# Rho-kinase inhibition prevents the progression of diabetic nephropathy by downregulating hypoxia-inducible factor 1α

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**The small GTPase Rho and its effector Rho-kinase are involved in the pathogenesis of diabetic nephropathy. Accumulating evidence shows that hypoxia-inducible factor-1α (HIF-1α) is a key regulator of renal sclerosis under diabetic conditions. However, the interactions of Rho-kinase and HIF-1α in the development of renal dysfunction have not been defined. Here, we assessed whether Rho-kinase blockade attenuates HIF-1α induction and the subsequent fibrotic response using type 2 diabetic mice and cultured mesangial cells. Fasudil, a Rho-kinase inhibitor, reduced urinary albumin excretion, mesangial matrix expansion, and the expression of fibrotic mediators in *db/db* mice. Mechanistically, HIF-1α accumulation and the expression of its target genes that contribute to diabetic glomerulosclerosis were also prevented by fasudil in the renal cortex. In mesangial cells, Rho/Rho-kinase signaling was activated under hypoxic conditions. Further *in vitro* studies showed that pharmacological and genetic inhibition of Rho-kinase promoted proteasomal HIF-1α degradation, which subsequently suppressed HIF-1-dependent profibrotic gene expression by upregulation of prolyl hydroxylase 2. Thus, we found a previously unrecognized renoprotective mechanism for the effects of Rho-kinase inhibition and this could be a potential therapeutic target for the treatment of diabetic nephropathy.**

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Diabetic nephropathy is a worldwide public health problem; it is the leading cause of end-stage renal disease and is associated with increased mortality from cardiovascular disease in diabetic patients.[1] The most remarkable characteristic of diabetic nephropathy is glomerular mesangial expansion caused by abnormal remodeling of the extracellular matrix. Preventive approaches to diabetic nephropathy are hampered by a poor understanding of the upstream abnormalities that lead to these morphological changes.

Chronic hypoxia has been implicated in the development and progression of various renal diseases.[2–4] The diabetic milieu also induces chronic renal hypoxia and cellular hypoxic responses.[5–8] A key regulator of oxygen homeostasis is hypoxia-inducible factor-1 (HIF-1), a basic helix-loop-helix transcription factor that consists of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, which is also known as the aryl hydrocarbon receptor nuclear translocator.[9] HIF-1α expression has been identified in glomerular and tubulointerstitial lesions of diabetic animal models and in patients with diabetic nephropathy.[8,10–12] Accumulation of HIF-1α is particularly evident in the medullary region, where oxygen tensions are known to be low.[8,10] Recent studies have demonstrated that HIF-1α expression is associated with tubulointerstitial injury in several renal disease models.[10,12] In addition, a significant increase in HIF-1α expression has been found in glomerular mesangial cells from diabetic mouse model.[11] Moreover, hyperglycemia promotes expression of HIF-1α in cultured mesangial cells, resulting in transactivation of downstream target genes, such as those encoding connective tissue growth factor and plasminogen activator inhibitor 1, which are involved in renal fibrosis.[11]

Rho GTPase and its downstream effector Rho-kinase (Rho-associated, coiled-coil-containing protein kinase (ROCK)), which regulate a variety of cellular functions such as contraction, motility, and proliferation, play important roles in the pathogenesis of systemic vascular diseases.[13–15] Recently, the Rho/Rho-kinase pathway has also

been implicated in several renal pathophysiological conditions including hypertensive glomerulosclerosis,[16] subtotal nephrectomy,[17] and unilateral ureteral obstruction.[18] A previous study in our laboratory indicated that treatment with fasudil, a Rho-kinase inhibitor, could prevent diabetic albuminuria via suppression of transforming growth factor-β (TGF-β) and connective tissue growth factor in streptozotocin-induced diabetic rats, a rodent model of type 1 diabetes.[19] We have also reported that Rho-kinase is involved in the regulation of chemokine production in mesangial cells, suggesting the participation of Rho-kinase in glomerular inflammation.[20] Although several other studies have also documented the renoprotective effects of Rho-kinase inhibition in rodent model of diabetic nephropathy,[21–23] the underlying mechanisms of these effects remain to be understood. Rho has however been shown to mediate hypoxia-induced HIF-1α expression in certain cell types,[24,25] and we therefore hypothesized that blockade of Rho-kinase may prevent the development of diabetic nephropathy by inhibiting HIF-1α activation and effecting suppression of its target genes. Here, we present data identifying a role of Rho-kinase in the regulation of HIF-1α accumulation and subsequent glomerular fibrosis in diabetic nephropathy.

## RESULTS

### Rho/Rho-kinase activity is elevated in renal cortex of *db/db* mice

As shown in Figure 1a and b, guanosine-5'-triphosphate (GTP)-bound active RhoA and the phosphorylated form of myosin phosphatase target subunit 1 (MYPT1), the substrate of Rho-kinase, were increased in non-treated *db/db* mice as compared with control animals. On the contrary, in *db/db* mice treated with fasudil, phosphorylated MYPT1 was significantly lower than nontreated counterparts.

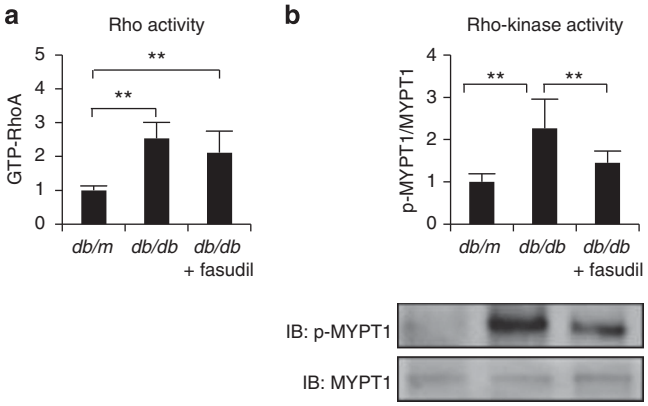


**Figure 1 | Rho and Rho-kinase are activated in renal cortex of *db/db* mice.** (**a**) Levels of GTP-bound RhoA in renal cortical tissue assessed by RhoA G-LISA assay. (**b**) Quantitative analysis and representative blots of the phosphorylated form of MYPT1 (p-MYPT1) and MYPT1 in protein extracts from the renal cortex of each group. Data are means ± s.d. ($n = 8$ in each group, $^{**}P < 0.01$). GTP, guanosine-5'-triphosphate; IB, immunoblotting; MYPT1, myosin phosphatase target subunit 1.

### Rho-kinase inhibitor improves albuminuria in *db/db* mice without affecting metabolic parameters

Fasudil treatment significantly attenuated the increase in albuminuria observed in *db/db* mice (Figure 2a). Furthermore, significant reductions in the serum levels of urea nitrogen and creatinine were observed in the fasudil-treated *db/db* mice compared with nontreated *db/db* mice, consistent with improvement in renal function (Table 1). Other physiological data including body weight, blood glucose levels, blood pressure, and lipid profiles were not significantly different between the *db/db* mice and fasudil-treated *db/db* mice (Figure 2b-d and Table 1).

### Rho-kinase inhibitor ameliorates mesangial matrix expansion in *db/db* mice

The glomerular structure in *db/db* mice showed accelerated mesangial expansion characterized by an increase in periodic acid–Schiff (PAS)–positive mesangial matrix area compared with that observed in *db/m* mice (Figure 3a). This mesangial expansion was noticeably ameliorated in fasudil-treated *db/db* mice. The glomerular area (defined by tracing along the outline of the capillary loop) was also increased in *db/db* mice compared with *db/m* mice, suggesting glomerular hypertrophy, and was reduced by fasudil treatment (Figure 3c). Similarly, the matrix fraction calculated by the ratio of the mesangial area to the glomerular area increased in *db/db* mice compared with *db/m* mice, and administration of fasudil significantly ameliorated the increase in matrix fraction (Figure 3d). These findings demonstrate that blockade of Rho-kinase prevents glomerular sclerosis of *db/db* mice.

It should be noted that albuminuria can also result from decreased proximal tubular uptake.[26] To further evaluate the therapeutic effect of Rho-kinase inhibition in tubulointerstitium, we assessed PAS and Masson's trichrome-stained samples. PAS-stained sections showed widened tubular lumen and fine vacuoles in the cortex of *db/db* mice (Supplementary Figure S1a online). Masson's trichrome staining identified collagen deposition (blue color) in diabetic mice compared with *db/m* mice (Supplementary Figure S1b and c online). However, these tubulointerstitial changes were not affected by administration of fasudil.

### Rho-kinase inhibitor attenuates the expression of profibrotic mediators and extracellular matrix proteins in renal cortex of *db/db* mice

To clarify the mechanism by which fasudil prevented the development of glomerular sclerosis in *db/db* mice, the expression levels of profibrotic mediators and extracellular matrix proteins were estimated by quantitative real-time PCR (Figure 4). Expression of TGF-β1, connective tissue growth factor and plasminogen activator inhibitor 1 was elevated in the renal cortex of *db/db* mice compared with *db/m* mice, and these increases were significantly suppressed in fasudil-treated *db/db* mice (Figure 4a–c). Furthermore, fasudil treatment significantly ameliorated enhancement of

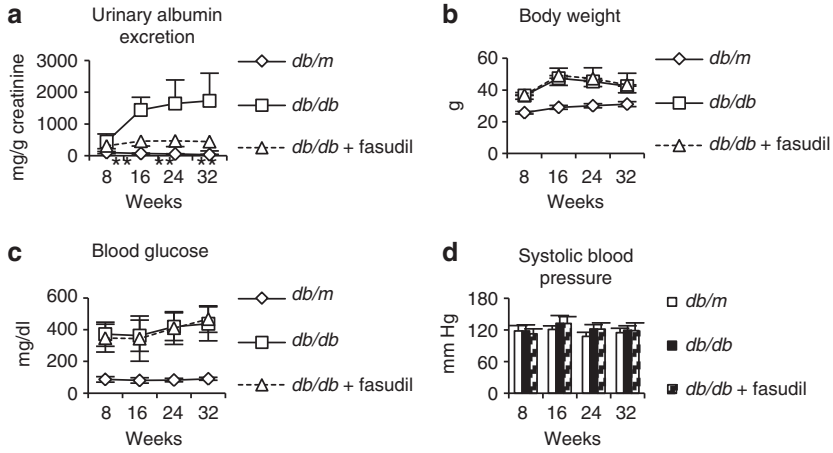


**Figure 2 | Fasudil treatment reduces urinary albumin excretion without affecting metabolic control.** Time course of changes in (**a**) urinary albumin-to-creatinine ratio, (**b**) body weight, and (**c**) fasting blood glucose in control *db/m* mice, *db/db* mice, and *db/db* mice treated with fasudil. (**d**) Systolic blood pressure was also determined every 8 weeks ($n = 8$ in each group, $^{**}P < 0.01$ vs. nontreated *db/db* mice).

**Table 1 | Metabolic and physiologic parameters**

| Parameter | *db/m* | *db/db* | *db/db* + fasudil |
|---|---|---|---|
| Kidney weight (g) | 0.54 ± 0.04 | 0.66 ± 0.18 | 0.61 ± 0.04 |
| Kidney/body weight (g) | 1.7 ± 0.1 | 1.6 ± 0.2 | 1.4 ± 0.5 |
| Serum urea nitrogen (mg/dl) | 29 ± 7 | 51 ± 19** | 34 ± 11† |
| Serum creatinine (mg/dl) | 0.22 ± 0.06 | 0.28 ± 0.10 | 0.19 ± 0.04† |
| Food intake (g/day) | 5.2 ± 0.6 | 7.4 ± 1.7** | 7.1 ± 1.6** |
| Water intake (g/day) | 8.5 ± 1.5 | 22.9 ± 8.2** | 23.0 ± 9.2** |
| Serum total cholesterol (mg/dl) | 79 ± 10 | 145 ± 47** | 143 ± 17** |
| Serum triglyceride (mg/dl) | 64 ± 20 | 147 ± 59** | 178 ± 78** |
| Serum free fatty acid (mEQ/l) | 0.7 ± 0.1 | 1.4 ± 0.5** | 1.3 ± 0.4** |

Data are means ± s.d. ($n = 8$ in each group).
$^{**}P < 0.01$ versus *db/m* mice.
$^{\dagger}P < 0.05$ versus nontreated *db/db* mice.

the expression of extracellular matrix proteins including type I collagen, type IV collagen, and fibronectin (Figure 4d–f).

### Rho-kinase inhibitor suppresses the accumulation of HIF-1α protein and the expression of its target genes in the renal cortex of *db/db* mice

It is known that connective tissue growth factor and plasminogen activator inhibitor 1 are regulated by HIF-1α and play crucial roles in renal fibrosis.[27,28] Western blot analysis showed that HIF-1α was more abundant in total cortical tissue lysate in *db/db* mice compared with control mice, and that fasudil strongly suppressed this increase in HIF-1α protein (Figure 5a). Similar results were also obtained with nuclear extract from renal cortical tissue (Figure 5b). Hexokinase II, glucose transporter 1 (GLUT-1), vascular endothelial growth factor, and adrenomedullin are major targets of HIF-1 and contribute to the progression of diabetic glomerular sclerosis.[29–32] These genes were upregulated in the renal cortex of *db/db* mice, and fasudil strongly attenuated the increase in expression of these genes (Figure 5c–f). Our findings indicate that the expression of HIF-1α in diabetic kidney is attenuated by Rho-kinase blockade.

### Hypoxia upregulates Rho/Rho-kinase activity in mesangial cells

It has been suggested that HIF-1α in mesangial cells plays a role in the development of diabetic nephropathy.[11] To gain a more profound understanding of the mechanism by which Rho-kinase may be involved in regulation of HIF-1α, *in vitro* experiments were performed using cultured mesangial cells. As shown in Figure 6a and b, GTP-bound active RhoA and phosphorylated MYPT1 were increased under hypoxic conditions. Y-27632, a specific Rho-kinase inhibitor, significantly attenuated the hypoxia-induced activation of Rho-kinase.

### Rho-kinase is involved in the degradation of HIF-1α under hypoxic conditions in mesangial cells

To substantiate the role of Rho-kinase in hypoxia-mediated gene expression, we next investigated the effect of Y-27632 on the expression of HIF-1-target genes. Hypoxic challenge significantly increased mRNA levels of these genes, which were also markedly repressed upon administration of Y-27632 (Figure 7a-f). As shown in Figure 7g, exposure of mesangial cells to hypoxia remarkably stimulated the production of HIF-1α, whereas Y-27632 attenuated the hypoxia-driven accumulation of HIF-1α protein. These changes in HIF-1α protein were not associated with alteration of mRNA levels of HIF-1α (Supplementary Figure S2 online). Importantly, in the presence of proteasome inhibitor (MG132), the inhibitory effect of Y-27632 on hypoxia-induced HIF-1α production was canceled (Figure 7g). Similar results were obtained with deferoxamine, a well-established hypoxia mimetic that results in the stabilization of HIF-1α through the inactivation of prolyl hydroxylase (PHD) activity[28] (Supplementary Figure S3 online), suggesting that the effect of Rho-kinase inhibition is mediated through enhanced proteasome-dependent degradation of HIF-1α protein. Supporting the results of western blot analysis, immunoprecipitation showed that accumulation of polyubiquitinated HIF-1α in the presence

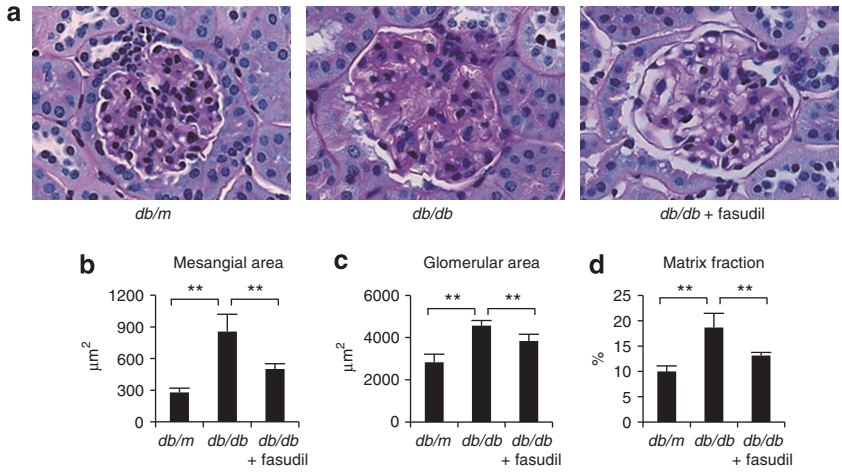


**Figure 3 | Treatment of *db/db* mice with fasudil prevents mesangial matrix expansion.** (**a**) Representative photomicrographs of periodic acid–Schiff (PAS)–stained kidney from *db/m* mice, *db/db* mice, and *db/db* mice given fasudil. Original magnification ×400. Quantitative analysis of (**b**) mesangial area, (**c**) glomerular area, and (**d**) matrix fraction. Data are means ± s.d. ($n = 8$ in each group, $^{**}P < 0.01$).

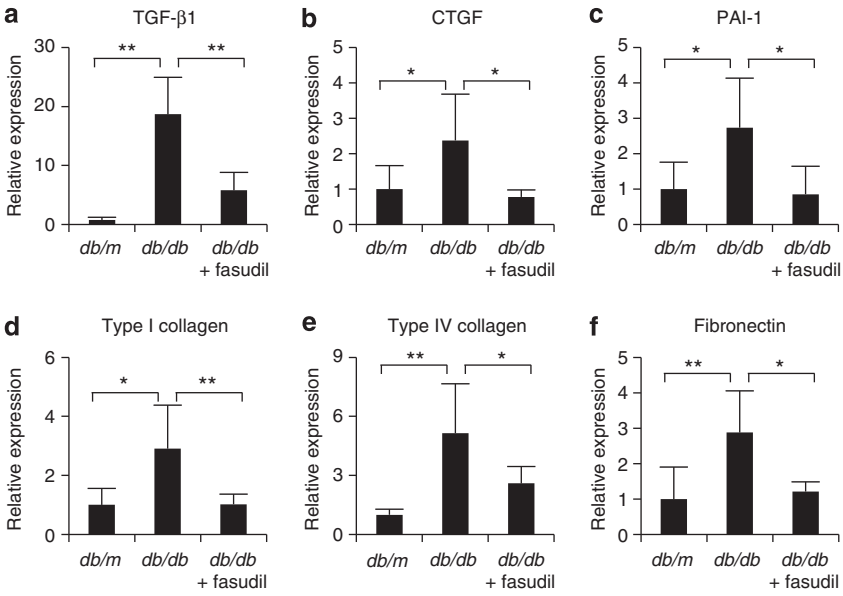


**Figure 4 | Fasudil attenuates the expression of profibrotic growth factors and extracellular matrix components in the renal cortex of *db/db* mice.** (**a**–**c**) Profibrotic and (**d**–**f**) extracellular matrix protein gene expression levels measured by quantitative real-time PCR analysis. mRNA expression was normalized to the level of 36B4. Data are means ± s.d. ($n = 8$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor 1; TGF-β1, transforming growth factor-β1.

of MG132 was increased by administration of Rho-kinase inhibitor (Supplementary Figure S4 online). These results indicate that Rho-kinase blockade accelerates proteasomal HIF-1α degradation via enhancement of polyubiquitination.

We next used small interfering RNA (siRNA) to target the Rho-kinase isoforms (ROCK1 and ROCK2). To confirm the efficacy of siRNA knockdown of ROCK1 and ROCK2, western blot was performed. A decrease in protein expression was observed in mesangial cells transfected with the specific siRNA (Figure 7h). Silencing of ROCK1 or ROCK2 was sufficient to cause reduction of HIF-1α induced by hypoxia (Figure 7i). These data indicate that Rho-kinase is involved in hypoxia-mediated HIF-1α generation, and that both ROCK1 and ROCK2 play a crucial role in this process.

### PHD2 activity is upregulated by Rho-kinase inhibition in mesangial cells

Degradation of HIF-1α protein is tightly controlled by site-specific hydroxylation mediated by PHD isoforms. Hydroxylation is a nonreversible process, and PHD activity is a determinant of the degradation of HIF-1α. Accordingly, changes in the expression of PHD isoforms were investigated

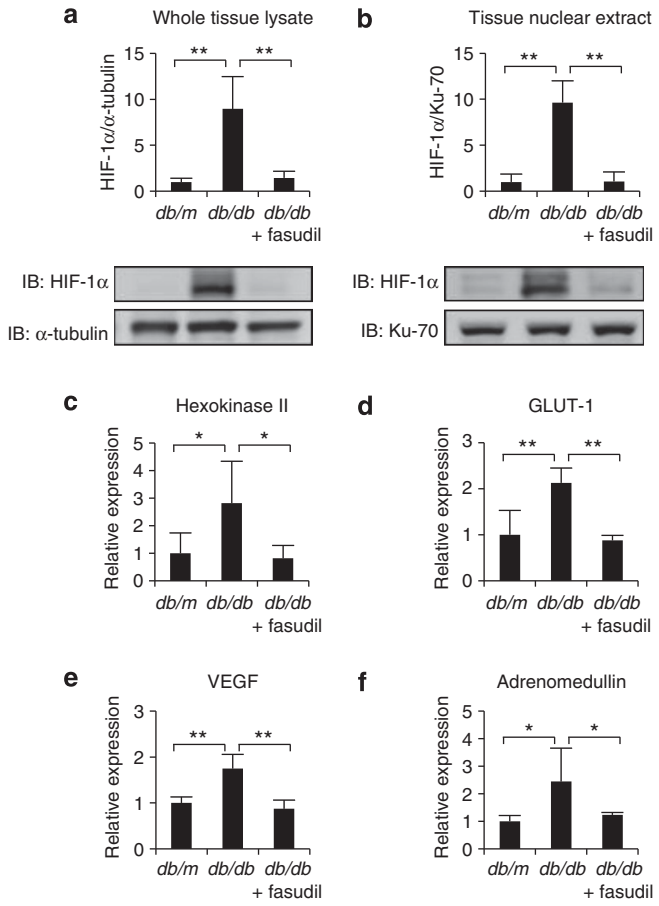


**Figure 5 | Treatment with fasudil inhibits hypoxia-inducible factor-1α (HIF-1α) accumulation in the renal cortex of *db/db* mice.** HIF-1α protein of (**a**) whole renal cortical tissue and (**b**) nuclear fractions analyzed by western blot. α-Tubulin and Ku-70 were loaded as internal control, respectively. The results are quantified and shown in the bar graph. (**c**–**f**) Quantitative real-time PCR analysis of HIF-1-regulated genes. mRNA expression was normalized to the level of 36B4. Data are means ± s.d. ($n = 8$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). GLUT-1, glucose transporter 1; IB, immunoblotting; VEGF, vascular endothelial growth factor.

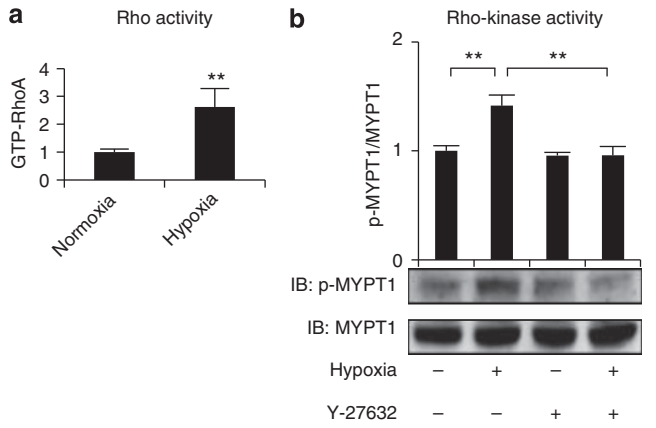


**Figure 6 | Rho/Rho-kinase activity is upregulated in hypoxic mesangial cells.** (**a**) Starved MES-13 cells were placed in normoxic (21% $O_2$) or hypoxic (1% $O_2$) conditions for 10 min, and GTP-bound active RhoA was analyzed by G-LISA. (**b**) Starved MES-13 cells were cultured under hypoxia (1% $O_2$) for 1 h. Phosphorylated MYPT1 (p-MYPT1) was quantified by western blot analysis. Data are means ± s.d. ($n = 3$, $^{**}P < 0.01$). GTP, guanosine-5′-triphosphate; IB, immunoblotting; MYPT1, myosin phosphatase target subunit 1.

under both normoxic and hypoxic conditions. Under normoxic conditions, expression of PHD1 and PHD3 mRNA remained stable for 120 min and was not affected by Y-27632, whereas blockade of Rho-kinase upregulated the mRNA levels of PHD2 (Figure 8a). Under hypoxic conditions, levels of PHD1 and PHD3 mRNA were unchanged (Figure 8b). In contrast, expression of PHD2 mRNA suppressed, and this effect was reversed by Y-27632. Importantly, *in vivo* data showed an increase in PHD2 mRNA levels in *db/db* mice treated with fasudil (Figure 8c). In addition, increases in both the levels of PHD2 protein and the ratio of hydroxylated to nonhydroxylated HIF-1α were detected upon administration of Y-27632 in mesangial cells (Figure 8d and e). These data suggest that Rho-kinase inhibition may promote hydroxylation of HIF-1α via activation of renal PHD2.

## DISCUSSION

In this study, we showed that the activities of Rho and Rho-kinase are increased in the renal cortex of *db/db* mice, a model of type 2 diabetes. Suppression of Rho-kinase with fasudil ameliorated albuminuria, mesangial expansion, and glomerular hypertrophy, which are the most striking morphologic characteristics of diabetic nephropathy, via downregulation of HIF-1α. Although proteinuria is also associated with tubular damage,[26] histological analysis indicated that fasudil does not affect tubulointerstitial injury in *db/db* mice. These data indicate the importance of Rho/Rho-kinase signaling pathway as a major contributor to the development of diabetic glomerulosclerosis rather than interstitial fibrosis. However, several recent studies suggest that Rho-kinase may play a crucial role in tubulointerstitial fibrosis by enhancing signaling pathways including the TGF-β/Smad pathway.[33–35] Given the significance of HIF-1α in the fibrotic reaction of the tubulointerstitium,[12] a greater understanding of the cross-talk between Rho-kinase and HIF-1α in tubular epithelial cells is an important goal of future research.

Our findings that fasudil attenuated glomerulosclerosis via reductions in the levels of HIF-1α and its target genes led us to investigate whether Rho-kinase regulates hypoxia-mediated fibrotic response in *in vitro* study using cultured mesangial cells. Consistent with data obtained from mice, activities of Rho and Rho-kinase were increased in mesangial cells exposed to hypoxic conditions. HIF-1α target genes were also upregulated under hypoxic conditions, and repressed by Y-27632, a specific Rho-kinase inhibitor, suggesting the involvement of the Rho/Rho-kinase pathway in HIF-1α-mediated gene expression. Indeed, hypoxia-induced HIF-1α accumulation was suppressed by pharmacological and genetic inhibition of Rho-kinase. This change in HIF-1α protein was,

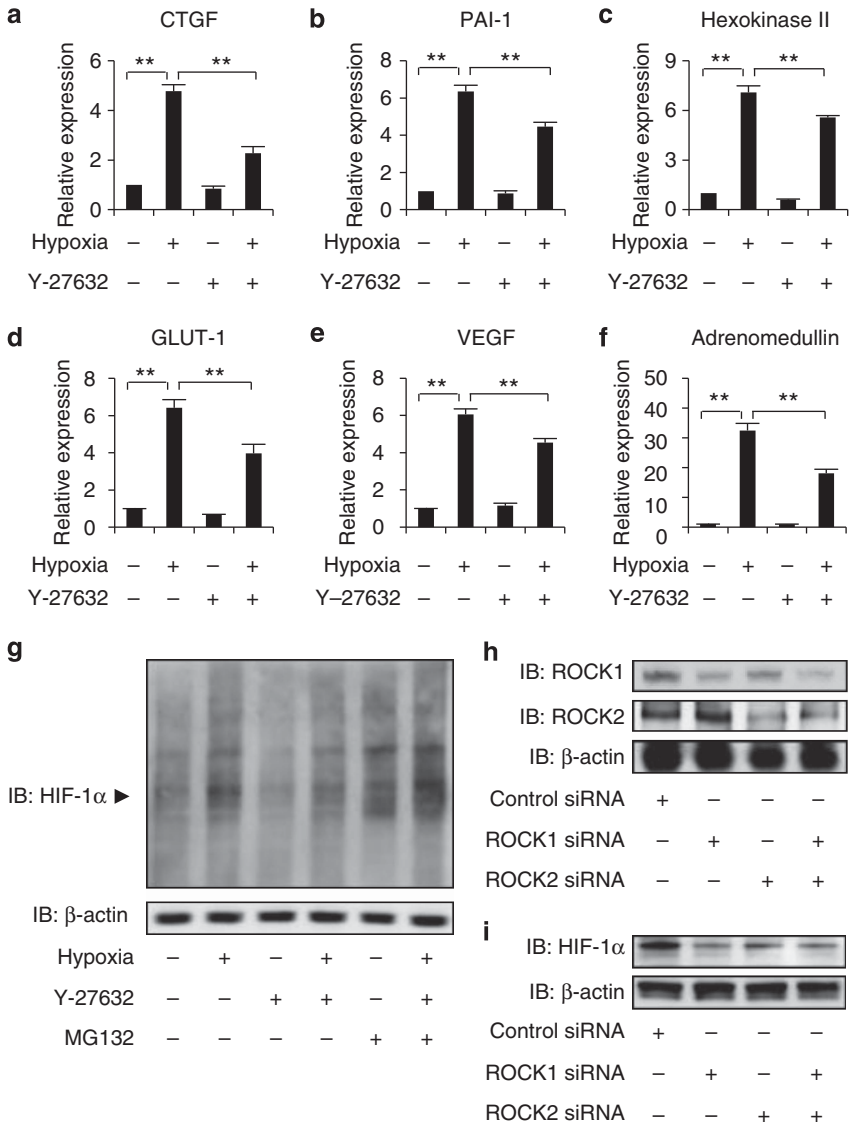


**Figure 7 | Rho-kinase inhibition promotes hypoxia-induced hypoxia-inducible factor-1α (HIF-1α) degradation in a proteasome-dependent manner in mesangial cells.** (**a**–**f**) Quantitative real-time PCR analysis of HIF-1 target genes. Starved MES-13 cells were treated with or without 10 μmol/l Y-27632 under normoxic (21% $O_2$) or hypoxic (1% $O_2$) conditions for 18 h. mRNA expression was normalized to the level of 36B4. (**g**) Western blot analysis of HIF-1α in MES-13 cells. Starved cells were pretreated with or without 10 μmol/l Y-27632 and/or 10 μmol/l MG132 under normoxic (21% $O_2$) or hypoxic (1% $O_2$) conditions for 1 h. Whole-cell lysates were subjected to western blot analysis using HIF-1α antibody. (**h**) MES-13 cells were transfected with control, ROCK1, ROCK2, or a mixture of ROCK1 and ROCK2 siRNA. The gene silencing effect was analyzed by western blot analysis. (**i**) MES-13 cells were treated with control, ROCK1, ROCK2, or a mixture of ROCK1 and ROCK2 siRNA and then stimulated by hypoxia (1% $O_2$) for 1 h. Data are means ± s.d. ($n = 3$, $^{**}P < 0.01$). CTGF, connective tissue growth factor; IB, immunoblotting; GLUT-1, glucose transporter 1; PAI-1, plasminogen activator inhibitor 1; ROCK, Rho-associated, coiled-coil-containing protein kinase; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor.

however, not associated with change in mRNA expression. Based on the fact that Y-27632-mediated HIF-1α degradation was rescued by MG132 and that accumulation of poly-ubiquitinated HIF-1α was promoted by Rho-kinase inhibition, we concluded that at least part of the proteolysis was mediated by the ubiquitin–proteasome pathway.

The notion that HIF-1α is regulated by Rho-kinase at the posttranslational level is consistent with a recent study reporting an interaction between Rho-kinase and HIF-1α induction in endothelial cells.[36] This report also described that fasudil inhibited hypoxia-induced HIF-1α protein expression by enhancing proteasomal degradation without altering the abundance of HIF-1α mRNA. Under aerobic conditions, HIF-1α is hydroxylated by PHD domain proteins, which use oxygen and α-ketoglutarate as substrates and contain ferric ion in their catalytic center. Hydroxylated HIF-1α interacts with the von Hippel–Lindau protein that leads to polyubiquitination and proteasomal degradation. Although the mechanism by which HIF-1α stability is regulated by Rho/Rho-kinase signaling remains a matter of debate, several

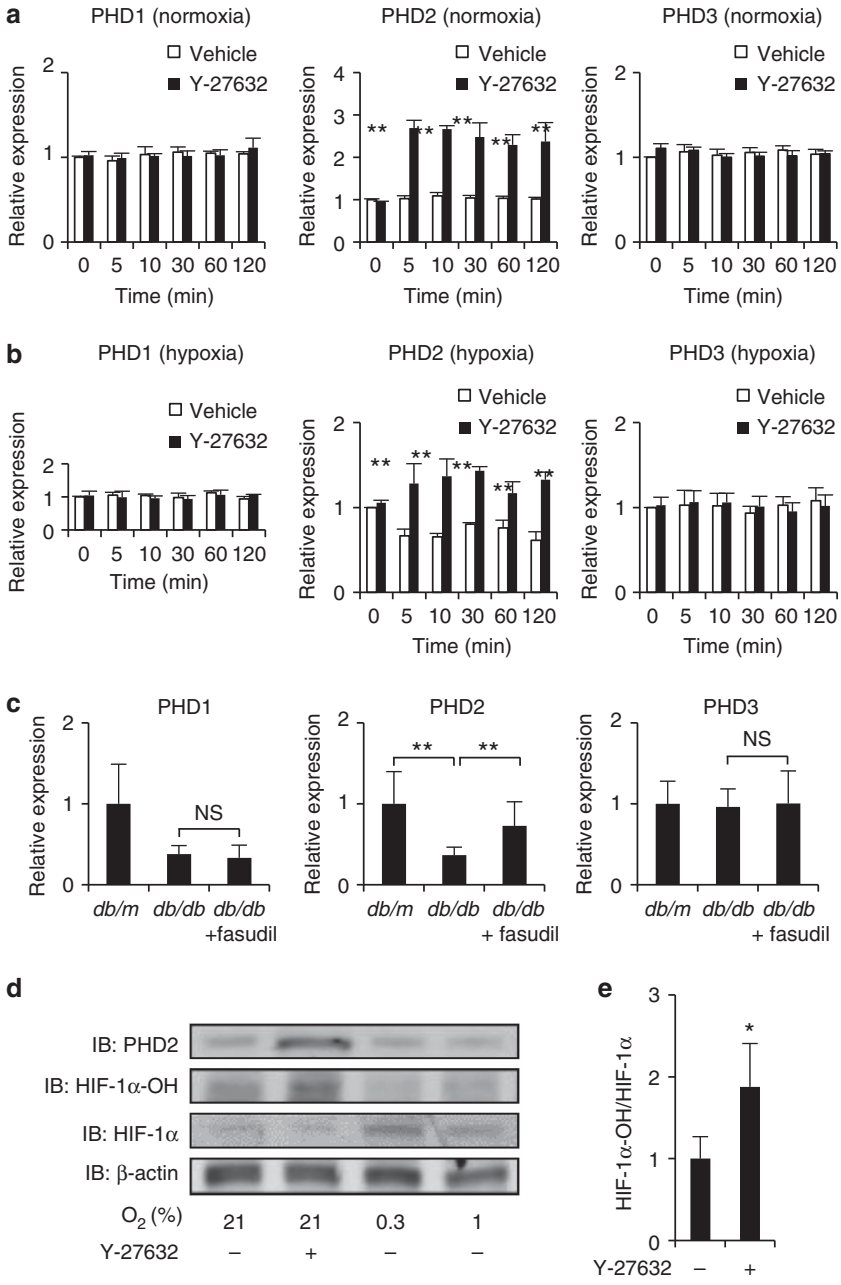


**Figure 8 | Rho-kinase regulates prolyl hydroxylase domain protein 2 (PHD2) activity.** Starved MES-13 cells were treated with 10 μmol/l Y-27632 or vehicle (water) for the indicated duration at (**a**) 21% $O_2$ or (**b**) 1% $O_2$ conditions. The mRNA expression levels of PHD isoforms PHD1, PHD2, and PHD3 were evaluated by quantitative real-time PCR analysis. The data were normalized to the level of 36B4. Data are means ± s.d. ($n = 3$, $^{**}P < 0.01$ vs. vehicle-treated cells at the same time point). (**c**) Real-time PCR analysis of PHD isoforms from the renal cortex of *db/m* mice, *db/db* mice, and *db/db* mice given fasudil. Data are means ± s.d. ($n = 8$ in each group, $^{**}P < 0.01$). (**d**) PHD2, hydroxylated hypoxia-inducible factor-1α (HIF-1α-OH), and HIF-1α proteins were quantified by western blot analysis treated with 10 μmol/l Y-27632 for 10 min at 21% $O_2$. Graded hypoxia (0.3 and 1% $O_2$) is included as control condition. β-Actin was loaded as an internal control. (**e**) Levels of total and hydroxylated HIF-1α were quantified by densitometry. The ratio of hydroxylated/total HIF-1α was determined. Data are means ± s.d. ($n = 3$, $^{*}P < 0.05$). IB, immunoblotting; NS, not statistically significant.

plausible mechanisms implicating Rho-kinase in this process may be proposed. Intriguingly, our data suggested the interaction between Rho-kinase and PHD2 activity both *in vitro* and *in vivo*. In mammalian cells, three PHD isoforms, PHD1, PHD2, and PHD3, have been characterized and have been shown to hydroxylate key proline residues (Pro402 and Pro564) in HIF-1α.[37] Although all isoforms are expressed in the kidneys,[38,39] PHD2 is thought to be the critical regulator of HIF-1α because transiently silencing of PHD2, but not PHD1 or PHD3, results in stabilization of HIF-1α *in vitro*.[40] Moreover, PHD2 knockout mice exhibit enhanced angiogenesis and erythropoiesis, whereas PHD1 and PHD3

knockout mice appear normal.[41] These studies suggest a key role for PHD2 in the regulation of HIF-1α stability. Importantly, fasudil offset decreases in PHD2 mRNA levels in *db/db* mice. Because little is known concerning the transcriptional regulation of PHD2, it is unclear why PHD2 is downregulated in diabetic kidney and hypoxic mesangial cells. However, it has been shown that TGF-β1 is a negative regulator of PHD2,[42] and we have demonstrated increased TGF-β1 expression in the renal cortex of *db/db* mice. Interestingly, TGF-β1 expression was induced under hypoxic conditions, and PHD2 mRNA was significantly suppressed by administration of recombinant TGF-β1 in mesangial cells (data not shown). These results indicate that inhibition of Rho-kinase might have affected PHD2 expression via downregulation of TGF-β1.

Although future research is needed to clarify the molecular basis of both the regulation of PHD2 expression and Rho-kinase modulation of the abundance of PHD2, this study suggests that hypoxia-mediated HIF-1α protein stabilization by Rho-kinase plays a pivotal role in mesangial expansion and the progression of diabetic nephropathy (Figure 9).

Rho GTPase and Rho-kinase have also been shown to mediate hypoxia-induced HIF-1α activation in several cell types,[24,25,36] and to promote the production of HIF-1-regulated genes. The underlying mechanisms by which hypoxia activates Rho/Rho-kinase signaling are not clear. It is possible that reactive oxygen species (ROS) detect low oxygen content and are involved in the activation of Rho under hypoxic conditions. Turcotte *et al.*[24] demonstrated that activation of Rho is dependent on ROS production under low-oxygen conditions. The cross-talk of ROS and Rho activation is also suggested by our previous observation that the expression of nicotinamide adenine dinucleotide phosphate oxidase, a major source of oxidative stress, is suppressed by fasudil in the kidney of diabetic rat.[19] A recent genetic study using mice with podocyte-specific overexpression of Rho-kinase demonstrated increased mitochondrial fission that results in ROS production.[23] These data suggest an important role for Rho/Rho-kinase signaling in generating a vicious cycle for ROS augmentation and in hypoxia-mediated ROS production. Although the impact of ROS on HIF-1α is a matter of debate, given that increased ROS formation contributes to nonhypoxic HIF-1α stabilization,[43] fasudil may have promoted HIF-1α downregulation via inhibition of mitochondrial ROS production besides the induction of PHD2. In addition to ROS, it is possible that HIF-1α activates Rho-kinase under hypoxia. However, Rho-kinase activity was not altered by HIF-1α stabilization in mesangial cells (Supplementary Figure S5 online). Therefore, in our experimental models, at least *in vitro*, we propose that reciprocal impact of HIF-1α upon Rho-kinase is not significant in this context.

In conclusion, this study demonstrated that Rho-kinase blockade attenuates the progression of diabetic glomerulosclerosis via downregulation of HIF-1α. These findings provide new insights into the mechanisms of renoprotection associated with Rho-kinase inhibition for the treatment and prevention of diabetic nephropathy.

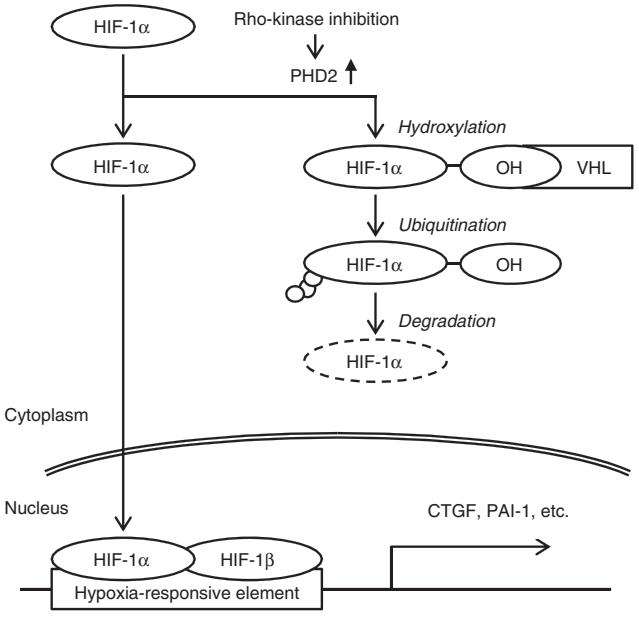


**Figure 9 | Rho-kinase inhibition accelerates proteasomal degradation of hypoxia-inducible factor-1α (HIF-1α).** Blockade of Rho-kinase promotes HIF-1α degradation by activating prolyl hydroxylase domain protein 2 (PHD2), resulting in hydroxylation (OH) and polyubiquitination of HIF-1α. Subsequently, nuclear translocation of HIF-1α is suppressed, which promotes the expression of profibrotic mediators. CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor 1; VHL, von Hippel–Lindau protein.

## MATERIALS AND METHODS

### Animal studies

The 5-week-old male BKS.Cg-+*Leprdb*/+*Leprdb*/Jcl (*db/db*) mice and their age-matched heterozygous male littermates BKS.Cg-m+/+*Leprdb*/Jcl (*db/m*) mice were obtained from CLEA (Tokyo, Japan). At 8 weeks of age, these mice were divided into the following groups: nondiabetic *db/m* mice, *db/db* mice, and *db/db* mice treated with the Rho-kinase inhibitor fasudil. Fasudil was kindly provided by Asahi Kasei Pharma (Tokyo, Japan) and administered in drinking water (100 mg/kg/day). Fasudil treatment was continued for 24 weeks, at which time tissues were obtained for biochemical and histopathological evaluation. After collection of blood samples from the left cardiac ventricle, the kidney was perfused with ice-cold phosphate-buffered saline and rapidly dissected. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals at Jikei University School of Medicine.

### Histological and morphometric analysis

Renal cortical tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin (data not shown), PAS, and Masson's trichrome for light microscopic observation. In each animal of the three experimental groups, 20 glomeruli cut at their vascular pole in the cortex were used for analysis of mesangial expansion. The mesangial area was determined by the presence of PAS-positive and nuclei-free area in the mesangium. The glomerular tuft area was also traced along the outline of capillary loop. The mesangial matrix fraction was calculated as the ratio of the mesangial area to the glomerular

tuft area. Interstitial fibrosis was assessed in 10 random non-overlapping fields of Masson's trichrome-stained cross-sections in Adobe PhotoShop (San Jose, CA) to determine the percentage of collagen (blue) staining area to total tissue area.

### Cell culture

Murine mesangial cells (MES-13) obtained from the American Type Cell Collection (Manassas, VA) were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum in a humidified air/5% $CO_2$ atmosphere at 37 °C. Cells were synchronized for 24 h in serum-free Dulbecco's modified Eagle's medium at 80 to 90% confluence before the experiments. For hypoxia experiments, cells were incubated at 1 or 0.3% $O_2$ conditions. Levels of $O_2$ were maintained by the gas oxygen controller (ProOx Model 110, BioSpherix, Redfield, NY) using a premixed gas composed of 5% $CO_2$ and 95% $N_2$. Rho-kinase inhibitor (Y-27632, Wako, Osaka, Japan) and proteasome inhibitor (MG132, Calbiochem, San Diego, CA) were added before hypoxic treatment. Deferoxamine (Sigma, St Louis, MO) was used to mimic hypoxic effects at 21% $O_2$. In this study, we used two different Rho-kinase inhibitors. As fasudil is the only Rho-kinase inhibitor approved for clinical use, we used this drug for our *in vivo* studies. When given orally, fasudil is metabolized into the more potent inhibitor hydroxyfasudil.[44] Both hydroxyfasudil and Y-27632 are more selective for Rho-kinase over other serine/threonine kinases such as protein kinase A, whereas fasudil inhibits both enzymes with equal potency. Accordingly, we investigated the inhibitory effects of Rho-kinase *in vitro* using Y-27632, which is used to assess the effects of Rho-kinase inhibition in a variety of cellular models.

### RNA isolation and quantitative real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) followed by chloroform–isopropanol extraction and ethanol precipitation as reported previously,[20] and 1 μg of total RNA was reverse-transcribed using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative real-time PCR was performed using the Thermal Cycler Dice Real Time System TP800 (Takara Bio) using SYBR Green I fluorescence signals. Levels of mRNA were normalized with 36B4, which is known to be not regulated by $O_2$ tension.

### GTP-RhoA activity assay

RhoA activation was determined by the quantification of GTP-bound RhoA, using G-LISA RhoA activation biochem kit according to the manufacturer's recommendations (Cytoskeleton, Denver, CO). Briefly, collected renal cortex and mesangial cells were immediately snap-frozen to prevent degradation of active Rho and then lysed in ice-cold lysis buffer. The protein samples were added to a 96-well microplate coated with Rho-GTP binding protein. The plates were then incubated with RhoA antibody and secondary horseradish peroxidase–conjugated antibody. The luminescence signal was detected by measuring absorbance at 490 nm using a microplate spectrophotometer.

### Western blot analysis

The dissected cortical tissues and cultured mesangial cells were homogenized in ice-cold radioimmunoprecipitation assay buffer. Nuclear extracts from the cortex were obtained using a NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL). Equal amounts of protein samples were loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, electrophoresed, and transferred onto nitrocellulose membranes (Invitrogen). After blocking in 5% nonfat milk, membranes were incubated with phospho-MYPT1 at Thr850 (Millipore, Temecula, CA), MYPT1 (Cell Signaling Technology, Beverly, MA), HIF-1α antibody (R&D Systems, Minneapolis, MN), PHD2 antibody (Cell Signaling Technology), hydroxylated HIF-1α antibody at Pro564 (Novus Biologicals, Littleton, CO); or with an α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin antibody (Cosmo Bio, Tokyo, Japan), or Ku-70 antibody (Santa Cruz Biotechnology). Subsequently, the blots were washed and incubated with a horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized with the ECL system (Amersham, Buckinghamshire, UK).

### Immunoprecipitation

Whole-cell lysates (500 μg) of mesangial cells were prepared with lysis buffer. The immunoprecipitation assay was performed using Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) with 2 μg HIF-1α antibody according to the manufacturer's protocol. Accumulation of polyubiquitinated HIF-1α was detected by western blot analysis with ubiquitin antibody (Cell Signaling Technology).

### Silencing of Rho-kinase

Mesangial cells were transfected with ROCK1 and/or ROCK2 siRNA or with a nontargeting siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h after siRNA transfection, cells were harvested to determine the level of ROCK1 and ROCK2 proteins by western blot, or were stimulated with hypoxia for 1 h.

### Statistical analysis

Data are expressed as mean ± s.d. Statistical evaluation of the data was performed using Student's *t*-test or analysis of variance followed by Bonferroni's *post hoc* correction. A value of $P<0.05$ was considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

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### SUPPLEMENTARY MATERIAL

**Figure S1.** Rho-kinase inhibitor does not affect tubular damage in diabetic mice.
**Figure S2.** HIF-1α mRNA is not altered by Rho-kinase inhibition in mesangial cells.
**Figure S3.** DFX-induced HIF-1α accumulation is prevented by Rho-kinase inhibition in mesangial cells.
**Figure S4.** Rho-kinase inhibition accelerates polyubiquitination of HIF-1α.
**Figure S5.** HIF-1α stabilization does not affect Rho-kinase activity in mesangial cells.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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