Original Article
Therapeutic effect of rhein on high glucose-induced podocyte injury via GSK3β-wnt/β-Catenin-PPARγ signaling pathway

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Abstract: This study investigated the therapeutic effects of rhein (RH) as a traditional Chinese herbal medication on human podocytes in high glucose (HG) condition and the molecular mechanism involved. Differentiated podocytes were divided into: the normal glucose group (NG: 5.5 mM of glucose), the high glucose groups (HG: 30 mM of glucose), and the osmotic control group (NG+M: 5.5 mM of glucose and 24.5 mM of mannitol). Those groups were cultured for a range of times. RH (25 µg/ml) and GSK-3β inhibitor (LiCl) were used to treat podocytes in HG condition. GSK-3β, β-catenin and PPARγ were assessed with RT-PCR, western blot. HG promotes GSK3β phosphorylation and activates wnt/β-catenin signaling in cultured podocytes. Meanwhile PPARγ mRNA and protein expressions are significantly decreased in podocytes exposed to HG which also induced reduction in podocin and nephrin expression indicating podocytes injury. RH down-regulating GSK-3β, β-catenin expression similar to the effect of LiCl and strengthened PPARγ expression to improve podocytes structural and functional abnormalities. These findings suggest that RH repair high glucose (HG)-induced podocyte injury through the GSK3β-wnt/β-Catenin/PPARγ pathway and thus exert its therapeutic effect in diabetic nephropathy (DN).

Keywords: Rhein, GSK3β, β-Catenin, PPARγ, high glucose, podocyte

Introduction

As one of the most serious diabetic microvascular diseases, diabetic nephropathy (DN) has become the most important cause of end-stage renal disease worldwide [1, 2]. In its early stages, podocyte number is markedly reduced, which in turn leads to injury to the integrity of the glomerular filtration barrier. Podocyte injury has been identified as a key role in processes of diabetic nephropathy, which is characterized by decreased expression of slit diaphragm-associated proteins, nephrin and podocin and increased albumin filtration [3-5]. Thus, high glucose (HG)-induced podocyte structure and function changes may provide a promising therapeutic target for treatment of diabetic nephropathy.

Rhein (RH) is a traditional Chinese herbal medication that has been used for more than 2,000 years. Numerous studies demonstrate that RH has the ability to improve glucose metabolism disorders in diabetic mice, and its effect on reducing blood glucose level was even stronger than PPARγ agonist rosiglitazone and benazepril [6-9]. So RH is assumed to be a novel therapeutic agent for hyperglycemia treatment. Moreover, many in vivo and in vitro experiments suggest that RH mediates multiple molecular targets, particularly oxidative stress and inflammation, which makes RH a potential candidate for the therapy of DN. In our previous study [10], we have demonstrated the relationship between wnt/β-catenin signaling pathway and kidney impairment in diabetic nephropathy (DN) mice as well as the renoprotective effect of RH. Furthermore, the involvement of GSK3β and PPARγ on podocyte injury of DN is likely contributing to the protection of podocyte and against albuminuria by RH. Therefore, we initiated this study to examine the effects of RH on HG-induced human podocyte injury and the molecular mechanism involved. Our results in vitro data shed light on DN pathogenesis and new ways to prevent or treat it.
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Materials and methods

Podocyte culture

Conditionally immortalised human podocytes were derived and cultured as described previously [11]. The cells were grown at the permissive temperature of 33°C in the presence of ITS in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml) (Gibco, USA) to promote cell propagation with a cobblestone phenotype (undifferentiated) and then moved to the nonpermissive culture temperature of 37°C under 5% CO₂ and 95% air for 10 to 20 days to allow them to differentiate. Differentiated podocytes were used for the proposed experiments.

For a proportion of experiments, differentiated podocytes were divided into three groups: the normal glucose group (NG: 5.5 mM of glucose), the high glucose groups (HG: 30 mM of glucose), and the osmotic control group (NG+M: 5.5 mM of glucose and 24.5 mM of mannitol (sigma, USA). And those groups were cultured for a range of times (0 h, 24 h, 48 h). For some experiments, podocytes were also treated with RH (25 µg/ml [10], kindly provided by Zhihong Liu, Research Institute of Nephrology, Jingling Hospital, Nanjing University School of Medicine, Nanjing, PR China) and water-soluble LiCl (sigma, USA), a GSK-3β inhibitor, a final concentration of 10 mM of LiCl.

Cell viability assay

Cell viability was assessed using the colorimetric reagent, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT) (Sigma, USA). Briefly, cells were seeded in 96-well plates, 1×10⁴ cells in 200 µl medium per well, and cultured for 24 h for cell stabilization. Cultures were incubated with NG, NG+M, HG for 0 h, 12 h, 24, 48 h, 72 h, 96 h. Then, cultures were incubated with MTT (20 µl, 5 mg/ml) for 4 h at 37°C. Medium was removed and 150 µl DMSO was added to each well to dissolve the formazan product and finally, formazan absorbance was assessed using a microplate reader (at 490 nm). Each group of cells was set up in six parallel wells, and experiments were repeated three times. Results are presented as mean ± SD.

Caspase-3/9 activity assay

The activity of caspase-3/9 were determined using the caspase-3 and Caspase-9 activity kits (Beyotime Biotechnology, Nantong, China) according to the manufacturer’s instruction 6 h after microwave exposure. Briefly, cells were lysed, and then the supernatant was mixed with buffer containing the substrate peptides for caspase-3/9 attached to p-nitroanilide (pNA). After incubation at 37°C for 2 h, samples were measured with an ELISA reader at 405 nm. Caspase-3/9 activity was expressed as percentage compared to control.

Cell morphology observation

Four groups of podocytes were created: NG, HG for 24 h, and HG for 48 h and 25 µg/ml RH for 48 h. Those podocytes were observed under light microscopy (Olympus1*71, China) at 10×10 magnification.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from cultured podocytes was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol as described by the manufacturer. Aliquots of total RNA (1 µg) from each sample were reverse-transcribed into cDNA according to the instructions of the first strand cDNA synthesis kit manufacturer (Bio-Rad, USA). Equal amounts of the reverse transcriptional products were subjected to PCR amplification using SYBR Green as the fluorescence indicator on a Bio-Rad iCycler system (Bio-Rad, USA). The mRNA levels of target genes were normalized to the β-actin mRNA levels. The primers used in this study were synthesized by Operon (Generay biotech, China) and the sequences were: for PPARγ, sense CGCTGATGCACTGCTATGA, anti-sense AGAGGTTCACAGAGCTGATTCC; for Wnt3a, sense CGCGCTCTCCAGGCACTCT, antisense TCCGTTGCACACACCCTGT; for β-catenin, sense AGCCATCAGACACTGCATA, antisense GCTTGCTCTTGTGACATGGCA; for nephrin, sense CCCAGGTCACAGACACAAACATCTACA and for β-actin, sense TCAGTCCTCGGTCGTC, antisense GCCCTGTACCCACATAGGA.

Western blot analysis

Total proteins were obtained from each group using the RIPA lysis buffer (KeyGEN Company, China). Proteins were quantified using a BCA protein assay kit (KeyGEN Company, China).
Figure 1. HG induced injury to podocytes. Podocytes were treated with different conditions (NG, NG+M, HG) for a range of periods of time. NG: the normal glucose group (5.5 mM of glucose), NG+M: the osmotic control group (5.5 mM of glucose and 24.5 mM of mannitol), HG: the high glucose groups (30 mM of glucose). Cell viability was assessed after incubation in (A). The optical density (OD) represents the viability of podocytes. Caspase-3/9 activity (B) was detected using assay kits with an ELISA reader. The data was from three independent experiments (n=3). (C) Shows western blot analysis of nephrin and podocin protein expression in response to different conditions. Podocytes
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Proteins (15-20 μg) were separated using 10% SDS-PAGE, and were transferred on PVDF membranes (Roche, Germany). Non-specific sites were blocked using 5% powdered milk diluted in TBS with 0.05% Tween 20 (TBST) for 1 h. Primary antibody was incubated overnight at 4°C. After washing the membranes, the secondary antibody was incubated at 37°C for 2 h. An ECL chemiluminescence reagent kit (Millipore Company, USA) was used to reveal the bands. Blots were analyzed using the ImageJ software. Antibodies were: mouse polyclonal antibody against GAPDH (Wuhan Boster company, China), rabbit polyclonal antibody against GSK3β (phospho S9), rabbit monoclonal [Y174] antibody to GSK3β, antibody against wnt 3a (Abcam, USA), anti-β-actin, anti-nephrin, anti-podocin, anti-GAPDH (Santa Cruz Biotechnology, USA), and horse-radish peroxidase-labeled goat anti-mouse secondary antibody (Wuhan Boster Company, China).

Statistical analysis

All data were obtained from three independent experiments, and are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SAS 6.12 software (SAS Institute Inc., USA). Data are given as the mean ± SD analysed using one-way ANOVA with Dunnett’s significance correction test. Differences were considered significant at P < 0.05.

Results

Cultured immortalized human podocytes were treated with high glucose (HG) for various periods of time. Viability of cells was examined using the MTT assay and results showed that HG reduced cell viability (P < 0.05) in a time manner compared to control cells and leveled off after 48 hours (Figure 1A). Moreover, activity of caspase-3/9 was significantly increased in podocytes in HG condition at 48 hours while the activity was significantly inhibited by the effect of RH in those HG-treated cells (Figure 1B). Western blot and Real time PCR results
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Figure 3. Activation of wnt/β-catenin signaling by HG is GSK3β dependent in podocytes. Podocytes were incubated with NG or NG+M or HG for 0 h, 24 h and 48 h respectively. (A) Protein levels of wnt3a and β-catenin first analysed to confirm effective activation of Wnt/β-catenin pathway. Total GSK3β and its phosphorylated form (p-GSK3β) were revealed by specific antibodies in a western blot analysis. The intensities of the protein bands were quantified and calculated by a densitometer shown in (B-D) respectively. Gene expression levels wnt3a and β-catenin were analyzed by RT-PCR, as shown in (E and F). Each example shown is representative of 3 independent experiments. Values are means ± SE of 3 experiments for each condition determined from densitometry relative to β-actin. *P < 0.05 compared to the control group (NG, NG+M, HG for 0 h). Δ,*P < 0.05 HG compared to the NG, NG+M for 48 h.

HG promotes GSK3β phosphorylation and activates wnt/β-catenin signaling in cultured podocytes

To ascertain activation status of wnt signaling in pathogenesis of early DN injury, activation of the canonical wnt signaling pathway was detected by western blot and RT-PCR. As shown in Figure 3, protein and mRNA expression of Wnt3a was induced significantly by 48 h after HG treatment. Since activation of the canonical Wnt signaling pathway is known to dephosphorylation and nuclear translocation of β-catenin, we then analyzed its regulation in HG induced
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Inhibition of podocyte injury by GSK3β and PPARγ

Western blot and RT-PCR analyses revealed a dramatic increase in podocyte β-catenin protein by 48 h after HG treatment.

As GSK3β phosphorylates β-catenin, we therefore next investigated whether the activation of wnt/β-catenin signaling mediated by HG involves GSK3β phosphorylation in podocytes. Podocytes were treated with different conditions for 24-28 h, lysed for immunoblotting using anti-phospho-GSK3β (Ser-9) and GSK3β antibodies. As shown in Figure 3, HG significantly enhanced the phosphorylation levels of GSK3β Ser9 at 48 hours. These data suggest that the activation of wnt/β-catenin signaling induced by HG is dependent on GSK3β activity.

Inhibition of PPARγ induced by HG in podocytes

To assess the potential role of PPARγ in podocyte injury of HG, expression of PPARγ was examined in podocytes with different conditions (NG, NG+M, HG) for a range of periods of time. As shown in Figure 4, expression of PPARγ protein and mRNA was examined. PPARγ Protein expression in podocytes started to decrease at 24 h, and down at the bottom by 48 h.

Effects of RH on HG induced podocyte injury were detected. Cells incubated with NG, NG+M, HG for 48 h and HG combined with RH (25 µg/ml) for 48 h. Dosage of RH used in our experiments was selected according to previous studies [10, 11]. As shown in Figure 5, the levels of PPARγ protein expression were significantly increased with RH treatment as compared in HG induced podocyte damage. The protein in the wnt/β-catenin signaling pathway, including wnt3a, β-catenin, were significantly down-regulated by RH treatment (Figure 5). Furthermore, RH treatment efficiently suppressed p-GSK3β, while the total GSK3β showed no difference.

The effects of GSK-3β inhibitor (LiCl) on the expressions of GSK3β, β-catenin and PPARγ in the presence or absence of RH

To evaluate the essential role of GSK3β in relation with the therapeutic effect of RH on HG-induced podocyte injury, GSK-3β inhibitor (LiCl) was incubated with podocytes in HG group for 36 h, with or without the treatment of RH. The protein expression of GSK3β and β-catenin were effectively decreased by the LiCl or RH, RH upregulates PPARγ expression via suppressing GSK3β-Wnt/β-catenin signaling pathway in HG induced podocytes

Figure 4. Inhibition of PPARγ induced by HG in podocytes. Podocytes were incubated with NG or NG+M or HG for 0 h, 24 h and 48 h respectively. Podocyte lysate proteins (60 µg) were separated by SDS-PAGE, immunoblotted using anti-PPARγ and anti-actin antibodies. A. Western blot assay of PPARγ. B. Corresponding histogram of PPARγ protein expression. C. Real time PCR analysis of PPARγ mRNA expression. Data representative of three independent experiments. Values are means ± SE of 3 experiments for each condition determined from densitometry relative to β-actin. *P < 0.05 compared to the control group (NG, NG+M, HG for 0 h). Δ,*P < 0.05 HG compared to the NG, NG+M for 48 h.
and RH functioned as synergistic effects to LiCl. In addition, RH treatment up-regulated the protein expression of the adipogenic transcription factors PPARγ, the same function as LiCl (Figure 6). The results confirm that GSK3β is involved in the anti-HG effects of RH in podocytes.

Discussion

In the present study, we not only confirmed the role of RH in HG induced podocytes structure and function changes, but also explored the mechanisms mediating this pathological process. Firstly, we demonstrated that HG promotes GSK3β phosphorylation and activates wnt/β-catenin signaling in cultured podocytes. Meanwhile PPARγ mRNA and protein expressions are significantly decreased in podocytes exposed to HG which also induced reduction in podocin and nephrin expression indicating podocytes injury. RH attenuated HG induced GSK3β-Wnt/β-catenin signaling pathway and strengthened PPARγ expression to improve podocytes structural and functional abnormalities. Furthermore, to confirm the mechanism underlying RH’s effects, we used GSK-3β inhibitor (LiCl) as a positive control. The results showed RH exert synergistic effect with LiCl in HG-induced podocytes injury.

At first, we confirmed that HG-induced podocyte injury is mediated by the serine/threonine protein kinase GSK-3β phosphorylation-Wnt/β-catenin signaling pathway. GSK3β is a protein kinase involved in a variety of signaling pathways including the wnt pathways. Wu and Pan reported that GSK3β phosphorylates and...
promotes the degradation of β-catenin in quiescent cells [12]. Mao et al, Fukumoto et al and pan showed that phosphorylation of GSK-3β leads to inhibition of GSK-3β thus resulting in the stimulation of the wnt pathway [13, 14]. In addition, the rationale for this process is supported by the fact that GSK-3β participates in podocytes’ EMT in the HG environment [15]. Down-regulating GSK-3β expression decreased β-catenin and Snail expression and reversed HG-induced podocytes EMT. Our data clearly showed increased expression of Wnt3a and β-catenin in podocytes exposed to HG, an effect that was attenuated by downregulation of GSK3β expression. To be noted, GSK-3β inhibitor doesn’t decrease β-catenin expression as significant as the effect of RH in HG condition, which coincides with Guo’s report that β-catenin expression is not entirely modulated by GSK-3β through the classical wnt/β-catenin pathway, but even more complex regulatory mechanism are involved [16].

Next, we demonstrated how activation of Wnt3a-β-catenin signal pathway caused podocytes injury. PPARγ, one of downstream target genes of Wnt/β-catenin signaling pathway, is a promising target in the therapy of DN [17]. A number of in vivo and in vitro studies demonstrated that PPARγ benefits all kinds of kidney cells including the glomerular mesangial cells, endothelial cells, podocytes, and tubular epithelial cells under the diabetic condition [18] with more research emphasis on the podocytes.
Many basic studies performed in diabetic animals and in vitro cells also proved the beneficial action of PPARγ in diabetic kidney disease [23-25]. Moreover, numerous reports including a meta-analysis of 15 original clinical studies involving 2860 patients convincingly demonstrated the significant efficacy of PPARγ agonists on diabetic proteinuria [26]. Among the research about the effect of PPARγ agonists on GSK-3β and β-catenin activation, troglitazone stimulates the degradation of β-catenin, depending on GSK-3β activity during adipogenesis [27]. In addition, the antiproliferative effect of rosiglitazone is mediated by inhibition of ERK and activation of GSK-3β [28]. Lee reported that PPARγ agonist ameliorates HG-induced EMT through PI3K/Akt, GSK-3, Snai1, and β-catenin in renal proximal tubule cells [29]. In our study, we observed that down regulation of GSK3β inhibited HG induced PPARγ mRNA and protein expression. These results suggested that PPARγ gene is one of target effectors of GSK3β-Wnt/β-catenin signaling pathway, which contributes to podocytes injury and dysfunction on exposure to HG. In addition, it is reasonable that activation of PPARγ leads to the renal-protective effect in the HG condition.

Although previous in vitro and animal studies have confirmed the biological activity and therapeutic effects of RH in DN, the exact mechanism of RH working on podocyte signal pathways to protect HG-induced podocytes damage is still unclear. RH, an anthraquinone compound isolated from rhubarb, a traditional Chinese medicinal plant, has been proved effective in treatment of experimental DN [30]. It improves cell metabolism through glucose transporter 1 and decreases mesangial cell hypertrophy and extracellular matrix synthesis. Treatment of db/db diabetic mice using RH has been shown to decrease the levels of extracellular matrix and expression of transforming growth factor-β1 and fibronectin in the kidney [31, 32]. In addition, combination therapy with RH and an angiotensin-converting enzyme inhibitor in db/db mice provided additional renal protection that was more than either therapy by itself, as reflected in the reduction of urinary albumin excretion and improvement of renal function and histology [33]. Understanding the mechanism of RH is essential in establishing novel therapeutic strategies for the prevention or arrest of progressive DN. In strategies to disrupt any one of the HG steps, RH has shown promise. Currently, there are very few reports on the relationship of GSK3β, wnt/β-Catenin, PPARγ and RH in podocytes in literature. For the first time, we elaborate on the therapeutic effect of RH on HG-induced podocyte injury via GSK3β-wnt/β-Catenin-PPARγ signaling Pathway. Our results suggest that RH serves as a GSK3β antagonist to activate GSK3β phosphorylation, leading to accumulated downstream wnt3a and β-catenin decreased and the target PPARγ increased in HG destroyed podocytes. Moreover, the effect of RH is similar to PPARγ agonists which had been thought to be a promising candidate for strengthening the therapy of DN. More detailed mechanistic studies and clinical studies are needed to elucidate the relationship of RH and those proven PPARγ agonists in DN.

In conclusion, our results identify RH exert therapeutic effect by activating the GSK3β-dependent wnt signaling pathway, resulting in the phosphorylation of GSK3β and the accumulation of β-catenin, which subsequently activated the downstream wnt-targeted PPARγ elevation for renoprotection. Thus, results from the present study provide refinements about DN pathogenesis, as well as clues for new treatment targets.

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Disclosure of conflict of interest

None.

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