

Original Article

Nicousamide protects kidney podocyte by inhibiting the TGF β receptor II phosphorylation and AGE-RAGE signaling

Sen Zhang, Dongjie Wang, Nina Xue, Fangfang Lai, Ming Ji, Jing Jin, Xiaoguang Chen

State Key Laboratory Of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, P. R. China

Received August 24, 2016; Accepted December 21, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Nicousamide, a clinical phase II renal protective new drug, has been demonstrated to have renal protective effect on diabetic nephropathy (DN) by experimental animal model. Its known molecular mechanisms include AGE formation blocking and moderately decreasing the blood pressure. Nicousamide shows potential on attenuating albuminuria, thereby suggests it might have protective effect on podocytes. The aim of present study was to investigate whether nicousamide could protect integrity of podocytes, and further its protection mechanisms. Sprague-Dawley (SD) rats were induced to DN by streptozotocin, and nicousamide (20 and 40 mg/kg) was orally administrated for 20 weeks. Every five weeks, the albuminuria was measured, and renal pathology was evaluated at the end of experiment. Real-time PCR and immunofluorescence were used to test expression of podocyte marker nephrin, CD2AP and podocin in rat kidney tissues. Western blot was used to test the activation and phosphorylation of TGF β 1-smad signaling pathway. surface plasmon resonance (SPR) technology was used to analyze whether nicousamide can interact with TGF β 1 receptor II (TGF β RII) and receptor for advanced glycation endproducts (RAGE). Results demonstrate that nicousamide significantly reduces albuminuria and ameliorate the glomerulosclerosis in DN rats. RT-PCR and immunofluorescence demonstrate that nicousamide can increase the expression of podocyte markers and keep podocyte effacement. Phosphorylation of TGF β RII and smad2 in rat kidney was inhibited by nicousamide dose dependently. SPR demonstrate that nicousamide have strong binding capability with hRAGE with Kd approximate 6 μ M. These results indicate a protective effect of nicousamide against podocyte injury, and this effect might contribute from suppression of TGF β -involved fibrosis and AGE-RAGE signaling activation.

Keywords: Nicousamide, diabetic nephropathy, podocyte, nephrin, TGF β receptor II, RAGE

Introduction

Nicousamide, a promising renal protective agent, has been moved forward to stage II clinical trial for diabetic nephropathy (DN) in China. Nicousamide is a novel coumarin-aspirin derivative, and in the preclinical study, nicousamide could significantly slow down the progression of DN by reducing the albuminuria and blood urea nitrogen (BUN), increasing the creatinine clearance, and ameliorating the glomerulosclerosis [1]. The current mechanism study demonstrates that nicousamide can significantly inhibit the production of advanced glycation end products (AGEs) and reduce the AGE-stimulated overexpression of transforming growth factor (TGF- β 1) and connective tissue growth factor (CTGF) in renal mesangial cells, which in turn

induce renal hypertrophy, sclerosis and functional failure [2]. In addition, by in vitro experimental study, nicousamide also shows it could inhibit the phosphorylation of TGF β receptor II, consequently block the TGF-smad signaling over-activation, which is key event in renal end-stage fibrosis [3]. Besides attenuating the renal impairment in DN, nicousamide also shows renal protective in hypertensive nephropathy in spontaneously hypertensive rats [4] and normalizes renovascular hypertension in two-kidney one-clip hypertensive rats [5].

Proteinuria and albuminuria are most pathological events in DN and podocyte play a key role in urine protein infiltration [6]. Podocytes, endothelial cells and the glomerular basement membrane (GBM) constitute the kidney filtra-

tion barrier, a highly specialized structure for selective ultrafiltration. The common denominator in a variety of kidney diseases is podocyte dysfunction involving proteinuria [7-9]. Although we have demonstrated that nicousamide can significantly reduce the proteinuria and albuminuria in progression of DN, its beneficial effect on podocyte structure, permeability and integrity is unknown; besides that, although nicousamide could inhibit the phosphorylation of TGF β RII in vitro, and inhibits the AGE-stimulated fibronectin secretion, and G1 phase arrest in human proximal tubular epithelial cell line (HK2) [1-3], the related in vivo study has not been performed yet. In the current study, we established streptozotocin-induced DN model again, aim to investigate the protective effect of nicousamide on podocyte, and further investigate the underlying mechanisms by studying the TGF β -smad and AGE-RAGE signaling pathway.

Materials and methods

Streptozotocin-induced diabetes model

The SD rats (male, 7-week old, 165-180 g) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College. All animal procedures were in conformity with national and international laws for the care and use of laboratory animals, and the Animal Research Committee of Institute of Materia Medica approved the experimental protocols. Streptozotocin-induced DN model was established with guidance of previous experimental protocol from our laboratory [1]. Briefly, the rats were rendered diabetic with a single i.p. 60 mg/kg streptozotocin (Sigma, MO, USA) in 0.1 M sodium citrate buffer (pH 4.5), and animals with blood glucose excess of 12 mM one week after injection were enclosed in the study. Sham-injected animals (0.1 M sodium citrate buffer, pH 4.5) were followed concurrently and served as normal control. The diabetic rats were randomly divided into three groups (8 rats each), which served as DN model group and two nicousamide-treated groups which received 20 and 40 mg/kg, p.o. per day, respectively. Nicousamide was dissolved in 0.5% sodium carboxymethyl cellulose and was daily-administered using gavage 6 days per week till 20 weeks. All rats were allowed free access to

standard chow and water. During the period of experiments, insulin was subcutaneously injected 1-2 units to prevent ketoacidosis if the rat blood glucose was over 40 mM. Protein and albumin concentration from urine were measured using commercial kits (Nanjing Jiancheng Bio, Nanjing, China). Urinary albumin excretion (UAE) per day was calculated from the urine samples collected in metabolic cage for 24 h, using urine albumin concentration plus 24 h urine volume. Once all the rats received euthanasia, partial kidney tissues from each rat were embedded in paraffin for PAS staining to evaluate the pathological injury. The remaining fresh kidney tissues were prepared for RNA isolation, total protein extraction and making frozen sections.

Immunofluorescence

Frozen kidney sections (5 μ m) were treated with a rabbit monoclonal antibody anti-rat nephrin (1:100 dilution; Abcam Biotechnology, Cambridge, MA, USA) and a rabbit monoclonal antibody anti-rat podocin (1:100 dilution; Abcam Biotechnology, Cambridge, MA, USA), followed by a donkey anti-goat IgG-FITC (1:100 dilution; Santa Cruz Biotechnology, CA, USA). Negative controls consisted of histological sections incubated with PBS rather than the primary antibody. Immunofluorescence was visualized on a ZEISS Observer A1 fluorescence microscope equipped with a high-pressure mercury lamp (ZEISS, Germany). The percentage of positive staining of podocin and nephrin on total glomerular area was analyzed and calculated by Image pro plus software (USA).

Podocytes genes mRNA determination

Total RNA was extracted using Trizol reagent (Invitrogen, life technology Inc, USA) from renal cortical tissues and the purity of the RNA was evaluated by measuring the ratio of A260/A280. First-strand cDNA was generated by ReverTra Ace[®] qPCR RT Kit (Code No. FSQ-101, TOYOBO, Inc, Japan). Briefly, first strand cDNA was obtained by adding 2.4 μ g total RNA, 2 \times PrimeScript Buffer, 10 μ l; PrimeScript RT Enzyme Mix I, 1.5 μ l; and finally RNase Free dH₂O were used to reach a total reaction volume of 20 μ l. The condition of reverse transcription was as follows: 15 min at 37°C and 95°C for 15 s.

Table 1. Sequences of the primers for RT-PCR

Genes	Sense primers (5' to 3')	Antisense primers (5' to 3')	Product size (bp)
Nephrin	ATGGGCGCTAAGAGAGTCAC	CGCAGTCAGGTTTTCAGACA	171
Podocin	TCTTGTCTCTCTCCCTGA	AGACGGAGGTCAACCTTGTC	195
CD2AP	GCTGGTGGAAGGTGAAGT	CATCTCTGTCTCCGCCTTC	192
GAPDH	AAACCCATCACCCTCTCCA	GTGGTTCACCCATCACAA	198

All the primers were designed to span an intron and the information of primer were shown in **Table 1** [10]. The PCR reaction system was combined in a master mix composed of SYBR Premix Ex Taq II (2×, TOYOBO, Inc, Japan), 10 µl; PCR Forward Primer (10 µM), 0.8 µl; PCR Reverse Primer (10 µM), 0.8 µl; dH₂O, 6 µl; cDNA, 2 µl. The real-time quantitative PCR was performed by thermo cycler instrument (ABI 7500, Applied Biosystems, USA) and the cycling program was set at 1 cycle of pre-denaturation at 93°C for 2 min, and then 40 cycles at 93°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by 1 cycle at 72°C for 5 min. The relative expression of mRNA was calculated by methods mentioned by Schmittgen: Fold = $2^{-\Delta\Delta Ct}$, and $\Delta\Delta Ct = Ct \text{ target mRNA} - Ct \text{ GAPDH}$. Ct refers to the number of cycles experienced by fluorescent signals that reached the threshold inside the reactor. All qRT-PCR products were replicated three times.

Western blot to analyze the status of components in TGFβ-smad signal pathway

Kidney tissues from four groups were homogenated for western blot analysis. TGFβ1 (Immunoway, China), TGFβ RI (Santa Cruz, USA), TGFβ RII (Santa Cruz, USA), phosphorylated-TGFβ RII (Santa Cruz, USA), smad2 (Cell signaling technology, USA), p-smad2 (Cell signaling technology, USA), RAGE (Santa Cruz, USA) were used as primary antibodies.

The brief description of western blotting procedure was following. Renal tissue lysate was prepared using a lysis buffer containing 25 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), 5 mmol/l ethylenediamine tetraacetic acid (EDTA), 10 mmol/l NaF, 1 mmol/l phenylmethyl sulfonyl fluoride (PMSF), 1% TritonX-100, 0.5% Nonidet P40, 10 mg/l aprotinin, 10 mg/l leupeptin and quantified by Bradford dye-binding procedure. Equal amounts of proteins were separated through electro-

phoresis on a sodium dodecyl sulphate (SDS)- polyacrylamide gel (5% stacking gel and 10% separating gel for target proteins) and electroblotted onto a piece of nitrocellulose membrane. After blocking with 5% bovine serum albumin (BSA),

the membrane was incubated with appropriate antibodies overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody against rabbit or mouse immunoglobulin G (IgG) at a 1:2000 dilution for 1 h at room temperature after washing. The signals were detected by Enhanced chemiluminescence (ECL) (ImageQuant LAS 4000 Mini, GE healthcare, America). The intensity of the detected bands was analyzed using Image J program (USA).

Surface plasmon resonance measurement

Purified human TGFβ RII-Fc and RAGE-Fc protein were purchased from Acro Biosystems, Inc (Newark, USA). The binding activity of nicousamide to immobilized hRAGE and TGFβ RII was analyzed using surface plasmon resonance (SPR) technology. We used hRAGE as a sample to describe the procedure of SPR. Briefly, a new CM5 chip (Biacore; GE Bioscience) with four binding surfaces/channels was placed into the Biacore T100 system (GE Healthcare), followed by binding surface activation using 400 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 100 mM N-hydroxysuccinimide, according to the standard protocol in the Biacore Sensor Surface Handbook (GE Healthcare). After activation, one channel was used as the reference, and three were used for binding experiments. To immobilize human RAGE-Fc on the chip, purified hRAGE-Fc (50 µg/ml) in NaAc/HAc buffer (pH 4.5) was injected into activated channels. The final response unit change with immobilized protein was ~7500. After protein coating, 1 M ethanolamine was injected into the channels to neutralize residual activated carboxyl groups. The reference channel was blocked directly with ethanolamine. For nicousamide interaction experiments, nicousamide dissolved in deionized water (0 µM, 0.1 µM, 0.5 µM, 1.0 µM, 5 µM, 10 µM) were injected into the channels at a flow rate of 10 µl/min, which was

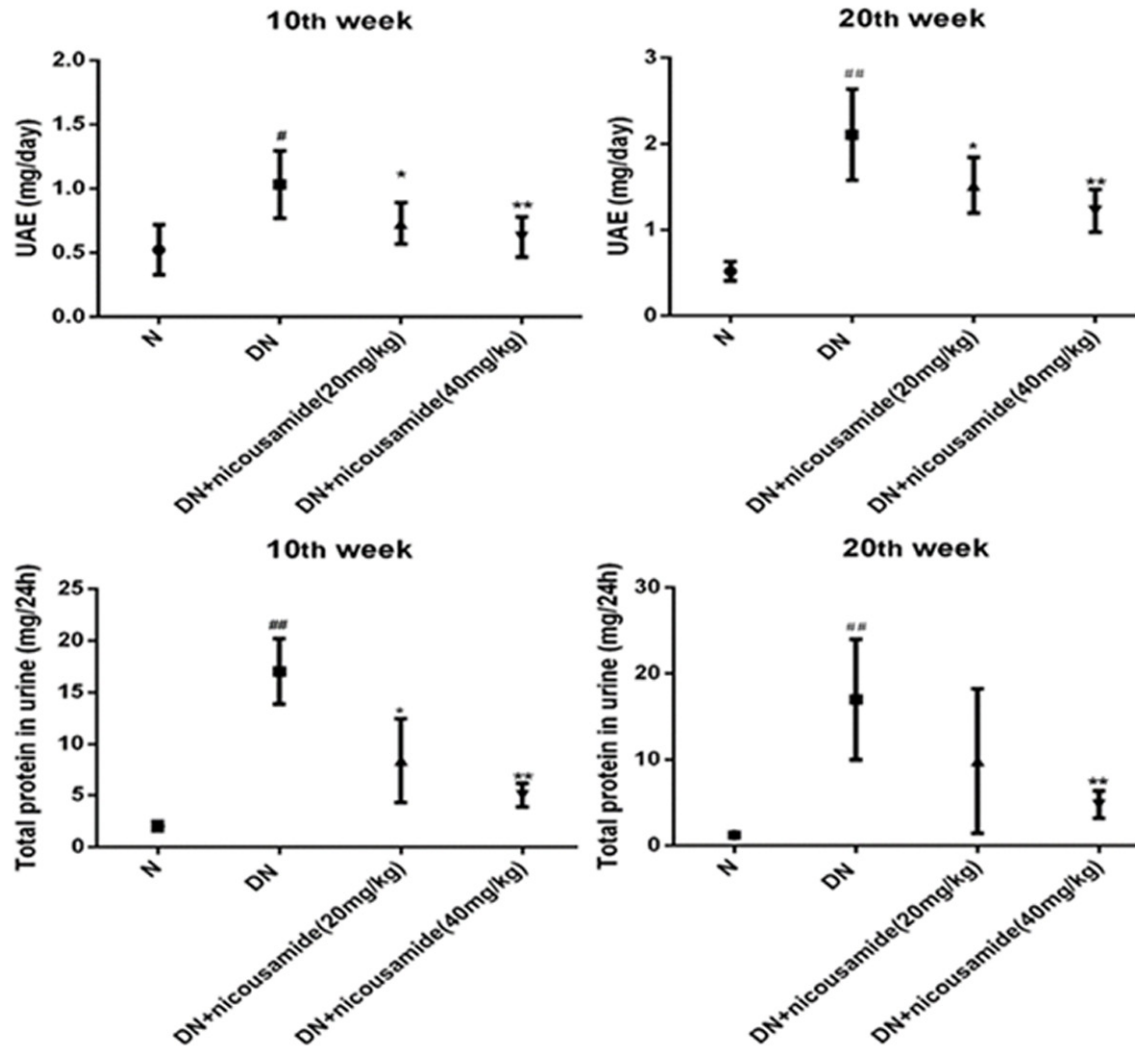


Figure 1. Nicousamide reduced urinary albumin excretions (UAE) and urinary total protein in diabetic SD rats at 10th and 20th week (n=8; [#]P<0.05, ^{##}P<0.01 vs. normal group; ^{*}P<0.05, ^{**}P<0.01 vs. DN model group). All the SD rats were induced to diabetic nephropathy by streptozotocin intraperitoneal injection, and nicousamide was orally administrated for up to 20 weeks.

lasted six minutes. Continual flow of deionized water at the same rate was provided after the nicousamide injection. To terminate compound-protein binding, the channels were washed/regenerated by running 5 M LiCl in HEPES (pH 7.3) for 10 min at a flow rate of 3 ml/min. Same procedure was also used for the interaction study between nicousamide and hTGF β RII.

Statistical analysis

Data were shown as Mean \pm Standard deviation (SD). Comparisons among groups were carried out using oneway analysis of variance

(ANOVA) followed by post-hoc Duncan's test. P<0.05 was considered statistically significant.

Results

Nicousamide reduced the proteinuria and albuminuria and ameliorated the glomerulosclerosis in diabetic nephropathy in rats

One week after streptozotocin injection, a significant increase in blood glucose was detected in the streptozotocin-injected SD rats as compared with the normal control rats, confirming successful induction of experimental diabetes. After 20-week duration, all the diabetic rats

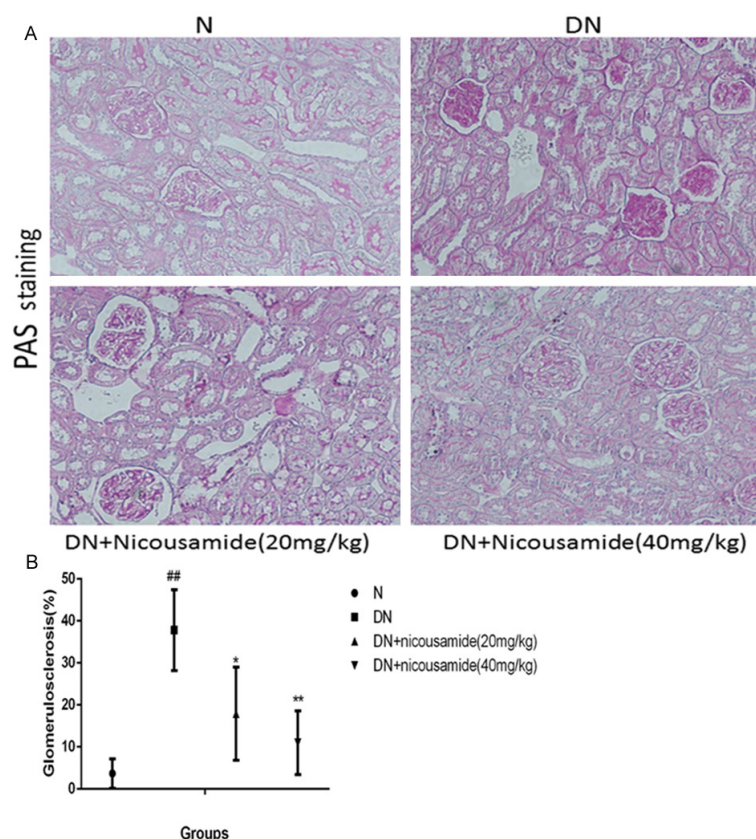


Figure 2. Nicousamide ameliorated the glomerulosclerosis in diabetic rats dose dependently. A. Representations of PAS-stained kidneys of normal rats, diabetes model rats and nicousamide-treated diabetes rats ($\times 200$). As shown in DN model group, severe sclerosis in glomerulus segments, segmental and diffuse mesangial expansion of the glomeruli, and tubule vacuolar degeneration in proximal tubules had been observed, while nicousamide treatment could effectively ameliorate these pathological injuries. B. Glomerulosclerosis quantification, $\#P < 0.05$, $\#\#P < 0.01$ vs. normal group; $*P < 0.05$, $**P < 0.01$ vs. diabetic nephropathy model group.

established severe proteinuria and albuminuria. Every five weeks, we collected 24-hour urine to measure total albumin and protein in the urine. In this present study, nicousamide could significantly reduce total protein and albumin in the 24-hour urine compared with vehicle-treated DN group in a dose dependent manner (**Figure 1**, 10-week and 20-week time point data were provided). PAS staining and pathological analysis also demonstrated that nicousamide administration could attenuate the glomerulosclerosis and pathological injury significantly in a dose dependent manner (**Figure 2**).

Effects of nicousamide on mRNA expression of podocyte genes in renal tissues

Nephrin, podocin and CD2 associated protein (CD2AP) are three of the functional genes for

podocytes, which have been demonstrated to be involved in the development of proteinuria. In order to disclose the beneficial effect of nicousamide on podocyte, mRNA levels of nephrin, podocin and CD2AP were determined. The results showed that nephrin, podocin and CD2AP mRNA in the kidneys of diabetic rats were highly down-regulated, which compared with normal group. However, nicousamide could enhanced the expression of renal nephrin, podocin and CD2AP mRNA in a dose dependent manner (**Figure 3**).

Nicousamide increased podocyte biomarker by immunofluorescence

Podocin expression is restored in kidney after treatment with nicousamide. As detected by immunofluorescence, diabetic rats from model group displayed a dramatic decrease in glomerular podocin expression compared with the saline-treated normal group ($P < 0.05$; $n = 8$; **Figure 4**). However, the expression of the slit membrane protein podocin was significantly increased in the nicousamide-treated group in

a dose-dependent manner. Expression of nephrin in diabetic rats by nicousamide treatment also appeared similar pattern as podocin (**Figure 4**).

Nicousamide inhibit the TGF- β smad signaling pathway but not RAGE protein level

The kidney tissue homogenate was used for western blot to analyze the protein level of TGF β 1, TGF β RII, phosphorylated-TGF β RII, total smad2 and phosphorylated smad2, as well as RAGE. As shown in **Figure 5**, higher levels of TGF β 1, and phosphorylation of TGF β RII and smad2 was observed in diabetic model group, while nicousamide significantly reduced the TGF β 1 content in the kidney tissues, as well as expression of p-TGF β RII and p-smad2. However, nicousamide did not influence the expression of total TGF β RII and smad2.

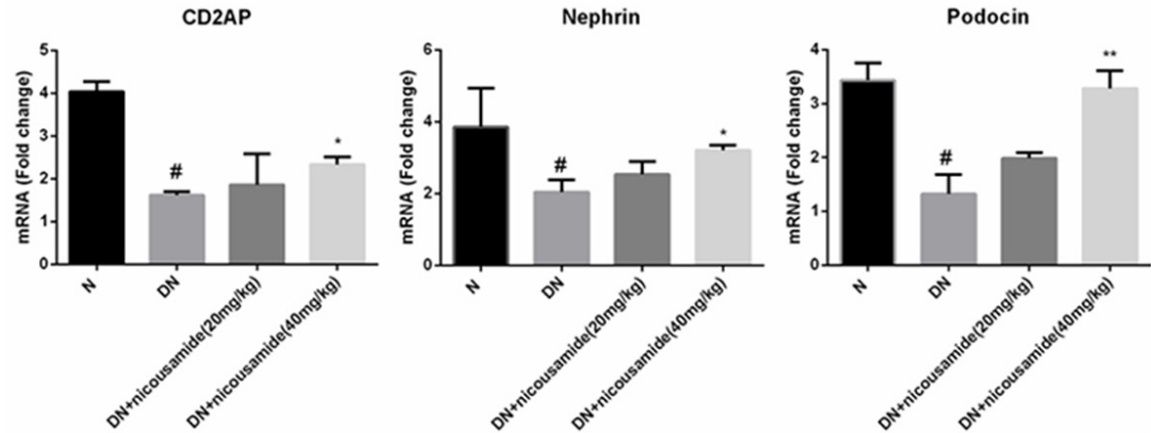


Figure 3. Bar graphs representing the nephrin, podocin and CD2AP mRNA levels in the kidney tissues among four groups. #P<0.05 vs. Normal; *P<0.05, **P<0.01 vs. DN model. All values are means \pm SD (n=8). Real-time PCR demonstrated that nicousamide could significantly increase the mRNA levels of nephrin, podocin and CD2A in kidney tissues from diabetic rats compared to DN model.

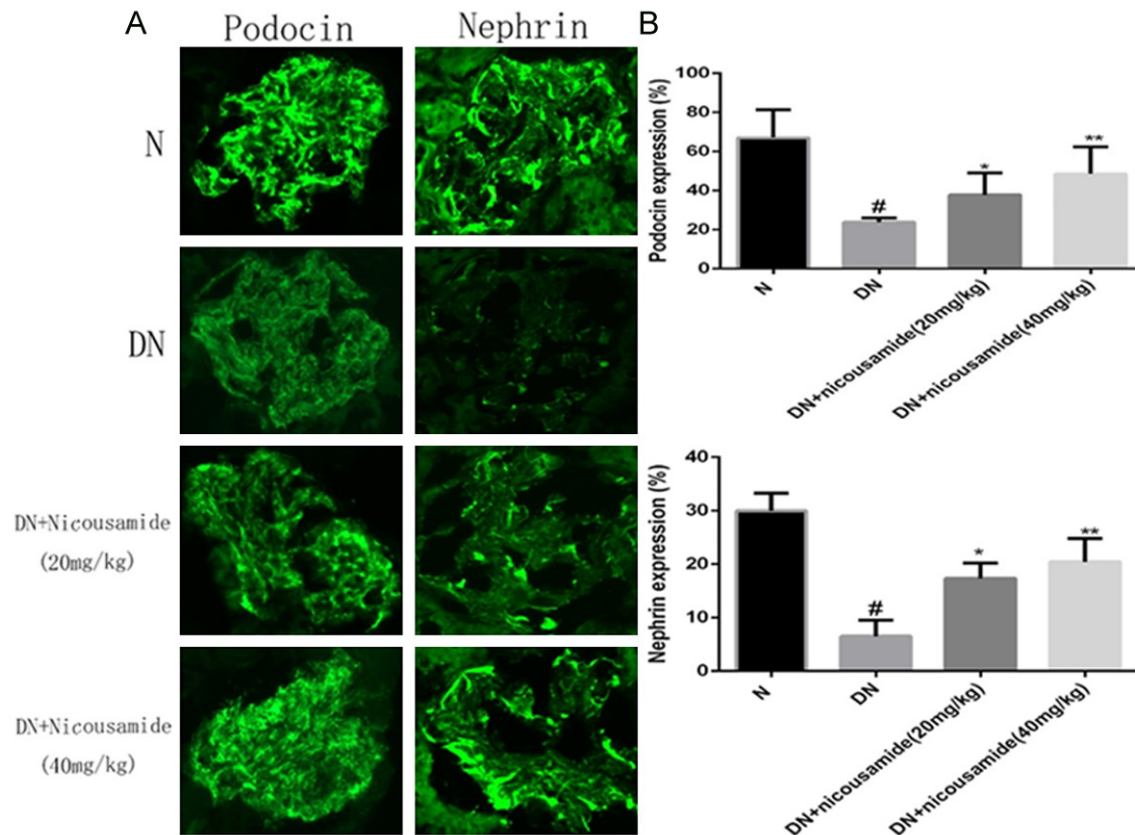


Figure 4. Immunofluorescence microscopy demonstrated that nicousamide could significantly restore the expression of nephrin and podocin in glomerulus from diabetic rats, and kept the podocyte integrity, compared to weak staining from DN model group. A. Nephlin and podocin staining with monoclonal antibodies and FITC-conjugated second antibody. B. Bars show quantification of nephrin and podocin stained area. %: percentage of total glomerular area. Data are means \pm SD. #P<0.05 vs. Normal; *P<0.05, **P<0.01 vs. DN model.

Nicousamide protects podocyte by inhibiting TGF β RII and RAGE

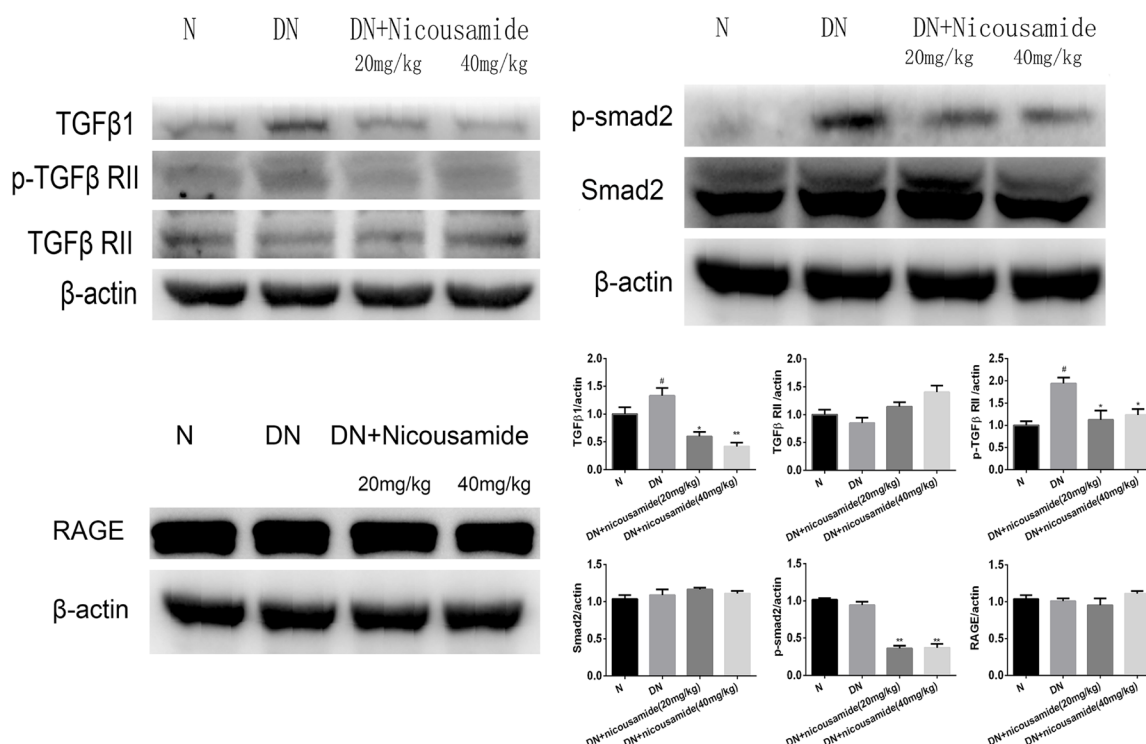


Figure 5. Western blot results of nicousamide on protein expression of TGF β -smad signaling pathway and RAGE. Compared to DN model group, nicousamide treatment significantly decreased the protein level of TGF β 1, p-TGF β RII and p-smad2, but no remarkable influence on total TGF β RII, total smad2 and RAGE. The figure includes representative figures of western blot and quantification analysis of protein levels by Image J.

The current study also suggested that nicousamide did not influence the protein level of RAGE in diabetic rats, although in vitro study demonstrated that nicousamide could block the AGE-stimulated down-stream pathway and transduction cascades by previous study from our laboratory [2] (**Figure 5**).

Nicousamide directly binds to hRAGE protein in vitro by SPR assay but not to hTGF β RII

SPR has been widely used to measure the binding between mobile analytes and immobilized biomolecules without the use of labels. The signals can be acquired qualitatively and quantitatively. It was previously reported that Biacore 3000 (GE Healthcare, USA) could be used to provide kinetic data for the interactions between a compound and its target protein, which prompts us to examine whether nicousamide directly binds to TGF β RII or RAGE.

We firstly analyzed whether different concentrations of nicousamide could adhere to immobilized hRAGE, using SPR. For this experiment,

different concentrations of nicousamide were set to flow through CM5 chip surfaces coated with hRAGE-Fc. As shown in **Figure 6**, nicousamide exhibited rapid attachment/retention to the chip and demonstrated effective and rapid adhesion to RAGE in a dose-dependent manner with $KD=6.86 \mu M$. These results show that nicousamide have the ability to bind to immobilized hRAGE and were washed away by the flow shear force. However, when the same condition was performed for hTGF β RII, we did not find significant binding evidence for nicousamide with hTGF β RII.

Discussion

The current report suggests that nicousamide possesses antiproteinuric properties, and this antiproteinuric effect can be attributed to the drug's protection of kidney podocyte integrity to some extent.

Proteinuria is a major health care problem that affects several 100 million people worldwide. It is a cardinal sign and a prognostic marker of

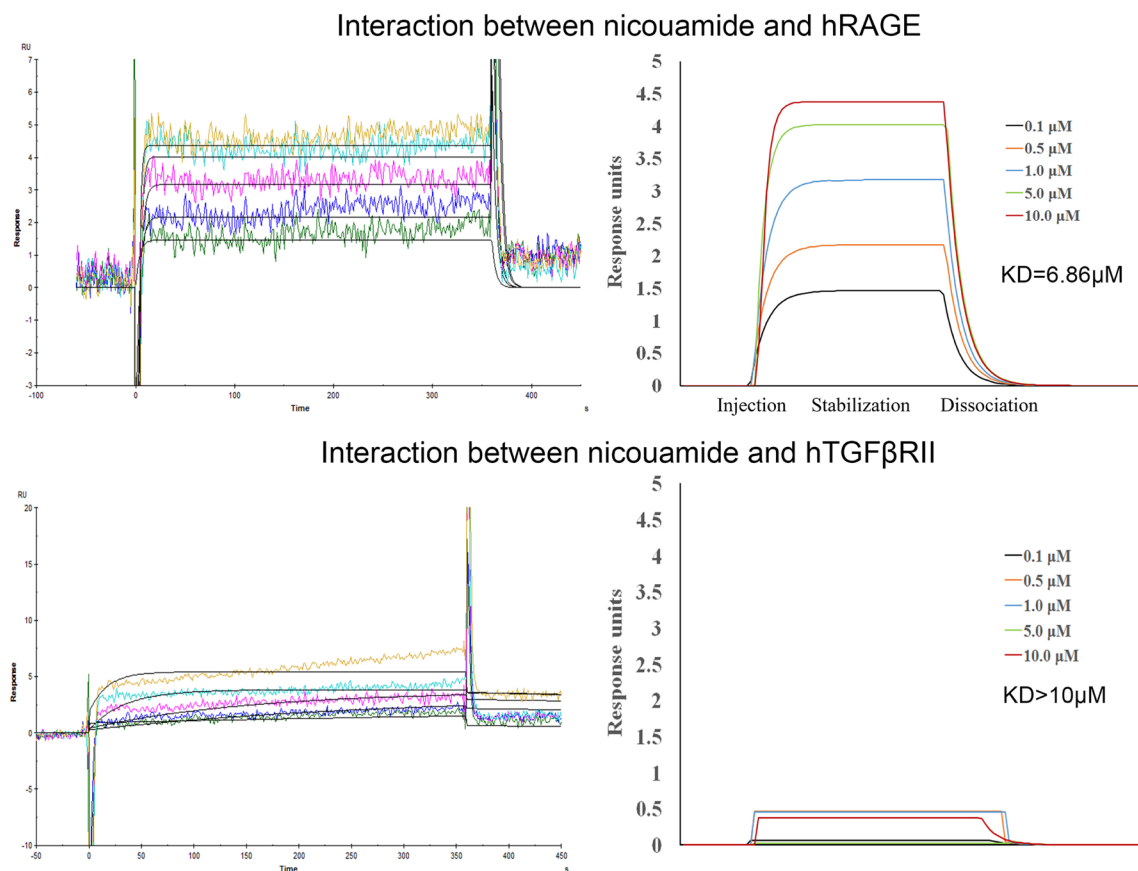


Figure 6. The interaction between nicousamide and hTGF β RII or hRAGE by SPR assay. nicousamide exhibited rapid attachment/retention to the chip and demonstrated effective and rapid adhesion to RAGE in a dose-dependent manner with KD=6.86 μ M, but no significant binding with hTGF β RII. The adhesion curves represent similar results from three independent experiments.

kidney disease and also an independent risk factor for cardiovascular morbidity and mortality [11]. Therefore, the reduction or prevention of proteinuria is highly desirable. Although the glomerular endothelium, GBM and podocytes all contribute to the filtration barrier, the podocytes seem to be the most critical part of the filtration unit [12]. Pathogenic pathways activated in podocytes during proteinuria have been identified, and these findings pinpoint the podocyte as the most obvious candidate for therapeutic intervention [9, 13].

Podocyte function of maintaining the filtration barrier of glomerulus depend on nephrin, podocin and CD2AP, which are demonstrated to be involved in maintaining the structural integrity of the slit diaphragms [14]. This present study shows that nephrin, podocin, and CD2AP are all down-regulated in diabetic nephropathy rats on mRNA level, compared with normal rats, which

suggests that the imbalance of nephrin, podocin, and CD2AP expression will lead to a dysregulated ultrafiltration of glomerulus. By immunofluorescence, we also confirmed that protein amount of nephrin and podocin in glomeruli from diabetic nephropathy rats is significantly lower than normal rats. By drug intervention, nicousamide could restore the imbalances of the gene and protein expression of these key proteins, thereby prevent the podocytes from being injured.

Previous studies from our laboratory demonstrated that nicousamide could inhibit the AGE formation by some unknown mechanisms [2]. Besides that, nicousamide also block the AGE stimulated fibrogenesis in HKC cells, which means that nicousamide might has more inhibitory effect on AGE-mediated pathogenesis. Kidney is a target of for AGE-mediated damage and also the main contributor to increasing the

circulating AGE concentration via a decrease in renal function, by the clearance of AGEs [15, 16]. The effect of AGEs can be classified as receptor-independent and -dependent. Although AGEs can directly cause dysfunction of many functional proteins [17], AGE-RAGE signaling pathway is another important pathway to deteriorate progress of DN. In the human kidney, RAGE protein is found in tubular epithelial cells [18], mesangial cells [19], podocytes [20, 21] and within vascular and neural compartments. In diabetes, RAGE expression is increased at sites of macrovascular and microvascular injury. This is supported by AGE and RAGE colocalization in susceptible organs in diabetes [22].

RAGE binding by AGEs or activates diverse signal transduction cascades including p21ras, p38, p44/p42 (erk1/2, extracellular signal-related kinase), and stress-activated protein kinase/c-Jun N-terminal kinase mitogenactivated protein (MAP) kinases, the Janus kinase/signal transducers and activators of transcription pathway, and protein kinase C (PKC) pathway. Signal transduction leads to downstream consequences including generation of reactive oxygen species (ROS) and activation of transcription factors such as nuclear factor kappa B (NF-κB) [23]. One important consequence of NF-κB translocation is the up-regulation of RAGE itself because the promoter region of RAGE contains functional binding elements for NF-κB [24]. AGE-RAGE induction of NF-κB or other pathways contributes to the release of proinflammatory cytokines, and the expression of adhesion molecules and growth factors that are implicated in the pathogenesis of diabetic complications. These include TGFβ1, vascular endothelial growth factor, connective tissue growth factor, interleukin-1β and -6, insulin-like growth factor-1, platelet-derived growth factor, tumor necrosis factor (TNF)-α, and vascular cell adhesion molecule (VCAM)-1 [23].

Based on these knowledge, RAGE inhibitors might have beneficial effect on DN, especially on podocytes, fortunately, several studies have proved that. Severe renal pathologic changes can be diminished by treating the diabetic animals with soluble RAGE [20] or a RAGE-specific neutralizing antibody [25]. Besides that, genetic manipulation of RAGE expression influences the renal phenotype in the setting of diabetes. For example, diabetic transgenic mice that overexpress human RAGE have more advanced

renal disease when compared with diabetic wild-type mice. These changes included increases in albuminuria and serum creatinine levels, mesangial expansion, and advanced glomerulosclerosis [26]. In our present study, by SPR assay, we demonstrate that nicousamide can bind hRAGE effectively, together with our previous in vitro data, we postulate that nicousamide not only inhibit the AGE formation but also block AGE-RAGE signaling pathway.

Numerous studies have proved that TGFβ-smad signaling pathway is key mediator for glomerulosclerosis of DN, and also induce depletion, apoptosis, hypertrophy and abnormalities of podocyte foot processes, which ultimately result in renal dysfunction [27, 28]. In vitro study from our laboratory has proved that nicousamide can inhibit the TGFβ RII phosphorylation in human proximal tubule epithelial cell line (HK2) stimulated by TGFβ1 [3], and in vivo study from the current study once again obtains the same results. Nicousamide significantly decrease TGFβ RII phosphorylation, and its down-stream transmitter smad2 phosphorylation in animal kidney tissues. Although SPR assay does not find that nicousamide can interact with TGFβ RII directly, the TGFβ RII in the current research is not full-length protein, it only covers catalytic domain region (sequence from Thr 23-Asp 159) of TGFβ RII, thus we can't exclude possibility that nicousamide can influence the non-catalytic region of TGFβ RII. We have tried to use the full-length TGFβ RII to attach the CM5 chip, but big protein space size limits its kinase activity in vitro, so we have to use the catalytic sequence, that is one limitation for this study.

In conclusion, nicousamide may have a protective effect on podocytes in DN animal model by intervening both AGE-RAGE and TGFβ-smad signaling pathway, which is possible to be helpful for its undergoing stage II clinical trial.

Acknowledgements

We greatly appreciate the funding support from National natural science foundation of P. R. China (No. 81302830) and CAMS Initiative for Innovative Medicine (No. 2016-I2M-3-011).

Disclosure of conflict of interest

None.

Address correspondence to: Xiaoguang Chen, State Key Laboratory of Bioactive Substances and Functions of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, P. R. China. Tel: 86-10-63165207; Fax: 86-10-63165207; E-mail: chxg@imm.ac.cn

References

- [1] Li H, Zheng X, Wang H, Zhang Y, Xin H and Chen X. XLF-III-43, a novel coumarin-aspirin compound, prevents diabetic nephropathy in rats via inhibiting advanced glycation end products. *Eur J Pharmacol* 2010; 627: 340-347.
- [2] Li H, Zhang Y, Wang H, Zheng X and Chen X. Nicousamide blocks the effects of advanced glycation end products on renal cells. *Eur J Pharmacol* 2012; 674: 455-459.
- [3] Zhang HJ, Jin J, Zhou WQ, Zhang Y, Li Y, Liu JY, Zhang S and Chen XG. Nicousamide, a potent inhibitor of phosphorylation by TGF- β receptor II. *Acta Pharmaceutica Sinica B* 2011; 1: 160-165.
- [4] Zhang S, Li Y, Li H, Zheng X and Chen X. Renal-protective effect of nicousamide on hypertensive nephropathy in spontaneously hypertensive rats. *Biomed Rep* 2013; 1: 34-40.
- [5] Zhang S, Li H, Li Y, Zhang F, Liu Y and Chen X. Nicousamide normalizes renovascular hypertension in two-kidney one-clip hypertensive rats. *Biomed Rep* 2013; 1: 89-92.
- [6] Bondeva T, Ruster C, Franke S, Hammerschmid E, Klagsbrun M, Cohen CD and Wolf G. Advanced glycation end-products suppress neuropilin-1 expression in podocytes. *Kidney Int* 2009; 75: 605-616.
- [7] Zhang B, Xie S, Shi W and Yang Y. Amiloride off-target effect inhibits podocyte urokinase receptor expression and reduces proteinuria. *Nephrol Dialysis Transplant* 2012; 27: 1746-1755.
- [8] Somlo S and Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* 2000; 24: 333-335.
- [9] Tryggvason K, Patrakka J and Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med* 2006; 354: 1387-1401.
- [10] Wu JB, Ye SF, Liang CL, Li YC, Yu YJ, Lai JM, Lin H, Zheng J and Zhou JY. Qi-Dan Fang ameliorates adriamycin-induced nephrotic syndrome rat model by enhancing renal function and inhibiting podocyte injury. *J Ethnopharmacol* 2014; 151: 1124-1132.
- [11] Zandi-Nejad K, Eddy AA, Glassock RJ and Brenner BM. Why is proteinuria an ominous biomarker of progressive kidney disease? *Kidney Int Suppl* 2004; S76-89.
- [12] Patrakka J and Tryggvason K. New insights into the role of podocytes in proteinuria. *Nat Rev Nephrol* 2009; 5: 463-468.
- [13] Mundel P and Reiser J. Proteinuria: an enzymatic disease of the podocyte? *Kidney Int* 2010; 77: 571-580.
- [14] Maezawa Y, Takemoto M and Yokote K. Cell biology of diabetic nephropathy: Roles of endothelial cells, tubulointerstitial cells and podocytes. *J Diabetes Investig* 2015; 6: 3-15.
- [15] Tan AL, Forbes JM and Cooper ME. AGE, RAGE, and ROS in diabetic nephropathy. *Semin Nephrol* 2007; 27: 130-143.
- [16] Miyata T, Ueda Y, Horie K, Nangaku M, Tanaka S, van Ypersele de Strihou C and Kurokawa K. Renal catabolism of advanced glycation end products: the fate of pentosidine. *Kidney Int* 1998; 53: 416-422.
- [17] Liu X, Liu K, Wang Z, Liu C, Han Z, Tao J, Lu P, Wang J, Wu B, Huang Z, Yin C, Gu M and Tan R. Advanced glycation end products accelerate arteriosclerosis after renal transplantation through the AGE/RAGE/ILK pathway. *Exp Mol Pathol* 2015; 99: 312-319.
- [18] Morcos M, Sayed AA, Bierhaus A, Yard B, Waldherr R, Merz W, Kloeting I, Schleicher E, Mentz S, Abd el Baki RF, Tritschler H, Kasper M, Schwenger V, Hamann A, Dugi KA, Schmidt AM, Stern D, Ziegler R, Haering HU, Andrassy M, van der Woude F and Nawroth PP. Activation of tubular epithelial cells in diabetic nephropathy. *Diabetes* 2002; 51: 3532-3544.
- [19] Geoffroy K, Wiernsperger N, Lagarde M and El Bawab S. Bimodal effect of advanced glycation end products on mesangial cell proliferation is mediated by neutral ceramidase regulation and endogenous sphingolipids. *J Biol Chem* 2004; 279: 34343-34352.
- [20] Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, Bucciarelli LG, Rong LL, Moser B, Markowitz GS, Stein G, Bierhaus A, Liliensiek B, Arnold B, Nawroth PP, Stern DM, D'Agati VD and Schmidt AM. RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. *Am J Pathol* 2003; 162: 1123-1137.
- [21] Tanji N, Markowitz GS, Fu C, Kislinger T, Taguchi A, Pischetsrieder M, Stern D, Schmidt AM and D'Agati VD. Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. *J Am Soc Nephrol* 2000; 11: 1656-1666.
- [22] Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP and Cooper

- ME. Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. *Diabetologia* 1997; 40: 619-628.
- [23] Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM and Nawroth PP. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005; 83: 876-886.
- [24] Li J and Schmidt AM. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *J Biol Chem* 1997; 272: 16498-16506.
- [25] Flyvbjerg A, Denner L, Schrijvers BF, Tilton RG, Mogensen TH, Paludan SR and Rasch R. Long-term renal effects of a neutralizing RAGE antibody in obese type 2 diabetic mice. *Diabetes* 2004; 53: 166-172.
- [26] Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S, Okamoto H and Yamamoto H. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* 2001; 108: 261-268.
- [27] Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P and Bottinger EP. Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* 2001; 108: 807-816.
- [28] Loeffler I and Wolf G. Transforming growth factor-beta and the progression of renal disease. *Nephrol Dial Transplant* 2014; 29 Suppl 1: i37-i45.