Therapeutic effect of Keap1-Nrf2-ARE pathway-related drugs on age-related eye diseases through anti-oxidative stress

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Abstract

- Age-related eye diseases, including cataract, glaucoma, diabetic retinopathy (DR), and age-related macular degeneration (AMD), are the main causes of blindness worldwide¹¹. As a degenerative lesion of the eye, several studies have shown that the occurrence and development of these diseases have an important relationship with oxidative stress in the eye. The Keap1-Nrf2-ARE pathway is a classical pathway that resists oxidative stress and inflammation in the body. This pathway is also active in the development of age-related eye diseases. A variety of drugs have been shown to treat age-related eye diseases through the Keap1-Nrf2-ARE pathway, such as calcium dobesilate (CaD), polyphenols, alpha-lipoic acid (ALA), Lutein/zeaxanthin isomers (L/Zi), probucol and coumarins¹⁶–⁸. This review describes the role of oxidative stress in the development of age-related eye diseases, the function and regulation of the Keap1-Nrf2-ARE pathway, and the therapeutic effects of drugs associated with this pathway on age-related eye diseases.

- KEYWORDS: oxidative stress; Keap1-Nrf2-ARE pathway; age-related eye diseases

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INTRODUCTION

Age-related eye diseases, including cataract, glaucoma, diabetic retinopathy (DR), and age-related macular degeneration (AMD) are the main causes of blindness worldwide¹¹. As a degenerative lesion of the eye, several studies have shown that the occurrence and development of these diseases have an important relationship with oxidative stress in the eye¹–⁴. Keap1-Nrf2-ARE (Kelch-like ECH-Associating protein 1- nuclear factor erythroid 2 related factor 2-antioxidant response element) pathway is a classic pathway to resist oxidative stress in the body. Under normal conditions, Nrf2 binds to Keap1 and is inactivated by Keap1-dependent ubiquitination, thereby remaining at a low level. Under oxidative stress, the cysteine residue of Keap1 is modified and loses ubiquitin activity. Then, Nrf2 transfers to the nucleus, where it binds to ARE and mediates the expression of a series of antioxidant protein genes⁵. Modulation of this pathway has been widely recognized as a new pharmacological target for the treatment of many diseases. In recent years, the role of this pathway in the development of age-related eye diseases has also gained attention among researchers. A variety of drugs have been shown to treat age-related eye diseases or delay the progression through the Keap1-Nrf2-ARE pathway, such as calcium dobesilate (CaD), polyphenols, alpha-lipoic acid (ALA), Lutein/zeaxanthin isomers (L/Zi), probucol and coumarins¹⁶–⁸. This review describes the role of oxidative stress in the development of age-related eye diseases, the function and regulation of the Keap1-Nrf2-ARE pathway, and the therapeutic effects of drugs associated with this pathway on age-related eye diseases.

OXIDATIVE STRESS AND AGE-RELATED EYE DISEASE

Oxidative stress occurs with the interference between the antioxidant and the pro-oxidant⁹. Antioxidant enzymes cannot quench free radicals, causing the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which interact with many macromolecules in the cell. The excessive ROS and RNS injury cellular components including proteins, lipids, and DNA. These changes lead to a variety of diseases, including cancer, aging, cardiovascular and metabolic diseases⁹. In recent years, an increasing number of studies have shown that oxidative stress plays a key role in the occurrence and development of age-related eye diseases, such as cataracts, glaucoma, DR, and AMD⁹.
**Cataract**  Cataract is the leading cause of global vision loss[10]. The etiology is the deposition of aggregated proteins in the lens and plasma membrane injury of lens fiber cells, leading to lens opacity, light scattering, and visual impairment[11]. The main symptoms include decreased vision, glare, and monocular diplopia[12]. The pathogenesis of cataract involves multiple mechanisms and oxidative stress is important in this process[13]. The lens is sensitive to oxidative stress from ultraviolet radiation, metabolic changes, and osmotic pressure[14]. Studies have shown that under oxidative stress, calcium ATPase in the plasma membrane is oxidized and loses its ion-transfer function. Calcium ions cannot be transported out of the lens epithelium, resulting in the activation of the m-calpain. The activated m-calpain causes dysfunction and precipitation of lens proteins[15]. Oxidative stress causes ROS to accumulate in the lens, leading to apoptosis and autophagy of the epithelial cells, and the opacity of lens cortex[15]. Apoptosis associated with diabetic cataract formation occurs in human lens epithelial cells (HLE) after exposed to high glucose-induced oxidative stress for 24h[16]. The expression of let-7c-3p (a miRNA capable of inhibiting apoptosis and autophagy) is also downregulated in HLE[17]. Oxidative stress can also promote the development of cataract by destroying intracellular macromolecules, especially mitochondrial DNA, and disrupting the balance of electrolyte homeostasis[18]. Furthermore, the polyol pathway, which is also an important mechanism for the onset of diabetes, has crosstalk with oxidative stress. The accumulated polyol upregulates the level of aldose reductase enzyme (AR). Excessive consumption of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) by AR inhibits the reduction of oxygen and increases the generation of ROS[13,18].

**Glaucoma**  Glaucoma is a neurodegenerative disease characterized by progressive visual field defects. The pathological changes include progressive loss of retinal ganglion cells (RGCs) and structural changes in the optic papilla and disc[19]. Pathological increase in intraocular pressure (IOP) is an primary risk factor for glaucoma[20]. The common cause of hypertensive glaucoma is the oxidative stress of the trabecular meshwork (TM)[21], which is the classic aqueous outflow pathway and is important in regulation of IOP[22]. ROS causes TM mitochondrial dysfunction, inflammatory factor release, and extracellular matrix (ECM) composition changes, which causing dysfunction of TM[23]. Rotenone is a drug for establishing glaucoma model, it can inhibit mitochondrial electron chain complex I, cause the production of ROS and induce the senescence of human trabecular meshwork (HTM) cells[23]. Defects in mitochondrial complex I have been reported to be associated with TM cell degeneration in patients with primary open angle glaucoma (POAG)[24-25]. Under oxidative stress, F-actin cytoskeletal is reorganized, which changes the morphology and contractile function of TM tissues and increases aqueous outflow resistance[21-26]. Oxidative stress-induced death of RGCs is also one of the pathogeneses of glaucoma[27]. Elevated level of ROS causes protein damage, lipid peroxidation, calcium overload, and mitochondrial dysfunction, ultimately leading to RGCs death and apoptosis[28]. The nuclear protein siruin 6 (SIRT6) promotes Nrf2/ARE signaling and decreases ROS generation via deacetylation of Nrf2, prevents hydrogen peroxide-induced apoptosis in RGCs[29]. Sestrin2 protein (a stress-inducible antioxidant protein) can downregulate Keap1 expression and activate Nrf2, alleviate RGC cell apoptosis under oxidative stress[29]. Lycium barbarum polysaccharides (LBp) protects RGC from chloride (CoCl2)-induced oxidative stress through inhibiting ROS generation and reducing RGC mitochondrial membrane potential[30].

**Diabetic Retinopathy**  DR is a common microvascular complications of diabetes and is also an important cause of non-traumatic blindness in adults[31]. The mechanism is hyperglycemia-induced inflammation and rupture of retinal microvasculature, causing distortion of the microvascular system and ultimately leading to retinal detachment[32-33]. Oxidative stress is the main pathological process of DR[34-35]. Persistent hyperglycemia induces many metabolic abnormalities in the retina, including inflammation, activation of protein kinase C (PKC), activation of hexosamine and polyol pathways[7]. These processes are all associated with mitochondrial overproduction of ROS, ultimately leading to increased oxidative stress[32]. Persistent hyperglycemia elevates the enzymatic activity of arginase. The activated arginase promotes activation of retinal angiogenesis through restoring NO and increasing oxidative stress[32]. Hyperglycemia-induced oxidative stress upregulates the expression of inflammatory factors[32,33] and induce apoptosis of pericytes through stimulating PKC-δ signaling[36]. Several studies demonstrated that hyperglycemia-induced oxidative stress can also increase mitochondrial permeability by opening permeability transition pores (MTP), leading to mitochondrial swelling and cytochrome C release[37]. Cheng *et al.*[31] found that pancreatic kallikrein can significantly improve retinal pathological features in mouse models of type 2 diabetes by decreasing ROS production, downregulating NADPH oxidase 2 (NOX2), and upregulating the antioxidant superoxide dismutase 2 (SOD2). In addition, studies have shown that specific antioxidant supplementation can reduce the rate of DR progression by enhancing antioxidant defense[38-39].

**Age-related Macular Degeneration**  AMD is a cumulative disease of the central retina. It is a major cause of blindness in the elderly and the prevalence increases with age[40]. It is characterized by injury to the retinal pigment epithelium...
(RPE) cells in the macula, leading to foveal sensation damage\textsuperscript{[41]}. RPE degeneration is mainly caused by long-term oxidative stress\textsuperscript{[42]}. Retina is susceptible to oxidative stress on account of the high concentration of oxygen, the exposure to light and the high content of unsaturated fatty acids\textsuperscript{[43-44]}. Excessive ROS and declining antioxidant systems result in oxidative stress, causing damage and apoptosis of RPE cells, photoreceptor cells, and choroidal capillary layers\textsuperscript{[1]}. The mitochondrial DNA in human RPE cells exposed to oxidative stress is damaged and not effectively repaired\textsuperscript{[45]}. The study by Alamri et al\textsuperscript{[46]} showed that the loss of translocator protein (TSPO) can injury RPE cells through affecting multiple metabolism, while TSPO regulates mitochondria-mediated oxidative stress in RPE cells. Oxidative stress also causes lipid peroxidation and protein oxidation. The levels of malondialdehyde (MDA) and advanced oxidation protein products (AOPP) in the blood of AMD patients are higher than normal\textsuperscript{[47]}. The activity of SOD (an antioxidant enzyme which can quench ROS) in the blood of AMD patients also decreases, aggravating oxidative stress\textsuperscript{[42]}. In addition, studies by Sheu et al\textsuperscript{[48]} and Du et al\textsuperscript{[49]} found that the apoptosis of RPE cells can be reduced through inhibiting oxidative stress and activating antioxidant defense.

**KEAP1-NRF2-ARE PATHWAY AND OXIDATIVE STRESS**

To protect macromolecules from the adverse effects of oxidative stress, cells have developed multiple defense systems\textsuperscript{[33]}, as well as a range of antioxidant stress signaling pathways, including the JAK/STAT (Janus kinase-signaling transducer and activator of transcription) signaling pathway, the PI3K/Akt (phosphatidylinositol-3-kinase-protein kinase B) signaling pathway, and Keap1-Nrf2-ARE pathway, etc. The Keap1-Nrf2-ARE signaling pathway plays a central role in the body's antioxidant stress. Studies have shown that, through the expression of Akt protein, the JAK/STAT signaling pathway induces the phosphorylation of Nrf2, which is then translocated to the nucleus, combined with an ARE, and finally activates the transcription of downstream genes\textsuperscript{[49]}. When the PI3K/Akt signaling pathway is activated, phosphorylated Nrf2 can be released from Keap1 and translocated to the nucleus to activate expression of downstream antioxidant proteins\textsuperscript{[50]}. The Keap1-Nrf2-ARE pathway is a multi-organ protection chain that is resistant to both exogenous and endogenous oxidative stress. It protects the body from damage or delays the progression of related diseases and is the main defense mechanism against oxidative stress and inflammation\textsuperscript{[51-52]}. This pathway has already been a research hotspot in the development mechanism of oxidative stress diseases both at home and abroad.

Nrf2 is a transcription factor of the leucine zipper family that mediates the expression of a variety of antioxidant defense protein genes\textsuperscript{[53-54]}. These genes all contain the cis-regulator element sequence ARE (5-GTTACnnnGC-3)\textsuperscript{[55]} as a binding target for Nrf2. Human Nrf2 has six conserved domains, Neh1 to Neh6. A bZip motif in the Neh1 helps Nrf2 form a heterodimer with small Maf proteins, and then heterodimer binds to ARE, activating antioxidant gene transcription. The Neh2 contains a DLG and ETGE motif that binds to the Kelch domain of Keap1 with different affinities, downregulating the transcriptional activity of Nrf2\textsuperscript{[56,57]}. Keap1 is known as a Nrf2 inhibitor (INrf2). It consists of five domains: N-terminal region (NTR), bric-'a-brac domain (BTB), intervening region (IVR), Kelch region (also known as double glycine repeat, DGR), and C-terminal region (CTR)\textsuperscript{[1,57]}. The Keap1 forms a dimer and binds to the Cullin3 (Cul3) through the BTB region\textsuperscript{[57]}. Upon normal circumstances, Nrf2 binds to Keap1\textsuperscript{[58]}, inactivated by Keap1-dependent ubiquitination of Nrf2, and thus remains at a low level\textsuperscript{[5,59]}. The IVR region contains key cysteine residues that can sense oxidative signals and react with endogenous and exogenous electrophiles\textsuperscript{[40-61]}. Under oxidative stress, cysteine residues in IVR are chemically modified by ROS or other electrophiles, preventing the ubiquitination and degradation of the Nrf2 protein. Nrf2 accumulates in the cytoplasm and transfers to the nucleus, where it binds to ARE and mediates a range of gene expressions of antioxidant and cytoprotective proteins\textsuperscript{[44,52,62-63]}, including glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), NAD(P)H : quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCL), and glutathione transferase\textsuperscript{[64,65]}. The Nrf2 protein is known to regulate nearly 200-600 cell protection genes\textsuperscript{[66]} (Figure 1). Regarding this regulation of Keap1 on Nrf2, there are several models explaining its mechanism, and the more widely accepted one is the “hinge and latch” model\textsuperscript{[5]}. In this model, the high-affinity ETGE motif acts as a “hinge” to fix Nrf2 to Keap1, and the low-affinity DLG motif is a “latch”. Upon normal condition, both the ETCG and DLG bind to keap1, and nrf2 is ubiquitinated and degraded\textsuperscript{[67-68]}. Under oxidative stress, the covalently modification of the cysteine residues in Keap1’s BTB and IVR domains cause the low-affinity DLG motif to detach from Keap1, while the high-affinity ETGE motif keeps binding to Keap1\textsuperscript{[10]}. The Keap1-cul3 complex loses ubiquitin ligase activity to Nrf2. Nrf2 accumulates in the cytoplasm and transfers to the nucleus, mediating transcription of downstream genes\textsuperscript{[5,70]} (Figure 2).

**THERAPEUTIC EFFECT OF KEAP1-NRF2-ARE PATHWAY-RELATED DRUGS IN AGE-RELATED EYE DISEASES**

The Keap1-Nrf2-ARE pathway, a major defense mechanism against oxidative stress and inflammation, has been widely recognized as a new pharmacological target for the treatment of...
many diseases, including cancer, neurodegenerative diseases, diabetes, reproductive system diseases, septic kidney injury, prion disease, and osteoarthritis[59,71-76]. As the core pathway against oxidative stress, the role of regulation of this pathway in the treatment of age-related eye diseases has also attracted the attention of researchers. Cheng et al[77] evaluated the level of Nrf2 expression in glaucoma trabecular mesh (GTM) and HTM cells. Nrf2 was found to be downregulated in GTM cells relative to HTM cells, and the following experiments have shown that the over-expression of Nrf2 could stimulate cell proliferation and inhibit apoptosis in both GTM and HTM. Multiple studies have also indicated that upregulation of Nrf2 signaling and reduction of ROS production protect RGCs, retinal epithelial cells, and lenses from oxidative stress[2-3,61,78-80]. A variety of drugs have been proved to treat and protect age-related eye diseases through the Keap1-Nrf2-ARE pathway, including CaD, polyphenols, ALA, L/Zi, probucol, and coumarins.

**Figure 1** Protein domains of Keap1 and Nrf2
Keap1 consists of five domains: NTR, BTB, IVR, Kelch region (also known as DGR), and CTR. The BTB domain can facilitate Keap1 forming a dimer and interacting with Cul3. The DGR and CTR are also known as the DC domain, which is the Keap1- and Nrf2-binding region. Nrf2 contains six Nrf2-ECH homology (Neh) domains. Neh1 contains a CNC-type basic leucine zipper DNA-binding motif, which is required for the heterodimerization of Nrf2 and proteins of the small Maf family. The Neh2 contains DLG and ETGE motifs. They bind to the Kelch domain of Keap1 with different affinities, regulating the activity of Nrf2. The Neh3 domain helps stabilize the Nrf2 protein. Neh4 and Neh5 are related to transcriptional activity. Neh6 contains a serine-rich conserved region that inhibits Nrf2 independent of Keap1.

**Figure 2** The mechanisms involved in the Keap1-Nrf2-ARE pathway: the “hinge and latch” model
The ETGE and DLG motifs in the Neh2 domains bind to Keap1’s DC domain with different affinities. Under normal circumstances, Nrf2 is isolated in the cytosol and keeps a low level through Keap1-dependent ubiquitination and protease degradation. Under oxidative stress, electrophiles (such as ROS) covalently modify the cysteine residues in Keap1’s BTB and IVR domains, resulting in a conformational change in Keap1. The low-affinity DLG motif separates from Keap1, while the high-affinity ETGE motif keeps binding to Keap1. The Keap1-cul3 complex loses ubiquitin ligase activity to Nrf2. Nrf2 accumulates in the cytoplasm and metastasizes to the nucleus, upregulating transcription of downstream genes.

**Calcium Dobesilate**
CaD is widely used as an angioprotective drug in the treatment of DR, diabetic nephropathy, and many other complications of diabetes. It can reduce blood viscosity, platelet activity and capillary permeability by inhibiting a variety of vasoactive substances[81]. Several studies have shown that CaD can inhibit the signaling of vascular endothelial growth factor (VEGF) and the activation of the autophagy-related PI3K/Akt/mTOR pathway, thereby inhibits endothelial cell migration and permeability change, reduces vascular damage in diabetic nephropathy[82-85].
The antioxidant effect of CaD has been demonstrated in several experiments. Jafarey et al. demonstrated that CaD can alleviate the oxidative stress in kidney tissue and protect rats against gentamicin-induced nephrotoxicity. They thought this protective effect of CaD comes from its antioxidant properties. Bogdanov et al. reported that CaD significantly decreased the level of dihydroethidium (DHE) and MDA (two oxidative stress markers), in the retina of db/db mice (a diabetic mouse model). It also inhibited interleukin (IL)-6, IL-8, nuclear factor (NF)-κB, tumor necrosis factor (TNF)-α, and other inflammatory factors expression. Leaf et al. found that CaD can inhibit changes in the level and organization of tight junction protein and leukocyte adhesion to restore excessive vascular permeability in diabetic patients' retina. This protective effect of CaD may be related to the inhibition of p38 mitogen-activated protein kinase (MAPK) and NF-κB activation through alleviating oxidative/nitrosative stress.

Regarding the antioxidant mechanism of CaD, recent studies have shown that this may be related to the Keap1-Nrf2-ARE pathway. Sun et al. found that CaD can increase the level of lens Nrf2 and HO-1, and inhibit the level of Keap1 in diabetic cataract rats, significantly decrease the degree of lens opacity of D-galactose-induced cataract rat model. This suggests that CaD may be able to achieve therapeutic effects by adjusting the oxidant-antioxidant balance of the lens.

**Polyphenols** Polyphenolic compounds are a class of organic substances derived from plants, with several phenol structures in their molecular structure. Polyphenols can be divided into different sub-categories, including four families: flavonoids, tannins, phenolic acids, and anthocyanins. Many of these subtypes have been shown to have anti-inflammatory, antioxidant, anti-allergic, antibacterial, and anti-viral effects. Recent studies have found that polyphenols can prevent the development of age-related eye diseases through the Keap1-Nrf2-ARE pathway. The polyphenol-enriched fraction of Vaccinium uliginosum L. (FH), which contains several flavonoids and anthocyanins, can significantly inhibit the decrease of Nrf2 and HO-1 in the lens of sulphate-induced cataract SD rats. In addition, FH also inhibits the consumption of glutathione (GSH) and the decrease in SOD and GPx activity. Myricetin derivatives from syzygium malaccense is a flavonoid. It activates Nrf2 in ARPE-19 cells, increases the level of the downstream antioxidant protein SOD2, and reduces the production of hydrogen peroxide (H₂O₂) and ROS in cells. It also downregulates the expression of nitric oxide production factor (NOS2) at the transcriptional level, attenuate oxidative stress-induced apoptosis of ARPE-19 cells.

Hesperetin, which also belongs to flavonoid, can prevent H₂O₂-triggered oxidative damage through upregulating the Keap1-Nrf2/HO-1 signaling pathway in ARPE-19 cells. It inhibits ROS production, enhances the level of SOD and GSH, and reduces the formation of malondialdehyde (MDA). Hesperetin may play a role in the treatment of AMD and prevent further development.

Epigallocatechin gallate (EGCG) is a tannin that accounts for more than 50% of the whole tea polyphenols. Short-term supplementation with EGCG has been shown to treat glaucoma by protecting injured retinal neurons due to high IOP. Tea polyphenols can protect the optic nerve by inducing the activation of Nrf2, regulating the activity of antioxidant enzymes and inhibiting the production of ROS. One plausible mechanism of EGCG-induced Nrf2 activation is that modification of cysteine thiols in Keap1 by active EGCG causes Keap1 to lose ubiquitin activity, resulting in activation of Nrf2.

**Alpha-lipoic Acid** ALA is a natural mitochondrial disulfide complex. It is an important coenzyme of pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase. ALA can resist oxidative stress-mediated tissue damage by promoting the formation of endogenous antioxidants via activation of Nrf2. Gu et al. reported that ALA alleviated oxidative damage of the liver induced by microcystins (MCs). ALA can decrease the level of MCs-induced ROS and promote the reduction of oxidized glutathione (GSSG), the oxidation product of GSH to reduced GSH (rGSH). However, knockdown of Nrf2 expression with shRNA remarkably attenuated α-LA-mediated rGSH regeneration. They speculated that Nrf2 is involved in the antioxidant process of ALA. Lee et al. confirmed that ALA can partially restore the functionality of retinal neurocircuitry and significantly improve the visual response properties of RGCs by activating the antioxidant pathway. Several theories explain the mechanism of ALA antioxidants. Some evidences supported ALA form lipoyl-cysteinyln mixed disulfides on Keap1, which increase Nrf2 activity and promote the transcription of downstream antioxidant genes. Koriyama et al. demonstrated that ALA produced H₂O₂, which activated Keap1/Nrf2 signaling in RGC-5 cells, and induced the expression of the downstream antioxidant protein HO-1. Choi et al. established Nrf2-deficient epithelial cell models and found that ALA can still reduce ROS-induced cytotoxicity in these cells regardless of the Nrf2 status. The result suggested that ALA can activate the Nrf2-independent ROS detoxification pathway.

Zhao et al. found that lipoamide (LM), a neutral amide derivative of ALA, is more effective than ALA in protecting...
RPE cells from the acrolein-induced oxidative stress. They found that LM can more potently upregulate the expression of PPARC1a (the pivotal transcriptional coactivator for mitochondrial biogenesis), increasing mitochondrial biosynthesis. It also more effectively induced the expression of Nrf2 and its translocation to the nucleus, leading to an increase in expression of phase II antioxidant enzymes. This may due to the stable structure of LM, and LM has a lower acidity, which makes it more suitable for physiological pH than ALA[97].

**Lutein/zeaxanthin Isomers** L/Zi are oxidized forms of carotenoids (oxyxaroenoids) that belong to natural pigments. In addition to a basic C40 isoprenoid structure similar to other carotenoids, they also have an ionone ring at each end[98]. The concentration of L/Zi in the retina, especially the fovea, is much higher than in other tissues, recommending the significant role of L/Zi in retina function and viability[98]. The Age-Related Eye Disease Study 2 (AREDS2) was a phase 3 study and controlled clinical trial performed by the National Institutes of Health’s Eye Institute, which was conducted from 2006-2012, involving 4203 patients aged 50 to 85y. This study used L/Zi as a dietary supplement and found the risk of progression to advanced AMD has been reduced by 10% in the patients who received L/Zi compared with those who did not[99]. Until now, L/Zi is the only therapeutic intervention for AMD that successfully underwent clinical trials.

Many studies have shown that carotenoids can react with reactive ROS/RNS as a direct antioxidant, but because of the kinetic limitations due to their low scavenging rate constants, they cannot fully exert their antioxidant activity in vivo[100-101]. It is assumed that the major way carotenoids protect cells is inducing endogenous antioxidant defense through the Nrf2-Keap1 pathway[102-103]. Structural-functional studies have demonstrated that an electrophilic α, β-unsaturated carbonyl group (a C=C bond conjugated to -CHO aldehyde group) of the carotenoids or their cleavage and oxidation derivatives can undergo Michael reaction with Keap1, causing modification of redox sensitive SH groups in Keap1, which allows for Nrf2 release into the cytoplasm, activating downstream signaling[100]. Sahin et al[100] reported that supplementation of L/Zi attenuated the retina edema in mice induced by intense light emitting diode (LED) illumination. L/Zi supplementation also reduced the level of MDA and significantly increased the levels of Nrf2 and HO-1 in the retina ONL layer in BALB/cJ mice. ERS can activate the unfolded protein response (UPR)[104]. It has been reported that excessive activation of UPR in the retina of elderly rats can significantly decrease the expression of Nrf2 and its downstream proteins, including rhodopsin and heme oxygenase-1 (HO-1)[105].

**Probucol** Probucol is a bisphenol compound and has anti-inflammatory and antioxidant properties[105-108]. It is a potent free radical scavenger, which can inhibit lipid oxidation and increases low-density lipoprotein (LDL) catabolism[109-110], and is widely used in the treatment of atherosclerosis, xanthoma, and cardiovascular diseases[111-112]. Probucol can increase the expression of endogenous antioxidant enzymes and potentially eliminate oxygen radical, thus mitigating oxidative stress-induced tissue damage[113-115]. Several studies have shown that probucol can treat some eye diseases through antioxidant[109,113-115]. The combined treatment with probucol and insulin can prevent the development of diabetic cataract[116]. Probucol alone decreases the degree of lens opacity and suppresses the increase in protein carbonyls (a marker of reactive oxygen-mediated protein oxidation) in the lens of hyperglycemic rats[115]. The anti-cataract effect of probucol was due to its antioxidant capacity, not by ameliorating hyperglycemia and hyperlipidemia[115]. Experiments have shown that probucol can also inhibit high glucose-induced ROS generation, promote proliferation, and decrease apoptosis in Müller cells[117].

Probucol’s antioxidant effect is related to Keap1/Nrf2 signaling pathway[109,112]. Huang et al[112] demonstrated that probucol can decrease oxidative stress via upregulating the Keap1/Nrf2 signaling pathway, relieve mice cognitive deficits induced by D-galactose. Liu et al[109] found that probucol significantly inhibited retinopathy and retinal neuron apoptosis in streptozotocin (STZ)-induced diabetic mice. It upregulated the expression of Nrf2 in diabetic retina and inhibited the increase of ROS, indicating that probucol may reduce oxidative stress via upregulating the Nrf2 signaling pathway.

Regarding the antioxidant mechanism of probucol, Xiao et al[114] demonstrated that probucol can downregulate the expression of Keap1 in cells and upregulate the expression of nrf2 to alleviate oxidative stress. Huang et al[112] reported that probucol can increase the expression of Nrf2 in the brain of D-galactose-induced cognitive impairment mice, but does not affect the level of Keap1. They demonstrated that probucol can also promote the dissociation of Keap1/Nrf2 complex, preventing Nrf2 from degradation and upregulating gene expression of antioxidant enzymes. The underlying mechanism of this process requires further research.
Treat age-related eye diseases by Keap1-Nrf2-ARE

Coumarins are secondary compounds with a benzopyrone skeleton, which are widely distributed in the fruits, leaves and rhizomes of plants. Coumarins mainly contain 4 major sub-types: simple coumarins, furanocoumarins, pyran-substituted coumarins, and pyranocoumarins. Coumarins have many pharmacological activities including anti-inflammatory, antioxidant, anti-coagulant, and anticancer activities, and have been widely used in complementary and alternative medicine. Several studies have found that coumarins can act as modulators of the Keap1-Nrf2-ARE signaling pathway and regulate oxidative stress in the body. Yao *et al.* found that coumarin derivatives can mitigate high-glucose induced oxidative stress and fibrosis in mesangial cells via activating Nrf2. The experiment of Hassanein *et al.* demonstrated that Escletin (the 6,7-dihydroxy derivative of coumarin) can directly bind to Keap1 and up-regulate Nrf2, restore GSH and SOD activity in kidney cells, and reduce the kidney damage induced by methotrexate. Arora *et al.* demonstrated that Esclenet (the 6,7-dihydroxy derivative of coumarin) can down-regulate Keap1 and up-regulate Nrf2, restore GSH and SOD activity in kidney cells, and reduce the kidney damage induced by methotrexate. Arora *et al.* reported that imperatorin (a coumarin derivative) can induce Nrf2 activation, effectively upregulate the expression of downstream antioxidants, and protect cardiomyocytes from arsenic trioxide-induced damage. Regarding the mechanism by which coumarins regulate the Keap1-Nrf2-ARE pathway, Hassanein *et al.* found that UMB can down-regulate the expression of Keap1 and up-regulate that of Nrf2. They thought that UMB causes the stabilization of Nrf2, thereby accumulating in the nucleus and activating antioxidant and cytoprotective enzymes, terminally inhibiting oxidative damage. Using western blot analysis, Arora *et al.* found a remarkable loss of the interaction between Keap1 and Nrf2 after treatment with esclenet. In vitro experiments also showed that under esclenet treatment, Nrf2 can be released from Keap1, phosphorylated and transported to the nucleus. However, their results showed that esclenet did not change the levels of the two proteins in the cell. Hassanein *et al.* used AutoDockVina 1.5.6 for the molecular docking of coumarin derivatives and Keap1 protein. They found that coumarins can form hydrogen bonds with the side chains of polar, positively and negatively charged amino acids, binding to the binding pocket of Keap1 with high affinity and disrupting the interaction between Keap1 and Nrf2. This mechanism of action is considered to be superior to the modification of cysteine residues in Keap1 for the less toxic. The latter may modify thiol groups of other non-target proteins, leading to unpredictable toxic effects.

In recent years, many researches have studied the therapeutic effects of coumarins in ophthalmic diseases. Kim *et al.* found that escletin reduced activity of rat lens aldose reductase *in vitro* and inhibited the occurrence of galactose-induced cataract in rats. Angapelly *et al.* synthesized 4-sulfamoylphenyl/sulfocoumarin benzamides and evaluated its inhibitory effect on carbonic anhydrase. The results showed that this coumarin derivative can effectively inhibit carbonic anhydrase subtypes hCAII and XII. They thought the molecular structure of this compound can be further modified to develop effective carbonic anhydrase inhibitors for the treatment of epilepsy, cancer and glaucoma. Bucolo *et al.* found that cloricromene, a novel coumarin derivative, can reduce retinal inflammation in diabetic rats and preserve the blood-retinal barrier. However, there are few studies on the anti-oxidative stress ability of coumarins in the treatment of ophthalmic diseases. Ozal *et al.* reported that esclenet can reduce the inflammatory response of human RPE cells induced by lipopolysaccharide. They found that esclenet can inhibit NF-kB activation, extracellular signal-regulated kinase (ERK)1/2 phosphorylation and decrease the level of VEGF to undermine inflammation and oxidative stress. They also found that esclenet can significantly up-regulate the expression of GPx and SOD2, which are downstream antioxidant enzymes of Nrf2. While they did not conduct further studies on whether this upregulation is through the Keap1-Nrf2-ARE pathway. Considering the ability of coumarins to regulate the Keap1-Nrf2-ARE pathway in other tissues and the crosstalk between the inflammation pathway and the anti-oxidative stress pathway, we believe that the ability of coumarins to regulate the Keap1-Nrf2-ARE pathway may take an important role in its treatment of ophthalmic diseases.

**DISCUSSION**

Age-related eye diseases, such as cataracts, AMD, glaucoma, and DR are the leading causes of progressive and irreversible vision loss all over the world. Although there are several surgical operations, such as intraocular lens (IOL) implantation, aqueous drainage device implant, and vitrectomy to treat these diseases, complications caused by surgery, including inflammation, hyphema, and corneal edema, cannot be ignored. Therefore, it is necessary to find new therapeutic pathways and drug targets.

As an important mechanism of age-related eye diseases, oxidative stress has become a new research hotspot. Keap1-Nrf2-ARE, a classical pathway against oxidative stress and inflammation, plays a key role in combating oxidative stress and protecting the body from damage. It has been recognized as a new pharmacological target for the treatment of many diseases. However, the specific mechanism of antioxidants related to this pathway at the molecular level has yet to be further explored. It is currently known that probucol can upregulate the expression of Nrf2 and promote the
transcription of downstream antioxidant genes, but whether it affects the expression of keap1 is controversial\textsuperscript{[112,114]}. EGCG, a tea polyphenol, is thought to modify cysteine thiols in Keap1, causing Keap1 to lose ubiquitin activity\textsuperscript{[1]}. The mechanism of other phenols antioxidant effect remains unclear. We assume that the mechanism by which other phenols with similar structure to tea polyphenols activate Keap1-Nrf2 pathway is similar to that of EGCG.

In addition to activating the keap1-Nrf2-ARE pathway, many antioxidants can also affect inflammatory, apoptosis, autophagy pathways to protect the body. CalD can inhibit the activation of the autophagy-related PI3K/Akt/mTOR pathway\textsuperscript{[82-83]}, as well as downregulate the p38 MAPK/NF-κB inflammatory pathway through decreasing oxidative stress\textsuperscript{[84]}. FH can inhibit the transcriptional activity of NF-κB and AP-1 induced by oxidative stress\textsuperscript{[132]}. L/Zi alleviates inflammation by quenching free radicals and blocking NF-κB pathway\textsuperscript{[105]}. It can also reduce light-induced ERS in the retina of BALB/cJ mice, inhibit the activation of UPR and apoptosis\textsuperscript{[104]}. Coumarins have been proven to be able to inhibit the activation of NF-κB, and have anti-inflammatory, anti-cancer biological activities\textsuperscript{[120-123]}. Moreover, metformin, which is widely used as a first-line medication for type 2 diabetes for decades, has been found that it can active nrf2 through AMPK/Sirt1 pathway and reduce oxidative stress in ARPE-19 cells induced by glyoxal. Metformin has been proposed as a candidate drug for AMD by both preclinical and clinical studies\textsuperscript{[133]}. We speculate that this is because Keap1-Nrf 2-ARE is not a single antioxidant stress pathway, but has crosstalk with multiple pathways related to inflammation, apoptosis, and autophagy. The NF-κB pathway, which is widely present in a variety of metazoan cells, is a classic inflammation pathway and is critical in the regulation of many physiological and pathological processes including apoptosis, viral replication, tumorigenesis and inflammation\textsuperscript{[134-135]}. NF-κB is normally linked to the inhibitory protein IκB and remains an inactive state. Under the stimulation of pro-inflammatory substances, rapid phosphorylation of IκB by IκB kinase (IKK) causes IκB to dissociate from NF-κB and subsequently NF-κB translocate to the nucleus. In the nucleus, NF-κB induces the transcription of a variety of inflammatory factors including IL-6, COX-2, iNOS\textsuperscript{[136-137]}. Keap1 can degrade IKKβ through ubiquitination, thereby inhibiting the activity of NF-κB\textsuperscript{[138]}. The IKK complex is not only the key to NF-κB activation, but also an interface for crosstalk between NF-κB pathways and other physiological processes. Several inflammatory mediators produced in the inflammatory process can react with Keap1 and activate Nrf2, causing downstream gene transcription initiating and inhibition of NF-κB activity\textsuperscript{[134]}. NF-κB can also bind to the Nrf2 competitive transcription co-activator CBP\textsuperscript{[134]}.

However, the study of Lampiasi and Montana\textsuperscript{[136]} showed that IKK/NF-κB did not affect the transcription and nuclear translocation of Nrf2 in LPS-stimulated RAW 264.7 cells. Chen et al\textsuperscript{[139]} found human aortic endothelial cells treated with adenovirus vector Nrf2 can inhibit NF-κB downstream genes without affecting the activity of NF-κB. Therefore, whether there is crosstalk between Keap1-Nrf2-ARE pathway and NF-κB pathway remains to be further studied. The PI3K/Akt signaling pathway plays a key role in the regulation of apoptosis and autophagy\textsuperscript{[140-141]}. It protects cells from apoptosis and autophagy through phosphorylation of Akt\textsuperscript{[142]}. Besides activating Akt phosphorylation, PI3K can also promote nuclear translocation of Nrf2, increase the expression of downstream target genes of Nrf2 and enhance cell antioxidant capacity, thus promotes cell survival\textsuperscript{[143]}. PI3K inhibitors can block the nuclear translocation of Nrf2 and the induction of stress proteins, indicates that the crosstalk between PI3K/Akt and Nrf2 pathway is very important to the cellular defense antioxidant system\textsuperscript{[144]}. In addition, some antioxidants, such as dimethyl fumarate, have been reported to have some systemic side effects, such as lymphopenia\textsuperscript{[52]}.

Potent antioxidant drugs are usually hydrophobic and have low water solubility\textsuperscript{[110]}. Probucol is a non-ionizable, highly lipophilic, hydrophobic drug compound. It is the most poorly soluble hydrosoluble antioxidant with an oral bioavailability <10\textsuperscript{[146]}. Mooranian et al\textsuperscript{[147]} found that using ursodeoxycholic acid (UCDA) for the nano and micro encapsulation of probucol can improve probucol pharmacological properties, enhance probucol delivery and biological effects. Tale et al\textsuperscript{[148]} used diblock terpolymers as an excipient platform to increase the solubility of probucol. Evidence suggested that at the same concentration, LM, a neutral amide derivative of ALA, can more effectively attenuate H2O2 or 6-hydroxydopamine-induced oxidative injury to PC12 cells than ALA\textsuperscript{[96]}. This is considered to be due to the presence of the -CONH2 structure in LM. The resonance stabilization of CO-N helps stable LM. These researches suggest that we can improve bioavailability and drug efficacy through encapsulating or modifying drugs or adding excipients.
In conclusion, Keap1-Nrf2-ARE, an important pathway against oxidative stress, can be used as a new drug target for the treatment of age-related eye diseases. Further research is needed to determine the specific mechanism of drugs involved in this pathway inside and outside the cell. How to clear drug toxicity, how to avoid or reduce side effects, and how to modify the molecular structure of the drug to improve efficacy may be future research directions.

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