Oxidative Stress Suppresses Cellular Autophagy in Corneal Epithelium

Yuanyuan Yin,[1–3] Rongrong Zong,[1–3] Xiaorui Bao,[1–3] Xiling Zheng,[1,3–5] Huixia Cui,[1–3] Zuguo Liu,[1–5] and Yueping Zhou[1–3]

[1]Eye Institute of Xiamen University, Xiamen, Fujian, China
[2]Medical College of Xiamen University, Xiamen, Fujian, China
[3]Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Xiamen, Fujian, China
[4]Department of Ophthalmology, The Affiliated Xiang'an Hospital of Xiamen University, Xiamen, Fujian, China
[5]Xiamen University Affiliated Xiamen Eye Center, Xiamen, Fujian, China

Correspondence: Yueping Zhou, Eye Institute of Xiamen University, 168 Daxue Road, Xiamen, Fujian 361005, China;
ypzhou@yahoo.com.

YY and RZ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: February 08, 2018
Accepted: May 20, 2018

Citation: Yin Y, Zong R, Bao X, et al. Oxidative stress suppresses cellular autophagy in corneal epithelium. *Invest Ophthalmol Vis Sci*. 2018;59:3286–3293. https://doi.org/10.1167/iovs.18-24057

Purpose. Oxidative stress is a major pathogenesis of certain ocular surface diseases. This study investigated the association of oxidative stress and cellular autophagy in corneal epithelium.

Methods. We applied hydrogen peroxide (H_2O_2) to induce oxidative damage to cultured human corneal epithelial (HCE) cells and rat corneas. Cell viability, Western blotting of caspase 8, and TUNEL staining were conducted to measure the cellular injury. The production of reactive oxygen species (ROS) was measured and the levels of the following marker and key factors of ROS were also measured to detect oxidative stress: 3-nitrotyrosine, nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), superoxide dismutase, catalase, and glutathione S-transferase P. The following key factors of autophagy were measured: LC3, beclin 1, Atg 12, and P62. We also applied an agonist of autophagy, rapamycin, in the experiment.

Results. Cellular injury and oxidant damage were induced after exposure to H_2O_2 in HCE cells and rat corneas, such as increases of cell death and production of ROS; upregulation of a ROS generation enzyme, NOX4; and downregulation of degradation factors of ROS, superoxide dismutase, catalase, and glutathione S-transferase P. However, the process of cellular autophagy was suppressed by the measurements of LC3, beclin 1, Atg 12, and P62. Furthermore, application of rapamycin antagonized the cellular and oxidant injury induced by H_2O_2 but increased the level of autophagy in HCE cells.

Conclusions. The oxidative stress of corneal epithelium is associated with the inhibition of cellular autophagy.

Keywords: ROS, autophagy, cornea, rapamycin

It is known that oxidative stress contributes to the causes of some corneal and ocular surface diseases, such as keratoconus, Fuchs' endothelial corneal dystrophy, pterygium, and dry eyes.[1–3] Better illustration and further investigation about the roles and mechanisms of oxidative stress in corneal and ocular surface diseases is required. Recently, this topic has attracted a lot of attention to further understand the association between oxidative stress and other pathologic mechanisms, such as apoptosis, inflammation, and angiogenesis in the field of corneal research.[4,5]

The excessive production of reactive oxygen species (ROS) or an imbalance of ROS can cause oxidative stress or induce oxidative injury or damage in the human body. ROS can induce cellular injury by targeting DNA, protein, and cytolipin.[6–8] The ROS system is very complicated and can be divided into ROS generation and ROS degradation.[9,10] Among the key factors of the ROS system, nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) is a major generation enzyme,[11–13] whereas the ROS degradation factors include superoxide dismutase (SOD), catalase, and glutathione S-transferase P (GSTP).[14,15]

Autophagy is a major cellular mechanism that recycles and degrades cell components to maintain normal cellular function and metabolism.[16–18] Autophagy is a complex cellular process and can be divided into three major forms: macroautophagy, microautophagy, and chaperone-mediated autophagy. There are several key factors that play important roles in the process of cellular autophagy, for example, LC3, bechlin1, Atg, and P62.[19–23] Among these key factors, LC3 is a major autophagy marker and P62 is known as a linker molecule between ubiquitinated proteins and the autophagy system and plays a major role in the clearance of autophagy.[20,21] Further investigations are needed about the roles of autophagy in the pathogenesis of corneal and ocular surface diseases, which are largely unknown.

The association or crosstalk between ROS and autophagy is not completely understood. Little evidence is reported about the association between ROS and autophagy in corneal diseases or ocular surface diseases.[24]

In this investigation, we performed an oxidant injury setting in corneal epithelium both in cultured human corneal epithelial (HCE) cells and in corneal epithelium of rats to elucidate the association between oxidative stress and cellular autophagy.

Materials and Methods

Materials

The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Company (Tokyo, Japan). A TUNEL assay kit was purchased from Beyotime Biotechnology (Haimen, China). The following antibodies were purchased from Abcam (Cambridge, UK): anti-3-nitrotyrosine (3-NT), anti–NOX4, anti-SOD2, anti-catalase, anti-LC3, anti-beclin1, and anti-P62. The antibody of anti-Atg12 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody of anti-GSTP was purchased from Santa Cruz (Cambridge, MA, USA). The antibody specific for β-actin was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and HRP-conjugated rabbit were purchased from Sigma-Aldrich Corp. AlexaFluor488 donkey anti-mouse IgG was purchased from Invitrogen (Carlsbad, CA, USA).

Cell Culture

We obtained simian virus (SV) 40 immortalized HCE cells from RIKEN BioResource Center (Tokyo, Japan) and passaged HCE cells in Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM-F12 basic) media (Invitrogen, Carlsbad, CA, USA) supplemented with recombinant human epidermal growth factor (10 ng/mL), 1% penicillin and streptomycin, and 6% heat-inactivated fetal bovine serum.

TUNEL fluorescent staining was conducted by following the protocol of the manufacturer.

The procedure of the cell viability assay was as follows. The HCE cells were cultured at a density of 8,000 to 10,000 cells per well in 96-well culture plates. When the HCE cells reached 70% confluency, the media was removed and changed to DMEM-F12 basic media (serum free), containing a specific concentration of H_2O_2, and then cultured for different lengths of time (0, 2, 4, 8, 12, and 24 hours). For all other experiments, the HCE cells were cultured at a density of 30×10^4 cells per well in 6-well plate or 10×10^4 cells per well in 12-well culture plates, and then cultured in specific concentrations of H_2O_2 for different time, followed by the CCK-8 assay, immunofluorescent staining, and Western blot, as instructed by the methods and procedures described below.

In the rapamycin interference experiment, HCE cells were treated with H_2O_2 at a concentration of 0.25 mM. Simultaneously, rapamycin at concentrations of 0.5 μM and 1 μM were given to the treated groups.

Cell Viability Assay

We detected cell viability with CCK-8 as instructed by the protocol from the manufacturer. After HCE cells were cultured for different time in the conditional media, then the conditional media was replaced by CCK-8 constituted in culture media and incubated for 4 hours at 37°C in the dark. After incubation, the cell viability of the solution was measured. We detected the absorbance at 450 nm spectrophotometrically with a Bio Tek ELX800 microplate reader (Bio Tek Instruments, Winooski, VT, USA).

ROS Assay

The production of ROS in HCE cells was measured following the protocol of the manufacturer. After the treatment of the conditioned medium containing H_2O_2 or rapamycin, the HCE cells were washed with PBS once, and then were incubated with 2′,7′-dichlorofluorescein diacetate (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 minutes. Dichlorofluorescein fluorescence distribution of 1×10^4 cells was ultimately detected by flow cytometry at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

In Vivo Experimental Procedures

We obtained Wistar rats (male, aged 4–6 weeks, 180–220 g) from Shanghai Shilaike Laboratory Animal Co., Ltd., Shanghai, China. We conducted the animal experiments carefully following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experimental protocol was approved by the Animal Ethics Committee of Xiamen University School of Medicine (approval ID, 2016-12-10). The animals were maintained in an air-conditioned facility. We randomly divided the rats into two groups ($n = 6$): (1) control group without any treatment (saline containing same amount of vehicle) and (2) H_2O_2 treatment group.

We anesthetized rats with pentobarbital (50 mg/kg intraperitoneally), then administered 10 μL saline or H_2O_2 (0.5 mM) topically. After 0 hours and 4 hours, the rats were killed, and then removal and dissection of the eyeballs or cornea followed. The method for the dissection of corneal tissue for Western blot was previously published.[25,26] The whole corneal tissue was carefully dissected immediately under a surgical microscope by an experienced person.

Western Blot Analysis

We lysed the collected HCE cells or corneal tissues and measured total cellular protein concentrations by a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein were resolved by electrophoresis through 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 1% (wt/vol) BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies of anti-3NT (1:200), NOX4 (1:1000), anti-SOD2 (1:5000), anti-GSTP (1:1000), anti-catalase (1:2000), anti-LC3 (1:1000), anti-beclin1 (1:1000), anti-Atg12 (1:1000), and anti-P62 (1:1000). After washing three times with TBST, the membranes were incubated with a 1:10,000 dilution of an HRP-conjugated IgG antibody in TBST containing 1% BSA for 1 hour. After another three washes with TBST, the bands were detected by a commercial imaging system (Molecular Imager ChemiDoc XRS; Bio-Rad Laboratories, Hercules, CA, USA). As needed, the membrane was stripped for 30 minutes in stripping buffer (CWBio, Beijing, China) and reblotted with an antibody specific for β-actin for loading control. We semiquantified the band intensities by using densitometry with analytical software (Quantity-One; Bio-Rad Laboratories).

Statistical Analysis

We analyzed the data from the CCK-8 assay, ROS assay, and Western blotting with the 1-way ANOVA test and performed a post hoc analysis Tukey test to compare the differences between the groups or a Student's *t*-test. A value of P less than 0.05 was considered significant statistically.

Results

Hydrogen Peroxide-Induced Cellular and Oxidant Injury

Hydrogen Peroxide (H_2O_2) is widely applied and reported as a typical experimental tool to induce oxidative stress.[27] We first

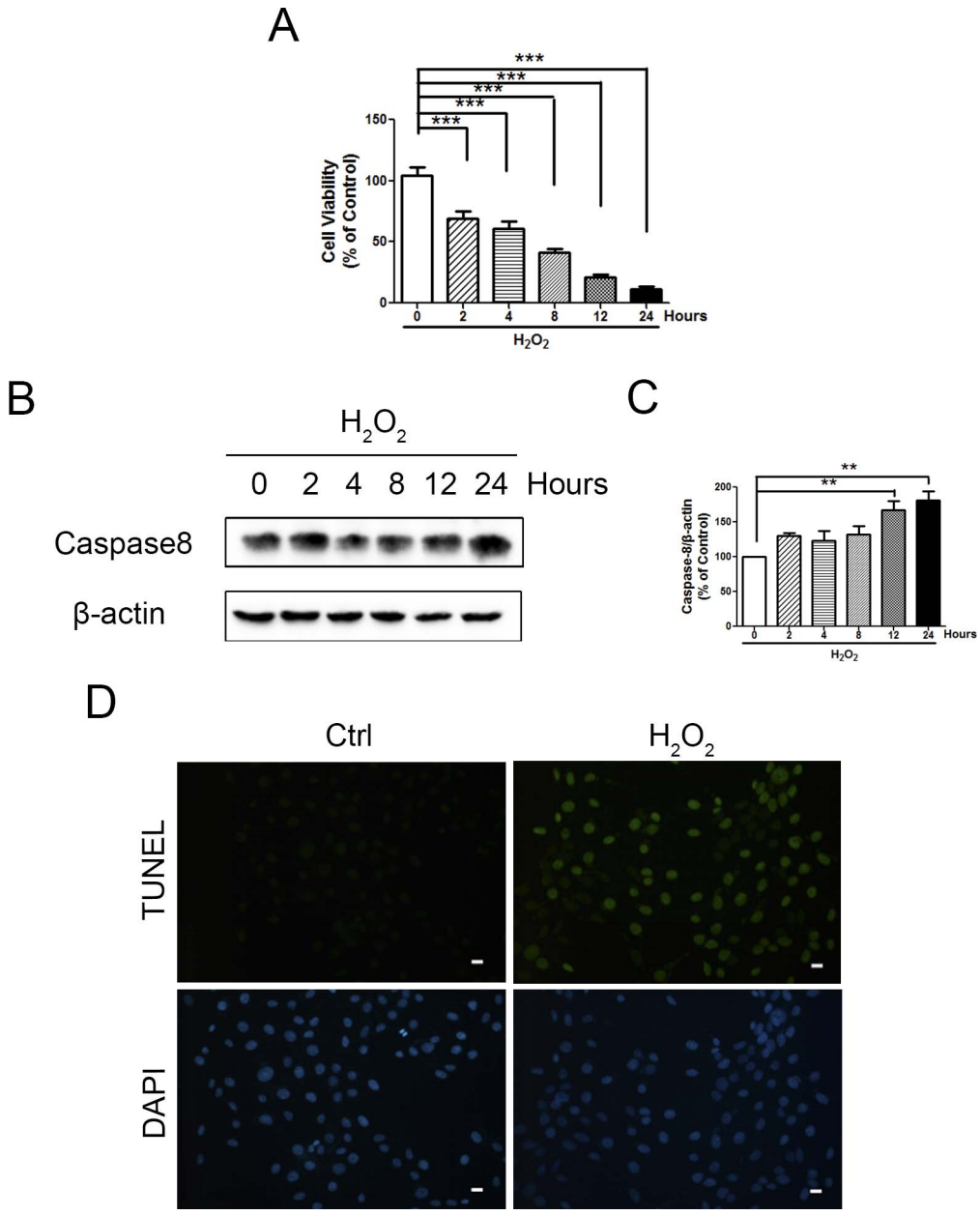

Figure 1. Hydrogen peroxide-induced cell injury in cultured HCE cells. (**A**) Cell viability of HCE cells after treatment of 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours. Data are presented as mean ± SEM, $n = 4$ in each group. $^{***}P < 0.001$. (**B**) Representative images of Western blotting of caspase 8, a marker of apoptosis, in HCE cells after treatment of 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours. (**C**) Statistical analysis of Western blotting data of caspase 8. Data are presented as mean ± SEM, $n = 3$ in each group. $^{**}P < 0.01$. (**D**) Representative images of TUNEL staining in HCE cells after treatment of H_2O_2 for 24 hours. *Scale bars*: 50 μm.

administered 0.25 mM H_2O_2 in cultured HCE cells for 2, 4, 8, 12, and 24 hours to examine the dynamic changes of cellular and oxidant damages. As shown in Figure 1A, the cell viability was significantly decreased in a time-dependent manner after treatment of H_2O_2, whereas the level of caspase 8, a marker of apoptosis, was increased (Figs. 1B, 1C). The positive TUNEL staining signals also showed increases of cell loss after exposure to H_2O_2 (Fig. 1D).

Meanwhile, it was demonstrated that the production of ROS was significantly increased after treatment of H_2O_2 in HCE cells (Fig. 2A). It was also shown that the level of 3-NT, a major marker of ROS, was significantly elevated after exposure of H_2O_2 both in HCE cells (Figs. 2B, 2C) and rat corneas (Figs. 2D, 2E). In addition, the level of NOX4, a key generation enzyme of ROS, was increased significantly in HCE cells (Figs. 2F, 2G). The application of H_2O_2 also significantly increased the level of NOX4 in rat corneas. (Figs. 2H, 2I). Furthermore, the levels of ROS degradation factors, such as SOD, catalase, and GSTP, were decreased significantly in a time-dependent manner in HCE cells (Supplementary Figs. S1A–F).

Oxidative Stress Suppressed the Cellular Autophagy

The association of oxidative stress and cellular autophagy is not completely elucidated yet. Autophagy is a complicated process in the cell and can be divided into macroautophagy, microautophagy, and chaperone-mediated autophagy. There are

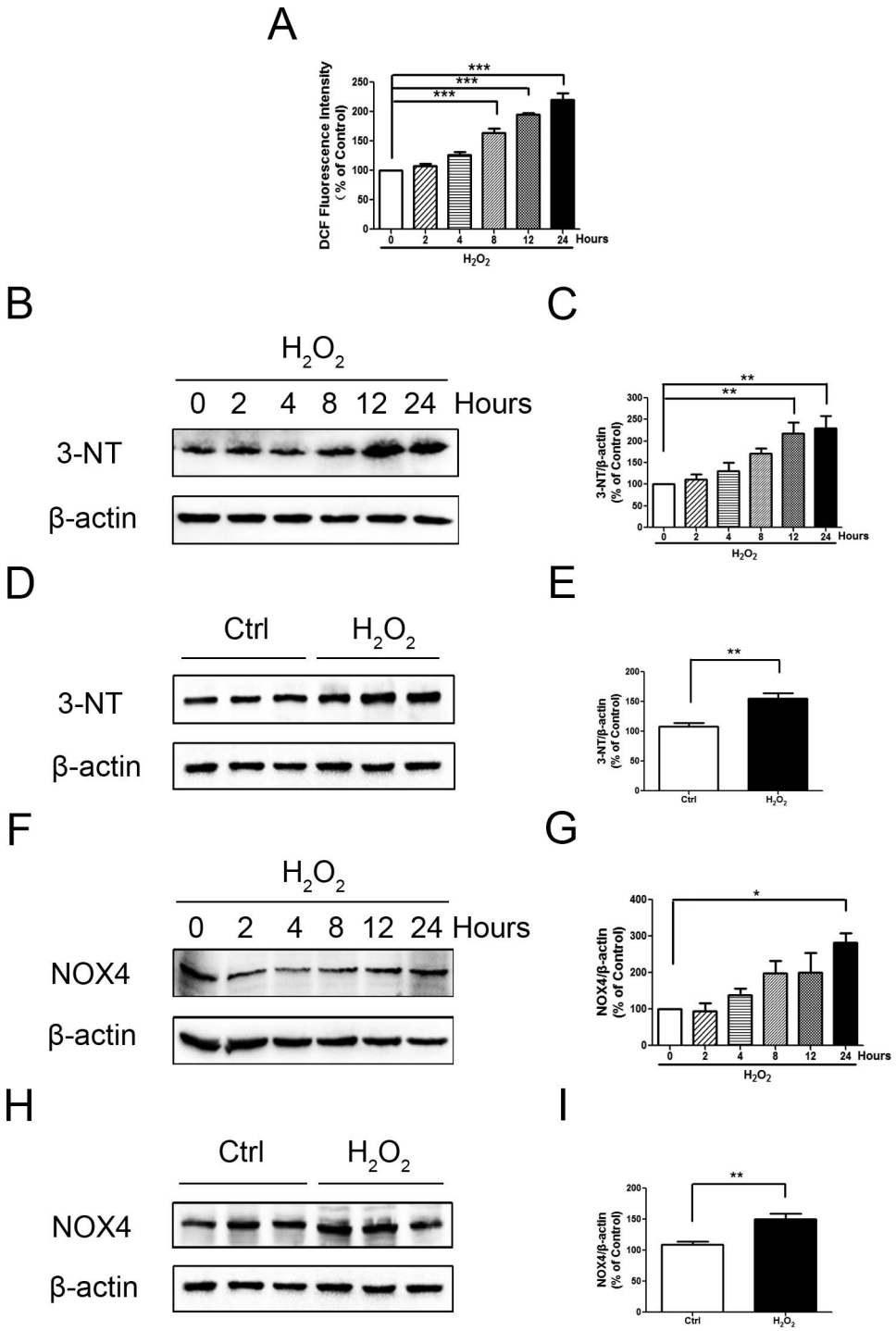

Figure 2. Hydrogen peroxide-induced oxidative stress in HCE cells and rat corneas. (**A**) Production of ROS in HCE cells after treatment of 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours. Data are presented as mean ± SEM, $n = 3$ in each group. $^{***}P < 0.001$. (**B**) Representative images of Western blotting of 3-NT, a marker of ROS, in HCE cells after treatment of 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours. (**C**) Statistical analysis of Western blotting data of 3-NT in HCE cells. Data are presented as mean ± SEM, $n = 3$ in each group. $^{**}P < 0.01$. (**D**) Representative images of Western blotting of 3-NT in corneal epithelium of rat after treatment of H_2O_2 for 24 hours. (**E**) Statistical analysis of Western blotting data of 3-NT in rat corneas. Data are presented as mean ± SEM, $n = 6$ in each group. $^{**}P < 0.01$. (**F**) Representative images of Western blotting of NOX4, a major generation enzyme of ROS, in HCE cells after treatment of 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours. (**G**) Statistical analysis of Western blotting data of NOX4 in HCE cells. Data are presented as mean ± SEM, $n = 3$ in each group. $^{*}P < 0.05$. (**H**) Representative images of Western blotting of NOX4 in the corneal epithelium of rat after treatment of H_2O_2 for 24 hours. (**I**) Statistical analysis of Western blotting data of NOX4 in rat corneas. Data are presented as mean ± SEM, $n = 6$ in each group. $^{**}P < 0.01$.

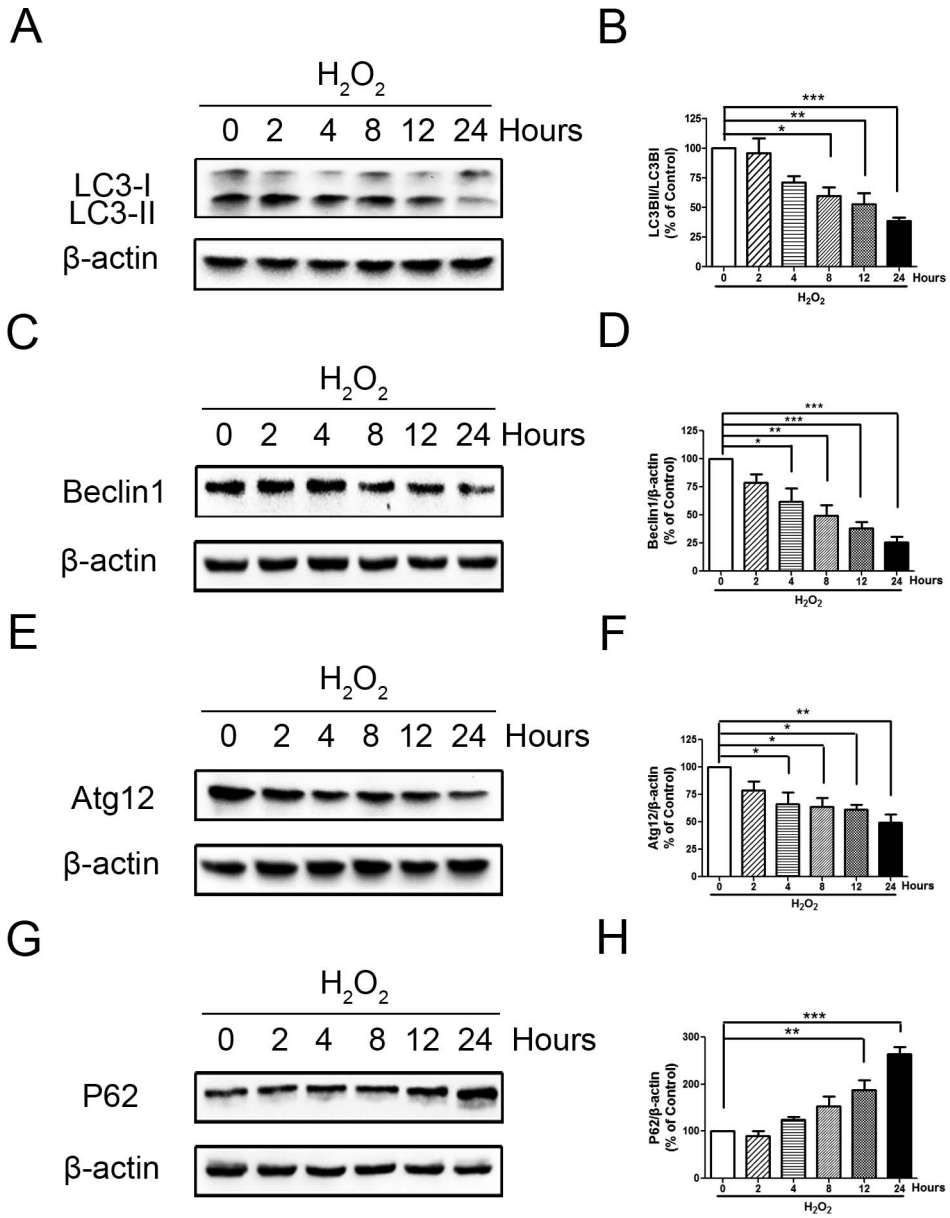

Figure 3. Hydrogen peroxide-suppressed cellular autophagy in HCE cells. (**A**–**H**) Representative images of Western blotting and statistical analysis of Western blotting data of ratio of LC3-I and LC3-II (**A**, **B**), beclin1 (**C**, **D**), Atg12 (**E**, **F**), and P62 (**G**, **H**) in HCE cells after treatment with 0.25 mM H_2O_2 for 2, 4, 8, 12, and 24 hours respectively. Data are presented as mean ± SEM, $n = 3$ in LC3, $n = 4$ in beclin 1, $n = 4$ in Atg 12, and $n = 3$ in P62. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

several key factors that play important roles in the process of cellular autophagy, such as LC3, bechlin1, Atg, and P62. We detected theses key factors of autophagy both in the HCE cells and rat corneas after treatment with H_2O_2.[19–23]

It was shown by Western blot results that the induction of H_2O_2 significantly induced reduction of the ratios of LC3-I and LC3-II, and beclin 1 and Atg12 in a time-dependent manner in HCE cells (Figs. 3A–F). Meanwhile, H_2O_2 increased the level of P62, which was shown by Western blot results (Figs. 3G, 3H). On the other hand, the topical application of H_2O_2 in rat corneas significantly downregulated the ratio of LC3-I and LC3-II and the level of beclin 1 (Supplementary Figs. S2A–D), but upregulated the level of P62 (Supplementary Figs. S2E–F).

These results suggested that corneal oxidant injury suppressed the corneal cellular autophagy at the early stage, and, in other words, the corneal oxidative stress is associated with the inhibition of autophagy.

Rapamycin-Antagonized Corneal Oxidative Stress

To further prove and support the association of corneal oxidative stress and corneal autophagy, we applied the pharmacologic interference approach and investigated the blocking effects of rapamycin, which is an agonist of autophagy and targets the mTOR signaling pathway,[28] on the oxidant damage induced by H_2O_2.

We demonstrated that rapamycin at 0.5 and 1 μM protected the cell viability against H_2O_2 in HCE cells (Fig. 4A) and rapamycin also significantly decreased the production of ROS (Fig. 4B). Meanwhile, it was shown by Western blot results that the levels of the marker of ROS, 3-NT, and the major generation

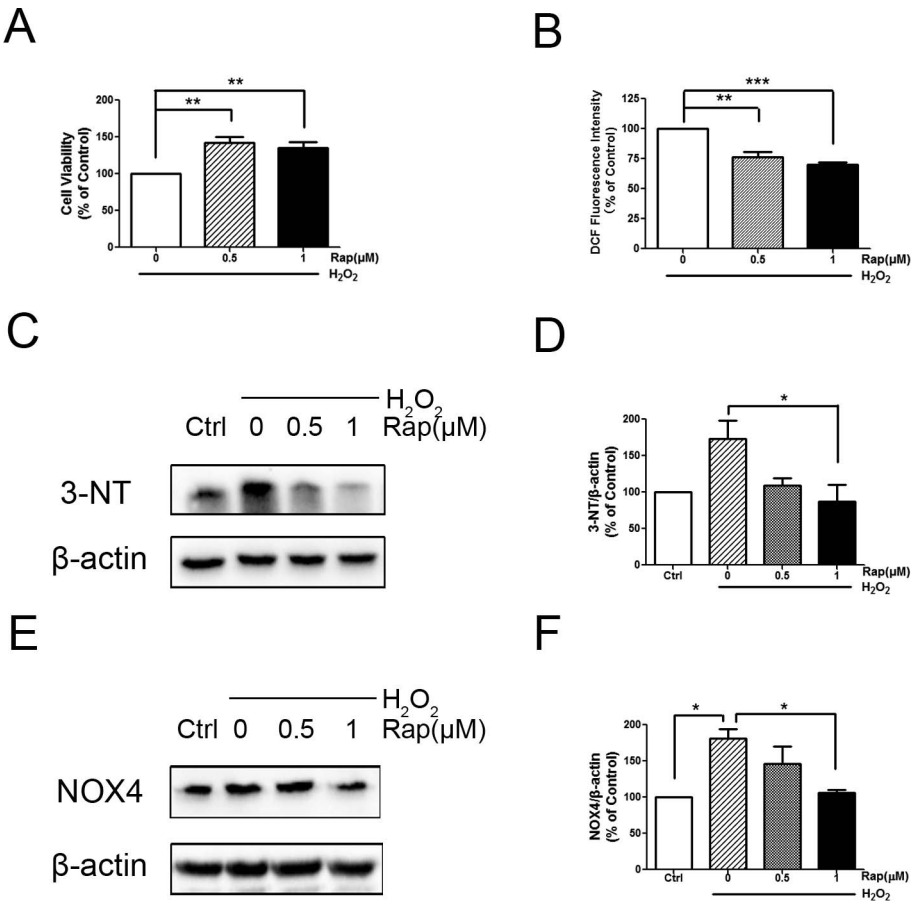

Figure 4. Rapamycin protected the cell injury and oxidant damage induced by H_2O_2. (**A**) Rapamycin at 0.5 and 1 μM significantly ameliorated cell injury of HCE cells after treatment with H_2O_2. Data are presented as mean ± SEM, $n = 4$ in each group. $^{**}P < 0.01$. (**B**) Rapamycin at 0.5 and 1 μM significantly decreased production of ROS in HCE cells after treatment with H_2O_2. Data are presented as mean ± SEM, $n = 3$ in each group. $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) Representative images of Western blotting of 3-NT, a marker of ROS, in HCE cells. (**D**) Statistical analysis of Western blotting data of 3-NT. Data are presented as mean ± SEM, $n = 3$ in each group. $^{*}P < 0.05$. (**E**) Representative images of Western blotting of NOX4, a major generation enzyme of ROS, in HCE cells. (**F**) Statistical analysis of Western blotting data of NOX4. Data are presented as mean ± SEM, $n = 3$ in each group. $^{*}P < 0.05$.

enzyme of ROS, NOX4, were significantly decreased after treatment of rapamycin in HCE cells exposed to H_2O_2 (Figs. 4C–F). On the other hand, we also measured the key factors of degradation of ROS, SOD, catalase, and GSTP by Western blot. It was demonstrated that rapamycin significantly upregulated the levels of SOD, catalase, and GSTP in HCE cells after exposure to H_2O_2 (Supplementary Figs. S3A–F). Furthermore, it was also demonstrated by Western blot results that rapamycin activated cellular autophagy, such as the increases of levels of LC3, beclin 1, and Atg12 (Supplementary Figs. S4A–F) and the reduction of P62 (Supplementary Figs. S4G–H) in cultured HCE cells.

Collectively, the results above indicated that the activation of cellular autophagy induced by rapamycin can antagonize the cellular and oxidant damage induced by H_2O_2.

Discussions

In this study, we offered novel experimental evidence that oxidative stress can suppress autophagy in corneal epithelium. This evidence will help to better understand the association between corneal oxidative injury and other cellular mechanisms. The blocking effects of rapamycin on corneal oxidant injury will provide a clue for new directions for the treatment of corneal diseases caused mainly by oxidative stress.

The crosstalk between ROS and autophagy has drawn increasing attention recently, although the mechanism is not completely illustrated. It is suggested that the interplay between ROS and autophagy may play roles in the neurologic disorders, diabetes, and other diseases.[29,30] The crosstalk between ROS and autophagy is complicated. It has been reported that ROS induces autophagy[31,32]; on the other hand, autophagy may reduce oxidative damage.[33,34] Few evidence demonstrate the role of association between ROS and autophagy in the corneal diseases and ocular surface diseases.[24] In this study, we report that the corneal oxidative injury experimentally induced by H_2O_2 can inhibit the corneal autophagy process, suggesting that cellular autophagy is associated with the oxidative stress, which is a major pathogenesis of certain corneal and ocular surface diseases. Interestingly, it is revealed from this study that the agonist of the process of autophagy, rapamycin, can reverse and ameliorate the cellular oxidant damage in cultured corneal epithelial cells, supporting the involvement of ROS and autophagy in the corneas. Further investigation is needed to identify the blocking effects of rapamycin on corneal oxidative stress in animals and in other experimental oxidant models to explore the potential therapeutic role of rapamycin in the corneal diseases.

Autophagy is an important cellular catabolic process in cells. The major function of autophagy is to help remove the damaged cellular organelles and to promote the survival of cells.[16–18] Autophagy is a very complex process and may play a different or "double-edged sword" role in the different states of cells or in different cells. There are three major forms of the process of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. There are also key factors that play vital roles in process of autophagy. In this investigation, we examined the following key factors: LC3, beclin 1, Atg 12, and P62. LC3 is an autophagy marker and protein that is synthesized in the form of LC3-I in the cytoplasm. Upon autophagy formation, LC3-I is converted to LC3-II and bound to the autophagosome membrane.[17,18] Bechlin1 is an essential molecule in autophagosome formation. It can mediate the autophagy protein autophagic vacuoles, which regulate mammalian autophagosome formation and maturation. Bechlin1 expression tends to increase during autophagy.[19] The Atg family is composed of very important proteins in the process of autophagy. P62 acts as a receptor for vesicles to be degraded by autophagy and as a receptor for ubiquitinated protein aggregates to be eliminated. Therefore, P62 is mainly involved in the clearance of autophagy.[20,21] We demonstrated that corneal oxidative stress can elevate the levels of LC3, beclin 1, and Atg12 but downregulated the level of P62, suggesting an inhibitory role on the process of autophagy by oxidant damage. It will be helpful to better elucidate the interplay of ROS and autophagy in corneas by further illustration on the different signaling pathways, such as the mTOR signaling pathway.[35]

Complete elucidation of the mechanism of autophagy and the interplay with other pathologic events, such as inflammation and oxidative stress in corneal epithelium will help to better understand the physiology of the cornea as well as the pathogenesis of corneal diseases and ocular surface diseases.

Acknowledgments

Supported by National Key R&D Program of China (Grant no. 2018YFA0107304; Beijing, China) and Natural Science Foundation of China (Grant no. 81170818, 81400379; Beijing, China). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure: **Y. Yin**, None; **R. Zong**, None; **X. Bao**, None; **X. Zhen**, None; **H. Cui**, None; **Z. Liu**, None; **Y. Zhou**, None

References

1. Wojcik KA, Kaminska A, Blasiak J, et al. Oxidative stress in the pathogenesis of keratoconus and Fuchs endothelial corneal dystrophy. *Int J Mol Sci*. 2013;14:19294–19308.
2. Zhu C, Pan F, Ge L, et al. SERPINA3K plays antioxidant roles in cultured pterygial epithelial cells through regulating ROS system. *PLoS One*. 2014;9:e108859.
3. Kruk J, Kubasik-Kladna K, Aboul-Enein HY. The role oxidative stress in the pathogenesis of eye diseases: current status and a dual role of physical activity. *Mini Rev Med Chem*. 2015;16:241–257.
4. Huo YN, Chen W, Zheng XX. ROS, MAPK/ERK and PKC play distinct roles in EGF-stimulated human corneal cell proliferation and migration. *Cell Mol Biol* (*Noisy-le-grand*). 2015;61:6–11.
5. Cheung IM, McGhee CN, Sherwin T. Beneficial effect of the antioxidant riboflavin on gene expression of extracellular matrix elements, antioxidants and oxidases in keratoconic stromal cells. *Clin Exp Optom*. 2014;97:349–55.
6. Wei R, Zhang R, Xie Y, Shen L, Chen F. Hydrogen suppresses hypoxia/reoxygenation-induced cell death in hippocampal neurons through reducing oxidative stress. *Cell Physiol Biochem*. 2015;36:585–598.
7. Sozou PD, Kirkwood TB. A stochastic model of cell replicative senescence based on telomere shortening, oxidative stress, and somatic mutations in nuclear and mitochondrial DNA. *J Theor Biol*. 2001;213:573–586.
8. Louis M, Rosato RR, Brault L, et al. The histone deacetylase inhibitor sodium butyrate induces breast cancer cell apoptosis through diverse cytotoxic actions including glutathione depletion and oxidative stress. *Int J Oncol*. 2004;25:1701–1711.
9. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol*. 2004;55:373–399.
10. Sinha N, Dabla PK. Oxidative stress and antioxidants in hypertension—a current review. *Curr Hypertens Rev*. 2015;11:132–142.
11. Geiszt M, Leto TL. The Nox family of NAD(P)H oxidases: host defense and beyond. *J Biol Chem*. 2004;279:51715–51718.
12. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;87:245–313.
13. Frey RS, Ushio-Fukai M, Malik AB. NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. *Antioxid Redox Signal*. 2009;11:791–810.
14. Bresciani G, da Cruz IB, Gonz'alez-Gallego J. Manganese superoxide dismutase and oxidative stress modulation. *Adv Clin Chem*. 2015;68:87–130.
15. Nishikawa M, Hashida M, Takakura Y. Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. *Adv Drug Deliv Rev*. 2009;61:319–326.
16. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol*. 2014;15:81–94.
17. Ren F, Zhang L, Zhang X, et al. Inhibition of glycogen synthase kinase 3beta promotes autophagy to protect mice from acute liver failure mediated by peroxisome proliferator-activated receptor alpha. *Cell Death Dis*. 2016;7:e2151.
18. Rikiishi H. Possible role of autophagy in the treatment of pancreatic cancer with histone deacetylase inhibitors. *Cancers* (*Basel*). 2010;2:2026–2043.
19. Atala A. Re: VHL-Regulated miR-204 suppresses tumor growth through inhibition of LC3B-mediated autophagy in renal clear cell carcinoma. *J Urol*. 2012;188:2434.
20. Mikhaylova O, Stratton Y, Hall D, et al. VHL-regulated MiR-204 suppresses tumor growth through inhibition of LC3B-mediated autophagy in renal clear cell carcinoma. *Cancer Cell*. 2012;21:532–546.
21. Xu W, Jiang H, Hu X, Fu W. Effects of high-mobility group box 1 on the expression of Beclin-1 and LC3 proteins following hypoxia and reoxygenation injury in rat cardiomyocytes. *Int J Clin Exp Med*. 2014;7:5353–5357.
22. Kessler SM, Laggai S, Barghash A, et al. IMP2/p62 induces genomic instability and an aggressive hepatocellular carcinoma phenotype. *Cell Death Dis*. 2015;6:e1894.
23. Zhang J, Chan EK. Autoantibodies to IGF-II mRNA binding protein p62 and overexpression of p62 in human hepatocellular carcinoma. *Autoimmun Rev*. 2002;1:146–153.
24. Shetty R, Sharma A, Pahuja N, et al. Oxidative stress induces dysregulated autophagy in corneal epithelium of keratoconus patients. *PLoS One*. 2017;12:e0184628.
25. Zhou T, Zong R, Zhang Z, et al. SERPINA3K protects against oxidative stress via modulating ROS generation/degradation and KEAP1-NRF2 pathway in the corneal epithelium. *Invest Ophthalmol Vis Sci*. 2012;53:5033–5043.

Investigative Ophthalmology & Visual Science

26. Chen L, Zong R, Zhou J, et al. The oxidant role of 4-hydroxynonenal in corneal epithelium. *Sci Rep*. 2015;5:10630.
27. Melov S. Animal models of oxidative stress, aging, and therapeutic antioxidant interventions. *Int J Biochem Cell Biol*. 2002;34:1395–1400.
28. Back JH, Rezvani HR, Zhu Y, et al. Cancer cell survival following DNA damage-mediated premature senescence is regulated by mammalian target of rapamycin (mTOR)-dependent Inhibition of sirtuin 1. *J Biol Chem*. 2011;286:19100–19108.
29. Fang C, Gu L, Smerin D, Mao S, Xiong X. The interrelation between reactive oxygen species and autophagy in neurological disorders. *Oxid Med Cell Longev*. 2017;2017:8495160.
30. Dehdashtian E, Mehrzadi S, Yousefi B, et al. Diabetic retinopathy pathogenesis and the ameliorating effects of melatonin; involvement of autophagy, inflammation and oxidative stress. *Life Sci*. 2018;193:20–33.
31. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci*. 2011;36:30–38.
32. Liu Z, Lenardo MJ. Reactive oxygen species regulate autophagy through redox-sensitive proteases. *Dev Cell*. 2007;12:484–485.
33. Gurusamy N, Lekli I, Gorbunov NV, et al. Cardioprotection by adaptation to ischaemia augments autophagy in association with BAG-1 protein. *J Cell Mol Med*. 2009;13:373–387.
34. Li L, Tan J, Miao Y, Lei P, Zhang Q. ROS and autophagy: interactions and molecular regulatory mechanisms. *Cell Mol Neurobiol*. 2015;35:615–621.
35. Paquette M, El-Houjeiri L, Pause A. mTOR pathways in cancer and autophagy. *Cancers* (*Basel*). 2018;10:E18.

Investigative Ophthalmology & Visual Science