Therapeutic effect of MG-132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities: roles of Nrf2 and NF-κB

Yuehui Wang,1 Weixia Sun,2,3 Bing Du,2 Xiao Miao,1,3 Yang Bai,2,3 Ying Xin,3,4 Yi Tan,3,5 Wenpeng Cui,1,3 Bin Liu,1 Taixing Cui,5,6 Paul N. Epstein,3,7 Yaowen Fu,2 and Lu Cai3,5,7

1The Second Hospital, Jilin University, Jilin, China; 2The First Hospital, Jilin University, Jilin, China; 3Kosair Children’s Hospital Research Institute, Department of Pediatrics, University of Louisville, Louisville, Kentucky; 4Department of Pediatrics, University of Louisville, Louisville, Kentucky; 5Chinese-American Research Institute for Diabetic Complications, Wenzhou Medical College, Wenzhou, China; 6Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, South Carolina; and 7Departments of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky

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Wang Y, Sun W, Du B, Miao X, Bai Y, Xin Y, Tan Y, Cui W, Liu B, Cui T, Epstein PN, Fu Y, Cai L. Therapeutic effect of MG-132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities: roles of Nrf2 and NF-κB. Am J Physiol Heart Circ Physiol 304: H567–H578, 2013. First published December 7, 2012; doi:10.1152/ajpheart.00650.2012.—MG-132, a proteasome inhibitor, can upregulate nuclear factor (NF) erythroid 2-related factor 2 (Nrf2)-mediated antioxidative function and downregulate NF-κB-mediated inflammation. The present study investigated whether through the above two mechanisms MG-132 could provide a therapeutic effect on diabetic cardiomyopathy in the OVE26 type 1 diabetic mouse model. OVE26 mice develop hyperglycemia at 2–3 wk after birth and exhibit albuminuria and cardiac dysfunction at 3 mo of age. Therefore, 3-mo-old OVE26 diabetic and age-matched control mice were intraperitoneally treated with MG-132 at 10 μg/kg daily for 3 mo. Before and after MG-132 treatment, cardiac function was measured by echocardiography, and cardiac tissues were subjected to pathological and biochemical examination. Diabetic mice showed significant cardiac dysfunction, including increased left ventricular systolic diameter and wall thickness and decreased left ventricular ejection fraction with an increase of the heart weight-to-tibia length ratio. Diabetic hearts exhibited structural derangement and remodeling (fibrosis and hypertrophy). In diabetic mice, there was also increased systemic and cardiac oxidative damage and inflammation. All of these pathogenic changes were reversed by MG-132 treatment. MG-132 treatment significantly increased the cardiac expression of Nrf2 and its downstream antioxidant genes with a significant increase of total antioxidant capacity and also significantly decreased the expression of IκB and the nuclear accumulation and DNA-binding activity of NF-κB in the heart. These results suggest that MG-132 has a therapeutic effect on diabetic cardiomyopathy by upregulating Nrf2-dependent antioxidant function and downregulation of NF-κB-mediated inflammation.

diabetic cardiomyopathy; nuclear factor erythroid 2-related factor 2; nuclear factor-κB; proteasome inhibitor; MG132; therapeutic effect contribute to the pathogenesis and progression of several cardiac diseases, and inhibition of proteasome activity has become an interesting approach to prevent various diseases, including cardiac diseases.

Bortezomib (velcade) was the first proteasome inhibitor approved by the Federal Drug Administration in 2003 (8). However, side effects of Bortezomib and the development of resistance in some patients with tumors prompted the study of new proteasome inhibitors (8). The cell-permeable MG-132 tripeptide (Z-Leu-Leu-Leu-aldehyde) is a peptide aldehyde proteasome inhibitor that also inhibits other proteases, including calpains and cathepsins. Reportedly nontoxic concentrations of MG-132 inhibit Nrf2 proteasomal degradation and stimulate nuclear factor (NF) erythroid 2-related factor 2 (Nrf2) translocation into the nucleus (5, 28). By blocking the proteasome, this tripeptide has been shown to induce the expression of cell-protective proteins, such as heat shock proteins, in vitro and in vivo. MG-132 was found to play an important role in cardiovascular protection from various stresses and pathogeneses (4, 19, 28).

One mechanism responsible for the cardiac protection by MG-132 is its upregulation of antioxidants via activation of Nrf2 (28). Nrf2 is a key transcription factor in the regulation of multiple antioxidant proteins, including NADPH-quinone oxidoreductase (NQO)-1, heme oxygenase (HO)-1, catalase (CAT), SOD, glutathione peroxidase, and glutamate-cysteine ligase. The actin-tethered protein kelch-like ECH-associated protein 1 is a cytosolic repressor that binds to and retains Nrf2 in the cytoplasm, which promotes Nrf2 proteasomal degradation and results in prevention of Nrf2 transcription (14, 30). Therefore, inhibition of proteasome activity is one method to maintain cytoplasm Nrf2 available to nuclear translocation and activation in response to oxidative stress (15).

Another important mechanism underlying MG-132 cardiovascular protection is its inactivation of NF-κB, a nuclear transcription factor that regulates proinflammatory cytokine expression. Activation of NF-κB has been documented in myocardial ischemia-reperfusion (13), and specific inhibition of NF-κB is cardioprotective (25). NF-κB usually is inhibited via IκB by forming a complex with NF-κB. However, IκB is degraded by proteasome ubiquitination, resulting in the release of NF-κB and nuclear translocation to turn on inflammatory genes. Therefore, proteasomal inhibitors such as MG-132 can inhibit proteasome activity to provide anti-inflammation function, leading to cardiac protection from ischemic damage.
Diabetic cardiomyopathy is also associated with early cardiac oxidative stress and inflammation, which initiates the late development of cardiac remodeling and dysfunction (1, 12, 27, 34). A previous report (16) has shown that diabetes increased cardiac proteasome function (11, 22), which was associated with diabetic complications. Therefore, we hypothesized that MG-132 could be one of the proteasome inhibitors that may be potentially used for the prevention of diabetic cardiomyopathy via upregulation of Nrf2 to reduce oxidative stress and downregulation of NF-κB-mediated inflammation (16). A few studies (3, 4, 17, 19, 28, 31) have shown cardiac and renal protection by proteasome inhibitor from other pathogeneses. Dreger et al. (9) demonstrated the protective effect of low-dose MG-132 on H2O2-induced oxidative stress and damage in cardiomyocytes and also showed increased sensitivity in myocytes from Nrf2 knockout mice, suggesting the important role of Nrf2 in cardiac protection. Using MG-132, several studies (16, 32) have also shown the preventive effect of inhibition of proteasome activity on diabetic vascular injury. Results from a pilot study (18) indicated that a proteasomal inhibitor that upregulates Nrf2 activity and inhibits NF-κB-mediated inflammation provided a preventive effect on diabetes-induced renal dysfunction.

From a clinical setting, there is an urgent need for an efficient approach that can provide a therapeutic effect rather than only prevention, since it is not easily accepted that diabetic patients will receive preventive medical treatment once diagnosed as diabetic. So far, however, there is no report regarding the therapeutic effect of MG-132 on diabetic complications; in the present study, therefore, we tried to address the question of whether MG-132 can provide a therapeutic effect on diabetic cardiomyopathy by testing if MG-132 could reserve or slow the progression of established cardiac dysfunction in the OVE26 mouse model of severe type 1 diabetes. In addition, mechanistic experiments were focused on the upregulation of Nrf2-mediated antioxidants and downregulation of NF-κB-mediated inflammation.

**MATERIALS AND METHODS**

**Animals**

The transgenic type 1 diabetic OVE26 mouse model on a FVB background has been characterized in a previous study (38). All mice were housed at 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, particularly in female OVE26 mice (38). Sixteen 3-mo-old female OVE26 mice were randomly divided into two groups: a diabetic group (DM group; n = 10) and a MG-132-treated OVE26 group (DM/MG-132 group; n = 6). Sixteen age- and sex-matched nondiabetic FVB mice were randomly divided into two groups: a control group (n = 10) and a MG-132-treated group (MG-132 group; n = 6). MG-132 (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO at a concentration of 0.0025 μg/ml and diluted with saline for injection. For MG-132 and DM/MG-132 mice, MG-132 was given intraperitoneally at a dose of 10 μg/kg body wt daily for 3 mo, based on a recent study (18), starting at 3 mo old in OVE26 mice when these mice already displayed significant renal dysfunction. For control and DM mice, equal amounts of physiological saline solution containing 0.0025 μg DMSO/ml were given. MG-132 treatment for 3 mo in OVE26 diabetic mice did not affect their blood glucose levels (7).

Four mice from both control and DM groups were killed at 3 mo of age, and other mice (n = 6) were treated with MG-132 for 3 mo and killed at 6 mo of age. After heart weight and tibia length had been measured, whole heart tissue was harvested for protein and mRNA analysis. Histopathological observations were predominantly based on the left ventricle (LV).

**Echocardiography**

To assess cardiac function, echocardiography was performed in mice using a Visual Sonics Vevo 770 high-resolution imaging system, as previously described (29, 39). Under sedation with Avertin (2,2,2-tribromoethanol, 240 mg/kg ip), mice were placed in the supine position on a heating pad to maintain body temperature at 36–37°C. Heart rates were kept 400–550 beats/min. Two-dimensional and M-mode echocardiography were used to assess wall motion, chamber dimensions, and cardiac function. Directly measured indexes included LV internal dimensions (LVID) at diastole and systole, LV posterior wall thickness (LVPW at diastole and systole), and interventricular septal thickness (IVS) at diastole and systole. LV fractional shortening (FS) was determined as follows: FS = [(LVID at diastole − LVID at systole) × 100]. LV ejection fraction (EF) was determined as follows: EF = [(LV volume at diastole − LV volume at systole)/LV volume at diastole] × 100.

**Western Blot Analysis**

Western blot analysis was performed as described in our previous studies (2, 39). The primary antibodies used included 3-nitrotyrosine (3-NT; 1:1,000 dilution), 4-hydroxy-2-nonenal (4-HNE; 1:1,000 dilution), TNF-α (1:500 dilution), ICAM-1 (1:500 dilution), plasminogen activator inhibitor (PAI)-1 (1:1,000 dilution), transforming growth factor (TGF)–β1 (1:500 dilution), fibroactin (1:500 dilution), Nrf2 (1:500 dilution), NQO-1 (1:500 dilution), HO-1 (1:500 dilution), CAT (1:500 dilution), atrial natriuretic peptide (ANP; 1:1,000 dilution), NF-κB (1:1,000 dilution), IκB-α (1:1,000 dilution), α-tubulin (1:2,000 dilution), and β-actin (1:2,000 dilution), all of which were purchased from Santa Cruz Biotechnology except for TNF-α (Abcam), 3-NT (Millipore), NF-κB, IκB-α, and α-tubulin (Cell Signaling), and 4-HNE (Alpha Diagnostic). All antibodies were polyclonal antibodies except for TNF-α and PAI-1 antibodies, which were monoclonal.

**Isolation of RNA and Real-Time RT-PCR**

Isolation of RNA and real-time RT-PCR were performed as described in our previous study (39) for Nrf2 (primer: Mm00477784_m1), ANP (primer: Mm01255748_g1), β-myosin heavy chain (β-MHC; primer: Mm00605555_m1), HO-1 (primer: Mm00516005_m1), NQO-1 (primer: Mm253561_m1), CAT (primer: Mm00437229_m1), and the housekeeping gene β-actin (primer: Mm00607939_s1). All primers were purchased from Applied Biosystems (Foster City, CA). Total RNA was extracted from whole heart tissues using TRIzol reagent (RNA STAT 60 Tel-Test, Ambion, Austin, TX). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer (Biolab, Ontario, CA). First-strand cDNA was synthesized from total RNA according to the RNA PCR kit (Promega, Madison, WI) following the manufacturer’s protocol.

**Cardiac Histopathological Examination and Immunohistochemical Staining**

Whole cardiac tissue was fixed overnight in 10% phosphate-buffered formalin, dehydrated in a graded alcohol series, cleared with xylene, embedded in paraffin, and then sectioned at 5 μm thickness for pathological and immunohistochemical staining. Paraffin sections were dewaxed followed by an incubation in 1X target retrieval solution (Dako, Carpinteria, CA) for 15 min at 98°C for antigen
Sections were then treated with 3% H₂O₂ for 15 min at room temperature followed by blocking with 5% BSA for 30 min. Cardiac sections were stained with hematoxylin and eosin and Sirius red, respectively, as previously described (2, 39). For immunohistochemical staining, sections were incubated with the primary antibodies of TGF-β₁ (1:100 dilution, Santa Cruz Biotechnology) and PAI-1 (1:100 dilution, BD Biosciences) overnight at 4°C. After being washed with PBS, sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:300–400 dilutions in PBS) for 2 h at room temperature. For the development of color, sections were treated with the peroxidase substrate 3,3-diaminobenzidine in the developing system (Vector Laboratories, Burlingame, CA).

**Proteasome Activity**

The 20S proteasome, the catalytic core of the 26S proteasome complex, is responsible for the breakdown of short-lived regulatory proteins, including Nrf2 and NF-κB (6, 9). Since MG-132 mainly

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Fig. 1. Therapeutic effects of MG-132 on diabetes-induced cardiac dysfunction. Three-mo-old female OVE26 mice and FVB control mice were given MG-132 (10 μg/kg) or an equal volume of physiological saline solution daily for 3 mo. Mice were divided into the following groups: control FVB mice (Ctrl group), control mice with MG-132 treatment (MG-132 group), diabetic OVE26 mice (DM group), and diabetic mice with MG-132 treatment (DM/MG-132 group). Cardiac functional (A) and structural (B) changes were evaluated by echocardiography. LVID;d and LVID;s, left ventricular (LV) internal dimension at diastole and at systole, respectively; EF%, ejection fraction (in %); FS%, fractional shortening (in %); IVS;d and IVS;s, interventricular septal thickness at diastole and at systole, respectively; LVPW;d and LVPW;s, LV posterior wall thickness at diastole and at systole, respectively; 3m and 6m, 3 mo and 6 mo, respectively. Data are presented as means ± SD. *P < 0.05 vs. the Ctrl group; #P < 0.05 vs. the DM group.
inhibits proteasome chymotrypsin-like activity (24), we determined 20S proteasome activity by quantifying the hydrolysis of the SLLVY-AMC-a fluorogenic substrate for chymotrypsin-like activity. 20S proteasome activity was measured with the 20S proteasome activity assay kit (Millipore).

Isolation of Nuclei

Cardiac nuclei were isolated using the nuclei isolation kit (Sigma-Aldrich). Whole cardiac tissue (60 mg) from each mouse was homogenized for 45 s with 300 μl cold lysis buffer containing 1 μl DTT and 0.1% Triton X-100. After that, 600 μl of cold 1.8 mol/l cushion solution (sucrose cushion solution: sucrose cushion buffer:DTT (900:100:1)] were added to the lysis solution. The mixture was transferred to a new tube preloaded with 300 μl of 1.8 mol/l sucrose cushion solution followed by centrifugation at 13,000 rpm for 45 min. The supernatant contained the cytosolic components, and nuclei were visible as a thin pellet at the bottom of the tube.

Measurements of Serum TNF-α and Monocyte Chemoattractant Protein-I and Cardiac Nuclear NF-κB DNA-Binding Activity

Serum levels of TNF-α and monocyte chemoattractant (MCP)-1 were performed using mouse TNF-α and MCP-1 ELISA kits (Invitrogen, Camarillo, CA), as previously described (21). The low detection limits were 3 and 9 pg/ml for serum TNF-α and MCP-1, respectively. Nuclear p65 DNA-binding activity was determined by an ELISA-based NF-κB activity assay using cardiac tissue from the whole heart (Cayman Chemical, Ann Arbor, MI), according to manufacturer instructions.

Total Antioxidant Capacity Assay

The cardiac total antioxidant capacity (TAC) assay was performed using a commercially available assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s instructions. Briefly, whole heart tissues were homogenized in cold PBS and then centrifuged at 10,000 g at 4°C for 10 min to collect the supernatant for the TAC assay. Values were calculated using optical density at 490 nm and expressed as micromoles per gram of protein for TAC.

Statistical Analysis

Data were expressed as means ± SD. For statistical analysis, one-way ANOVA was used as appropriate. An overall F-test was performed to test the significance of the ANOVA models. The significance of the interactions and main effects were taken into consideration, and multiple comparisons were then performed with a
Bonferroni test. P values of <0.05 were considered as statistically significant.

RESULTS

Therapeutic Effect of MG-132 on Diabetes-Induced Cardiac Dysfunction and Hypertrophy

Transgenic OVE26 type 1 DM mice at 3 mo showed increased LVID at systole and decreased EF and FS percentages, suggesting the exist of cardiac dilation and decreased systolic function (Fig. 1A). Hypertrophic measurements of IVS, LVPW, and LV mass were significantly increased in these mice, with a progressive manner from 3 to 6 mo (P < 0.05; Fig. 1B). However, when some of the DM mice at 3 mo of age were treated with low-dose MG-132 for 3 mo (DM/MG-132 group), diabetes-induced cardiac dysfunction was almost completely reversed (P < 0.05; Fig. 1).

The therapeutic effects of MG-132 on diabetes-induced cardiac hypertrophy were also reflected by the ratio of heart weight to tibia length (Fig. 2A), which was significantly increased in an age-dependent manner in the DM group from 3 to 6 mo but not in the DM/MG-132 group.

To further confirm cardiac hypertrophy, cardiac morphology and molecular hypertrophy makers were also examined. Hematoxylin and eosin staining showed that, compared with the control and MG-132 groups, the main pathological changes in the DM group included 1) myocardial hypertrophy, 2) a few cardiac cells that showed features of degeneration and 3) proliferation of interstitial collagen fibers. Those pathological changes were alleviated or not observed in DM/MG-132 mice (Fig. 2B). Real-time PCR analysis showed that DM induced a significant increase of the cardiac mRNA expression of ANP (Fig. 2C) and β-MHC (Fig. 2D), an effect that was completely abolished by MG-132 treatment in the DM/MG-132 group. Consistent with the ANP mRNA findings, Western blot analysis showed a significant increase of cardiac ANP protein expression at 6 mo of age in DM mice but not in DM/MG-132 mice (Fig. 2E). These results suggest that 3-mo treatment with low-dose MG-132 can significantly or even completely pre-

Fig. 3. Therapeutic effect of MG-132 on diabetes-induced cardiac fibrosis. A: cardiac tissue was stained with Sirius red for collagen. Arrows indicate the proliferation of interstitial collagen fibers. Original magnification: ×200. Bar = 50 μm. B: semiquantitative analysis was done by a computer imaging system. C: cardiac expression of transforming growth factor (TGF)-β1, fibronectin, and plasminogen activator inhibitor (PAI)-1 was examined by Western blot analysis. D: cardiac expression of TGF-β1 and PAI-1 was also tested by immunohistochemical staining. Arrows indicate positive staining. Data are presented as means ± SD. *P < 0.05 vs. the Ctrl group; #P < 0.05 vs. the DM group.
vent the progression of the cardiac hypertrophy induced by diabetes.

Therapeutic Effect of MG-132 on Diabetes-Induced Cardiac Fibrosis

Cardiac tissue was examined with Sirius red staining for collagen (Fig. 3A) followed by semiquantitative analysis with a computer imaging system (Fig. 3B), which showed a significant increase in collagen accumulation in the DM group but not in the DM/MG-132 group. Western blot analyses of TGF-β1, fibronectin, and PAI-1 as important profibrotic mediators confirmed the Sirius red staining results: there was a significant increase in cardiac fibrosis in the DM group but not in the DM/MG-132 group (Fig. 3C). Furthermore, the increased expression of TGF-β1 and PAI-1 was supported by immunohistochemical staining results (Fig. 3D).

Therapeutic Effect of MG-132 on Diabetes-Induced Cardiac Inflammation and Oxidative Stress

Since PAI-1 acts as both a profibrotic and inflammatory mediator, its increase in the hearts of DM mice suggests possible cardiac inflammation. Thus, we next examined the protein expression of TNF-α (Fig. 4A) and MCP-1 (Fig. 4B) in serum by ELISA and in the heart by Western blot assay (Fig. 4, C and D). Serum TNF-α and MCP-1 levels were progressively elevated from 3 to 6 mo in the DM group and were completely prevented by MG-132 treatment in the DM/MG-132 group. Increased cardiac TNF-α and MCP-1 contents were also observed only in the DM group and not in the DM/MG-132 group.

Considering that inflammation in target organs often causes oxidative stress and that oxidative stress is also able to induce inflammation, we examined oxidative stress by measuring 3-NT accumulation as an index of protein nitration (Fig. 5A) and 4-HNE as an index of lipid peroxidation (a measure of oxidative damage; Fig. 5B). Both 3-NT and 4-HNE contents were significantly increased in the hearts of DM mice but not DM/MG-132 mice. TAC in heart tissue (Fig. 5C) was slightly decreased (P < 0.05) in the DM group at both 3 and 6 mo but was not changed in the DM/MG-132 group at 6 mo.

Possible Mechanisms for the Therapeutic Effect of MG-132 on Diabetic Cardiomyopathy

Diabetes increases cardiac proteasomal activity, an effect prevented by MG-132. Since tetrahydrobiopterin deficiency has been reported to uncouple the enzymatic activity of endothelial nitric oxide synthase in DM hearts triggered by DM-
increased proteasome-dependent mechanisms (35), we examined whether cardiac proteasome activity was increased in diabetic mice. The results shown in Fig. 6 demonstrate that MG-132 decreased, and diabetes increased, cardiac 20S proteasomal activity. Compared with the DM group, cardiac 20S proteasomal activity was significantly reduced, even to control levels, in the DM/MG-132 group (45% DM, $P < 0.05$ vs. the DM group).

MG-132 inhibits cardiac proteasomal activity, resulting in an upregulation of cardiac Nrf2 and its downstream antioxidants. Proteasomal degradation of Nrf2 has been delineated as the major of mechanism responsible for Nrf2’s negative regulation (14, 30). Our finding that MG-132 completely inhibited diabetes-upregulated proteasomal activity implies a possibility for MG-132 treatment to decrease the degradation of Nrf2 protein. Therefore, expression of Nrf2 at mRNA and protein levels was examined with real-time PCR and Western blot assays, respectively. We found that MG-132 had no impact on, but diabetes had a significant increase in, the mRNA expression of Nrf2 (Fig. 7A). The DM/MG-132 group had a similar expression of Nrf2 mRNA as in the DM group (Fig. 7A). The results shown in Fig. 7B demonstrate that both MG-132 and DM increased the cardiac expression of Nrf2 protein, so that Nrf2 protein expression was synergistically increased in the DM/MG-132 group.

Furthermore, the upregulated Nrf2 transcription activity was reflected by the increase of several downstream antioxidant genes (Fig. 7, C and D). MG-132 treatment in control mice significantly increased the expression of NQO-1, HO-1, and
CAT at the mRNA and protein levels compared with the control group. Diabetes also increased the expression of these antioxidants at the mRNA and protein levels. Expression of these Nrf2 downstream antioxidant genes in the hearts of DM/MG-132 mice was synergistically increased at both the mRNA and protein levels compared with both MG-132 or DM mice.

MG-132 inhibits cardiac proteasomal activity, resulting in a reduction of cardiac inflammation by preventing NF-κB nuclear translocation. Since NF-κB is bound by IκB-α in the cytoplasm, inhibiting NF-κB nuclear translocation to transcriptionally upregulate inflammatory cytokines, a reduction of IκB-α will indirectly upregulate NF-κB-mediated inflammation. Western blot analysis showed that the expression of IκB-α was significantly decreased in hearts of the DM group but not the DM/MG-132 group (Fig. 8A). This suggests that MG-132 inhibits the proteasomal degradation of IκB-α. To define whether preservation of a normal level of IκB-α is able to prevent NF-κB nuclear translocation, cardiac proteins were separated into nuclear and cytoplasmic parts, which showed that the nuclear accumulation of NF-κB was significantly increased in the DM group but not in the DM/MG-132 group (Fig. 8B). Furthermore, the increased cardiac nuclear NF-κB DNA-binding activity only in the hearts of DM mice at both 3 and 6 mo was further confirmed by an ELISA-based NF-κB activity assay (Fig. 8C).

DISCUSSION

The present study is the first to report the therapeutic effects of chronic treatment with low-dose MG-132 on diabetes-induced cardiomyopathy using the OVE26 diabetic mouse model. We demonstrated that the therapeutic effect of MG-132 on diabetic cardiomyopathy is associated with its suppression
of diabetic upregulation of proteasome activity, which may increase the proteasomal degradation of IκB-α and Nrf2. Increased degradation of IκB-α would release its binding and restricting NF-κB in the cytoplasm, leading to an increase of NF-κB nuclear translocation to generate inflammatory cytokines, as shown in Fig. 9. These cytokines then initiate an overgeneration of ROS/reactive nitrogen species, cardiac oxidative stress and damage, and remodeling and dysfunction. In addition, increased proteasomal degradation of Nrf2 will reduce the transcription of Nrf2 to generate its downstream antioxidants, which will exacerbate cardiac pathogenic alterations, leading to an acceleration of cardiomyopathy development, as shown in Fig. 9.

Several in vivo and in vitro studies have provided evidence for the increase in proteasomes under diabetic conditions. For example, exposure of human umbilical vein endothelial cells to a high level of glucose significantly increased 26S proteasome activity (35). Proteasomal activity was also found to be increased in the hearts of type 1 diabetic mice (11, 22) and in the gastrocnemius muscles of spontaneous type 2 diabetic (db/db) mice (33). Measurements of proteasomal activity in the kidneys of diabetic rats showed an increase of 26S proteasomal activity in streptozotocin-induced diabetic rats (18).

An in vivo pilot study (18) has shown the preventive effect of MG-132 on diabetes-induced renal damage. In that study, shortly after the induction of diabetes, rats were treated with MG-132 at 10 μg/kg daily for 3 mo. This regimen produced renal prevention, as indicated by reductions in proteinuria, basement membrane thickening, and glomerular mesangial expansion. MG-132 also reduced kidney markers of oxidative stress and increased protein levels of Nrf2 and several antioxidant enzymes. These experiments demonstrated a potential for
MG-132 to prevent the development of diabetic complications. However, whether Nrf2 activators have therapeutic effects on the heart and/or kidney of diabetic subjects remains unknown. Here, we report, for the first time, the therapeutic effects of MG-132 on diabetic cardiomyopathy in the transgenic type 1 diabetic OVE26 mouse model. When these diabetic mice at 3 mo old began to exhibit albuminuria as an index of renal dysfunction (38) and cardiac dysfunction (Fig. 1), some of them were treated with MG-132 at a very low dose for 3 mo, which offered a significant therapeutic outcome, as indicated by the complete prevention of diabetes-induced cardiac inflammation and oxidative stress and damage, leading to a complete reverse of cardiac remodeling (fibrosis and hypertrophy) and dysfunction. Diabetic mice without treatment with MG-132 showed the progressive development of cardiac structural remodeling and functional abnormalities.

In terms of the mechanisms underlying the therapeutic effects of MG-132 on diabetic cardiomyopathy, we assumed that they are associated with the significant upregulation of Nrf2 expression and transcriptional increases of its downstream antioxidants and the complete prevention of diabetes-reduced IκB content and diabetes-increased NF-κB nuclear accumulation, as shown in Fig. 9.

Reportedly nontoxic concentrations of MG-132 inhibited the proteasomal degradation of Nrf2 to stimulate Nrf2 translocation into the nucleus (5, 28). An in vitro study (9) has shown that treatment with 0.5 μM MG-132 for 48 h protected neonatal rat cardiac myocytes against H2O2-mediated oxidative stress. This was correlated with reduced levels of intracellular ROS and significant upregulation of superoxide, HO-1, and CAT expression. This demonstrated that nontoxic concentrations of MG-132 could upregulate Nrf2-mediated antioxidant
enzymes to confer cardiomyocyte protection (9). We (10) have demonstrated the high susceptibility of cardiomyocytes from Nrf2-null mice to high-glucose-induced ROS generation and damage compared with those from Nrf2 wild-type mice. Therefore, we assumed that the preventive effect of the proteasomal inhibitor MG-132 was due to elevated Nrf2 protein content, which increased the expression of multiple downstream antioxidant enzymes.

RT-PCR analysis revealed that diabetes, but not MG-132, significantly increased Nrf2 mRNA expression (Fig. 7A), suggesting the existence of a compensatory response of the heart to diabetes-induced oxidative stress by upregulation of Nrf2 mRNA expression. However, diabetes also increased 20S proteasome activity, which should have reduce Nrf2 protein levels and resulted in less nuclear translocation; therefore, the final outcome of Nrf2 protein expression in the diabetic heart remained at a relatively high level compared with the control group. In contrast to the DM group, DM/MG-132 mice showed increased Nrf2 mRNA expression, which should be attributed to an effect of diabetes (Fig. 7A), and also synergistically increased Nrf2 protein levels, which should be attributed to both an effect of diabetes on mRNA expression as well as the inhibitory effect of MG-132 on proteasomal degradation of the Nrf2 protein level. Expression profiles of Nrf2 downstream antioxidants at the mRNA and protein levels (Fig. 7, C and D) were similar to the profile of Nrf2 protein expression among the groups (Fig. 7B). These upregulated Nrf2-mediated antioxidants seem to be responsible for the therapeutic effects on the heart, based on a previous study (37) reporting that upregulated Nrf2 expression in the heart or kidney provided significant prevention of diabetes-induced damage.

However, due to the fact that the expression of Nrf2 and its downstream antioxidants was not significantly decreased in the hearts of DM mice compared with control mice, we assumed that the increased expression of Nrf2 and its downstream antioxidants may be not the sole mechanism underlying the therapeutic effects of MG-132 on diabetic cardiomyopathy. The role of an excessive inflammatory response in the initiation of diabetic cardiomyopathy has been discussed recently (27, 34). As a key transcription factor to control inflammation, NF-κB activation was found to play a pivotal role in the development of cardiomyopathy (20). Recent studies (19, 23) have demonstrated the protection of MG-132 in the other organs via inhibition of NF-κB. Here, we demonstrated the activation of NF-κB in the hearts of OVE26 diabetic mice, which was significantly inhibited by treatment with MG-132 in the DM/MG-132 group. This suggests that the effective inhibition of proteasome activity by MG-132 may be attributed to its effective inhibition of cardiac inflammation, as we observed in this study. As shown in Fig. 9, NF-κB triggers the transcription of many inflammation cytokines. These inflammatory cytokines stimulate the generation of ROS and/or reactive nitrogen species, which induce cardiac oxidative stress and damage and remodeling, leading to the development of diabetic cardiomyopathy. Therefore, inhibition of cardiac inflammation activation should provide cardiac protection from diabetes. We demonstrated that diabetes significantly suppressed the expression of IkBα and increased the nuclear accumulation of NF-κB and DNA-binding capacity. All these effects were almost completely abolished by MG-132 treatment, which was accompanied with a complete reverse of diabetes-induced cardiac inflammation and oxidative damage.

A potential limitation of the present study may be the route (intraperitoneal injection) by which MG-132 was given, which may not directly applicable to clinics. In the present study, we used intraperitoneal injection because we wanted to ensure the precise dose given for the low dose of MG-132. Although we cannot directly expect that what we do in this study will be directly extrapolated to clinics, the eventual oral administration of the MG-132 for diabetic patients with the appearance of albuminuria will be easily established if we confirm the therapeutic effect, safety, and underlying mechanisms. The latter two will be further investigated in future studies.

In summary, the present study demonstrated that the proteasomal inhibitor MG-132 at a low dose (10 μg/kg) can provide a therapeutic effect on diabetic cardiomyopathy. Here, we used the transgenic type 1 diabetic OVE26 mouse model. When these diabetic mice began to exhibit both albuminuria as an index of renal dysfunction and cardiac dysfunction by echocardiography, some of them were treated with MG-132 at a very low dose for 3 mo, which offered a significant therapeutic outcome, as indicated by the complete prevention of diabetes-induced cardiac inflammation and oxidative damage, leading to a reversal of cardiac remodeling and dysfunction. In contrast, diabetic mice without treatment with MG-132 showed the progressive development of cardiac inflammation, structural remodeling, and dysfunction. These therapeutic changes were associated with both significant upregulation of Nrf2 expression and transcriptional increases of its downstream antioxidants and significant suppression of NF-κB-mediated inflammation (Fig. 9). Therefore, MG-132 has great potential as a therapeutic agent for diabetic patients, including those with diabetic cardiomyopathy.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
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