Hyperglycemia Impairs Proteasome Function by Methylglyoxal

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OBJECTIVE—The ubiquitin-proteasome system is the main degradation machinery for intracellularly altered proteins. Hyperglycemia has been shown to increase intracellular levels of the reactive dicarbonyl methylglyoxal (MGO) in cells damaged by diabetes, resulting in modification of proteins and alterations of their function. In this study, the influence of MGO-derived advanced glycation end product (AGE) formation on the activity of the proteasome was investigated in vitro and in vivo.

RESEARCH DESIGN AND METHODS—MGO-derived AGE modification of proteasome subunits was analyzed by mass spectrometry, immunoprecipitation, and Western blots. Proteasome activity was analyzed using proteasome-specific fluorogenic substrates. Experimental models included bovine retinal endothelial cells, diabetic *Ins2*^{Akita} mice, glyoxalase 1 (GLO1) knockdown mice, and streptozotocin (STZ)-injected diabetic mice.

RESULTS—In vitro incubation with MGO caused adduct formation on several 20S proteasomal subunit proteins. In cultured endothelial cells, the expression level of the catalytic 20S proteasome subunit was not altered but proteasomal chymotrypsin-like activity was significantly reduced. In contrast, levels of regulatory 19S proteasomal proteins were decreased. In diabetic *Ins2*^{Akita}, STZ diabetic, and nondiabetic and diabetic G101 knockdown mice, chymotrypsin-like activity was also reduced and MGO modification of the 20S-β2 subunit was increased.

CONCLUSIONS—Hyperglycemia-induced formation of MGO covalently modifies the 20S proteasome, decreasing its activity in the diabetic kidney and reducing the polyubiquitin receptor 19S-S5a. The results indicate a new link between hyperglycemia and impairment of cell functions. ***Diabetes*** **59:670–678, 2010**

Chronic hyperglycemia in diabetic patients is characterized by increased intracellular glucose concentration in cells damaged by diabetes complications, such as endothelial cells. Intracellular hyperglycemia activates four major pathways implicated in the pathogenesis of diabetes complications: the hexosamine and polyol pathways, protein kinase C isoforms, and formation of the reactive carbonyl compounds methylglyoxal (MGO) and glyoxal (1). These reactive carbonyl compounds form covalent adducts with particular lysine and arginine residues in proteins, altering their function (2,3). MGO is the major source of intracellular advanced glycation end products (AGEs) (4). MGO is metabolized by glyoxalase (GLO)1 and -2 to the end product D-lactate. This system efficiently detoxifies MGO and its two-carbon analog glyoxal. GLO1 deficiency is associated with high levels of intracellular AGE (5). MGO-derived AGEs, which are particularly abundant in vascular tissue, are thought to contribute to the observed vasculopathies in late complications of diabetes. In particular, these pathological conditions are associated with chronic inflammation, atherosclerosis, and diabetic nephropathy (6).

Several studies have reported that intracellular protein glycation by MGO caused pathological changes in particular proteins (7–10). However, the possibility that MGO modifies protein subunits of the proteasome has not yet been examined. The ubiquitin-proteasome system is responsible for the protein quality control in a given cell in general through degradation or removal of misfolded or damaged proteins that would adversely affect cell function (11,12). Damaged or modified intracellular proteins become targeted for proteolysis via Lys-48–connected polyubiquitin chains followed by degradation in the 26S proteasome. The 26S proteasome itself is comprised of two main multiprotein components: the 20S core proteasome containing the proteolytic activities and the 19S regulatory complex. The 19S regulator complex binds ubiquitinated proteins via the ubiquitin-interacting motifs of the S5a-subunit (Rpn10), unfolds them, and directs them into the 20S core proteasome for subsequent degradation (13–15). The 20S core complex contains three proteolytic activities: a trypsin-, a chymotrypsin- and a peptidyl-glutamyl (caspase)–like activity. As a protection mechanism against damaged and aggregated proteins, the proteasome rapidly eliminates oxidized, damaged proteins. Oxidative stress is known to upregulate the ubiquitin-proteasome machinery (16,17).

We hypothesized that MGO induced by chronic hyperglycemia could modify the proteasome and thereby impair proteasome function. Thus, the influence of MGO modification on proteasome function was studied in vascular endothelial cells, diabetic *Ins2*^{Akita} mice, streptozotocin (STZ)-injected diabetic mice, nondiabetic GLO1-knockdown mice, and STZ diabetic/GLO1-knockdown mice. Our results indicate that hyperglycemia-induced MGO formation causes covalent modification of the proteasome, which results in decreased chymotrypsin-like proteasome activity and reduced levels of the polyubiquitin receptor 19S-S5a.

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RESEARCH DESIGN AND METHODS

All chemicals and materials were purchased from Calbiochem (EMD Biosciences, San Diego, CA) unless otherwise stated. *Ins2*^{Akita} mice and C57BL/6 wild-type mice were purchased from The Jackson Laboratories (Bar Habor, ME). Six-month-old male Akita mice were killed and organs were removed in accordance with the guidelines of the ethics committee of the University of Heidelberg, School of Medicine Mannheim, and the procedures were approved by local and national authorities. GLO1-knockdown mice on a C57/B6 background were created as previously described (18). In brief, short oligonucleotides with a target sequence to mouse *glo1* in a hairpin sequence were subcloned into a lentiviral vector. The recombined plasmids were used to generate lentiviral particles. shRNA lentivirus was injected into the perivitelline space of single-cell C57/B6 mouse embryos. After incubation for 4–6 h, embryos were implanted into pseudopregnant females and were carried to term. Mice whose genome contained a single copy of the insert were identified by Southern blotting and used to establish founder lines. *glo1* mRNA and protein levels were determined by quantitative PCR and Western blot analysis and further confirmed by measurement of GLO activity. Heterozygous offspring of the founder had a 45–65% decrease in tissue GLO1 activity, and these mice were used in all experiments.

Insulin deficiency in GLO1-knockdown and wild-type mice was induced by STZ injection. In brief, 7-week-old mice were rendered diabetic via intraperitoneal injections of 50 mg · kg^{-1} · day^{-1} STZ (MP Biomedicals, Eschwege, Germany). Mice developed hyperglycemia after 10–12 weeks, with reduced insulin levels and increased glucose levels. Eight-month-old GLO1-knockdown male mice were killed and organs removed in accordance with the guidelines of the institutional animal care and use committee of the Albert Einstein College of Medicine, New York, New York.

Cell culture. Human endothelial cell line EA-hy.926 and bovine retinal endothelial cells were kindly provided by Dr. Sigrid Zink (Diabetes Research Institute, University of Düsseldorf, Germany). Cells were cultured at 37°C in a 5% CO_2 atmosphere with a minimal volume of Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FBS; 100 units/ml penicillin; 100 μg/ml streptomycin; and, for EA-hy.926 cells, additionally with 100 μmol/l hypoxanthine, 0.4 μmol/l aminopterin, and 16 μmol/l thymidine (HAT) obtained from Sigma-Aldrich (Saint Louis, MO). All culture media were from Gibco-Invitrogen (Carlsberg, CA). Cells were cultured in 5.5 mmol/l and 30 mmol/l glucose or mannitol for 14 days prior to analysis. Culture medium was changed daily.

AGE preparation. AGE-BSA was prepared as previously described (19). Briefly, 12 mg/ml BSA was incubated with 250 mmol/l glucose and 50 mmol/l sodium phosphate buffer, pH 7.4, for 6 weeks at 37°C under sterile conditions and dialyzed against in sodium phosphate buffer, pH 7.4. Preparations of differently modified forms of AGE-BSA were prepared by incubation with MGO as previously described (19). Briefly, 12 mg/ml BSA was incubated in sodium phosphate buffer, pH 7.4, in the presence of 1 mmol/l MGO for 1–6 days at 50°C.

20S proteasome purification. The 20S proteasome was purified from EA-hy.926 cell extract–essentially as previously described but with some modifications (20–22). Briefly, proteasomes were eluted from DEAE-Sephacel (GE, Waukesha, WI) with 500 mmol/l KCl and then further purified by filtration and ultracentrifugation in a sucrose gradient for 16 h at 100,000*g*. Fractions containing 20S proteasomes were pooled and concentrated by filtration and chromatographed on a Mono-Q column HR5/5 (GE) with a 80–500 mmol/l KCl gradient. All proteasome preparations were tested for proteasome activity as described below and analyzed by SDS-PAGE.

Proteasome activity assay. Tissue homogenates and cells were lysed using lysis buffer containing 25 mmol/l Tris-HCl, pH 7.5; 100 mmol/l KCl; 1 mmol/l EDTA; 0.1% (vol/vol) Triton X-100; 1 mmol/l phenylmethane sulfonylfluoride; and protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany). Tissue or cells were homogenized with liquid nitrogen and a mortar. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Thirty-microgram cells or tissue lysate was suspended in 25 mmol/l Tris-HCl, pH 7.4; 1 mmol/l dithiothreitol; and 20 mmol/l KCl and tested for proteasome activity in the absence or presence of 25 μmol/l proteasome inhibitor MG-132 with 100 μmol/l each of fluorogenic substrates ($activity_{proteasome} = activity_{total} - activity_{inhibitor}$): Z-Leu-Leu-Glu-7-amino-4-methylcoumarin (AMC) for caspase-like, Succ-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like, and Boc-Leu-Ser-Thr-Arg-AMC (Sigma-Aldrich) for trypsin-like activity. Experiments were performed for 3 h at 37°C in microtiter plates using a FLx800 microplate fluorescence reader (ex., 380 nm; em., 460 nm). V_{max} of proteasomal activity ($activity_{total} - activity_{inhibitor}$) was determined by KC4 software (BIO-TEK, Bad Friedrichshall, Germany).

20S proteasome inhibition assay. Twenty-five micrograms of purified 20S proteasomes were preincubated with 5 μg AGE-BSA or 250 μmol/l MGO, glyoxal, or glyceraldehyde for 30 min at 4°C before the 20S proteasome activity was measured as described above.

Western blot analysis. Thirty micrograms of total cell lysate was resolved by SDS-PAGE on 10% gel and blotted onto a polyvinylidine fluoride (PVDF) membrane. Polyclonal rabbit anti-20S proteasome β4-subunit antibody (Calbiochem), polyclonal rabbit anti-19S antibody from Abcam (Cambridge, MA), and polyclonal rabbit anti-ubiquitin antibody from Sanra Cruz (Santa Cruz, CA) were used for detection. The membrane was stripped and reprobed with anti–β-actin antibody clone AC-74 (Sigma-Aldrich).

Immunoprecipitation. The lysates from mice tissues were precleared by preimmune IgG plus protein A agarose beads for 2 h, and the supernatants were immunoprecipitated by the indicated antibodies and a 50% slurry of protein A agarose beads overnight at 4°C. After a washing with buffer containing 50 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; 1% NP-40; and 0.5% deoxycholate with protease inhibitors, proteins were released and the 40-μg proteins were run in NUPAGE 4–12% Bis-Tris gels (Invitrogen) and then transferred to PVDF membranes, then blotted by primary antibodies, and then simultaneously incubated with the differentially labeled species-specific secondary antibodies: anti-rabbit (or anti-goat) IRDye 800CW (green) and anti-mouse ALEXA680 (red). Membranes were scanned and quantified by the ODYSSEY infrared imaging system (LI-COR). Antibody for 19S-S5a was obtained from Abcam, and antibodies for 20S proteasome β1, β2, and β5 were obtained from Santa Cruz Biotech. Antibody for specific ubiquitin-Lys48 chain was obtained from Millipore (Bedford, MA).

Matrix-assisted laser desorption/ionization–time of flight mass spectometry. Purified 20S proteasome was short-term modified (30 min) with MGO, purified by SDS-PAGE, and stained with coomassie, and protein bands were exercised and digested with trypsin at 37°C overnight. Matrix-assisted laser desorption/ionization–time of flight mass spectometry (MALDI-TOF-MS) analysis was performed as previously described (23). Briefly, samples were extracted with 1% trifluoroacetic acid solution and mixed on target with 2.4-dihydroxy benzoic acid/methylenbisphosphonic acid (each 5 mg/ml in water), air-dried, and analyzed by a MALDI-TOF-MS (Ultraflex I; Bruker Daltonik, Bremen, Germany).

Statistics. All data are expressed as means ± SD ($n \geq 3$) unless otherwise indicated. Experimental conditions were compared with Student's t test for single measurements; those containing multiple comparisons were analyzed using ANOVA. Differences were statistically significant at $P < 0.05$.

RESULTS

High glucose causes decreased proteasomal chymotrypsin-like activity. To investigate proteasomal activity under hyperglycemic conditions, endothelial cells were incubated with high (30 mmol/l) or low (5.5 mmol/l) glucose for 14 days and proteasome activity was examined. While proteasomal trypsin-like and caspase-like activities in cells incubated with high-glucose medium were found to be unaltered (Fig. 1*A* and *B*), the chymotrypsin-like proteasomal activity was significantly reduced both in macro- and microvascular endothelial cells (Fig. 1*C*). Western blots showed that the expression level of 20S proteasome subunits was not altered. Interestingly, the expression level of the 19S regulatory complex was downregulated after incubation with high glucose (Fig. 1*D*).

Glycated albumin does not affect proteasomal activity in vitro. To determine whether MGO-AGE affects proteasome activity, an in vitro digestion of MGO-modified BSA by purified 20S proteasome was performed. Proteasomes were incubated with increasing doses of glycated AGE-BSA (0–33%) followed by analysis of proteasomal activities. These were not altered by MGO-modified BSA at any of the concentrations tested (Fig. 2). These results indicate that MGO-modified substrates do not influence proteasomal activity.

Glucose-derived carbonyl compounds inhibit the proteasomal chymotrypsin-like activity in vitro. To study the influence of glucose-derived reactive carbonyl compounds on proteasome activities, isolated 20S proteasome was incubated with 250 μmol/l MGO, glyoxal, or glyceraldehyde for 30 min prior to activity testing. Short-term

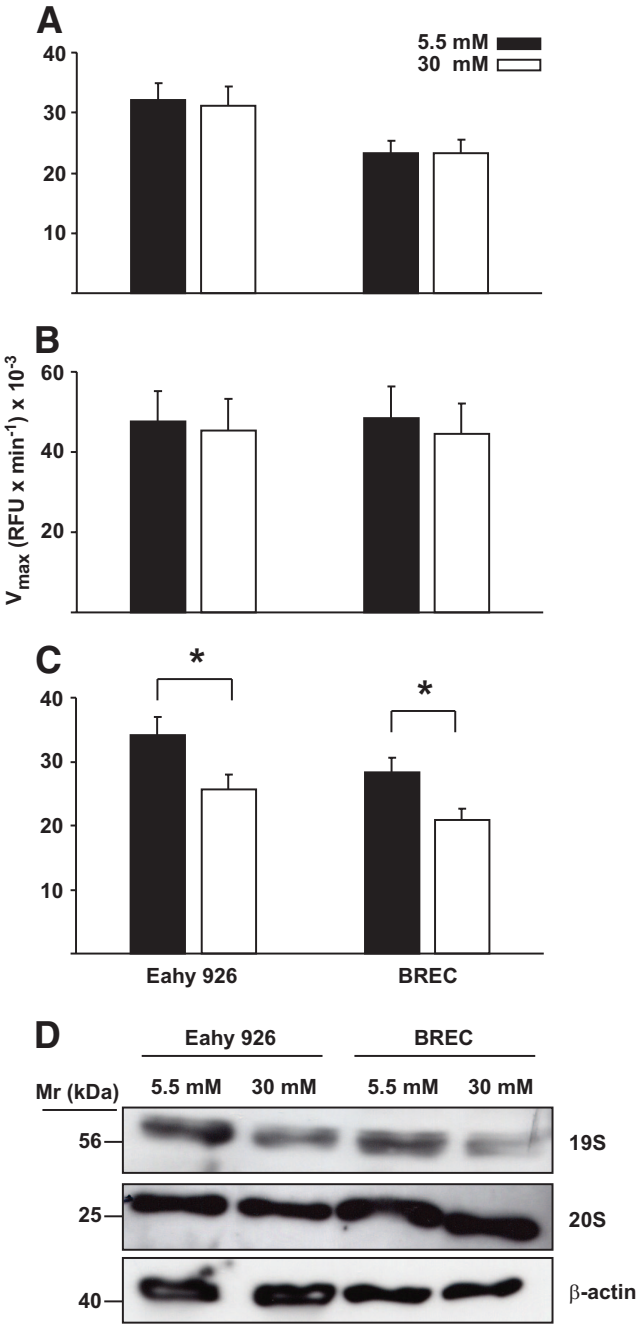

FIG. 1. Proteasome activity in endothelial cells under chronic hyperglycemia. Proteasome activity and 20S proteasome expression levels were analyzed in immortalized human endothelial Eahy.926 cells and in primary bovine retinal endothelial cells (BRECs) following incubation with low- and high-glucose medium for 14 days. Proteasomal trypsin-like (*A*) and caspase-like (*B*) activities following incubation with high-glucose medium were not significantly changed in comparison with those following low-glucose medium, whereas the proteasomal chymotrypsin-like activity was significantly reduced (*C*). The level of the catalytic 20S proteasome remained constant, while the 19S regulatory complex expression was decreased, as shown by Western blot (*D*). Data represent means ± SD from at least five separate experiments. **P* ≤ 0.05. RFU, relative fluorescence unit.

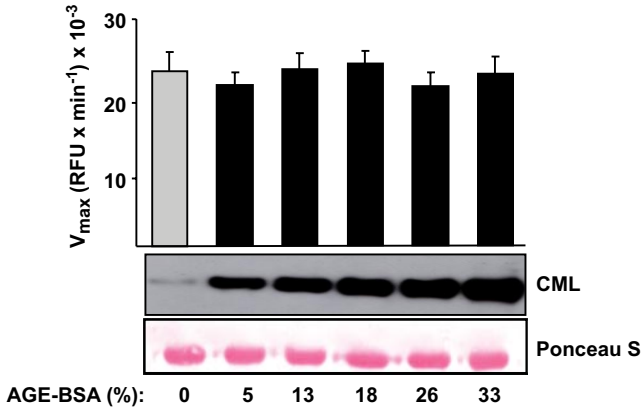

FIG. 2. Resistance of glycated albumin against proteasomal degradation in vitro. AGE-BSA (10 μg) was incubated with isolated 20S proteasome to examine the influence on proteasomal activity. The degree in carboxy-methyl-lysine modification of BSA (as demonstrated by Western blotting) did not significantly affect the chymotrypsin-like activity of the 20S proteasome. Ponceau S staining is shown as a loading control. Data represent means ± SD from at least three separate experiments. RFU, relative fluorescence unit.

incubation with MGO, glyoxal, or glyceraldehyde showed strong inhibition of chymotrypsin-like activity but no influence on the trypsin- or caspase-like activities of the 20S proteasome (Fig. 3*A*–*C*). Similarly, endothelial cells incubated with 500 μmol/l MGO, glyoxal, or glyceraldehyde for 12 h exhibited reduced chymotrypsin-like proteasomal activity, but no change in the other proteasome activities occurred (Fig. 4*A*–*C*). Furthermore, increased accumulation of ubiquitinated proteins was observed in endothelial cells exposed to reactive carbonyl compounds (Fig. 4*D*).

To investigate the potential mechanism of proteasome inhibition, purified 20S proteasomes were incubated with MGO for 30 min in vitro, purified by SDS-PAGE, and analyzed by mass spectrometry. The data demonstrated primary MGO modification of the catalytic proteasome β2-, β4-, and β5-subunits, resulting in the formation of methylimidazolone [*N*-acetyl-*N*-(5-hydro-5-methyl)-4-imidazolone], carboxyethyl-lysine, and argpyrimidine. In contrast, modifications in the α-subunits could not be detected (Table 1). These results suggest that endogenously produced reactive carbonyl compounds might be responsible for the reduction of chymotrypsin-like activity via modification of specific β-subunits.

Diabetic *Ins2*^{Akita} mice exhibit reduced proteasomal activity. For analysis of the effect of hyperglycemia on proteasomal activity in vivo, 6-month-old diabetic *Ins2*^{Akita} mice (n = 6), a model for type 1 diabetes that also manifests IgA nephropathy, were killed and tissue homogenates were subjected to proteasome analysis (Fig. 5). Trypsin- and caspase-like activities were not altered in tissues from aorta and the heart (Fig. 5*A* and *B*), whereas chymotrypsin-like activity was greatly reduced in kidney and to a smaller extent in aorta of diabetic animals in comparison with wild-type controls (Fig. 5*C*). Surprisingly, all three proteasomal activities were reduced in the kidney. The heart showed no significant changes in general, indicating that heart proteasomes were more resistant to hyperglycemia. Western blot analysis revealed that the protein-expression level of the catalytic 20S core proteasome in tissue samples did not significantly differ between diabetic and nondiabetic animals (Fig. 5*D* and *E*). These data indicate that proteasomal activity is impaired in diabetic animals without affecting the protein levels of the 20S proteasome.

Nondiabetic GLO1-knockdown mice and STZ diabetic mice demonstrate increased 20S MGO modification resulting in impaired proteasomal activity. To test the hypothesis that reactive carbonyl compounds, especially

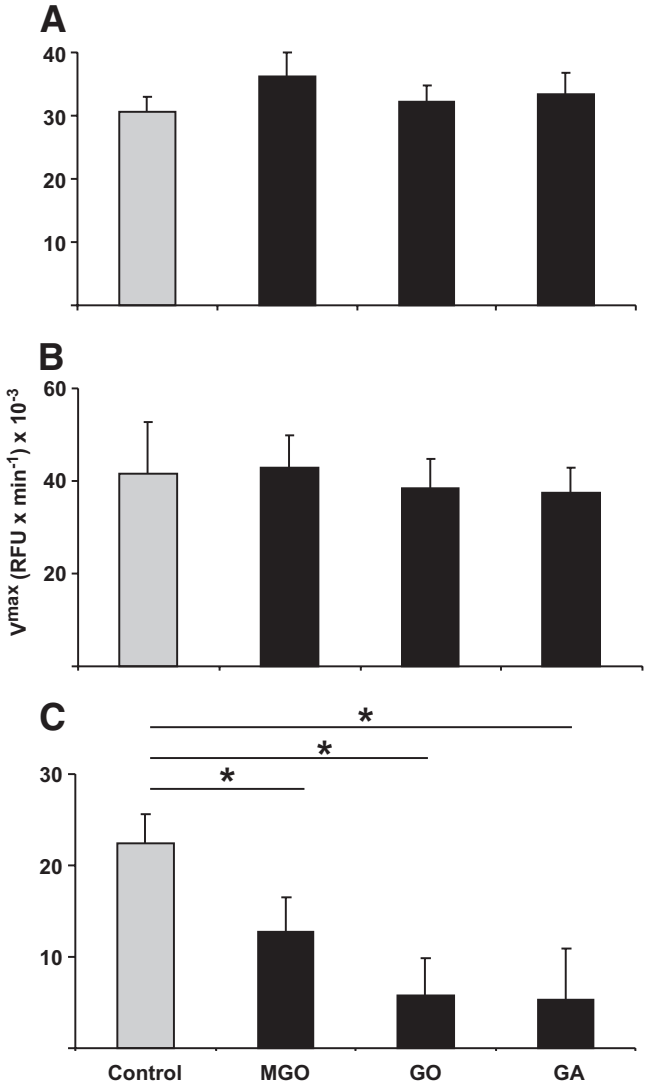

FIG. 3. Inhibition of proteasomal chymotrypsin-like activity in vitro by reactive carbonyl compounds. Reactive carbonyl compounds were incubated with the 20S proteasome and the enzymatic activities determined. Trypsin-like (*A*) and caspase-like (*B*) activities were not significantly changed, whereas the chymotrypsin-like activity was significantly reduced (*C*). Data represent means ± SD from at least three separate experiments. **P* ≤ 0.01. GA, glyceraldehyde; GO, glyoxal; RFU, relative fluorescence unit.

MGO, are responsible for decreased proteasome activity, nondiabetic GLO1 knockdown mice and STZ diabetic/GLO1-knockdown mice were analyzed for MGO-modified proteins and proteasome activity (Fig. 6). Nondiabetic GLO1-knockdown mice exhibited a twofold increase in MGO-hydroimidazolone staining; the same level of increase was seen in STZ diabetic mice. STZ diabetic GLO1-knockdown mice had a threefold increase compared with nondiabetic wild-type mice—consistent with an additive effect of a diabetes-induced increased MGO production and a knockdown-induced decrease in MGO degradation by GLO1 (Fig. 6*A*). In addition, GLO1-knockdown mice had an increase in specific Ub-Lys48 staining and a more dramatic increase was seen in STZ diabetic GLO1-knockdown mice, indicating that proteasome-specific polyubiquitinated proteins are accumulating in diabetic mice (Fig. 6*B*). Furthermore, chymotrypsin-like activity in kidney homogenates of GLO1-knockdown mice and STZ-injected mice was significantly decreased (Fig. 6*C*).

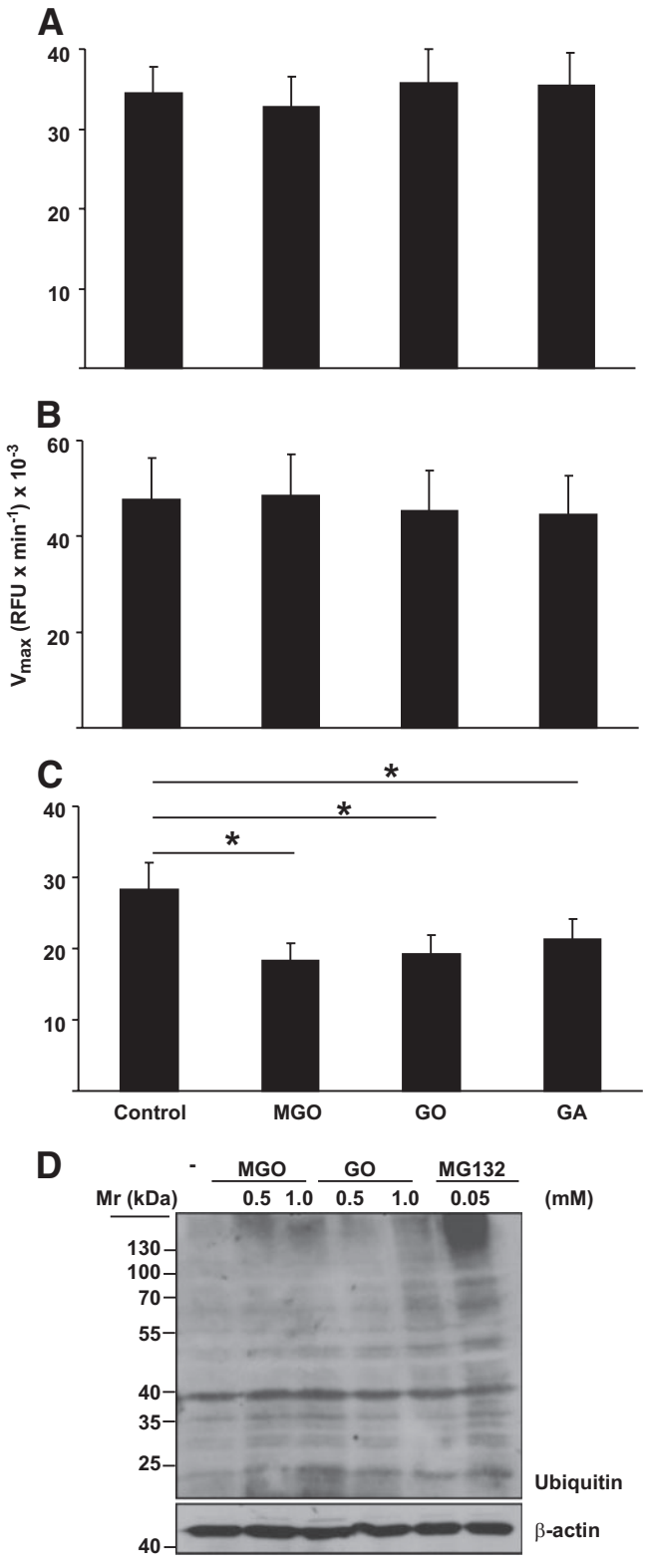

FIG. 4. Inhibition of proteasomal chymotrypsin-like activity in endothelial cells and accumulation of ubiquitinated proteins. Immortalized human endothelial Eahy.926 cells and primary bovine retinal endothelial cells were each incubated for 12 h with 500 μmol/l reactive carbonyl compounds. Trypsin-like (*A*) and caspase-like (*B*) activities were not changed, whereas the chymotrypsin-like activity was significantly reduced after incubation with each of the three reactive carbonyl compounds (*C*). Incubation with reactive carbonyl compounds exhibited accumulation of ubiquitinated proteins in a dose-dependent manner (*D*). Data represent means ± SD from at least three separate experiments. **P* ≤ 0.05. GA, glyceraldehyde; GO, glyoxal; RFU, relative fluorescence unit.

TABLE 1
20S proteasome modification after incubation with MGO in vitro

Subunit	Peptide	Sequence	Modification
β5	117–123	LLARQCR	7:methylimidazolone
β5	124–130	IYELRNK	5:methylimidazolone
β2	30–41	DDHDKMFKMSEK	8:carboxyethyllysine
β2	69–85	MRNGYELSPTAAANFTR	17:methylimidazolone
β4	217–227	CMRVLYYRDAR	8:arg-pyrimidin
β4	220–231	VLYYRDARSYNR	5:methylimidazolone
β4	225–240	DARSYNRFQIATVTEK	7:methylimidazolone

In addition, the expression of the polyubiquitin binding subunit 19S-S5a was assessed. 19S-S5a protein level was decreased more than 50% in GLO1-knockdown and STZ diabetic mice, respectively (Fig. 7*A*). We then assessed MGO modification of 20S proteasome by performing immunoprecipitation of specific 20S proteasomal components, which were then immunoblotted for MGO modification. There was no difference in protein amount of these subunits in kidney lysates for wild-type, GLO1-knockdown, STZ diabetic, or STZ diabetic/GLO1-knockdown mice. However, there was a twofold increase in MGO modification of the 20S β2-subunit in lysates from nondiabetic GLO1-knockdown mice and a two- to fivefold increase in lysates from STZ diabetic and STZ diabetic/GLO1-knockdown mice (Fig. 7*B*). This finding confirms in vivo our observation (Table 1) that the 20S proteasome β2-subunit is modified by methylimidazolone after incubation with MGO in vitro. However, at MGO levels found in vivo, there was no detectable modification of the β5-subunit.

DISCUSSION

An undisturbed function of the ubiquitin-proteasome system is essential for cellular processes—such as cell-cycle progression, signal transduction, and antigen processing—to occur normally (20,21,24). Different pathologies can influence cellular signal transduction systems and may also affect the functional activities of the proteasome. The influence on the ubiquitin-proteasome system of hyperglycemia associated with diabetes in this regard has yielded seemingly contradictory results. Several reports have demonstrated that high glucose levels lead to an acceleration of proteasomal activity with the consequences of muscle wasting and sustained nuclear factor-κB activation (19,25–27), while other reports have shown impaired proteasome function in diabetes and arteriosclerosis (28,29). Previous reports demonstrated that AGEs influence proteolytic degradation through decreased cathepsin activity, suggesting that AGEs affect proteolysis (30,31).

Here, we demonstrate that proteasome activity is decreased in vitro and in vivo under conditions of chronic hyperglycemia. Incubation of endothelial cells with high glucose (14 days) led to reduction of the proteasomal chymotrypsin-like activity in vitro. This defect was replicated by exposure of the cells to the dicarbonyl MGO, which covalently modified 20S proteasome subunits. Similar, results were obtained using kidney from nondiabetic GLO1-knockdown mice, indicating that increased MGO alone is sufficient to cause proteasome dysfunction. Likewise, results were obtained from kidneys of diabetic mice, demonstrating that the increased levels of MGO induced by diabetic hyperglycemia are sufficient to cause these defects. In separate experiments, we have observed that upregulation of the chymotryptic-like activity occurs in the acute phase of high glucose (3–5 days) (M.A. Queisser, unpublished data), suggesting that hyperglycemia affects the proteasomal activity in a biphasic manner. These observations may explain the apparent discrepancies reported in the literature. Interestingly, stimulation with only reactive carbonyl compounds affected the chymotrypsin-like activity. However, this treatment was sufficient to cause accumulation of ubiquitinated proteins. This is consistent with data indicating that the chymotrypsin-like activity is the proteolysis rate-limiting function (32).

The 26S proteasome with the ubiquitin/ATP-dependent 19S complex is more sensitive to oxidative stress than the ubiquitin/ATP-independent 20S core complex. However, mildly oxidized proteins are rapidly degraded by the 20S proteasome, which does not require assembly with the 19S complex. Here, high glucose also led to downregulation of the proteasomal 19S regulatory complex, suggesting that the ubiquitin-proteasome system may initially respond to increased oxidative stress by increasing the 20S proteasome and thus facilitating the degradation of oxidized and aggregated proteins as an early event under hyperglycemic conditions. More chronically, however, this reduction of the ubiquitin/ATP-dependent 19S complex may cause accumulation of polyubiquitinated intracellular proteins.

Previously, it was reported that protein glycation has an inhibitory effect on protein ubiquitination because for both, modifications occur on epsilon-amino groups in lysine residues (33). However, the major intracellular AGE precursor is MGO, which, along with its analog glyoxal, preferentially modifies arginine residues. Consistent with this, ubiquitinated AGEs were immunoprecipitated from glyoxal-treated fibroblasts, although it is not clear whether glycated proteins were mono- or polyubiquitinated (34). To evaluate the effects of diabetic hyperglycemia and increased intracellular MGO in vivo, we used three mouse models.

In insulin-deficient *Ins2*^{Akita} mice, which develop hyperglycemia soon after weaning (35), proteasomal activity measurements in aorta confirmed the in vitro finding that exclusively the chymotrypsin-activity was reduced, whereas the trypsin- and caspase-like activities were not changed. In contrast, no significant change in proteasome activation was noted in heart tissue, indicating that the cardiac proteasome might have specific properties for responding to cellular stress such as hyperglycemia. This may reflect the fact that the cardiac proteasome complex contains special inducible subunits (36). Surprisingly, the kidney revealed a highly significant decrease in all three proteasomal activities.

We next examined kidneys from nondiabetic GLO1-knockdown mice, which exhibited reduced chymotrypsin-like activity only. This observation indicated that

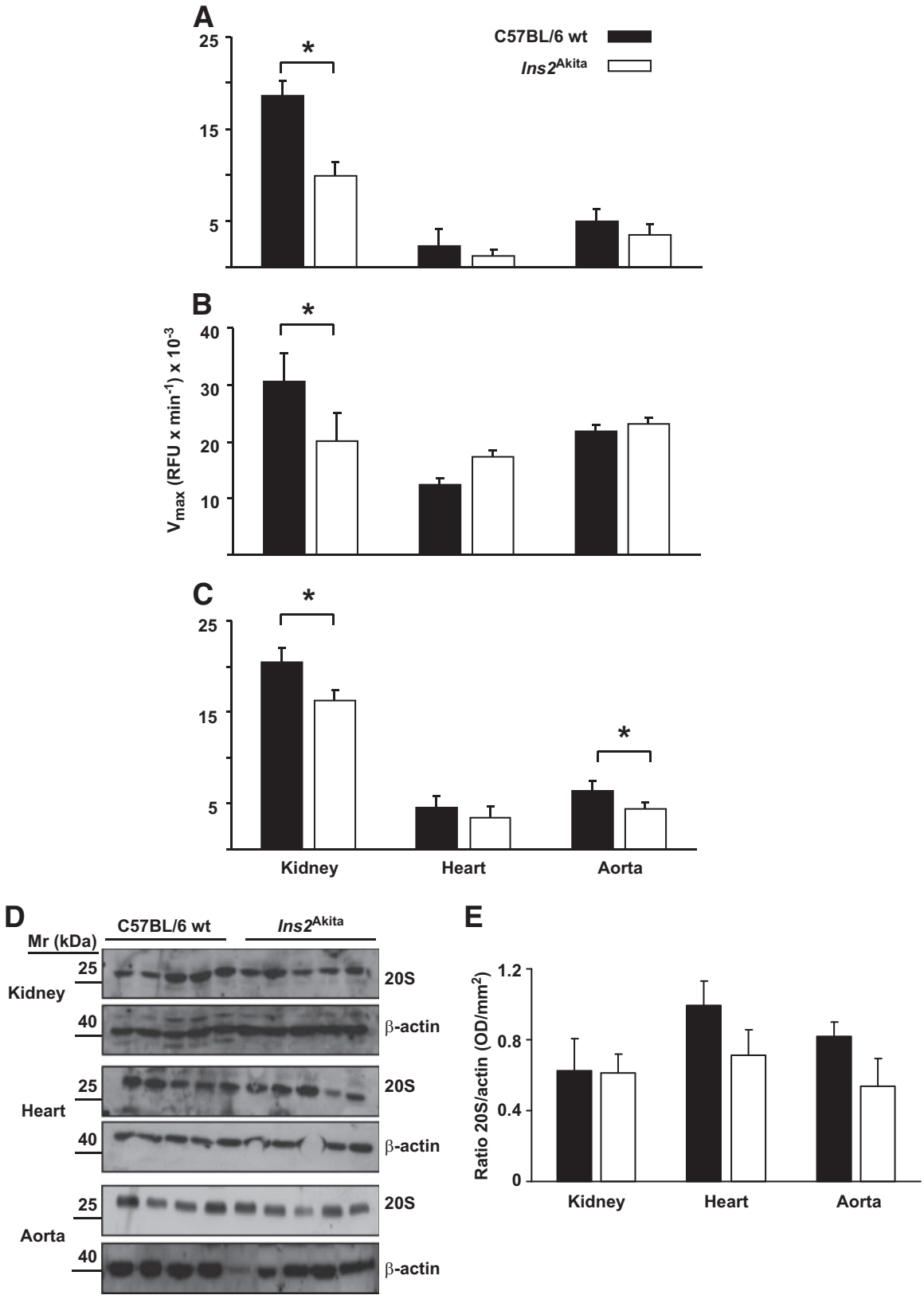

FIG. 5. Decreased proteasomal activity in organs from diabetic *Ins2*^{Akita} mice. Proteasomal activity and proteasome expression was determined in kidney, heart, and aorta from diabetic *Ins2*^{Akita} mice (n = 6) and control wild-type mice (n = 6). The three proteolytic activities of the proteasome were measured with fluorogenic substrates. Trypsin-like (*A*) and caspase-like (*B*) activities were significantly reduced in kidneys from diabetic mice. The chymotrypsin-like activity (*C*) was significantly reduced in kidney and aorta, whereas in the heart no significant changes were seen. The 20S proteasome protein levels were not significantly changed in any organs, as shown by Western blotting (*D* and *E*). Data represent means ± SD from at least three separate experiments. *$P \leq 0.05$.

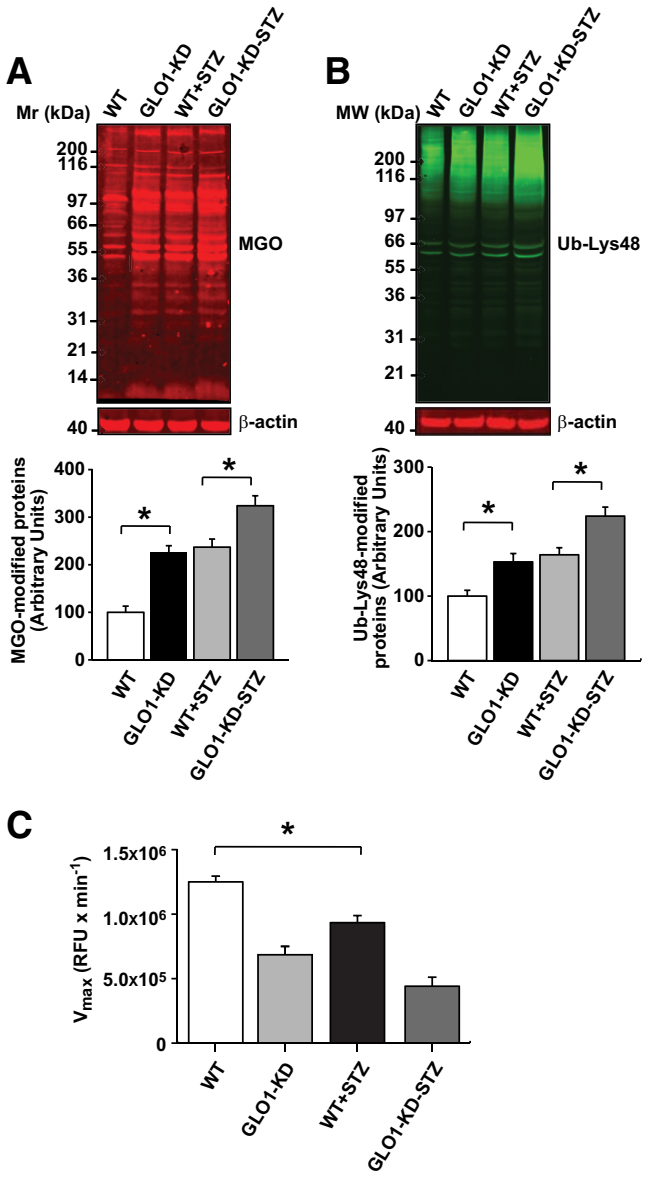

FIG. 6. Increased MGO- and ubiquitin-modified proteins with impaired proteasomal activity in GLO1 knockdown and diabetic mice. Kidney lysate protein (40 μg) from wild-type (WT), GLO1-knockdown (GLO1-KD), and STZ diabetic and GLO1-knockdown diabetic (GLO1-KD-STZ) mice were analyzed by Western blotting. GLO1-knockdown mice exhibited a significant increase in MGO- and ubiquitin-modified proteins in kidney homogenates compared with levels in wild-type mice (*A* and *B*). GLO1-knockdown and STZ-injected mice exhibited significantly reduced proteasomal chymotrypsin-like activity in kidney homogenates compared with that in wild-type mice (*C*). The protein band densities were scanned and quantified using the Odyssey imaging system. Data are expressed as means ± SD. **P* < 0.05 vs. wild type; *n* = 4. RFU, relative fluorescence unit. (A high-quality digital representation of this figure is available in the online issue.)

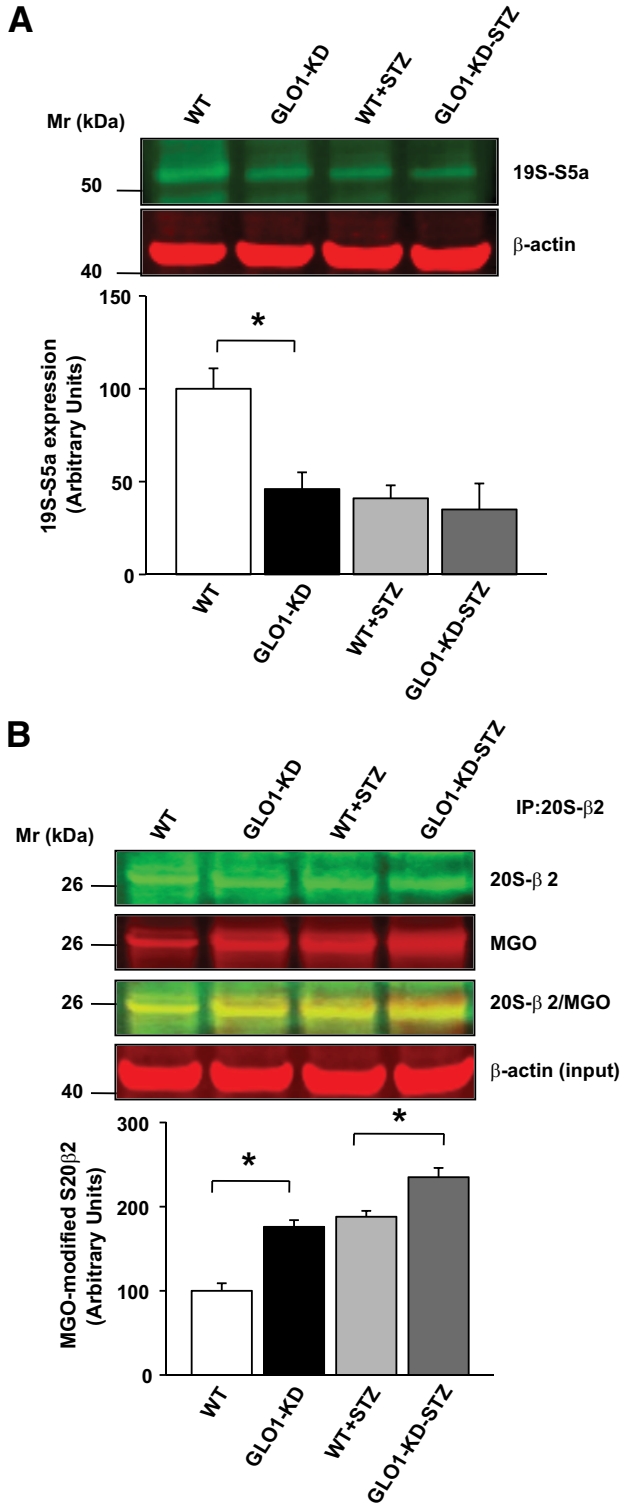

FIG. 7. Decreased 19S-S5a protein levels and MGO-modified 20S-β2 proteasome subunit in GLO1-knockdown (GL01-KD) and STZ diabetic mice. Kidney lysate protein (40 μg) from indicated mice were analyzed on NUPAGE 4–12% Bis-Tris gels, transferred to PVDF membranes, and blotted by 19S-S5a antibody (*A*). The lysates from indicated mice kidney tissues were immunoprecipitated with 20S-β2 antibody and analyzed by Western blotting, and 10% whole lysates were used for β-actin detection as input. MGO modification of the 20S proteasome β2-subunit was detected in kidneys from GLO1-knockdown and STZ diabetic mice (*B*). The protein band densities were scanned and quantified using the Odyssey imaging system. Data are expressed as means ± SD. **P* < 0.05 vs. wild type; *n* = 4. RFU, relative fluorescence unit. (A high-quality digital representation of this figure is available in the online issue.)

increased MGO in the absence of hyperglycemia was sufficient to induce decreased proteasome function. STZ diabetes, a chemical model of type 1 diabetes, also showed reduced chymotrysin-like activity. In both models, MGO modification of the 20S proteasome subunit was detected. Both of these changes were increased further in STZ diabetic/GLO1- knockdown mice. Both nondiabetic GLO1-knockdown and STZ diabetic mice showed decreased chymotrypsin-like activity only, while Akita mice exhibited reductions in all three proteasomal activities in the kidney. This may reflect the fact that *Ins2*^{Akita} mice, in

addition to diabetes, also exhibit IgA nephropathy, an immune complex-mediated pathology. Genetic background differences between the strains may also be important.

Collectively, these results show that reactive carbonyl compounds selectively inhibited the chymotrypsin-like activity of the proteasome in cell culture and in vivo. Although it was previously described that glyoxal inhibited all three proteasomal activities (34), the findings documented here are consistent with other reports observing that chemical compounds selectively target the chymotrypsin-like activity of the proteasome (37,38). In addition to decreasing chymotrypsin-like activity in the 20S proteasome, we found that increased intracellular MGO caused a marked decrease in protein levels of the regulatory 19S component of the 26S proteasome. Previously, Zhang et al. (39) described that the O-linked N-acetylglucosamine (O-GlcNAc) transferase was able to modify the 19S regulatory subunit of the proteasome. The Rpt2 ATPase (19S-S4) in the mammalian proteasome 19S cap is modified by O-GlcNAc in vitro and in vivo, and as its modification increases, 26S proteasome function decreases. Since hyperglycemia increases intracellular protein modification by O-GlcNAc (10), this change in diabetic tissues would further augment the inhibitory action of hyperglycemia on proteasome function. The relationship between increased MGO and increased O-GlcNAc modification remains to be clarified. However, O-GlcNAc modification of 19S-S5a was increased in kidneys of nondiabetic GLO1-knockdown mice, suggesting that MGO increases this modification (D. Yao, unpublished data).

Based on the presented data and previous publications, we propose the following mechanism: Chronic high glucose causes increased intracellular reactive oxygen species in cells that cannot downregulate glucose transport. This intracellular hyperglycemia increases MGO concentration in microvascular endothelial cells in retina and kidney with the formation and accumulation of reactive carbonyl compounds. MGO covalently modifies 20S proteasome subunits, which decreases the proteasomal chymotrypsin-like activity. In addition, increased MGO reduces 19S protein levels. Decreased levels of the polyubiquitin receptor 19S-S5a cause polyubiquitinated proteins to accumulate in the cell, which may interfere with the function of their nonubiquitinated counterparts. The decrease of the proteolysis rate-limiting step may influence other proteasomal activities, the decline of which over time was detected in kidney from *Ins2*^{Akita} mice. However, the precise contribution of impaired proteasome function to the functional and structural changes of diabetic nephropathy remains to be elucidated.

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