Rhein Inhibits Integrin-Linked Kinase Expression and Regulates Matrix Metalloproteinase-9/Tissue Inhibitor of Metalloproteinase-1 Ratio in High Glucose-Induced Epithelial-Mesenchymal Transition of Renal Tubular Cell

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Studies have found overexpressed integrin-linked kinase (ILK) and disturbed matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 (MMP-9/TIMP-1) ratio in diabetic nephropathy epithelial-mesenchymal transition (EMT). However, the underlying mechanisms of EMT and the inhibiting effect of rhein need further understanding. The aim of this study was to investigate the possible regulating effects of ILK towards MMP-9/TIMP-1 ratio in EMT and the inhibiting effect of rhein. The characteristic epithelial marker and mesenchymal marker of EMT were examined by cytokeratin and vimentin immunostaining. The results showed that in high glucose conditions, overexpression of ILK and an abnormal changing of MMP-9/TIMP-1 ratio occurred; ILK inhibition by siRNA could adjust MMP-9/TIMP-1 ratio to near normal. Meanwhile, rhein inhibited the overexpressing ILK and inhibits high glucose-induced EMT; the effect was similar to that of ILK-siRNA. The decreased expression of ILK regulated by rhein contributed to the adjustment of the MMP-9/TIMP-1 ratio. Our data indicate that rhein inhibits high glucose-induced-EMT partially through the inhibition of ILK expression and regulates the MMP-9/TIMP-1 ratio in HK-2 cells. This mechanism may be associated with rhein's effect of ILK suppression.

Key words epithelial-mesenchymal transition; rhein; diabetic nephropathy; small interfering RNA; integrin-linked kinase; matrix metalloproteinase-9

Tubulointerstitial lesions are thought to play important roles in the progression of diabetic nephropathy. Pathological analysis has revealed that the impairment of renal function correlates better with the extent of tubulointerstitial damage rather than with the degree of glomerular damage.\(^1,2\) Epithelial-mesenchymal transition (EMT) in renal tubular epithelial cells has been considered a crucial step in diabetic nephropathy tubulointerstitial damage. Numerous in-depth studies have been made to reveal the large and complex network of molecular signals in EMT; among these key molecules, integrin-linked kinase (ILK), a downstream factor of transforming growth factor β1 (TGF-β1), participates in integrin, TGF-β1/Smad, mitogen-activated protein kinases (MAPK) and other signal transduction pathways, and has been proved to regulate cell adhesion, migration, extracellular matrix accumulation.\(^3,4\)

Generally the promoting roles of matrix metalloproteinase-9 (MMP-9) in EMT are considered to be: (1) Decomposes normal tissue basement membrane type IV collagen, and enhances the migration ability of epithelial cells, facilitates the migration of transited epithelial cells through the damaged basement membrane;\(^5,6\) (2) Hinders the degradation and promotes the accumulation of extracellular matrix, thereby aggravates renal interstitial fibrosis.\(^7,8\) Recent studies have shown the imbalance of MMP-9 and its tissue inhibitor TIMP-1 (tissue inhibitor of metalloproteinase-1) in EMT,\(^9,10\) and there was evidence about the disordered ratio of MMP-9/TIMP-1 in diabetic nephropathy patients.\(^11\) Our previous study has shown similar results in EMT of diabetic nephropathy rats.\(^12\) However, its mechanism and the up-stream regulating factors remain unclear. Report has shown that ILK could induce an invasive phenotype via activator protein 1 (AP-1)-dependent upregulation of MMP-9 in IEC18 (intestinal epithelial cell 18) cell lines.\(^13\) In this study, we examined the changes of MMP-9/TIMP-1 and the role of ILK in high glucose-induced EMT in human renal epithelial cells; furthermore, the inhibiting role of rhein in this process was discussed. Rhein is the main renal protective ingredient of traditional Chinese medicine rhubarb. Studies have reported that rhein could inhibit high glucose-induced mesangial cell proliferation, hypertrophy and increased extracellular matrix synthesis; suppress TGF-β1-induced tubular epithelial cell hypertrophy and extracellular matrix production; inhibit fibroblast proliferation and promote its apoptosis.\(^14-17\) Our results indicate that rhein could inhibit ILK and regulate abnormal MMP-9/TIMP-1 ratio in EMT; rhein’s effect of regulating imbalanced MMP-9/TIMP-1 ratio might involve ILK suppression.

MATERIALS AND METHODS

Materials Rhein (purity 99%, contributed by Professor Zhihong Liu of the Nanjing General Hospital of Nanjing Military Command) was purified and identified by the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and diluted with 0.1 mol/L sodium hydroxide (NaOH), then added Dulbecco’s modified...
Eagle’s medium/F12 (DMEM/F12) as a secondary dilution solvent. The final dilutions in culture medium were 25 µg/mL, 50 µg/mL, and 100 µg/mL. DMEM/F12, trypsin and fetal bovine serum (FBS) were from Invitrogen, U.S.A.

The molecular structure and physicochemical properties of rhein are shown in Fig. 1, Table 1, respectively.

**Cell Culture and Treatment** HK-2 (human kidney 2) is an immortalized proximal tubular cell (PTC) line derived from normal kidney of adult male, and can reproduce experimental results obtained with freshly isolated PTCs. The HK-2 cells (kindly provided by Professor Ruihong Liu of the Second Xiangya Hospital, Central South University) were cultured in DMEM/F12 containing 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FBS at 37°C in a humidified 5% CO₂ atmosphere. After digested with 0.25% trypsin, 5×10⁵ cells were grown in 100 mm culture dish. For experiments, HK-2 cells were cultured overnight in DMEM/F12+10% FBS, then media were changed to fresh DMEM/F12 containing 30 mmol/L d-glucose+10% FBS and rhein with different concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL; these concentrations were based on preliminary experiment), with positive (10% FBS+5 nmol/L ILK-small interfering RNA (ILK-siRNA)), hypertonic (10% FBS+24.5 mmol/L mannitol), and negative (10% FBS) controls. Cultures were continued for a further 48 h for Western blot, real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) and cytoimmunostaining.

**Transfection of ILK-siRNA** Cells were grown in six-well plates (Costar, Cambridge, U.K.) and transfected with a mixture of 5 nmol/L specific ILK-siRNA or nonsilencing (negative control) siRNA (QIAGEN, Germany), serum-free DMEM/F12 medium and Hiperfect Transfection Reagent (QIAGEN). After 8 h, cells were washed with phosphate buffer solution (PBS) for 3 times and cultured for 48 h in complete medium with 10% FBS and 30 mmol/L glucose. This time point was selected from a time course of decreasing ILK mRNA expression in response to siRNA. A negative control scrambled siRNA provided by the manufacturer did not reduce ILK mRNA.

**Cytoimmunostaining** To determine whether rhein can inhibit high glucose-induced phenotypic alterations, HK-2 cells were grown on chamber slides (Thermo, U.S.A.) for 3 d until ca. 70% confluent and cells were stimulated as described above for 48 h, washed with PBS and fixed with 4% para-formaldehyde. Endogenous peroxidases were removed by 30 min incubation in 3% peroxide and biotin was blocked using the sheep serum working solution (Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China). The slides were then incubated with a mouse monoclonal anti-α-smooth muscle actin (α-SMA) antibody (Ab) (1:100, Abcam, Cambridge, U.K.) diluted in PBS containing 3% bovine serum albumin (BSA) for 1 h at 37°C. After washing in PBS, slides were incubated with a horse radish peroxidase (HRP) coupled goat anti-mouse Ab (Proteintech, U.S.A) for 30 min at room temperature, and washed in PBS for 3 times. Slides were then developed with peroxidase substrate kit 3′,3′-diaminobenzendine (DAB) (Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). HK-2 cells were counterstained with Harris’ hematoxylin and dehydrated with absolute alcohol. Images were acquired using a Leica TCS SP confocal microscope (Heidelberg, Germany). Substitution of the primary Ab with PBS was used as a negative control.

Cytoimmunostaining of E-cadherin (E-cad) was processed with a mouse monoclonal anti-E-cadherin Ab (1:100, AbCam) and the laboratory procedure was processed as mentioned above.

**Semi-quantitative Real-Time RT-PCR Analysis** Total RNA was extracted from the HK-2 cells using TRizol reagent according to the manufacturer’s instructions. Reverse transcription (RT) was performed using the SYBR PrimeScript RT-PCR kit (TaKaRa, Japan) according to the manufacturer’s protocols. mRNA levels of α-SMA, E-cadherin, ILK, MMP-9 and TIMP-1 were analyzed, while house keeping gene—“glyceraldehyde-3-phosphate dehydrogenase (GAPDH)” was used.

![Fig. 1. The Chemical Structure of Rhein (C15H8O6)](image)

Table 1. The Physical and Chemical Properties of Rhein

<table>
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<th>Name</th>
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<th>4,5-Dihydroxyanthraquinone-2-carboxylic acid</th>
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as the internal standard. The oligonucleotide primers used are seen in Table 2. Real-time PCR amplification was performed using the Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, U.S.A.). Cycling conditions were 95°C for 30 s followed by 40 repeats of 95°C for 5 s and 60°C for 30 s. The mRNA levels of target genes were normalized to the GAPDH mRNA levels and calculated using the delta–delta method from threshold cycle numbers. Data are mean±S.D. of four independent experiments.

**Western Blot Analysis** Confluent HK-2 was stimulated as described before and harvested using radio immunoprecipitation assay (RIPA) buffer (Dingguo Changsheng Biotechnology Co., Ltd.) following the manufacturer’s instructions. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology, Shanghai, China), against a BSA standard curve.

Thirty to fifty microgram protein samples were separated on 8–15% Bis-Tris gels in sodium dodecyl sulfate (SDS) Running Buffer and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories, U.S.A.). After transferring, all incubations were conducted on a rocking platform. The membrane was blocked in 5% skim milk/TBST (Tris buffered saline with Tween 20) for 1.5 h, and then incubated overnight at 4°C with a mouse monoclonal anti-α-SMA Ab (1:2000, AbCam). Immunoblots were then washed with TBST for 3 times and incubated with peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (1:5000, Proteintech), and washed with TBST for 3 times. Immunoreactivity was detected using an enhanced electrochemiluminescence (ECL) kit (Pierce, U.S.A.) and visualized on Kodak Omat X-ray films (Kodak, U.S.A.). Blots were stripped and re-probed with a rabbit polyclonal anti-β-tubulin antibody (1:2000, Proteintech), as the internal standard.

Western blot of E-cadherin was processed with a mouse monoclonal anti-E-cadherin Ab (1:500, AbCam); ILK was processed with a rabbit polyclonal anti-ILK Ab (1:5000, AbCam); MMP-9 was processed with a goat polyclonal anti-MMP-9 Ab (1:200, Santa Cruz); TIMP-1 was processed with a rabbit polyclonal anti-TIMP-1 Ab (1:500, AbCam). All these primary Ab were incubated with corresponding peroxidase-conjugated IgG as secondary Ab.

Densitometric analysis of the images obtained from X-ray films was performed using the Image J software (NIH, U.S.A.), and data are mean±S.D. of four independent experiments.

**Statistical Analysis** Data were analyzed by SPSS 13.0 Programs for Windows. Different two groups of values were compared using Student’s t test. A value of p<0.05 was considered significant.

**RESULTS**

**High Glucose Induces Epithelial-Mesenchymal Transition and Up-Regulates ILK in HK-2 Cells** Epithelial-mesenchymal transition is of great importance in tissue remodeling, wound healing, and fibrotic disorders in kidney and the contribution of high glucose to this phenomenon in vitro is well established. In this research, the effect of high glucose on the induction of a myofibroblast-like phenotype in normal human renal epithelial cells was determined, so as to prove the existence of EMT. In culture, HK-2 showed basally almost no expression of α-SMA as demonstrated by cytoimmuno-histochemistry. Stimulation with high glucose led to a discernable increase in α-SMA+ cell number. In contrast, HK-2 expressed high level of E-cadherin basically and stimulation with high glucose led to E-cadherin+ cells number decreasing. In the meantime, 24.5 mmol/L mannitol, which has an equal osmotic pressure with 30 mmol/L d-glucose, could not promote the phenomenon mentioned above, which indicated that hypertonic culture environment could not induce phenotypic change of HK-2 (Fig. 2A). Western blot of HK-2 cell lysates and real-time RT-PCR of HK-2 cell mRNA confirmed our observations. HK-2 cultured with high glucose medium showed a significant increase in α-SMA expression and a remarkable decrease in E-cadherin expression, while hypertonic environment could not promote this myofibroblast-like phenotypic change (Figs. 2B, 2C).

As a downstream factor of TGF-β1, ILK was up-regulated when EMT occurred in tubular epithelial cells. This has been reported by various researches18–20 and we got the same results in high glucose-induced EMT. Western blot and real-time RT-PCR showed significantly increased ILK expression in HK-2 cultured in high glucose compared to normal HK-2, while hypertonic medium could not induce this phenomenon (Figs. 3A, 3B).

**ILK-siRNA Inhibits ILK Expression in HK-2 Cells and Attenuates High Glucose-Induced EMT** siRNA experiments were performed to decrease the mRNA expression of ILK in cultured HK-2 cells. As shown in Fig. 4A, a majority of HK-2 cells expressed spots of rhodamine fluorescence,
which confirmed successful transfection. Meanwhile, cultured HK-2 with empty vector (Hiperfect) could not show fluorescence expression. In high glucose stimulated cells, silencing of ILK was obvious at 48 h after transfection and reached 70% to 80% reduction. 48h after transfection, the mRNA expression of ILK in high glucose stimulated cells was significantly below the normal level. The cells transfected with nonsilencing siRNA served as negative controls were also cultured in high glucose medium after transfection for 48 h. ILK expression in these cells showed no significant inhibition compared to high glucose stimulated cells (Fig. 4B). Similar results of protein expression were obtained in Western blot analysis (Fig. 4C).

As shown in Fig. 5, the negative control cells developed similar EMT after 48h high glucose stimulation and demonstrated downregulated E-cadherin and upregulated α-SMA, both in mRNA and protein levels. The HK-2 cells transfected with ILK-specific siRNA demonstrated resistance to high glucose-induced EMT. This was evident because increased levels of E-cadherin, reduced α-SMA expression in cytoimmunostaining, mRNA and protein levels, compared with negative controls (Figs. 5, 7A).

Inhibition of ILK Regulates Abnormal MMP-9/TIMP-1 Ratio in High Glucose-Induced EMT MMP-9 and its tissue inhibitor TIMP-1 expression of HK-2 cells were changed after stimulated with high glucose, whereas the ratio of MMP-9/TIMP-1 showed a trend of up-shifting in these cells, compared with normal HK-2 (Fig. 9). Similarly the negative control cells showed an up-shifted MMP-9/TIMP-1 ratio (Fig. 6). After ILK-siRNA transfection, the ratio of MMP-9 and TIMP-1 was almost the same with normal cells, which demonstrated that inhibition of ILK could regulate the abnormal ratio of MMP-9/TIMP-1 in high glucose-induced EMT (Figs. 6, 9).
Rhein Inhibits High Glucose-Induced EMT and Partially Inhibits ILK Expression

HK-2 cells were incubated in high glucose medium with rhein of different concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL). The results indicated rhein inhibited high glucose-induced EMT in a concentration-dependent way. α-SMA decreasing became more obvious as the medium concentration increased, meanwhile E-cadherin expression enhanced (Figs. 7B, 7C). In addition, 100 µg/mL rhein significantly decreased α-SMA+ cell number and increased the expression of E-cadherin (Fig. 7A).

Suppression of over-expressed ILK stimulated by high glucose was also shown in a concentration-dependent way in HK-2 co-cultured with high glucose and different concentrations of rhein (Fig. 8). Therefore we chose 100 µg/mL rhein as an optimal concentration for further studies.

Abnormal MMP-9/TIMP-1 Ratio was Corrected by Rhein in High Glucose-Induced EMT

As mentioned above, 100 µg/mL rhein was chosen to make further observations. We compared MMP-9/TIMP-1 ratio in high glucose and 100 µg/mL rhein co-cultured HK-2, with specific ILK-siRNA transfected HK-2, which was stimulated by high glucose. Relative quantity determined by Western blot and real-time RT-PCR showed the abnormal MMP-9/TIMP-1 ratio caused by high glucose-induced EMT was corrected by rhein, the effect of which was similar to ILK-siRNA transfection (Fig. 9).

DISCUSSION

Previous studies have confirmed that tubulointerstitial lesions were more important in prognosis of diabetic nephropathy rather than glomerular lesions.21,22) Epithelial-mesenchymal transition was commonly believed as one of the most important factors that could lead to renal fibrosis in diabetic nephropathy. Up-regulated TGF-β1 caused by high glucose...
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was considered an important cause for EMT of renal tubular cells.

As the one and only stimulating factor that involved in this study, high glucose is generally believed to alter ILK expression by regulating its up-stream factor TGF-β. Overexpressed ILK was observed in EMT, which was confirmed in our study. ILK expression was up-regulated in HK-2 cells cultured in high glucose. Moreover, HK-2 cells cultured in high glucose displayed a significant increase in α-SMA+ cell number, while hypertonic environment could not induce phenotypic change of HK-2. Studies have shown that ILK inhibition could attenuate EMT in tubular cells, podocytes and tumor cells. In our study, similar results were noted. Successful ILK inhibition through specific ILK-siRNA reverses tubular cell EMT by decreasing α-SMA expression and increasing E-cadherin expression in HK-2 cells.

The MMP-9/TIMP-1 ratio was altered in EMT in this study. MMP-9, also called 92-kDa type IV collagenase or gelatinase, decomposes specifically type IV collagen in vivo. The tissue inhibitor of metalloproteinase-1 (TIMP-1), a natural inhibitor of MMP-9, normally regulates and counterbalances the proteolytic activity of MMP-9 by binding to both the latent and the active forms of MMPs in a 1 : 1 stoichiometry. MMP-9 expression and activity was increased in EMT of diabetic nephropathy, which would be due to raised TGF-β level induced by high glucose. On the other hand, studies have shown that both the MMP-9 and the TIMP-1 expression increased in the process of renal fibrosis. The conflicting results indicate that further understanding of the MMP-9/TIMP-1 interplay is required. Recently the ratio of MMP-9/TIMP-1 has been considered as a potential index for evaluating MMP-9 activity in the process of renal fibrosis. Under

Fig. 5. ILK-siRNA Attenuates High Glucose-Induced EMT via ILK Inhibition

Since a significant ILK inhibition was created by specific ILK-siRNA, characteristic markers in EMT, α-SMA and E-cadherin, were detected by real-time RT-PCR (A) and Western blot (B). α-SMA was significantly reduced in ILK-siRNA intervened cells, and E-cadherin was up-regulated. **p<0.01.

Fig. 6. Inhibition of ILK Regulates MMP-9/TIMP-1 Ratio in High Glucose-Induced EMT, While Nonsilencing siRNA Can Not

Real-time RT-PCR (A) and Western blot (B) were composed to determine the MMP-9 and TIMP-1 expression. The results shown in (A) are expressed as the mean ratio of each transcript adjusted by the expression of MMP-9 or TIMP-1 mRNA in normal group. MMP-9 or TIMP-1 protein expression are expressed as the ratio of each transcript relative to the protein expression of the housekeeping gene β-tubulin, then the ratio of MMP-9/TIMP-1 was calculated and presented in (B). **p<0.01.
Fig. 7. Rhein Inhibits High Glucose-Induced EMT

HK-2 cells were cultured in high glucose medium with different concentrations of rhein: 25 µg/mL, 50 µg/mL, and 100 µg/mL. Expression of α-SMA and E-cadherin by cytoimmunohistochemistry is shown in (A). Real-time RT-PCR of total cell RNA for α-SMA and E-cadherin is shown in (B) and Western blot of cell lysates for α-SMA and E-cadherin is shown in (C). *p < 0.05; **p < 0.01.
normal conditions, MMP-9 and its tissue inhibitor TIMP-1 are in a state of homeostasis, and the ratio of MMP-9/TIMP-1 fluctuates around 1. In systemic diseases, the MMP-9/TIMP-1 ratio is disturbed. Studies have shown disordered MMP-9/TIMP-1 ratio in sepsis and tumor patients, which was considered to be related with EMT.9,10) Troussard et al. found ILK could enhance the MMP-9 promoter activity.13) In our study, inhibition of ILK turned the MMP-9/TIMP-1 ratio back to normal (around 1), suggesting a possible relationship between ILK and the MMP-9/TIMP-1 ratio. This relationship would be due to the regulating effect of ILK on MMP-9.

Rhein is a precursor component isolated from rhubarb anthraquinone derivatives, and it is a main renoprotective ingredient of rhubarb. Increasing evidence suggests that some Chinese herbal medicines, including rhubarb, have a beneficial role in slowing the progression of chronic kidney disease (CKD).30,31) Experimental evidence suggests that rhein not only antagonizes the effects of TGF-β1 in mesangial cells,15) but also inhibits the hypertrophy of renal tubular epithelial cells and the accumulation of extracellular matrix (ECM) induced by TGF-β1.16) Further studies are needed to explain whether there is a TGF-β1 inhibiting effect of rhein in renal tubular cells, and this may help us to better understand rhein’s renoprotective effects.

To explore the mechanism underlying rhein’s effects, we used specific ILK-siRNA as a positive control. The results showed significant ILK inhibition by ILK-siRNA. Rhein also inhibited high glucose-induced ILK in a concentration-dependent manner. As 100µg/mL of rhein did not show significant toxicity to HK-2 cells in our preliminary study (data not shown), we chose this concentration in the present study, with an aim to investigate whether rhein would also influence MMP-9/TIMP-1 ratio in high glucose-induced EMT. The results indicated that 100µg/mL of rhein could also adjust abnormal MMP-9/TIMP-1 ratio, and acted identically to 5ng/mL of ILK-siRNA.

Our results showed that ILK expression was reduced by rhein. ILK elicits its biologic activities through two principal
properties: as a scaffolding protein and as a protein kinase. As a scaffolding protein, ILK interacts with integrins and numerous intracellular proteins, such as α-parvin and particulary interesting new cysteine–histidine rich protein (PINCH). As a protein kinase, the catalytic activity of ILK renders it to directly phosphorylate several physiologically important downstream effector kinases including Akt (protein kinase B) and glycogen synthase kinase-3β (GSK-3β), leading to the stabilization of β-catenin. All these processes would be potential targets of rhein. Further studies are needed to find out whether the renoprotective effect of rhein was a direct result of rhein or ILK inhibition by rhein. In the present study, our results do not differentiate whether the MMP-9/TIMP-1 regulating role was a direct effect of rhein, or an indirect effect achieved by its ILK inhibiting ability, which partially limits the significance of this study. Nevertheless, our results clearly indicate that rhein could regulate the MMP-9/TIMP-1 ratio, which may be a novel renal protective mechanism of rhein.

In conclusion, specific ILK inhibition could adjust increased MMP-9/TIMP-1 ratio back to near normal level in high glucose-induced EMT in HK-2 cells. Rhein inhibited the overexpression of ILK and inhibits high glucose-induced EMT. As overexpression of ILK is one of the mechanisms underlying renal damage in diabetic nephropathy, the effect of rhein in inhibiting the expression of ILK and regulating the MMP-9/TIMP-1 ratio may partially explain the renoprotective effects of rhein in experimental diabetic nephropathy.

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