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Rapamycin Removes Damaged Mitochondria and Protects Human Trabecular Meshwork (TM-1) Cells from Chronic Oxidative Stress

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Abstract

Glaucoma is a chronic optic neuropathy that could lead to permanent vision loss. Primary open-angle glaucoma (POAG) is the most common type of glaucoma, with elevated intraocular pressure (IOP) as a major risk factor. IOP is mainly regulated by trabecular meshwork (TM), an important component of the conventional aqueous humor (AH) outflow pathway. TM cells are constantly subjected to oxidative stress. Long-term exposure to oxidative stress has been shown to cause elevation of AH outflow resistance, leading to higher IOP. In this study, we induced chronic oxidative stress in human trabecular meshwork (TM-1) cells with 1 μM rotenone and investigated the levels of reactive oxygen species (ROS), autophagy, and mitochondrial functions. Protective effects of rapamycin, an inducer of autophagy, were also investigated. Our data indicated that rotenone significantly increased oxidative stress, but not autophagy, in TM-1 cells. Rapamycin at 10 nM effectively suppressed the rotenone-induced cell apoptosis, as well as the ROS elevation. The protective effects of rapamycin could be associated to the induction of autophagy and removal of damaged mitochondria in TM-1 cells. Our results suggest autophagy has important roles in protecting TM-1 cells from oxidative stress, which could be further developed into a novel treatment to POAG.

Keywords Trabecular meshwork · POAG · Oxidative stress · Autophagy

Introduction

Glaucoma is a heterogeneous group of optic neuropathies characterized by progressive degeneration of the optic nerve and could be detected as cupping of nerve head, loss of retinal ganglion cells, and thinning of the retinal nerve fiber layer [1]. In 2010, glaucoma caused 2.1 million (6.5%) in 32.4 million blind individuals worldwide [2]. Primary open-angle glaucoma (POAG) is the most common type of glaucoma. Elevated intraocular pressure (IOP) is a major risk factor for high-tension POAG and lowering IOP is currently the only intervention method that has been proven to be able to delay the progression of the disease [1, 3].

Trabecular meshwork/Schlemm's canal (TM/SC) conventional aqueous humor (AH) outflow pathway plays a key role in the regulation of IOP [4]. The major resistance to AH outflow is generated by the cribriform meshwork layer and the inner wall of endothelial cells of the SC [4]. These trabecular meshwork cells in the TM/SC outflow pathway are constantly subjected to oxidative stress attack generated from physiological cellular metabolism [5]. Oxidative stress represents increased intracellular reactive oxygen species (ROS) levels that cause damage to DNA, proteins, and lipids [6]. The lack of effective antioxidant mechanism in the TM may lead to cell decay, causing both morphological and functional alterations of TM tissues. This scenario subsequently results in increased AH outflow resistance and eventually elevated IOP [4, 5]. Autophagy is emerging as an essential and adaptive cellular survival mechanism against various cellular stress, including oxidative damage [5]. Autophagy can remove damaged protein and organelles like mitochondria and lysosomes to reduce oxidative stress [5]. Previous report showed that acute oxidative stress can initiate autophagosome formation and autophagic degradation in glioma cells [7]. However, the pathological processes of glaucoma are long-term

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chronic procedures instead of acute changes [1]. Indeed, previous study showed that there was no induction of autophagy levels in normal and glaucomatous TM cells under chronic oxidative stress, suggesting the acute and chronic oxidative stress would have different effects on autophagy [8]. These observations led to a hypothesis that inducing autophagy biochemically may relieve chronic oxidative stress in the TM and therefore alleviate the IOP elevation and glaucoma pathology [9, 10]. Currently, the IOP-lowering agents mainly involve reducing production of AH or promoting AH outflow through the unconventional intercellular spaces of the ciliary muscle cells. These agents can only resolve the high IOP temporarily [11]. On the other hand, as the TM/SC conventional AH outflow pathway controls more than 75% of the AH outflow [11], the knowledge about autophagy in response to chronic oxidative stress in TM cells may help to develop new therapeutic strategies to POAG.

In this study, we reported the induction of chronic oxidative stress, oxidative stress levels that showed no detectable elevation of autophagy, in human trabecular meshwork TM-1 cells by using low-dose rotenone as previously reported [12]. Protective effects of rapamycin, an mTOR inhibitor and autophagy inducer, on the chronic oxidative stress–induced TM-1 cell damage were investigated.

Materials and Methods

Cell Culture and Drug Treatments

Primary human trabecular meshwork cells were isolated by Polansky et al. [13]. These primary cells were transfected with a SV40 origin defective vector by Filla et al. to establish the immortalized TM-1 cell line [14]. We followed a consensus recommendation to characterize the TM-1 cells by treating with 500 nM dexamethasone (Sigma-Aldrich, MO, USA) for 7 days and the expression of myocilin was evaluated by Western blot [15]. TM-1 cells were cultured in DMEM medium (Gibco, MD, USA) containing 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco, MD, USA) at 37 °C in an incubator with 5% CO_2 and 95% O_2. Rotenone and rapamycin were dissolved in dimethyl sulfoxide (DMSO). DMSO was used as the solvent control. Rapamycin or DMSO solvent control was co-incubated with rotenone for 24 h. Bafilomycin A1 was also dissolved in DMSO and co-treated with rapamycin and rotenone for 6 h. All reagents were obtained from Sigma-Aldrich Corporation (MO, USA).

Cell Viability

TM-1 cells were seeded in triplicate at 10^4 cells/well in a 96-well plate. Rapamycin or DMSO was treated to cells for 24 h. Five milligrams per milliliter of MTT (Invitrogen, MA, USA) was then incubated with cells at 37 °C for 3 h. The medium was then carefully removed and DMSO was added to each well to dissolve the crystals for 5 min. Absorbance was measured at 570 nm in an ELISA reader (PowerWave™ XS Microplate Reader, BioTek, VT, USA).

Western Blot

Cells were lysed in buffer containing 62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, and 0.01% bromophenol blue. Protein concentration was determined using BCA Protein Assay Kit (Bio-Rad, USA). One hundred micrograms of total protein was analyzed on a 15% SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, IL, USA). The membrane was probed with indicated antibodies (MAP LC3β, 1:200, Santa Cruz sc-271,625, TX, USA; Myocilin, 1:1000, Imgenex, CO, USA; p62, 1:2500, Abcam ab56416, MA, USA; GAPDH, 1:2000, Sigma G9295, MO, USA). Western blot membrane was developed with enhanced chemiluminescence detection system (Thermo Fisher, MA, USA). Images were analyzed by ImageJ software. Relative expression levels of LC3 II/I bands were determined by densitometry. LC3 II/I ratios were normalized with the untreated cells.

Detection of Intracellular ROS Level

Intracellular ROS was determined by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Thermo Fisher, MA, USA). After 24-h exposure to rotenone with or without rapamycin, cells were collected and incubated with 4 μM H_2DCF-DA at 37 °C for 30 min in the cell culture incubator. Flow cytometry was used to quantify the fluorescence signals. Fluorescence signals were normalized with the signals from the untreated cells.

Apoptosis Assay

Cells were harvested by trypsin and fixed in 70% ethanol and kept at − 20 °C overnight. Cells were then resuspended in phosphate-buffered saline (PBS) containing 0.01% Triton and incubated on ice for 10 min. Cells were then stained with 40 μg/mL propidium iodide (Sigma-Aldrich, MO, USA) with 100 μg/mL RNaseA 30 min at 37 °C. Flow cytometry was used to quantify sub-G1 cells.

Measurement of Mitochondrial Membrane Potential

To measure damaged mitochondria in live cells, MitoTracker™ Red CM-H2Xros (Thermo Fisher, MA, USA) was used. Cells were harvested by trypsin and incubated with 300 nM MitoTracker at 37 °C for 30 min in the cell culture incubator. After washing with PBS, flow cytometry was used to measure the fluorescence signals. Percentages of

cells with damaged mitochondria were normalized with the untreated cells.

Statistics

At least 50,000 cells were counted in flow cytometry. All data were expressed as mean ± SEM from at least three independent experiments. Statistical analyses were performed with the Mann-Whitney test using SPSS software (IBM, version 24.0). Differences were considered statistically significant at a p value of less than 0.05.

Results

Rapamycin Alleviated the Rotenone-Induced Oxidative Stress in Rotenone-Treated TM-1 Cells

TM-1 cells can be characterized by treating dexamethasone to induce the expression of myocilin [15]. The Western blot result showed the induction of myocilin after treating 500 nM dexamethasone for 7 days, which confirmed the identity of human trabecular meshwork cells (Fig. 1). We followed a published protocol to induce chronic oxidative stress by treating TM-1 cells with 1 μM rotenone for 24 h [12]. To investigate the protective effects of rapamycin, various doses of rapamycin (0, 2.5, 5, 10, and 20 nM) were co-treated with 1 μM rotenone for 24 h. The oxidative stress levels in TM-1 cells were then quantified by using the H_2DCF-DA method. The oxidative stress levels were reduced with increasing concentrations of rapamycin (Fig. 2a).

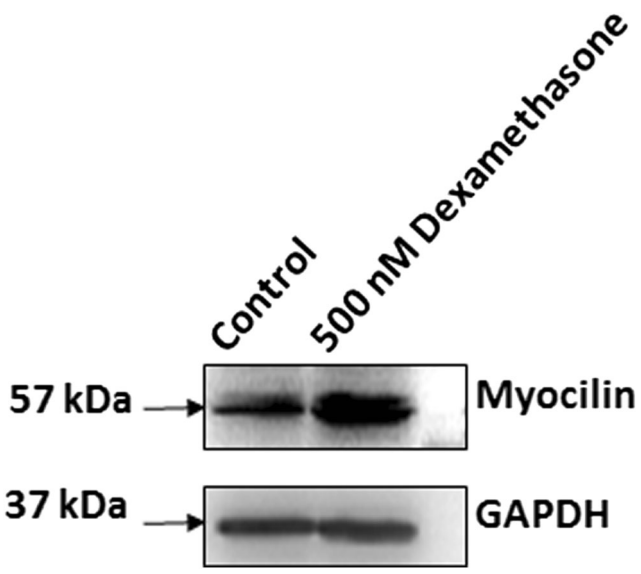

Fig. 1 Dexamethasone treatment induced myocilin expression in TM-1 cells. TM-1 cells were treated with 500 nM dexamethasone for 7 days. Myocilin level was analyzed by Western blot. GAPDH was served as a loading control

There was a significant difference in ROS levels between 5- and 10-nM rapamycin treatments (Fig. 2a). No further significant reduction of oxidative stress was found at 20 nM rapamycin (Fig. 2a). Therefore, we treated TM-1 cells with 5 nM and 10 nM rapamycin in our subsequent experiments. Intracellular ROS in TM-1 cells, as indicated by the fluorescence of H_2DCF-DA, was significantly elevated after 24 h of exposure to 1 μM rotenone (1.26-fold induction compared with the solvent control–treated cells, $p < 0.05$) (Fig. 2b). When TM-1 cells were co-treated with rotenone and 5 and 10 nM rapamycin, there was a significant reduction in the intracellular oxidative stress comparing with the 1-μM rotenone–treated cells (Fig. 2b).

In cells without rotenone treatment, cells treated only with rapamycin (5 nM and 10 nM) also showed significantly decreased intracellular ROS compared with solvent control–treated cells (0.71- and 0.65-fold of the control, respectively; $p < 0.05$ in both cases) (Fig. 2b).

Furthermore, there was no significant difference in oxidative stress levels between solvent control–treated cells and cells co-treated with 1 μM rotenone and 10 nM rapamycin, which indicated the oxidative stress–inducing effect could be fully suppressed by 10 nM rapamycin (Fig. 2b).

We also studied the impact of rapamycin on cell viability. According to cell viability results, there was no significant difference between DMSO and both the 5-nM and 10-nM rapamycin–treated groups (Fig. 2c).

Rapamycin Upregulated Autophagy both Under Control-Treated and Rotenone-Treated Conditions in TM-1 Cells

To validate the induction of autophagy by rapamycin, TM-1 cells were treated with or without 1 μM rotenone and co-treated with 5 and 10 nM rapamycin for 6 h. In TM-1 cells with and without rotenone treatment, the microtubule-associated protein light chain 3 isoforms II and I (LC3 II/I) ratio was increased significantly in both the 5- and 10-nM rapamycin–treated cells (Fig. 3a, b). But there were no significant differences of the LC3 II/I ratio between DMSO and rotenone treatment groups with or without bafilomycin A1 treatment (Fig. 3a, c). These results were similar to the findings reported by Porter et al. that no significant induction of autophagy in TM cells isolated from glaucomatous and age-matched donor eyes after treating chronic oxidative stress of 40% O_2 and 5% CO_2 for 2 weeks [8]. To further confirm the induction in autophagosome formation and autophagic flux, cells were co-treated with 10 nM bafilomycin A1 for 6 h. Bafilomycin A1 is an inhibitor of autophagosome-lysosome fusion. The ratio of LC3 II/I (Fig. 3a, d) and the p62 level (Fig. 3e, f) [16] increased significantly in the presence of bafilomycin A1, which indicated that rapamycin could induce autophagic flux and not simply by the accumulation of

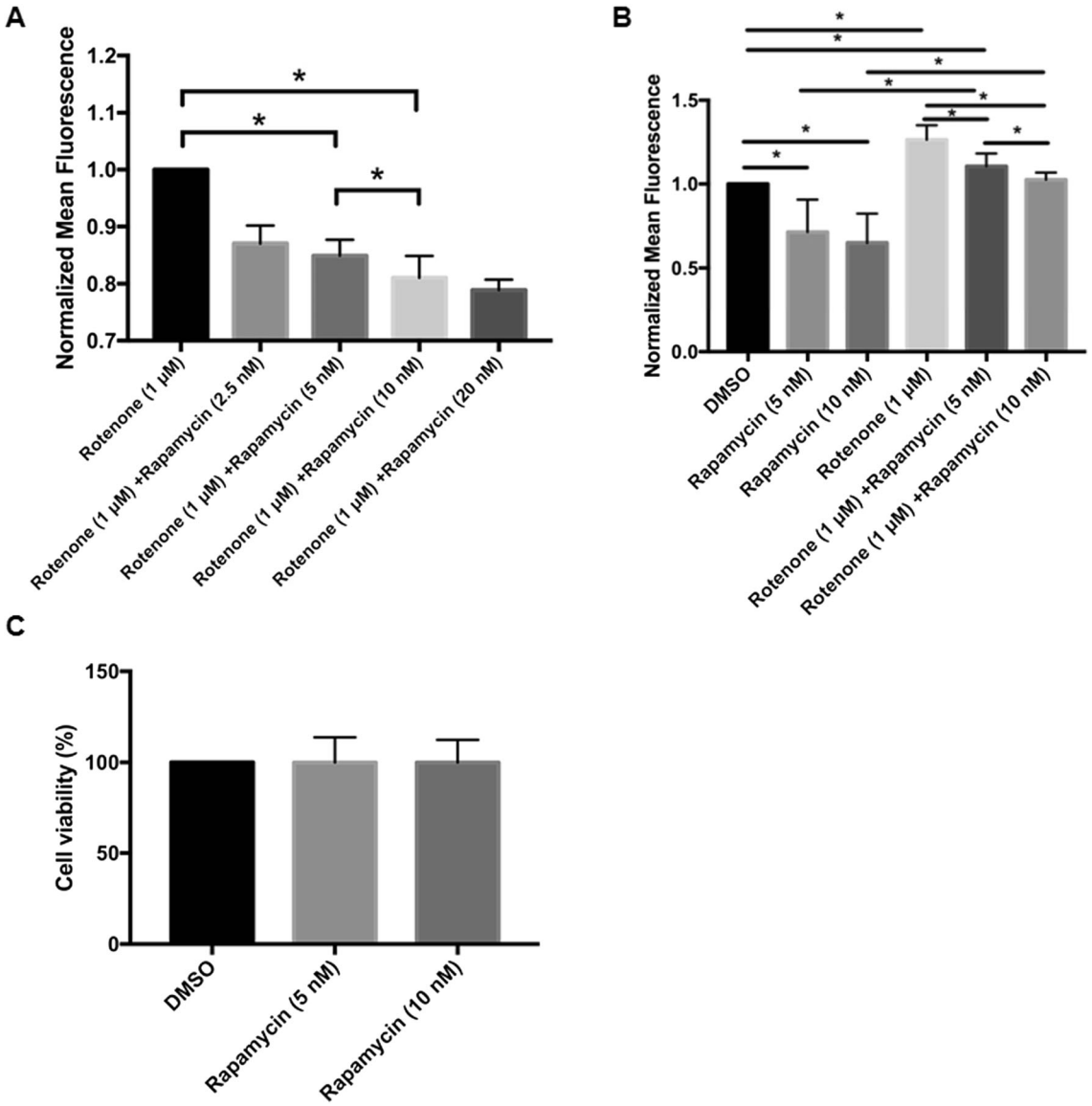

Fig. 2 Rapamycin reduced chronic oxidative stress in rotenone-treated TM-1 cells. **a** TM-1 cells were treated with 1 μM rotenone for 24 h. Different dosages (0, 2.5, 5, 10, and 20 nM) of rapamycin was co-treated with rotenone to TM-1 cells. Mean fluorescence was normalized with the 1-μM rotenone–treated group to represent oxidative stress level. **b** TM-1 cells were treated with or without 1 μM rotenone and co-treated with 5 and 10 nM rapamycin for 24 h. Mean fluorescence was normalized with the DMSO-treated group to represent oxidative stress level. **c** TM-1 cells were treated with DMSO or rapamycin (5 and 10 nM). Cell viability was determined by MTT assay. Mean fluorescence was normalized with the DMSO treated group to represent cell viability. Mean ± standard deviation of three independent experiments were shown. *$p < 0.05$

autophagosome in both untreated and rotenone-treated TM-1 cells.

Rapamycin Reduced Apoptosis in Rotenone-Treated TM-1 Cells

Oxidative stress has been widely reported to induce apoptosis [17]. Therefore, we investigated the roles of rapamycin in regulating apoptosis. The apoptosis levels were similar between solvent control-treated cells and the 5-nM and 10-nM rapamycin–treated cells (Fig. 4). A 24-h exposure to rotenone significantly elevated the apoptosis level in TM-1 cells (Fig. 4). Rapamycin at 5 nM and 10 nM significantly reduced the rotenone-induced apoptosis. However, the amount of apoptotic cells was still higher than those in solvent control–treated cells (Fig. 4). These results indicated that rapamycin could reduce apoptosis in rotenone-treated TM-1 cells.

Rapamycin Cleared Up Damaged Mitochondria in Rotenone-Treated TM-1 Cells

Our data demonstrated that rapamycin could induce autophagy, as well as reducing oxidative stress and apoptosis. Recent studies suggested autophagy could remove intracellular-

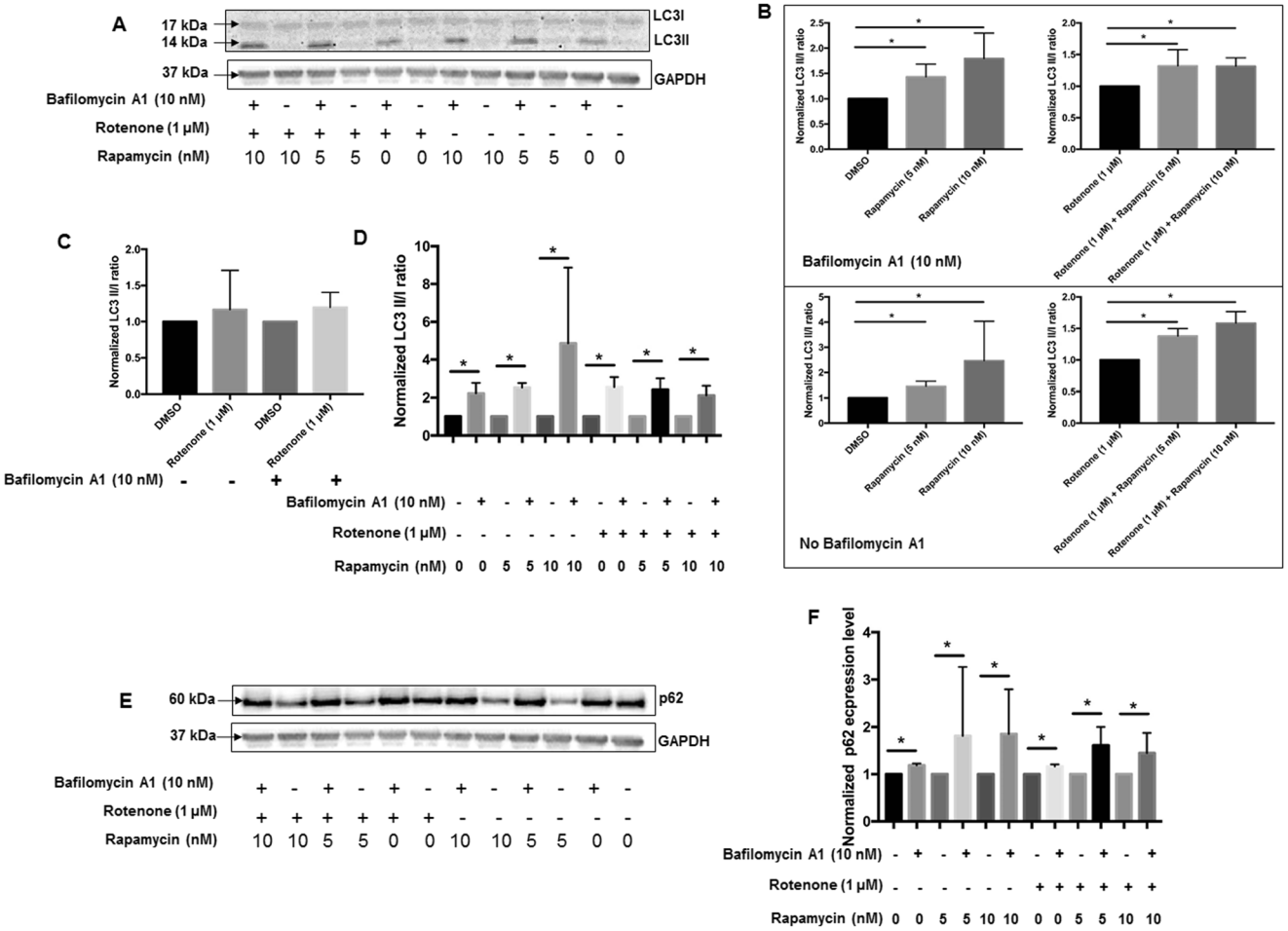

Fig. 3 Rapamycin increased autophagy level. **a** TM-1 cells were treated with or without 1 μM rotenone and 10 nM bafilomycin A1 and co-treated with 5 and 10 nM rapamycin for 6 h. LC3 levels were analyzed by Western blot. GAPDH was served as a loading control. **b**–**d** The autophagy levels were quantified by measuring the normalized ratio of LC3 II and I. The ratios in **b** were normalized with DMSO or rotenone treatment group, respectively. In **c**, the ratios were normalized with DMSO treatment group. In **d**, the ratios were normalized with the corresponding groups without bafilomycin A1 treatment. **e** p62 levels were analyzed by Western blot. GAPDH was served as a loading control. **f** The autophagy levels were quantified by measuring the normalized ratio of p62. The ratios were normalized with the corresponding groups without bafilomycin A1 treatment. Mean ± standard deviation of three independent experiments were analyzed by using *t* test. $^{*}p < 0.05$

damaged mitochondria to reduce oxidative stress [18]. To quantify the number of damaged mitochondria, we used MitoTracker to measure the mitochondrial membrane potential. When mitochondria are damaged, the mitochondrial membrane would be disrupted that leads to leakage, which causes reduction of the membrane potential, resulting in a reduction of the MitoTracker fluorescence signal. The percentage of cells carrying damaged mitochondria was similar between solvent control–treated cells and cells treated with 5 nM or 10 nM rapamycin (Fig. 5). After rotenone treatment in TM-1 cells, the percentage of cells carrying damaged mitochondria significantly increased compared with the solvent control–treated cells (Fig. 5). The 5-nM and 10-nM rapamycin treatments could significantly reduce the percentage of cells carrying damaged mitochondria in rotenone-treated TM-1 cells (Fig. 5).

Discussion

While most complex lives on Earth requires oxygen for their existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS) [19]. The ROS generated in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion ($O2^{-}$) [20]. Hydrogen peroxide (H_2O_2) is a short-lived product in biochemical processes and is toxic to cells [21]. The toxicity is due to oxidation damage to cellular components like proteins, membrane lipids, and DNA by the peroxide ions [21]. H_2O_2 has been used to induce oxidative stress in some disease models as oxidative stress is well established as an important cause of cellular damage associated with the initiation and progression of many diseases [21]. However, H_2O_2 is an unstable chemical compound and could be slowly decomposed under

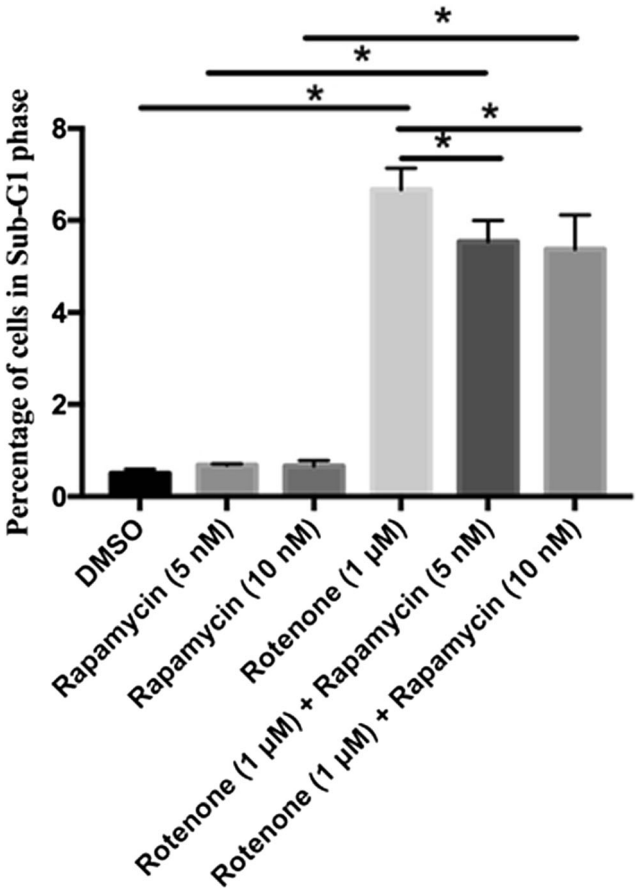

Fig. 4 Rapamycin decreased apoptosis induced by chronic oxidative stress. TM-1 cells were treated with or without 1 μM rotenone and co-treated with 5 and 10 nM rapamycin for 24 h. Cells were then stained with propidium iodide and sub-G1 cells were quantified by flow cytometry. Mean ± standard deviation of three independent experiments were analyzed by using *t* test. $*p < 0.05$

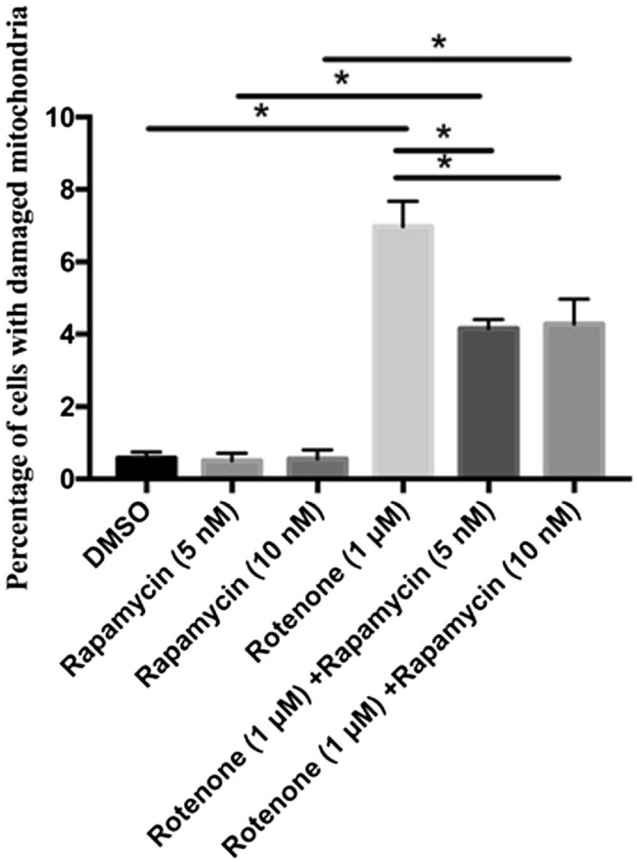

Fig. 5 Rapamycin cleared out damaged mitochondria induced by chronic oxidative stress. TM-1 cells were treated with or without 1 μM rotenone and co-treated with 5 and 10 nM rapamycin for 24 h. Cells were then stained with Mito-Tracker and analyzed with flow cytometry. Mean ± standard deviation of three independent experiments were analyzed by using *t* test. $*p < 0.05$

physiological conditions. To obtain consistent experimental results, we induced chronic oxidative stress by using rotenone. Rotenone, a common pesticide, is an inhibitor of the mitochondrial electron transport chain complex I, which leads to the generation of ROS [22]. Rotenone has been widely used to establish high oxidative stress animal models to study neurodegeneration diseases such as Parkinson's disease [23, 24]. It has been reported that a mitochondrial complex I defect is associated with the degeneration of trabecular meshwork cells in POAG patients [25]. Recently, rotenone has been used to induce senescence in a human trabecular meshwork cells to mimic a glaucomatous condition [12]. In this current study, we employ this rotenone-treated TM-1 cells to study the protective effects of rapamycin. This immortalized TM-1 cell line has been employed in other studies investigating the proteome, mitochondrial function, cytoskeleton, and responses to dexamethasone [26–29]. And the TM-1 cells showed elevated myocilin expression after dexamethasone treatment, which is comparable with the primary human trabecular meshwork cells [15]. Future studies could be done to explore the protective effects of rapamycin in rotenone-treated primary human trabecular meshwork cells isolated from normal and POAG patients.

To resolve oxidative damage, organisms possess a complex network of antioxidant metabolites and enzymes [30]. These antioxidant metabolites and enzymes could prevent the generation of ROS or remove the ROS to avoid damages in vital cellular components [30]. However, our study showed that chronic oxidative stress alone could not induce autophagy. Instead, rapamycin treatment could significantly induce autophagy and suppress oxidative stress and apoptosis, probably by removing damaged mitochondria. It has been reported that rapamycin could protect human corneal endothelial cells from oxidative-induced cell death by lowering ROS, improving redox status, and attenuating oxidative stress in rats [31, 32]. Rapamycin was isolated from the mycelium of the *Streptomyces hygroscopicus* from the soil of Easter Island, also called Rapa Nui [33]. Subsequent studies found the protein target inhibited by rapamycin. It was named mTOR, mammalian target of rapamycin [34]. There are two complexes of mTOR, mTOR complex 1 (mTORC1) and mTOR complex

2 (mTORC2). It has been reported that mTOR inhibits autophagy [35–37]. Hence, rapamycin is able to inhibit mTOR to activate autophagy.

Mutations in the autophagy pathway have been reported to associate with POAG. In 2002, Sarfarazi et al. identified mutations in the *FIP-2* gene as causal mutations for some of the POAG [38]. This gene was later renamed as *Optineurin* (*OPTN*) [38]. Further investigation showed that OPTN is involved in autophagy [39]. There is an LC3-interacting region (LIR) and an ubiquitin-binding domain (UBD) in OPTN, which could guide the transportation of ubiquitylated cargos into autophagosome, a specialized organelle to degrade targeted cargos. There is a specific form of autophagy called mitophagy, which eliminates damaged mitochondria [40]. Our results indicate rapamycin could induce mitophagy to reduce oxidative stress and apoptosis in rotenone-treated TM-1 cells. It would be informative to study the responses to chronic oxidative stress in TM-1 cells carrying POAG-related OPTN mutations.

Conclusions

In summary, we have shown rotenone could induce chronic oxidative stress in TM-1 cells. Rapamycin could successfully induce autophagic flux and remove damaged mitochondria. Our findings indicate rapamycin could be developed into a novel treatment to protect the trabecular meshwork and the Schlemm's canal from oxidative stress, which in turn maintain the AH outflow to lower IOP. Further studies of testing rapamycin in in vivo animal models are needed to investigate the effects of rapamycin in treating POAG.

Author Contributions S.D.Z., J.N.H., C.P.P. and W.K.C. designed research; J.N.H. and W.K.C. performed research; All authors analyzed data; J.N.H., S.D.Z., C.P.P. and W.K.C. wrote the manuscript; and C.P.P. and W.K.C. supervised the project.

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