Current Medical Science
DOI https://doi.org/10.1007/s11596-019-2057-8
39(3):442-448,2019

4-HNE Induces Apoptosis of Human Retinal Pigment Epithelial Cells by Modifying HSP70*

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Summary: The role of heat shock protein 70 (HSP70) in apoptosis of human retinal pigment epithelial cells (ARPE-19) induced by 4-hydroxy-2-nonenal (4-HNE) was explored. Different concentrations of 4-HNE were used to stimulate ARPE-19 cells, and apoptosis was measured by flow cytometry. The expression of apoptotic-related proteins, HSP70, X-linked inhibitor-of-apoptosis (XIAP), Bcl-2, and Bax were quantified by Western blotting. HSP70 and XIAP overexpression plasmids, or their corresponding siRNAs were transfected into ARPE-19 cells using Lipofectamine™ 2000. Co-immunoprecipitation and Western blotting were used to detect the effect of 4-HNE on the expression of HSP70 and the binding level between 4-HNE and HSP70. The results showed that 4-HNE induced late apoptosis in ARPE-19 cells, accompanied by elevated levels of 4-HNE-modified HSP70, but it did not affect HSP70 protein expression. 4-HNE-modified HSP70 down-regulated the expression of the apoptosis inhibitory protein XIAP. Overexpression of HSP70 or XIAP inhibited 4-HNE-induced apoptosis of ARPE-19 cells. It was suggested that 4-HNE could promote XIAP degradation by modification of HSP70 to induce late apoptosis of human retinal pigment epithelial cells.
Key words: 4-hydroxy-2-nonenal; HSP70; XIAP; human retinal pigment epithelial cells; apoptosis

Age-related macular degeneration (AMD) is the leading cause of vision loss and blindness in individuals over 50 years old and ranks the third among causes for blindness in China with the aging population[1]. Currently, oxidative stress has been proposed as the fundamental factor for AMD occurrence, which can induce apoptosis of retinal pigment epithelial cells and chronic inflammatory reaction, leading to secondary pathological changes such as natural drusen formation, retinal pigment epithelial map atrophy and choroidal neovascularization, finally resulting in the vision loss or blindness[2, 3]. However, so far, the detailed mechanisms of oxidative stress leading to apoptosis of retinal pigment epithelial cells remain unclear.

4-Hydroxy-2-nonenal (4-HNE) is one of the primary end products during lipid peroxidation. It can covalently modify histidine, cysteine, and lysine residues of proteins, leading to stable HNE-protein adducts, and directly causing functional impairment of proteins involved in induction of apoptosis, inhibition of enzyme activity and other biological behaviors[4]. Thus, 4-HNE modification is confirmed to be associated with many diseases, such as atherosclerosis, ischemia-reperfusion injury, Parkinson's syndrome and Alzheimer's disease[5]. In the retinal pigment epithelial cells of AMD patients, 4-HNE-modified proteins are also widely present, such as heat shock protein 70 (HSP70) and Trx[6, 7]. HSP70 is a member of heat shock protein families, which exerts various biological functions such as anti-oxidation, inhibition of apoptosis and inflammatory response by inhibiting ROS production induced by oxidative stress or cell survival-related protein degradation[8, 9]. However,

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*This project was supported by grants from the Natural Science Foundation of Hubei Province, China (No. 2009CDB115, and No. 2012FKB02444), and Natural Science Foundation of Health and Family Planning Commission of Wuhan Municipality (No. WX18Q03, and No. WX18Q27).

whether 4-HNE induces apoptosis of retinal pigment epithelial cells by modifying HSP70 in AMD is still unclear.

Our previous studies found that exogenous administration of 4-HNE significantly decreased the activity of RPE cells, while Nrf2-mediated antioxidant activation blocked the toxicity of 4-HNE[10]. In the present study, we aimed to investigate the effect and related molecular mechanisms of HSP70 in apoptosis of retinal pigment epithelial cells induced by 4-HNE, which will provide new strategies and potential intervention targets for AMD prevention and treatment.

1 MATERIALS AND METHODS

1.1 Reagents

4-HNE was purchased from BioVision (USA). The FITC-labeled Annexin V/PI apoptosis detection kit was purchased from Biolegend (USA). 4-HNE, HSP70, XIAP, Bcl-2 and Bax antibodies were purchased from Abcam (USA). Protein G magnetic beads were from MCE (China). Lipofectamine™ 2000 Transfection Reagent, HSP70 siRNA and XIAP siRNA, as well as their respective negative controls were purchased from ThermoFisher (USA). The pcDNA3.1-HSP70 plasmid, pcDNA3.1-XIAP plasmid and pcDNA3.1 empty plasmid were constructed and identified by Hunan Aijia Biotechnology Co., Ltd. (China).

1.2 Cell Culture

Human retinal pigment epithelial cell line ARPE-19 was purchased from the American Model Collection Center (USA)[10] and cultured in DMEM/Ham F12 (50/50) medium containing 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% non-essential amino acids (ThermoFisher). Cells in log phase were seeded into 6-well plates at 2.5×10^5 cells/well, and treated with 4-HNE at a final concentration of 0, 20, and 40 μmol/L for 24 h.

1.3 Apoptosis Detection by Flow Cytometry

Different concentrations of 4-HNE-treated ARPE-19 cells were trypsinized with Trypsin (Beyotime, China), collected in a 5-mL flow tube, centrifuged at 300 *g* for 5 min, and the supernatant was removed. Cells were washed twice with pre-cooled PBS containing 1% BSA and then resuspend in 100 μL Annexin V Binding Buffer. After adding 5 μL of FITC-labeled Annexin V and 10 μL of PI, cells were incubated in a dark room at room temperature (25°C) for 15 min. Apoptosis was detected using BD FACSCanto™ Ⅱ.

1.4 Cell Transfection

ARPE-19 cells were seeded into 6-well plates at 2.5×10^5 cells/well, and the serum-free medium was replaced after the cell confluence reached 80%–90%. A total of 6 μL of Lipofectamine™ 2000 transfection reagent was dissolved in 150 μL of serum-free medium, and 2.5 μg of pcDNA3.1-HSP70 or pcDNA3.1-XIAP overexpression plasmid was also added to 150 μL of serum-free medium. Then, these two media were mixed at a ratio of 1:1. After incubation for 5 min at room temperature, 250 μL was added to the cell culture medium, and transfection efficiency was measured 48 h later. The siRNA co-transfection procedure was performed according to the Lipofectamine™ 2000 instructions.

1.5 Co-immunoprecipitation

ARPE-19 cells were washed twice with PBS, then added to cell lysis buffer, simultaneously added with the protease inhibitor, mixed and placed in ice water for 10 min. The cells were centrifuged at 14 000 *g* (4°C, 10 min), and the supernatant was collected after cells were lysed. 50 μL of protein G-conjugated magnetic beads were added to the cell lysate, and then protein that was non-specifically bound to protein G was removed by magnetic separation after incubation at 4°C for 2 h. The antibody (4-HNE or HSP70 antibody) was diluted with a binding/washing buffer to a final concentration of 50 μg/mL, and incubated with protein G-conjugated magnetic beads. Then the antibody-bound protein G-modified magnetic beads were magnetically separated and incubated at 4°C for 2 h with prepared cell lysate (400 μL) on a tumbler, and the supernatant was moved by magnetic separation. The precipitation was subjected to Western blotting for detecting the related protein expression.

1.6 Statistical Analysis

Measurement data were presented as mean±standard deviation (SD) and analyzed by GraphPad Prism V 6.0 for Windows (GraphPad Software, USA). Statistical significance was determined by Student *t* test or ANOVA followed by Neuman-Keuls post hoc test. *P* value less than 0.05 was considered as statistically significant.

2 RESULTS

2.1 4-HNE Promotes Late Apoptosis of ARPE-19 Cells in a Concentration-dependent Manner

ARPE-19 cells were stimulated with different concentrations (0, 20, 40 μmol/L) of 4-HNE for 24 h, and then apoptosis was detected by flow cytometry. The proportion of late apoptotic cells gradually increased with the rise of 4-HNE concentration (3.01%±0.75%, 6.61%±0.69% and 14.27%±1.20), and the difference was statistically significant. There was no significant change in early apoptosis, necrosis, and viable cell ratio (fig. 1A and 1B). Detection of apoptosis-related proteins revealed that 4-HNE promoted the expression of the proapoptotic protein Bax, while down-regulated the expression of apoptotic inhibitory proteins XIAP and Bcl-2 (fig. 1C). The above results indicated that 4-HNE can promote late apoptosis of ARPE-19 cells.

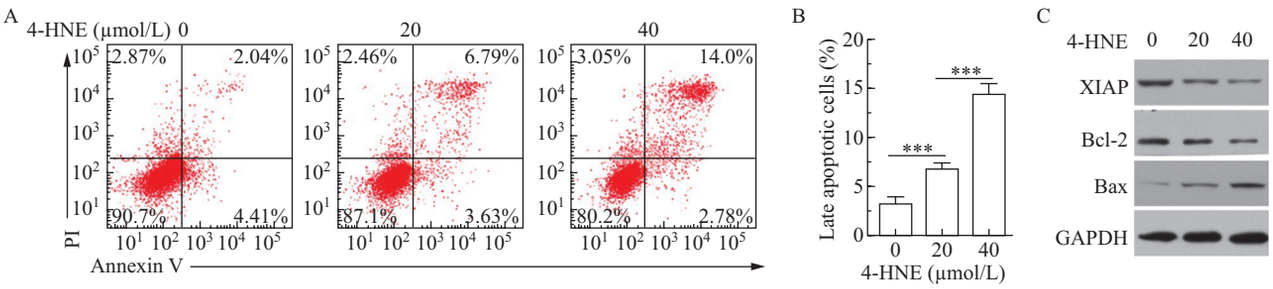

Fig. 1 4-HNE-induced apoptosis of ARPE-19 cells

A: ARPE-19 cells were stimulated with different concentrations of 4-HNE for 24 h, and apoptosis was detected by flow cytometry. B: statistical plot of late apoptosis. C: ARPE-19 cells were stimulated with different concentrations of 4-HNE for 24 h, and the expression of XIAP, Bcl-2 and Bax was detected by Western blotting. GAPDH was used as an internal control. Data were presented as mean±SD. $^{***}P<0.0001$

2.2 4-HNE Promotes Apoptosis of ARPE-19 Cells by Modifying HSP70

Western blotting showed that 4-HNE stimulation increased its intracellular accumulation in ARPE-19 cells, but had no significant influence on the total protein expression of HSP70 (fig. 2A). However, after immunoprecipitation with 4-HNE antibody, the amount of HSP70 protein bound was gradually increased with the increase of 4-HNE concentration. The same result was obtained by reverse immunoprecipitation with HSP70 antibody (fig. 2B). The above results indicated that 4-HNE does not affect HSP70 protein expression but can modify HSP70 protein.

To detect the effect of HSP70 on apoptosis of ARPER-19 cells induced by 4-HNE, HSP70 was exogenously overexpressed with pcDNA3.1-HSP70 plasmid transfection or interfered with HSP70 siRNA (fig. 2C). The exogenous overexpression of HSP70 did not affect the intracellular level of 4-HNE, and there was no significant change in the amount of 4-HNE-bound HSP70 protein. However, we observed that the HSP70 overexpression decreased the ratio of 4-HNE-modified HSP70 protein (fig. 2C and 2D). Apoptosis assay revealed that the HSP70 overexpression significantly inhibit 4-HNE-induced late apoptosis of ARPE-19 cells (13.92%±0.78% *vs.* 6.24%±0.71%) (fig. 2E and 2F). Furthermore, it also upregulated the XIAP and Bcl-2 expression, and down-regulated the Bax expression in ARPE-19 cells (fig. 2G). The interference of HSP70 expression with siRNA decreased the level of 4-HNE-modified and total protein level of HSP70 protein (fig. 2C and 2D). HSP70 siRNA also promoted late apoptosis of ARPE-19 cells (12.88%±1.24% *vs.* 24.83%±2.47%) (fig. 2E and 2F), downregulated the expression of XIAP and Bcl-2 and upregulated the Bax expression (fig. 2G). The above results indicated that 4-HNE promotes apoptosis of ARPE-19 cells by modifying HSP70.

2.3 4-HNE-modified HSP70 Induces Apoptosis of ARPE-19 Cells by Promoting XIAP Degradation

HSP70 binds to a variety of cell survival-associated proteins, inhibiting its degradation and protecting cells from apoptosis, such as XIAP[11]. The qRT-PCR assays revealed that neither 4-HNE treatment nor HSP70 overexpression or interference affected the XIAP mRNA expression (fig. 3A and 3B), which indicated that 4-HNE promotes XIAP protein degradation by modifying HSP70.

Transfection of ARPE-19 cells with XIAP overexpression plasmid promoted the XIAP expression (fig. 3C), and significantly inhibited 4-HNE-induced late apoptosis (13.18%±1.20% *vs.* 6.44%±0.50%), and blocked the promoting effect of interfering HSP70 on the late apoptosis of ARPE-19 cells (26.90%±1.21% *vs.* 14.95%±1.26%), with the difference being statistically significant (fig. 3D and 3E). More importantly, XIAP siRNA significantly promoted 4-HNE-induced late apoptosis (12.40%±1.18% *vs.* 27.35%±2.04%), and HSP70 overexpression only partially reversed the effect of XIAp siRNA (27.35%±2.04% *vs.* 22.58%±2.00%) (fig. 3D and 3E). These results indicated that XIAP is an important downstream molecule of HSP70, and 4-HNE promotes apoptosis of ARPE-19 cells by modifying HSP70, which inhibits the protective effect of HSP70 on XIAP and promotes XIAP degradation.

3 DISCUSSION

The mechanism of AMD mainly includes three aspects: oxidative stress, neovascularization, and inflammatory injury. To this reason, the current clinical treatments of AMD mainly include palliative treatment (photodynamic, anti-vascular factor, etc.), antioxidant drugs for the formation of new blood vessels, and food therapy[12]. However, the therapeutic effects of these methods are still limited or controversial, and some patients still have progressive vision loss after treatment[13]. Loss of retinal pigment epithelial cells due to apoptosis is the main cause of retinal pattern atrophy and vision loss in AMD patients[14]. Oxidative stress, due to accumulation of lipofuscin, H_2O_2, tBH, and propionaldehyde, can lead to shortening

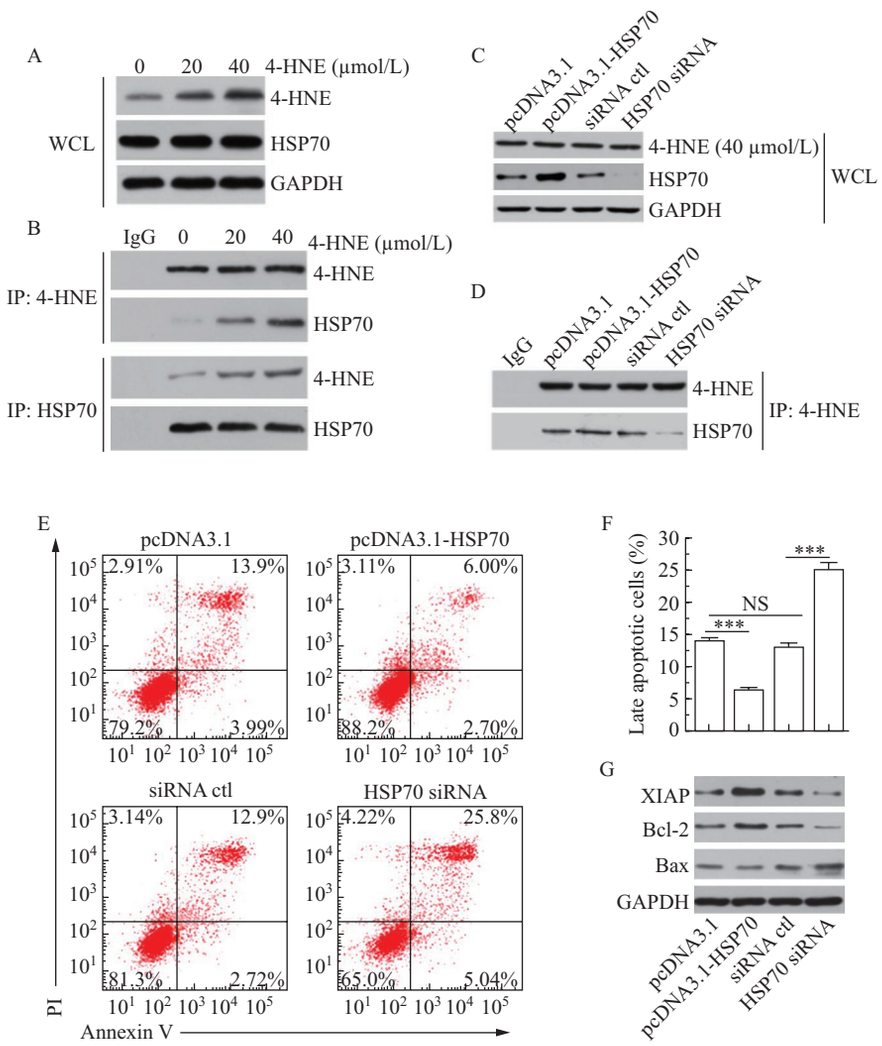

Fig. 2 4-HNE-induced apoptosis of ARPE-19 cells by modifying HSP70

A: Western blotting was used to detect the expression of 4-HNE and HSP70 in ARPE-19 cells stimulated by different concentrations of 4-HNE. B: ARPE-19 cells were stimulated with different concentrations of 4-HNE for 24 h, and the binding between 4-HNE and HSP70 was determined by co-immunoprecipitation and Western blotting. Antibody isotype (IgG) was used as a control. C: ARPE-19 cells were transfected with HSP70 overexpression plasmid or interfering RNA for 48 h, then cells were treated with 4-HNE (40 μmol/L) for 24 h. The expression of HSP70 and 4-HNE was detected by Western blotting. D: ARPE-19 cells were treated as (C), then the binding between 4-HNE and HSP70 was detected by co-immunoprecipitation and Western blotting. E: ARPE-19 cells were treated as (C), and apoptosis was detected by flow cytometry. F: The late apoptosis was counted. G: The expression of XIAP, Bcl-2 and Bax was detected by Western blotting. Data are presented as mean±SD. NS: no statistical significance between groups. $^{***}P<0.0001$. WCL: whole cell lysate

and disintegration of photoreceptor outer and inner segments, induce Bruch membrane thickening, retinal pigment epithelial cell degeneration, and ultimately lead to apoptosis. It has been confirmed to be one of the main pathogenic factors of AMD[15]. Therefore, further research on the mechanism of oxidative stress leading to apoptosis of retinal pigment epithelial cells will be of great significance for the prevention and treatment of AMD.

As an essential oxidative stress product, 4-HNE plasma concentration gradually increases with age, and accumulation in retinal pigment epithelial cells is associated with multiple age-related retinal diseases[16]. Previous studies have shown that 4-HNE can induce apoptosis of retinal pigment epithelial cells via activating multiple pathways such as mitochondrial membrane depolarization by activating NF-κB, p53, Caspase-3 or up-regulating NOX4, and exogenously overexpressing Trx2 or Nrf2 can protect cells from apoptosis by reducing the oxidative damage caused by 4-HNE[17]. In this study, 4-HNE also induced apoptosis of human retinal pigment epithelial cell line ARPE-19 cells, but mainly in late apoptosis, which was different from the previous report that 4-HNE could simultaneously induce early apoptosis and late apoptosis[17]. Besides, 4-HNE also inhibited the expression of cell survival

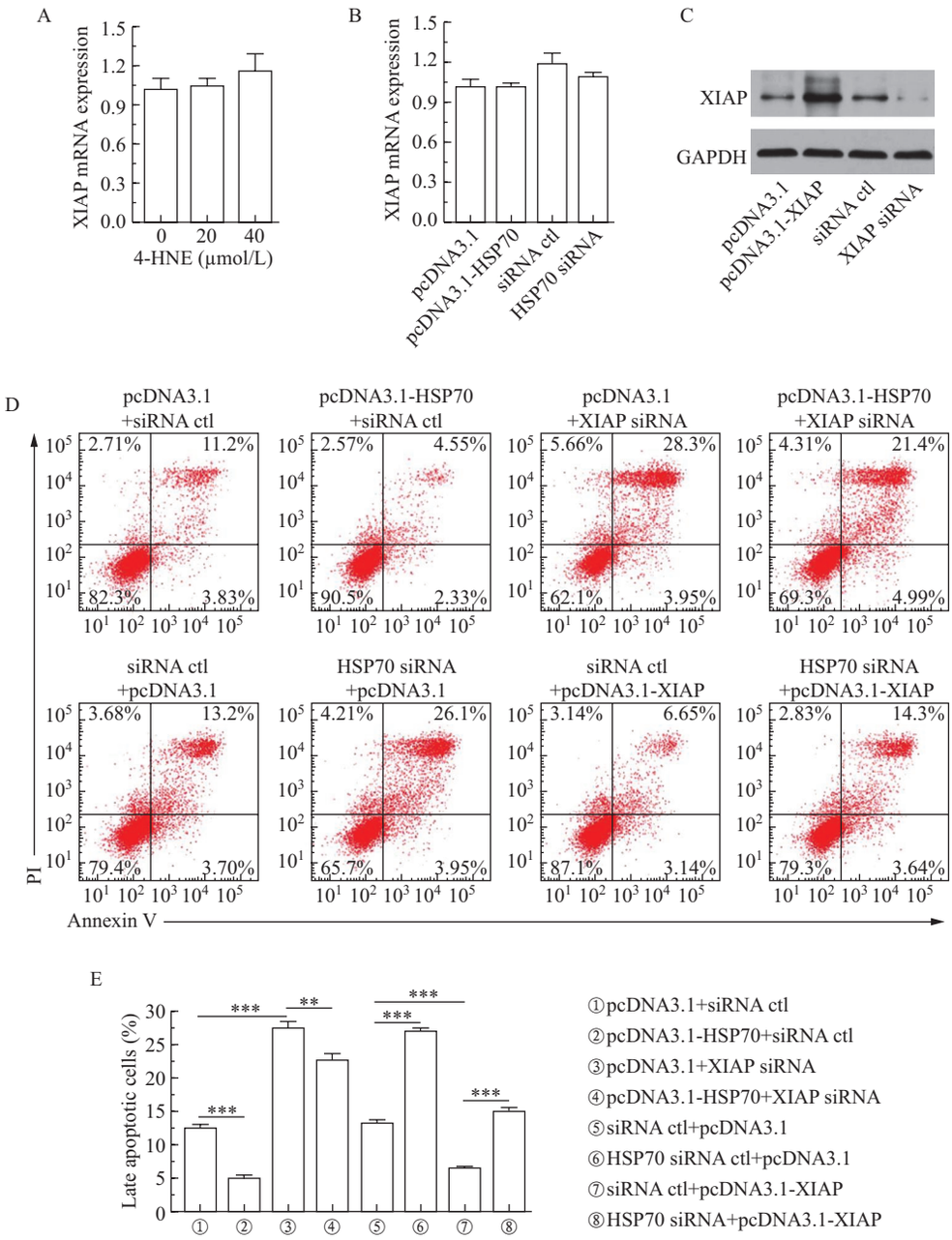

Fig. 3 4-HNE-modified HSP70 induces apoptosis of ARPE-19 cells by promoting XIAP degradation

A: The qRT-PCR was used to detect the expression of XIAP mRNA in ARPE-19 cells stimulated by different concentrations of 4-HNE for 24 h. B: ARPE-19 cells were transfected with HSP70 overexpression plasmid or interfering RNA, and 48 h later, cells were treated with 4-HNE (40 μmol/L) for 24 h. Then, the XIAP mRNA expression was detected by qRT-PCR. C: ARPE cells were transfected with XIAP overexpression plasmid or interfering RNA, and 48 h later, the XIAP expression was detected by Western blotting. D: ARPE-19 cells were co-transfected with HSP70 overexpression plasmid and XIAP interfering RNA, or HSP70 interfering RNA and XIAP overexpression plasmid. 48 h later, ARPE-19 cells were further treated with 4-HNE (40 μmol/L) for 24 h, then apoptosis was detected by flow cytometry. E: statistical plot of late apoptosis of ARPE-19 cells. Data are presented as mean±SD. $^{**}P<0.01$, $^{***}P<0.0001$

related proteins XIAP and Bcl-2 and promoted the expression of the proapoptotic protein Bax in a dose-dependent manner. 4-HNE mainly binds to proteins through the Michael addition reaction and changes its spatial structure to affect the function of the protein[5]. Strong light irradiation, hypoxia, and other factors can cause acute elevation of 4-HNE-modified protein expression in retinal pigment epithelial cells (about 3 h)[18], which suggests that the modification of 4-HNE protein may be an early event in the process of retinal pigment epithelial cell apoptosis, and elucidation of this mechanism may provide new strategies and potential

intervention targets for the prevention and treatment of AMD.

Ethen *et al*[7] screened a variety of 4-HNE modified proteins, such as Trx, Daxx, and HSP70, by two-dimensional electrophoresis and mass spectrometry after retinal co-immunoprecipitation in AMD patients. In the development of AMD, HSP70 has important functions such as anti-oxidation, inhibition of apoptosis and excessive inflammatory response[19]. Although studies have reported that 4-HNE modification can alter the HSP70 conformation, the effect on HSP70 function is still unclear[20]. In this study, 4-HNE gradient stimulation had no significant effect on HSP70 protein expression, but co-immunoprecipitation showed that HSP70 was modified by 4-HNE in a dose-dependent manner. In view of the fact that the HSP70 and 4-HNE binding sites are still not clear, we investigated the effect of 4-HNE modification on HSP70 function by reducing the ratio of 4-HNE-modified HSP70 in total HSP70 protein pool. Actually, overexpression of HSP70 reduced the proportion of 4-HNE-modified HSP70, and flow cytometry results showed that it also inhibited 4-HNE-induced apoptosis in ARPE-19 cells. Western blotting also confirmed that overexpression of HSP70 promoted the expression of XIAP and Bcl-2 and inhibited the expression of Bax. The siRNA interference with HSP70 expression promoted late apoptosis and down-regulated the XIAP and BCL-2 protein expression in ARPE-19 cells. Therefore, 4-HNE can block the protective effect of HSP70 on apoptosis of retinal pigment epithelial cells by modifying HSP70.

As a molecular chaperone, HSP70 protects a variety of proteins that inhibit apoptosis from degradation, thereby promoting cell survival, including XIAP[11]. XIAP is a key protein that links p53 and caspase3 and plays a vital role in inhibiting apoptosis[21]. The qRT-PCR results showed that neither HSP70 nor 4-HNE affected XIAP mRNA expression levels. Therefore, It is highly possible that 4-HNE promotes apoptosis by modifying HSP70 to inhibit the protective effect of HSP70 on XIAP. The interference or overexpression of XIAP followed by 4-HNE stimulation showed that the interference with XIAP expression significantly increased the proportion of late apoptosis in ARPE-19 cells induced by 4-HNE, and meanwhile, the exogenous overexpression of HSP70 could only slightly reverse the pro-apoptotic effect of XIAP interference. The exogenous overexpression of XIAP can effectively inhibit 4-HNE-induced late apoptosis, which was almost totally blocked by interfering HSP70 expression. These results demonstrate that 4-HNE induce late apoptosis of ARPE-19 cells by modifying HSP70, which in turn promotes XIAP degradation.

HSP70 recombinant protein has been reported with the ability to inhibit the progression of AMD and sphingolipidosis by reducing lipid deposition, oxidative stress damage, and its oral small molecule agonist arimoclomol has entered the clinical trial phase for the treatment of type C Niemanneck disease[19, 22]. This study demonstrated for the first time that the oxidative stress terminal product 4-HNE can reduce the protective effect of HSP70 on XIAP and induce retinal pigment epithelial cell apoptosis by modifying HSP70, which further confirmed the key role of HSP70 in AMD and provided an important theoretical basis for the application of HSP70 recombinant protein or agonist in the treatment of AMD.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest or financial disclosures to report.

REFERENCES

1 Jin M, Dai H, Zhang X, *et al*. A Traditional Chinese Patent Medicine ZQMT for Neovascular Age-Related Macular Degeneration: a multicenter randomized clinical trial. Curr Mol Med, 2018,18(9):622-629

2 Cheng Z, Yao W, Zheng J, *et al*. A derivative of betulinic acid protects human retinal pigment epithelial (RPE) cells from cobalt chloride-induced acute hypoxic stress. Exp Eye Res, 2018,180:92-101

3 Dieguez HH, Romeo HE, Alaimo A, *et al*. Oxidative stress damage circumscribed to the central temporal retinal pigment epithelium in early experimental non-exudative age-related macular degeneration. Free Radic Biol Med, 2018,131:72-80

4 Czerwinska J, Poznanski J, Debski J, *et al*. Catalytic activities of Werner protein are affected by adduction with 4-hydroxy-2-nonenal. Nucleic Acids Res, 2014,42(17):11 119-11 135

5 Castro JP, Jung T, Grune T, *et al*. 4-Hydroxynonenal (HNE) modified proteins in metabolic diseases. Free Radic Biol Med, 2017,111:309-315

6 Baek JH, Lim D, Park KH, *et al*. Quantitative proteomic analysis of aqueous humor from patients with drusen and reticular pseudodrusen in age-related macular degeneration. BMC Ophthalmol, 2018,18(1):289

7 Ethen CM, Reilly C, Feng X, *et al*. Age-related macular degeneration and retinal protein modification by 4-hydroxy-2-nonenal. Invest Ophthalmol Vis Sci, 2007,48(8):3469-3479

8 Reeg S, Jung T, Castro JP, *et al*. The molecular chaperone Hsp70 promotes the proteolytic removal of oxidatively damaged proteins by the proteasome. Free Radic Biol Med, 2016,99:153-166

9 Li X, Yu Y, Gorshkov B, *et al*. Hsp70 Suppresses Mitochondrial Reactive Oxygen Species and Preserves Pulmonary Microvascular Barrier Integrity Following Exposure to Bacterial Toxins. Front Immunol, 2018,9:1309

10 Chen J, Wang L, Chen Y, *et al*. Phosphatidylinositol 3 kinase pathway and 4-hydroxy-2-nonenal-induced oxidative injury in the RPE. Invest Ophthalmol Vis Sci, 2009,50(2):936-942

11 Cesa LC, Shao H, Srinivasan SR, *et al*. X-linked inhibitor of apoptosis protein (XIAP) is a client of heat shock protein 70 (Hsp70) and a biomarker of its

inhibition. J Biol Chem, 2018,293(7):2370-2380
12 Van Lookeren Campagne M, LeCouter J, Yaspan BL, *et al*. Mechanisms of age-related macular degeneration and therapeutic opportunities. J Pathol, 2014,232(2):151-164
13 Nguyen CL, Gillies MC, Nguyen V, *et al*. Characterisation of poor visual outcomes of neovascular age-related macular degeneration treated with anti-vascular endothelial growth factor agents. Ophthalmology, 2019, 126(5):735-742
14 Totsuka K, Ueta T, Uchida T, *et al*. Oxidative stress induces ferroptotic cell death in retinal pigment epithelial cells. Exp Eye Res, 2019,181:316-324
15 Bellezza I. Oxidative Stress in Age-Related Macular Degeneration: Nrf2 as Therapeutic Target. Front Pharmacol, 2018,9:1280
16 Hytti M, Piippo N, Salminen A, *et al*. Quercetin alleviates 4-hydroxynonenal-induced cytotoxicity and inflammation in ARPE-19 cells. Exp Eye Res, 2015,132: 208-215
17 Sugano E, Murayama N, Takahashi M, *et al*. Essential role of thioredoxin 2 in mitigating oxidative stress in retinal epithelial cells. J Ophthalmol, 2013,2013:185825
18 Masuda T, Shimazawa M, Takata S, *et al*. Edaravone is a free radical scavenger that protects against laser-induced choroidal neovascularization in mice and common marmosets. Exp Eye Res, 2016,146:196-205
19 Subrizi A, Toropainen E, Ramsay E, *et al*. Oxidative stress protection by exogenous delivery of rhHsp70 chaperone to the retinal pigment epithelium (RPE), a possible therapeutic strategy against RPE degeneration. Pharm Res, 2015,32(1):211-221
20 Carbone DL, Doorn JA, Kiebler Z, *et al*. Inhibition of Hsp72-mediated protein refolding by 4-hydroxy-2-nonenal. Chem Res Toxicol, 2004,17(11):1459-1467
21 Gao C, Yu H, Yan C, *et al*. X-linked inhibitor of apoptosis inhibits apoptosis and preserves the blood-brain barrier after experimental subarachnoid hemorrhage. Sci Rep, 2017,7:44 918
22 Kirkegaard T, Gray J, Priestman DA, *et al*. Heat shock protein-based therapy as a potential candidate for treating the sphingolipidoses. Sci Transl Med, 2016,8(355):355ra118

(Received Jan. 10, 2019; revised Mar. 27, 2019)