Full Length Article

Naringin ameliorates diabetic nephropathy by inhibiting NADPH oxidase 4

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A B S T R A C T

Naringin, a naturally flavanone glycoside, has been previously demonstrated to alleviate diabetic kidney disease by inhibiting oxidative stress and inflammatory reaction. However, the underlying mechanism of naringin in diabetic nephropathy (DN) has not been fully elucidated. Here, the beneficial effect of naringin on DN in streptozotocin (STZ)-induced DN rats and high glucose (HG)-induced podocytes and its underlying mechanism were elaborated. The result revealed that naringin alleviated STZ-induced renal dysfunction and injury in DN rats, relieved STZ-induced oxidative stress in vivo and inhibited HG-induced apoptosis and reactive oxygen species level in vitro. More importantly, naringin inhibited NOX4 expression at mRNA and protein levels in STZ-induced DN rats and HG-induced podocytes. Loss of function indicated that NADPH oxidases 4 (NOX4) down-regulation suppressed apoptosis and reactive oxygen species level in HG-treated podocytes. Take together, this study demonstrated that naringin ameliorates diabetic nephropathy by inhibiting NOX4, contributing to a better understanding of the progression of DN.

1. Introduction

As the leading cause of end-stage kidney disease, diabetic nephropathy (DN) is one of the most common and severe chronic complications of type 1 and type 2 diabetes (Wang et al., 2011). The annual incidence of DN is increasing worldwide along with the epidemic rise in diabetes (Chen et al., 2015b). DN is morphologically characterized by glomerulosclerosis, excessive accumulation of extracellular matrix (ECM), glomerular hypertrophy and basement membrane thickening (Hou et al., 2014). It is well known that oxidative stresses involving in metabolic dysregulation in diabetes cause many diabetic complications (Noh and Ha, 2011). Accumulating evidence indicates that oxidative stress acts as a crucial mediator in the development and progression of DN and an abnormal increase of reactive oxygen species contributes to the pathogenesis of DN (Kitada et al., 2003; Wolf and Ziyadeh, 2007). Previous studies have demonstrated that the number of podocytes is remarkably reduced in DN, and podocyte damage and loss are related to the pathogenesis of DN (Toyoda et al., 2007; Wei and Dong, 2014). Although extensive researches have been made, the pathogenesis of DN is sophistication and has not been fully explained.

Naringin, a natural flavanone glycoside, is widely distributed in grapefruit and oranges and has been demonstrated to possess many kinds of therapeutic and pharmacological activities, such as anti-inflammatory, antiapoptotic, and antioxidant/free radical scavenging properties (Pereira et al., 2007). It has been revealed that naringin prevents oxidative damage by promoting glutathione (GSH) synthesis and decreasing malondialdehyde (MDA) level through a novel antioxidant defense mechanism against reactive oxygen species production (Dhanya et al., 2015). Recently, increasing attention has been focused on the effect of naringin on ameliorating diabetes and diabetic complications. For example, Mahmoud et al. found that naringin had antidiabetic effects in high fat fed/streptozotocin (STZ)-induced type 2 diabetes mellitus rats by attenuating hyperglycemia-mediated oxidative stress and proinflammatory cytokine production (Mahmoud et al., 2012). Chen et al. exhibited that naringin could protect against high glucose-induced injuries by inhibiting oxidative stress in H9c2 cardiac cells (Chen et al., 2014). Besides, naringin was also reported to show the antihyperglycemic and antioxidant effects in STZ-induced diabetic rats by increasing plasma insulin levels (Punithavathi et al., 2008). Notably, naringin has been previously demonstrated to alleviate diabetic kidney disease by inhibiting oxidative stress and inflammatory reaction (Chen et al., 2015a). However, the underlying mechanism of naringin in DN had not been fully elucidated.

NADPH oxidases 4 (NOX4), initially termed Renox, is the major source of reactive oxygen species production in renal cells including glomerular mesangial and podocyte cells and generate superoxide anion (O2⁻) by catalyzing the transfer of electrons from NADPH to molecular oxygen (Asaba et al., 2005; Yong et al., 2013). The elevated levels of reactive oxygen species derived from NOX4 are recognized as enhancing ECM accumulation in mesangial and tubular cells and

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mediating renal hypertrophy (Barnes and Gorin, 2011; Block et al., 2008). It is reported that NOX4 localizes to membranes and mitochondria in the renal cortex in STZ-induced DN, where it is abundantly expressed (Block et al., 2009). Moreover, NOX4 has been recently suggested to contribute to the initiation and development of renal injury in animal models of DN (Holterman et al., 2015; Susztak et al., 2006).

In the present study, the animal model of DN by STZ and DN cell model by high glucose were established to examine the effects of naringin on DN and to investigate whether naringin ameliorated DN by regulating NOX4 in DN.

2. Materials and methods

2.1. Animals and treatment

All animal experiments were performed with approval of Ethical Committee and Institutional Animal Care and Use Committee of Huaile Hospital of Henan University. Male Sprague-Dawley (SD) rats (aged 8–9 weeks and weighing 200–250 g) were obtained from BioLasCO (Taipei, Taiwan) and were housed at 24 ± 2 °C in cages under a 12 h light/dark cycle with access to normal basal diet and water ad libitum. The rats were randomly divided into five groups (n =10/per group): control, DN, DN + naringin (20 mg/kg; Sigma, St. Louis, MO, USA), DN + naringin (40 mg/kg), and DN + naringin (80 mg/kg). The rats receiving a tail vein injection of 0.1 M citrate buffer (pH 4.5) was considered as controls. To induce rat models of DN, the other groups were treated with a tail vein injection of STZ (80 mg/kg). The rats receiving a tail vein injection of 0.1 M citrate buffer (pH 4.5) were considered as controls. To induce rat models of DN, the other groups were treated with a tail vein injection of STZ (80 mg/kg body weight; Sigma) dissolving in 0.1 M citrate buffer (pH 4.5). After consecutive injection for 5 days, rats with blood glucose levels >16.7 mM were regarded as being diabetic and selected for the subsequent experiments. One week later, the diabetic rats in DN + naringin groups were treated with injection of different dosage of naringin into tail vein daily for 12 weeks. At the same time point, the rats in the control and DN group were injected with equivalent volume of sterile normal saline via tail vein. At the end of the experiment, all animals were killed in deep anaesthesia and peripheral blood, urine and kidney were harvested for further analysis.

2.2. Biochemical analysis

The kidney weight and body weight were measured at 12 weeks after administration of naringin injection. Blood samples of rats were centrifuged and prepared for biochemical test. The measurement of blood urea nitrogen (BUN) and serum creatinine (Scr) in peripheral blood were performed by using commercially-available kits (Jiancheng BioTech, China). The concentration of blood glucose was estimated by Glutest E II (Kyoto Daiichi Kagaku, Kyoto, Japan). The 24 h-urinary protein level was determined by Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

2.3. Enzyme-linked immunosorbent assay (ELISA)

Kidney tissues were cut into small pieces and grinded in a homogenization buffer at 4 °C. To assess oxidative stress injury, the levels of oxidative stress markers superoxide dismutase (SOD), MDA, glutathione peroxidase (GSH-Px) and reactive oxygen species production in the kidney tissue homogenate were detected by ELISA using relevant test kits (Beyotime, Haimen, China) for protein extraction. Equal amounts of proteins (30 μg) were separated by 10% SDS-PAGE, electrotransferred into polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA, USA) membranes and blocked with 5% non-fat dry milk in PBS for 1 h. The membrane was then incubated with the primary antibodies against Cleaved caspase3, NOX4 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by incubating with a horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA, USA) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence reagents (Amersham Biosciences, Inc., Little Chalfont, UK).

2.4. Cell culture, model and transfection

Conditionally immortalized mouse podocytes were purchased from the Cell Culture Center, Peking Union Medical College (Beijing, China) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) at 33 °C containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 100 U/ml penicillin G/streptomycin, 5 mM glucose and 10 U/ml recombinant murine interferon-γ (Sigma).

Cell transfection with NOX4 siRNA or siRNA control (con siRNA) (GenePharma, Shanghai, China) was performed by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). After incubated with 10 μM naringin for 2 h, non-transfected or transfected podocytes were exposed to 30 mM d-glucose (high glucose, HG) for 24 h to induce cell model of DN.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from kidney cortex homogenate or podocytes by using TRIzol reagent (Invitrogen). qRT-PCR was performed to measure the expression of NOX4 using a LightCycler 350 s RT-PCR system (Roche, Mannheim, Germany) with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche). NOX4 primers were as follows: forward 5'-GGCTGGAGGCATTGGAGTAA-3' and reverse 5'-CCAGTCATCACAACAGGTGT-TT-3'. The expression of NOX4 was normalized to β-actin mRNA and calculated by the 2^−ΔΔCt method.

2.6. Western blot analysis

Podocytes and kidney cortex homogenate were rapidly lysed in cell lysis buffer (Beyotime, Haimen, China) for protein extraction. Equal amounts of proteins (30 μg) were separated by 10% SDS-PAGE, electrotransferred into polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA, USA) membranes and blocked with 5% non-fat dry milk in PBS for 1 h. The membrane was then incubated with the primary antibodies against Cleaved caspase3, NOX4 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight, followed by incubating with a horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA, USA) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence reagents (Amersham Biosciences, Inc., Little Chalfont, UK).

2.7. Histological examination for tubular damage

Periodic-acid schiff (PAS) were performed for histological examination of tubular damage. The renal tissues of 2 μm thickness were fixed in Methyl Carnoy’s solution and embedded in paraffin. Then the tissues were stained with PAS reagent (Carlo Erba, Milan, Italy) and counterstained with hematoxylin. Tubular damage in randomly selected PAS-stained sections was examined under a light microscope (Olympus PM-10AQ, Japan) (×400 magnification).

2.8. Assessment of reactive oxygen species

The oxidation-sensitive fluorescent probe DCFH-DA was used to analyze intracellular reactive oxygen species levels using the Reactive Oxygen Species Assay Kit (Beyotime, Jiangsu, China). Podocytes were incubated with DCFH-DA (10 μM) for 30 min at 37 °C and analyzed using a Becton Dickinson FACScalibur instrument (Franklin Lakes, NJ, USA) equipped with CellQuest software. The cellular fluorescence intensities were obtained at excitation and emission wavelengths of 485 nm and 530 nm.

2.9. Flow cytometry analysis

Apoptosis in confluent podocytes was assessed by an annexin V/propidium iodide (PI) double staining apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. The cells that were annexin V-positive and PI-negative for early stages of apoptosis were counted with a FACScalibur flow cytometer (Beckman-Coulter, Indianapolis, IN, USA).
Fig. 1. Effect of naringin on renal functional parameters and injury in STZ-induced DN rats. (A) The ratio of kidney/body weight of treated rats. The serum levels of BUN (B), blood glucose (C), 24 h-urinary protein level (D) and Scr (E) were detected in treated rats. (F) Extracellular matrix deposition (indicated by arrows) as determined by PAS staining (400×). *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 2. Effect of naringin on oxidative stress and apoptosis in STZ-induced DN rats or HG-treated podocytes. The concentrations of MDA (A), SOD (B), reactive oxygen species (C) and GSH-Px (D) in kidney tissues of treated rats were detected by ELISA. (E) Apoptosis of treated podocytes was examined by flow cytometry. (F) The level of Cleaved caspase3 in treated podocytes was determined by western blot. (G) The intracellular reactive oxygen species level in treated podocytes was evaluated by DCFH-DA fluorescent probe and quantified by the fluorescence intensities. *p < 0.05, **p < 0.01, ***p < 0.001.
2.10. Statistical analysis

All data are presented as means ± standard deviation (S.D.) and analyzed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences were analyzed by Student’s t-test or one-way ANOVA. A P-value of less than 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Naringin alleviated STZ-induced renal dysfunction and injury in DN rats

In order to confirm the effects of naringin on renal system of STZ-induced DN rats, the kidney index and renal functional parameters were monitored including the kidney/body weight, BUN, blood glucose, 24 h-urinary protein level and Scr. Compared with the NC groups, the ratio of kidney/body weight (Fig. 1A), serum levels of BUN (Fig. 1B), blood glucose (Fig. 1C), 24 h-urinary protein (Fig. 1D) and Scr (Fig. 1E) were all significantly increased in the DN groups. However, naringin treatment exhibited a dose-dependent decrease in these renal functional parameters in STZ-induced DN rats. In addition, to assess the kidney tissue injury, extracellular matrix deposition was determined by PAS staining. The results showed that the PAS staining (indicated by arrows) was increased in STZ-induced DN rats compared with the NC group, whereas naringin treatment markedly decreased PAS staining in DN rats (Fig. 1F). These data indicated that naringin mitigated STZ-induced renal dysfunction and injury in DN rats.

3.2. Naringin relieved STZ-induced oxidative stress in vivo and inhibited HG-induced apoptosis and reactive oxygen species level in vitro

To evaluate the effects of naringin on oxidative stress injury in DN, the levels of MDA, SOD, GSH-Px, and reactive oxygen species were measured. STZ treatment resulted in a significant increase of the levels of MDA and reactive oxygen species, and a remarkable decrease of the levels of SOD and GSH-Px in kidney tissues of rats, indicating an elevation of oxidative stress in DN rats. However, naringin treatment dramatically reduced the levels of MDA (Fig. 2A) and reactive oxygen species (Fig. 2C) and improved SOD (Fig. 2B) and GSH-Px (Fig. 2D) levels in a dose-dependent manner in the kidney of STZ-induced DN rats. As demonstrated by flow cytometry analysis, the apoptosis rate of HG-induced podocytes was significantly promoted with respect to the NC group, while naringin treatment conspicuously alleviated this effect (Fig. 2E). Accordingly, HG led to an obvious increase of the level of Cleaved caspase 3 in podocytes in comparison with NC group, while naringin strikingly inhibited this effect (Fig. 2F). Cellular reactive oxygen species formation in podocytes was monitored by DCFH-DA conversion to DCF and it is noticed that DCF fluorescence was considerably strong in HG-induced podocytes when compared to the NC group, indicating an elevation of reactive oxygen species production. However, the fluorescence intensity was dramatically reduced in HG-induced podocytes in the presence of naringin (Fig. 2G). Taken together, these results demonstrated that naringin inhibited STZ-induced oxidative stress injury in vivo and suppressed HG-induced apoptosis and reactive oxygen species level in vitro.

3.3. Naringin inhibited NOX4 expression at mRNA and protein levels in STZ-induced DN rats and HG-induced podocytes

In order to investigate the interaction between naringin and NOX4, the influence of naringin on expression of NOX4 at mRNA and protein levels was detected by qRT-PCR and western blot. The results indicated that STZ and HG both markedly increased the expression of NOX4 at mRNA and protein levels. However, naringin significantly restrained the expression of NOX4 at mRNA and protein levels in STZ-induced DN rats (Fig. 3A and B) in a dose-dependent manner and in HG-treated podocytes (Fig. 3C and D), suggesting that naringin repressed NOX4 expression at mRNA and protein levels in STZ-induced DN rats and HG-induced podocytes.

3.4. NOX4 down-regulation inhibited apoptosis and reactive oxygen species level in HG-treated podocytes

To explore the role of NOX4 in DN, siRNA-mediated NOX4 down-regulation was performed in HG-treated podocytes. The expression of NOX4 at mRNA (Fig. 4A) and protein (Fig. 4B) levels in NOX4 siRNA-
transfected podocytes in the presence of HG was confirmed by qRT-PCR and western blot, indicating the effectiveness of NOX4 siRNA knockdown. As compared with the con siRNA group, flow cytometry analyses implied that NOX4 down-regulation remarkably inhibited apoptosis in HG-treated podocytes (Fig. 4C). Besides, NOX4 down-regulation markedly reduced the level of Cleaved caspase3 in HG-treated podocytes in comparison with con siRNA-transfected cells (Fig. 4D). Furthermore, the DCF fluorescence intensity was obviously reduced in NOX4 siRNA-transfected podocytes in the presence of HG compared to con siRNA-transfected cells, revealing that NOX4 down-regulation decreased reactive oxygen species level in HG-treated podocytes. These findings implicated that NOX4 down-regulation led to a remarkable suppression of apoptosis and reactive oxygen species level in HG-treated podocytes.

4. Discussion

As one of the leading causes of chronic renal failure worldwide, DN is a high risk factor for vascular disease, affecting approximately 25% and 40% of all patients with type 1 and type 2 diabetes, respectively (Chen et al., 2015a; Eid et al., 2016). Generally, DN is functionally characterized by glomerular hyperfiltration and glomerular hypertrophy, followed by the elevated urinary albumin excretion (Chiarelli et al., 2009; Xu et al., 2016). The increased urinary albumin excretion, Scr accumulation and kidney dysfunction are regarded as the important indexes in DN patients (Zhang et al., 2014). The STZ-induced diabetic rat model has been extensively used in the field of diabetes and diabetic complications (Kitada et al., 2016). In addition, it is well known that HG is an initiating factor that contributes to tubular damage and apoptosis in DN (Xiao et al., 2014). Consequently, HG-induced podocytes are used as an in vitro model for studies of early-stage DN (Liu et al., 2016). In the present study, the rat and cell models of DN were successfully established to evaluate the effect of naringin on DN. The present study confirmed naringin could alleviate STZ-induced renal injuries, consistent with the previous study (Chen et al., 2015a).

A large body of evidence has suggested that chronic hyperglycemia-induced oxidative stress played pivotal roles in the initiation and development of DN (Kashihara et al., 2010; Singh et al., 2011). The degree of oxidative stress mainly depends on the balance between reactive oxygen species generation, especially O$_2^-$ and hydrogen peroxide (H$_2$O$_2$), and the antioxidant defense system that includes SOD and peroxidases (Abdo et al., 2015). It is reported that endogenous antioxidant molecules which diminish reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009). Kidney is susceptible to oxidative stress and is directly damaged by reactive oxygen species-induced renal injury, including SOD and GSH-Px, are significantly reduced in patients with DN (Wagener et al., 2009).
A previous study indicated that NOX4, the main source of reactive oxygen species in the kidney during early stages of diabetes, was upregulated in podocytes in response to HG treatment and NOX4 deletion afforded renoprotection in a mouse model of DN (Jha et al., 2016). Besides, NOX4 deletion alleviated oxidative stress and renal injury via PKC-dependent mechanism in DN (Thallas-Bonke et al., 2014). Furthermore, the NOX1/NOX4 inhibitor GKT1377831 attenuated renal pathology in DN by reducing glomerular hypertrophy, mesangial matrix expansion, urinary albumin excretion, and podocyte loss (Gorin et al., 2015). Consistently, the present study further revealed that NOX4 down-regulation repressed apoptosis and reactive oxygen species level in HG-stimulated podocytes. Mechanistic analyses further demonstrated that naringin could inhibit NOX4 expression at mRNA and protein levels in both animal and cell models of DN, suggesting that naringin attenuated STZ-induced oxidative stress damage and HG-induced apoptosis and reactive oxygen species level by inhibiting NOX4.

In conclusion, this study demonstrated naringin attenuated kidney injury and oxidative stress in STZ-induced DN rats, and suppressed apoptosis and reactive oxygen species level in HG-induced podocytes. Naringin inhibited NOX4 expression in STZ-induced DN rats and HG-induced podocytes. Moreover, down-regulation of NOX4 decreased apoptosis and reactive oxygen species level in HG-induced podocytes. This study suggested that naringin exerted its protective roles in DN by inhibiting NOX4 expression, contributing to a better understanding of the progression of DN.

Author contributions
Hui Cong Li designed the experiments. Fang Chen and Jun Shi performed the experiments and acquired the data. Junwei Zhang and Suzia Yang analyzed the data and prepared the manuscript.

Conflicts of interest
The authors have no conflict of interest to declare.

References
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