Restoration of Mimecan Expression by Grape Seed Procyanidin B2 Through Regulation of Nuclear Factor-kappaB in Mice With Diabetic Nephropathy

Yi Zhou,[1,2] Bao-ying Li,[1] Xiao-li Li,[1] Ya-juan Wang,[1] Zhen Zhang,[1] Fei Pei,[1] Quan-zhen Wang,[1] Jun Zhang,[1] Ya-wei Cai,[1,3] Mei Cheng,[1] Hai-qing Gao[1]

[1]Department of Geriatrics, Qi-Lu Hospital of Shandong University, Key Laboratory of Cardiovascular Proteomics of Shandong Province, Jinan, Shandong, China
[2]Department of Cardiovasology, Taian City Central Hospital, Tai'an, Shandong, China
[3]Department of Geriatrics, Ningbo No 2 Hospital, Ningbo, Zhejiang, China

Keywords. grape seed procyanidin B2, mimecan, nuclear factor-κB, mice, diabetic nephropathy

Grape seed procyanidin B2 (GSPB2) exerts a variety of potent protective pharmacological effects on diabetic complications. The renal protective effects of GSPB2 and the target protein mimecan regulated by GSPB2, discovered in a previous quantitative proteomic analysis, were assessed in mice with diabetic nephropathy Twenty-four db/db mice were divided into 2 groups of the vehicle-treated and GSPB2-treated (30 mg/kg/d) diabetic groups. All animals were observed for 10 weeks. Treatment with GSPB2 resulted in an improvement in body weight increase and serum levels of triglyceride, total cholesterol, advanced glycation end products, and urinary albumin excretion in comparison with the vehicle-treated diabetic mice ($P < .05$), although these levels were still higher than those in the control group. Treatment with GSPB2 significantly reduced the extent of glomerular basement membranes thickening, mesangial expansion, and glomerular area as well. Mimecan protein expressions in diabetes mellitus were decreased approximately by 28% when compared with those in the control group ($P < .05$), and restored remarkably after GSPB2 treatment ($P < .05$). The expression of nuclear factor-κB (NF-κB) p65 in nuclear extracts, markedly higher in the diabetic mice than in the controls, was significantly suppressed by GSPB2. The findings of this study revealed that mimecan might become a new therapeutic target in the future and indicated that GSPB2 had beneficial effects not only on oxidative stress, but also on renal fibrosis, particularly in the diabetic kidney.

IJKD 2016;10:325-31
www.ijkd.org

Diabetic nephropathy (DN) occurs in about 35% of diabetic patients and is becoming a major health problem worldwide.[1-3] Numerous studies have pointed to the role of long-term hyperglycemia-induced oxidative stress in the development of DN.[4,5] Therefore, alleviating oxidative stress may be a novel prospective approach for DN.[6] Our previous studies demonstrated that grape seed proanthocyanidin extract exerts a variety of potent protective pharmacological effects on diabetic complications, especially on diabetic kidney,[7-9] attributed to antioxidant, anti-inflammatory, anti-atherosclerosis, and free-radical scavenging effects.[10,11] Grape seed procyanidin B2 (GSPB2) is one of the main dimeric forms, which has higher biological activities than other water-soluble polyphenos. We investigated the protective effects of GSPB2, on renal complications of mice.

In our team previous study,[12] we resorted to a quantitative proteomic assay to get the differential

renal protein profiles in GSPB2-treated and untreated diabetic mice. Of all the differentially expressed proteins, mimecan showed a 0.232-fold decrease in db/db mice versus db/m mice, and backregulated by GSPB2 treatment at a ratio of 2.064. Mimecan is closely related to atherosclerosis,[13,14] cardiomyopathies,[15] neovascularization,[16] and decreased heart dilation risk.[17] Therefore, we assessed the significance of mimecan as a marker to renal dysfunction and investigated whether GSPB2 could prevent renal injury through an anti-inflammatory mechanism in DN.

Grape seed procyanidin B2 (Lot No: 20100915, purity > 92%; Jianfeng Inc, Tianjin, China) was extracted from red grape with concentration of 6.75 mg/mL. Male C57BLKS/J db/db (n = 24, 7 weeks old) and db/m littermates (n = 12, 7 weeks old) were supplied by Model Animal Research Center, Nanjing University (Jiangsu, China). The experimental protocol was approved by the animal ethics committee of Shandong University. The db/m mice were assigned to a normal control group and the db/db mice were divided into 2 groups of the vehicle-treated (normal saline solution) diabetic group (n = 12) and GSPB2-treated diabetic group (n = 12) that was given daily with 30 mg/kg of GSPB2 in normal saline solution orally. All animals were observed for 10 weeks without any intervention of hypoglycemic therapy, and assessed weekly. Serum advanced glycation end products (AGEs) specific fluorescence emissions were detected at 440 nm by exciting at 370 nm with a fluorescence spectrophotometer (Hitachi F-2500, Japan). At the end of the administration, urinary albumin excretion was measured in 24-hour urine samples. After that, all the animals, fasted overnight, were sacrificed. Fasting blood was sampled and the serum was stored at -80°C for various laboratory test and quantification. Perfused kidneys were rapidly dissected and then frozen in liquid nitrogen until further analysis. Renal tissues were examined for histopathological changes by light microscopy. A specimen of the kidney cortex was homogenized and centrifuged (4°C, 12 000 g for 10 minutes). The nuclear pellet was washed twice in buffer A and resuspended in 200 µL of ice-cold buffer B for 10 minutes. The tube was shaken vigorously (4°C, 15 minutes). After being centrifuged, the supernatant was collected and frozen at -80 °C for later measurement. The protein content was evaluated using the Bradford method.

To demonstrate the effects of GSPB2 on the DN, 40 µg of nuclear extracts were loaded on a 12% Tris-glycine gel and separated under reducing conditions. Then the proteins were transferred in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The blots were blocked with 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20 containing 5% non-fat dry milk powder for an hour at room temperature before being immunoblotted with each primary antibody overnight at 4°C. After washing in Tris-buffered saline with Tween, the antigen-antibody binding was detected using appropriate secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK). The intensities were analyzed with the NIH software ImageJ. Each experiment was repeated at least three times.

At the beginning of the experiment, the average body weights were similar for the vehicle-treated and GSPB2-treated groups, which exhibited substantial obesity compared with the normal db/m mice. During the observation period, as shown in Table 1, body weights in the diabetic group and

Table 1. Effect of Grape Seed Procyanidin B2 (GSPB2) on db/db Mice Body Weight*

Body Weight, g	Control Group (n = 12)	Diabetic Group (n = 12)	GSPB2-treated Diabetic Group (n = 12)
Time of Measurement			
8 weeks old	19.19 ± 2.25	39.20 ± 2.34†	38.88 ± 3.51†
12 weeks old	23.14 ± 2.15	47. 53 ± 1.35†	44.41 ± 2.01†‡
16 weeks old	24.69 ± 1.53	53.19 ± 2.31†	47.47 ± 3.42†‡
18 weeks old	26.63 ± 2.45	58.49 ± 3.04†	52.28 ± 2.57†‡
Body weight gain	7.44 ± 2.09	19.29 ± 2.26†	13.40 ± 2.88†‡

*Values are mean ± standard deviation.
†P < .05 compared to the controls
‡P < .05 compared to the diabetic group

GSPB2 diabetic group were consistently larger than in the control group ($P < .05$). However, from the 2nd week and onwards, the increase in the body weight was significantly lower in the GSPB2 group as compared to the diabetic group ($P < .05$).

The effects of GSPB2 on the fasting blood glucose, lipid profile, AGEs and 24-hour urinary albumin excretion rate are shown in Table 2. During of the experiment, db/db diabetic mice exhibited a dramatically elevated levels of these factors at the age of 18 weeks old compared with the control group ($P < .05$). In addition, the development of diabetes mellitus in the db/db mice resulted in increased urinary albumin levels compared to the normal control group ($P < .05$). Treatment for 10 weeks with GSPB2 in the db/db mice resulted in an improvement of triglyceride, total cholesterol, AGEs, and urinary albumin excretion levels in comparison with the vehicle-treated diabetic mice ($P < .05$), although these levels were still higher than those in the control group ($P < 0.05$). However, GSPB2-treatment showed a slight decrease in serum blood glucose that did not achieve statistical significance compared to the vehicle-treated db/db mice.

Renal histological sections stained with period acid-Schiff were depicted under light microscope in Figure 1. Thickened glomerular basement membranes, diffuse mesangial expansion, and an increase in the glomerular size related to matric accumulation and mesangial cell proliferation were observed in the db/db mice in comparison with the control mice. Treatment with GSPB2 significantly reduced the extent of glomerular basement membranes thickening, mesangial expansion, and glomerular area seen in the diabetic mice.

We carried out western blot to confirm mimecan protein expression in db/db mice and in their nondiabetic db/m littermates. As shown in Figure 2, the mimecan protein expressions in diabetes mellitus were decreased approximately by 28% when compared with those in the control group ($P < .05$), and restored remarkably after GSPB2 treatment ($P < .05$), corresponding to the quantitative proteomic assay measurement. We further examined whether GSPB2 affected nuclear factor-κB (NF-κB) p65 activation by western blotting. The expression of NF-κB p65 in nuclear extracts was markedly higher in the diabetic mice than in the controls ($P < 0.05$), and was significantly suppressed by treatment with GSPB2 (Figure 3), although the level of NF-κB p65 in GSPB2 group was still higher than that in the control group ($P < .05$). These results suggested that high glucose activated NF-κB p65, and GSPB2 downregulated the activation of NF-κB p65 in diabetic kidney.

Our present study suggested that GSPB2 has an anti-obesity effect on type 2 diabetes mellitus.

Table 2. Effect of Grape Seed Procyanidin B2 (GSPB2) on Laboratory Factors*

Factor	Control Group (n = 12)	Diabetic Group (n = 12)	GSPB2-treated Diabetic Group (n = 12)
Fasting blood glucose, mmol/L	8.76 ± 1.32	33.48 ± 4.17†	28.79 ± 3.54†
Triglyceride, mmol/L	0.78 ± 0.18	1.45 ± 0.25†	1.05 ± 0.13†‡
Total cholesterol, mmol/L	2.34 ± 0.31	3.89 ± 0.42†	3.01 ± 0.40†‡
Advanced glycation end products, AU/mg	0.18 ± 0.03	0.38 ± 0.06†	0.29 ± 0.02†‡
24-hour urinary albumin, μg	18.92 ± 4.18	204.66. ± 22.36†	130.59 ± 29.31†‡

*Values are mean ± standard deviation.
†P < .05 compared to the controls
‡P < .05 compared to the diabetic group

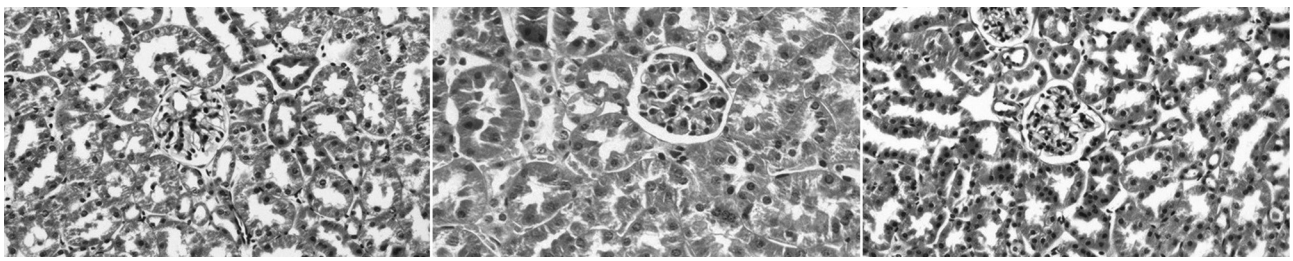

Figure 1. Renal histological sections stained with periodic acid-Schiff.

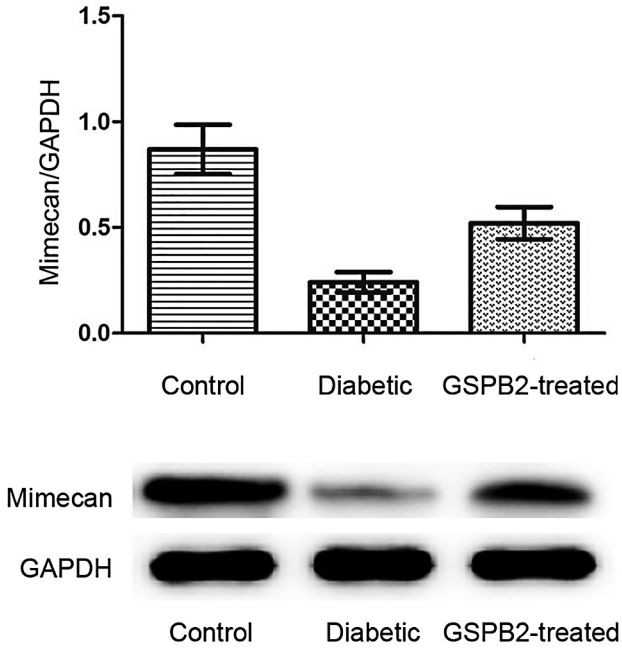

Figure 2. Mimecan protein expression in db/db mice with and without grape seed procyanidin B2 (GSPB2) and in their nondiabetic db/m littermates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control.

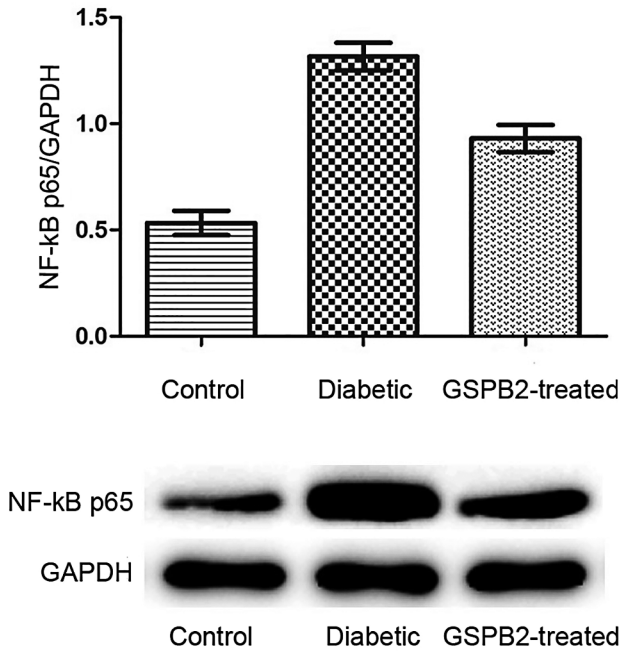

Figure 3. Expression of nuclear factor (NF)-κB p65 in nuclear extracts from db/db mice with and without grape seed procyanidin B2 (GSPB2) and their nondiabetic db/m littermates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control.

In addition, GSPB2 decreased lipid levels in mice, which was thought to be related to reduction of lipid formation.[18] Previous studies have reported that GSPB2 treatment reduced the plasma glucose levels of db/db mice, but very close to that of vehicle-treated db/db mice without significant alteration,[7] suggesting its renal protective effects to be independent of anti-hyperglycemia.

Accumulation of AGEs resulted from excessive oxidative stress, a pathogenic factor in sustained hyperglycemia-mediated kidney injuries, accounts for the pathogenesis of diabetic vascular complications.[12] In our present study, GSPB2 showed significant decreases in the serum AGEs levels in diabetic mice, suggesting that GSPB2 would alleviate the oxidative stress under diabetes mellitus and improve kidney function through the inhibition of AGEs. Furthermore, GSPB2 treatment decreased the levels of triglyceride and cholesterol, which are upregulated under diabetes. These findings indicate that oxidative stress is increased in the diabetic mice kidney and that GSPB2 can prevent renal damage associated with diabetes by attenuating the oxidative stress.

Diabetic nephropathy is characterized with an excessive increase of albuminuria, thickened glomerular and tubular basement membranes, glomerular hypertrophy and sclerosis, mesangial expansion and increased amount of mesangial matrix.[19,20] Mesangial expansion and albuminuria, which are considered as the most striking characteristic of DN, are strongly related to ESRD in human diabetics.[21] The db/db mice used in the present study is a rodent model for type 2 diabetes mellitus, closely simulates the pathophysiological abnormalities and deteriorates renal alterations similar to that of human DN.[22] The rodent model has been used in studying the pathophysiology or in evaluating therapeutic plans for type 2 diabetes mellitus in many laboratories.[23] In this study, the elevated levels of albuminuria and exacerbated renal histological alterations in diabetic mice which are resulted from abnormal glycometabolism indicated the development of renal injury and dysfunction. Our study showed that GSPB2 treatment depressed urine albumin excretion in diabetic mice and significantly attenuated glomerular hypertrophy, mesangial expansion, and glomerular basement membranes thickening in db/db mice similar to the control mice, indicating GSPB2 may effectively reverse diabetic kidney dysfunction and protect the kidneys, especially the glomeruli, against renal deteriorative progression to ESRD.

Mimecan has not been linked directly with diabetic nephropathy, although progress has recently been made in establishing of the relationships between mimecan and chronic cardiovascular disease. We, therefore, performed western blot analysis to evaluate mimecan expression with or without GSPB2 treatment in db/db mice. A signal pathway that exhibits increased expression levels in diabetic mice could be a clue to help us find molecular pathogenesis of chronic kidney disease. Mimecan is the 7th member of small leucine-rich proteoglycan family that was originally named osteoinductive factor,[24] also known as osteoglycin. The small leucine-rich proteoglycans share a mutual protein structure that is formed by 6 to 11 repeats of leucine-rich regions and have core proteins region in size from 25 to 62 kDa. Mimecan is a well characterized secretory protein in the cornea, bone, kidney, and other tissues,[25] and appears to undergo proteolytic process in vivo. Mimecan, which is known to be an essential constituent of the normal extracellular matrix, plays an important role in promoting the degrading of type I collagen.[26] Cornea and subcutaneous collagen fiber layers were markedly thicker in mice with the osteoglycin genes knocked down than in those of the normal counterparts, which was mainly caused by type I collagen metabolic disorders.[27]

In our western blot analysis, mimecan was significantly reduced in diabetic kidneys, and restored after GSPB2 treatment, which was consistent with our previous quantitative proteomic analysis. This result demonstrated that mimecan may be involved in the pathogenesis of diabetic fibrotic complication and played a beneficial role in the development of diabetic nephropathy; meanwhile GSPB2 was reported to possess an extra excellent effect on renal prevention. In mice with chronic kidney disease, decreased serum mimecan level is positively associated with deteriorated renal function and thickened basement, indicating the degrading of type I collagen is declined by reduced expression of mimecan. After GSPB2 treatment, restored mimecan can increase the degradation of collagen and reduce the thickening of basement membrane, which is consistent with our histological results.

We established the underlying molecular mechanism by which declined mimecan accelerated diabetic kidney injury, and uncovered that mimecan was weakened by activation of signaling pathway. We found the level of NF-κB p65 was elevated in the nuclear extract of diabetic mice, whereas GSPB2 deactivated over- activation of NF-κB p65 in the kidney caused by hyperglycemia. Hence, we deduced that reduced mimecan may aggravate diabetic renal fibrosis partially by increased level of NF-κB p65.

As a ubiquitous transcription factor, NF-κB, one of the cross-talk points of multiple inflammatory signal transduction pathways, plays a pivotal role in the regulation of many inflammatory molecules.[28] It is demonstrated that the nuclear translocation of NF-κB has increased in human and experimental kidney diseases.[29] However, the correlation of NF-κB and DN has not been well investigated and detecting the role of NF-κB activation in GSPB2-pretreatment db/db mice renal impairment is very limited, because the potential link between the NF-κB and GSPB2 has not been valued. We demonstrated in present work that activated nuclear NF-κB has been detected in db/db mice, and NF-κB activation took part in renal fibrosis induced by high glucose, meanwhile GSPB2 could inhibit NF-κB-mediated inflammatory response in diabetic mice. This research had confirmed not only that NF-κB had a close relationship with the diabetes mellitus and fibrosis, but also that the inflammatory response of diabetes was effectively attenuated by GSPB2. This suggests that GSPB2 mediates mimecan protein expression restoration, at least in part, through NF-κB p65 pathway.

Mimecan was determined to be downregulated in db/db mice. Decreased mimecan as well as activation of NF-κB p65 signaling pathway was correlated with the pathogenesis of diabetic nephropathy using a diabetic mouse model. Our research for the first time identified that GSPB2 could protect diabetic kidneys by reversing mimecan protein expression and inhibiting NF-κB p65 signaling pathway. Treatment with GSPB2 has beneficial effects not only on oxidative stress but also on renal fibrosis, particularly in the diabetic kidney.

FINANCIAL SUPPORT

This work was supported by the National Natural Science Foundation of China (81100595), Science and Technology Development Plan of Shandong Province (2014GGH218028), Natural Science

Foundation of Shandong Province (ZR2014HM071), and Fundamental Research Funds of Shandong University (2014QLKY28).

ACKNOWLEDGMENTS

The authors acknowledge the personnel of the Medical Science Academy of Shandong, the personnel the Research Center for the proteome analysis, and the Shanghai Institute for Biological Sciences for their technical support.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Heerspink HJ, de Zeeuw D. The kidney in type 2 diabetes therapy. Rev Diabet Stud. 2011;8:392-402.
2. Luis-Rodriguez D, Martinez-Castelao A, Gorriz JL, De-Alvaro F, Navarro-Gonzalez JF. Pathophysiological role and therapeutic implications of inflammation in diabetic nephropathy. World J Diabetes. 2012;3:7-18.
3. Young BA, Maynard C, Boyko EJ. Racial differences in diabetic nephropathy, cardiovascular disease, and mortality in a national population of veterans. Diabetes Care. 2003;26:2392-9.
4. Forbes JM, Coughlan MT, Cooper ME. Oxidative stress as a major culprit in kidney disease in diabetes. Diabetes. 2008;57:1446-54.
5. Takayanagi R, Inoguchi T, Ohnaka K. Clinical and experimental evidence for oxidative stress as an exacerbating factor of diabetes mellitus. J Clin Biochem Nutr. 2011;48:72-7.
6. Palsamy P, Subramanian S. Resveratrol protects diabetic kidney by attenuating hyperglycemia-mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling. Biochim Biophys Acta. 2011;1812:719-31.
7. Li XL, Li BY, Gao HQ, et al. Proteomics approach to study the mechanism of action of grape seed proanthocyanidin extracts on arterial remodeling in diabetic rats. Int J Mol Med. 2010;25:237-48.
8. Li X, Xiao Y, Gao H, et al. Grape seed proanthocyanidins ameliorate diabetic nephropathy via modulation of levels of AGE, RAGE and CTGF. Nephron Exp Nephrol. 2009;111:e31-41.
9. Li BY, Cheng M, Gao HQ, et al. Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. J Cell Biochem. 2008;104:668-79.
10. Houde V, Grenier D, Chandad F. Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharides of periodontopathogens. J Periodontol. 2006;77:1371-9.
11. Shao ZH, Becker LB, Vanden Hoek TL, et al. Grape seed proanthocyanidin extract attenuates oxidant injury in cardiomyocytes. Pharmacol Res. 2003;47:463-9.
12. Zhang Z, Li BY, Li XL, et al. Proteomic analysis of kidney and protective effects of grape seed procyanidin B2 in db/db mice indicate MFG-E8 as a key molecule in the development of diabetic nephropathy. Biochim Biophys Acta. 2013;1832:805-16.
13. Kwon HM, Hong BK, Kang TS, et al. Expression of osteopontin in calcified coronary atherosclerotic plaques. J Korean Med Sci. 2000;15:485-93.
14. Fernandez B, Kampmann A, Pipp F, Zimmermann R, Schaper W. Osteoglycin expression and localization in rabbit tissues and atherosclerotic plaques. Mol Cell Biochem. 2003;246:3-11.
15. Petretto E, Sarwar R, Grieve I, et al. Integrated genomic approaches implicate osteoglycin (Ogn) in the regulation of left ventricular mass. Nat Genet. 2008;40:546-52.
16. Shanahan CM, Cary NR, Osbourn JK, Weissberg PL. Identification of osteoglycin as a component of the vascular matrix. Differential expression by vascular smooth muscle cells during neointima formation and in atherosclerotic plaques. Arterioscler Thromb Vasc Biol. 1997;17:2437-47.
17. Ma QY, Zuo CL, Ma JH, et al. Glucocorticoid up-regulates mimecan expression in corticotroph cells. Mol Cell Endocrinol. 2010;321:239-44.
18. Lu WD, Li BY, Yu F, et al. Quantitative proteomics study on the protective mechanism of phlorizin on hepatic damage in diabetic db/db mice. Mol Med Rep. 2012;5:1285-94.
19. Dong YF, Liu L, Lai ZF, et al. Aliskiren enhances protective effects of valsartan against type 2 diabetic nephropathy in mice. J Hypertens. 2010;28:1554-65.
20. Yu R, Mao J, Yang Y, Zhang Y, Tian Y, Zhu J. Protective effects of calcitriol on diabetic nephropathy are mediated by down regulation of TGF-beta1 and CIP4 in diabetic nephropathy rat. Int J Clin Exp Pathol. 2015;8:3503-12.
21. Koya D, Haneda M, Nakagawa H, et al. Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC beta inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. FASEB J. 2000;14:439-47.
22. Tesch GH, Lim AK. Recent insights into diabetic renal injury from the db/db mouse model of type 2 diabetic nephropathy. Am J Physiol Renal Physiol. 2011;300:F301-10.
23. Wang HJ, Jin YX, Shen W, et al. Low dose streptozotocin (STZ) combined with high energy intake can effectively induce type 2 diabetes through altering the related gene expression. Asia Pac J Clin Nutr. 2007;16 Suppl 1:412-7.
24. Tanaka K, Matsumoto E, Higashimaki Y, et al. Role of osteoglycin in the linkage between muscle and bone. J Biol Chem. 2012;287:11616-28.
25. Hocking AM, Shinomura T, McQuillan DJ. Leucine-rich repeat glycoproteins of the extracellular matrix. Matrix Biol. 1998;17:1-19.
26. Ge G, Seo NS, Liang X, Hopkins DR, Hook M, Greenspan DS. Bone morphogenetic protein-1/tolloid-related metalloproteinases process osteoglycin and enhance its ability to regulate collagen fibrillogenesis. J Biol Chem. 2004;279:41626-33.
27. Moali C, Font B, Ruggiero F, et al. Substrate-specific modulation of a multisubstrate proteinase. C-terminal processing of fibrillar procollagens is the only BMP-1-dependent activity to be enhanced by PCPE-1. J Biol

Chem. 2005;280:24188-94.

28. Wang ZP, Cai SX, Liu DB, Xu X, Liang HP. Anti-inflammatory effects of a novel peptide designed to bind with NF-kappaB p50 subunit. Acta Pharmacol Sin. 2006;27:1474-8.
29. Schmid H, Boucherot A, Yasuda Y, et al. Modular activation of nuclear factor-kappaB transcriptional programs in human diabetic nephropathy. Diabetes. 2006;55:2993-3003.

Correspondence to:
Hai-Qing Gao, MD
Key Laboratory of Cardiovascular Proteomics of Shandong Province, Department of Geriatrics, Qi-Lu Hospital of Shandong University, 107 Wenhuaxi Rd, Jinan, Shandong 250012, China
E-mail: haiqinggao1@sina.com

Received March 2016
Revised June 2016
Accepted June 2016