely linked to the development of renal fibrosis. To evaluate further the mechanisms of beneficial effects of fasudil in tubulointerstitial compartment in uninephrectomized diabetic rats, we have also evaluated the effects on EMT [38]. Uninephrectomized diabetic rats displayed increased expression of the EMT markers fibroblast specific protein 1, α-smooth muscle actin and vimentin, which were attenuated with fasudil treatment in conjunction with beneficial effects on renal fibrosis and reduced expression of TGF-β and CTGF.

In the first study describing the long term nephroprotective effect of fasudil in a model of type 2 diabetes (OLETF rats), Kikuchi *et al*. [41] reported beneficial effects of the high dose (100 mg kg^{-1}) on proteinuria, glomerulosclerosis and interstitial fibrosis. However, this treatment also cured type 2 diabetes in OLETF rats, and it remained unclear whether the described beneficial effects were attributable specifically to ROCKi or to dramatic improvements in metabolic status.

Therefore, more evidence was needed to evaluate nephroprotective potential of ROCKi in type 2 diabetic nephropathy. Kolavennu *et al*. [9] addressed this issue in a study conducted in db/db mice. Fasudil, administered for 16 weeks, attenuated the development of mesangial expansion, thickening of glomerular basement membrane, albuminuria and expression of ECM proteins, in conjunction with reductions of diabetes-induced increases in RhoA/ROCK activity. The effects of ROCKi were comparable with those induced by simvastatin. Unlike the report by Kikuchi *et al*. [41], fasudil did not influence metabolic parameters in db/db mice. The results of studies with pharmacological inhibitors of the pathway have been reproduced, at least in part, in diabetic mice with ROCK deletion (ROCK knockout) [11, 42]. This evidence is important with respect to known imperfect specificity of fasudil towards ROCKs.

In addition to beneficial effects on hallmark structural changes and prosclerotic/profibrotic signalling, studies in experimental type 2 diabetes reported reduced number of infiltrating inflammatory cells in the kidney suggesting anti-inflammatory actions of ROCKi. Indeed, RhoA-ROCK are known to mediate inflammatory actions of AngII [43] and play a pivotal role in the signalling of strong inflammatory factors such as tumour necrosis factor α (TNFα) [44]. Both AngII [45] and TNFα [46] have been implicated in diabetic renal pathophysiology and inhibition of their proinflammatory actions is likely to contribute to protective effects of ROCKi. In studies conducted specifically in the diabetic context, RhoA-ROCK were identified as mediators of another important inflammatory factor monocyte chemoattractant protein-1 (MCP-1), its receptor (CCR2), and their deleterious effect on podocytic expression of nephrin and consequent albuminuria [47].

The following acute studies focused on additional pathophysiological mechanisms mediated by RhoA-ROCK, and addressed previously only in the non-diabetic context.

Axelsson *et al*. [48] have investigated the role of RhoA-ROCK in changes of glomerular permeability, determined as passage of intravenously administered FITC-Ficoll 70/400 into urine. In normal rats, acute glucose infusion to achieve hyperglycaemia (20–25 mM) caused rapid, reversible increases in permeability for large Ficolls, which was abrogated by co-administration of ROCKi Y27632. These observations are consistent with the formation of an increased number of large pores in the glomerular filter and their prevention by ROCK inhibition via regulation of cytoskeleton in cells forming glomerular filtration barrier and correspond to a large body of evidence in non-renal endothelial cells that identified Rho-ROCK as mediators of increased permeability in response to a variety of stimuli [15, 26]. Thus, Rho-ROCK might contribute to increased permeability of glomerular endothelial cells and consequently to albuminuria.

In addition to other functions, RhoA-ROCK contribute to the control of vascular tone in the cardiovascular system and in the kidney [49]. When activated, ROCK phosphorylate the myosin binding subunit (MBS) of the myosin light chain phosphatase (MLCP) leading to its inhibition, myosin light chain (MLC) phosphorylation, and contraction [13]. Consequently, RhoA-ROCK may play a role in the pathogenesis of renal haemodynamic changes in diabetes, which are known to contribute to the development of nephropathy [50]. To test this hypothesis, we have studied acute effects of the ROCKi Y27632 and fasudil in hyperfiltering STZ-diabetic rats and in non-diabetic control animals [51]. Inhibition of ROCKs decreased blood pressure (BP) in both control and diabetic rats. In contrast to comparable effects on BP, the renal haemodynamic response to ROCKi, characterized by renal vasodilation, stable glomerular filtration rate (GFR), and a decrease in filtration fraction (FF), was observed only in diabetic animals. The enhanced renal response to ROCKi indicates greater dependence of renal haemodynamics on RhoA-ROCK in diabetes, in agreement with evidence indicating activation of RhoA-ROCK in the diabetic kidney [9, 10].

The whole kidney haemodynamic pattern induced by ROCKi in diabetic rats suggested not only afferent but also an important efferent effect of these compounds. Consequently, the responses to ROCKi indicate a contribution of the RhoA-ROCK pathway to haemodynamic changes in the diabetic kidney and a possible haemodynamic component of the nephroprotective actions of ROCKi.

Most recently, the knowledge about pathogenic effects of ROCKs in the DKD has been expanded in two major

directions. First, impressive studies by Danesh's group [42] searched for the mechanism of beneficial actions of ROCK1 deletion on the development of DKD in mice and mechanisms of accelerated nephropathy in diabetic mice with podocyte-specific ROCK1 knock-in. They linked ROCK1 activation in the diabetic milieu to mitochondrial fragmentation in podocytes and glomerular endothelial cells, resulting in marked mitochondrial dysfunction, associated with enhanced ROS production and podocytic apoptosis. Mitochondrial generation of ROS has been identified as one of general mechanisms of pathogenesis of diabetic microvascular complications [52]. Second, RhoA-ROCK seem to have important interactions with micro RNAs (miRNA). Studies indicate the role of miRNAs in pathways implicated in the pathophysiology of DKD, in particular in the control of renal profibrotic signalling. Long *et al*. [53] reported that kidney developmental protein Sprouty homolog 1 (Spry1), which also acts as an endogenous negative regulator of RhoA, is under the control of miR-29c. Treatment with miR-29c antisense nucleotide reduced albuminuria and mesangial ECM accumulation via partial inhibition of ROCK activity.

In parallel, it has become apparent that TGF-β, the major driver of diabetic renal injury, regulates the expression of certain miRNA families such as miR-200, miR-192, miR 215 or 216 in kidney cells [54–59], which then regulate expression of TGF-β-dependent genes and production of ECM proteins in the diabetic kidney. In this context, Wang and coworkers [60] have recently demonstrated the role of the miR-29 family as a negative regulator of TGF-β-induced renal collagen synthesis and fibrosis. Interestingly, miR29 were found to be decreased in three models of renal tubulointerstitial fibrosis (TIF) *in vivo* including the UNx diabetic rats, and the treatments with fasudil that in parallel ameliorated TIF in UNx diabetic rats also restored low renal miR-29 levels [60]. More recent preliminary data indicate a similar pattern for miR-21 [61].

It should be noted that results by Wang *et al*. [60] are not, with respect to protective roles of miR29 and the involvement of ROCKs, in agreement with the study by Long *et al*. [53]. However, both these studies suggest that modulation of miRNAs may contribute to nephroprotective actions of ROCKi. Further studies are needed to elucidate these mechanisms.

Another controversy remains with respect to podocytic actions of ROCKi. Studies in STZ-diabetic rats have suggested beneficial effects of ROCKi in podocytes *in vivo* [10], supported by some previous observations in proteinuric non-diabetic kidney disease [62]. These effects of ROCKi included prevention of foot process effacement [10], up-regulation of nephrin [38] and, more recently, amelioration of mitochondrial dysfunction and apoptosis [42]. Further support for the pathogenic roles of RhoA-ROCK in podocytes has been provided by studies showing that the selective podocytic expression of constitutively active RhoA causes segmental foot-process effacement and histological features of focal segmental glomerulosclerosis (FSGS) [63]. However, these effects are difficult to reconcile with the evidence indicating important roles for RhoA in podocyte biology. The cytoskeleton is one of crucial elements in podocyte integrity. Derangements of the cytoskeleton contribute to podocyte foot process effacement, a structural feature observed in a number of proteinuric glomerulopathies [64]. Therefore, as a regulator of the cytoskeleton, the Rho-ROCK pathway might be important for maintaining podocyte shape and function. Indeed, as reported by [65] RhoA is linked to synaptopodin, a protein essential for the integrity of the podocyte actin cytoskeleton. Synaptopodin protects RhoA from ubiquitination. Morecently, Wang *et al*. [66] further developed this concept. The authors compared transgenic mice overexpressing inactive RhoA with those expressing constitutively active RhoA. Both models developed proteinuria and foot process effacement. The mechanisms of these adverse effects were different. While active RhoA enhanced actin polymerization, caused a reduction in nephrin and promoted podocyte apoptosis, expression of the dominant-negative RhoA caused a loss of podocyte stress fibres, did not alter the expression of either nephrin or Rho A, and did not cause podocyte apoptosis. These findings suggest that RhoA is pivotal for maintaining of the integrity of podocytes under basal conditions, and its activation or inhibition promotes podocyte injury. Thus, the beneficial effects of ROCKi described above are most likely associated with prevention of increased activity of RhoA-ROCK, but beneficial effects of ROCKi in established lesions associated with podocytes effacement has not been documented. Indeed, when initiated later in the course of experimental nephropathy, beneficial effects of ROCKi in the diabetic kidney are substantially weaker [38]. It is possible that intact RhoA-ROCK activity and signalling is essential for reversal or healing of podocyte effacement. Further studies focusing on this issue are needed to determine whether ROCKi is still beneficial or even harmful in this situation. In addition, the beneficial effect of ROCKi on podocytes in *in vivo* studies, could occur due to indirect effects on podocytes related, for example, to the effects of ROCKi on glomerular haemodynamics or attenuation of effects of AngII.

Clinical evidence

The experience with ROCKi is not limited to studies in experimental models of disease. Over the past decade, clinical studies with fasudil have suggested that ROCKi may be useful for treatment of various cardiovascular diseases.

Fasudil has been approved for clinical use in Japan and China, for treatment of cerebral vasospasms in its intravenous form. Intracoronary administration of fasudil markedly inhibits acetylcholine-induced coronary spasm and

related myocardial ischaemia [67]. Fasudil is also effective in treating patients with microvascular angina, indicating an involvement of Rho-kinase-mediated hyper-reactivity of coronary microvessels [68]. Intracoronary fasudil was also effective for the treatment of coronary spasm resistant to maximal vasodilator therapy with calcium channel blockers and nitrates after coronary artery bypass surgery [69]. Acute intra-arterial administration of fasudil also caused enhanced forearm arterial vasodilator responses in hypertensive patients [70] and in patients with heart failure [71] as compared with control subjects, and intravenous fasudil reduced pulmonary vascular resistance in patients with pulmonary hypertension [72]. Most recently, another interesting acute study [73] showed that intra-arterial fasudil enhanced both endothelium-dependent and -independent vasodilation in patients with metabolic syndrome when infused with insulin. This phenomenon was not observed in control subjects with normal insulin sensitivity. These studies have suggested activation of RhoA-ROCK in a variety of human cardiovascular disorders and identified these molecules as potential treatment targets in the clinical context.

More relevant with respect to treatment of complications of diabetes are clinical studies with prolonged administration of oral fasudil. The clinical trials exploring the anti-anginal effects of fasudil in Japanese patients with stable effort angina have demonstrated that the treatment improves exercise tolerance [74]. In a parallel multicentre, double-blind, placebo-controlled, randomized trial, conducted in the US in 84 stable angina patients for 8 weeks fasudil significantly increased the ischaemic threshold of angina patients during exercise with a trend toward increased exercise duration [75]. The incidence of adverse events in patients treated with fasudil in these two studies was similar to those receiving placebo.

Conclusions

Available experimental evidence indicates that ROCKi is a promising approach in the treatment of DKD that interferes with multiple mechanisms underlying the development and progression of this disorder. Considering the mentioned preclinical studies, promising data generated by studies in patients with cardiovascular disorders and the favourable safety profile of fasudil, there is a strong argument emanating from this research for the clinical testing of ROCKi in DKD. The need for add-on treatments complementing current options cannot be overemphasized. In some situations, ROCKi may even have some theoretical advantages over RAAS inhibitors. For example, renal haemodynamic actions of ROCKi in diabetes as described in experimental settings [51] might be beneficial in diabetic patients with low GFR or concomitant renovascular disease who cannot be treated with sufficient doses of RAAS inhibitors due to risks of acute kidney insufficiency or hyperkalaemia. Moreover, unlike the RAAS inhibitors, ROCKi do not act on the receptor level, but inhibit intracellular signalling of most of the vasocontrictors. This phenomenon suggests that in some situations ROCKi may have a stronger impact on glomerular haemodynamics than RAAS inhibitors.

One of possible reasons for the lack of clinical testing of available ROCKi may lie in their low specificity towards their major target [6], an issue that could be resolved by introduction of new compounds. It should be also noted that the described favourable safety profile of fasudil relies on studies with relatively short term patient follow-up. However, these questions will not be answered without further clinical testing of fasudil and possibly newer ROCKi. In the meantime basic research should focus, in addition to already discussed problems, on synthesis and testing of inhibitors with higher selectivity for ROCKs and on inhibitors selective for individual ROCK isoforms.

Competing Interests

The author has completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf and declares he had support from the Juvenile Diabetes Research Foundation for parts the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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