Biochemical and Biophysical Research Communications 463 (2015) 1071–1076

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Inhibition of autophagy induces IL-1β release from ARPE-19 cells via ROS mediated NLRP3 inflammasome activation under high glucose stress

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ARTICLE INFO

*Article history:*
Received 4 June 2015
Accepted 9 June 2015
Available online 20 June 2015

*Keywords:*
Diabetic retinopathy
Autophagy
Retinal pigment epithelium
NLRP3 inflammasome

ABSTRACT

Autophagy plays an important role in the development of diabetic retinopathy (DR). Retinal pigment epithelial (RPE) cells are the main cells involved in DR, a process in which hyperglycemia plays a crucial role. This study was conducted to investigate the protective effect of autophagy against high glucose-induced inflammatory response in ARPE-19 cells and its underlying mechanism. In the present study we subjected ARPE-19 cells to high glucose stress and showed that ARPE-19 cells respond to high glucose with an increase in autophagy. 3-methyladenine (3-MA) inhibited occurrence of autophagy and it leaded to the accumulation of damaged-mitochondria-producing-ROS, and the activation of NLRP3 inflammasome, and subsequently, caused IL-1β secretion.



## 1. Introduction

Retinopathy is the most common microvascular complication of diabetes, resulting in blindness for over 10,000 people with diabetes every year. Diabetic retinopathy (DR) remains a leading cause of preventable blindness worldwide [1,2]. The mechanisms involved in the development of DR are complex and incompletely understood. Retinal cells are vulnerable to damage in diabetes, significant of which are retinal pigment epithelium (RPE) cells. RPE cells are specialized epithelium lying in the interface between neural retina and choriocapillaris where they form the outer blood-retinal barrier (BRB). RPE cells play an important role in the pathologic process of DR. Metabolic changes cause the RPE cells disorder, which lead to microvascular leakage in diabetes. Most of the research on the development of DR has been focused on the impairment of the neuroretina and the inner blood retinal barrier (BRB). By contrast, the effects of diabetes on the RPE cells have received less attention and the molecular mechanisms responsible for these early changes in the RPE cells remain unclear [8]. Hyperglycemia is one of the most outstanding initiators in DR. Early inflammatory response, disruption of the blood–retinal barrier, accelerated microvascular cell death, and pathological angiogenesis are all marks of DR. The increase in pro-inflammatory cytokines plays a key role in the development of DR [3–5]. As a crucial pro-inflammatory cytokine secreted by lymphocytes and macrophages, interleukin-1β (IL-1β) can trigger and amplify inflammatory response during DR progression [6,7].

Elimination of cytosolic components inside cells such as damaged organelles can occur through autophagy. Mitophagy is a form of autophagy in which damaged mitochondria are specifically targeted for autophagic degradation by the lysosomes to stop the proinflammatory activation. It has been shown recently that autophagy is capable of regulating inflammasomes, and it probably inhibits the intracellular signaling by removing dysfunctional mitochondria that would otherwise produce increased amounts of intracellular reactive oxygen species ROS [9]. ROS may also lead to the formation of NLRP3 inflammasomes, which activate the IL-1β and IL-18 [10]. In vivo, several studies using genetically modified mice that lack inflammasome components NLRP3, ASC, and Caspase-1 displaying improved insulin sensitivity provides initial evidence that activation of the NLRP3 inflammasome is a key mechanism that induces inflammatory response. To further elucidate the mechanism of DR, in the present study we subjected ARPE-19 cells to high glucose and showed that ARPE-19 cells respond to

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http://dx.doi.org/10.1016/j.bbrc.2015.06.060
0006-291X/

high glucose with an increase in autophagy. The autophagy inhibitor 3-methyladenine (3-MA) inhibited occurrence of autophagy and it leaded to the accumulation of damaged-mitochondria-producing-ROS, and the activation of NLRP3 dependent proinflammatory responses, and subsequently, increase of IL-1β secretion. These results indicate autophagy is a crucial cytoprotective process that prevents the accumulation of damaged organelles in ARPE-19 cells. Thus, activation autophagy may be a potential therapeutic target for preventing the development and progression of DR.

## 2. Materials and methods

### 2.1. Cell culture

The human retinal pigment epithelium cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC, Mantissa, VA). It was cultured in Dulbecco's modified essential medium/Ham's F12 medium (DMEM/F12, Gibco, Grand Island, NY) and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY), 100 μg/ml of streptomycin, and 100 U/ml of penicillin. The cells were cultured at 37 °C in a humidified chamber of 5%$CO_2$ and were used at passages 10–15. The culture medium was replaced with fresh medium every other day.

### 2.2. High glucose treatment of ARPE-19 cells

ARPE-19 cells were seeded in 6 well culture dishes with 5.5 mM glucose (normal glucose) condition until 70–80% confluent, and then the serum-free DMEM/F-12 was added to the cells for 24 h before switching to high glucose treatment (30 mM, D-glucose). The cells were then incubated in 37 °C in a humidified chamber of 5% $CO_2$ for 48 h.

### 2.3. Cell viability assay

A CCK-8 assay (Roche, Mannheim, Germany) was performed to determine cell viability. Briefly, cells were plated at a density of $2 \times 10^3$ cells/well in 96-well microplates. After incubation for 24 h, 3-MA (Sigma, St Louis, MO, USA) was added to the culture medium at different concentrations (5, 10 mM). After 1 h, glucose solution was added to a final concentration of 5.5, 30, 50, 70 mM. The cells were cultured for an additional 48 h at 37 °C. CCK-8 solution was added (10 μl/well), and the cells were further incubated for 3 h at 37 °C in a 5% (v/v) $CO_2$ atmosphere. The absorbance was measured at 450 nm using a microplate reader, with a background control as a blank. The cell survival ratio was expressed as the percentage of the control.

### 2.4. Transmission electron microscopy

TEM were performed with reference to a previous report [11]. ARPE-19 cells were fixed in 2.5% glutaradehyde in 0.1 M sodium cacodylate buffer (PH7.0) for 1 h, fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for another 1 h, dehydrated with increased doses of ethanol, and infiltrated with araldite resin. Ultrathin sections (70–80 nm) were made using an ultramicrotome. Sections were stained with uranylacetate and lead citrate, and then analyzed by transmission electron microscopy (Hitachi H-7500).

### 2.5. ROS detection

The ROS were measured using the DCFH-DA (Sigma, St Louis, MO, USA) molecular probes. ARPE-19 cells were incubated with 10 μM DCFH-DA for 30 min at 37 °C, then washed three times, and resuspended in PBS at $1 \times 10^6$ cells/ml. The cells were monitored using flow cytometry at excitation and emission wavelengths of 488 and 525 nm, respectively. Untreated cells served as the control. ROS generation was determined using a FACScan flow cytometer and CellQuest software (Becton Dickinson). The results were expressed as fluorescence intensity of dichlorofluorescein (DCF) compared with control.

### 2.6. ELISA

After different treatment conditions, cell culture supernatants were collected. Concentrations of pro-inflammatory cytokines IL-1β was measured using a high-sensitive ELISA kit (eBioscience, California, USA), according to the manufacturer's instruction.

### 2.7. Western blot analysis

ARPE-19 cells were incubated with 30 mM high glucose for 48 h with or without pretreatment with 5 mM 3-MA for 1 h. After treatments, the cells were washed twice gently with ice-cold PBS and then lysed using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Haimen, China) according to the manufacturer's instruction. Lysates were centrifuged at 15,000× *g* for 10 min at 4 °C. Protein concentrations were determined with the Bicinchoninic Acid Protein Assay kit (Pierce, IL). Protein samples were separated with SDS-PAGE and then transferred to a polyvinylidene difluoride membranes (Millipore, USA) at 15 V for 30 min. After blocking with 5% (v/v) nonfat dry milk for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4 °C and then incubated with the corresponding horseradish peroxidase-linked secondary antibodies for 1 h at room temperature. The signals were developed using a standard enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham Biosciences, NJ) and exposed to X-ray film. Densitometric analysis was performed with Quantity One software (Bio-Rad Laboratories).

### 2.8. Statistical analysis

All statistical analyses were performed using SPSS (version 10.0). Data were expressed as mean ± standard deviation (SD). All data were analyzed with one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test for multiple comparisons. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Inhibition of autophagy decreases ARPE-19 cell viability under high glucose stress

Initial experiments were performed with a hyperglucose cell model using glucose in ARPE-19 cells. It is known that high glucose conditions exhibited cytotoxicity on ARPE-19 cells [12]. We confirmed this in our study. We exposed cultured ARPE-19 cells to increasing concentration of glucose (5.5, 30, 50, and 70 mM) for 48 h. Cell viability was examined using CCK-8 assay. As shown in Fig. 1A, ARPE-19 cells underwent cell death after glucose treatment in a concentration of 50, 70 mM. At 30 mM, high glucose caused an approximate 100% in cell viability, and this dosage was used in our study to investigate the protective effect of autophagy on high glucose-induced cell death. As shown in Fig. 1B, ARPE-19 cells underwent cell death after 3-MA treatment in a dose of 10 mM. At 5 mM, 3-MA caused an approximate 85% in cell viability, and this dosage was used in our study. We found that compared with the control group, high glucose treatment did not significantly alter cell

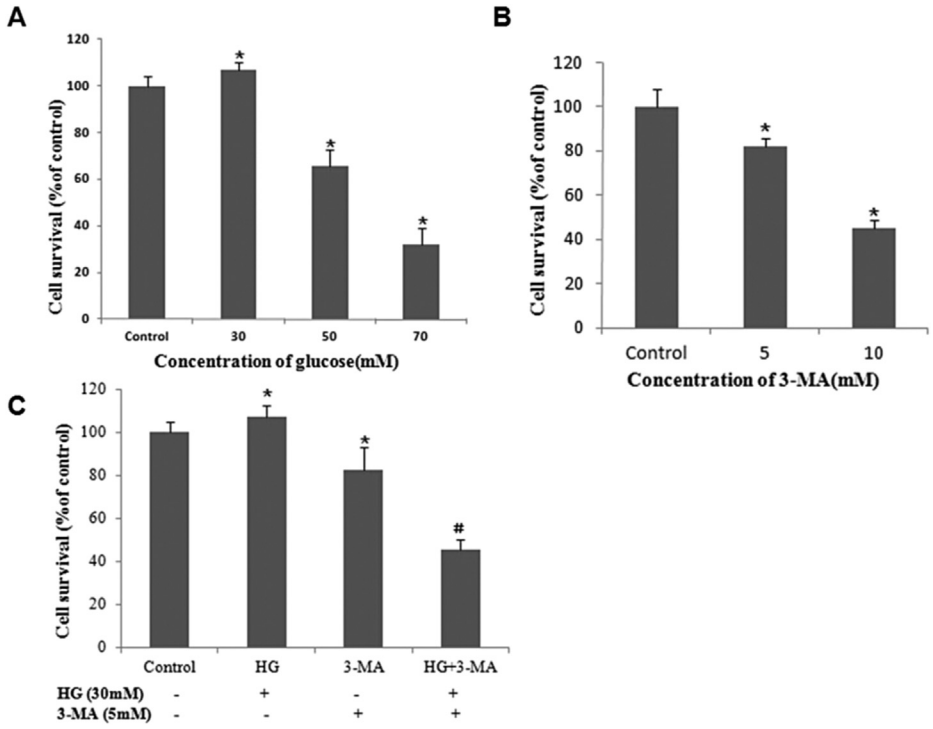


**Fig. 1.** Effects of autophagy on high glucose-induced ARPE-19 cell viability. The cell viability was determined by CCK-8 assay. The ARPE-19 cells were incubated with different concentrations of glucose (5.5, 30, 50, 70 mM) for 48 h (*P < 0.05 vs control, n = 6) (A). The cells were treated with 3-MA at doses of 0, 5, and 10 mM for 48 h (*P < 0.05 vs control, n = 6) (B). The cells were pretreated with 3-MA (5 mM) for 1 h, then exposed to glucose (30 mM) for 48 h (*P < 0.05 vs control, #P < 0.05 vs HG, n = 6) (C). Data were shown as mean ± standard deviation (SD) and expressed as a percentage of the untreated control.

viability (Fig. 1C). By contrast, high glucose resulted in an obvious reduction in cell viability after autophagy inhibiting by 3-MA, suggesting that autophagy plays a protective role in ARPE-19 cells upon high glucose stress.

### 3.2. High glucose treatment induces ARPE-19 cells autophagy

To determine the effect of high glucose on autophagy, ARPE-19 cells were incubated in the medium containing normal glucose (5.5 mM) and high glucose (30 mM), respectively. Transmission electron microscopy observation showed that high levels of glucose resulted in a significant increase in the number of double-membrane vacuoles, which was typical of autophagosomes (Fig. 2A).

### 3.3. 3-MA inhibits the expression pattern of autophagic markers under high glucose

The primary biochemical marker of autophagy activation and autophagosome formation is the protein LC3, an essential component of the autophagosome complex [13]. As shown in Fig. 2B, Endogenous LC3 migrates is two bands on polyacrylamide gel electrophoresis. The upper band represents LC3-I, which is cytosolic, and the lower, faster-migrating band represents LC3-II. LC3-II is conjugated with phosphatidylethanolamine and is present on autophagosome. LC3-II levels increase in the high glucose condition, suggesting that the degradation of LC3 protein by autophagy. 3-MA inhibits both LC3 conversion and LC3 turnover compare to high glucose conditions (Fig. 2C and D).

### 3.4. Inhibition of autophagy increases intracellular generation of ROS under high glucose stress

The ARPE-19 cells were pretreated with or without 5 mM 3-MA for 1 h and then incubated with 5.5 mM and 30 mM glucose for 48 h. The levels of intracellular ROS were modest increased in the high glucose-induced and 3-MA treated separately cells in comparison with those of the control group. However, treatment together with 3-MA and high glucose resulted in a significant increase of intracellular production of ROS (Fig. 3).

### 3.5. Inhibition of autophagy increases IL-1β release in ARPE-19 cells under high glucose stress

In order to study the relationship of autophagy with inflammasome activation in ARPE-19 cells, we measured the IL-1β activity from cell supernatants. As shown in Fig. 4A, high glucose+3-MA treatment significantly increased the IL-1β activity. Treatment with the 3-MA alone or high glucose did not have any significant effect on IL-1β activity.

### 3.6. Involvement of the NLRP3 inflammasomes in autophagy-induced cytoprotection under high glucose stress

Previous studies suggest that decline in intracellular degradation systems results in the activation of NLRP3 inflammasomes, arisen as a result of elevated production of biologically active IL-1β in RPE cells [14,15]. To better understand the protective mechanism of autophagy on ARPE-19 cells, we examined intracellular IL-β and NLRP3 protein using a western blot analysis. As shown in Fig. 4B, high glucose activated NLRP3 inflammasomes and induced the release of IL-1β in ARPE-19 cells. Furthermore, pretreatment for 1 h with 3-MA (5 mM), a specific inhibitor of autophagy, increased the production of intracellular IL-1β and NLRP3 inflammasomes induced by high glucose (Fig. 4C and D). These data indicated that autophagy participated in the activation of NLRP3 inflammasomes in ARPE-19 cells.

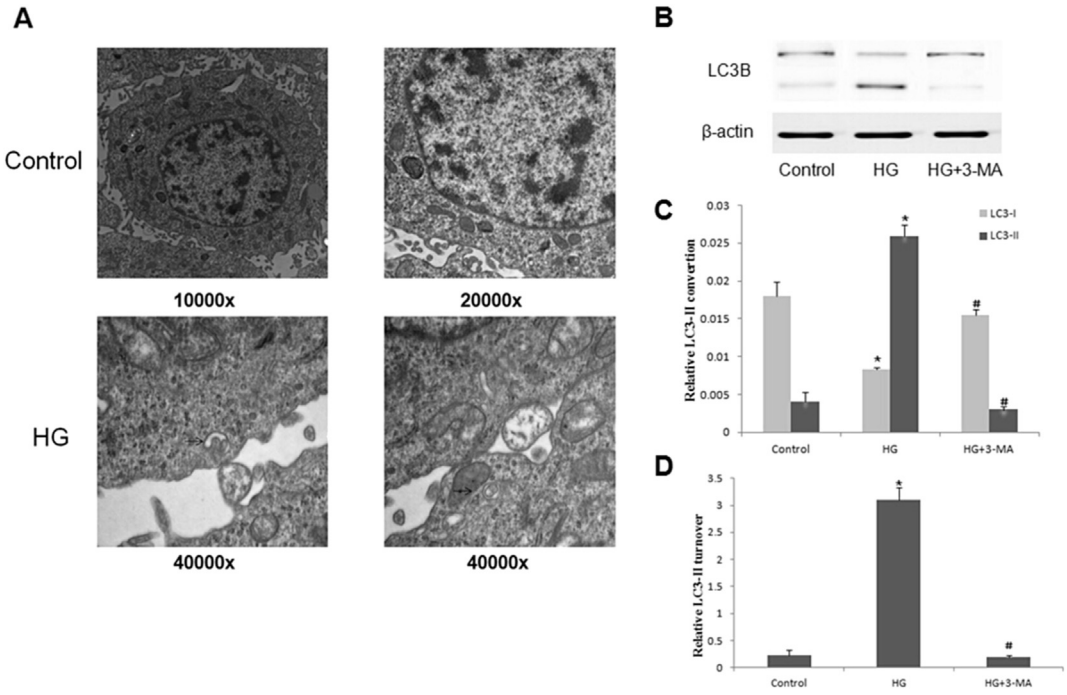


**Fig. 2.** Effects of high glucose treatment on autophagy in ARPE-19 cells. ARPE-19 cells were incubated in the medium containing normal glucose (5.5 mM) or high glucose (30 mM) for 48 h and then these cells were prepared for transmission electron microscopy observation. Arrow indicates autophagosome in ARPE-19 cells (A). The cells are incubated with 30 mM of glucose for 48 h with/without pretreatment with 5 mM 3-MA for 1 h. The protein levels of LC3-I and LC3-II are measured using Western blot analyses. β-actin expression was detected as the loading control (B). The group treated with normal glucose was taken as the control group. Densitometric analyses of Western blot were presented as the mean ± SD for three independent experiments performed in triplicate. The relative amount of LC3-II conversion and LC3-II turnover was expressed compared with the control group(C, D) (*P < 0.05 vs control, #P < 0.05 vs HG, n = 3).

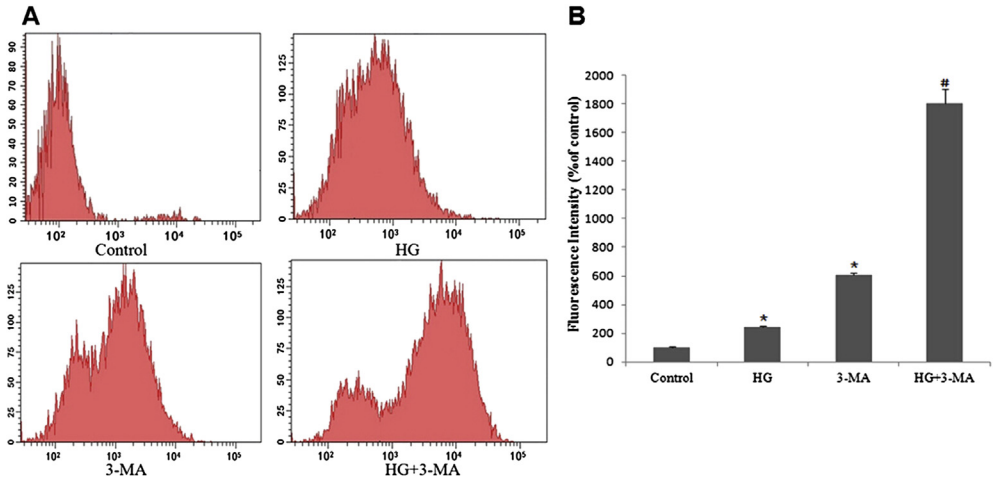


**Fig. 3.** Effects of autophagy on high glucose-induced intracellular generation of ROS in ARPE-19 cells. The ARPE-19 cells were pretreated with 5 mM 3-MA for 1 h and then exposed to 30 mM high glucose for 48 h. The intracellular ROS was measured with flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (A). The results shown were one representative example of the three separate experiments. HG+3-MA increased the generation of ROS in ARPE-19 cells significantly (B). Data were expressed as a percentage of cells control (*P < 0.05 vs control, #P < 0.05 vs HG, n = 3).

## 4. Discussion

There is an increasing number of studies investigating the role of autophagy in DR [16]. Ample studies have shown that autophagy is capable of regulating inflammasomes in extracellular environments [9]. However, whether it has an effect against high glucose-induced cell damage in RPE cells is unknown. In the present work, we demonstrated that inhibition of autophagy increased high glucose-induced cell death, intracellular ROS production, and IL-1β secretion, and that the mechanism of autophagy-induced cytoprotection was probably related to the NLRP3 inflammasome activation in ARPE-19 cells.

Chronic hyperglycemia associated with reactive oxygen/nitrogen species (ROS/RNS) stress and low grade inflammation are considered to play a critical role in the development of DR [17]. Excess glucose metabolic flux through the aldose reductase/polyol

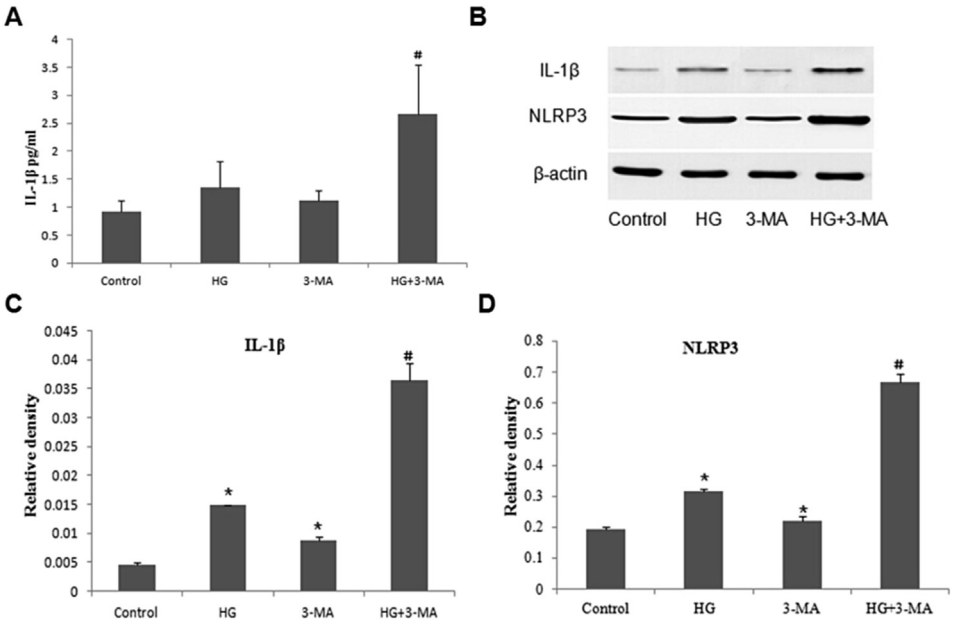


**Fig. 4.** Activation of the NLRP3 inflammasome was involved in the protection of autophagy on ARPE-19 cells. The ARPE-19 cells were pretreated with or without 5 mM 3-MA for 1 h and then treated with or without 30 mM high glucose for 48 h. Cell supernatants (A) were collected for analysis by ELISA (*P < 0.05 vs control, n = 6). Western blot analysis was done using the corresponding antibodies (B). Nuclear fractions were immunoblotted with anti-IL-1β, and anti-NLRP3 antibodies. Quantitative analysis of the relative protein levels in ARPE-19 cells (C, D). Data were shown as mean ± SD (*P < 0.05 vs control, #P < 0.05 vs HG, n = 3).

pathway, advanced glycation end (AGE) production, elevated hexosamine biosynthesis pathway (HBP), diacyl glycerol/PKC activation, and mitochondrial ROS generation are all implicated in DR [18]. Initial experiments were performed with DR model using high glucose in ARPE-19 cells. We observed the autophagosome in this part successfully.

Autophagy is a central lysosomal clearance system that may play an important role in DR development. Damaged cellular components, which are no longer functional, should be degraded by cellular clearance systems including autophagy, which is a self-eating process [19]. If the autophagic degradative pathway is faulty, an accumulation of damaged proteins as aggregated deposits takes place that may cause anatomical obstacles to physiological processes. To avoid cellular damage, ROS-generating mitochondria are constantly removed by autophagy and mitophagy [20]. Therefore we speculated that inhibition of mitophagy/autophagy should lead to the accumulation of ROS-producing mitochondria damaged, and as a consequence, to the activation of the NLRP3 inflammasome in ARPE-19 cells. To this end, the mitophagy/autophagy inhibitor 3-methyladenine (3-MA) was added to ARPE-19 cells, which, as expected, resulted in the accumulation of damaged mitochondria and increased generation of mitochondrial ROS. A critical component of the autophagosome, LC3 has been regarded as a primary biochemical marker for autophagy activation. During autophagy, LC3-I, the cytosolic form, is conjugated to phosphatidylethanolamine to form the phosphatidylethanolamine conjugate (LC3-II), which is then recruited to membranes. Therefore, monitoring the conversion of LC3-I to LC3-II is indicative of autophagic flux and has been regarded as a reliable autophagy marker [19,21]. In our study, we had also shown that the activation of autophagy in response to high glucose stress was inhibited by 3-MA. In the present work, we found that high glucose-treated cells showed an increased production of intracellular ROS. However, 3-MA pretreatment could significantly increase production of intracellular ROS production. These data strongly support the hypothesis that inhibiting autophagosome formation by 3-MA increased concentrations of mitochondrial ROS. The mechanism of autophagy-induced cytoprotection was most likely related to the NLRP3 inflammasome activation.

The role of the NLRP3 inflammasome in the development of DR is a very new area. NLRP3 activity has been demonstrated in a wide variety of other diseases, including Alzheimer's disease, asbestosis, gout, and atherosclerosis [22–24]. The NLR family, NLRP3 inflammasome is a multiprotein complex that activates caspase-1, leading to the process and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Three models for activation of the NLRP3 inflammasome have been proposed. They are the P2X7ATP-gated ion channel, the lysosome rupture model and the ROS model. The generation of ROS results in NLRP3 inflammasome activation through release of the ROS-sensitive NLRP3 ligand thioredoxin-interacting protein (TXNIP) from its inhibitor thioredoxin (TRX) [25]. In our study, the presence of high glucose increase the ability of RPE cells to remove damaged or nonfunctional proteins by autophagy. However, intracellular ROS production and IL-1β secretion is not increased under the environment of high glucose at the same time. But Inhibition of autophagy by 3-MA results in a remarkable production of intracellular ROS and release of IL-1β. These data suggest that autophagy could protect the stability of the cell in a certain extent.

In summary, our studies showed that autophagy played a protective role in DR, partly through regulation of NLRP3 inflammasome activation in ARPE-19 cells under high glucose stress. Although the contributions of autophagy to DR are complicated and have yet to be fully understood, our findings may facilitate a better understanding about the development of DR, and further studies with in vivo models investigating the exact role of autophagy in DR are needed.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.060.

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