Targeting of RhoA/ROCK Signaling Ameliorates Progression of Diabetic Nephropathy Independent of Glucose Control

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Running title: RhoA/ROCK and diabetic nephropathy

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ABSTRACT

Objective: RhoA, a small GTPase protein, and its immediate downstream target, ROCK, control a wide variety of signal transduction pathways. Recent studies have shown that fasudil, a selective ROCK inhibitor, may play a pivotal role in a number of pathological conditions, ranging from cardiovascular diseases to pulmonary hypertension and erectile dysfunction. Considerable evidence suggests that some of the beneficial effects of statins may also stem from their modulatory effects on RhoA/ROCK signaling. In the current study, we hypothesized that pharmacological blockade of the RhoA/ROCK pathway with either fasudil or simvastatin would ameliorate progression of diabetic nephropathy.

Research Design and Methods: In two separate experiments, diabetic db/db mice received fasudil (10 mg/kg/day i.p.) or simvastatin (40 mg/kg/day p.o.) for 16 weeks. Untreated db/db and db/m mice served as controls.

Results: The kidney cortices of untreated db/db mice displayed increased ROCK activity compared to db/m mice. The fasudil-treated mice exhibited a significant reduction in ROCK activity, albuminuria, glomerular collagen IV accumulation, and urinary collagen IV excretion compared to untreated db/db mice. Interestingly, blood glucose was unaffected by fasudil administration. Treatment with simvastatin significantly attenuated RhoA activation in the kidney cortices of db/db mice, and also resulted in a significant reduction of albuminuria and mesangial matrix expansion.

Conclusions: Based on these results, we propose that RhoA/ROCK blockade constitutes a novel approach to the treatment of diabetic nephropathy. Our data also suggest a critical role for RhoA/ROCK activation in the pathogenesis of diabetic nephropathy.
The Rho family of small GTPases are 20- to 40-kD monomeric G proteins that regulate a number of cell functions, including cytoskeletal reorganization, cell motility, and gene expression (1). They cycle between two conformational states: an active GTP-bound state and an inactive GDP-bound state (2). One of the best recognized members of the Rho GTPase family is RhoA. Rho kinase (ROCK), a downstream effector of RhoA, is a serine/threonine kinase of approximately 160 kDa that exists in two isoforms in mammals: ROCK1 and ROCK2 (3). ROCK is comprised of an amino-terminal kinase domain, followed by a coiled-coil region that contains the Rho-binding domain (RBD). The carboxy-terminal consists of a pleckstrin-homology (PH) domain which contains an internal cysteine-rich domain. ROCK1 and ROCK2 are highly homologous, sharing an identity of 65% in their overall amino acid sequences and 92% in their kinase domains.

The RhoA/ROCK pathway has recently received considerable attention for its involvement in a wide variety of pathological states ranging from cardiovascular disease and pulmonary hypertension to Alzheimer’s disease and glaucoma (4-7). A growing body of evidence also suggests that RhoA/ROCK pathway may also have renomodulatory effects. Indeed, several recent studies have proposed that RhoA/ROCK may play an important role in renal fibrosis by enhancing signaling pathways involving TGF-β, angiotensin II, and NFκB (8-10). Furthermore, since the discovery of fasudil and Y-27632, selective ROCK inhibitors, RhoA and ROCK have generated great interest as key regulators in a variety of renal injury models, including unilateral ureteral obstruction (11; 12), hypertensive glomerulosclerosis (13; 14), and ischemia/reperfusion-induced acute renal failure (15).

Another reason for the growing attention on this pathway is the recent experimental observations suggesting that some of the beneficial effects of statins may stem from their effects on the RhoA/ROCK pathway. Statins have recently emerged as novel renoprotective drugs in several models of kidney injury including diabetic nephropathy (DN), and for this reason are currently being investigated in several large clinical trials (16-19). A recent review of the effect of statins on renal function suggested that statins reduced the rate of kidney function loss by about 75% and reduced albuminuria by 47% in people with baseline albuminuria of at least 300 mg/dL (20-22). Although the benefits of statins had initially been attributed to their lipid-lowering effects (21; 23; 24), recent studies have proposed that statins have direct modulatory effects on the Rho GTPase signaling pathways (25; 26). These pleiotropic, or lipid-independent, effects of statins primarily involve inhibition of the mevalonate pathway with subsequent inactivation of several signaling proteins including RhoA (27-29). Indeed, inhibition of RhoA/ROCK by statins or other selective inhibitors leads to the upregulation and activation of endothelial nitric oxide synthase (eNOS) and reduction of vascular inflammation and atherosclerosis (30).

We have previously shown that Rho GTPases modulate several signaling cascades relevant to mesangial and glomerular endothelial cell remodeling in the diabetic milieu in vitro (31-35). However, the role of RhoA/ROCK pathway in DN remains to be elucidated in vivo. In the present study, we hypothesized that pharmacological blockade of the RhoA/ROCK pathway would slow the progression of DN in the db/db mouse, an established model of type 2 diabetes. To this end, we used two separate approaches to inhibit this pathway. First, we studied the effect of fasudil administration on DN in vivo to elucidate the role of ROCK in the pathogenesis of DN. Subsequently, we examined the pleiotropic effects of simvastatin on RhoA activation and the progression of DN.

**MATERIALS AND METHODS**

Fasudil was a kind gift of Asahi-Kasei Pharma Corporation (Shizuoka, Japan). Simvastatin was obtained from Merck & Co.,
Inc. (West Point, PA). Simvastatin was activated by alkaline hydrolysis as previously described (35). Y27632 was obtained from Calbiochem (San Diego, CA).

**Cell Culture and Transfection.** Early passaged (passage 3-10) rat mesangial cells were grown in DMEM/F12 medium containing 10% FBS, 100U/ml penicillin, and 100µg/ml streptomycin in a humidified incubator at 37°C under 5% CO₂. Transfections of RhoA mutants were performed as described previously (35).

**Animal Studies.** All animal studies were carried out with the review and approval of the Animal Care and Use Committee (ACUC) of Northwestern University. In two separate experiments, db/db mice received fasudil (10 mg/kg/day by i.p. injection, n=8) or simvastatin (40 mg/kg/day p.o., n=8) starting at 8 weeks of age. After 16 weeks of treatment (24 weeks age), the mice were housed in individual metabolic cages for collection of urine. Blood glucose was measured after a 12-hr fast using the OneTouch UltraSmart Blood Glucose Meter (Lifescan, Inc., Milpitas, CA). Urinary albumin and collagen IV concentrations were measured using the Albuwell M and Collagen IV M assay kits (Exocell, Philadelphia, PA). Serum and urine creatinine were measured using High Performance Liquid Chromatography (HPLC). Total cholesterol was determined by the Vanderbilt Mouse Metabolic Phenotyping Center (Nashville, TN) using a microtiter plate assay.

**Morphometric Studies.** For morphometric studies, the kidneys were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. The 4-µm sections of paraffin-embedded tissues were stained with periodic acid-Schiff (PAS). Light microscopic views of 40 consecutive glomerular cross-sections per mouse were scanned into a computer. Glomerular and mesangial matrix areas were quantified in a blinded fashion using an image analysis system (MetaMorph version 6.1, Universal Imaging Corp.). Mesangial Matrix Index (MMI) was calculated as the ratio of mesangial area/glomerular area x 100 (% area).

**Immunohistochemistry.** Immunoperoxidase staining was carried out on 4-µm paraffin-embedded kidney tissue sections. The sections were pre-incubated in boiling sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. Specific primary antibodies, anti-fibronectin antibody and anti-collagen IV antibody (Chemicon, Temecula, CA) were incubated with the sections overnight. For visualization, the sections were developed with 3, 3'-diaminobenzidine to produce a brown color, counterstained with hematoxylin, and mounted in VectaMount mounting medium (Vector Laboratories).

**Transmission Electron Microscopy.** Small pieces of kidney cortex were fixed in 2.5% glutaraldehyde in 0.2 mol/L cacodylate buffer (pH 7.4). They were then dehydrated through graded ethanol and propylene oxide, embedded in epoxy resin, and polymerized at 60°C overnight by standard procedures. Ultrathin sections were stained with uranyl acetate and lead citrate. The specimens were observed using a Jeol 1220 transmission electron microscope (JEOL, USA). For each specimen, images of the glomerular basement membrane (GBM) in 10 consecutive glomeruli were captured onto a computer.

**Confocal Laser Scanning Fluorescence Microscopy.** The 4-µm sections of snap-frozen kidneys were permeabilized for 10 min using cold acetone, rehydrated in 1xTBS, and preincubated in 5% bovine serum albumin, 0.2% saponin, 0.001% tween in TBS for 45 min at room temperature. Primary antibodies included anti-RhoA (Abcam, Cambridge, MA), anti-vWF antibody (Sigma, St Louis, MO), and collagen IV (Abcam, Cambridge, MA). The actin cytoskeleton was stained with rhodamine phalloidin and visualized by using scanning confocal fluorescence microscopy (Zeiss LSM510).

**RhoA Activation Assay.** RhoA activity was determined by a pull-down assay according to the instructions by the manufacturer (Upstate Biotechnology). In brief, fresh kidney cortex tissues were washed in cold PBS, and lysed in ice-cold MLB buffer. Samples were centrifuged and incubated for 60 min at 4°C.
with 100 µl of Rhotekin agarose to precipitate GTP-bound RhoA. Precipitated complexes were washed three times in MLB buffer and resuspended in 30 µl of 2x Laemmli buffer. Total lysates and precipitates were analyzed by Western blot analysis using rabbit polyclonal antibody against RhoA (Santa Cruz, CA).

Rho kinase (ROCK) Activity. ROCK phosphorylates the myosin phosphatase target subunit 1 (MYPT1), also known as myosin-binding subunit (MBS), of myosin light chain phosphatase at Thr853. ROCK activity was measured as previously described(36). Briefly, equal amounts of kidney cortical tissue extracts were analyzed by Western blot analysis by using rabbit phospho-MBS/MYPT1-Thr853 (CycLex Co., Ltd, Japan) and rabbit anti-MYPT1 (Santa Cruz Biotechnology, CA). ROCK activity was expressed as the ratio of phospho-MYPT1/total MYPT1.

Statistical Analysis. The values are reported as mean±SE. Statistical significance was determined by using a one-way analysis of variance (ANOVA) followed by Tukey’s Multiple Comparisons Test. A value of p<0.05 (two tailed) was considered statistically significant.

RESULTS
To establish the potential role of RhoA/ROCK in the pathophysiology of DN, we investigated the effect of either fasudil (10 mg/kg/day i.p.) or simvastatin (40 mg/kg/day p.o.) in two separate experiments. After 16 weeks of therapy, animals were sacrificed and various biochemical and histological parameters were evaluated.

Increased Rho kinase activity in the kidney cortex of db/db mouse. Rho kinase (ROCK) activity in the kidney cortex was directly assessed by the amount of phospho-Thr853 in the myosin phosphatase target subunit 1 (MYPT1) of myosin light chain phosphatase, a downstream target of ROCK (36). As shown in Figure 1-A, Rho kinase activity was significantly increased in the kidney cortex of diabetic db/db mice while treatment with fasudil inhibited the increased Rho kinase activity in diabetic mice. However, there were no significant changes in total MYPT1 in different groups. Figure 1-B depicts a semiquantitative measure of the percentage differences in Rho kinase activity in db/m, db/db, and fasudil-allocated mice (100±4, 152±10, 120±3 respectively, *p<0.05).

Effect of fasudil on metabolic & renal parameters of db/db mice. Figure 2 depicts several metabolic and renal parameters of fasudil-treated db/db mice at 24 weeks of age as compared to non-diabetic db/m mice. These parameters include blood glucose levels, serum creatinine and cholesterol levels, and urine albumin/creatinine ratio. As expected, the mean blood glucose level of the diabetic db/db mice (n=8) was significantly higher than that of db/m mice (n=8) (Figure 2-A). Treatment of diabetic animals with fasudil did not significantly lower blood glucose levels (n=8). However, after 16 weeks of therapy with fasudil, the diabetic mice exhibited a significant reduction in albuminuria (Figure 2-C, 417±120 vs. 150±20 µg/mg, p<0.01). As shown in Figure 2-B and 2-D, there were no significant changes in the serum creatinine and cholesterol levels between fasudil-treated and db/db mice (P>0.05).

Decreased mesangial matrix and type IV collagen accumulation with fasudil. Mesangial matrix expansion is considered a hallmark of DN. Figure 3-A suggests accelerated mesangial matrix expansion in db/db mice as characterized by an increase in PAS-stained area compared with that observed in db/m mice at 24 weeks of age. However, treatment with fasudil prevented mesangial expansion in db/db mice. To assess the effect of fasudil therapy on mesangial matrix accumulation, we evaluated Mesangial Matrix Index (MMI). As shown in Figure 3-B, the MMI in db/db diabetic animals was significantly increased as compared to non-diabetic db/m mice (23±0.5 vs. 7±0.2%, p<0.01). Treatment with fasudil markedly reduced MMI in the glomeruli of db/db mice (23±0.5 vs. 14±0.3%, p<0.01).

Furthermore, to evaluate for the effect of fasudil on extracellular matrix proteins, we performed immunohistochemical studies of type IV collagen and fibronectin (Figure 3-C).
Consistent with our previous results for mesangial matrix expansion, there was a strong staining for type IV collagen and fibronectin in the diabetic db/db mice as compared with db/m mice. In contrast, the expression of collagen IV and fibronectin were reduced in the glomeruli of fasudil-treated db/db mice. Figure 3-D demonstrates the effect of fasudil on collagen IV protein levels using Western blot analysis. Consistent with previous reports, type IV collagen protein expression was significantly increased in the kidney cortices of db/db mice. Treatment with fasudil significantly reduced type IV collagen protein expression.

A number of previous studies have suggested that changes in collagen IV accumulation may also be accompanied by increased excretion of urinary collagen IV in db/db mice (37). To address the effect of fasudil on urinary collagen IV excretion, we monitored urinary collagen IV excretion as previously described (37). The data presented in Figure 3-E indicate that urinary collagen IV increased in db/db mice but not in non-diabetic db/m mice (1.2±0.25 vs 0.18±0.03 ng/24hrs, p<0.01). Furthermore, the fasudil-treated animals exhibited a significant reduction in urinary collagen IV excretion after 16 weeks of therapy (0.46±0.081 vs 1.2±0.25 ng/24hrs, p<0.05).

To assess the effect of fasudil on glomerular basement membrane (GBM), we performed transmission electron microscopy. As shown in Figure 3-F and 3-G, the GBM thickness in db/db diabetic animals was increased compared with that in the normal animals (236±4 nm vs. 150±2 nm, p<0.001). Interestingly, fasudil-treated animals showed a significant reduction in GBM thickness as compared with untreated db/db mice (171±2 nm vs. 236±4 nm, p<0.001).

**Simvastatin therapy blocks RhoA protein activation in vivo.** We and others have previously shown that statins, by inhibiting mevalonate pathway, also inhibit isoprenoids which are critical for the proper function and activity of RhoA GTPases. Thus, to assess the effect of simvastatin on RhoA activation in vivo, we initially assessed a quantitative measure of activated RhoA in db/db mice. As depicted in Figure 4-A, membrane-bound RhoA (active form) was increased in the diabetic kidney cortex when compared to that in the normal control group (n=8/group). However, total RhoA protein (active and inactive form) was similar among the two groups. Moreover, RhoA activity in the kidney cortex was also directly assessed by an affinity pull-down assay using GST fusion protein rhotekin, which recognizes only the active form of RhoA (GTP-RhoA). As shown in Figure 4-B, RhoA pull-down assay further confirmed that GTP-RhoA (active form) is highly expressed in the kidney cortex of diabetic db/db mice. Treatment with simvastatin suppressed the increased activity of RhoA in diabetic mice which is demonstrated in the semiquantitative analysis displayed in Figure 4-C.

**Effect of simvastatin on metabolic & renal parameters.** Figure 5 depicts several metabolic and renal parameters of simvastatin-treated db/db mice at 24 weeks age as compared to non-diabetic db/m mice and PBS-treated diabetic db/db mice. As expected, the mean blood glucose level of the diabetic db/db mice was significantly higher than that of db/m mice (Figure 5-A). Treatment of diabetic animals with simvastatin lowered blood glucose levels although it did not reach statistical significance (397±23 vs. 452±18 mg/dL, p>0.05). Urinary albumin excretion was significantly greater in db/db animals than in db/m animals (Figure 5-B). After 16 weeks of therapy with simvastatin, the diabetic mice exhibited a significant reduction in albuminuria (400±37 vs. 717±76. µg/mg, p<0.001). As shown in Figure 5-C, there were no significant changes in the plasma creatinine in different groups. And finally, db/db mice allocated to simvastatin exhibited lower cholesterol levels (249±24 vs. 223±14 mg/dl, p<0.05, n=8/group) as compared to PBS-treated db/db mice.

**Effect of simvastatin on ECM proteins.** The representative glomerular histology of PAS-stained sections is shown in Figure 6-A. The glomerular accumulation of PAS-positive matrix was prominent in PBS-treated diabetic animals. In simvastatin-treated animals, matrix expansion was significantly less than
that in PBS-treated animals. Similarly, as shown in Figure 6-A, the increases in fibronectin and type IV collagen protein expression were prominent in the glomeruli of diabetic db/db mice. Treatment with simvastatin significantly reduced the accumulation of these proteins in the glomeruli of diabetic db/db mice. As shown in Figures 6-B and 6-C, simvastatin-treated animals also showed a significant reduction in GBM thickness compared to PBS-treated db/db mice (176 ± 2 vs. 236 ± 4 nm, p<0.001).

Figure 6-D depicts that the MMI in simvastatin-treated animals was also significantly lower than that of the vehicle-treated group.

Effect of high glucose (HG) on RhoA kinase activity and collagen accumulation. We have previously provided data that HG mediates RhoA activation in mesangial cells (MC) (32). We also reported that RhoA activation plays an important role in collagen IV accumulation in MCs via cytoskeletal remodeling (33). To decipher whether RhoA/ROCK pathway mediates HG-induced mesangial matrix expansion and collagen IV accumulation, cultured rat MCs were exposed to HG (30 mM), and Rho kinase activity was measured as previously described. As shown in Figure 7-A, HG significantly increased Rho kinase activity, as measured by MYPT1-phospho Thr853, a downstream target of ROCK, after 8hrs of stimulation. To test whether RhoA/ROCK activation mediates HG-induced type IV collagen protein levels via cytoskeletal remodeling, MCs were transfected with Myc-tagged dominant-negative mutant (N19RhoA) and wild-type (wt) RhoA. Cells were then stimulated with HG milieu (30 mM). Cytoskeletal remodeling was evaluated by confocal laser microscopy. As shown in Figure 7-B (upper panel), HG caused a significant increase in actin stress fiber formation. However, there was a significant reduction in HG-induced actin stress fiber formation in cells co-treated with Y-27632 (10 nM), a specific inhibitor of ROCK, as well as in N19RhoA-transfected MCs, suggesting a pivotal role for RhoA/ROCK activation in HG-induced cytoskeletal remodeling. The data in Figure 7-B (lower panel) also show that HG stimulation led to an increase in cell-associated collagen IV protein levels as evaluated by confocal microscopy. Collagen IV protein levels did not increase in cells co-treated with Y-27632 (10 nM) and in N19RhoA-transfected cells, indicating a critical role for RhoA/ROCK activation in HG-induced collagen IV accumulation. Consistent with these results, a Western blot analysis in MCs stimulated with HG also indicated that HG-induced collagen IV protein accumulation is significantly reduced in dominant-negative RhoA-transfected cells and in Y-27632 co-treated MCs.

DISCUSSION

The results of this study implicate RhoA/ROCK in the signaling pathways contributory to the development of DN. A principal finding of this study is that RhoA/ROCK activation is increased in the kidneys of db/db mice, and that the inhibition of RhoA/ROCK pathway can protect against the progression of DN.

The RhoA/ROCK pathway has recently attracted a great deal of attention in various research fields, particularly diabetes-related research for several reasons. First, at the cellular level, the RhoA/ROCK pathway has a prominent role in various signaling pathways that are potentially implicated in the pathogenesis of microvascular complications of diabetes. For instance, our laboratory and several others have recently unraveled the role of RhoA/ROCK in VEGF signaling, an important mediator of microvascular complications of diabetes (34; 35). We determined that VEGF-induced glomerular endothelial cell hyperpermeability is mediated by an increase in RhoA/ROCK activation, which leads to phosphorylation of myosin regulatory light chain, a substrate of ROCK. The second reason for the emergence of ROCK as a novel target in several research areas is because it has been suggested that the pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are mediated, at least in part, by their inhibitory effects on
RhoA/ROCK signaling (28; 32; 38). Indeed, we and others have previously shown that the inhibitory effects of statins on RhoA/ROCK signaling pathways may play an important role in the pleiotropic effects of statins (31; 33; 34). The final reason for the recent focus on RhoA/ROCK pathway may be based on the involvement of ROCK in ECM accumulation. For instance, we have recently shown that RhoA activation mediates actin cytoskeleton remodeling, intracellular FAK phosphorylation, and β1 integrin activation leading to increased type IV collagen synthesis (33). However, despite a growing body of evidence suggesting a pathogenic role for RhoA and ROCK activation in vitro, the role of RhoA/ROCK activation in vivo in DN is not well-defined.

In the current study, we established the effect of pharmacological blockade of RhoA and ROCK pathway in the progression of DN. We found that RhoA and ROCK activity is increased in the kidney cortices of db/db mice. The pathogenic role of RhoA/ROCK activation was evident when pharmacological inhibition of the pathway resulted in a significant reduction in RhoA/ROCK activity, mesangial matrix expansion, collagen IV accumulation, and albuminuria. Thus, our data indicate that targeting RhoA/ROCK is an effective and novel strategy in preventing the progression of experimental diabetes nephropathy.

The effect of fasudil on albuminuria in this study is consistent with recent reports indicating that fasudil may reduce albuminuria in different animal models of diabetes (39, 40). For instance, in a previous report, high-dose fasudil administration significantly attenuated albuminuria and glomerulosclerosis, but also led to a significant decrease in blood glucose (39). The authors concluded that fasudil at least in part, by improving glucose control, ameliorates progression of renal disease. Our results differ from this report in that the beneficial effects of fasudil in our study were independent of its potential effect on glucose control. Indeed, the fasudil-allocated db/db mice in our experiments did not exhibit significant changes in their blood glucose levels compared with untreated db/db mice. This finding suggests that ROCK inhibition may have renoprotective effects independent of its potential effects on glucose levels. We also recognize that differences in the observed effects of fasudil may be explained, at least in part, by the experimental model of diabetes and dose of fasudil employed.

Currently, two major families of ROCK inhibitors are used: isoquinoline derivatives and 4-aminopyridine derivatives. Fasudil is one of the most prominent isoquinoline derivatives which is used clinically to treat cerebral vasospasm in Japan, and several clinical trials are currently examining its anti-anginal effects (41, 42). The 4-aminopyridine derivative, Y-27632, is widely utilized in biological and pharmacological experiments. Both Y-27632 and fasudil are selective ROCK inhibitors that target its ATP-dependent kinase domain. Hydroxyfasudil, an active metabolite of fasudil after oral administration, has been shown to have greater selectivity for ROCK than fasudil itself (43). Several efforts have been made to develop more specific and more potent ROCK inhibitors. Recently, the 3D crystal structure of ROCK and its binding site for fasudil have been determined, which should facilitate the development of more selective ROCK inhibitors in the future (44).

Because of the ability of statins to attenuate several experimental models of inflammatory diseases, considerable interest has arisen in their therapeutic potential for treating a broad spectrum of chronic kidney diseases including DN. In support of a beneficial role for statins in ameliorating the progression of DN, several clinical studies have reported findings of a preserved glomerular filtration rate (GFR) and diminished urine albumin excretion in diabetic patients with proteinuria treated with statins (18; 20; 22; 24; 45; 46). However, the molecular mechanism(s) responsible for the renoprotective effects of statins has been the subject of much debate. Several mechanisms may account for the possible beneficial effects of statins in DN. For instance, because of their direct lipid-lowering
properties, statins may diminish Ox-LDL–induced nephrotoxicity (47). It has become increasingly apparent, however, that the effects of statins cannot be ascribed solely to their lipid-lowering properties. For instance, Usui et al. have suggested that the key anti-fibrotic effects of statins lie in their inhibitory effect on NF-κB activation (48). Kim et al. have also reported that the anti-fibrotic effect of lovastatin occurs through modulation of TGF-β1 signaling (49). And Yokota et al. have proposed that the renoprotective effects of statins in diabetic milieu is via suppression of Ras-MAPK cascade (50).

Our data also suggest that the therapeutic potential of RhoA and ROCK inhibition in DN may, at least in part, stem from their inhibitory effect on mesangial matrix accumulation. Indeed, the present study indicates that RhoA/ROCK blockade effectively prevented the increase in collagen IV accumulation in the cortical kidneys of db/db mice. These findings are of significant interest since they provide not only a rationale for the renoprotective effects of fasudil and simvastatin, but they also offer a novel therapeutic targets in DN, namely RhoA and its downstream effector, ROCK.

Our results provide strong evidence that fasudil and simvastatin protect against the progression of DN in an experimental model of type 2 diabetes. Further studies elucidating the potential benefits of targeting specifically RhoA/ROCK activation via pharmacological and genetic manipulations are underway in our laboratory. Elucidating signaling pathways by which RhoA/ROCK exert their pathogenic effects may not only enable us to understand the mechanisms of microvascular complications of diabetes, but also may lead us to strategies for targeted therapy and protection against DN.

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REFERENCES


47. Chen HC, Guh JY, Shin SJ, Lai YH: Pravastatin suppress superoxide and fibronectin production of glomerular mesangial cells induced by oxidized-LDL and high glucose. Atherosclerosis 160:141-146, 2002
FIGURE LEGENDS

Figure 1: A) Representative immunoblot of Rho kinase activity in kidney cortices of db/m, db/db, and fasudil-treated animals. Rho kinase activity was measured by immunoblotting with an antibody that recognizes MYPT1 at Thr 853 residue. B) Rho kinase activity is expressed as p-MYPT1/total MYPT1. *p<0.05. (n=8 in each group).

Figure 2: Treatment with fasudil resulted in a significant decrease in the urinary albumin excretion levels (2-C) in db/db mice (p<0.01, n=8/each group) without significant changes in the blood glucose (2-A) levels. Serum cholesterol and serum creatinine were not significantly different between fasudil-treated and db/db mice (2-b, and 2-D).

Figure 3: A) Representative photomicrograph of immunohistochemical staining with periodic acid Schiff (PAS). B) Mesangial matrix expansion in PAS-stained glomeruli was semiquantified as described in Materials & Methods. Data are presented as mean±SE. C) Representative photomicrographs of immunohistochemical staining with fibronectin and collagen IV in control, diabetic, and fasudil-allocated diabetic mice. D) Western blot analysis of collagen IV protein levels in kidney cortex of db/m, db/db, and fasudil-treated mice. Western blots were performed under nonreducing conditions for collagen IV. E) Urinary collagen IV excretion at the conclusion of experimental protocol in db/m, db/db, and fasudil-treated animals. Results are mean±SE (n=5 in each experimental group). F) Representative electron photomicrographs. Diabetic db/db mice developed significant GBM thickening which was ameliorated in fasudil-treated diabetic animals. G) GBM thickness was quantified according to Materials & Methods. The data are presented as mean±SE (n=3 in each experimental group).

Figure 4: A) Western Blot of membrane-bound RhoA (active form) and total RhoA protein in the kidney cortex. B: RhoA activity in the kidney cortex was also directly assessed by a RhoA pull-down assay. The figures are each representatives of 3 separate experiments. Figure 4-C depicts a semiquantitative analysis of RhoA-GTP/ RhoA-total in db/m, db/db, and fasudil-allocated mice (n=8/group, *p<0.05).

Figure 5: Treatment with simvastatin resulted in a significant decrease in the urinary albumin excretion (5-B) and cholesterol levels (5-D) in db/db mice (p<0.001 and p<0.05, respectively). NS: non-significant. (n=8/group).

Figure 6: A) Representative photomicrographs of immunohistochemical staining for periodic acid Schiff (PAS), fibronectin and collagen IV in control, diabetic, and simvastatin-allocated diabetic mice. B) Effect of simvastatin on GBM. C) GBM thickness was quantified according to Materials & Methods. The data are presented as mean±SE. D) Mesangial Matrix Index (MMI) was calculated based on the ratio of mesangial area/glomerulus (% area). Data are presented as mean±SE.

Figure 7: A) ROCK activity as measured by MYPT1-Thr 853 in HG milieu (30 mM) as compared to normal glucose (NG, 5.5 mM) after 8 hrs of stimulation. The figure depicts a representative of 4 separate experiments. B) Confocal microscopy was used to evaluate the effect of Y-27632 or N19RhoA transfected cells on HG-induced actin cytoskeletal remodeling in MCs. C: Representative Western blot for collagen IV-associated protein. *p<0.05 compared with un-stimulated control cells. (n=3)
FIGURE 1

A

p-MYPT1 →
Total MYPT1 →

db/m  db/db  db/db + fasudil

B

Rho kinase activity (% of db/m mice)

db/m  db/db  db/db + fasudil
FIGURE 2
FIGURE 3
FIGURE 4

A

RhoA-membrane  
RhoA-total

db/m  db/db

B

RhoA-GTP  
RhoA-total

db/m  db/db  db/db + PBS  db/db + simvastatin

C

RhoA-GTP/RhoA-total

p < 0.05

db/m  db/db  db/db + simvastatin
RhoA/ROCK and diabetic nephropathy

FIGURE 5
FIGURE 6
FIGURE 7

A

B

C

RhoA/ROCK and diabetic nephropathy