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Rho kinase inhibition protects kidneys from diabetic nephropathy without reducing blood pressure

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Rho-associated kinases (ROCK) are activated in the kidney as well as in cultured cells of diabetic models and have been implicated in renal pathophysiology. To explore whether inhibition of ROCK is protective, we studied its role in a model of accelerated diabetic nephropathy where uninephrectomized rats were made diabetic by streptozotocin. After establishing diabetes, rats were treated with the ROCK inhibitor fasudil continuously or for the final 6 weeks of an 18-week experimental period. The results were compared to similar rats given losartan, an established treatment of clinical and experimental diabetic nephropathy, or a combination of both agents. Vehicle-treated diabetic and non-diabetic uninephrectomized rats served as controls. Diabetes resulted in a rapid development of albuminuria, higher glomerulosclerosis and interstitial fibrosis scores, lower glomerular filtration rates, and increased expression of several molecular markers of diabetic nephropathy. Eighteen weeks of fasudil treatment reduced renal ROCK activity, and ameliorated diabetes-induced structural changes in the kidney and expression of the molecular markers in association with a modest anti-proteinuric effect but no change in blood pressure. Late intervention with fasudil reduced glomerulosclerosis, but did not influence proteinuria. Most effects of fasudil were comparable to those of losartan, although losartan lowered blood pressure and further lowered proteinuria. The combination of both treatments was no different than losartan alone. Thus, ROCK inhibition protected the kidney from diabetic nephropathy even though it did not reduce the blood pressure.

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Despite progress in the prevention and treatment of diabetic nephropathy (DN), reversal and even stabilization of the progressive course of DN are still difficult to achieve, and many patients still progress to end-stage renal disease. New approaches that would broaden the spectrum of available treatments for DN are needed to improve prognosis in these patients.

RhoA, a member of the Ras superfamily of small GTP-binding proteins, and its downstream effectors Rho-associated kinases (ROCKs), are signaling molecules implicated in a variety of biological functions, including cell contraction, cell migration, cell adhesion, cell cycle progression, and gene expression.[1] The RhoA/ROCK pathway is stimulated by agonists acting via G-protein-coupled, tyrosine kinase, and cytokine receptors, cell adhesion and integrin clustering, as well as by mechanical stress, which regulate the activity of RhoA guanine nucleotide exchange factors and GTP loading of RhoA.[1] RhoA/ROCK have emerged as important players in cardiovascular and renal pathophysiology. This pathway is activated in the vasculature and kidney in different models of hypertension, hypertensive end-organ damage, and kidney disease,[2–7] and studies with ROCK inhibitors (ROCKi), such as fasudil or Y27632, indicate protective renal actions of these compounds.[4–10] Importantly, several clinical studies have also documented beneficial effects of ROCKi in patients with cardiovascular disorders.[11,12]

RhoA/ROCK are activated in the kidney and cardiovascular system in models of diabetes both *in vitro* and *in vivo*.[6,7,13–16] The pathway converges numerous pathophysiological signals triggered by the diabetic milieu, and mediates processes implicated in the pathophysiology of nephropathy, such as upregulation of prosclerotic cytokines, PAI-1 (plasminogen activator inhibitor 1), osteopontin, and production of extracellular/mesangial matrix (ECM).[6,7,13–17]

Based on this evidence, the RhoA/ROCK pathway appears to be a promising target for pharmacological intervention to prevent the development and progression of nephropathy. To address this issue, we explored the nephroprotective potential and mechanisms of action of the ROCKi fasudil in uninephrectomized diabetic rats with an accelerated course

of nephropathy,[18] including the effects later in the course of this disorder. The effects of fasudil were compared with those of the angiotensin receptor blocker (ARB) losartan, an established treatment for both clinical and experimental DN, and to a combination of both agents.

RESULTS

Physical and metabolic parameters

As summarized in Table 1, all diabetic groups demonstrated lower body weight gains. The right kidney weight was increased in diabetic animals, except the group of diabetic rats treated with fasudil (D-FA; fasudil from Calbiochem, San Diego, CA), and the kidney/body weight ratios were significantly higher in all groups of diabetic rats when compared with non-diabetic controls. Diabetic rats had hyperglycemia and corresponding increases in HBA1c (glycosylated hemoglobin) when compared with non-diabetic rats ($P<0.001$). The metabolic parameters were not influenced by treatment.

Blood pressure and renal functional parameters

At baseline, there were no differences in systolic blood pressure among the groups of rats (Table 2). Systolic blood pressure did not significantly change throughout the study in control rats receiving vehicle (C-VE) and diabetic rats receiving vehicle (D-VE), and in D-FA rats. In contrast, rats treated with losartan both alone or in combination with fasudil demonstrated lower systolic blood pressure at all time points during follow-up, with a significant difference at weeks 12 and 18 when compared with vehicle-treated rats, and diabetic rats receiving fasudil as late treatment (D-FAlate) at weeks 6 and 12.

As shown in Figure 1, 24-h urinary albumin excretion ($U_{alb}V$) was increased in D-VE rats when compared with C-VE rats at weeks 6 and 12. Treatment with fasudil was associated with a significant antialbuminuric effect at weeks 6 and 12, similar to the effects of losartan and the combination of both agents. However, at the end of the study, a significant reduction in $U_{alb}V$ was observed only in diabetic rats treated with losartan (D-LOS; losartan from Merck, Whitehouse Station, NJ). Moreover, $U_{alb}V$ was lower in D-LOS when compared with D-FA rats. The course of $U_{alb}V$ in D-FAlate was similar to that in D-VE; $U_{alb}V$ was higher than in C-VE throughout the study, and also higher than losartan-treated animals at weeks 12 and 18 ($P<0.05$).

Compared with non-diabetic controls, D-VE rats demonstrated lower glomerular filtration rate (GFR), determined as creatinine clearance at week 18 (Figure 2). This decrease in creatinine clearance was not observed in diabetic groups treated with fasudil, losartan, or late fasudil; all demonstrated significantly higher creatinine clearance values than those observed in D-VE.

Histological analysis

The glomerular sclerosis score and proportion of severely affected glomeruli were increased in D-VE compared with non-diabetic controls (Figure 3). In all fasudil- and losartan-treated groups, the glomerular sclerosis score and proportion of severely affected glomeruli were not different from controls, and significantly lower when compared with D-VE rats. The tubulointerstitial fibrosis score was significantly higher in D-VE when compared with controls, and reduced to values observed in C-VE by treatment with fasudil, losartan, or the combination (Figure 3). Unlike the other groups of rats, in which the treatment was initiated at the onset of diabetes, the reduction in tubulointerstitial fibrosis score in D-FAlate rats did not reach statistical significance.

Determination of renal ROCK activity

Compared with C-VE, vehicle-treated diabetic rats displayed increased phosphorylation of MYPT, a downstream substrate

Table 2 | Effect of fasudil and losartan on systolic blood pressure (mm Hg)

Group	Baseline	Week 6	Week 12	Week 18
C-VE	135 ± 7	133 ± 3	141 ± 7	147 ± 7
D-VE	148 ± 3	141 ± 6	142 ± 4	147 ± 3
D-FA	143 ± 6	136 ± 6	142 ± 6	145 ± 8
D-LOS	146 ± 6	127 ± 6[c]	125 ± 6[*,a]	127 ± 4[*,a]
D-FA+LOS	145 ± 6	127 ± 5[c]	122 ± 5[*,b,c]	131 ± 4[a]
D-FAlate	147 ± 6	147 ± 5	150 ± 4	141 ± 6

Abbreviations: C-VE, control-vehicle; D-FA, diabetic-fasudil; D-FA+LOS, diabetic-fasudil+losartan; D-FAlate, diabetic-late fasudil treatment; D-LOS, diabetic-losartan; D-VE, diabetic-vehicle.
*$P<0.05$ vs C-VE.
[a]$P<0.05$ vs D-VE.
[b]$P<0.01$ vs D-VE.
[c]$P<0.05$ vs D-FAlate.

Table 1 | Physical and metabolic parameters

Group	BWT (g)	RKW (g)	RKW/BWT (g per 100 g bwt)	BG (mg/dl)	HBA1c (%)
C-VE	460 ± 12	2.7 ± 0.1	0.58 ± 0.02	72 ± 6	3.2 ± 0.1
D-VE	400 ± 6[†]	3.3 ± 0.1*	0.82 ± 0.03[†]	295 ± 27[†]	4.9 ± 0.2[†]
D-FA	403 ± 9[†]	3.1 ± 0.1	0.77 ± 0.03*	321 ± 20[†]	4.9 ± 0.2[†]
D-LOS	414 ± 8*	3.4 ± 0.2*	0.83 ± 0.06[†]	327 ± 18[†]	5.0 ± 0.2[†]
D-FA+LOS	417 ± 12*	3.6 ± 0.2[†]	0.86 ± 0.04[†]	319 ± 25[†]	5.2 ± 0.2[†]
D-FAlate	410 ± 4[†]	3.5 ± 0.1[†]	0.85 ± 0.04[†]	291 ± 29[†]	5.2 ± 0.2[†]

Abbreviations: BG, blood glucose; BWT, body weight; C-VE, control-vehicle; D-FA, diabetic-fasudil; D-FA+LOS, diabetic-fasudil+losartan; D-FAlate, diabetic-late fasudil treatment; D-LOS, diabetic-losartan; D-VE, diabetic-vehicle; HBA1c, glycosylated hemoglobin; RKW, right kidney weight.
*$P<0.05$.
[†]$P<0.01$ vs C-VE.

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