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Rhein inhibits renal tubular epithelial cell hypertrophy and extracellular matrix accumulation induced by transforming growth factor β_1

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KEY WORDS rhein; epithelial cells; transforming growth factor beta; extracellular matrix

ABSTRACT

AIM: To investigate the effects of rhein on cell hypertrophy and accumulation of extracellular matrix (ECM) in the renal tubular epithelial cells. **METHODS**: LLC-PK1 cells were incubated with transforming growth factor β_1 (TGFβ_1) 2 μg/L for 24 h to induce cell hypertrophy and production of ECM. To evaluate the effects of rhein on inhibiting the action of TGFβ_1, cell volume, cellular protein level, and [^{3}H]leucine incorporation in LLC-PK1 cells treated with rhein at different concentrations were measured. In addition, the [^{3}H] proline incorporation, level of fibronectin (FN) in supernatant, and mRNA expression of collagen IV and FN were also detected in rhein treated cells. **RESULTS**: The cell volume, cellular protein content, and [^{3}H] leucine incorporation were markedly increased in LLC-PK1 cells after TGFβ_1 stimulation as compared with control ($P < 0.01$), and this TGFβ_1-stimulated cell hypertrophy was ameliorated by rhein. It was observed that TGFβ_1 not only increased the production of FN and [^{3}H]proline incorporation in LLC-PK1 cells ($P < 0.01$), but also enhanced the mRNA expression of collagen IV and FN. Rhein significantly decreased the protein production and mRNA expression of ECM in LLC-PK1 cells stimulated by TGFβ_1. **CONCLUSION**: Rhein can inhibit cell hypertrophy and ECM accumulation in LLC-PK1 cells induced by TGFβ_1, which may partly account for the role of rhein in preventing and retarding the progression of diabetic nephropathy.

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INTRODUCTION

Diabetic nephropathy is a major complication of diabetes mellitus and the leading cause of end-stage renal disease in developed countries. Nephromegaly is a prominent feature of diabetic nephropathy and predominantly reflects increased renal tubule mass, mostly due to cell hypertrophy[1]. It comes to be known that hypertrophy of proximal tubular cells is one of an early hallmarks of diabetic renal involvement[2]. Such cell hypertrophy is characterized by cell cycle arrest in the G_1 phase and is often followed by increased accumulation of extracellular matrix (ECM) components[3]. These alterations contribute to the late abnormalities of end-stage diabetic kidneys such as tubular basement membrane width, tubular atrophy and interstitial fibrosis. Increased evidences from *in vivo* and *in vitro* studies have demonstrated that transforming growth factor β_1 (TGFβ_1) is the key cytokine that mediates the hypertrophy of renal cells[4-9]. Accordingly, halt of cell hypertrophy will be used to prevent or treat diabetic nephropathy effectively.

Rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid) is one of anthraquinone derivatives isolated mainly from Chinese rhubarb, which has being broadly used in the treatment of diabetic nephropathy and other chronic renal diseases in experiment and clinic[10-12], but underlying mechanisms are not completely understood. Here, we examined the effects of rhein on LLC-PK1 cells under TGFβ_1 stimulation.

MATERIALS AND METHODS

Cell cultures and preparation LLC-PK1 cell, which is a porcine kidney cell line analogous to the proximal tubule cell, was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown in 24-well plates in RPMI-1640 (Gibco, Shanghai, China) with glucose 5.5 mmol/

L and supplemented with 10 % fetal cow serum (FCS) (Sijiqing, Hangzhou, China). At confluence, medium was replaced with RPMI-1640 contained 1 % FCS for 24 h. Cells were cultured for an additional 24 h with or without TGFβ_1 (2 μg/L) (Sigma, St Lousis, USA) and rhein (Sigma) at different concentrations (0, 3.125, 12.25, 25 mg/L). Cells were washed three times before used for experiments. Every experiment was divided into five groups (one TGFβ_1-stimulated, three treated with rhein, and one control) and each group was quadruplicated.

Cell sizes Cells were digested with 0.25 % trypsin/0.02 % ethylene diamine tetraacetic acid (EDTA) and stopped with FCS. Single cell suspensions at a density of 1×10^9/L in PBS were subjected to flow cytometry (Epics XL, Coulter, Brea, CA, USA). Relative cell sizes for 10 000 cells in each sample were determined by quantification of forward light scattering[13].

Cellular protein content Cells were solubilized with NaOH 1 mol/L. Protein content was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratatories, Richmond, CA, USA) with Bovine Serum Albumin as the standards.

[^{3}H]Leucine incorporation [^{3}H]leucine (0.925 GBq/L, Amesham, Buckinghamshire, England) was added meanwhile when cells were incubated with TGFβ_1 and rhein. Cells were washed three times with ice-cold PBS before solubilization with NaOH 1 mol/L, and counted in liquid scintillation β counter (LS 6500, Beckmen, Brea, CA, USA).

[^{3}H]Proline incorporation study Proline is one of the major constituents of collagen, and it preferentially incorporates into collagens type I and IV. For these experiments, cells were plated into 24-well cell culture plates in RPMI-1640 containing glucose 5.5 mmol/L and 10 % FCS. At confluence, medium was changed to RPMI-1640 containing glucose 5.5 mmol/L with 1 % FCS without proline for 24 h. TGFβ_1, [^{3}H]proline (0.925 GBq/L, AAmesham, Buckinghamshire, England) and rhein were added to each well before additional 24 h incubation. Cells were washed and dissolved with NaOH 1 mol/L, and counted in liquid scintillation β counter.

ELISA Production of fibronectin (FN) was quantified by enzyme-linked immunosorbent assay (ELISA) as the total amount accumulated in the cell culture medium during 24 h of incubation. Samples of culture medium (100 μL) were added in quadruplicate to wells of a 96-well ELASA plate and incubated for 18 h at 4 ℃. Purified human FN (Sigma), diluted in the same medium, was added (0.5 ng – 1 μg per well) to each assay plate as standards. At the end of this incubation period, the medium was removed, and the unoccupied sites blocked by a 2-h treatment with 5 % nonfat dry milk in PBS containing 0.05 % Tween. Wells were then washed and incubated for 3 h with 100 μL of rabbit antibody for human FN (Gibco, Shanghai, China). After extensive washing of the wells, an enzyme-linked alkaline phosphatase-labeled goat anti-rabbit IgG (Dako, Glostrup, Denmark) was added and the plates were incubated for an additional 3-h period, then followed by extensive washing and the addition of a phosphatase substrate solution. *OD* intensity at 410 nm wavelengh was measured.

RT-PCR The mRNA expression of collagen IV and FN was measured by RT-PCR. Total RNA in cells was extracted by Trizol Reagent kit (Biobasic, Scarborough Ontario, Canada). Pooled RNA was reverse transcribed, and the resulting cDNA was amplified with a commercial kit (Promega, Wisconsin, USA) using specific primers designed according to the published sequences[14]. The house-keeping gene of β-actin was co-amplified with collagen IV or FN as intra-control. Primer sequences are detailed in Tab 1.

PCR conditions were 35 cycles with denaturation at 94 ℃ (1 min), annealing at 54 ℃ (1 min) and extension at 72 ℃ (1 min) for collagen IV cDNA, whereas FN PCR annealing was done at 55 ℃ and 28 cycles. Aliquots of PCR products were run on 2 % agarose gels, and the relative densities of the bands (type IV collagen or FN over β-actin) were calculated.

Statistical analyses All results were expressed as $\bar{x} \pm s$. Student's t-test was used when compare was performed during two groups; F test for multiple comparisons.

RESULTS

Rhein inhibited LLC-PK1 cell hypertrophy

Cell hypertrophy was evaluated by cellular protein levels, [^{3}H]leucine incorporation, and relative cell sizes. As shown in Tab 2, after LLC-PK1 was incubated with TGFβ_1 for 24 h, cells sizes markedly increased [(536 ± 29) *vs* (466 ± 28) in control group, $P < 0.01$]. Similarly, cellular protein content and [^{3}H]leucine

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Tab 1. Primer sequences and their amplified size.

	Primer sequence		Size
Human collagen IV	Sense	5′-AGC ACA ATG CCC TTC-3′	437 bp
	Antisense	5′-TTG AAC ATC TCG CTC-3′	
Human FN	Sense	5′-GCA GCC CAC AGT GGA GTA-TGT-3′	226 bp
	Antisense	5′-TTC TTT CAT TGG TCC GGT CTT-3′	
β-actin	Sense	5′-AAC GCA GCT CAG TAA CAG-TC-3′	287 bp
	Antisense	5′-ATC CGT AAA GAC CTC TAT GC-3′	

incorporation were also increased as compared with control cells, rising 2 times for cellular protein [(374 ± 77) *vs* (178 ± 28) pg/cell, $P < 0.01$, $n = 4$] and 3 times for [^{3}H]leucine incorporation [(0.349 ± 0.049) *vs* (0.118 ± 0.012) Bq/cell, $P < 0.01$, $n = 4$] respectively (Tab 2). After rhein (25 mg/L) treatment for 24 h, cell sizes significantly decreased (482 ± 36, $P < 0.05$ *vs* TGFβ$_1$-treated group). In addition, rhein also diminished an increase in cellular protein content and [^{3}H]leucine incorporation induced by TGFβ$_1$ in a dose manner (Tab 2).

Rhein inhibited accumulation of extracellular matrix in LLC-PK1 cells TGFβ$_1$ induced a significant increase in [^{3}H]proline incorporation and FN secretion as compared with control cells after LLC-PK1 cells stimulated with TGFβ$_1$ for 24 h [(1.39 ± 0.13) *vs* (0.48 ± 0.07) Bq/cell, (10.7 ± 1.4) *vs* (21.3 ± 0.8) mg/g protein, respectively, $P < 0.01$, $n = 4$] as shown in Tab 2. TGFβ$_1$-stiminated [^{3}H]proline incorporation was 184 % higher than control cells, and FN secretion 199 %. The results from RT-PCR demonstrated that TGFβ$_1$ induced a significant increase in mRNA expression of FN and collagen IV in LLC-PK1 cells as compared with the control (relative density was 0.74 *vs* 0.43 and 0.45 *vs* 0.20 respectively, $P < 0.01$, Tab 3).

Rhein (25 mg/L) treatment diminished TGFβ1-stiminated increase in [^{3}H]proline incorporation and FN secretion [(1.12 ± 0.07) Bq/cell, (9.8 ± 0.6) mg/g protein, respectively, $P < 0.01$ *vs* TGFβ$_1$-treated group] (Tab 2), as well as mRNA expression of FN and collagen IV in LLC-PK1 cells (relative density was 0.58 and 0.26 respectively, $P < 0.05$ *vs* TGFβ$_1$-treated group) (Tab 3).

DISCUSSION

Our previous work demonstrated that rhein was effective to treat diabetic nephropathy. It has been proved that rhein can eliminate renal hypertrophy, reduce the expansion of the glomerular mesangium, inhibit the development of glomerulosclerosis and tubulointerstitial fibrosis[10-12]. The present study further shows that rhein alleviates cell hypertrophy induced by TGFβ$_1$, as well as ECM accumulation both in the mRNA expression and protein synthesis. This may be one of the mechanisms of rhein improving the abnormalities of renal lesions and delaying the progression of diabetic nephropathy.

TGFβ$_1$ plays a central role in the development of renal hypertrophy and accumulation of ECM components

Tab 2. Effects of Rhein on TGFβ$_1$-induced phenotypic changes in LLC-PK1 cells. $n = 4$ wells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Control group. $^eP < 0.05$, $^fP < 0.01$ *vs* TGFβ$_1$ group.

	Relative cell volume	[^{3}H]Leucine incorporation/ Bq·cell^{-1}	Cellular protein content/ pg·cell^{-1}	[^{3}H]Proline incorporation/ Bq·cell^{-1}	FN concentration/ mg·g^{-1} protein
Control	466 ± 28	0.118 ± 0.012	178 ± 28	0.48 ± 0.07	10.7 ± 1.4
TGFβ$_1$ (2 μg/L)	536 ± 29^c	0.349 ± 0.049^c	374 ± 77^c	1.39 ± 0.13^c	21.3 ± 0.8^c
TGFβ$_1$ + rhein (3.125 mg/L)	510 ± 19	0.297 ± 0.026	277 ± 40^e	1.25 ± 0.21	20.5 ± 2.3
TGFβ$_1$ + rhein (12.5 mg/L)	499 ± 31	0.270 ± 0.019^e	195 ± 16^f	1.13 ± 0.18^e	14.8 ± 3.7^e
TGFβ$_1$ + rhein (25 mg/L)	482 ± 36^e	0.192 ± 0.017^f	189 ± 27^f	1.12 ± 0.07^f	9.8 ± 0.6^f

Tab 3. The level of mRNA expression of collagen IV and FN, showed by the relative densities of the bands of PCR product (collagen IV or FN over β-actin) on 2 % agarose gels. $n = 4$ wells. $x \pm s$. $^cP < 0.01$ *vs* Control group. $^eP < 0.05$ *vs* TGFβ1 group.

	Type IV collagen	FN
Control	0.200 ± 0.020	0.43 ± 0.04
$TGF\beta_1$ (2 μg/L)	0.45 ± 0.07^c	0.74 ± 0.08^c
$TGF\beta_1$ + rhein 25 mg/L	0.26 ± 0.05^e	0.58 ± 0.06^e

in diabetes mellitus through an autocrine or paracrine pathway[15]. In our experiment, when stimulated by $TGF\beta_1$ (2 μg/L) for 24 h, LLC-PK1 cells became hypertrophy characterized by an increase in cell volume, cellular protein synthesis, and content. The molecular mechanisms of $TGF\beta_1$-induced cell hypertrophy involve preventing activation of cyclin-dependent kinase 2 (CDK2)/cyclin E kinase, increasing dephosphorylation of retinoblastoma protein (pRB), and decreasing phosphorylation and ubiquitination of CDK-inhibitors (such as $p27^{Kipl}$ and p21), consequentially leading to a G_0/G_1 phase arrest[16-18]. While, which step of cell hypertrophy was interfered by rhein is still uncertain.

The results from our study also showed that $TGF\beta_1$ stimulation can induce ECM accumulation in LLC-PK1 cells by stimulating synthesis and expression of collagens and FN. Rhein not only inhibited the mRNA expression of type IV collagen and FN, but also reduced the synthesis of ECM, thus rhein acts at both translation and transcription level. It has been reported that $TGF\beta_1$ stimulates transcription and production of ECM, and also inhibits matrix degradation by inhibiting the secretion of proteases as well as increasing the synthesis of protease inhibitors[19]. Whether or not rhein also works on the step of ECM degradation need to be explored.

Our works reported previously, rhein inhibited the uptake of glucose stimulated by $TGF\beta_1$ in mesangial cell, and cell hypertrophy and ECM accumulation in mesangial cell transfected with glucose transporter 1 gene[20,21]. Therefore, in addition of interfering with the downstream of TGFβ signal pathway, rhein may also impose on the upstream of TGFβ signal pathway, such as at the level of endogenous TGFβ synthesis and cell glucose uptake under high ambient glucose.

In conclusion, we first demonstrated that rhein effected on the hypertrophy and ECM accumulation in renal tubular cells stimulated by $TGF\beta_1$, and this effect of rhein may contribute to its therapeutic mechanisms of diabetic nephropathy. Further studies are needed to explore the molecular mechanisms of rhein and its clinical implications.

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大黄酸抑制转化生长因子 β_1 诱导的肾小管上皮细胞肥大及细胞外基质积聚

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关键词：大黄酸；上皮细胞；转化生长因子 β；细胞外基质

目的：观察大黄酸对 $TGF\beta_1$ 诱导的肾近端小管上皮细胞肥大及细胞外基质的影响. **方法**：在体外以 $TGF\beta_1$(2 μg/L)刺激 LLC-PK1 细胞，诱导细胞肥大和细胞外基质合成的增加. 同时，以不同浓度的大黄酸处理细胞，检测细胞体积、蛋白质含量以及 $[^3H]$亮氨酸掺入以观察细胞肥大的变化. 此外，检测细胞培养上清液中的纤维素增生(FN)含量. $[^3H]$脯氨酸掺入以及细胞胶原 IV 及 FN mRNA 的表达以观察大黄酸对细胞外基质的影响. **结果**：$TGF\beta_1$(2 μg/L)刺激可以导致 LLC-PK1 细胞出现细胞肥大，表现为细胞体积、细胞内蛋白量及 $[^3H]$亮氨酸掺入量明显增加. 大黄酸治疗后细胞体积及细胞内蛋白量降低. $TGF\beta_1$ 也能明显增加 LLC-PK1 细胞 $[^3H]$脯氨酸掺入量，培养上清液中 FN 含量，以及细胞内胶原 IV 和 FN mRNA 的表达. 大黄酸则能抑制上述细胞外基质合成的增加，明显降低细胞内胶原 IV 和 FN mRNA 表达水平. **结论**：大黄酸可以逆转 $TGF\beta_1$ 诱导的近端肾小管上皮细胞肥大，抑制 $TGF\beta_1$ 刺激的细胞外基质合成. 这可能是大黄酸预防或改善糖尿病肾脏病变、延缓糖尿病肾病进展的作用机制之一.

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