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RESEARCH PAPER

Effects of systemic inhibition of Rho kinase on blood pressure and renal haemodynamics in diabetic rats

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BACKGROUND AND PURPOSE
The RhoA/Rho associated kinases (ROCK) pathway has been implicated in the pathophysiology of diabetic nephropathy (DN). Early stages of diabetes are associated with renal haemodynamic changes, contributing to later development of DN. However, the role of RhoA/ROCK, known regulators of vascular tone, in this process has not been studied.

EXPERIMENTAL APPROACH
Blood pressure (BP), glomerular filtration (GFR), effective renal plasma flow and filtration fraction (FF) in response to the ROCK inhibitors Y27632 (0.1 and 0.5 mg·kg^{-1}) and fasudil (0.3 and 1.5 mg·kg^{-1}) were examined in streptozotocin-diabetic rats and non-diabetic controls.

KEY RESULTS
Diabetic rats demonstrated baseline increases in GFR and FF. In contrast to similar decreases in BP in diabetic and control rats, renal vasodilator effects and a decrease in FF, following ROCK inhibition were observed only in diabetic rats. The vasodilator effects of Y27632 and a further decrease in FF, were also detected in diabetic rats pretreated with the angiotensin antagonist losartan. The effects of ROCK inhibitors in diabetic rats were modulated by prior protein kinase C (PKC)β inhibition with ruboxistaurin, which abolished their effects on FF. Consistent with the renal vasodilator effects, the ROCK inhibitors reduced phosphorylation of myosin light chain in diabetic kidneys.

CONCLUSIONS AND IMPLICATIONS
The results indicate greater dependence of renal haemodynamics on RhoA/ROCK and beneficial haemodynamic effects of ROCK inhibitors in diabetes, which were additive to the effects of losartan. In this process, the RhoA/ROCK pathway operated downstream of or interacted with, PKCβ in some segments of the renal vascular tree.

Abbreviations
ARB, angiotensin receptor blocker; BP, blood pressure; CHF, congestive heart failure; DN, diabetic nephropathy; ERPF, effective renal plasma flow; FF, filtration fraction; GFR, glomerular filtration; HBA1c, glycosylated haemoglobin; Hct, haematocrit; MAP, mean arterial pressure; MBS, myosin binding subunit; MLC, myosin light chain; MLCP, myosin light chain phosphatase; PAH, *para*-aminohippurate; Pgc, glomerular capillary pressure; PKCβ, protein kinase C-β; ROCK, Rho associated kinases; RVR, renal vascular resistance

Introduction

RhoA, a member of the Ras superfamily of small GTP-binding proteins, and its down-stream effectors Rho associated kinases (ROCK) are signalling molecules implicated in a variety of biological functions including cell contraction, cell migration, cell adhesion, cell cycle progression and gene expression (Loirand *et al.*, 2005). The RhoA/ROCK pathway is stimulated by agonists acting via G protein-coupled, tyrosine kinase, and cytokine receptors, cell adhesion and integrin clustering, and by mechanical

stress, which essentially regulate the activity of RhoA guanine nucleotide exchange factors and GTP loading of the RhoA (Loirand *et al.*, 2005). The pathway has been found to be activated in different models of hypertension and hypertensive end-organ damage (Kanda *et al.*, 2005), cardiovascular disease (Kataoka *et al.*, 2002; Sanada *et al.*, 2004; Hamid *et al.*, 2007) and kidney disease (Kataoka *et al.*, 2002; Kanda *et al.*, 2003; Sun *et al.*, 2006; Kolavennu *et al.*, 2008; Peng *et al.*, 2008). Studies with ROCK inhibitors, such as fasudil or Y27632, indicate protective cardiovascular and renal actions of these compounds (Kanda *et al.*, 2003; Nishikimi *et al.*, 2004; Ishikawa *et al.*, 2006; Sun *et al.*, 2006; Ozawa and Kobori, 2007; Kolavennu *et al.*, 2008; Peng *et al.*, 2008).

In addition to other functions, RhoA/ROCK plays a role in the control of vascular tone in the cardiovascular system and in the kidney. ROCK inhibitors decrease basal vascular tone and attenuate vascular smooth muscle contraction induced by agonists of GPCRs including angiotensin II (Uehata *et al.*, 1997; Bauer and Parekh, 2003; Cavarape *et al.*, 2003a; Nakamura *et al.*, 2003; Winaver *et al.*, 2006). 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 (Somlyo and Somlyo, 2003).

Early stages of nephropathy in diabetes are characterized by specific haemodynamic changes characterized by elevations in glomerular filtration rate (GFR) and increases in filtration fraction (FF) (Hannedouche *et al.*, 1990). On the single-nephron level, the major renal haemodynamic alteration in diabetes is a disproportionate decrease in afferent as compared with efferent arteriolar resistance, resulting in elevated glomerular capillary pressure (Pgc) (Anderson and Komers, 2004), which initiates a cascade of events that contribute to the development of structural changes in the kidney and ultimately to kidney failure (Anderson and Komers, 2004). Therapeutic interventions that result in reductions of Pgc are nephroprotective in progressive glomerulosclerosis (Zatz *et al.*, 1986). As indicated in multiple studies from the past three decades and summarized in a recent meta-analysis (Magee *et al.*, 2009), this 'diabetic hyperfiltration' is considered to be an early risk factor for future development of diabetic nephropathy (DN).

In addition to other mechanisms, the complex pathophysiology underlying the renal haemodynamic changes in diabetes involves activation of vasoconstrictor (renin-angiotensin system, endothelin, vasoconstrictor prostanoids) and vasodilator (vasodilator prostanoids, nitric oxide, atrial natriuretic peptide) systems and an imbalance in their actions on glomerular vasculature and contractile components (Anderson and Komers, 2004).

RhoA/ROCK is another vasoactive system that has been recently found to be activated in the diabetic kidney and cardiovascular system both *in vitro* and *in vivo* (Miao *et al.*, 2002; Kawamura *et al.*, 2004; Rikitake and Liao, 2005; Kolavennu *et al.*, 2008; Peng *et al.*, 2008). The RhoA/ROCK pathway converges numerous pathophysiological signals triggered by the diabetic milieu, including those implicated in the pathophysiology of nephropathy. Recent studies (Kolavennu *et al.*, 2008; Peng *et al.*, 2008), including our data (Komers *et al.*, 2010), have suggested nephroprotective effects of inhibitors of ROCK in experimental DN. However, the contribution of the RhoA/ROCK pathway to the development of renal haemodynamic changes in diabetes remains unknown. Moreover, despite the fact that ROCK inhibitors are now being clinically tested in cardiovascular disease (Fukumoto *et al.*, 2007), there is no information about the effects of ROCK inhibitors on individual renal haemodynamic parameters in diabetes.

We hypothesized that the RhoA/ROCK pathway was involved in the regulation of blood pressure (BP) and renal haemodynamics in diabetes. To test this hypothesis, acute effects of the ROCK inhibitors Y27632 and fasudil were examined in hyperfiltering streptozotocin-diabetic rats, a model of Type 1 diabetes, and compared with non-diabetic animals. Furthermore, to elucidate whether haemodynamic responses to ROCK inhibitors in Type 1 diabetes could be modulated by inhibition of established mediators of diabetes-induced renal injury (Anderson, 1998; Koya and King, 1998), we also studied the effects of ROCK inhibitors in diabetic rats pretreated with the angiotensin receptor blocker (ARB) losartan or the protein kinase C-β (PKCβ) inhibitor, ruboxistaurin.

Methods

The diabetic rat model

All animal care and experimental procedures in these studies were approved by the Portland Veteran Affairs Institutional Animal Care and Use Subcommittee. Studies were conducted in adult male Sprague-Dawley rats with initial weights of ~300 g. The rats were made diabetic by intraperitoneal injection of streptozotocin (Sigma Chemical Co., St. Louis, MO, USA, 65 $mg \cdot kg^{-1}$). Three days later, induction of diabetes was confirmed by measurement of glucose levels in tail blood, using a reflectance meter (One Touch II; Lifescan, Milpetas, CA,

USA). Rats with blood glucose >15 mmol·L^{-1} were considered diabetic. Diabetic rats received daily evening injections of long-acting insulin (Lantus, Sanofi-Aventis, Bridgewater, NJ, USA). Blood glucose levels were monitored at least weekly and the doses of insulin were individually adjusted to maintain blood glucose levels ~20 mmol·L^{-1}. Age-matched non-diabetic Sprague-Dawley rats served as controls. All rats were fed standard rat chow (Rodent Laboratory Chow 5010; Ralston Purina, Richmond, IN, USA) *ad libitum*.

Study design

To explore the acute systemic and renal haemodynamic effects of ROCK inhibitors, control and hyperfiltering diabetic rats (4 weeks of diabetes) were first studied before and after administration of the ROCK inhibitors, Y27632 (Calbiochem, San Diego, CA, USA). After surgical preparation and 60 min of equilibration, all rats underwent baseline measurements of mean arterial pressure (MAP), GFR, effective renal plasma flow (ERPF), FF and renal vascular resistance (RVR). Thereafter, rats received a continuous 20 min infusion of Y27632 (0.1 mg·kg^{-1}) or the same volume of vehicle (0.9% NaCl), and all measurements were repeated to assess changes from baseline (Period 1). The clearance period was started at minute 5 of the Y27632 infusion. After these measurements, the effects of a higher dose of Y27632 (0.5 mg·kg^{-1}) were assessed in a similar manner, including the effects of vehicle alone (Period 2). An additional group of diabetic rats was studied to assess whether Y27632-induced effects can be reproduced by a structurally different ROCK inhibitors, fasudil (Calbiochem, 0.3 and 1.5 mg·kg^{-1}). The doses of Y27632 were selected based on published observations in normal rats and our pilot studies. The lower dose of Y27632 (0.1 mg·kg^{-1}) has been reported as the lowest to decrease BP after systemic i.v. administration in normal rats (Winaver *et al.*, 2006). The dose of fasudil was selected from reports indicating that an approximately three times higher dose of fasudil as compared with Y27632 is required to induce a similar vasodilator response (Uehata *et al.*, 1997; Bauer and Parekh, 2003).

To assess whether RhoA/ROCK operate as part of the angiotensin II or PKCβ signalling pathways, effects of Y27632, tested as described above, were explored in additional groups of diabetic rats pretreated with the ARB losartan (receptor nomenclature follows Alexander *et al.*, 2009; supplied by Merck, Whitehouse Station, NJ, USA, 20 mg·kg^{-1} (Qin *et al.*, 2003) or ruboxistaurin (Elli-Lilly, Indianapolis, IN, USA, 10 mg·kg^{-1}; (Koya *et al.*, 1997) administered by gavage 48, 24 and 1 h before the haemodynamic measurements. After completion of haemodynamic measurements, the right kidney in control and diabetic rats treated with vehicle or Y27632 was rapidly excised, weighed, divided into cortical and medullary portions, and snap frozen in liquid nitrogen for further analyses by Western blotting. Blood samples for measurement of glycosylated haemoglobin (HBA1c) were obtained from the abdominal aorta.

Functional studies

Surgical preparation and renal function studies were performed under general anaesthesia (Inactin, 50 mg·kg^{-1} i.p.) as previously described (Komers *et al.*, 2000). In brief, the left femoral artery was catheterized and used for subsequent periodic blood sampling and measurement of MAP using an electronic transducer connected to a direct-writing recorder. After tracheostomy, jugular venous catheters were inserted for infusions of inulin (10%, Questcor, Carlsbad, CA, USA), *para*-aminohippurate (PAH) (0.8%, PAH; Merck, West Point, PA, USA) and rat serum. Intravenous infusions of rat serum and inulin/PAH solution in 0.9% NaCl were started at rates of 6.0 and 1.2 mL·h^{-1} respectively. The left ureter was catheterized for urine collection. Euvolemia was maintained by infusing iso-oncotic rat serum at 6 mL·h^{-1} in a total amount equal to 1% of the body weight, followed by a reduction in infusion rate to 1.6 mL·kg^{-1}·h^{-1} to maintain haematocrit (Hct) constant. Diabetic rats received extra saline to match the excessive urinary losses during the procedure. GFR was measured as inulin clearance and ERPF was measured as PAH clearance. The FF was determined as GFR/ERPF. The RVR was calculated as MAP/(ERPF/1-Hct).

Immunoblotting

The kidneys were homogenized in lysis buffer containing protease and phosphatase inhibitors and analysed by immunoblotting as previously described (Komers *et al.*, 2006), using primary antibodies raised against phospho-serine 19-MLC (P-MLC, 1:800, Cell Signaling, Beverly, MA, USA cat. #3671); and ROCK1 and ROCK2 (1:500, BD Transduction Laboratories™, 2350 Qume Drive, San Jose, CA, USA, cat. # 611137 and 610623). Following detection of P-MLC, membranes were stripped and reincubated with an antibody against total MLC (Cell Signaling, 1:800, cat. # 3672), and further processed as described above. To confirm equality of loading, all membranes were stripped and reanalysed for actin expression (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Table 1
Physical and metabolic characteristics in control and diabetic rats

Group	*n*	BW (g)	LKW (g)	LKW/BW (g·100 g^{-1})	BG (mmol·L^{-1})	HBA1c (%)
Control-vehicle	8	361 ± 5	1.30 ± 0.05	0.36 ± 0.02	4.1 ± 0.2	2.5 ± 0.2
Control-Y27632	8	377 ± 7	1.33 ± 0.02	0.35 ± 0.01	3.8 ± 0.1	2.5 ± 0.1
Diabetic-vehicle	10	332 ± 13†	1.74 ± 0.09†	0.54 ± 0.02†	19.4 ± 1.2†	4.6 ± 0.2†
Diabetic -Y27632	8	324 ± 9†	1.71 ± 0.03†	0.53 ± 0.01†	18.2 ± 2.0†	4.5 ± 0.2†
Diabetic-FASUDIL	8	348 ± 7*	1.95 ± 0.10†	0.59 ± 0.03†	21.9 ± 2.0†	4.9 ± 0.2†
Diabetic-LOS-Y27632	9	344 ± 7†	1.82 ± 0.08†	0.53 ± 0.02†	20.0 ± 1.7†	4.8 ± 0.2†
Diabetic-RBX- Y27632	8	324 ± 6†	1.73 ± 0.06†	0.53 ± 0.01†	19.2 ± 0.9†	4.8 ± 0.2†

*$P < 0.05$; $^{\dagger}P < 0.01$ versus control rats.
BW, body weight; LKW, left kidney weight; LKW/BW, left kidney/body weight ratio; BG, blood glucose; HBA1c, glycosylated haemoglobin; LOS, losartan; RBX, ruboxistaurin.

Biochemical methods

Inulin concentrations were measured by using a macroanthrone method. A solution of anthrone (0.5%)/sulphuric acid (70%) was added to 250 µL aliquots of samples and allowed to incubate for 10 min. Samples were then read on a spectrophotometer (Milton Roy 21D) at 620 nm. Unknown values were then calculated against known standard concentrations run in the same assay. PAH levels were determined using a colorimetric assay. Reagents used in this assay include 1 N HCl, $NaNO_2$, NH_4 sulfamate and a Coupling reagent (N-1-naphthyl ethylenediamine HCl). After a 10 min incubation, solutions were read at 540 nm on a spectrophotometer (Beckman DU 40) and values were again calculated against known standards. HBA1c was determined by a Nycocard Reader (Axis-Shield, Oslo, Norway).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was defined as a $P < 0.05$. Significant differences between experimental periods within one group were evaluated using ANOVA for repeated measures. Significant differences between groups were examined with the use of ANOVA factorial and the Bonferroni post-test.

Results

General characteristics of control and diabetic rats are shown in Table 1. Diabetic rats demonstrated reduced weight gain, renal hypertrophy (as assessed by kidney weight and kidney/body weight ratio), hyperglycemia and increases in HBA1c.

Effects of ROCK inhibition on BP and renal haemodynamics in control and diabetic rats

We first studied the effects of the ROCK inhibitors Y27632 on BP and renal haemodynamics in control and diabetic rats. Both control and diabetic animals displayed no differences in baseline MAP (Table 2, Figure 1) and renal haemodynamic parameters between the vehicle-treated groups and their ROCK inhibitor-treated counterparts (Figure 1, Tables 2–4). There were also no changes in MAP and renal haemodynamics in control and diabetic rats during the administration of vehicle. The groups of diabetic rats demonstrated baseline increases in GFR and FF as compared with non-diabetic animals ($P < 0.01$). In non-diabetic animals, Y27632 infusion resulted in a dose-dependent decrease in MAP (Table 2, Figure 1). Over this dose range, Y27632 did not lead to changes in renal haemodynamics as compared with baseline (Figure 1, left panels, Tables 2–4). However, a mild renal vasodilator effect of ROCK inhibition was suggested by the lower RVR in Y27632-treated compared with vehicle-treated animals (Table 2).

In diabetic rats, the MAP response to Y27632 was similar to that in control animals, resulting in a dose-dependent reduction (Figure 1, Table 2). In contrast to non-diabetic animals, diabetic rats demonstrated significant increases in ERPF and reductions in RVR and FF in response to a higher dose of the inhibitor ($P < 0.01$ vs. baseline and low-dose), while GFR remained stable (Figure 1, right panels; Tables 2–4). The effects of Y27632 on MAP and renal haemodynamic parameters in diabetic rats were reproduced by the structurally dissimilar ROCK inhibitor fasudil (Figure 1, right panels, Tables 2–4).

Table 2

Effects of ROCK inhibitors on mean arterial blood pressure and renal vascular resistance in control and diabetic rats

Group	n	Mean arterial pressure (mmHg) Baseline	Period 1	Period 2	Renal vascular resistance (mmHg·mL^{-1}·min^{-1}) Baseline	Period 1	Period 2
Control-vehicle	8	137 ± 3	136 ± 4	134 ± 4	12.4 ± 1.0	12.0 ± 1.1	12.9 ± 1.3
Control-Y27632	8	132 ± 4	128 ± 4*	116 ± 3$^{\dagger b}$	10.9 ± 0.6	11.3 ± 0.7	10.0 ± 0.5^{a}
Diabetic-vehicle	10	137 ± 3	136 ± 4	135 ± 4	12.5 ± 0.9	12.4 ± 1.0	13.2 ± 1.4
Diabetic-Y27632	8	132 ± 4	128 ± 3*	115 ± 4$^{\dagger\P b}$	13.2 ± 0.7	12.4 ± 0.9	10.1 ± 1.0†¶
Diabetic-FASUDIL	8	133 ± 6	132 ± 6	123 ± 5$^{\dagger\P a}$	12.2 ± 0.9	12.6 ± 1.1	10.2 ± 0.8†¶
Diabetic-LOS-Y27632	9	121 ± 5^{a}	121 ± 5^{a}	108 ± 5$^{\dagger\P b}$	8.0 ± 0.5^{b}	7.7 ± 0.4^{b}	6.7 ± 0.3$^{\dagger\P b}$
Diabetic-RBX-Y27632	8	136 ± 4	132 ± 3*	119 ± 3$^{\dagger\P b}$	15.0 ± 1.0	14.6 ± 0.8	11.9 ± 0.7†¶

*$P < 0.05$, $^{\dagger}P < 0.01$ versus Baseline; $^{\ddagger}P < 0.05$, $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated animals of the same period.
LOS, losartan; RBX, ruboxistaurin.

Table 3

Effects of ROCK inhibitors on glomerular filtration rate and effective renal plasma flow in control and diabetic rats

Group	n	Glomerular filtration rate (mL·min^{-1}) Baseline	Period 1	Period 2	Effective renal plasma flow (mL·min^{-1}) Baseline	Period 1	Period 2
Control-vehicle	8	1.81 ± 0.13	1.67 ± 0.09	1.60 ± 0.04	6.85 ± 0.65	6.70 ± 0.55	6.30 ± 0.41
Control-Y27632	8	1.87 ± 0.07	1.79 ± 0.14	1.74 ± 0.15	6.94 ± 0.31	6.70 ± 0.32	7.00 ± 0.46
Diabetic-Vehicle	10	2.45 ± 0.11	2.46 ± 0.14	2.37 ± 0.14	6.50 ± 0.50	6.60 ± 0.55	6.40 ± 0.50
Diabetic-Y27632	8	2.25 ± 0.11	2.35 ± 0.14	2.29 ± 0.10	5.80 ± 0.36	6.06 ± 0.48	7.00 ± 0.57†¶
Diabetic-FASUDIL	8	2.55 ± 0.20	2.33 ± 0.22	2.51 ± 0.20	6.25 ± 0.49	6.10 ± 0.39	7.20 ± 0.60†¶
Diabetic-LOS-Y27632	9	2.50 ± 0.17	2.56 ± 0.17	2.41 ± 0.21	8.74 ± 0.38^{b}	8.94 ± 0.44^{b}	9.67 ± 0.46$^{\dagger\P a}$
Diabetic-RBX-Y27632	8	1.80 ± 0.18^{a}	2.07 ± 0.15†	2.02 ± 0.15†	5.38 ± 0.38	5.50 ± 0.37	6.16 ± 0.40†¶

*$P < 0.05$, $^{\dagger}P < 0.01$ versus Baseline; $^{\ddagger}P < 0.05$, $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated animals of the same period.
LOS, losartan; RBX, ruboxistaurin.

Table 4

Effects of ROCK inhibitors on filtration fraction in control and diabetic rats

Group	n	Baseline	Period 1	Period 2
Control-vehicle	8	0.276 ± 0.019	0.256 ± 0.017	0.263 ± 0.016
Control-Y27632	8	0.273 ± 0.015	0.280 ± 0.026	0.250 ± 0.016
Diabetic-vehicle	10	0.385 ± 0.025	0.372 ± 0.023	0.371 ± 0.019
Diabetic-Y27632	8	0.394 ± 0.023	0.395 ± 0.025	0.340 ± 0.026†¶
Diabetic-FASUDIL	8	0.399 ± 0.030	0.390 ± 0.030	0.339 ± 0.031†¶
Diabetic-LOS-Y27632	9	0.290 ± 0.020^{a}	0.289 ± 0.020^{a}	0.251 ± 0.019$^{\dagger\P a}$
Diabetic-RBX-Y27632	8	0.337 ± 0.018	0.378 ± 0.020†	0.329 ± 0.020¶

$^{\dagger}P < 0.01$ versus Baseline; $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated animals of the same period.
LOS, losartan; RBX, ruboxistaurin.

Effects of ROCK inhibitors on BP and renal haemodynamics in diabetic rats with angiotensin II or PKCβ inhibition

The next experiments examined haemodynamic responses to ROCK inhibitors in diabetic rats pretreated with the ARB losartan. For clarity, the ERPF, GFR and FF in losartan-pretreated rats in comparison with the vehicle- or Y27632-treated diabetic animals without pretreatment are shown separately in Figure 2 and Tables 2–4. These rats displayed

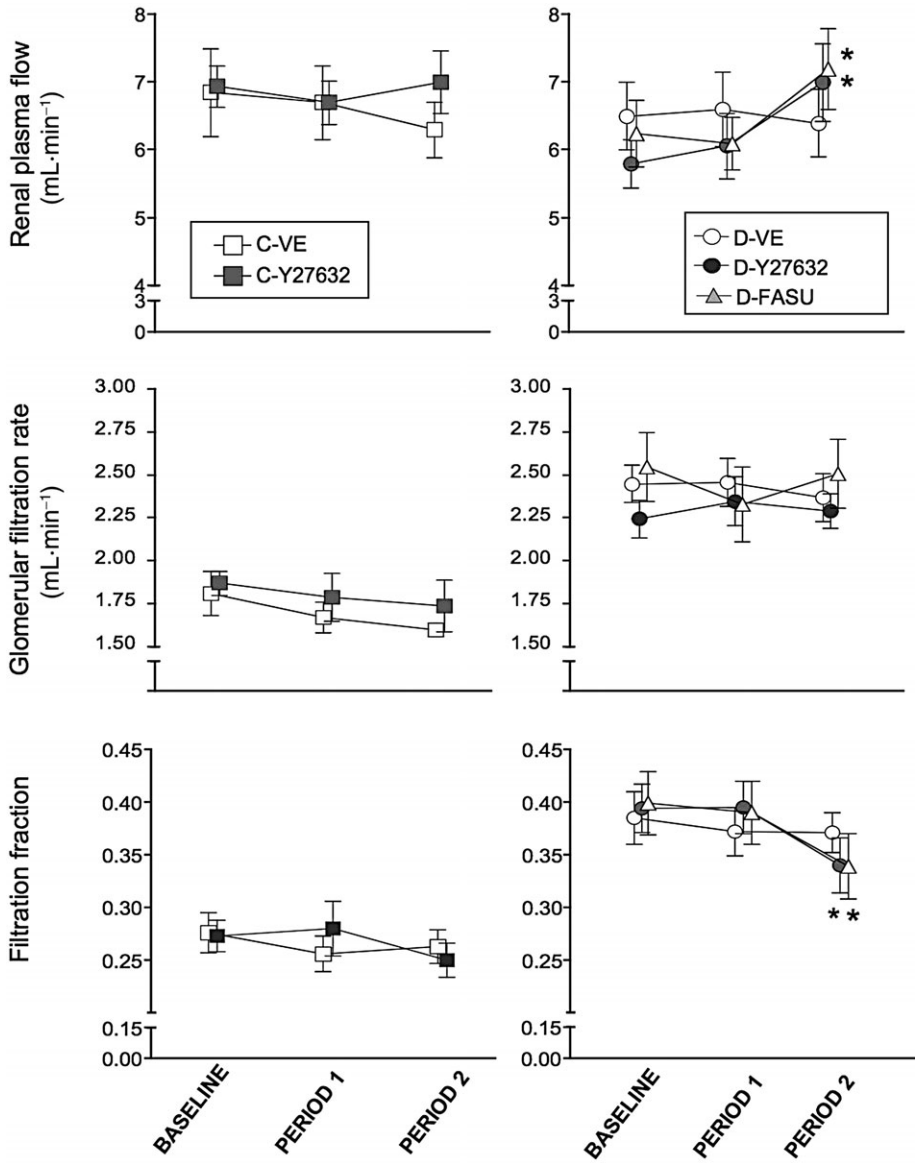

Figure 1

Effects of Rho associated kinases (ROCK) inhibitors on mean arterial pressure and renal haemodynamics in control and diabetic rats. After completion of baseline measurements, control (C; left panels) and diabetic rats (D; right panels) received a 20-minute infusion of the ROCK inhibitor Y27632 (0.1 mg·kg^{-1}) or the same volume of vehicle (VE, 0.9% NaCl), and all measurements were repeated to assess changes from baseline (PERIOD 1). After these measurements, the effects of a higher dose of Y27632 (0.5 mg·kg^{-1}) were assessed, including effects of vehicle alone (PERIOD 2). An additional group of diabetic rats was studied to assess whether Y27632-induced changes could be reproduced by a dissimilar ROCK inhibitor, fasudil (FASU, 0.3 and 1.5 mg·kg^{-1}). *$P < 0.05$; $^{\dagger}P < 0.01$ versus Baseline; $^{\ddagger}P < 0.05$; $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated animals of the same period.

lower baseline MAP (Table 2; Figure 2), higher ERPF and lower FF as compared with vehicle- or Y27632-treated groups of diabetic rats (Figure 2, Tables 2–4). When given Y27632, these animals displayed further decreases in MAP and FF, and renal vasodilation in response to the higher dose of the compound, comparable to animals treated with Y27632 alone (Figure 2; Tables 2–4).

To explore the possibility that RhoA/ROCK acts downstream of PKCβ, an additional group of diabetic rats was pretreated with the PKCβ inhibitor ruboxistaurin. Pretreatment with ruboxistaurin did not influence baseline MAP (Table 2, Figure 3), but did affect baseline renal haemodynamics. The ruboxistaurin-treated animals displayed lower baseline GFR and FF, compared with the diabetic-vehicle or diabetic-Y27632 groups (Figure 3, Tables 3 and 4). As in the other diabetic groups, acute administration of Y27632 resulted in a dose-dependent decrease in MAP (Table 2, Figure 3) and an increase

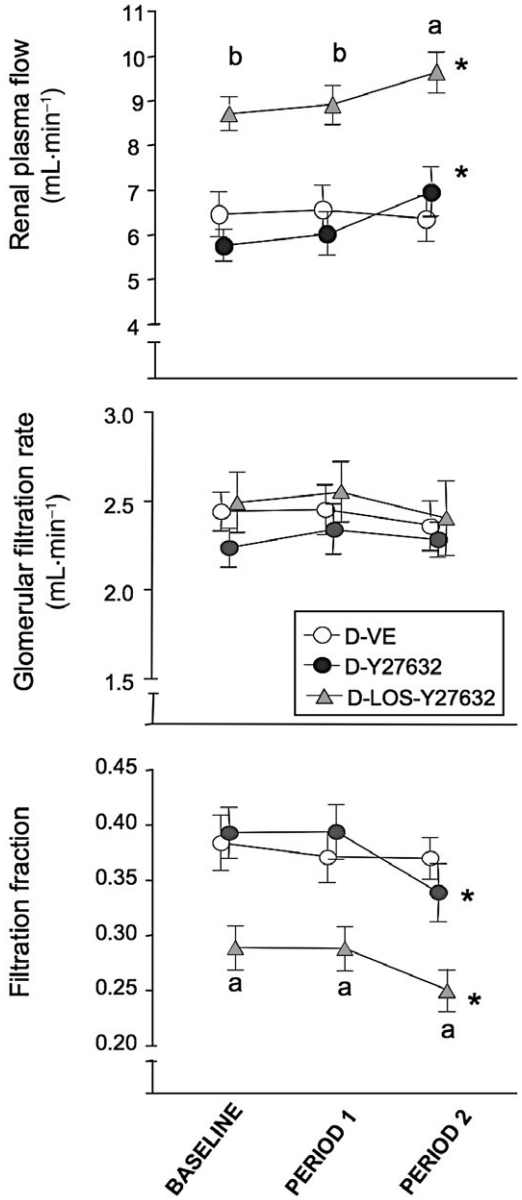

Figure 2

Effects of losartan on mean arterial pressure and renal haemodynamic responses to Rho associated kinases (ROCK) inhibitor in diabetic rats. The rats received losartan (20 mg·kg^{-1} p.o.) 48, 24, and 1 h before the haemodynamic studies. After completion of baseline measurements, the rats (D-LOS-Y27632) received a 20-minute infusion of the ROCK inhibitor Y27632 (0.1 mg·kg^{-1}), and all measurements were repeated to assess changes from baseline (PERIOD 1). After these measurements, the effects of a higher dose of Y27632 (0.5 mg·kg^{-1}) were assessed in a similar manner (PERIOD 2). The results were compared with vehicle-treated (D-VE) and Y27632-treated (D-Y27632) diabetic rats with no pretreatment. *$P < 0.05$; $^{\dagger}P < 0.01$ versus Baseline; $^{\ddagger}P < 0.05$; $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated animals of the same period.

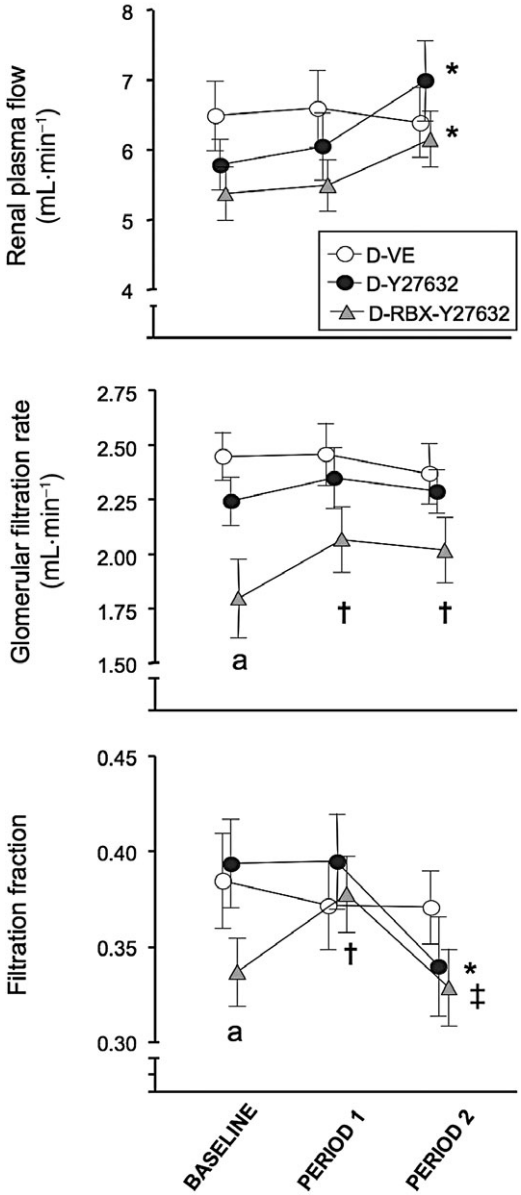

Figure 3

Effects of protein kinase C-β (PKCβ) inhibition with ruboxistaurin on mean arterial pressure and renal haemodynamic responses to Rho associated kinases (ROCK) inhibitor in diabetic rats. Rats received treatment with ruboxistaurin (RBX; 10 mg·kg^{-1} p.o.) 48, 24, and 1 h before the haemodynamic studies. Following the baseline measurements, the rats (D-RBX-Y27632) received a 20-minute infusion of the ROCK inhibitor Y27632 (0.1 mg·kg^{-1}), and all measurements were repeated to assess changes from baseline (PERIOD 1). After these measurements, the effects of a higher dose of Y27632 (0.5 mg·kg^{-1}) were assessed in a similar manner (PERIOD 2). The results were compared with vehicle-treated (D-VE) and Y27632-treated (D-Y27632) diabetic rats with no pretreatment. *$P < 0.05$; $^{\dagger}P < 0.01$ versus Baseline; $^{\ddagger}P < 0.05$; $^{\P}P < 0.01$ versus. Period 1; $^{\P}P < 0.01$ versus Period 1; $^{a}P < 0.05$; $^{b}P < 0.01$ versus vehicle-treated animals of the same period.

in ERPF (Table 3, Figure 3), associated with a lower RVR in response to the higher dose of the compound (Table 2). In contrast to other ROCK inhibitor-treated diabetic animals, which demonstrated no changes in GFR, the rats pretreated with ruboxistaurin responded to Y27632 with an increase in GFR. This effect was already detectable after the lower dose of Y27632. GFR also remained elevated after the higher dose of the inhibitor (Figure 3, Table 3). These changes in GFR in ruboxistaurin-pretreated animals were associated with a rise in FF after the lower dose of Y27632. However, the higher dose of the inhibitor resulted in return of FF to baseline levels (Figure 3, Table 4).

Effects of diabetes and ROCK inhibitors on expression of MLC and ROCKs

To determine the effects of ROCK inhibitors at the molecular level, renal cortical homogenates harvested from control and diabetic rats treated with either vehicle or Y27632 were analysed by Western blotting. MLC is a molecular target of ROCKs which is involved in the process of vasoconstriction (Somlyo and Somlyo, 2003). As shown in Figure 4, vehicle-treated diabetic rats demonstrated increased renal MLC phosphorylation as compared to control animals. In diabetic rats given Y27632, P-MLC expression was significantly lower than in vehicle-treated counterparts. This effect of ROCK inhibitors was not observed in non-diabetic animals. Unlike the differences in MLC phosphorylation, total MLC protein expression was similar in all groups of rats. Similarly, there were no differences in expression of ROCK1 between the groups of rats. ROCK2 protein expression tended to be higher in diabetic animals as compared to controls, but there were no differences between the vehicle- and Y27632-treated animals.

Discussion

In the present studies, acute administration of the ROCK inhibitor Y27632 decreased BP in both control and diabetic rats. This observation was compatible with previous evidence indicating ability of ROCK inhibitors to acutely decrease BP in various experimental models, including normotensive animals (Uehata *et al.*, 1997; Winaver *et al.*, 2006). Although the systemic haemodynamic actions of ROCK inhibitors have been well studied in non-diabetic models, this area has been far less investigated in the diabetic context. Studies have indicated activation of the RhoA/ROCK pathway in the cardiovascular system in experimental diabetes (Miao *et al.*, 2002; Kawamura *et al.*, 2004; Rikitake and

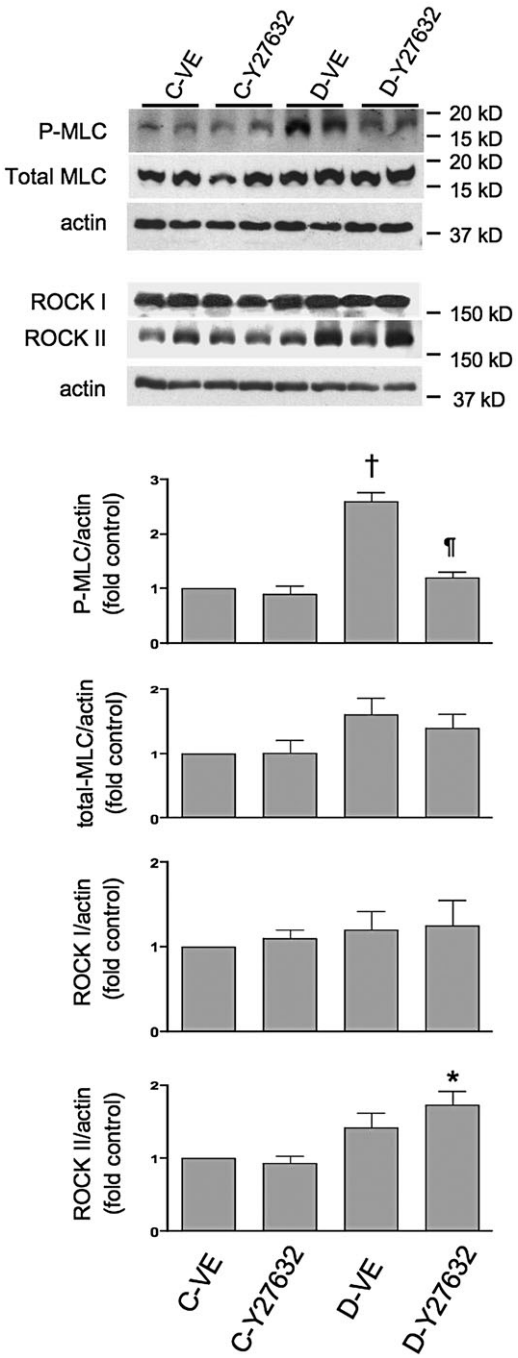

Figure 4

Renal expression of phosphorylated and total myosin light chain (MLC), and Rho associated kinases (ROCK1 and ROCK2) in control and diabetic rats treated with vehicle (C-VE, D-VE) or Y27632 (C-Y27632, D-Y27632). Renal homogenates were analysed by Western blotting using primary antibodies raised against phosphoserine19-MLC (P-MLC), total MLC, ROCK1, and ROCK2. The upper panel shows representative blots. The lower panels depict densitometric analysis of Western blots. Data are presented as protein/actin ratios plotted on *Y* axis. $n = 4$ per group. $^{*}P < 0.05$ versus C-VE and C-Y27632; $^{\dagger}P < 0.01$ versus C-VE and C-Y27632; $^{\P}P < 0.01$ versus D-VE.

Liao, 2005). Consequently, we expected enhanced BP responses to ROCK inhibitors in diabetic rats. However, the BP response to ROCK inhibitors in diabetic rats was similar to that in controls, suggesting no specific role for RhoA/ROCK in the control of BP in diabetes.

In contrast to comparable effects on BP, the renal haemodynamic response to ROCK inhibitors was observed only in diabetic animals, at given doses of these compounds. This response was characterized by renal vasodilation, indicated by increases in ERPF, reductions in RVR and a decrease in FF. The enhanced renal response to ROCK inhibitors was in agreement with accumulating evidence indicating activation of RhoA/ROCK in the diabetic kidney *in vitro* and *in vivo* (Massey *et al.*, 2003; Banes-Berceli *et al.*, 2006; Kolavennu *et al.*, 2008; Peng *et al.*, 2008), and indicated greater dependence of renal haemodynamics on RhoA/ROCK in diabetes.

The effects of ROCK inhibitors on renal haemodynamics have been previously investigated in non-diabetic models. Studies using local administration of inhibitors in intact rats have reported ROCK inhibitor-induced dose-dependent increases renal blood flow (Bauer and Parekh, 2003; Cavarape *et al.*, 2003b). At the glomerular level, measurements utilizing videomicroscopy in isolated split rat hydronephrotic kidneys or the *in vitro* blood-perfused juxtamedullary nephron technique have determined that ROCK inhibitors induce predominantly afferent and lesser efferent, arteriolar dilation (Cavarape *et al.*, 2003b; Nakamura *et al.*, 2003; Inscho *et al.*, 2009). In the present studies, the whole kidney haemodynamic pattern induced by ROCK inhibitors in diabetic rats suggests not only afferent but also an important efferent effect of these compounds in diabetic rats.

Although hyperfiltration was not acutely ameliorated by the ROCK inhibitors, the FF, which was measured as an indirect indicator of Pgc at the whole kidney level (Numabe *et al.*, 1994), was significantly reduced. Consequently, the responses to ROCK inhibitors indicate a contribution of the RhoA/ROCK pathway to haemodynamic changes in the diabetic kidney. Moreover, the observed pattern of ROCK inhibitor-induced renal changes in diabetic animals suggests that the recently described nephro-protective actions of ROCK inhibitors in diabetes (Kolavennu *et al.*, 2008; Peng *et al.*, 2008; Komers *et al.*, 2010) might have a haemodynamically-mediated component.

The responses to ROCK inhibitors in diabetic rats correspond to those previously observed in rats with congestive heart failure (CHF) (Winaver *et al.*, 2006). Using experimental settings comparable to our present experiments, that is, systemic administration of Y27632 and similar surgical preparation, the authors reported no changes in renal blood flow after bolus administration (0.3 $mg \cdot kg^{-1}$) of the inhibitor in normal rats. In contrast, animals with CHF responded with renal vasodilation. In contrast to renal blood flow, and similar to our studies, the authors did not report changes in GFR in animals with CHF indicating both afferent and efferent effects of ROCK inhibitors. Of note, similar to CHF, diabetes is a state of enhanced renal activity of the renin-angiotensin system (Anderson *et al.*, 1993).

Angiotensin II plays an important role in the pathophysiology of nephropathy, and inhibition of angiotensin II actions with an angiotensin converting enzyme inhibitor or an ARB has become a key component of treatment of this disorder. Therefore, we sought to determine the effects of a combination of ARB and ROCK inhibitors, that is, an established treatment of nephropathy and an emerging treatment of this disorder. Theoretically, RhoA/ROCK could be activated by angiotensin II in the diabetic kidney and operate in angiotensin II post-receptor signalling. *In vitro*, studies suggest synergism in high glucose- and angiotensin II-induced RhoA-ROCK signalling (Banes-Berceli *et al.*, 2006). Consequently, treatment with the ARB could attenuate the effects of ROCK inhibitors in our model. Indeed, previous studies have shown attenuation of glomerular microvascular actions of angiotensin II by ROCK inhibitors (Cavarape *et al.*, 2003a; Nakamura *et al.*, 2003).

The rats treated with losartan demonstrated lower baseline BP, increases in ERPF and lower FF, effects similar to our previously published acute studies (Komers *et al.*, 2000). However, the vasodilator effects of Y27632 and a further decrease in FF, were still detected in losartan-treated animals, indicating additive effects of ARB and ROCK inhibitors on BP and renal haemodynamics in experimental diabetes. This phenomenon also suggests that, in addition to angiotensin II, other molecules that are activated in the diabetic kidney (Anderson and Komers, 2004) and operate upstream of RhoA/ROCK in the control of renal haemodynamics, such as thromboxane A_2 or endothelin-1 (Cavarape *et al.*, 2003a,b) could contribute.

The RhoA/ROCK pathway seems to have complex interactions with PKC isoforms in the control of a variety of cell functions (Strassheim *et al.*, 1999; Mehta *et al.*, 2001; Holinstat *et al.*, 2003; Rikitake and Liao, 2005) including vasoconstrictor signals (Strassheim *et al.*, 1999; Somlyo and Somlyo, 2003). PKCs, in particular its β isoform, are activated in the diabetic kidney, and play a role in the pathophysiology of nephropathy (Ishii *et al.*, 1996; Koya

et al., 2000; Kelly *et al.*, 2003) by mediating signals leading to local alterations in vascular tone, cell growth, extracellular matrix production and oxidative stress (Das Evcimen and King, 2007). PKCβ inhibition has been shown to be nephroprotective in various models of diabetes (Ishii *et al.*, 1996; Koya *et al.*, 2000; Kelly *et al.*, 2003), with beneficial haemodynamic actions in the kidney (Ishii *et al.*, 1996). More recently, clinical testing of ruboxistaurin has been conducted in patients with DN (Tuttle *et al.*, 2005), and Cherney *et al.* (Cherney *et al.*, 2009) reported a decrease in GFR in hyperfiltering Type 1 diabetic patients treated with ruboxistaurin. However, the possible interaction of PKC and RhoA/ROCK has not been sufficiently studied in the diabetic context and remains to be tested in the kidney. In this context, we evaluated whether the haemodynamic effects of Y27632 in diabetic rats could be modulated by prior treatment with the PKCβ inhibitor ruboxistaurin.

In agreement with the previous evidence (Ishii *et al.*, 1996), rats pretreated with ruboxistaurin had lower baseline GFR and FF as compared with vehicle-treated animals. Notably, PKCβ inhibition modulated the renal haemodynamic response to ROCK inhibitors. In contrast to the previous experiments, GFR and FF increased sharply with the lower dose of the Y27632 in ruboxistaurin-treated diabetic animals. Although FF decreased after the higher dose in parallel with the rise in ERPF, it did not fall below the baseline levels.

This combination of changes indicates that unlike the findings in diabetic animals with no pretreatment (which suggested actions of ROCK inhibitors both on preglomerular and postglomerular vasculature), the efferent effects of ROCK inhibitors in ruboxistaurin-pretreated rats were substantially blunted. Consequently, RhoA/ROCK appears to act downstream of PKCβ in postglomerular segments of the renal vascular tree in diabetes.

Although the ROCK inhibitors induced clear haemodynamic effects in diabetic rats, we further investigated the impact of ROCK inhibitors at the molecular level. RhoA/ROCK act as mediators of agonist-induced vascular smooth muscle contraction (Somlyo and Somlyo, 2003) as well as in the control of myogenic tone of resistance arteries (Bolz *et al.*, 2003). The ROCKs phosphorylate MBS of the MLCP leading to its inhibition, MLC phosphorylation, and contraction. In the present studies, renal cortical MLC phosphorylation was higher in diabetic rats as compared with controls, most likely reflecting activation of the RhoA/ROCK pathway. Renal vasodilator effects of Y27632 in diabetic rats were associated with a reduction in renal MLC phosphorylation, while total MLC expression and abundance of ROCK1 remained unchanged. Of note, expression of ROCK2 was higher in diabetic animals. We can only speculate about the significance of this finding. One of the original reports exploring the role of ROCKs in smooth muscle contraction indicated that the ROCK2 isoform phosphorylates Ser19 of MLC (Amano *et al.*, 1996). More recently Wang *et al.* (Wang *et al.*, 2009) expanded those earlier observations and demonstrated a predominant role of ROCK2 in vascular smooth muscle cell contractility. Therefore, diabetes-induced increases in ROCK2 in the kidney might predispose the kidney vasculature to enhanced acute actions of ROCK inhibitors.

In conclusion, acute ROCK inhibition induced similar BP responses in control and diabetic animals. In contrast, a renal vasodilator response to ROCK inhibitors, and a decrease in FF, was observed only in diabetic animals, indicating a contribution of the RhoA/ROCK pathway to altered haemodynamics at early stages of DN. The effects of the ROCK inhibitors were observed even after prior treatment with an ARB and the combination of ARB and ROCK inhibitors had additive affects on ERPF and FF. In contrast to the ARB, the effects of ROCK inhibitors were modulated by prior PKCβ inhibition. This finding suggests that RhoA/ROCK operate downstream of or interact with, PKCβ at least in some segments of the renal vascular tree. From the treatment perspective, the ROCK inhibitor-induced haemodynamic pattern is compatible with nephroprotective effects of these compounds, and combination with an ARB may enhance the beneficial haemodynamic actions of ROCK inhibitors.

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Conflicts of interest

No conflicts of interest are declared by the authors.

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