Defective Autophagy in Diabetic Retinopathy

Jacqueline M. Lopes de Faria, Diego A. Duarte, Chiara Montemurro, Alexandros Papadimitriou, Silvio Roberto Consonni, and José B. Lopes de Faria

1Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil
2Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, Brazil
3Brazilian Biosciences National Laboratory, Brazilian Center for Research in Energy and Materials, Campinas, Brazil

Correspondence: Jacqueline M. Lopes de Faria, Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil. jmlfaria@fcm.unicamp.br.

RESULTS. High glucose induces increase of early and late autophagic markers, accumulation of p62/SQTSM1 and endoplasmic reticulum (ER) stress response associated with apoptosis augmentation (P < 0.01). The inhibition of autophagy in HG leads to higher rMC apoptotic rate (P < 0.001). By silencing the p62/SQTSM1, ER stress is ameliorated (p<0.0001), preventing apoptosis. Retinal MCs in HG treated with rapamycin (mTOR inhibitor) show autophagy machinery activation and reestablishment of cargo degradation, protecting cells from apoptosis (P < 0.0001). Rapamycin improves lysosomal proteolytic activity by improving cathepsin L activity restoring autophagic cargo degradation, and preventing increased VEGF release (P < 0.0001). In experimental model of diabetes, Beclin-1 and p62/SQTSM-1 were found to be marked increased in retinas from diabetic Wystar Kyoto rats compared with control group (P < 0.003) with reduction of cathepsin L activity.

CONCLUSIONS. High glucose upregulates autophagy but accumulates p62/SQTSM1 cargo due to lysosomal dysfunction, leading to massive VEGF release and cell death of rMCs. Lysosomal impairment and autophagic dysfunction are early events present in the pathogenesis of diabetic retinopathy (DR). This might be valuable for developing a novel therapeutic strategy to treat DR.

Keywords: Müller cells, autophagy, VEGF, diabetes, lysosome

The prevalence of degenerative retinal diseases is increasing worldwide. Retinal neurodegeneration is an early feature in the pathogenesis of diabetic retinopathy (DR) and contributes to the development of retinal microvasculopathy.1–4 Therefore, a neuroprotective strategy is needed to prevent the development and progression of diabetic retinal diseases.

The retinal Müller cells (rMCs), the predominant glial cells in the retina, reside in the inner nuclear layer but span all retinal layers, interact with neighboring neurons, and are part of the inner and outer limiting membranes.5 In this way, rMCs monitor retinal structures and functions. Its processes wrap around retinal blood vessels, thus controlling the retinal barrier and serving as a conduit for the transference of molecules between retinal cells.6–9 Retinal MCs also maintain neurons by releasing trophic factors, recycling neurotransmitters, and controlling the ionic balance in the extracellular space.8 In addition, photoreceptor outer segments are phagocytized by Müller cells, thus participating in the visual cycle. Under different stresses such as high glucose (HG), MCs respond through reactive glyosis, resulting in morphologic, biochemical, and physiological changes. One of those changes is overexpression of vascular endothelial growth factor (VEGF),10 a known therapeutic target for pharmacologic treatment of diabetic retinopathy and macular edema.11

Macroautophagy (hereafter referred to as “autophagy”) is an evolutionarily conserved lysosomal degradation pathway that controls cellular bioenergetics (by recycling cytoplasmic constituents) and cytoplasmic quality (by eliminating protein aggregates, damaged organelles, lipid droplets, and intracellular pathogens).12 Cell survival and homeostasis depend on the synthesis and degradation of cellular proteins and organelles. If autophagy fails, this may lead to accumulation of harmful damaged organelles and protein aggregates.13,14 Autophagy is regulated by a range of stresses and stimuli such as endoplasmic reticulum (ER) stress, starvation, hypoxia, cytotoxicity, and infection. Depending upon the stimulus, the cell achieves a number of functions through autophagic degradation of specific elements or interaction with other pathways, which leads to apoptosis. Therefore, cell death and survival depends on autophagic control. The autophagy pathway plays a key role in protection against aging and certain cancers, infections, neurodegenerative disorders, and metabolic, inflammatory, and muscle diseases.15–19 Recently, the development of autophagy inducers in medicine has caused them to be considered a potential therapeutic tool.20
The endoplasmic reticulum facilitates proper folding of proteins and provides cells with calcium reservoirs. In various physiologic and pathologic conditions, when the capacity of the ER to fold proteins becomes saturated, unfolded proteins begin to accumulate in the cytoplasm. This situation triggers the ER to nucleus signaling pathway, called the unfolded protein response (UPR), which reduces global protein synthesis and induces synthesis of chaperones and other proteins to improve the capacity of ER to fold proteins. Under diabetic conditions, when the levels of intracellular reactive oxygen species (ROS) are increased, the levels of unfolded/ misfolded proteins are also augmented and the UPR mechanism is triggered. Studies have shown that ER stress markers, phospho-eif2α, and CHOP are elevated in retinas from diabetic rats and in human retinal capillary endothelial cells (HRCECs) and immortalized human RPE cell lines (ARPE-19) cultured under diabetic conditions. However, the sequence of events by which diabetic milieu conditions lead to insufficient ER stress response and cell death is not completely understood. In the present study, we investigated the role of autophagy in modulating rMC response under HG conditions (mimicking diabetic milieu). We revealed that MCs exposed to HG upregulate autophagy initiation, but due to a lysosomal dysfunction, the p62/SQSTM1 cargo is not degraded. This accumulation leads to massive VEGF release and cell death. This new concept may be of great value for the development of a novel therapeutic strategy for treating diabetic retinal diseases.

**MATERIALS AND METHODS**

**Reagents**

All reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

**Rat Retinal Müller Cell (rMC-1) Culture**

Rat retinal Müller cells (rMC-1), provided by Vijay J. Sarthy, PhD (Northwestern University, Evanston, IL, USA), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Northwestern University, Evanston, IL, USA) unless otherwise stated.

**In Vitro Study**

Cultures of rMC-1 at 70% confluence were exposed to 5.5 mM D-glucose (NG) and 25 mM D-glucose (HG) with or without 1 mM 3-methyladenine (3MA, a type III PI3 kinase complex inhibitor) or 1 μM bafilomycin-A1 (BAF, autophagosome-lysosome fusion blocker); 2 μM rapamycin (RAP, mTOR blocker) as an autophagy inducer; 10 μM GSK2606414, a protein kinase RNA-like ER kinase (PERK) inhibitor I; small interfering RNA (siRNA); and scramble (scrRNA) for p62/SQSTM1 (200 nM) for 24, 48, and 72 hours. The concentrations of treatments used in all experiments were chosen after carrying out a thiazolyl blue tetrazolium bromide assay (data not shown). The highest nontoxic dose (below 10%) was chosen for the experiments.

**Transient Transfection With siRNAs**

The duplexes of siRNA and scrambled siRNA corresponding to rat p62/SQSTM1 were obtained from Dharmacon, Inc. (Boulder, CO, USA). The transient transfection of siRNAs for rat p62/SQSTM1 (200 nM) was carried out using a lipofectamine transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Western Blot Analysis**

The protein extracts obtained from total cell lysate were subjected to SDS-PAGE in a Bio-Rad slab gel apparatus (Mini-PROTEAN Tetra cell; Bio-Rad, Hercules, CA, USA) and electrophoretically transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies for anti-Beclin-1 (1:1000, Santa Cruz Biotechnology, Danvers, MA, USA); anti-LC3 (1:1000, Novus Biologicals, LLC, Littleton, CO, USA); anti-p62/SQSTM1 (1:100, Abcam, Cambridge, UK); anti-phospho-eif2α (peif2α, 1:500; Santa Cruz Biotechnology), and anti-eif2α (1:1000, Santa Cruz Biotechnology). Immunoreactive bands were visualized using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce Biotechnology, Rockford, IL, USA). Exposed films were scanned with a densitometer (Bio-Rad) and analyzed quantitatively with commercial equipment (Multi-Analyt Macintosh Software for Image Analysis Systems; Bio-Rad). At least three independent experiments were carried out.

**Immunofluorescence Assays**

The immunofluorescence assays in rMC-1 were performed as previously published. The cover glasses with fixed cells were incubated with the appropriate antibodies: anti-LC3 (1:10, Novus Biologicals, LLC, Littleton, CO, USA) and anti-p62/SQSTM1 (1:100, Santa Cruz Biotechnology) overnight at 4°C, and the appropriate secondary antibodies were applied for 1 hour at room temperature. The cover glasses were examined under a fluorescence microscope (Zeiss, Oberkochen, Germany). Digital images were captured using specific software (AxioVision; Carl Zeiss Microscopy, Thornwood, NY, USA).

**Caspase Activity**

Caspase 8 activity was analyzed in rMC-1 by a colorimetric assay, as previously described and expressed as the percentage of absorbance units in treated samples compared with the number of absorbance units in cells grown in NG conditions.

**Immunoprecipitation Assay in rMC-1**

Immunoprecipitation was performed as before. A total of 500 μg of protein diluted in extraction buffer were immunoprecipitated with goat anti-caspase 8 (Santa Cruz Biotechnology) using protein A/G agarose beads. The samples underwent Western blotting against p62/SQSTM1 (1:1000, Abcam, Cambridge, UK) followed by the appropriate secondary antibody.

**TUNEL Assay**

Fragmentation of DNA was evaluated by a TUNEL assay using a commercial TUNEL system (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI, USA), as previously described. At least 15 photos were taken on each cover glass using a fluorescence microscope (Carl Zeiss Microscopy). The number of cells emitting green fluorescence (TUNEL-positive) was recorded as the percentage of the total cells counted (4,6-diamidino-2-phenylindole [DAPI]-stained).
Detection of Acidic Vesicular Organelles With Acidine Orange Staining

Rat retinal MCs were stained using acidine orange (AO, 1 μg/mL) for 15 minutes and examined immediately by fluorescence microscopy. Acidine orange is accumulated inside acidic vesicles with prominent orange or red signals, thus serving as an acidic vesicle tracer. The mechanism by which AO is accumulated in vesicles involves the protonation of AO molecules in a low-pH environment.26

Cathepsin Activity

The activities of cathepsin L and D were measured using a fluorescent activity assay kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s instructions. By adding a specific cathepsin L or cathepsin D substrate (cell or retinal tissue lysates), the reaction sensitizes the developer, generating a fluorophore. Fluorescence was measured using a fluorescence plate reader (Victor 1420; PerkinElmer, Waltham, MA) at excitation and emission wavelengths of 488 and 520 nm, respectively, for cathepsin L activity and excitation and emission wavelengths of 400 and 505 nm, respectively, for cathepsin D activity. The relative fluorescence values were corrected by the amount of protein.

Measurement of VEGF-A Secretion in Supernatant

The rMC-1 culture medium was collected after 24 hours of treatment, followed by immediate centrifugation for 20 minutes at 1000g. The levels of VEGF-A of cell culture supernatant were measured by quantitative sandwich ELISA using a rat VEGF-A commercial kit (R&D Systems, Inc., Minneapolis, MN, USA).

Transmission Electron Microscopy

The cell monolayer grown over a glass coverslip was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate and 3 mM CaCl₂ buffer for 5 minutes at room temperature, followed by 1-hour incubation on ice. After fixation, the samples were washed three times in 0.1 M sodium cacodylate and 3 mM CaCl₂ buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate, 3 mM CaCl₂ and 0.8% potassium ferrocyanide for 30 minutes. Then, they were en bloc stained in ice-cold 2% uranyl acetate overnight. Cells were dehydrated in ethanol on ice and ending with four changes of 100% ethanol at room temperature. The dehydrated cells were infiltrated in resin (Epon; Hexxon, Columbus, OH, USA). After four changes of the resin solution, a fifth change was performed and the dish was immediately placed in a vacuum oven at 60°C to be polymerized for 72 hours. Ultrathin sections were cut with a microtome (Leica Ultracut; Leica Microsystems, Wetzlar, Germany), stained with 2% uranyl acetate and Reynolds’s lead citrate, and examined in a transmission electron microscopy (LEO 906-Zeiss; Carl Zeiss Microscopy) at an accelerating voltage of 60 kV.27

Animal Model and Experimental Design

The animal study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study protocol was approved by the local committee for ethics in animal research (CEEA/IB/UNICAMP, protocol number 3551-1). Experimental diabetes was induced in 12-week-old male Wistar Kyoto rats (WKY) by intravenous injection of streptozotocin (60 mg/kg in 0.5-M sodium citrate buffer, pH 4.5; Sigma-Aldrich Corp.); the control animals received vehicle alone. Blood glucose levels were measured 72 hours after the injection using an enzymatic colorimetric GOD-PAP assay (Merck, Darmstadt, Germany) and values ≥15 mmol/L were considered diabetic. The diabetic rats (DM, n = 10) and nondiabetic (CT, n = 10) were housed in a temperature-controlled room (22°C) with a 12-hour light:12-hour dark cycle with free access to food and water for 12 weeks, then euthanized; the eye globes and retinas were collected. During the study, the diabetic rats received 2 units of insulin (human insulin HJ-0310; Eli Lilly and Company, Indianapolis, IN, USA), 3 times per week, subcutaneously.

Immunofluorescence in Retinal Tissues

The eyes were enucleated and fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose in phosphate buffer, frozen in embedding medium (OCT; Sakura Finetek, Torrance, CA, USA). The 10-μm retinal sections were blocked with BSA and incubated with anti-p62/SQSTM1 (Abcam) or anti-Beclin-1 (Santa Cruz Biotechnology), overnight at 4°C then incubated with the appropriate secondary antibodies. Negative controls were established by omitting the primary antibody. The sections were then incubated with the appropriate secondary antibodies. Afterwards, the sections were rinsed and cover-slipped with antifading medium (Vectorshield; Vector Laboratories, Burlingame, CA, USA). The slides were examined under a fluorescence microscope (CLSM; Carl Zeiss Meditec AG, Oberkochen, Germany) with appropriate emission filters for fluorescein isothiocyanate 1 and rhodamine. The analyses were performed using commercial software (AxioVision; Carl Zeiss Meditec AG). Three images from three nonconsecutive retinal sections (100 μm apart) per animal and four animals per group were included into the analyses.

Statistical Analysis

The results were expressed as the mean ± SD of the mean (SEM), and comparisons between multiple groups were performed with a 1-way ANOVA followed by a Fisher’s test. Values of P lower than 0.05 were considered statistically significant. The analyses were performed using statistical software (StatView; SAS Institute, Inc., Cary, NC, USA).

RESULTS

HG Conditions Induce Autophagic Flux Dysfunction and ER Stress Response Associated With Augmentation of MCs, Leading to Apoptosis

Retinal MCs exposed to HG increase Beclin-1, which marks the induction of autophagosome formation, and LC3I (MAP1LC3B/LC3-microtubule-associated protein 1 light chain 3), which marks the existence of autophagosomes in the cell. Cells cultured under NG conditions (Fig. 1A, P = 0.005 and 0.003, respectively) do not increase these markers, indicating that HG conditions induce initiation of autophagic machinery. To evaluate whether autophagic flux was functioning properly, we examined the autophagic substrate p62/SQSTM1, a cargo protein degraded by an autophagy–lysosome system.28 We observed increases in the p62/SQSTM1 levels of cells exposed to HG (P < 0.0001),
suggesting a reduction in autophagosome degradation by lysosome systems (Fig. 1A). Similar findings were observed after 48 and 72 hours of exposure to HG conditions and were steady across exposure times (Supplementary Figs. S1A, S1C). Stress markers of ER were also assessed under HG conditions. rMCs under HG conditions for 24 hours showed increased eif2α phosphorylation and CHOP expression, which are markers of ER stress (Fig. 1A, \( P = 0.01 \) and 0.02, respectively). Electron microscopy shows a massive increase in the autophagosome accumulation in rMCs exposed to HG conditions; in NG conditions, rMCs have normal autophagosome (AP) and autophagolysosome (APL) distribution throughout the cytosol (Fig. 1B). In order to investigate whether autophagy and ER stress are involved in cell death, caspase 8 activity, and TUNEL assays were performed (Figs. 1B, 1C). There was a clear increase in caspase 8 activity and TUNEL-positive cells in HG conditions \( (P = 0.02 \) and 0.004, respectively), but caspase 9 activity did not change in cells exposed to HG conditions (data not shown). A similar increase in TUNEL-positive cells was observed after 48 or 72 hours of HG exposure and was steady throughout those periods (Supplementary Fig. S1D). Collectively, these data show that rMCs in HG conditions show autophagic flux dysfunction and ER stress response, leading to increased risk of apoptosis.

The Inhibition of Autophagy Machinery Aggravates rMC Apoptosis in HG Conditions

To better understand the role of autophagic flux on rMC apoptosis, cells were treated with different autophagy inhibitors, such as 3MA and BAF. The former is a well-known inhibitor of the initial phase of the autophagic process, preventing the formation of autophagosomes by inhibiting class III PI3K/Vps34, and the latter inhibits the last phase of the autophagic process by preventing the fusion of lysosomes to autophagosomes, thus inhibiting degradation. As expected, cells treated with 3MA in NG or HG conditions showed reduced autophagosome formation, demonstrated by a significantly lower level of Beclin-1 than untreated cells (Fig. 2A, \( P = 0.04 \) and 0.005, respectively). Treatment with BAF also induced p62/SQSTM1 accumulation in rMCs under normal or diabetic milieu conditions (Fig. 2C, \( P < 0.01 \)). Inhibition of the initial stage of autophagy with 3MA or the final stage with BAF in NG or HG conditions increases caspase 8 activity and TUNEL-positive cells compared to untreated cells (Fig. 2B, 2C). These experiments indicate that blocking autophagy initiation or autophagy flux increases caspase 8 activity and TUNEL-positive cells under normal conditions, with further increases occurring when rMCs are exposed to diabetic...
milieu conditions. Hence, autophagy disturbances modulate rMC apoptosis under HG conditions.

**ER Stress Sensors Modulates Autophagy Initiation in MCs Exposed to HG Conditions but Does Not Restore Autophagic Flux**

To better understand the relationship between ER stress and autophagy, rMCs were treated with 3MA or GSK2606414, a PERK inhibitor. As PERK phosphorylates eif2α, the PERK inhibitor blocks the phosphorylation of eif2α by inhibiting the phosphorylation of PERK, thus inhibiting the ER stress response. Retinal MCs treated with 3MA under NG or HG conditions displayed increased eif2α phosphorylation compared to untreated cells (Fig. 3A). Retinal MCs treated with a PERK inhibitor in NG or HG conditions displayed reduced Beclin-1 (Fig. 3B, P < 0.05), leading to more p62/SQSTM1 accumulation than untreated cells (Fig. 3B, P < 0.005). Collectively, these findings suggest that ER stress response, assessed by eif2α phosphorylation, modulates the initial phase of autophagic machinery but does not improve p62/SQSTM1 degradation. Additionally, rMCs exposed to HG conditions and treated with PERK inhibitor decrease caspase 8 activity compared to cells treated in HG conditions (Fig. 3C, P < 0.0001). Endoplasmic reticulum provides the major intracellular Ca²⁺ storage, and blocking it affects intracellular processes, such as procaspase-8 cleavage, which depend on Ca²⁺ and do not activate caspase 8. 29

**Silencing of the p62 Sequestosome Ameliorates ER Stress and Cell Apoptosis in HG Conditions**

To better understand the possible involvement of autophagic flux in apoptosis and ER stress, p62 siRNA was applied to rMCs. Expression of p62 was reduced by 61% in rMCs silenced after 24 hours (Fig. 4A). In conditions of NG or HG, p62/SQSTM1 siRNA reduced ER stress response, evaluated through eif2α phosphorylation (Fig. 4B, P < 0.03). In addition, reduced caspase 8 activity and TUNEL-positive cells were observed (Fig. 4C, P < 0.0001). Immunoprecipitation of caspase 8 in all rMCs
was blotted against p62/SQTSM1, which showed a clear interaction between both proteins, indicating that p62/SQTSM1 directly binds to and activates caspase 8 (Fig. 4D, *P* < 0.0001).

**Blockage of Autophagy by Rapamycin, an mTOR Inhibitor, Improves Autophagic Flux and Protects Cells From Death**

Retinal MCs in normal or diabetic conditions were treated with rapamycin, which binds the FRB domain of mTOR, inducing downregulation and activating autophagy. The presence of rapamycin induced autophagosome formation, as demonstrated by upregulation of Beclin-1 and p62/SQTSM1 in rMC-1 treated with GSK2606414, a PERK inhibitor (10 μM). Equal loading and transfer for all proteins were ascertained by reprobing the membranes for β-actin. The bars represent the mean ± SD of band densities, expressed as the percentage of control in at least three independent experiments. PERK INH: PERK inhibitor, GSK2606414.

Rapamycin Improves Lysosomal Proteolytic Activity, Thus Restoring Autophagic Cargo Degradation

In order to address the role of lysosomes in rMCs in diabetic milieu conditions in or out of the presence of rapamycin, lysosome acidification (through AO assay) and cathepsin L and D activities were assessed. By treating the cells with AO, AO is accumulated in acidic vesicles, which yield prominent orange signals. Thus, AO is used as an acidic vesicle tracer. The mechanism of AO accumulation in vesicles involves the
protonation of AO molecules in a low-pH environment. In this immunofluorescence assay, rMCs in normal conditions were treated with BAF (an autophagosome–lysosome fusion blocker) as an internal control. Increased LC3 and p62/SQSTM1 and dramatic impairment of lysosome acidification were observed. Similar findings were observed in rMCs exposed to diabetic milieu conditions (Fig. 6A). Treatment with rapamycin restored lysosomal proteolytic activity, improving autophagy machinery (Fig. 6A). Next, we evaluated cathepsin activity in rMCs exposed to HG conditions. Cathepsins are lysosomal hydrolases and are subdivided into three groups based on the amino acids located in their active sites that perform catalytic activities as aspartyl (cathepsins D and E), cysteine (cathepsins B, C, F, H, K, L, N, O, S, T, U, W, and X) or serine (cathepsins A and G) catalytic sites. In this study, we evaluated cathepsin D, the most powerful lysosomal cysteine proteinase against protein substrates.32 There was a marked decrease in cathepsin L in cells exposed to HG conditions (Fig. 6B, $P < 0.0001$), which was restored with rapamycin treatment. Cathepsin D did not change when rMCs were exposed to NG or HG conditions (Fig. 6C, $P > 0.05$).

**Treatment With Rapamycin Prevents Massive Release of VEGF by MCs in HG Conditions**

As a proof of concept, VEGF levels were quantified in the supernatant from rMCs exposed to HG media both in and out of the presence of rapamycin. During the pathogenesis of DR, MCs are a major source of VEGF in retinal tissue and a therapeutic target when treating DR and diabetic macular edema. To investigate whether lysosome-mediated autophagic defects modulate the VEGF production of rMCs, VEGF levels were quantified in the supernatant from rMCs in NG and HG conditions in the presence of rapamycin (Fig. 6D). As expected, the presence of HG produced a 3-fold increase in VEGF levels ($P < 0.0001$), and treatment with rapamycin prevented an increase in VEGF levels ($P < 0.0001$).
Diabetes Increases Beclin-1 and p62/SQSTM-1 Immunostainings and Reduces Cathepsin L Activity in Retinal Tissue

As expected, the diabetic WKY rats presented lower final body weight and higher blood glucose levels compared to nondiabetic control group (CT = 326.3 ± 52.0 g versus DM = 262.8 ± 50.3 g, P = 0.004; CT = 5.51 ± 0.5 mmol/L versus DM = 23.8 ± 1.4 mmol/L, P < 0.0001, respectively).

In order to strengthen the findings observed in vitro using a Müller cell line, we performed in vivo experiments using Wystar Kyoto rats experimentally induced as diabetic for 12 weeks. We performed an immunofluorescence assay for Beclin-1 and p62/SQSTM1 markers and also estimated the proteolytic capacity of lysosome through the activity of cathepsin L in retinal tissue. Beclin-1 immunostaining was shown to be clearly increased in diabetic retinas at the outer plexiform, invading the outer and inner nuclear layers, and also at the RPE, inner plexiform, and ganglion cell layers (P < 0.05 compared with control animals, Fig. 7A). p62/SQSTM1 cargo (Fig. 7B) displayed a significant augmented expression at the RPE level, at the outer plexiform layer, invading the outer nuclear layer, and also around the nuclei of ganglion cells (P < 0.001). Finally, the retinal lysosomal proteolytic function, evaluated through the activity of cathepsin L assay, was deeply reduced in diabetic retina (approximately 20%) compared with control, although it did not reach conventional statistical significance (P = 0.0506; Fig. 7C).

**DISCUSSION**

For the first time, we found that lysosome-mediated autophagic dysfunction occurs in rMCs exposed to high glucose conditions, which leads to an increased apoptotic rate and massive VEGF release in MCs. Treatment with rapamycin, an mTORC1 blocker, prevents these alterations. In conditions of HG, oxidative stress leads to increased misfolded/unfolded protein levels, producing a sustained but insufficient ER stress response. When elf2a, an ER stress sensor, is phosphorylated, Beclin-1 signaling is upregulated. Consequently, autophagy machinery is activated, producing higher amounts of autophagosomes in the cytosol and accumulating p62/SQSTM1 cargo. Accumulation of p62/SQSTM1 occurs when autophagic flux is compromised. This study showed that lysosome proteolytic
activity is impaired because cathepsin L does not properly function in vitro conditions mimicking diabetes. This autophagic malfunction results in massive VEGF production and an increased apoptotic rate, both of which occur in the early phases of DR. In the presence of rapamycin, cathepsin L activity is restored, improving autophagic flux and reducing p62/SQSTM1 cargo accumulation. This novelty reveals lysosome as a central player in the early VEGF production by Müller cells under HG conditions. Similarly, in in vivo study, diabetic rats display a clear increase of Beclin-1 and p62/SQSTM1 accompanied by impairment of lysosomal function in retinal tissue. This is an indicative that lysosomal impairment and autophagic dysfunction are early events present in the pathogenesis of diabetic retinopathy.

As previously described by other groups, retinal Müller cells respond to a variety of insults such as trauma, ischemia, inflammation becoming activated. In this state, Müller cells express a specific protein profile including massive VEGF release to the retinal tissue. This later phenomenon may lead to breakdown of the blood retinal barrier, resulting initially in retinal edema and thereafter retinal neovascularization, and on the other hand, neurodegeneration (since VEGF is a neurotrophic factor). Presently, the massive VEGF Müller cell–dependent production is abrogated through reestablishment of autophagic machinery by improving the lysosomal function. Herein, we present a possible new therapeutic target for the treatment of DR not only in its vasculopathic stage (retinal edema and neovascularization) but also in its early phase, where no retinopathy is ophthalmoscopically detectable. Further experimental animal studies are needed to confirm this proposal.

Recently, Chang et al. described how the signaling of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) can be controlled through autophagy. The authors have shown that a TLR2-dependent stimulus leads to accumulation of ubiquitinated NF-κB in the cytosol, recruiting p62/SQSTM1. These vesicles are recognized as autophagosomes and are degraded by lysosomes. Impaired autophagy degradation prevents NF-κB degradation, which in turn transcribes the target genes, such as VEGF. Controlling cargo-selective autophagy in MCs in diabetic conditions may be a new treatment to combat the deleterious effects of HG in retinal tissue. The ubiquitin-binding nature of p62 has drawn the attention of many pathologists because it could be an additional diagnostic marker for diseases characterized by the inclusion of ubiquitin. Similar p62-positive cytoplasmic inclusions are also found in several neurodegenerative diseases, including Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis.

Protein p62/SQSTM1 is a functional protein that critically defines the fate of a cell. Through its ubiquitin binding–associated (UBA) domain and LC3 interacting region, p62/SQSTM1 links the autophagy and proteasome systems, shuttling the ubiquitinated substrates to autophagy degradation. Therefore, p62/SQSTM1 is a central regulator of unwanted protein degradation. In this study, we found that caspase 8, not caspase 9, is involved in the apoptosis of MCs exposed to HG conditions. The interaction between p62/SQSTM1 and caspase 8 routes to apoptosis suggests that p62/SQSTM1 determines MC death in diabetic conditions.

In a recent comprehensive review, Levine et al. discussed the rationale for developing autophagy inducers as potential therapeutic targets against a wide range of diseases such as
aging, certain cancers, infections, and neurodegenerative and metabolic diseases. In line with Settembre et al.\textsuperscript{36}—who revealed that transcriptional factor EB (TFEB), a master regulator of autophagic substrate degradation in lysosomal settings, is upregulated—our observations may support the idea that TFEB is a potential therapeutic target for treating early diabetic neurodegeneration.

Our findings suggest that in HG conditions, autophagy machinery is triggered in rMCs but autophagic flux is compromised. Due to its lysosomal storage nature, the autophagic substrate (p62/SQSTM1) is accumulated in the cytosol, signaling apoptosis and massive VEGF production. The presence of rapamycin restores lysosomal activity, improving autophagy and mitigating MC apoptosis and VEGF production.

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