Enhancement of 26S Proteasome Functionality Connects Oxidative Stress and Vascular Endothelial Inflammatory Response in Diabetes Mellitus

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Objective—Although the connection of oxidative stress and inflammation has been long recognized in diabetes mellitus, the underlying mechanisms are not fully elucidated. This study defined the role of 26S proteasomes in promoting vascular inflammatory response in early diabetes mellitus.

Methods and Results—The 26S proteasome functionality, markers of autophagy, and unfolded protein response were assessed in (1) cultured 26S proteasome reporter cells and endothelial cells challenged with high glucose, (2) transgenic reporter (UbG76V–green fluorescence protein) and wild-type (C57BL/6J) mice rendered diabetic, and (3) genetically diabetic (Akita and OVE26) mice. In glucose-challenged cells, and also in aortic, renal, and retinal tissues from diabetic mice, enhanced 26S proteasome functionality was observed, evidenced by augmentation of proteasome (chymotrypsin-like) activity and reduction in 26S proteasome reporter proteins, accompanied by increased nitrotyrosine-containing proteins. Also, whereas IκBα proteins were decreased, an increase was found in nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) nucleus translocation, which enhanced the NF-κB–mediated proinflammatory response, without affecting markers of autophagy or unfolded protein response. Importantly, the alterations were abolished by MG132 administration, small interfering RNA knockdown of PA700 (proteasome activator protein complex), or superoxide scavenging in vivo.

Conclusion—Early hyperglycemia enhances 26S proteasome functionality, not autophagy or unfolded protein response, through peroxynitrite/superoxide–mediated PA700-dependent proteasomal activation, which elevates NF-κB–mediated endothelial inflammatory response in early diabetes mellitus. (Arterioscler Thromb Vasc Biol. 2012;32:00–00.)

Key Words: diabetes mellitus • 26S proteasomes • oxidative stress • inflammatory response • nuclear factor-k-light-chain-enhancer of activated B cells

It has been widely accepted that oxidative stress is a common mechanism that links hyperglycemia to diabetic cardiovascular complications.1–4 Inflammation is a characteristic of both type 1 and type 2 diabetes mellitus.5,6 Overwhelming evidence implicates the association of oxidative stress with altered vascular inflammatory response in hyperglycemia. However, the underlying mechanisms are not fully elucidated. The ubiquitin proteasome system (UPS) is the major nonlysosomal degradative machinery for most intracellular proteins.6,7 As a key component in the UPS, the 26S proteasome functions to recognize, unfold, and ultimately destroy proteins.8 It is now recognized as a regulator of the cell cycle and cell division,9,10 immune responses and antigen presentation,11,12 apoptosis,13 and cell signaling.14,15 The 26S proteasome has been shown to be either overactivated in certain cancers (multiple myeloma)16 or dysfunctional in neurodegenerative disorders (eg, Alzheimer disease, Huntington disease)17 and amyotrophic lateral sclerosis.18,19 Although proteasome abnormalities continue to be implicated in these diseases,20 the elucidation of their roles in the pathogenesis of cardiovascular disease is in its infancy.21,22 Recent studies suggest that myocardial ischemia,23–25 certain mutant protein–associated cardiomyopathies,26–28 atherosclerosis,29,30 and even diabetes31 mellitus are proteasome deregulation disorders. Indeed, work from us26,29 and others30,31 in different conventional diabetic animal models document an altered 26S proteasome activity in tissues based on the protease-like activity assay in vitro. However, it remains unclear whether diabetes mellitus affects the 26S proteasome functionality in vivo. This is likely because of the lack of research on a proteasome reporter model in diabetes mellitus, so assessment of 26S proteasome functionality in vivo merely relies on in vitro approaches by measuring protease-like activity either in whole cell lysate or with purified 26S proteasomes. As a result, the contribution of...
26S proteasomes to diabetes mellitus and its complications is unknown.

Since the first report on imaging 26S proteasome in living cells, an effort has been made to generate models that allow assessment of 26S proteasome functionality in the whole animal. Ub\(^{G76V}\)-green fluorescence protein (GFP) mice have been engineered by expressing a surrogate protein substrate (GFP) fused with a ubiquitin mutant (Ub\(^{G76V}\)). In a similar manner, mice that express either a modified GFP (GFPdgn) or a different surrogate substrate (luciferase) have been produced. Although most of these models are designed to assess proteasome inhibition, none of them have been tested in diabetes mellitus. In addition, none of the mice are made commercially available for open investigation, except for the Ub\(^{G76V}\)-GFP mice (Jackson Laboratory, Bar Harbor, ME), which have been used to monitor proteasome functionality in models of amyotrophic lateral sclerosis, Alzheimer disease, and polyglutamine diseases. The aim of the present study was to define the role of 26S proteasome functionality in early vascular inflammatory response in diabetes mellitus with a Ub\(^{G76V}\)-GFP reporter in both cell and mouse models.

**Methods and Materials**

**Materials**

The antibodies included Grp94, LC3B, nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB; histone, and peroxidase-conjugated secondary antibodies (Cell Signaling; Danvers, MA); GFP, heat shock protein 70, heat shock protein 90, β-actin, and 3-nitrotyrosine (Santa Cruz Biotechnology; Santa Cruz, CA); and PA700/10B (ABcam, San Francisco, CA). The reagents were the following: control small interfering RNA (siRNA) and PA700 siRNA (Invitrogen, Carlsbad, CA); mito-TEMPO-H (mTempol) (Enzo Life Sciences, Farmingdale, NY); G-nitro-L-Arginine-Methyl Ester, MG132, and a ubiquitinated protein enrichment kit (EMD Chemicals, San Diego, CA); uric acid (Fisher scientific, Pittsburgh, PA); ProLong Gold and SlowFade Gold Antibody Reagents, and goat anti-rabbit IgG conjugates labeled with Alexa Fluor 594 (Invitrogen, Carlsbad CA); and fluorogenic substrate Suc-LLVY-7-amido-4-methylcoumarin for chymotrypsin-like activity assay (Sigma, St. Louis, MO). All drug concentrations are expressed as final concentrations in the buffer.

**Western blot analysis**

Cultured cells and tissues from aorta, kidney, retina, and lung were homogenized on ice in cell-lysis buffer. Protein quantification, Western blotting, and band densitometry were performed as previously reported.

**26S Proteasome Activity Assay**

The chymotrypsin-like activity was measured with a fluorogenic substrate as previously described.

**Mice and the Induction of Diabetes Mellitus With Streptozotocin**

Male mice of the C57BL/6J wild type and the Ub\(^{G76V}\)-GFP mice, as well as male genetic diabetic Akita mice, OVE26 mice (and their genetic control FVB mice), 8 to 12 weeks of age, 20 to 25 g, were obtained from the Jackson Laboratory. Mice (C57BL/6J or Ub\(^{G76V}\)-GFP) were rendered diabetic with a low-dose streptozotocin (STZ) regimen as previously described. Acute hyperglycemia was defined as a fasting (4 hours) blood glucose level of >250 mg/dL for >1 week after injection. After the final dose of STZ injection, some mice received an MG132 injection (5 mg/kg per day; 2 days IP with dimethyl sulfoxide, the vehicle, as a control), or an administration of mTempol (0.1 mmol/L in drinking water, 4 weeks; vehicle, normal drinking water) before tissue collections. Some Akita mice received insulin pellet implantation (LinShin, Canada Inc) to lower fasting blood glucose. The animal protocols were reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee (IACUC). The approved IACUC protocol numbers are as follows: 10-153-H, 11-072-H, and 11-045.

**Infection of Mice With siRNA**

siRNA was provided by Ambion. Briefly, 25 μg of PA700 or control siRNA was mixed with in vivo-jetPEI (Polyplus Transfection, France), an siRNA-delivering buffer developed for mice, at an N/P ratio of 5 at room temperature for 15 minutes. The procedure for administration of siRNA was performed as described.

**Chromatin Immunoprecipitation Assay in Tissue**

Tissue chromatin immunoprecipitation (ChIP) was performed with a commercial kit (EZ-ChIP; Millipore, Billerica, MA). Following the manufacturer instructions, 30 μg of lung tissue per mouse were the starting materials, with an NFκBp65 (Santa Cruz) antibody for ChIP, an IgG antibody for a negative control, and the total DNA before immunoprecipitation for an input. Sequences of promoter-specific primers for mouse Chemokine (C-C motif) ligand 5 (Ccl5) and interleukin-8 (IL-8), and L-selectin were kindly provided by Dr. Gioacchino Natoli and for mouse intracellular adhesion molecule 1 as reported. The primers were synthesized by Sigma (St. Louis, MO).

**Statistical Analysis**

Data are reported as means±SEM. ANOVA was used to compare means of different experimental groups, and Tukey tests were used as post hoc tests. P<0.05 was considered statistically significant.

**Results**

**High Glucose, Not the Osmotic Control, Reduces Poly-ub-GFP Protein Levels in Ub\(^{G76V}\)-GFP Cell**

We first tested to see whether high glucose affected reporter protein levels in 26S proteasome reporter (Ub\(^{G76V}\)-GFP) cells. We incubated the cells with a high concentration of d-glucose (25 mM/L, 4 hours), using mannitol (25 mM/L, 4 hours) and l-Glucose (25 mM/L, 4 hours), respectively, as an osmotic control. We found that high glucose, not the osmotic control, promoted a 2-fold reduction of a band located near 75kDa, but not that of another band located slightly above 26kDa (Figure 1A). Because the GFP-infused ubiquitin in the reporter has been mutated (G76V) to resist hydrolysis by ubiquitinase, the recognized bands were very likely the ubiquitinated GFP. To confirm this, we took 2 approaches of Western blotting: probing either the GFP immunoprecipitates with a GFP-infused ubiquitin antibody or the enriched ubiquitinated proteins (by an enrichment kit) with a GFP
antibody. We obtained same results in both the approaches (not shown), which were similar to Figure 1A. Given the molecular weights of a single GFP-infused ubiquitin (8.5 kDa) and GFP (27 kDa), the bands shown in Figure 1A represented a poly-ubiquitinated GFP ([poly-Ub-GFP] with 4–5 ubiquitin around the 75 kDa marker band) and a mono-ubiquitin GFP ([Ub-GFP] around 36 kDa, above the 26 kDa marker band). Importantly, preincubation of MG132 (5 µmol/L for 1 hour), a potent inhibitor of 26S proteasomes, blocked the reduction (Figure 1A), confirming both the identities of the ubiquitinated bands and the involvement of 26S proteasomes in high glucose-elicited impact.

To identify cellular locations of the targeted GFP proteins, we performed immunostaining staining with a GFP primary antibody and a secondary antibody conjugated with a red fluorescence dye (Alex Fluor 594). The rationale of using an antibody instead of detecting GFP fluorescence directly lies in the fact that the Ub<sup>G76V</sup>-GFP cell presents a very low fluorescent background, which favors the assay of 26S proteasome inhibition. Therefore, monitoring high glucose-decreased fluorescence (for proteasome activation) from an already low background would be hard to achieve. In contrast, immunostaining would be a more sensitive approach. Indeed, with a rabbit-derived GFP antibody, we were able to show that the GFP presented in both cytosol and nucleus (Figure 1B), consistent with previous reports. However, these signals were significantly decreased both in cytosol and nucleus in high glucose-treated cells, an effect that was abolished, at least in part, by preincubation of MG132 (Figure 1B).

High Glucose Promotes 26S Proteasome Activation Without Affecting the Markers of Autophagy and Unfolded Protein Response

We next investigated the possible mechanism(s) underlying enhanced 26S proteasome functionality. We measured 26S proteasome activity in Ub<sup>G76V</sup>-GFP cells being challenged with high glucose for up to 4 hours. The chymotrypsin-like activity was increased in high glucose-treated cells in a time-dependent fashion (Figure 1C). In a similar manner, the protein levels of poly-Ub-GFP, but not that of Ub-GFP, were reduced (Figure 1D), consistent with the 26S proteasome activity (Figure 1C). In contrast, protein levels of PA700 (S10B), Grp94, LC3B, heat shock protein (HSP) 90, HSP70, and β-actin, respectively. All blots shown are representative of the 3 independent experiments. The results (n=3) were analyzed with a 1-way ANOVA. *Significant difference vs control. Ub-GFP indicates mono-ubiquitin GFP; DAPI, 4',6-diamidino-2-phenylindole.
Scavenging of Peroxynitrite Prevents Both 26S Proteasome Activation and GFP Protein Reduction by High Glucose

We wondered whether the enhancement of 26S proteasome functionality was attributable to peroxynitrite (ONOO⁻), which affects 26S proteasome activity.²⁹ ONOO⁻ is formed during simultaneous generation of superoxide and NO in a cell; inhibition of superoxide or NO abolishes ONOO⁻ formation.³¹ We incubated the reporter cells with either G-nitro-L-Arginine-Methyl Ester (1 mmol/L for 1 hour), a nonselective inhibitor of endothelial nitric oxide synthase, mTempol (1 mmol/L for 1 hour), a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties,⁴² or uric acid (100 µmol/L for 1 hour), a potent scavenger of ONOO⁻, before high glucose stimulation. We found that preincubation with G-nitro-L-Arginine-Methyl Ester blocked the reduction in poly-Ub-GFP protein levels (Figure 2A). Similarly, the reduction was abolished by administration of either mTempol (Figure 2B) or uric acid (Figure 2C), suggesting an important role of ONOO⁻ in elevated 26S proteasome functionality.

High Glucose Activates NF-κB Nucleus Translocation in Both Reporter Cells and the Primary Endothelial Cells, Which Can Be Blocked by Either MG132 or mTempol Administration

We monitored NF-κB (p65) protein in Ub⁷⁶⁷⁶V-GFP cells to define the physiological outcome of activated 26S proteasome functionality. High glucose versus control-treated cells presented stronger NF-κB (p65) immunofluorescence in nucleus than in cytosol (Figure 3A), suggesting increased nucleus translocation of the protein. Consistent with its suppressive impact on 26S proteasome functionality, mTempol pretreatment prevented NF-κB (p65) nuclear translocation (Figure 3A). This was further confirmed by Western blotting on the nucleus preparations of the cells (Figure 3B), suggesting that high glucose elicited an mTempol-reversible NF-κB (p65) nucleus translocation. To exclude the possibility that the studied effects would be unique to the reporter cells, we repeated the experiments in human umbilicus vessel endothelial cells, as well as in bovine artery endothelial cells. Again, high glucose, but not the normal glucose control, increased NF-κB (p65) protein levels in the nucleus fraction both in human umbilicus vessel endothelial cells (Figure 3C) and bovine artery endothelial cells (not shown), which was blocked by MG132 (0.5 µmol/L, 1 hour) preincubation (Figure 3C).

UbG76V-GFP Mice Can Be Rendered Diabetic and Present Significant Reduction of Poly-Ub-GFP Protein Levels in Response to Acute Hyperglycemia

We further investigated whether diabetes mellitus enhanced 26S proteasome functionality. We first tested whether Ub⁷⁶⁷⁶V-GFP mice, which are generated on a C57BL/6J genetic background and appear phenotypically normal, could be rendered diabetic with a STZ regimen. Like C57BL/6J mice, Ub⁷⁶⁷⁶V-GFP mice became hyperglycemia by STZ injection (Figure 1A in the online-only Data Supplement). There was no significant difference in body weight between the wild-type and Ub⁷⁶⁷⁶V-GFP mice, either in vehicle- or STZ-treated groups on the day of tissue collection (Figure 1B in the online-only Data Supplement).

Furthermore, we detected both Ub-GFP (around 36 kDa) and poly-Ub-GFP (around 75 kDa proteins in aortic tissue (Figure 4A) with an immunoprecipitation combined Western blotting approach. When diabetes mellitus was present, the poly-Ub-GFP protein levels were significantly reduced (≈50%) (Figure 4A or 4B). Similar observations were also made in renal and retinal tissues (Figure 4A and 4B). In all cases, the GFP staining was absent in tissues of the wild-type (C57BL/6J) mice (Figure 4A). On the basis of the results of

Figure 2. Scavenging of peroxynitrite prevents both 26S proteasome activation and poly-ubiquitinated green fluorescence protein (poly-Ub-GFP) reduction by high glucose. The 26S proteasome reporter Ub⁷⁶⁷⁶V-GFP cells were incubated with a high concentration of d-glucose (25 mmol/L) for 4 hours, with preincubation for 1 hour with (A) G-nitro-L-Arginine-Methyl Ester (L-NAME) (2 mmol/L); (B) mito-TEMPO-H (mTempol) (1 mmol/L); or (C) uric acid (100 µmol/L), followed by cell lysates preparation for Western blotting with a rabbit-derived GFP antibody and a mouse-derived β-actin antibody. All blots shown are representative of the 3 independent experiments. The results (n=3) were analyzed with a 1-way ANOVA. *Significant difference vs control. Ub-GFP indicates mono-ubiquitin GFP; UA, uric acid.
to 26S proteasomes. To confirm that enhancement of functionality was a result of activity elevation, we chose to measure 26S proteasome activity (ATP-dependent chymotrypsin-like activity) in aortic, renal, and retinal tissues. The results showed that tissues from STZ- versus vehicle-treated UbG76V-GFP mice presented a 2-fold increase in 26S proteasome activity (Figure 4C); similar scales of increase were also detected in STZ-treated C57BL/6J mice (Figure 4C). However, protein levels of PA700 (S10B) were not affected in all settings (Figure IIA or IIB in the online-only Data Supplement). To exclude that STZ per se would induce 26S proteasome activation, we measured 26S proteasome activity of the same tissue types of genetic diabetic mice, specifically the Akita mice and OVE26 mice. Compared with their genetic controls, all the testing diabetic tissues presented enhanced 26S proteasome activity (Figure 4D). Furthermore, implantation of insulin pellets in Akita mice reduced blood glucose from 450±18 to 135±16 mg/dL, which was accompanied with the reduction in proteasome activity (Figure 4E). These data suggested that hyperglycemia was the trigger of 26S proteasome activation.

Diabetic UbG76V-GFP Mice Show Increased Protein Nitrotyrosine in Accordance With the Diabetic Wild-Type C57BL/6J Mice

We next asked whether diabetic UbG76V-GFP mice would manifest oxidative stress, an important pathogenetic factor for diabetic complications, like its wild-type counterparts under hyperglycemia. To this end, we assessed the levels of protein containing nitrotyrosine (3-NT), a hallmark for protein nitration caused by ONOOH. Like the wild-type mice (Figure 5A, C57BL/6J mice), STZ- versus vehicle-treated UbG76V-GFP mice had increased 3-NT levels in aortic, renal, and retinal tissues (Figure 5A, UbG76V-GFP mice). In both diabetic mice, an ~2-fold increase in 3-NT levels was evident (Figure 5A, right). To further establish the connection of tissue protein nitration levels and proteasome activation, we performed similar experiments with OVE26 mice at different durations of diabetes mellitus. As demonstrated in Figure 5, OVE26 at 8 weeks showed an increase in 3-NT levels compared with genetic nondiabetic control (Figure 5B, 8 weeks), which were kept increasing at 12 weeks of diabetes, but not for the control (Figure 5B, 12 weeks). The 3-NT levels appeared to be associated with increased 26S proteasome activities (Figure 5C).

Proteasome Activation in Early Diabetic UbG76V-GFP Mice Involves Superoxide–Mediated Tyrosine Nitration of PA700

Previously, we have shown in a nonreporter mouse model that diabetes-increased nitrination of PA700 is associated with proteasome activation. We wondered whether similar mechanism accounted for diabetes-enhanced 26S proteasome functionality in UbG76V-GFP mice. To this end, we compared PA700 nitrination in the nondiabetic and the diabetic UbG76V-GFP mice that received either vehicle or mTempol treatment. As depicted in Figure 5, diabetic versus nondiabetic UbG76V-GFP mice presented increased levels of PA700 nitrination (Figure 5D). However, mTempol versus vehicle treatment (mTempol, 0.1 mmol/L in drinking water; vehicle, normal drinking water,
Diabetic Mice Demonstrate 26S Proteasome Activation–Mediated IκBα Reductions Without Affecting the Markers of Autophagy and Unfolded Protein Response

We next examined whether enhanced 26S proteasome functionality would affect IκBα, a known proteasome substrate\(^{46,47}\) and a key component in the NF-κB pathway.\(^ {48}\) We found that STZ-diabetic versus vehicle-treated mice showed significantly reduced IκBα protein levels of all selected tissue types (Figure 6A, upper blots). However, these reductions occurred without affecting protein levels of Grp78 and LC3B (Figure 6A, bottom blots). To confirm that proteasome activation was required, we performed siRNA infection to knockdown the PA700 protein. As expected, PA700 (S10B) versus control siRNA treatment significantly reduced PA700 protein levels (Figure 6B) and 26S proteasome activity (Figure 6C) in aortic tissue. However, PA700 knockdown by siRNA suppressed 26S proteasome activity (Figure 6C) and blocked the IκBα reductions (Figure 6B). These data suggest a crucial role for PA700-dependent proteasome functionality in IκBα reduction–mediated NF-κB pathway activation, although other mechanisms, such as the enhanced IκBα ubiquitination for proteasomal degradation, might be involved. It is worth noting that the resultant proteasome inhibition did not affect hyperglycemia (data not shown), suggesting that the protective effects of the proteasome suppression may be independent of the hyperglycemic conditions.

Diabetic Mice Present Increased Promoter-Binding Capacity of NF-κB for Proinflammatory Genes, Which Can Be Reversed by Administration of Either MG132 or mTempol

To further explore the consequences of 26S proteasome–mediated IκBα reduction, a mechanism that leads to NF-κB pathway activation and promotes inflammatory response, we monitored promoter-binding capacity of NF-κB with a ChIP assay on NF-κB–regulated genes in diabetes mellitus. These genes included IκBα, a classic NF-κB–activated gene,\(^ {39}\) and the Ccl5/RANTES and intracellular adhesion molecule 1, 2 well-established proinflammatory genes that are also regulated by NF-κB in endothelial cell in vivo.\(^ {49,50}\) A conventional ChIP assay needs at least 30 mg of tissues, which weighs 3 times the mouse aorta. We used lung tissue as a substitute which is known to be rich in vascular endothelial cells and shared similar patterns of poly Ub-GFP reduction (not shown) to other tested diabetic tissues in this study. Compared with vehicle-treated mice, diabetic mice exhibited significantly increased promoter binding of NF-κB to the genes of IκBα, Ccl5/RANTES, and intracellular adhesion molecule 1, albeit at various degrees, suggesting the activation of inflammatory NF-κB pathway (Figure 6D).
To decide whether the activation was mediated by 26S proteasomes, we injected the STZ-diabetic mice with MG132 as previously reported. Compared with vehicle-treated diabetic mice, administration of proteasome inhibitor (MG132, 5 mg/kg; vehicle, dimethyl sulfoxide, 0.5%; IP, 2 days) significantly reduced the promoter-binding capacity of NF-κB to the genes of IκBα, Ccl5/RANTES, and intracellular adhesion molecule 1 (Figure 6D).

To further determine the involvement of oxidative stress as a mechanism in vivo, we treated the other diabetic mouse groups with superoxide scavenger mTempol. Compared with vehicle treatment, mTempol treatment (mTempol, 0.1 mmol/L in drinking water; vehicle, normal drinking water, 4 weeks) markedly reversed the diabetes-increased NF-κB binding to the genes of 1xBt, Ccl5/RANTES, and intracellular adhesion molecule 1 (Figure 6D).

To the best of our knowledge, this is the first study testing diabetic 26S proteasome functionality in vivo with a proteasome reporter model. It is worth noting that, like all mice that express a surrogate proteasome substrate, UbG76V-GFP mice were initially designed favoring in vivo assessment of 26S proteasome blockade, because when proteasome is inhibited, the otherwise degraded Ub-GFP would accumulate to a significant level that the GFP fluorescence could be quantified noninvasively with the whole animal. However, a basal GFP fluorescence is reported in untreated UbG76V-GFP mice when compared with the wild type. This suggests that there is a basic equilibrium for GFP protein turnover in the transgenic mice. Indeed, the present study revealed that with additional modification of the methodology, the reporter system is also ideal to detect 26S proteasome functional enhancement. Mechanistically, the 26S proteasome functional augmentation is likely operative involving ONOO−-mediated PA700-dependent 26S proteasome activation and NF-κB–activated inflammatory response in the early phase of diabetes mellitus.

Discussion

The present study provided evidence with cell (reporter or endothelial cell) and mouse (reporter or nonreporter) models to demonstrate that hyperglycemia-induced enhancement of 26S proteasome functionality manifests as an early event in diabetes mellitus independent of autophagy and unfolded protein response, which may account for the oxidative stress–mediated vascular inflammatory response in diabetes mellitus (Figure III in the online-only Data Supplement). Provided the uncertainty of 26S proteasome functionality in the development of cardiovascular disease, it is timely to revisit these areas with a functional reporting model.
Another novel aspect of this study is the first evidence that hyperglycemia enhances 26S proteasome functionality in vivo, which operates independent of autophagy and unfolded protein response. Furthermore, the enhancement of 26S proteasome functionality may underscore the oxidative stress–elicited vascular endothelial inflammatory response in diabetes mellitus. This article presented the major evidence for the following findings: (1) hyperglycemia enhances 26S proteasome functionality in diabetic aortic, renal, and retinal tissues; (2) such enhancement involves ONOO−-mediated 26S proteasome activation and PA700-dependent 26S proteasome activation; (3) the enhancement operates as an early event in diabetes mellitus without affecting markers of autophagy and unfolded protein response or PA700 levels; and (4) such an early event leads to activation of the NF-κB pathway and the resultant elevated inflammatory response. Emerging evidence implicates the involvement of 26S proteasomes in diabetes mellitus. An elevated proteolysis mediated by proteasome is reported to contribute, in part, to diabetes-associated muscle atrophy. In contrast, proteasome inhibition is recently found to stabilize glucose transporter 1 in oxidative stress–challenged retina cells. Depending on 26S proteasomes, hyperglycemia provokes a glycogen synthase kinase 3β–mediated degradation of insulin receptor substrate 1, leading to insulin resistance. A previous study has also linked the 26S proteasome to diabetic vascular endothelial dysfunction through GTPCH I, a potential proteasome substrate, which has been confirmed by other investigators. Interestingly, downregulation of the retinoid X receptor–α that reprograms PPARγ activity is shown to be proteasome dependent in obese mice and humans. Therefore, hyperglycemia-boosted 26S proteasome functionality in the early phase of diabetes mellitus, the mechanism identified in the present study, could be a shared trigger in these apparently unrelated events. Importantly, the elevated inflammatory response, observed as the physiological consequence of enhanced 26S proteasome activation, was reversed either by 26S proteasome inactivation or superoxide scavenging, without affecting markers of autophagy and unfolded protein response. Male and age-matched (10 weeks) wild-type (C57BL/6J) mice received streptozotocin (STZ) regimen (STZ, 50 mg/kg per day; vehicle, sodium citrate; pH 4.5; for 5 days; n=5 per group). Preparations of the indicated tissues obtained 7 days post-STZ regimen were subjected to Western blotting with (A) a rabbit-derived IκBα antibody or individual antibodies against Grp78 or LC3B. Additional groups were further administered with control small interfering RNA (siRNA) or PA700 (S10B) (vehicle/STZ mice, 25 µg per 100 µL of the control/PA700 siRNA in vivo) or PEI solution, single injection, retro-orbital; n=5 per group) on next day of the last STZ dose. Aortic tissue preparations collected 7 days after siRNA injections were subjected to (B) Western blotting with rabbit-derived antibodies respectively, against PA700 (S10B) and IκBα, and a mouse-derived β-actin antibody, followed by protein band densitometry or to (C) 26S proteasome (chymotrypsin–like) activity assay. Additional groups were either treated with (D) MG132 injections (MG132, 5 mmol/L/kg; vehicle, dimethyl sulfoxide; IP, 2 days) or (E) mito-TEMPO-H (mT), 1 mmol/L in drinking water; vehicle, normal drinking water, 4 weeks); 30 mg of lung tissue were subjected to chromatin immunoprecipitation assay (NF-κBp65), with an IgG antibody as the negative control and total DNA before immunoprecipitation as an input. Polymerase chain reaction products of promoter-specific primers were separated on a 1.2% agarose gel with sizes of 202 bp (IκBα), 164 bp (Ccl5), and 216 bp intracellular adhesion molecule 1 (ICAM-1). The results (n=3–5 per group) were analyzed with a 1-way ANOVA. *P<0.05 vs the vehicle or control siRNA treated.
proteasome functionality and NF-κB pathway activation, is often found in patients of type 1 and type 2 diabetes mellitus. Given the establishment of a 26S proteasome reporter diabetic mouse model and the demonstration of 26S proteasome functionality in the initial phase of diabetes mellitus in the present study, it remains to be confirmed that the enhanced endothelial inflammatory response causes diabetic complications, such as diabetic retinopathy and nephropathy. In addition, it is yet to be established whether chronic diabetes mellitus affects 26S proteasome functionality and what their roles are in the pathogenesis of diabetic complications. Because the development of diabetic complications involves many pathological factors, such as high insulin and hyperlipidemia, it will be important to know whether these factors affect diabetes mellitus through proteasome functionality. It merits further investigations with a proteasome reporter model.

In summary, the present study provided the first evidence of enhancement of 26S proteasome functionality in vivo and the resultant activation of NF-κB pathway leading to elevated inflammatory response as an early event in diabetes mellitus. These findings are expected to facilitate the identification of new targets for 26S proteasomes and therapeutic strategies in the pathogenesis of diabetic complications, including atherosclerosis and deregulated inflammation.

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Disclosures

None.

References


