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High-glucose（HG）triggers of Autophagy Affects the Expression of NF-KB and Apoptosis in Müller Cells

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Abstract

Purpose: Autophagy pathway might be involved in the production of pro-inflammatory cytokines and apoptosis in HG-stimulated Müller cells, though details of the mechanism remain largely not currently known.

Methods: In this experimental research, primary SD rat retinal Miller cells were exposed to normal glucose (NG) or 3 h, 6 h, 12 h, 24 h, 36 h of high glucose. LC3I/LC31I, P62, and Beclin-1 protein expression was examined by Western blot analysis in the various experimental groups. And in fluorescence microscopy experiments, autophagy was evaluated by the autophagy markers LC3I/LC3ll and P62. The formation of autophagosomes and autolysosomes were examined by electron microscopy. TUNEL assay was used to detect apoptosis in high glucose Müller cells. One-way analysis of variance was used to compare data between different groups.

Results: In the present study, the retinas Müller cell expose to HG for early stage (6h), HG increased autophagy by promoting the formation of autophagosomes, increasing lysosomal acidification, stimulating autophagic flux, meanwhile, protecting the cells from apoptosis and inflammation. However, decreased autophagy-related maker protein (Beclin-1 and LC3II/LC3I) and autophagic flux were detected in the HG-stimulated Müller cells at later time points (24 h) and 3MA, restrain autophagic can increase both apoptosis and NF-κB phosphorylation in the Müller cell.

Conclusions: These results highlight that HG regulates autophagy in different periods, and autophagy is a protective effect may account for the defense against Müller cell inflammation. This finding might be valuable for the study of DR pathogenesis.

Keywords: Müller cells; Autophagy; Diabetes retinopathy; Apoptosis; NF-KB

Introduction

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].

Müller cells are one of the primary glial cell types found in the retina and play a significant role in maintaining retinal function and health. In diabetes, it has been well established that Müller cells become activated[5-8]. Hyperglycemia-induced Müller cell goes hand in hand with stimulation of growth factor, cytokine, and chemokine[9-11]. Our research has previously found that hyperglycemia promotes release of cytokines and chemokines including interleukin-1β (IL-β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)[12]. In vitro studies have provided

ample evidence that Müller cells are a potential source for growth factors and cytokines when stimulated with elevated glucose levels. Considering that most of the cytokines released by Müller cells have been identified in the vitreous of diabetic patients it is fair to assume that Müller cells contribute to the overall synthesis of these factors in vivo[13-16].

Autophagy is a general term for pathways by which cytoplasmic material, including soluble macromolecules and organelles, is delivered to lysosomes for degradation[17]. It is now clear that autophagosomes can degrade substrates in a selective manner[18]. Some studies suggest that autophagy have diverse roles in membrane dynamics to benefit the host in the removal of invading pathogens[19, 20]. In addition to target intracellular pathogens for degradation, autophagy also accumulates numerous molecules involved in sensing and transduction of pathogen-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP) signals, and there is an increase in nuclear factor-κB (NF-κB)-dependent cytokine production. Indeed, in Müller cells, HG has been observed to disrupt autophagy and induce cargo accumulation due to lysosomal dysfunction[21]. And, the cargo accumulates in autophagy deficient cells, activate the pro-inflammatory transcription factor NF-κB [22].

Another interesting point is whether we should expect pro-survival or pro-death effects of autophagy in diabetic retinopathy[21, 23, 24]. Apoptosis and necrosis seem to establish a strict relationship with autophagy, which may function as a cell death route or may initiate a cell protective response[25]. Apoptotic pathways are known to participate in the death of retinal cells under different noxious stimuli, including diabetic stress, but autophagy, a catabolic pathway that promotes the degradation and recycling of cellular components, has also been recognized to be involved in the fate of stressed retinal neurons[26, 27]. A variety of studies has been concerned with the possible roles of autophagy in DR[21, 23, 28-30]. Although the modulation of autophagic signaling, with consequent autophagy dysregulation, is likely to be involved in the pathogenesis of DR[21, 31], whether high glucose (HG) levels determine an increase of the autophagic flux or, instead, inhibit autophagy, has not been established unambiguously. In addition to regulating inflammatory signaling, the autophagy pathway may prevent tissue inflammation through its role in apoptotic corpse clearance[32 33].

Therefore, it might be assumed that the autophagy pathway plays a role in Müller. However, autophagy formation and regulation in Müller, and its effect on the production of pro-inflammatory are largely unknown. In the present study, we demonstrated that Müller cells express both classical and modified autophagy processes, and the abnormal autophagy function affects the expression of NF-KB, IL6 and apoptosis in HG-stimulated Müller cells.

Materials and methods

Reagents

Green fluorescent protein (GFP)-light chain 3 (LC3) adenovirus was purchased from Hanbio Co. Ltd. (Shanghai, China). Primary antibodies against LC3 and P62, were purchased from Abcam Technology (Cambridge, UK), p-mTOR, mTOR, p-NF-κB and NF-κB were purchased from Cell Signaling Technologies (Danvers, MA, USA). Beclin-1 were purchased from Santa Cruz Biotechnology (Danvers, MA, USA). 3-Methyladenine (3-MA), chloroquine (CQ), and acridine orange (AO) were obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Other chemicals were purchased from Sigma, unless otherwise noted.

Rat Retinal Müller Cell Culture

Primary rat Müller cells were isolated and cultured as previously described[34]. Briefly, retinae from five-day-old rats were isolated and mechanically dissociated by careful homogenization, after digesting the retinal tissue with 0.05% trypsin in 0.5 mM EDTA (Gibco/Invitrogen, Carlsbad, CA, USA) and culturing in Dulbecco's minimal essential medium (DMEM)/15% fetal bovine serum (FBS). When the remaining adherent cells reached 80% confluence, they were replanted into new flasks. Müller cells were routinely evaluated by immunofluorescence staining using a monoclonal antibody against vimentin. Experiments with Müller cells were performed with cells at passages 4–8. Purified Müller cells were then plated on flasks at 600 cells/mm2 (37 °C, 5% CO2, 95% humidity). Only cultures of pure Müller cells showing no trace of glial contamination were selected for further studies.

In Vitro Study

Cultures of Müller cells at 70% to 80% confluence were exposed to 5.5 mM D-glucose (NG) and 40 mM D-glucose (HG) with or without 5 mM 3-methyladenine (3MA, a type III PI3 kinase complex inhibitor) as an autophagy inducer; chloroquine (CQ, Sigma-Aldrich, MO, USA) is a lysosomotropic agent that prevents acidification of the lysosomal lumen and inhibits the activity of lysosomal enzymes[35]. GFP-LC3-transfected Müller cells were established as previously described[36].

Western Blot Analysis

At the end of the treatment period, proteins were separated by SDS–PAGE under reducing

conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% FBS in phosphate-buffered saline with 0.1% tween 20 (PBST). The blots were incubated overnight at 4℃with primary antibodies. The membranes were incubated with primary antibodies for anti-Beclin-1 and cleaved-caspase3 (1:500, Santa Cruz Biotechnology, Danvers, MA, USA); for anti-LC3 and P62 (1:1000, Abcam, Cambridge, UK); for anti-p-mTOR and mTOR (1:1000, Cell Signaling Technologies, Danvers, MA, USA), and for anti-p-NF-κB and NF-κB (1:1000, Cell Signaling Technologies, Danvers, MA, USA). As an internal control for protein loadings, the membranes were hybridized against GAPDH (1:1000, Santa Cruz Biotechnology, Danvers, MA, USA). After incubation with an HRP-conjugated secondary antibody for 2 h at room temperature, the signals were developed using a chemiluminescence solution (ECL western blotting detection reagents; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Visualization of autophagic vacuoles

The Müller cells were transfected with GFP-light chain 3 (LC3) or GFP-RFP-LC3 adenovirus according to the manufacturer's instructions. After 24 h of transfection, the cells were incubated in NG or HG (6 h and 24h). Fluorescence images were visualized with a laser scanning confocal microscope (DM 400B, Leica, Germany). Autophagy was measured by quantifying the average number of autophagosomes per cell for each sample. A minimum of 100 cells per sample were counted.

Immunofluorescence Assays

The immunofluorescence assays in Müller glial cells were performed as previously published. Cells or retinal fragments were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS (3 min). After extensive washing with PBS, the fixed cells were incubated with the appropriate antibodies -- anti-LC3 (1:100, Abcam, Cambridge, UK) and anti-P62 (1:100, Abcam, Cambridge, UK) -- overnight at 4 °C, and the appropriate secondary antibodies were applied for 2 h at room temperature. The cover glasses were examined under a fluorescence microscope (DM 400B, Leica, Germany).

Cell Counting Kit-8 (CCK-8)

Müller glial cells were seeded at a density of 2×104 cells/ml onto 96-well plates with 100 ml of medium per well overnight before HG treatment. The next day, the Müller cells were treated with various concentrations of HG for 3, 6, 12, 24, 36 or 48 h in 96-well plates. We added 10 μl of the CCK-8 (Sigma-Aldrich，MO, USA) solution to each well of the plate. After

incubation in a humid atmosphere of 37 °C with 5% CO2 for 2 h, we finally measured the absorbance at 450 nm using a microplate reader (iMark Microplate Absorbance Reader; Bio-Rad, CA, USA).

5-Ethynyl-2'-Deoxyuridine (EdU) Analysis

Müller cells were seeded at a density of 1.5×104 cells/ml onto 24-well plates with 500 µl of medium per well overnight. To examine the HG and 3MA on cell proliferation, the Müller cells were exposed to HG or 3MA for 24 h. Then, half of the medium containing HG or 3MA was replaced with fresh medium containing 20 mM of EdU and was cultured with cells for 12 h. To detect cell proliferation, EdU-labelled cells were processed according to the manufacturer's protocol (Click-iT EdU Imaging Kit, Thermo Fisher, MA, USA). Finally, images were captured using a fluorescence microscope (DM 400B, Leica, Germany), The percentage of EdU+ cells within the total number were then quantified.

Enzyme-linked immunosorbent assay

The inflammatory cytokines in the Müller cells were measured using enzyme-linked immunosorbent assay (ELISA) kits for IL-6 (R&D Systems, Minneapolis, MN, USA). All spectrophotometric readings were performed using an absorption spectrometer (iMark Microplate Absorbance Reader; Bio-Rad, CA, USA). All procedures were performed according to the manufacturer's instructions.

TUNEL Assay

Fragmentation of DNA was evaluated by TUNEL assay using a commercial TUNEL system (In Situ Cell Death Detection Kit; Roche, Basel, Switzerland), as previously described. At least 15 photos were obtained from each cover glass using a fluorescence microscope (DM 400B, Leica, Germany). The number of cells emitting green fluorescence (TUNEL positive) was recorded as the percentage of the total cells counted.

Acridine Orange Staining

The Müller cells were incubated with 1 µg/ml acridine orange[37 38] (Sigma, St. Louis, MO, USA) for 15 min at 37 °C, followed by PBS washes, and then immediately observed under a fluorescence microscope (DM 400B, Leica, Germany).

Transmission Electron Microscopy

Müller cells were fixed at 4 °C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 1 h, followed by post-fixation with 1% osmium tetroxide. dehydrated with increasing

concentrations of ethanol and gradually infiltrated with Araldite resin. Ultrathin sections (70–80 nm) were obtained using an ultramicrotome (RMC MT6000-XL). The sections were stained with 2% uranyl acetate and lead citrate and examined by transmission electron microscopy (LEO 906-Zeiss; Carl Zeiss Microscopy)

Statistical Analysis

Data are expressed as the mean ± SD of at least 2–3 independent experiments. Data were analysed using GraphPad Prism software (GraphPad Software, CA, USA), and comparisons between multiple groups were performed with a one-way ANOVA, followed by a Fisher's test. P values < 0.05 was considered statistically significant.

Results

Autophagy in HG-treated Müller Cells

Müller cells were exposed to NG (5.5 mM) and HG (40 mM) for3, 6, 12, 24 and 36h. Beclin-1 and LC3II/LC3I protein expression showed a significant up-regulation, reached the peak at HG (6 h), and then gradually declined to the baseline from 24 h to 36 h（Fig1.A）. We detected expression of Beclin-1 and LC3II/LC3I in the NG groups. Beclin-1and LC3II is also known as autophagy-related maker protein. At the same time, the expression of P62 protein, a cargo receptor for the autophagic degradation of ubiquitinated substrates that is degraded during autophagy is related with autophagic flux, markedly accumulated in the cells exposed to HG at 24 h to 36 h. Our data suggested that HG increases Müller cell autophagy before 6 h and decreases after 24 h.

Analysis of the Autophagy in Müller cells during various time points in HG

Cytoplasmic LC3 puncta are characteristic of autophagosomal membrane formation; thus, we examined HG induction of LC3 puncta by ectopic expression of GFP-LC3 adenovirus in Müller cells. As shown in Fig.2.A, GFP-LC3 showed a certain distribution pattern in control cells and was increased in the HG for 6 h treatment group, whereas it showed a diffuse distribution pattern at 24 h.

Next, we observed the formation of autophagosomes and autolysosomes by electron microscopy. Interestingly, HG (6 h) stimulated increase in the Müller cells of autophagosomes but autolysosomes, HG (24 h) decreased formation of autophagoly some-like structure and made compartments that have less dense areas compared with the HG- Müller cell cellular

density (Coarse black arrow) and autophagosomes (Fig. 2B, Fine black arrow). Phagophore and double membrane structures indicate that active autophagy occurs with HG treatment. We suspect that HG stimulate he formation of autophagosomes at early phase, and HG inhibits the formation of autophagosomes and affects the function of autophagic lysosomes at later period.

Inhibition of Autophagy can restrain Lysosome Acidification, Thus Aggravating Restoring Autophagic Cargo Degradation

In this immunofluorescence assay, Compare with NG groups, HG increased LC3, but P62 and lysosome acidification has no obvious alteration for 6 h. More importantly, exposure to HG for 24 h, P62 accumulate, this suggests that autophagy is impaired. In order to address the role of lysosomes in Müller cells in diabetic milieu conditions, lysosome acidification (through Acridine Orange Staining assay) was assessed. By treating the cells with Acridine Orange (AO), AO is accumulated in acidic vesicles, which yield prominent orange signals. Thus, AO is used as an acidic vesicle tracer. Compare with NG groups, lysosome acidification has no obvious alteration, and dramatic impairment of lysosome acidification were observed in HG for 24 h (Fig. 3). Treatment with 3MA (a specific inhibitor of autophagy-regulating signaling molecule (PI3K-class III)) restrained lysosomal proteolytic activity, suppressing autophagy machinery and as negative control group (Fig. 3). We hold the opinion that lysosome acidification was retrained, thus aggravating restoring autophagic cargo degradation in HG-Stimulated Müller Cells

Inhibition of autophagy restrained cell proliferation and increased apoptosis in Müller cell exposed to HG

The results Fig. 4A reveal that the Müller cell exposed to HG (3, 6, 12, 24,36,48 h). Meanwhile, a time-dependent decrease in cell viability was observed when the Müller cell were exposed to HG after 24 h, cell viability showed a significant down-regulation. but it did not show significant effect to restrain cell damage in the early stage (before 12 h). As shown in Fig. 4F, the percentage of EdU+ cells was significantly decreased in the HG-exposed cells (24 h) and no significant effect in 6 h, We evaluated the expression of cleaved (active) caspase-3(Figs. 4 B) as a marker of apoptosis, Further increase cleaved (active) caspase-3was observed after HG exposure with 3MA.Additionally, TUNEL assays were performed (Fig.4 G). There was a clear increase cleaved (active) caspase-3 and TUNEL-positive cells in HG for 24 h conditions but 6 h.

To better understand the role of autophagic on Müller cell apoptosis, cells were treated with

autophagy inhibitors, such as 3MA.The former is a well-known inhibitor of the initial phase of the autophagic process. As expected, cells treated with 3MA in HG conditions showed reduced the percentage of EdU+ cells and increased TUNEL-positive cells. Collectively, these data show that Müller cell in HG conditions show increased risk of apoptosis at later period and inhibition of autophagy aggravated the condition

HG regulated autophagic flux by the mTOR-mediated pathway in Müller cells

Increased LC3II indicates an accumulation of autophagosomes, which does not always mean the upregulation of the autophagic flux. Thus. We next used several methods to assess whether HG facilitated autophagic flux at early phase. We compared the generation of LC3-II by HG alone or in combination with chloroquine (CQ), an inhibitor of autophagy. As shown in Fig. 3 A, CQ treatment induced a significant increase in LC3-II levels in the Müller cells treated with HG and an even greater increase in the Müller cells treated with both HG and CQ at 6 h. The analysis of the data by LC3-II turnover assay revealed that HG significantly increased the LC3-II net flux in Müller cells at early phase (Fig. 5 A). However, HG decreases Müller cell autophagy at later period.

We transfected tandem fluorescence RFP-GFP-LC3 adenovirus into cells to demonstrate autophagic flux. In this assay, GFP and RFP tagged to LC3 detects autophagosomes, whereas RFP detects only autolysosomes due to denaturation of GFP in the acidic environment of the autolysosome. Thus, in the merged images, yellow dots represent autophagosomes, and red dots represent autolysosomes. We observed that HG increased both autophagosome (yellow dots) and autolysosome (remaining red dots) formation in merged images at 6 h and decreased at 24 h (Fig. 5B).

To inspect the signaling pathways implicated in autophagy, we evaluated the activation of mTOR as a well-established signaling mediator of autophagy. Moreover, decreased phosphorylation of mTOR levels restrained the blockade of autophagy in HG-treated Müller cells at 6 h. The phosphorylation activities of mTOR were reinforced in HG-treated cells at 24 h (Fig 5C), showing that the mTOR pathway is a possible pathway in HG-mediated regulation of autophagic effects. Rapamycin (RAP, mTOR blocker as an autophagy inducer) was the positive control group.

Inhibition of autophagy facilitates HG -induced NF-κB activation

Given that NF-κB signaling pathway are involved in inflammatory pathway and play an essential role in diabetic retinopathy, our experiment measured HG -activated NF-κB

signaling pathway. As shown in Fig.6.A,Band C, HG decreased p-NF-κB translocation , p-NF-κB and IL-6 at 24h, as compared with 6h. To better investigate the effects of HG on the NF-κB activation nuclear translocation level, we evaluated the expression of NF-κB by western blot analysis, The results in Fig. 6 E revealed that HG treatment for 6 h no induced nuclear translocation of NF-κB, and the intracellular activated NF-κB level increased obviously for 24 h. And treatment of cells with 3MA significantly reduced HG-induced nuclear import of NF-κB ,p-NF-κB and IL-6 in Müller. Taken together, these results demonstrated that the decline in autophagy can stimulate NF-κB signaling and generate chronic inflammation.

Discussion

Diabetic retinopathy has been acknowledged as a microvascular complication; however, more and more studies have noted that neurodegeneration occurs before the onset of the vascular pathology and contributes to the initiation and progression of DR. In the present study, our evidence showed that autophagy in Müller cell (one of the primary glial cells) plays a vital role in DR development.

For the first time, we found that different contributions of autophagy to retinal Müller cell death in the diabetic in in different periods. when activated by HG, Müller cell expressed LC3-labeled dots and increase autophagic flux in early stage. However, HG-induced autophagic was blocked at later period.

No strong evidence for apoptosis was observed in HG early-stage Müller cell. Although one cannot exclude the possibility of undetected short-lasting apoptotic events, functional and morphological data point to a continuous progression of the damage during 6h after HG culture. Conversely, a substantial increase of autophagic activity was shown in diabetic retinas using a variety of tests. Autophagy is generally regarded as a survival mechanism, and its deregulation has been linked to non-apoptotic cell death[39, 40]. Here, we found remarkable increase in Beclin-1 and the ratio of LC3B-II-to-LC3B-I at early stage following induction of HG compared with the controls.

Although little is known of the balance between apoptosis and autophagy in retinal neurons during early phases of DR, a neuroprotective treatment may act by influencing this equilibrium. Our results reveal that activation of autophagy can increases the protective effect of Müller cells under HG condition at early stage. Indeed, there is clear evidence that neurons

possess the machinery for autophagy, while an efficient autophagic function has been found to be positively associated with neuroprotection in different regions of the central nervous system[41, 42 43], including the retina[27, 44]. However, after 24 h, our research shows that the level of autophagic flux decreased significantly, to a subnormal level. At the same time, the number of apoptotic Müller cells under HG condition at later stage. we speculate that in retinal Müller cell under mild stress related to DR, autophagy may confer protection, while under more severe stress it could promote cell death, it coincide with the research of D. Fu[31] in retinal pericytes.

In addition, to further prove the correlation between autophagy and apoptosis, we observed a further increase in the number of apoptotic Müller cells in the group treated with both HG and 3MA.Autophagy is a highly sensitive cellular process induced in response to a wide range of stressful conditions in order to maintain homeostasis. Autophagy occurs in all nucleated type cells. It is a process essential to many cells, including animals, plants, and yeasts[45-47]. Activation of autophagy has been shown to prevent cell death, while inhibition of autophagy can enhance reagent-induced cell death[48, 49]. As shown in many studies, autophagy promotes neuroprotection, whereas inhibiting autophagy reduces retinal degeneration caused by protein misfolding and stimulating autophagy has been reduced photoreceptor death[50], this is consistent with our experimental results. From the present data, we propose that the interplay between autophagy and apoptosis is critical for Müller cells survival. The balance between survival and death depends on the level of stress—minor stress may be countered by autophagy but severe stress leads to cell death. This concept is in concert with the findings of Piano et al regarding effects of early diabetic retinopathy on retinal neural cells[51].

Since an abnormal inflammation could disrupt cellular homeostasis, autophagy contributes to damp inflammatory responses[52]. Our results clearly showed that the autophagic flux, monitored by autophagy-related maker protein (Beclin-1 and LC3II) levels, was enhanced at 6 h, The activation of mTOR in the retina suppressed the NF-κB activation. NF-κB, which comprises the p50/p65 and the inhibitor of κB (IκB) protein, is essential for host defense and also mediates these pro-inflammatory mediators and cytokines expression. We also showed that the NF-κB activation, IL-6 levels as autophagy is inhibited at 24h under HG condition and 3MA. Our data demonstrates that inhibition of autophagy can promote NF-κB activation. Activation of autophagy suppressed NF-κB activation in the early stage, suggesting that the protective effects on the Müller cells during retinal inflammation were, at least partly, due to the suppression of retinal NF-κB activation.

In summary, we hypothesized that differences in autophagy could explain the differences in the retinal cell proliferation and chronic inflammation between early and later times. Therefore, in the retinas Müller cell expose to HG for early stage, activation of autophagy is a protection mechanism may account for the defense against neuroinflammation. It is intriguing what times patient or in vivo model of diabetic retinopathy is able to trigger autophagy as a protective mechanism aimed to preserve retinal structure and visual function. This response deserves further research in the context of neurodegeneration. Based on that, therapies aimed to increase the autophagic flux are promising to prevent the progression of neurodegenerative diseases, particularly those affecting the retina[53]

Figure Legends:

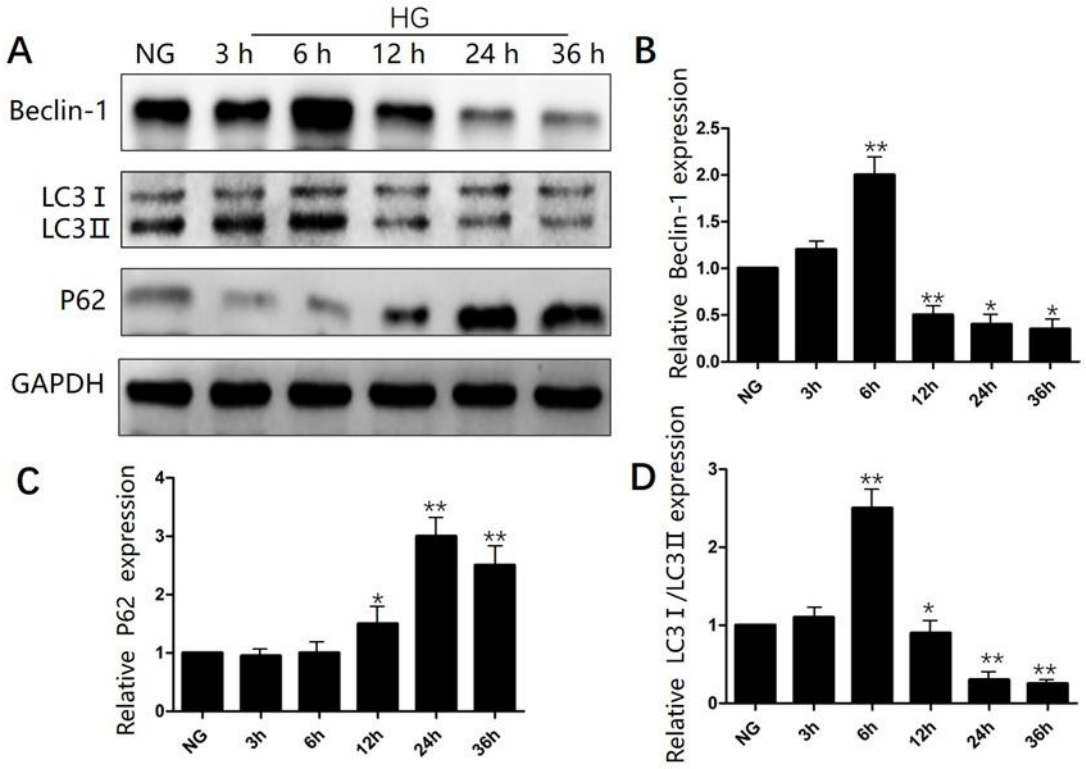

Fig. 1. Autophagy in HG-treated Müller cells. (A) LC3-II/LC3-I, P62 and Beclin-1 expression in NG and HG (3, 6, 12, 24, 36 h)-treated cells; GAPDH was used as an internal standard. (B-D) Quantification of relative protein expression was performed by densitometric analysis and GAPDH was acted as an internal control. Similar results were obtained from three independent experiments. All data are presented as means ± SEM (n = 5/group). *p<0.05, **p<0.01 vs. NG group.

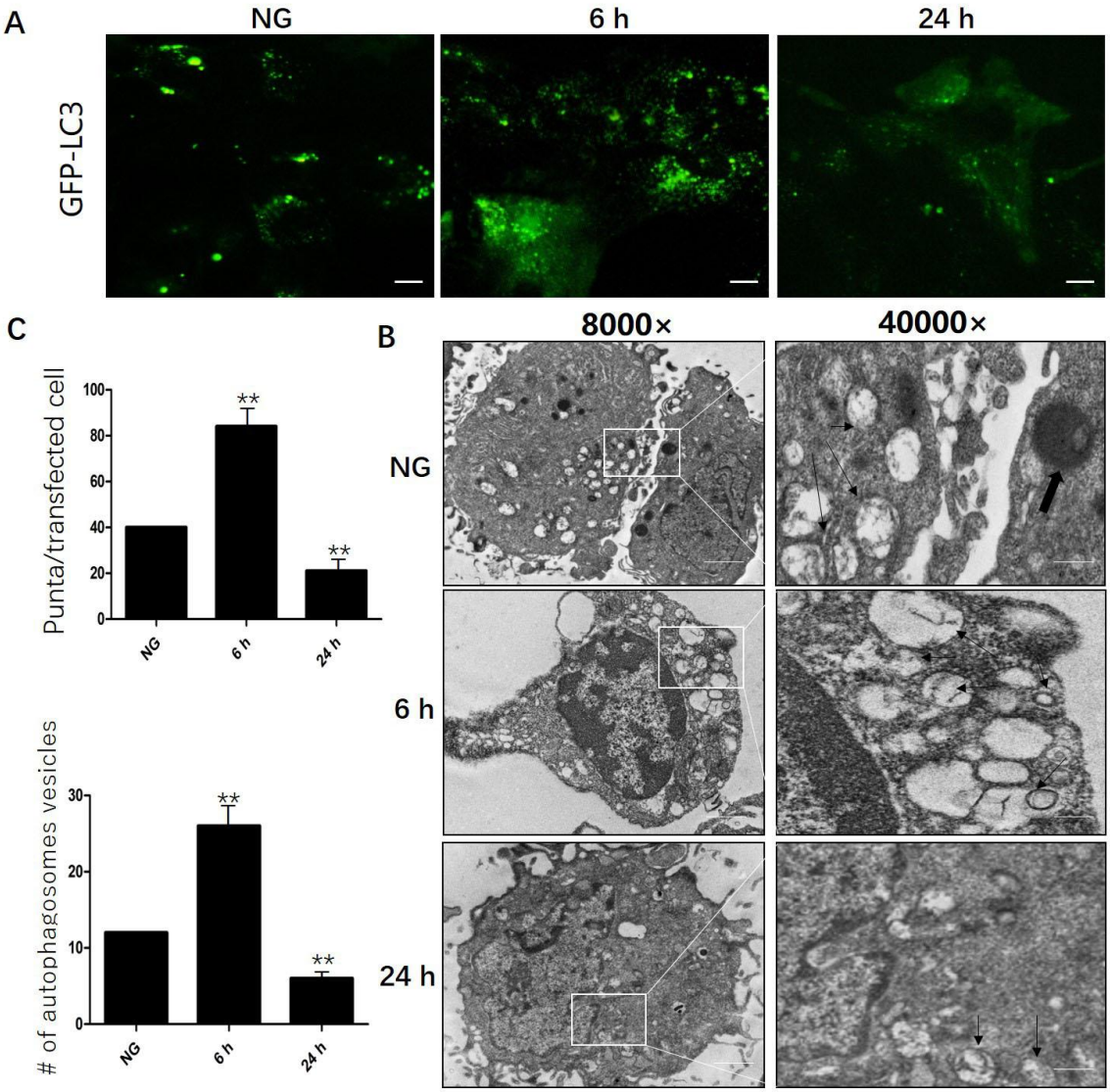

Fig. 2. Analysis of the Autophagy in Müller cells during various time points in HG.(A) A representative image of GFP-LC3 puncta in transfected Müller cells treated with NG and HG (6 and 24 h), Scale bars: 20 μm.(B) Electron micrographs images of the Müller cells. AP (Fine arrow) and electron-dense APL (Coarse arrow). Scale bars: 1 μm (C) Graphs showing numbers of GFP-LC3 puncta and autophagic vesicles. Scoring was done by counting 5 different cells in 5 random fields per condition. The results are expressed as fold changes in NG, *P < 0.05 and **P < 0.01 compared to NG.

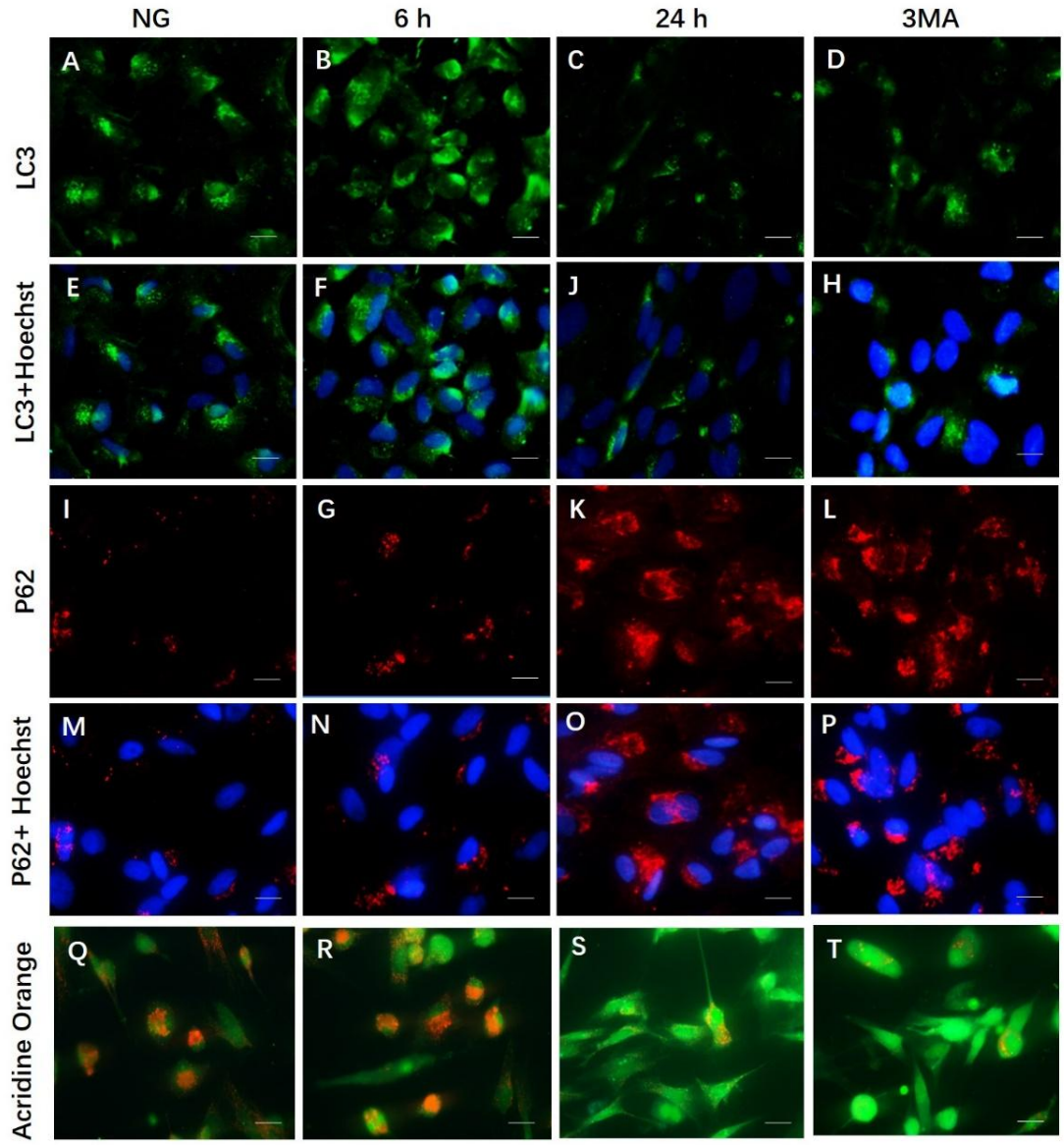

Fig. 3. Inhibition of Autophagy can restrain Lysosome Acidification, Thus Aggravating Restoring Autophagic Cargo Degradation. (A-P) A representative photomicrograph of LC3 and P62 immunofluorescence in Müller cells cultured NG, HG (6 and 24 h) and 3MA. Nuclear staining with LC3 (green), P62 (red), and Hoechst (blue) is shown. (Q-T) Further, acridine orange (AO) staining showed increased acidification in cells Scale bars: 20 μm.

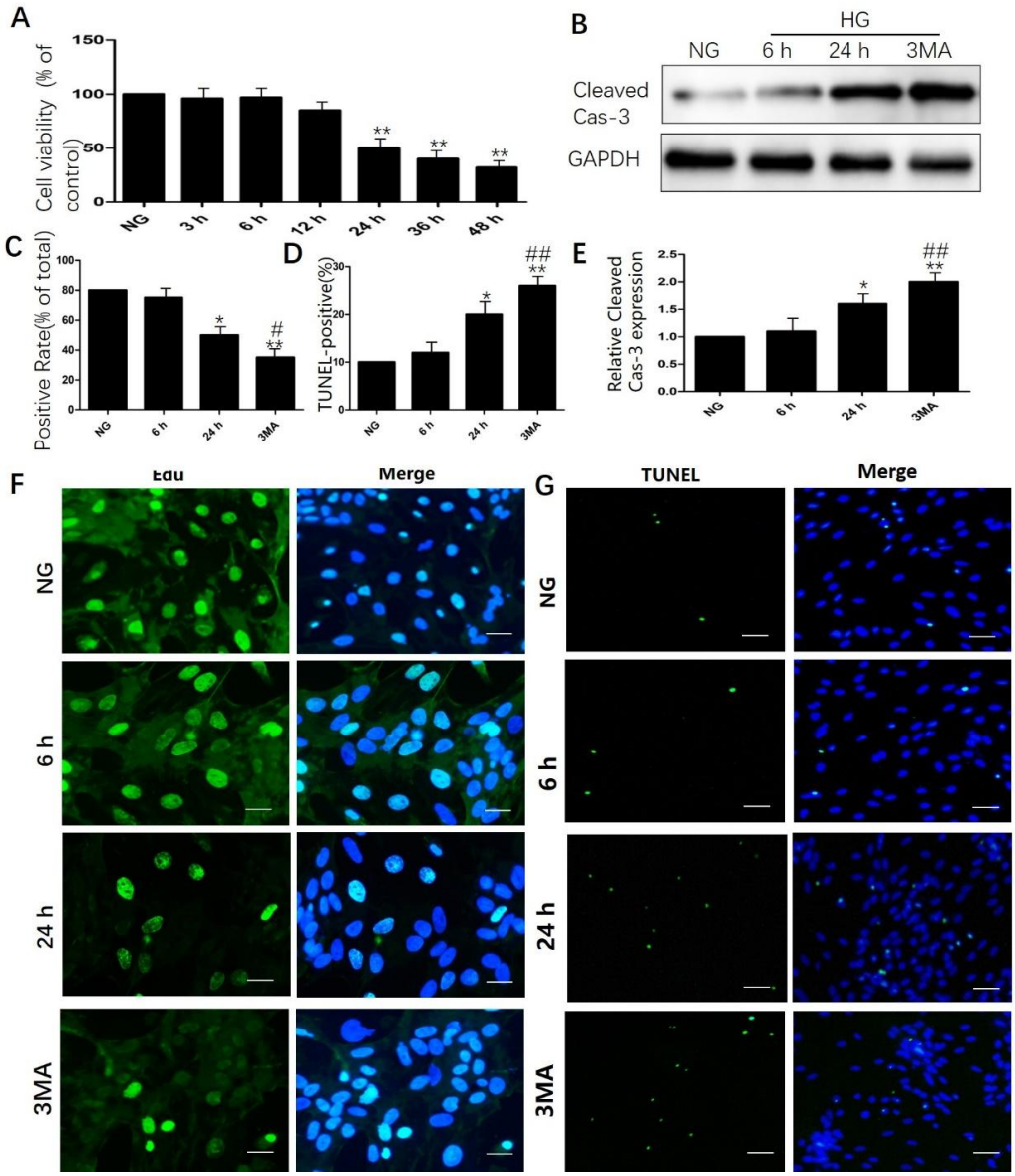

Fig. 4, Inhibition of autophagy restrained cell proliferation and increased apoptosis in Müller cell exposed to HG. (A) Cell viability in Müller cells exposed to NG and HG (3, 6, 12, 24, 36, 48 h) by CCK-8 assay. (B)Cleaved cas-3 expression in NG, HG (6, 24 h)-and 3MA treated cells; GAPDH was used as an internal standard.(C-E) Quantification of positive rate,TUNEL-positive cell and relative Cleaved cas-3 protein expression was performed by densitometric analysis, Ten fields (200 ×) from each group were randomly chosen for statistical analysis: NG, HG, and 3MA. All data are presented as the means ± SDs of at least three independent experiments (F) Images of EdU-labelled cells were detected by fluorescence microscope and treated with NG, HG (6, 24 h)-and 3MA (G) TUNEL assay. The positive cells are shown in green, and Hoechst for nuclear staining is shown in blue. Scale bars: 20 μm. The bars represent the mean ± SD of positive cells corrected by the number of total cells*P < 0.05 and **P < 0.01 compared to NG.

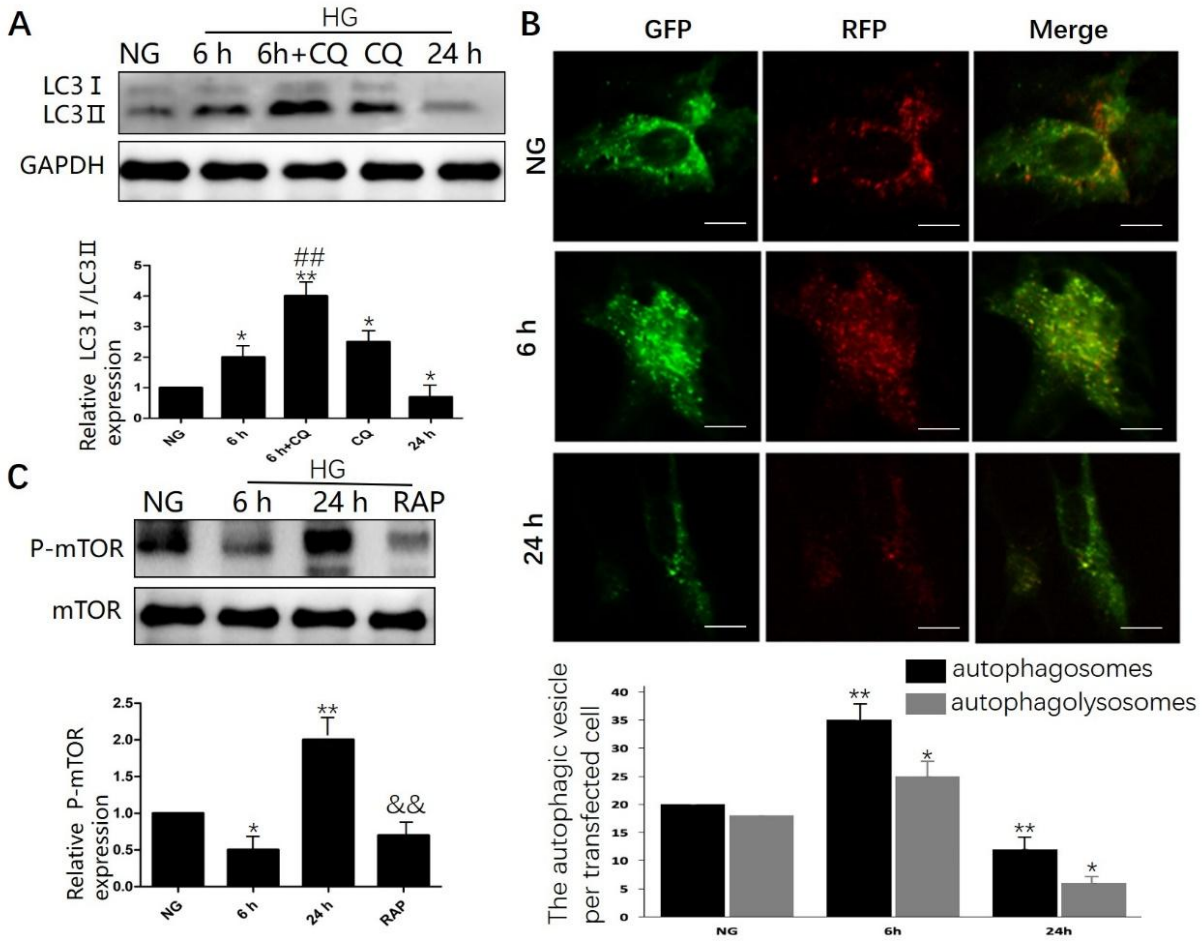

Fig. 5, HG regulated autophagic flux by the mTOR-mediated pathway in Müller cells.

(A) Densitometry analysis showed that LC3 II protein expression was upregulated or reduced in HG (6h, 24h)-induced autophagy, and it accumulated in the presence of chloroquine. (B) Quantification and representative images of autophagosomes (overlapping GFP + RFP puncta generating yellow puncta on overlay) shown as black bars and autolysosomes (RFP puncta) shown as grey bars after 6 and 24 h of HG treatment compared to NG in tandem RFP/GFP-tagged LC3 adenovirus-transfected

Müller cells. Scale bars: 10 μm. Bars represent the means of the respective individual ratios ± SEs. *P < 0.05, **P < 0.01 versus NG; (C) Protein levels of p-mTOR were measured by western blotting (normalized by GAPDH) and quantified by densitometry.

*P < 0.05, **P < 0.01 versus NG; ##P < 0.05 versus HG (6 h), && P < 0.05 versus HG (24h).

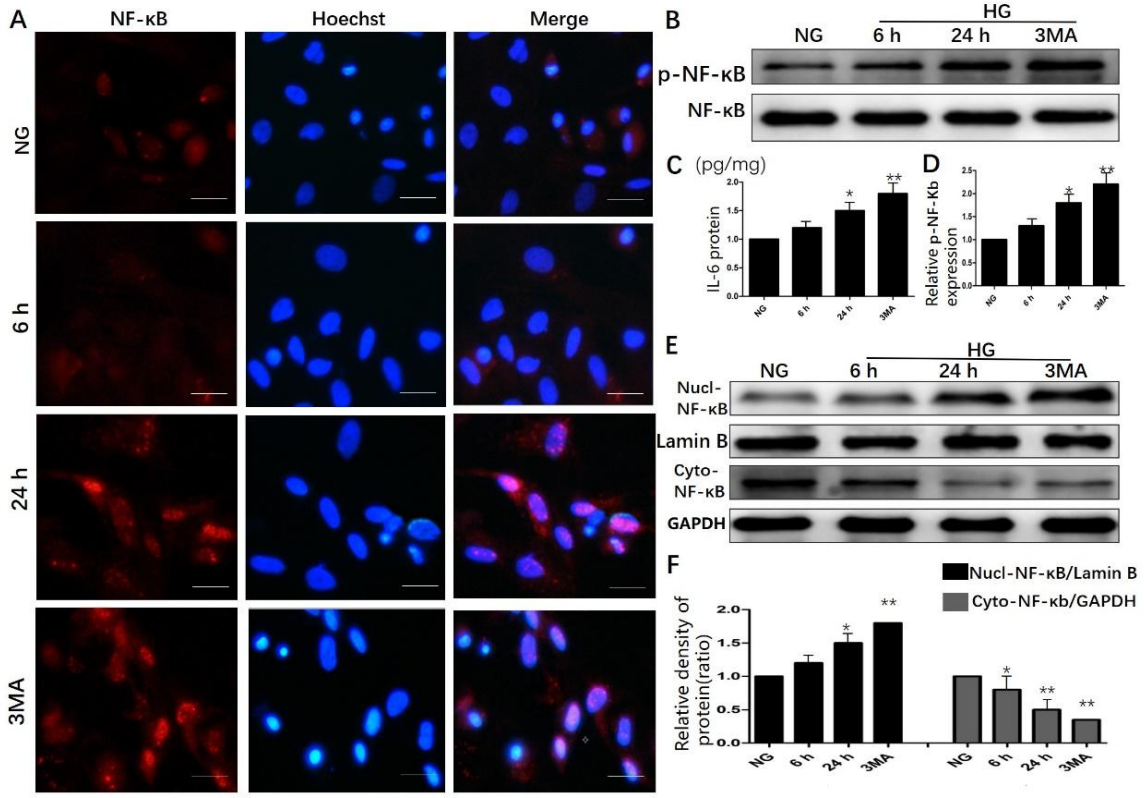

Fig. 6, Inhibition of autophagy facilitates HG -induced NF-κB activation. (A) The photomicrograph of NF-κB activation nuclear translocation immunofluorescence in Müller cells cultured in NG, HG (6 and 24 h) and 3MA. Nuclear staining NF-κB (red), and Hoechst (blue) is shown. Scale bars: 20 μm. (B, C) A representative Western blot and densitometric analysis of p-NF-κB in Müller cells treated with NG, HG (6 and 24 h) and 3MA. Equal loading and transfer for all proteins were ascertained by reprobing the membranes for GAPDH. (D) ELISA assays showed that the protein levels of interleukin-6 (IL-6) were increased in HG (24 h) after the induction of inflammation, and that these changes were attenuated by the 3MA treatmen. (E, F) Effects of NG, HG (6 and 24 h) and 3MA on the nuclear and cytoplasmic levels of NF-κB were examined by Western blotting analysis. Quantification of relative protein expression was performed by densitometric analysis. Similar results were obtained from three independent experiments. All data are presented as means ± SEM (n = 5/group). **p<0.01 vs.NG group; ##p<0.01 vs. HG (24h)

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Data Availability Statements

Due to the nature of this research, participants of this study did not agree for their data to be shared publicly, so supporting data is not available, at this time as the data also forms part of an ongoing study.

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