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High glucose and diabetes modulate cellular proteasome function: Implications in the pathogenesis of diabetes complications

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ABSTRACT

The precise link between hyperglycemia and its deleterious effects on retinal and kidney microvasculature, and more specifically loss of retinal perivascular supporting cells including smooth muscle cell/pericytes (SMC/PC), in diabetes are not completely understood. We hypothesized that differential cellular proteasome activity contributes to sensitivity of PC to high glucose-mediated oxidative stress and vascular rarefaction. Here we show that retinal endothelial cells (EC) have significantly higher proteasome peptidase activity compared to PC. High glucose treatment (HGT) increased the level of total ubiquitin-conjugated proteins in cultured retinal PC and EC, but not photoreceptor cells. In addition, *in vitro* proteasome activity assays showed significant impairment of proteasome chymotrypsin-like peptidase activity in PC, but not EC. The PA28-α/-β and PA28-β/-γ protein levels were also higher in the retina and kidney glomeruli of diabetic mice, respectively. Our results demonstrate, for the first time, that high glucose has direct biological effects on cellular proteasome function, and this modulation might be protective against cellular stress or damage induced by high glucose.

1. Introduction

Diabetic retinopathy (DR) and diabetic nephropathy (DN) are the leading causes of blindness and end-stage renal failure in diabetic patients, respectively [1–3]. Diminished number of retinal PC [4–5] and hypertrophy of the renal mesangial cells, as perivascular cells of the glomerulus with similar functions as PC, are hallmarks of established DR and DN [1–3,6–7]. The HGT of both retinal PC and EC promote oxidative stress, endoplasmic reticulum (ER) stress, and apoptosis in PC, but not in EC [8–9]. However, the molecular basis for selective vulnerability of PC in diabetic retinal and kidney complications, and higher sensitivity of retinal PC to high glucose compared to EC are not completely understood. We hypothesized that differential cellular proteasome activity contributes to selective sensitivity of PC to hyperglycemia.

Ubiquitin Proteasome System (UPS) is responsible for protein quality control and degradation. The conjugation of ubiquitin to target proteins primes them for UPS-mediated degradation [10]. The proteasomes are multiprotein barrel shaped assemblies with an approximate molecular weight of 2500 kDa and are considered the proteolytic machinery, which regulates the turnover of eukaryotic proteins. Proteasomes are composed of 20S core subunit and 19S or 11S regulatory subunits capable of binding to both ends of the barrel shaped core subunit and stimulating the proteolytic activity of the proteasomes. UPS also comprises many other enzymes involved in the ubiquitin activation (E1s), conjugation (E2s), ligation (E3s) and removal from target proteins by deubiquitylating enzymes (DUBs) [10].

The 11S proteasome regulatory subunit comprises the PA28-α, PA28-β and the PA28-γ proteins encoded by three different genes. All these proteins form a heptameric ring-shaped complex and, while the PA28-α and PA28-β proteins form a heptameric complex together (α3β4), the PA28-γ only exists in homoheptameric form. Although the important role of PA28 proteins in intracellular antigen processing and presentation to immune cells has been demonstrated, the PA28-α and PA28-β proteins can also mediate cellular response to oxidative stress [11–12].

Recent studies have demonstrated that high glucose, the glucose-mediated post-translational protein modifications, and diabetes are modulators of proteasome activity [13–15]. These reports link diabetes or hyperglycemia to UPS and raise many questions on how diabetes or hyperglycemia can modulate proteasome targeting and activity, and whether this modulation occurs in a cell-specific manner.

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2. Materials and methods

2.1. Cell culture

The isolation and culture of mouse retinal PC and EC were previously described [16–17]. Choroid EC (ChEC) were isolated from the mouse choroid using magnetic beads coated with anti-PECAM-1antibody and grown similar to retinal EC. NIH3T3 cells and 661W mouse photoreceptor cells were obtained from ATCC (Manassas, VA). Low glucose level was 5 mM and for HGT the cells were treated for 5 days with 30 mM D-glucose (Sigma, St. Louis, MO). For osmolarity control the cells were treated with 5 mM D-glucose and 25 mM L-glucose (Sigma). For cycloheximide (CHX; Sigma) treatment, cells were treated at final concentration of 100 μg/ml for indicated times.

2.2. Western blotting

For immunoblots, the cultured cells and the retina tissue were lysed in RIPA lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 5 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail (Roche, Nutley, NJ). The lysates were collected in 1.5 ml tubes, sonicated, and protein concentration was measured using BCA Protein Assay Kit (Pierce; Rockford, IL).Total protein (50 μg) was separated by SDS–PAGE (4-20% Tris-Glycine gels; Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Protein expression was analyzed by incubating with specific primary antibodies (Supplementary Table 1) at 4 °C overnight. Blots were washed, incubated with appropriate HRP-conjugated secondary antibody and developed using ECL. Mouse β-actin was used for loading control.

2.3. In vitro proteasome peptidase assay

Proteasome peptidase assays were performed as described by Bech-Otschir et al. [18]. The cells or tissues were lysed in the lysis buffer containing 50 mM Tris/HCl pH 7.4, 1 mM ATP, 10% glycerol, 0.1% NP40, 2 mM MgCl2, 1.5 mM DTT and 0.03% SDS. In total, 50 μg (1 μg/μl) of protein lysates were loaded into a dark 96-well microplate and probed with 100 μM of fluorogenic peptide substrates: Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like, Ac-Arg-Leu-Arg-AMC for trypsin-like, and Z-Leu-Leu-Glu-AMC for caspase-like (Enzo Life Sciences, Farmingdale, NY). The plates were then incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 200 μl of 100% ethanol. The fluorescence signal intensity of the samples was measured using a luminescence plate reader (PerkinElmer, Wellesley, MA). The excitation and emission wavelengths were 355 and 460 nm, respectively. Boiled protein lysates were used as blank samples and test values were normalized to the respective blanks.

2.4. Mice

The wild type and heterozygous Akita/+ ($Ins2^{Akita/+}$) male mice on C57BL/6j background were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our facility. Genotyping was performed by PCR using specific primers (Supplementary Table 2). All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin, School of Medicine and Public Health. Only male Akita/+ mice develop diabetes by 4-weeks of age and have an average life expectancy of 10 months. They develop some of the early non-proliferative retinopathies by six-months of age [19].

2.5. Immunofluorescence studies

The cryosections, prepared from eyes and kidneys harvested from age-matched male wild type and Akita/+ mice, were fixed in ice-cold acetone for 30 min, blocked (3% BSA, 0.1% Tween-20) for 1 h and incubated with the desired primary (Supplementary Table 1) and secondary antibodies (Alexa Fluor-488 or -594 conjugated; Invitrogen). Control samples were incubated with relevant IgG and nuclei were counterstained with DAPI (Invitrogen). Microscopic slides were photographed using a Zeiss fluorescence microscope in digital format (Carl Zeiss, Thornwood, NY).

2.6. Statistical analysis

For Western blot densitometry analysis the wand tool of ImageJ, and for statistics the GraphPad Prism software were used, respectively. Data are expressed as mean ± sem. Statistical significance was assessed by unpaired *t* test. Values were considered statistically significant at $p < 0.05$.

3. Results

3.1. Retinal EC exhibited enhanced proteasome peptidase activity

To test the effects of HGT on proteasomal degradation capacity of retinal PC, EC and retinas, synthetic fluorogenic proteasome substrate peptides were assayed in the presence of ATP [18]. Regardless of any change in the peptidase activity by HGT, we found that retinal EC had substantially higher peptidase activity for all the substrates compared to other cells (Fig. 1A–C). Retinal EC, compared to PC, showed approximately 3.4-, 8.1- and 6.5-fold higher chymotrypsin-like, trypsin-like and caspase-like peptidase activities, respectively (Fig. 1A–C). HGT of cultured cells resulted in a modest reduction of peptidase activity for most of the tested substrates, except for EC, which showed a significant rise in peptidase activity after HGT. Retinal PC after HGT showed a significant reduction in chymotrypsin-like peptide degradation. Similar to EC, but to a lower extent, the $Ins2^{Akita/+}$ retinas also showed a significant increase in chymotrypsin-like and trypsin-like peptidase activity compared to age-matched control retinas (Fig. 1A–C). Surprisingly, the ChEC responsible for the nourishment of outer retina did not show as high of peptidase activity as retinal EC (Fig. 1A–C). This may stem from the differences in the physiology of these EC, and suggest that ChEC are more susceptible to cytotoxic effects of hyperglycemia.

3.2. HGT increased the total levels of ubiquitinated proteins and PA28-α/-β levels in retinal vascular cells

Using Western blot to detect universal ubiquitination in cultured cells, we found that HGT resulted in elevation of total protein ubiquitination in retinal PC, EC, and NIH3T3 cells, but not in 661W photoreceptor cells (Fig. 2A). The observed rise in ubiquitination following HGT was similar to the increased ubiquitination reported for cultured myocytes incubated with oxidant reagent (H_2O_2), which was due to attenuation of proteasome activity [11].

We next determined whether HGT-mediated impairment of proteasome activity was responsible for increased levels of ubiquitinated proteins by analyzing the clearance of ubiquitinated proteins after CHX treatment and inhibition of new protein synthesis. Western blot for ubiquitin after 12 h of CHX treatment showed reduction in total amount of ubiquitinated proteins in HGT similar to normal glucose conditions in all cell types examined (Fig. 2A). Thus, HGT enhanced protein ubiquitination with minimal