Potential role for Nrf2 activation in the therapeutic effect of MG132 on diabetic nephropathy in OVE26 diabetic mice

Wenpeng Cui,1,2 Bing Li,1,2,3 Yang Bai,2,3,4 Xiao Miao,1,2 Qiang Chen,2,5 Weixia Sun,2,4 Yi Tan,2,6 Ping Luo,1 Chi Zhang,6 Shirong Zheng,2 Paul N. Epstein,2,7 Lining Miao,1,* and Lu Cai,2,6,7*  

1Second Hospital of Jilin University, Changchun, China; 2Kosair Children Hospital Research Institute, Department of Pediatrics, University of Louisville, Louisville, Kentucky; 3Jilin Province People’s Hospital, Changchun, China; 4First Hospital of Jilin University, Changchun, China; 5School of Public Health, Jilin University, Changchun, China; 6Chinese-American Research Institute for Diabetic Complications, Wenzhou Medical College, Wenzhou, China; and 7Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky  

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Cui W, Li B, Bai Y, Miao X, Chen Q, Sun W, Tan Y, Luo P, Zhang C, Zheng S, Epstein PN, Miao L, Cai L. Potential role for Nrf2 activation in the therapeutic effect of MG132 on diabetic nephropathy in OVE26 diabetic mice. Am J Physiol Endocrinol Metab 304: E87–E99, 2013. First published November 6, 2012; doi:10.1152/ajpendo.00430.2012.— Oxidative stress is a major cause of diabetic nephropathy. Upregulation of the key antioxidative transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2), was found to prevent the development of diabetic nephropathy. The present study was designed to explore the therapeutic effect of Nrf2 induced by proteasomal inhibitor MG132 at a low dose (10 μg/kg) on diabetic nephropathy. Transgenic type 1 diabetic (OVE26) mice displayed renal dysfunction with albuminuria by 3 mo of age, at which time MG132 treatment was started. After 3-mo treatment with MG132, renal function, morphology, and biochemical changes were examined with real-time PCR, Western blotting, and immunohistochemical examination. Compared with age-matched, nontreated diabetic mice, MG132-treated diabetic mice showed significant improvements in terms of renal structural and functional alterations. These therapeutic effects were associated with increased Nrf2 expression and transcriptional upregulation of Nrf2-regulated antioxidants. Mechanistic study using human renal tubular HK11 cells confirmed the role of Nrf2, as silencing the Nrf2 gene with its specific siRNA abolished MG132 prevention of high-glucose-induced pro-inflammatory response. Furthermore, diabetes was found to significantly increase proteasomal activity in the kidney, an effect that was significantly attenuated by 3 mo of treatment with MG132. These results suggest that MG132 upregulates Nrf2 function via inhibition of diabetes-increased proteasomal activity, which can provide the basis for the therapeutic effect of MG132 on the kidney against diabetes-induced oxidative damage, inflammation, fibrosis, and eventual dysfunction.

diabetic nephropathy; Nrf2; proteasome inhibitor; MG132; therapeutic effect

DIABETIC NEPHROPATHY IS CHARACTERIZED by oxidative stress, inflammation, thickening of basement membranes, expansion of mesangial matrix, interstitial fibrosis, and podocytes, and tubular cell death, leading to albuminuria and eventual loss of renal function (1, 15, 33). Reactive oxygen or nitrogen species (ROS or RNS) production in response to hyperglycemia, advanced glycosylation end products, hyperlipidemia, inflammatory cytokines including transforming growth factor (TGF)β1, and hypertension contributes to these renal pathogenic changes (15). However, supplying exogenous antioxidant compounds has been largely unsuccessful in reducing human diabetic complications (1, 5, 15, 33). Potentially upregulating multiple endogenous antioxidants may be a more efficient approach to prevent or treat diabetic complications (5).

Many endogenous enzymes catalyze antioxidant reactions, including glutathione peroxidase, superoxide dismutase (SOD), catalase (CAT), heme oxygenase (HO-1), NADPH-quinone oxidoreductase, and glutamate-cysteine ligase. These antioxidative enzymes are regulated by a key transcription factor, nuclear factor-erythroid 2-related factor 2 (Nrf2). Actin-tethered protein Keap1 is a cytosolic repressor that binds to and retains Nrf2 in the cytoplasm, which prevents Nrf2 activation of transcription and promotes Nrf2 proteasomal degradation (16, 32). Oxidative stress modifies specific cysteine residues of Keap1, resulting in conformational changes that render Keap1 unable to bind Nrf2. Consequently, Nrf2 is free from Keap1 and translocates into nuclear to activate its downstream target genes. A growing body of evidence indicates that compounds that upregulate Nrf2 activity can provide a potent approach to protect from diabetic complications including nephropathy (9, 17). Administration of proteasome inhibitors is one method that has been shown to be effective in increasing Nrf2 activity (17).

Proteasomal inhibition is effective for treatment of several human diseases. Bortezomib (velcade) was the first proteasome inhibitor approved by the FDA in 2003 (8). However, side effects of Bortezomib and the development of resistance in some tumor patients prompted study of a new generation of proteasome inhibitors (8). MG132 is another potent, reversible, and cell-permeable proteasome inhibitor, which reduces degradation of ubiquitin-conjugated proteins in mammalian cells. Reportedly, nontoxic concentrations of MG132 inhibit Nrf2 proteasomal degradation and stimulate Nrf2 translocation into the nucleus (6, 28). An in vitro study showed that treatment with 0.5 μM MG132 for 48 h protected neonatal rat cardiac myocytes against H2O2-mediated oxidative stress. This correlated with reduced levels of intracellular ROS and significant upregulation of SOD, HO-1, and CAT expression. This demonstrated that nontoxic concentrations of MG132 could upregulate Nrf2-regulated antioxidant enzymes and confer cardiomyocyte protection (10).

A recent study in rats made diabetic with a single dose of streptozotocin (STZ) indicated that MG132 was also protective against diabetic nephropathy (19). Shortly after diabetes induction, rats were treated with MG132 at 10 μg/kg daily for 3 mo.
This regimen produced renal protection, as indicated by reductions in proteinuria, basement membrane thickening, and glomerular mesangial expansion. MG132 also reduced kidney markers of oxidative stress and increased protein levels of Nrf2 and several antioxidant enzymes. These results suggested the hypothesis that the beneficial effects of the proteasomal inhibitor MG132 was due to elevated Nrf2 protein content, which induced higher expression of multiple downstream antioxidants.

In the present study, therefore, we have further explored the therapeutic effect of MG132 on diabetic nephropathy by testing whether MG132 could slow progression of the established nephropathy in the OVE26 murine model of severe type 1 diabetes. In addition, mechanistic studies with human renal tubular HK11 cells indicated that MG132 protects renal tubule cells from elevated lipid and glucose exposure and that this protection is dependent on upregulation of Nrf2.

**MATERIALS AND METHODS**

**Animals.** The transgenic type 1 diabetic OVE26 mouse model has been characterized in our previous studies (26, 38). The background strain of OVE26 mice is FVB. All mice were housed in the University of Louisville Research Resources Center at 22°C with a 12:12-h light-dark cycle and provided with free access to standard rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

OVE26 mice normally develop severe hyperglycemia before 3 wk of age and develop albuminuria by 3 mo of age. Sixteen 3-mo-old female OVE26 mice that exhibited significant albuminuria were randomly divided into two groups: diabetic group (DM, n = 10) and MG132-treated OVE26 group (DM/MG132, n = 6). Sixteen age- and sex-matched nondiabetic FVB mice were randomly divided into two groups: control group (n = 10) and MG132 group (MG132, n = 6). MG132 (Sigma-Aldich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0.0025 mM and diluted with saline for injection. For MG132 and DM/MG132 mice, MG132 was injected intraperitoneally at a dose of 10 μg/kg daily for 3 mo starting at 3 mo of age when OVE26 mice already displayed significant albuminuria.

For control and DM mice (n = 10), four mice were euthanized at 3 mo of age, and the remaining six were given physiological saline with DMSO at the concentration of 0.0025 μg/ml for 3 mo and then euthanized at 6 mo of age. After body weight, tibia length, and kidney weight were measured, renal tissue was snap-frozen in liquid nitrogen for protein and mRNA analyses, 20S proteasome activity assay, and histopathological examination.

**Blood and urine assays.** Plasma glucose (Sigma-Aldich), blood urea nitrogen (BUN; BioAssay Systems, Hayward, CA) and triglyceride (Cayman Chemical, Ann Arbor, MI) were assayed according to the manufacturers’ procedures described in the kits.

Mouse urine was collected once a month after the start of MG132 treatment. Urine albumin (Bethyl Laboratories, Montgomery, TX) and urinary creatinine (BioAssay Systems) were measured according to the manufacturers’ instructions. Urinary albumin-to-creatinine ratio (ACR) was calculated as ACR = urine albumin/urine creatinine (μg/mg).

**Cell culture, treatments, and measurements.** Human renal proximal tubular (HK11) cells (27) were maintained in Dulbecco’s modified Eagle’s medium (DMEM)-F12 supplemented with 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). To mimic diabetes, HK11 cells were exposed to d-glucose in a final concentration of 27.5 mM (high glucose, HG) for 48 h and the addition of palmitate (Pal, 300 μM) during the last 6 h. Cell culture medium with 2% bovine serum albumin (BSA, Sigma-Aldich) and 1% FBS was used during this period. To exclude a hyperosmotic effect, 5.5 mM d-glucose plus 22 mM d-mannitol (Sigma-Aldich) was added as osmotic control. MG132 dissolved in DMSO was added (2 μM final concentration) during the last 9 h. For vehicle control, DMSO alone was added. The role of Nrf2 in the HK11 cell response to HG/Pal treatment was tested by silencing Nrf2 gene expression with specific small interfering RNA (siRNA) added at a concentration of 40 nM for 48 h before cells were exposed to HG. Protein expression of connective tissue growth factor (CTGF), Nrf2, HO-1, and cystosolic SOD (SOD1) were measured by Western blotting in control and HG/Pal-treated HK11 cells.

Nrf2 expression and translocation into the nucleus were monitored by fluorescence microscopy. For this purpose, HK11 cells were cultured on chamber slides for 12 h to allow the cells to attach to the surface. They were then subjected to the experimental conditions described in the preceding paragraph. After treatment, cells were permeabilized with 0.1% Triton X-100 and then incubated with rabbit anti-Nrf2 antibody (1:50 dilution; Abcam, Cambridge, MA) overnight at 4°C followed by Cy3-conjugated goat anti-rabbit IgG antibody and counterstained with DAPI for visualization of nuclei. The fluorescent staining was analyzed with a Nikon eclipse E600 fluorescence microscope.

**Kidney histopathological examination and immunohistochemical and fluorescent staining.** Kidney tissue was fixed overnight in 10% phosphate-buffered formalin and then dehydrated in a graded alcohol series, cleared with xylene, embedded in paraffin, and sectioned at 5 μm thickness for pathological, immunohistochemical, and immunofluorescent staining. Paraffin sections were dewaxed, followed by incubation with 1× target retrieval solution (Dako, Carpinteria, CA) for 15 min at 98°C for antigen retrieval and then treated with 3% H2O2 for 15 min at room temperature followed by blocking with 5% BSA for 30 min.

To examine overall morphology, kidney sections were stained with hematoxylin and eosin (H&E). Periodic acid-Schiff (PAS) and Sirius red staining were used to examine collagen and tissue accumulations, as previously described (29, 30). For immunohistochemical staining, sections were incubated with the following primary antibodies: 4-hydroxy-2-nonenal (4-HNE, 1:400 dilution; Alpha Diagnostic International, San Antonio, TX), 3-nitrotyrosine (3-NT, 1:400 dilution; Millipore, Billerica, MA), tumor necrosis factor-α (TNFα, 1:100 dilution, Abcam), TGFβ1 (1:100 dilution, Santa Cruz Biotechnology), and CTGF (1:100 dilution, Santa Cruz Biotechnology) overnight at 4°C. After sections were washed with PBS, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1:300–400 dilutions in PBS) for 2 h at room temperature. For the detection of color, sections were treated with peroxidase substrate DAB (3,3’-diaminobenzidine) in the developing system (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. For immunofluorescence observation of Nrf2, sections were incubated with rabbit anti-Nrf2 antibody (1:50 dilution, Abcam) overnight at 4°C, followed by Cy3-conjugated goat anti-rabbit IgG antibody and DAPI. Digital fluorescent images were made and analyzed with Image Pro Plus 6.0 software.

**Real-time qPCR.** Renal tissues stored at −80°C were used to extract total RNA with TRizol reagent (Invitrogen). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA according to the manufacturer’s protocol from the RNA PCR kit (Promega, Madison, WI).

Real-time qPCR (quantitative PCR, qPCR) was carried out in a 20-μl reaction buffer that included 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primer, and 9 μl of cDNA and performed in duplicate for each sample in the ABI 7300 Real-Time PCR system. TaqMan primers for Nrf2, HO-1, SOD1, mitochondrial SOD (SOD2), CAT, and β-actin control primer were purchased from Applied Biosystems (Carlsbad, CA). The fluorescence intensity of each sample...
was measured at each temperature change to monitor amplification of the target gene. The comparative cycle time (Ct) method was used to determine fold differences between samples. The comparative Ct method determined the amount of target normalized to an endogenous reference (β-actin) and relative to a calibrator (2^-ΔΔCt).

siRNA transfection. HK11 cells were transfected with either negative control sense siRNA (Invitrogen) or human Nrf2 antisense siRNA (NFE2L2HS181505, Invitrogen), using Lipofectamine TM 2000 (Invitrogen) transfection reagent for 48 h as described by the manufacturer. Transfection was followed by addition of MG132 and HG/Pal as described above. The sequences of the sense and antisense siRNA were 5'-CAACUCAGAAGUUGACAAUUU-3' and 5'-AUAAUGUUCUACUGCUUGUUG-3'; respectively. The efficacy of siRNA treatment was assessed by Western blot analysis for Nrf2 protein expression. Effects of Nrf2 knockdown on the expression of CTGF and HO-1 were assessed by Western blotting analysis.

Isolation of nuclei. The renal nuclei were isolated using a nuclei isolation kit (Sigma-Aldrich). Kidney tissue (30 mg) from each mouse was homogenized for 45 s in 150 μl of cold lysis buffer containing 0.5 μl of dithiothreitol (DTT) and 0.1% Triton X-100. After that, 300 μl of cold 1.8 M Sucrose Cushion solution (Sucrose Cushion solution: Sucrose Cushion buffer: DTT = 900:100:1) was added to the lysis solution. The mixture was transferred to a new tube preloaded with 150 μl of 1.8 M Sucrose Cushion solution followed by centrifugation at 13,000 rpm for 45 min. The supernatant, containing cytosolic component and nuclei, was visible as a thin pellet at the bottom of the tube.

Western blotting. Western blots were performed as previously described (2). Briefly, kidney tissues were homogenized, and HK11 cells were sonicated in RIPA buffer. Total protein was extracted and described (2). Briefly, kidney tissues were homogenized, and HK11 cells were sonicated in RIPA buffer. Total protein was extracted and described (2). Western blots were performed as previously described (2). Brieﬂy, membrane-bound proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with a 5% nonfat dried milk for 1 h and then incubated overnight at 4°C with the appropriate primary and secondary antibodies for 1 h room temperature. Protein bands were visualized using ECL (Thermo scientiﬁc, Rockford, IL).

20S proteasome activity assay. The 20S proteasome, catalytic core of the 26S proteasome complex, is responsible for the breakdown of short-lived regulatory proteins, including Nrf2 (7, 10). Since MG132 mainly inhibits the proteasome chymotrypsin (ChT)-like activity (20), we determined 20S proteasome activity by quantifying the hydrolysis of SLLVY-AMC, a ﬂuorescent substrate for the ChT-like activity. 20S proteasome activity was measured with the 20S Proteasome Activity Assay Kit (Millipore). Brieﬂy, kidney tissues or HK11 cells were immersed in 1 ml of cold lysis buffer and homogenized. The homogenate was immediately centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected for measurement of protein concentration and proteasome activity. For each sample, the proteasome activity was measured by monitoring the release of 7-amino-4-methylcoumarin (AMC) with ﬂuorescence spectrophotometry at an excitation wavelength of 380 nm and an emission wavelength of 440 nm. All results were standardized by the fluorescence intensity of an equal volume of free AMC.

Statistical analysis. In vivo and in vitro data were collected from four to six animals or at least three separate cell cultures for each group and presented as means ± SD. Comparisons between groups were performed by one-way ANOVA, followed by Tukey’s post hoc test. Statistical analysis was performed with Origin 7.5 Laboratory data analysis and graphing software. Statistical significance was considered as P < 0.05.

RESULTS

Effects of MG132 on diabetes-induced general changes and on renal function. As shown in Fig. 1A, body weight gain in diabetic mice was slow compared with age-matched controls. Treatment with MG132 at 10 μg/kg daily for 3 mo did not affect body weight gain in control mice but signiﬁcantly increased body weight gain in diabetic mice (Fig. 1A).

Blood glucose levels of diabetic groups were significantly higher than those of age-matched controls (Fig. 1B), and MG132 treatment for 3 mo did not affect blood glucose in DM/MG132 mice compared with DM mice at 6 mo of age (i.e., at the end of 3-mo treatment). Serum triglyceride levels were elevated in both diabetic groups but were not changed by MG132 treatment (Fig. 1C). Diabetes increased kidney weight in an age-dependent manner, but MG132 treatment reduced the diabetes-induced elevation in kidney weight at 6 mo of age (Fig. 1D).

To evaluate renal function, BUN (Fig. 1E) and urinary albumin-to-creatinine ratio (ACR; Fig. 1F) were measured. BUN was elevated in 6-mo-old diabetic mice, but the increase was signiﬁcantly decreased by MG132 treatment. ACR was remarkably elevated in both diabetic groups at all ages tested. However, after 4 mo of age, just 1 mo of treatment, and continuing through 6 mo of age, MG132 signiﬁcantly reduced diabetic ACR.

Effect of MG132 on diabetes-induced pathological changes in the kidney. To investigate the therapeutic effect of MG132 on the kidney, renal pathology was examined with H&E (Fig. 2A) and PAS (Fig. 2B) staining. Pathological changes in the kidney of DM mice were obvious at 6 mo of age, including glomerular enlargement, increased mesangial matrix, and Kimmelstiel Wilson (K-W) nodules (representative pictures are shown in Fig. 2, A and B). Furthermore, renal tubular epithelium damage as well as a large number of protein casts could be found in the tubules of DM mice (Fig. 2, A and B). All these pathological alterations were less severe and less common after 3 mo of treatment with MG132 (Fig. 2, A and B).

Sirius red staining showed signiﬁcant accumulation of collagen in the kidney of DM mice at 6 mo of age, indicating the development of interstitial ﬁbrosis. This effect was signiﬁcantly decreased by MG132 treatment (Fig. 2C). The increased ﬁbrosis was further conﬁrmed by measuring the expression of the two profibrotic mediators CTGF and TGFβ1 by Western blotting assay (Fig. 3A) and immunohistochemical staining (Fig. 3B). Expression of both profibrotic mediators was signiﬁcantly increased in the kidney of 6-mo-old DM mice but signiﬁcantly less so in the kidney of 6-mo-old DM with 3-mo MG132 treatment.

Effect of MG132 on diabetes-induced renal inﬂammation and oxidative damage. Inflammation plays important roles in initiating renal ﬁbrosis under diabetic conditions; therefore, we examined renal inﬂammation by analyzing ICAM-1 and TNFα expression with Western blotting (Fig. 4A), which showed that diabetes signiﬁcantly increased the expression of both ICAM-1 and TNFα. Induction of renal inﬂammation was further conﬁrmed by immunohistochemical staining of TNFα (Fig. 3B). All these changes were alleviated by 3 mo of MG132 treatment (Figs. 3B and 4A).
It has been reported that oxidative damage often induces inflammation and inflammation induces oxidative stress (3, 11); therefore, the next study investigated the therapeutic effect of MG132 on diabetes-induced renal oxidative damage, measured by 3-NT as an index of nitrosative damage (Fig. 3B) and 4-HNE (lipid peroxide) as an index of oxidative damage (Fig. 4C) with Western blotting and immunohistochemical staining (Fig. 3B). Diabetes significantly increased 3-NT in multiple proteins from 22 to 98 kDa (Fig. 3B) and 4-HNE in proteins from 16 to 64 kDa (Fig. 4C). Immunohistochemical staining of 3-NT and 4-HNE showed accumulations of both predominantly in the renal tubules (Fig. 3B). All these changes were alleviated by MG132 treatment in the DM/MG132 group (Figs. 3B and Fig. 4, B and C).

Effect of MG132 on renal Nrf2 expression in vivo: a potential mechanism by which MG132 stops or slows the progression of diabetic nephropathy. Renal Nrf2 expression was examined by real-time qPCR (Fig. 5A) and Western blotting assay (Fig. 5B). The qPCR showed that diabetes significantly increased renal Nrf2 mRNA expression but that MG132 had no significant effect on Nrf2 mRNA in diabetic or control mice. However, Western blotting for Nrf2 protein showed that both MG132 treatment and diabetes were factors that significantly increased Nrf2 protein content. As a result, the DM/MG132 group, which had both the diabetes and the MG132 factors, had the highest renal content of Nrf2 protein of all the groups. Furthermore, treatment with MG132 not only increased the total content of Nrf2 protein but also increased its nuclear translocation in diabetic and nondiabetic mice, as shown by increased Nrf2 fluorescence and nuclear accumulation (Fig. 5C).

Effect of MG132 on Nrf2 expression and HG/pal-induced profibrotic response in human renal tubular cells in vitro: direct evidence for a role of Nrf2 in prevention of hyperglycemia/hyperlipidemia toxic effects. Our in vivo results and those of Luo et al. (19) showed only that there is an association between the beneficial effects MG132 on diabetic nephropathy and upregulation of renal Nrf2 content. The following studies done in human renal tubular HK11 cells (Figs. 6 and 7) provided direct, specific evidence for an essential role of Nrf2 in MG132 protection against diabetic damage. We first assessed whether HK11 cells treated with HG (27.5 mM) and Pal (300 μM) behaved similarly to OVE26 diabetic kidneys. CTGF was measured because it induces fibrosis, a critical feature of diabetic nephropathy. Western blotting of CTGF expression showed that HG/Pal treatment significantly increased CTGF expression in HK11 cells, and this was almost completely prevented by pretreatment with MG132 at 2 μM (Fig. 6A). MG132 prevention of HG/Pal-induced CTGF expression showed that HG/Pal treatment significantly increased CTGF expression in HK11 cells, and this was almost completely prevented by pretreatment with MG132 at 2 μM (Fig. 6A). MG132 prevention of HG/Pal-induced CTGF expression showed that HG/Pal treatment significantly increased CTGF expression in HK11 cells, and this was almost completely prevented by pretreatment with MG132 at 2 μM (Fig. 6A). MG132 prevention of HG/Pal-induced CTGF expression showed that HG/Pal treatment significantly increased CTGF expression in HK11 cells, and this was almost completely prevented by pretreatment with MG132 at 2 μM (Fig. 6A).
E91

THERAPEUTIC EFFECT OF MG132 ON DIABETIC NEPHROPATHY

A

Control | MG132 | DM | DM/MG132

H&E

B

Control | MG132 | DM | DM/MG132

PAS

C

Sirius-red

Sirius-red staining

Control | MG132 | DM | DM/MG132

* | #

Sirius-red staining

Control | MG132 | DM | DM/MG132

* | #
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E92

THERAPEUTIC EFFECT OF MG132 ON DIABETIC NEPHROPATHY

A

Control  MG132  DM  DM/MG132

CTGF  TGF-β1  Actin

B

Control  MG132  DM  DM/MG132

CTGF  TGF-β1  TNF-α  3-NT  4-HNE

*  #

Relative expression

Control  MG132  DM  DM/MG132

E92

THERAPEUTIC EFFECT OF MG132 ON DIABETIC NEPHROPATHY

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E92

THERAPEUTIC EFFECT OF MG132 ON DIABETIC NEPHROPATHY

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pression was accompanied by upregulation of Nrf2 (Fig. 6A). Immunofluorescent staining the Nrf2 (Fig. 6B) showed increases in Nrf2 expression and also nuclear accumulation, suggesting the activation of Nrf2 function by HG/Pal and MG132 treatments. Activation of Nrf2 was further supported by measuring protein expression of antioxidants downstream of Nrf2 such as HO-1 and SOD1 (Fig. 6A). These results for CTGF (Fig. 3) and Nrf2 (Fig. 5) in HK11 cells were very similar to what we had obtained in vivo for OVE26 diabetes and MG132 treatment. Assays of 20S proteasome activity (Fig. 7A) confirmed that MG132 at 2μM reduced proteasome activity in a time-dependent manner from 1 to 12 h in HK11 cells.

Finally, the direct role of Nrf2 in the MG132 therapeutic response was tested by silencing Nrf2 expression with its siRNA. We first demonstrated that Nrf2 siRNA effectively reduced Nrf2 protein in HK11 cells with or without 2μM MG132 present (Fig. 7B). We then tested whether Nrf2 was essential for the ability of MG132 to change the detrimental responses to HG/Pal treatment (Fig. 7C). Western blotting showed that knockdown of Nrf2 greatly reduced the ability of MG132 to reduce CTGF expression, which had been induced with HG/Pal. Similarly, Nrf2 knockdown suppressed the capacity of MG132 to raise HO-1 levels in HG/Pal treated HK11 cells. These results showed that Nrf2 was essential to the MG132 response, including its ability to decrease the HG/Pal-induced fibrotic response.

Validation of the in vitro finding in the kidney of diabetic mice. Figure 8A shows that diabetes increases and MG132 decreases 20S proteasomal activity in OVE26 kidney. Compared with control kidney these results show a slight decrease of 20S proteasomal activity in the kidney of DM/MG132 group was significantly reduced compared with DM group (Fig. 8A), con-

Fig. 3. Therapeutic effect of MG132 on diabetes-increased renal expression of profibrotic mediators, renal inflammation, and oxidative damage. A: intercellular adhesion molecule 1 (ICAM-1) and TNFα expression were examined by Western blotting assay. B: renal accumulation of 3-NT and 4-HNE were also examined with Western blotting assay. Data are presented as means ± SD (n = 6). *P < 0.05 vs. control; #P < 0.05 vs. DM group.
Fig. 5. Effect of MG132 on renal Nrf2 expression in vivo. Renal Nrf2 expression at both mRNA (A) and protein (B) levels was examined. Nrf2 expression and nuclear translocation were also visualized by immunofluorescent staining (C). Arrows, nuclear accumulation of Nrf2 (×1,000). Data are presented as means ± SD (n = 6). *P < 0.05 vs. control; #P < 0.05 vs. DM group.
consistent with the ability of MG132 treatment to decrease degradation of Nrf2 protein.

We further confirmed the increased nuclear accumulation of Nrf2 in MG132, DM, and DM/MG132 groups, which was indicated by immunohistochemical staining (Fig. 5C), by detecting Nrf2 protein by Western blotting in the nuclei (Fig. 8B). Furthermore, increased Nrf2 activity was reflected by the upregulation of several downstream antioxidant genes (Fig. 8, A).
C and D). MG132 treatment in control mice significantly increased the expression of HO-1, SOD1, SOD2, and CAT at mRNA and protein levels compared with untreated control. Diabetes increased the expression of only HO-1 mRNA and protein but significantly decreased the expression of SOD1, SOD2, and CAT mRNA and protein (Fig. 8, C and D). The expression of these Nrf2 downstream antioxidant genes in the kidney of the DM/MG132 group was significantly increased at both mRNA and protein levels compared with DM alone (Fig. 8, C and D).

**DISCUSSION**

These experiments are the first to demonstrate the therapeutic effects of chronic treatment with low-dose MG132 on diabetes-induced renal pathological and functional abnormalities by use of the OVE26 diabetic mouse model. We demonstrated with both in vivo and in vitro experiments that the therapeutic effect of MG132 on diabetic nephropathy is most likely mediated by its renal upregulation of Nrf2-mediated multiple antioxidants.

Emerging evidence has indicated that multiple antioxidants resulting from upregulated Nrf2 provide a remarkable prevention of diabetes-induced cardiac and renal complications. For instance, in Nrf2 gene-deficient mice, diabetes-induced cardiac and renal damage was significantly increased (12, 14, 36). Activation of Nrf2 by magnesium lithospermate B prevented diabetic atherosclerosis (13) by sulforaphane in vitro, and in vivo prevented diabetes-induced kidney (14, 37) and neuronal...
tissue damage (22), and by resveratrol prevented diabetes-induced renal damage (23). There was also an in vitro study showing the preventative effect of Nrf2 induced by MG132 on H2O2-induced cardiac cell damage (10), and an in vivo study showing the preventative effect on diabetes-induced renal damage (19). All of these studies suggest a critical role of upregulated Nrf2 by its activators in preventing the development of diabetic nephropathy.

However, it remains to be determined whether Nrf2 activators have therapeutic effects on the heart and/or kidney of diabetic subjects. In recent years, there were a few clinical trials that used the proteasomal inhibitor bardoxolone methyl (also known as CDDOMe) as a Nrf2 activator, which demonstrated certain therapeutic effects in patients with chronic kidney diseases and type 2 diabetes (24, 25). However, due to the lack of direct evidence that Nrf2 was increased in the kidney of these diabetic patients, it was not possible to attribute the renal therapeutic effect to Nrf2 upregulation by bardoxolone in these studies. To address such issues, animal studies should be done.

Our study has provided the following innovations to enhance our understanding of the mechanisms and features of the therapeutic effect of proteasomal inhibitors. First, in the clinical trials there was evidence of the potential therapeutic application of proteasomal inhibitor for type 2 diabetic patients. Our study utilized the OVE26 type 1 diabetic mouse model to show the therapeutic benefit of proteasomal inhibitor-induced renal Nrf2 upregulation in type 1 diabetes. To date, the OVE26 mouse model most closely displays the characteristics of human diabetic nephropathy (38).

Second, the present study showed that 3-mo treatment with low dose MG132 could significantly improve body weight gain in the DM/MG132 group compared with DM group (Fig. 1A), but this treatment did not significantly improve blood glucose level (Fig. 1B) and triglyceride level (Fig. 1C). Our finding is inconsistent with a previous study that showed a systemic improvement with MG132 when it was used as preventative of diabetes-induced renal pathological changes (19). We assume that the discrepancy between our study and the previous one...
E98 THERAPEUTIC EFFECT OF MG132 ON DIABETIC NEPHROPATHY

(19) is due mainly to the differences of animal models and MG132 administration times between the two studies. Lou et al. used diabetic rats induced by a signal dose of STZ, for which MG132 was given upon diabetes onset. Under this condition, there might still be β-cells in the pancreas that can be protected by MG132-induced pancreatic antioxidants against the further damage. Therefore, blood glucose levels were found to be decreased in diabetic rats with MG132 treatment compared with diabetic rats without MG132 treatment (19). In contrast to the previous study, we used OVE26 mice that had developed diabetes at around 3 wk, and MG132 was given when those diabetic mice began to exhibit albuminuria, as an index of renal dysfunction, at 3 mo of age. It was already too late for MG132 to prevent β-cells in the pancreas of OVE26 mice. The body weight improvement in the DM/MG132 group compared with the DM group may be due to the therapeutic effect on the kidneys, since any organ dysfunction will affect an animal’s general health status, including the decrease of body weight gain. These results suggest that the therapeutic effect of chronic treatment with low dose MG132 on diabetes-induced renal damage is not attributed to systemic improvement at least in the OVE26 diabetic model.

Third, several in vivo and in vitro studies have provided evidence for the increase in proteasomes in diabetes. For example, exposure of human umbilical vein endothelial cells to HG significantly increased the 26S proteasome activity (35). Proteasomal activity was increased in skeletal muscle and heart of STZ-induced diabetic rats (18, 21) and in gastrocnemius muscle of spontaneously diabetic (db/db) mice (34). However, whether diabetes increases proteasomal activity in the kidney has not been investigated until recently (19). Measurement of proteasomal activity in the kidney of diabetic rats (19) and of mice in the present study (Fig. 8A) showed a clear increase of 26S or 20S proteasomal activity induced by STZ or OVE26 diabetic animals. Furthermore, these two studies also demonstrated the significant inhibition of diabetes-increased proteasomal activity using preventive treatment (19) or therapeutic treatment (Fig. 8) with a low-dose level of MG132 for 3 mo. By RT-PCR analysis, we demonstrated that diabetes, but not MG132, significantly increased Nrf2 mRNA expression (Fig. 5A), suggesting the existence of a compensatory response of the kidney to diabetes-induced oxidative stress by upregulating Nrf2 mRNA expression. However, diabetes also increased the 20S proteasome activity that should reduce Nrf2 protein levels and lessen nuclear translocation; therefore, the final outcome of Nrf2 protein expression in diabetic kidney remained at a relatively high level compared with control (Fig. 5B). In contrast to the DM group, mice in the DM/MG132 group showed increased Nrf2 mRNA expression resulting from diabetes and also inhibition of proteasomal activity by MG132, both leading to a significant increase in Nrf2 protein expression in DM/MG132 kidney compared with DM or MG132 alone (Fig. 5, A and B).

Finally, our in vitro study further defined the direct role of Nrf2 in MG132 prevention of the profibrotic response induced by HG/Pal in renal tubule cells, since by silencing Nrf2 gene with its siRNA the prevention of HG/Pal-induced CTGF expression by MG132 could be completely abolished without upregulation of Nrf2 expression and its downstream genes (Fig. 7C).

It should be noted in the present study that initiating treatment with MG132 after diabetic mice began showing signs of renal dysfunction (Fig. 1) could significantly, but not completely, prevent the progression of diabetes-induced renal dysfunction (Fig. 1), including renal oxidative damage and inflammatory and fibrotic responses (Figs. 3 and 4). There are two possible explanations for this. 1) Unlike renal inflammation and oxidative damage, which are transient events, renal fibrosis once formed may be not easily be reversed by only upregulating antioxidants such as Nrf2 activation; therefore, the therapeutic approach may need to be applied early. 2) The dosage of MG132 of 10 μg/kg daily, the lowest reported in the literature for use in vivo, may be too low to efficiently inhibit proteasomal activity, as observed in Fig. 8A. Whether increasing MG132 doses can enhance its therapeutic effect needs further investigation.

One concern for the use of MG132 may be the nonspecific inhibition of proteasomes and whether global inhibition of proteasomes has other undesirable effects. In general, effective inhibition of the proteasome by high doses of proteasome inhibitors such as MG132 induces apoptotic cell death. In contrast, partial proteasome inhibition by low doses may also predominantly inhibit the elevated proteasomal activity that is caused by diabetes in multiple organs, but not inhibit proteasomal activity in normal tissues (31). Therefore, proteasome inhibitors at low doses may also predominantly inhibit the elevated proteasomal activity in tumor cells but not in normal tissues (31). Treatment with low doses of proteasome inhibitor mediates a protective response against oxidative stress (4). In addition, human cancers have elevated activity of proteasomes, and most proteasome inhibitors predominantly inhibit proteasome activity in tumor cells but not in normal tissues (31). Therefore, proteasome inhibitors at low doses may also predominantly inhibit the elevated proteasomal activity that is caused by diabetes in multiple organs, but not inhibit proteasomal activity in normal tissues. In support of our assumption, the clinical trials did not show significant side effects for the 52 wk during treatment (24, 25).

In summary, the present study has demonstrated that proteasomal inhibitor MG132 at low dose can provide a therapeutic effect on diabetes-induced nephropathy using transgenic type 1 diabetic OVE26 mouse model. When these diabetic mice began to exhibit albuminuria as an index of renal dysfunction, some of them were treated with MG132 for 3 mo, which offered a significant therapeutic outcome, indicated by marked slowing of the progression of diabetes-induced renal pathological and functional changes. These therapeutic changes were associated with significant upregulation of Nrf2 expression and transcriptional increases of its downstream antioxidants in vivo and in vitro. Experiments, both in vitro and in vivo, demonstrated the increase in proteasomal activity in the kidney and HG/Pal-treated renal cells, an effect that could be significantly attenuated by MG132. These results suggest that MG132 upregulation of Nrf2 function via inhibiting the increased proteasomal activity in diabetes provides therapeutic effects on the kidney against oxidative damage, inflammation, fibrosis, and eventually dysfunction. MG132, therefore, has great potential as a therapeutic agent for diabetic patients including those with diabetic nephropathy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
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AUTHOR CONTRIBUTIONS