BMP-7 enhances SnoN mRNA expression in renal tubular epithelial cells under high-glucose conditions

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Abstract. The present study aimed to identify any association between bone morphogenetic protein-7 (BMP-7) and the expression of the transcriptional co-repressor Ski-related novel protein N (SnoN), in addition to alterations in tubulointerstitial fibrosis, during the development and progression of diabetic nephropathy (DN). Streptozotocin was injected into the tail veins of 20 healthy and specific pathogen-free male Sprague-Dawley rats. The rats were sacrificed to detect the appropriate biochemical indicators of renal pathological alterations following 24 weeks. Then, various doses of human recombinant BMP-7 were added to high glucose-cultured NRK-52E cells. Immunohistochemistry, immunofluorescence staining and western blotting were used to determine the expression of SnoN, BMP-7, Smad ubiquitin regulatory factor (Smurf)2, Arkadia, E-cadherin, α-smooth muscle actin and Collagen III. Reverse transcription-quantitative polymerase chain reaction was used to detect SnoN mRNA expression. With the progression of DN, the expression of BMP-7 in rat renal tissue was downregulated, whereas the expression of Smurf2 and Arkadia increased. Furthermore, the expression of SnoN mRNA increased however the expression of SnoN protein decreased, accompanied by renal tubular epithelial cell mesenchymal transition, extracellular matrix (ECM) deposition and severe renal function disorder. The exogenous recombinant human BMP-7 alleviated high glucose-induced phenotypic transformation and ECM synthesis of NRK-52E in vitro and upregulated SnoN transcription and protein expression, however no effect was observed on the expression of Smurf2 and Arkadia. BMP-7 may ameliorate DN and renal fibrosis via increasing the expression of SnoN mRNA and protein in renal tubular epithelial cells, rather than directly inhibiting the degradation of SnoN by E3 ubiquitin ligase.

Introduction

The expression of transforming growth factor-β1 (TGF-β1) is upregulated in the course of multiple renal fibrosis and acts as an important cytokine in the renal fibrosis disease process (1-4). It induces the transition of renal tubular epithelial cells (RTECs) to mesenchymal cells and finally into myofibroblasts, increasing the deposition of extracellular matrix (ECM) (5,6). In recent years, many studies have demonstrated that transcriptional corepressor Ski-related novel protein N (SnoN), an important negative regulatory factor, inhibits the transcription activation of Smads-mediated TGF-1 target gene (7,8). It is well accepted that the diminution of SnoN protein is involved in the development and progression of renal fibrosis, and the downregulation of SnoN protein facilitates renal fibrotic lesions while the upregulation of which slows down the development of renal fibrosis (1,8).

Studies found that bone morphogenetic protein-7 (BMP-7), a member of TGF-β1 superfamily, is highly expressed in kidney and plays a renal protective effect in maintaining the normal development of kidney, reversing epithelial-to-mesenchymal transition (EMT) (3,9), and in restricting the deposition of ECM (4,10), but the exact mechanism is still unclear. Recent studies have shown that BMP-7 may play a role in anti-renal fibrosis by upregulating the protein expression of SnoN, which is subject to regulation strictly in activation of both transcription and protein stability (4,11,12). Therefore, this study aimed to explore the associations between and among BMP-7, the expression of transcriptional corepressor SnoN and the changes of tubulointerstitial fibrosis during the development and progression of diabetic nephropathy (DN). Furthermore, this study also aimed to demonstrate BMP-7 may have effect on the target of anti-fibrotic and potential mechanism by observing its influence on the expression of SnoN and fibrosis of RTECs while exogenous rhBMP-7 was added to RTECs cultured under hyperglycemic conditions.

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Materials and methods

Animal model. A total of 20 healthy and specific pathogen-free male Sprague-Dawley rats (weight: 180±20 g) were provided by Beijing HFK Bioscience Co., Ltd. (Beijing, China), and housed in the animal center of Guiyang Medical University, (Guizhou, China). The study was conducted in accordance with the guidelines of the National Health and Medical Research Council of China's Code for the care and use of animals for scientific purpose. All rats were randomly divided into diabetic group (DM group, n=10) and nomal control group (NC group, n=10). Diabetic rats were produced by injecting 0.01-mol/l streptozotocin (STZ, prepared with sterile citric acid-sodium citrate buffer, pH 4.5; Sigma, St. Louis, MO, USA) in the tail vein at a dose of 55-mg/kg. Fasting blood glucose level of all rats was detected after 48-h. The blood glucose level ≥16.7-mmol/l indicates that the diabetic rat model was established successfully. Rats in NC group were age-matched and injected an equal volume of solvent. Rats in each group were given normal diet and unlimited drinking water. After 24-weeks, 24-h urine of each rat was collected in metabolic cage, and the total volume of urine was recorded before rats were sacrificed. The rats were fasted for 6-8 h before being anesthetized by diethyl ether, and their femoral arteries were punctured to collect blood samples, which were centrifuged at 4°C to separate serum. Urine and serum were stored at -20°C for measuring urine protein and biochemical indices. Kidneys of each rats were harvested, one was fixed in 4% paraformaldehyde for paraffin sections, and the other one was snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extractions.

Tests of biochemical markers. The oxidase method was used to measure serum glucose, and the Coomassie Brilliant Blue method was used to measure urine protein. All tests were analyzed by 1650 automatic biochemical analyzer (Beckman Instruments, Inc., Brea, CA, USA), according to the manufacturer's instruction. Urine protein excretion (mg/24-h) was assessed as follows: urine protein (mg/ml) x urine volume (ml)/24-h.

Histopathological analysis. Kidneys were fixed in paraformaldehyde, embedded in paraffin, and cut by transversing transverse sections (4-µm) for staining. Renal tissue morphology was observed under a light microscope by hematoxylin-eosin (H&E) stain, while renal tissue fibrosis was observed by Masson's trichrome and Periodic Acid-Schiff (PAS) stain.

Cell culture and treatments. RTECs (NRK-52E cells) were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 5% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA) containing normal glucose (5.5-mmol/l glucose), seeded in 25-cm² culture flasks, and placed in the incubator with 5% CO₂ at 37°C. Before changing in a serum-free medium for 20-h to keep the same pace in growth, cells were treated with the following mediums: i) normal-glucose control group (NG group, 5.5-mmol/l glucose); ii) high-glucose control group (HG group) (5.5-mmol/l glucose +19.5-mmol/l D-glucose); iii) high glucose +10-ng/ml BMP-7 (HG+10-ng/ml rhBMP-7 group); and (iv) high glucose +20-ng/ml BMP-7 (HG +20-ng/ml rhBMP-7 group). Cells in each group were cultured for 48-h for further study.

Immunohistochemistry and immunofluorescence stain. The biotin-streptavidin-peroxidase method (ZSBIo, Beijing, China) was used to stain tissue sections which were which were incubated with different antibodies [rabbit-anti-BMP-7 1:200; rabbit-anti-E-cadherin 1:100; mouse-anti-α-SMA 1:100; rabbit-anti-Collagen III (Col-III) 1:150; and rabbit-anti-SnoN 1:200] (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) to show the distribution and expression of protein in kidney tissues. Phosphate-buffered saline (PBS) was substituted for the primary antibody, and the secondary antibodies were used affinity-purified biotinylated goat anti-rabbit or anti-mouse immunoglobulin G (IgG). The proteins were visualized by using dianaminobenzidine tetrahydrochloride as a chromogen. Tissue sections were counterstained with Mayer's hematoxylin.

Cells cultured on coverslips were washed with PBS twice and fixed with cold methanol: acetone (1:1) for 10 mins on ice. After washing extensively, cells were blocked with bovine serum antigen for 30 mins at room temperature and then incubated with the specific primary antibodies rabbit-anti-cytokeratin-18 (CK-18, 1:100; Biosynthesis, China), rabbit-anti-E-cadherin (1:200), and mouse-anti-α-SMA (1:100) overnight at 4°C. Then cells were stained with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG (1:400) or cyanine-3 (Cy3)-conjugated goat-anti-mouse IgG (1:400) (both from Beyotime, Haimen, China). After washing again, the cells were stained with 4’,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei, and then observed under an inverted fluorescence microscope. For colocalization of the tubular marker E-cadherin and α-SMA on NRK-52E cells, immunofluorescence stain was performed. After being incubated with primary antibodies rabbit-anti-E-cadherin (1:200) at 4°C overnight, the slides were then stained with FITC-conjugated goat-anti-rabbit IgG (1:400; Beyotime). After this, the slides were incubated with primary antibodies mouse-anti-α-SMA (1:100) at 4°C overnight, and then with Cy3-conjugated goat-anti-mouse IgG (1:400; Beyotime). The nucleus were re-dyed with DAPI. These stained slides were observed under an inverted fluorescence microscope and photographed.

Western blot analysis. The protein expression in renal cortex and cultured cells was analyzed by western blot analysis. Renal tissue and cultured cells were lysed in ice-cold lysis buffer. The protein concentration was determined using the BCA protein assay kit (Beyotime). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then the proteins were electro transferred onto a polyvinylidene difluoride membrane (Millipore, Germany). Non-specific binding to the membrane was blocked in 5% nonfat milk for 1 h at room temperature. The membranes were then incubated were different primary antibodies including BMP-7 (1:150), E-cadherin (1:200), α-SMA (1:150), Col-III (1:200), SnoN (1:200), Smad ubiquitin regulatory factor 2 (Smurf2; 1:600; Abcam, Cambridge, UK), Arkadia (1:300)
and β-actin (1:400) (both from Santa Cruz Biotechnology) for 16 h at 4°C. After washing extensively in Tris-buffered saline (TBS), the membranes were incubated with a secondary horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) in TBS containing 1% nonfat milk for 1 h at room temperature. The membranes were then washed with TBS buffer again. The signals were visualized by using the enhanced chemiluminescence system (Beyotime) and then detected on x-ray films. The Bio-Rad gel imaging system (Bio-Rad, Hercules, CA, USA) was used for image acquisition. The band intensity was quantified by using the Quantity One 4.6 software (Bio-Rad).

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA in rat renal cortex or NRK-52E cells was extracted by using the TRIzol (Tiangen, Beijing, China) method according to the manufacturer's protocol. First-strand cDNA, which was stored at -20°C, was synthesized from 5-µg total RNA in renal tissues or 3-µg total RNA in NRK-52E cells by using the revert Aid First Strand cDNA Synthesis kit (Fermentas, Lithuania). All quantitative real-time polymerase chain reaction (qRT-PCR) amplification was performed by iQ SYBR-Green SuperMix (Bio-Rad). The primer sequences were as follows: SnoN (ForteBio, Shanghai, China) 5'-CCA TTCATGGCCCCATCCT-3' (sense) and 5'-AGTTCGTGG CCGCAATAAAG-3' (antisense) (the size of the product was 81 bp); β-actin (ForteBio) 5'-GCCCAACACGTGCTGCT-3' (sense) and 5'-AGGAGCAATGATCTTGGATCCT-3' (antisense) (the size of the product was 114 bp). After 45 cycles of amplification, data was collected to plot kinetic curves, and the Ct value was acquired. The aforementioned steps were repeated for three times, and the ΔΔCt method was used to calculate the mRNA/β-actin ratio for each group.

Statistical analysis. The SPSS 13.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. All statistics were expressed as mean ± standard deviation (mean ± SD). Statistical analysis between the groups was performed using an unpaired Student's t-test, while comparison among multiple groups was performed using one-way analysis of variance followed by Student-Newman-Keuls q-test. Differences were considered significantly at P<0.05.

Results

Enhanced renal fibrosis in diabetic rats. The symptoms of diabetic rats, which had been injected with STZ, including polydipsia, diuresis, and polyphagia. The levels of 24-h urine protein and blood glucose were significantly higher in diabetic rats than those in normal rats (24-h protein: 305.36±42.49 vs. 39.41±11.31 mg; blood glucose: 27.42±3.930 vs. 6.84±1.58 mmol/l) (P<0.05).

H&E, PAS, and Masson stain showed an increase of mesangial matrix and thickness of glomerular basement membrane in 24-week diabetic rats. Moreover, part of RTECs were atrophied or lost, and the inflammatory cells increased and infiltrated into wider tubulointerstitium (Fig. 1A). The immunohistochemical analysis revealed that no positive α-SMA expression was detected in renal tubules except around the blood vessels in tubulointerstitium of normal kidneys, but it was remarkable in renal tubules of diabetic rats. Moreover, Col-III deposition was much more confined within the areas of tubulointerstitium in kidneys of diabetic rats when comparing with NC group. However, epithelial cell marker, E-cadherin, was much lower in the tubular epithelium of DM groups when comparing with NC group (Fig. 1B). Being consistent with the immunohistochemical analysis, the Western blot analysis revealed that the expression of α-SMA and Col-III increased, but E-cadherin in the kidneys of DM group decreased when comparing with NC group (Fig. 1C). This observation may indicate that RTECs show the transition from the epithelial cells to the mesenchymal cells.

Effect of diabetes on BMP-7, SnoN, Smurf2, and Arkadia expression in renal tissues of rats. The results of immunohistochemistry showed that, in rat renal tissues, BMP-7 mainly expressed in RTECs. Western blot indicated that the BMP-7 protein expression in the renal cortex of the DM group was lower than that in NC group (Fig. 2A). As shown in Fig. 2B and C, immunohistochemical stain and Western blot analyses...
showed that, in diabetic conditions, the SnoN protein expression was largely lower in vivo when comparing with that in normal rats. In contrast to the significant decrease of SnoN protein, the expression of SnoN mRNA was upregulated in kidneys of diabetic rats when comparing with that in normal rats. The western blot analysis revealed that Smurf2 and Arkadia proteins were weakly expressed in renal extracts from normal rat kidneys. In kidneys of diabetic rats, increased expression of Smurf2 and Arkadia was notable at the protein level (Fig. 2D).

Exogenous rhBMP-7 inhibited the transition from tubular epithelial cells to mesenchymal cells and the expression of Col-III in vitro. The immunofluorescence analysis showed that E-cadherin and CK-18 predominantly localized in the cytomembrane of NRK-52E cells. However, staining for α-SMA was hardly seen in NRK-52E cells (Fig. 3A). These evidences confirmed that the shape and growth of NRK-52E cells were normal. As shown in Fig. 3B, E-cadherin progressively diminished in NRK-52E cells cultured in high-glucose medium, when comparing with those cultured in normal-glucose medium for 48 h. However, the α-SMA expression increased significantly after high-glucose medium incubation.

Western blot analysis showed that renal fibrosis occurred in the high-glucose group (Fig. 3C). This was demonstrated by a significant upregulation of α-SMA and Col-III, and a remarkable downregulation of E-cadherin in NRK-52E cells cultured in high-glucose medium for 48 h. In contrast, these fibrotic changes were largely attenuated in NRK-52E cells co-treated with exogenous rhBMP-7. In high glucose conditions, treating NRK-52E cells with rhBMP-7 suppressed the expression of both α-SMA and Col-III, and restored the E-cadherin expression in a dose-dependent manner, peaking at a dose of 20-ng/ml.

Exogenous rhBMP-7 upregulated the expression of SnoN protein by promoting gene transcription independent of Smurf2 and Arkadia. qRT-PCR demonstrated that the upregulation of SnoN mRNA was inconsistent with the downregulation of SnoN protein as revealed by the Western blot analysis in NRK-52E cells in high-glucose medium when comparing with those in normal-glucose medium (Fig. 4A and B). Moreover, a significant increase in protein expression of Smurf2 and Arkadia was seen in the cells exposed to high glucose, similar to that in DM group (Fig. 4C). There results from Western blot analysis demonstrated that rhBMP-7 induced SnoN protein
expression in a dose-dependent manner, peaking at 20-ng/ml dose. In contrast, there was no effect on the expression of Smurf2 and Arkadia proteins in NRK-52E cells which were co-treated with or without exogenous rhBMP-7 exposed to high glucose (Fig. 4C). Further studies by qRT-PCR indicated that gene transcription of SnoN resulted in higher levels of SnoN mRNA in NRK-52E cells co-treated with exogenous BMP-7 when exposure to high glucose (Fig. 4B). All these results strongly suggested that BMP-7 upregulated the SnoN expression in NRK-52E cells.

Discussion

Numerous studies have suggested that the TGF-β1/Smads signaling pathway activates Smad2/3, which subsequently combine with Smad4 and translocate into the cell nucleus, and finally control the transcription of TGF-β1 gene, exerting its fibrotic effect on progression of DN (1,6). Thus, how to inhibit or block the TGF-β1/Smads signaling pathway has become the focus of recent research. SnoN was discovered early as an oncoprotein, due to the induction of transdifferentiation in chicken embryo fibroblasts (7). The most important mechanism of SnoN is switching the function of TGF-β1/Smads signal transduction pathways (6,7). Recently, SnoN was knocked down by using siRNA following high-glucose stimulation, which caused a significant reduction of E-cadherin in primary RTECs, while the upregulation of fibronectin (FN) and α-SMA could further increase RTECs fibrosis. On the contrary, the overexpression of SnoN could reduce high glucose-mediated RTECs fibrosis (1). So, the downregulation of SnoN protein may be an important pathogenesis of DN renal fibrosis. In this study, type 1 DM rat model was established by injecting STZ into tail vein. Continuous DM status combined with significant proteinuria and renal histological observation showed significant renal tubulointerstitial fibrosis, were regarded as the model with DN. The expression of SnoN and E-cadherin reduced in renal tissue of DM group rats, while α-SMA and Col-III increased significantly in renal tissue. This confirms that the reduction of SnoN proteins could promote the development of DN, which is the same as the preliminary findings of our study (1).

The present study found that the increase in the mRNA level of SnoN was not consistent with the protein expression in DM group. It was found that TGF-β1 upregulated the transcription of SnoN by binding directly to sno promoter's SBE (Smad-binding element) through the p-smad2/Smad4 complex. This induced the expression of SnoN mRNA (13), which acted as a negative feedback inhibitor for the TGF-β1/Smads...
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pathway. Another research showed that TGF-β1 activated the PI3K/AKT pathway and upregulated the expression of SnoN mRNA (14). Especially, TGF-β1 increased the SnoN transcription, strongly inducing the expression of SnoN mRNA in cells and kidney tissues of unilateral ureteral obstruction (UUO) rats (15). However, the level of protein was not coupled with the expression of SnoN mRNA, as TGF-β1 protein led to a reduction of SnoN rapidly and significantly. This decrease was mainly connected with Arkadia and Smurf2, an E3 ubiquitin enzyme, which specifically recognized SnoN and involved in activating Smads-mediated ubiquitination and degradation of SnoN (15-17). During DN, high expression of Smurf2 and Arkadia was observed in renal tissue, resulting in the TGF-β1-mediated interaction of Smad2, SnoN, and Smurf2, leading to the ubiquitin degradation of SnoN protein (17,18). However, TGF-β1-activated Smad3 interacted with Arkadia, inducing the degradation of SnoN and enhancing biological effects of p-smad3 (12,17,19). Thus, the expression of SnoN protein facilitates the balance of gene expression and degradation of ubiquitin: The protein level of SnoN, due to degradation by the ubiquitin-proteasome system, reverses the promotion of TGF-β1/Smads pathway on SnoN transcriptional activation in renal disease processes, which induces fibrosis cascade effects that enhance the DN fibrosis, similar to the report by Fukasawa et al in UUO (15).

BMP-7 has been proved to be anti-fibrotic as it counteracts the TGF-β1/Smads pathway in many fibrotic diseases including pulmonary fibrosis (20), liver fibrosis (21), and renal fibrosis (3-5,22). However, in a variety of fibrotic diseases, BMP-7 protein downregulation prompts the development of fibrosis. The present study showed that the expression of BMP-7 reduced gradually with the progression of DN. Transfection of recombinant BMP-7 with adenoviral gene or direct addition of rhBMP-7 into RTECs could reduce or counteract the occurrence of EMT and the synthesis of ECM induced by TGF-β1 (9,10,23). However, the mechanism of BMP-7 inhibiting the TGF-β1/Smad signaling pathway is not clear, only few studies explained the mechanism that BMP-7 does not affect the synthesis of TGF-β1 induced by high glucose in RTECs (24). And Luo et al found that SnoN, a negative regulator protein of the TGF-β1 signaling pathway, may be involved in the mechanism, and when SnoN was knocked down, the effect of BMP-7 inhibiting the TGF-β1/Smad signaling pathway was weakened (4). But the specific patterns and mechanisms were not clear. So, in this study, the tubular epithelial cells were cultivated in vitro (NRK-52E cells) in high glucose with various doses of rhBMP-7 (10- and 20-ng/ml) simultaneously, and the effects of rhBMP-7 were observed on the transcription and protein levels of SnoN and the expression of E3 ubiquitin ligases, Smurf2 and Arkadia, to clarify the influence and mechanism of BMP-7 on the expression of SnoN.

BMP-7 has a variety of biological effects, and the doses of rhBMP-7 that are applied for treating fibrotic diseases range from 5- to 400-ng/ml. In the present study, 10- and 20-ng/ml doses of rhBMP-7 were added to high glucose-cultured RTECs to observe whether the fibrosis effect
is being delayed. The results showed that rhBMP-7 depressed the phenotype transition of RTECs and synthesis of Col-III in a dose-dependent manner. The results suggested that 10- and 20-ng/ml rhBMP-7 have therapeutic implications. Moreover, although the high dose of glucose (in the high-glucose group) increased the expression of SnoN mRNA and Smurf2 and Arkadia proteins, exogenous rhBMP-7 (in the rhBMP-7 plus high-glucose group) further enhanced the level of SnoN mRNA significantly in NRK-52E cells under high glucose. And no significant difference was found between the expression of Smurf2 and Arkadia in the rhBMP-7 plus high-glucose group compared with the high-glucose group. In other words, BMP-7 could not inhibit the expression of Smurf2 and Arkadia, but upregulated SnoN at the transcriptional level significantly and even reversed part of the degraded protein, eventually restoring the expression of SnoN protein to block the TGF-β1/Smads signaling pathway.

In conclusion, the results showed that BMP-7 increased the expression of SnoN protein to inhibit the TGF-β1/Smads signaling pathway and delay the process of DN. And the effect of BMP-7 in upregulating the expression of SnoN protein is not mitigating E3 ubiquitin ligase protein-induced SnoN protein degradation, but enhancing the SnoN mRNA expression. The mechanism of BMP-7 regulating the expression of SnoN still requires further study.

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