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α-Tocopherol is an effective Phase II enzyme inducer: protective effects on acrolein-induced oxidative stress and mitochondrial dysfunction in human retinal pigment epithelial cells☆

Zhihui Feng[a,b], Zhongbo Liu[a,b], Xuesen Li[a,b], Haiqun Jia[a,b], Lijuan Sun[c], Chuan Tian[a,b], Lihong Jia[d], Jiankang Liu[e,*]

[a]Institute for Nutritional Science, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[b]Graduate School of the Chinese Academy of Sciences, Beijing 100081, China
[c]College of Physical Education and Health, East China Normal University, Shanghai 200241, China
[d]Department of Nutrition and Food Hygiene, School of Public Health, China Medical University, Shenyang 110001, China
[e]Institute of Mitochondrial Biology and Medicine, The Key Laboratory of Biomedical Information Engineering of Ministry of Education, Xi'an Jiaotong University School of Life Science and Technology, Xi'an 710049, China

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Abstract

Vitamin E has long been identified as a major lipid-soluble chain-breaking antioxidant in mammals. α-Tocopherol is a vitamin E component and the major form in the human body. We propose that, besides its direct chain-breaking antioxidant activity, α-tocopherol may exert an indirect antioxidant activity by enhancing the cell's antioxidant system as a Phase II enzyme inducer. We investigated α-tocopherol's inducing effect on Phase II enzymes and its protective effect on acrolein-induced toxicity in a human retinal pigment epithelial (RPE) cell line, ARPE-19. Acrolein, a major component of cigarette smoke and also a product of lipid peroxidation, at 75 μmol/L over 24 h, caused significant loss of ARPE-19 cell viability, increased oxidative damage, decreased antioxidant defense, inactivation of the Keap1/Nrf2 pathway, and mitochondrial dysfunction. ARPE-19 cells have been used as a model of smoking- and age-related macular degeneration. Pretreatment with α-tocopherol activated the Keap1/Nrf2 pathway by increasing Nrf2 expression and inducing its translocation to the nucleus. Consequently, the expression and/or activity of the following Phase II enzymes increased: glutamate cysteine ligase, NAD(P)H:quinone oxidoreductase 1, heme-oxygenase 1, glutathione S-transferase and superoxide dismutase; total antioxidant capacity and glutathione also increased. This antioxidant defense enhancement protected ARPE-19 cells from an acrolein-induced decrease in cell viability, lowered reactive oxygen species and protein oxidation levels, and improved mitochondrial function. These results suggest that α-tocopherol protects ARPE-19 cells from acrolein-induced cellular toxicity, not only as a chain-breaking antioxidant, but also as a Phase II enzyme inducer.

Keywords: Mitochondrial complex; Protein oxidation; Nuclear factor-E2-related factor 2 (Nrf2); Glutamate cysteine ligase (GCL); NAD(P)H:quinone oxidoreductase 1 (NQO1); Glutathione

1. Introduction

It has been more than 80 years since vitamin E was discovered in 1922 [1]. Originally, Evans and Bishop [2] concluded that "natural foods, as opposed to purified diets contained a substance not needed for normal growth, but essential for reproduction." In the following years, vitamin E studies were focused on its related antisterility function. Until 1936, Olcott from the Mattill group and Emerson from Evans' group described α-, β- and γ-tocopherols and their allophanates as being effective antioxidants for lard [3]. The discovery of vitamin E's antioxidant effects led to more research on its properties; up to now the components of vitamin E have been determined to include four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ) [4]. All of these have antioxidant activity *in vitro*. α-Tocopherol transfer protein is mainly expressed in liver; its highest affinity is for α-tocopherol relative to the other tocopherols and tocotrienols [5]. Therefore, of these eight compounds α-tocopherol is the major form in the human body.

The antioxidant function of α-tocopherol is defined in terms of its role as a peroxyl radical scavenger that terminates the chain reactions that oxidize polyunsaturated fatty acids [6]. This process has been

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* Corresponding author. Institute of Mitochondrial Biology and Medicine, Xi'an Jiaotong University School of Life Science and Technology, Xi'an 710049, China. Tel.: +85 29 8266 4232.
E-mail address: j.liu@mail.xjtu.edu.cn (J. Liu).

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described in great detail but α-tocopherol increasingly appears to be involved in molecular gene regulation in ways that cannot be accounted for by its antioxidant power [7]. α-Tocopherol was found to inhibit protein kinase C activity and vascular smooth muscle cell growth, and to diminish adhesion molecule, collagenase and scavenger receptor (SR-A and CD36) expression [5,7]. It has been indicated that there is a complex interplay between α-tocopherol and other antioxidant system components in human fibroblast cultures [8]. The reduction in NF-kappaB activation and inducible nitric oxide synthase expression, and the enhancement of PPAR-alpha and carnitine palmitoyl transferase gene expression by α-tocopherol may be relevant for cell survival [9]. Little by little, more and more molecular functions of α-tocopherol are revealed which are far different from the antioxidant activity discussed in textbooks. Even with respect to its antioxidant activity, we hypothesize that α-tocopherol exerts this activity not merely through peroxy radical scavenging, but also, even mostly, by activating additional antioxidant systems in the human body.

Cigarette smoke is now considered a strong risk factor for the development of age-related macular degeneration (AMD) [10,11]. Acrolein, a major component of the gas phase of cigarette smoke and also a product of lipid peroxidation *in vivo*, has been shown to be a mitochondrial toxicant that causes mitochondrial dysfunction in isolated liver mitochondria [12]. In primary hfRPE and ARPE-19 cells, acrolein was also shown to cause (1) significant loss of cell viability, (2) oxidative damage (*i.e.*, increases in oxidant generation and oxidative damage to proteins and DNA, decreases in antioxidants and antioxidant enzymes), (3) inactivation of the Keap1/Nrf2 pathway and (4) mitochondrial dysfunction (*i.e.*, decreases in membrane potential, activities of mitochondrial complexes, viable mitochondria, oxygen consumption and factors for mitochondrial biogenesis, and an increase in calcium) [13–15]. Therefore, acrolein-induced cellular oxidative mitochondrial dysfunction in retinal pigment epithelial (RPE) cells has been used as a cellular model to screen for effective antioxidants and mitochondrial protecting agents, including alpha-lipoic acid [14], hydroxytyrosol [13] and lipoamide [15].

Vitamin E has been used for treating AMD or other RPE degeneration. The strongest evidence showing a preventive effect of antioxidants on AMD is the Age-Related Eye Disease Study (AREDS). Compared to supplementation with placebo, researchers found a statistically significant reduction in the development of advanced AMD by antioxidants (vitamin C, 500 mg; vitamin E, 400 IU; and beta carotene, 15 mg), plus zinc (80 mg, as zinc oxide) by as much as 25% ($P<.01$) [16,17]. A pilot study showed that a combination of antioxidants and mitochondrial metabolites (acetyl-L-carnitine, polyunsaturated fatty acids, coenzyme Q10 and vitamin E) improves retinal function in early AMD [18]. In another study involving 976 subjects, fasting plasma levels of retinol, ascorbic acid, α-tocopherol and beta-carotene were measured and the degree of AMD determined; logistic regression analyses suggested that α-tocopherol is associated with a protective effect for AMD and that an antioxidant index, including ascorbic acid, α-tocopherol and beta-carotene, is also protective for AMD [19]. We propose that, besides its direct chain-breaking antioxidant activity, α-tocopherol may exert antioxidant activity by enhancing the cell's antioxidant system as a Phase II enzyme inducer. Therefore, in the present study, we have used this cellular model to investigate the new function of α-tocopherol as an inducer of Phase II enzymes.

2. Materials and methods

2.1. Chemicals

Acrolein was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DL-all-*rac*-α-Tocopherol was purchased from Sigma (St. Louis, MO, USA). Antibodies against Nrf2 and heme oxygenase 1 (HO-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-glutamate cysteine ligase (GCL) was purchased from NeoMarkers (Fremont, CA, USA). Anti-NAD(P)H:quinone oxidoreductase 1 (NQO1) was purchased from Cell Signaling Technology (MA, USA).

2.2. Cell culture

The human ARPE-19 cell line was obtained from Dr. Nancy J. Philip and was cultured according to her methods [20]. The ARPE cells were maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 3–4 days. ARPE-19 cells were used within 10 generations.

2.3. Acrolein exposure and α-tocopherol supplementation

All experiments were performed with an 80% confluent monolayer. Acrolein was dissolved in DMEM-F12 medium right before each experiment [14]. For toxicity experiments, cells were exposed to acrolein for 24 h [14]. α-Tocopherol was dissolved in ethanol. The highest final ethanol concentration was ≤0.02% and preliminary experiments showed it had no apparent effect. The protective effects of α-tocopherol were studied with an acute toxicity model by pretreating cells with α-tocopherol for 48 h.

2.4. Crystal violet assay for cell viability

Cells were cultured in 96-well plates. After treatments, cells were washed twice with PBS and then fixed with 4% formaldehyde for 30 min. After discarding the formaldehyde, 0.1% crystal violet was used to stain the cells for 10 min; then, the excess stain was washed away and the crystal violet bound to the cells was dissolved with 10% acetic acid. Optical densities of crystal violet were measured at 570 nm with a microplate spectrophotometer (Spectra Max 190; Molecular Devices).

2.5. JC-1 assay for mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was assessed in live APRE-19 cells using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [21]. For quantitative fluorescence measurements, cells were rinsed once after JC-1 staining and scanned with a multilabel counter (Wallac 1420; PerkinElmer Life Sciences, Wellesley, MA, USA) at 485 nm excitation, and 535- and 590 nm emission, to measure green and red JC-1 fluorescence, respectively. Each well was scanned by measuring the intensity of each of 25 squares (of 1 mm^2 area) arranged in a 5×5 rectangular array (bottom scanning).

2.6. MTT assay for mitochondrial dehydrogenase activity

The ARPE-19 cells were seeded at 4×10^4 per well in a 96-well plate. Cells were pretreated with different concentrations of α-tocopherol for 48 h and then treated with 75 μM acrolein for 24 h. The MTT assay was used as a qualitative index of mitochondrial dehydrogenase activity. The optical densities were read at 555 nm using a microplate spectrophotometer (Spectra Max 340, Molecular Devices, Sunnyvale, CA, USA) [22].

2.7. Assay for the activities of mitochondrial complexes

NADH-ubiquinone reductase (Complex I), succinate-CoQ oxidoreductase (Complex II), ubiquinol cytochrome *c* reductase (Complex III) and Mg^{2+}-ATPase (Complex V) were measured spectrometrically using conventional assays as described [12,23].

2.8. Determination of reactive oxygen species generation

The generation of intracellular reactive oxygen species (ROS) was determined by fluorescence of 2′,7′-dichlorofluorescein (DCF), upon oxidation of the nonfluorescent, reduced DCFH [24]. The fluorescence intensity of the supernatant was measured with a plate reader (Wallac; PerkinElmer) at 485 nm excitation and 535 nm emission. Cellular oxidant levels were expressed as relative DCF fluorescence per microgram of protein (BCA method).

2.9. Protein carbonyl detection

Protein carbonyls in soluble proteins were assayed with the Oxyblot protein oxidation detection kit (Cell Biolabs, San Diego, CA, USA). Protein carbonyls were labeled with 2,4-dinitrophenylhydrazine and detected by Western blot.

2.10. Total antioxidant capacity

The total antioxidant capacity was assayed with a commercially available assay kit (Jiancheng Biochemical, Inc., Nanjing, China). The principle of the test is to measure the color change upon reduction of Fe^{3+} to Fe^{2+} by the reducing components in the sample. The reducing components may include enzymatic and nonenzymatic molecules such as the lipid-soluble antioxidant vitamin E and the water-soluble antioxidants vitamin C,

uric acid, bilirubin, thiols and glutathione, *etc*. The optical density was measured at 520 nm by a microplate reader.

2.11. Intracellular GSH measurement

GSH levels were measured with 2,3-naphthalenedicarboxyaldehyde (NDA) by a published method [25]. A 20-μl sample and 180 μl of NDA derivatization solution [50 mM Tris (pH 10), 0.5N NaOH and 10 mM NDA in Me_2SO, v/v/v 1.4:0.2:0.2] were added to each well of a 96-well plate. The plate was covered to protect the wells from room light and allowed to incubate at room temperature for 30 min. The NDA-GSH fluorescence intensity was measured (472 ex/528 em) with a fluorescence plate reader (Wallac 1420; PerkinElmer Life Sciences, Wellesley, MA, USA).

2.12. Superoxide dismutase measurement

Intracellular superoxide dismutase (SOD) activity was assayed with a commercially available assay kit (Jiancheng Biochemical, Inc., Nanjing, China) using a xanthine and xanthine oxidase system to produce superoxide. Superoxide oxidizes hydroxylamine to nitrite to form a carmine color agent. The optical density at 550 nm was measured by a microplate reader.

2.13. NQO1 activity assay

NQO1 activity was measured as the dicoumarol-inhibitable fraction of 2,6-dichlorophenolindophenol (DCPIP) reduction in the cell cytosol in the presence or absence of activators of NQO1. DCPIP was used as the electron acceptor and its reduction was measured at 30°C for 1–2 min at 600 nm ($=21\times10^3\ M^{-1}\ cm^{-1}$) with or without 10 μmol/L dicoumarol. NQO1 activity is considered to be the dicoumarol-inhibitable part of DCPIP reduction. Activities are presented as micromoles of DCPIP reduced per minute per milligram of protein [26].

2.14. Glutathione S-transferase activity assay

Cells were cultured in six-well plates. After treatments, cells were lysed ultrasonically in 10 mM sodium phosphate buffer, pH 6.5. The total protein contents of the cell lysate were quantified by the BCA method. The glutathione S-transferase (GST) activity was measured with 5 mg protein, 1 mM GSH, 1 mM chloro-2,4-dinitrobenzene, 3 mg/ml bovine serum albumin in 10 mM sodium phosphate buffer. The mixture was scanned at 340 nm for 5 min at 25°C as previously described [27].

2.15. Western blots for expression of Nrf2, NQO1, GCL and HO-1

Samples were lysed with Western and IP lysis buffer (Beyotime, Jiangsu, China). The lysates were homogenized and the homogenates were centrifuged at 13,000×*g* for 15 min at 4°C. The supernatants were collected and protein concentrations were determined with the BCA Protein Assay kit (Pierce 23225). Equal aliquots (20 μg) of protein samples were applied to 10% SDS-PAGE gels, transferred to pure Nitrocellulose Membranes (PerkinElmer Life Sciences, Boston, MA, USA) and blocked with 5% nonfat milk. The membranes were incubated with anti-Nrf2 (1:500 Santa Cruz), anti-HO-1 (1:500 Santa Cruz), anti-NQO1 (1:1000 Cell Signaling), anti-histone H1 (1:1000 Upstate), anti-GCL (1:1000 NeoMarkers) or anti-β-actin (1:10000 Sigma) at 4°C overnight. Then the membranes were incubated with anti-rabbit or anti-mouse antibodies at room temperature for 1 h. Chemiluminescent detection was performed by an ECL Western blotting detection kit (Pierce).

2.16. Statistical analysis

Data are presented as means±S.E.M. Statistical significance was evaluated with one-way ANOVA followed by LSD *post hoc* analysis. In all comparisons, the level of significance was set at $P<.05$.

3. Results

3.1. Effect on cell viability of α-tocopherol pretreatment prior to acrolein exposure

Cell viability was assayed by the crystal violet method. α-Tocopherol had no obvious effect on cell viability. Acrolein (75 μM, 24 h) induced significant ARPE-19 cell death, while α-tocopherol pretreatment showed significant protection against the acrolein-induced decrease in cell viability at 1, 10 and 50 μM (Fig. 1).

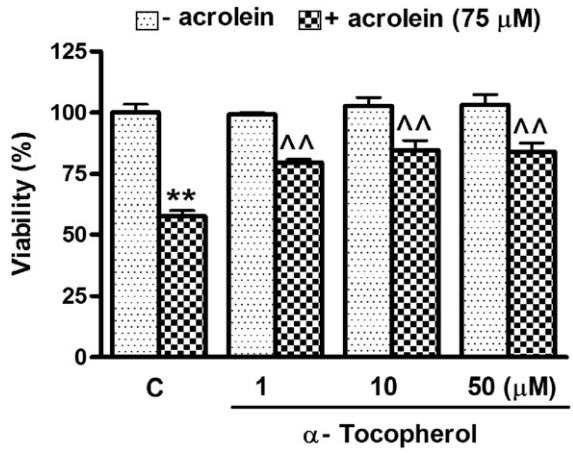

Fig. 1. Effects of α-tocopherol (α-T) on acrolein-induced decreases in cell viability. ARPE-19 cells were pretreated with α-tocopherol for 48 h and then exposed to an acrolein challenge (75 μM, 24 h). Cell viability was assayed with crystal violet. Values are mean±S.E.M. from three independent experiments, three wells each. **$P<.01$ vs. control (no acrolein, no α-T); ^^$P<.01$ vs. acrolein.

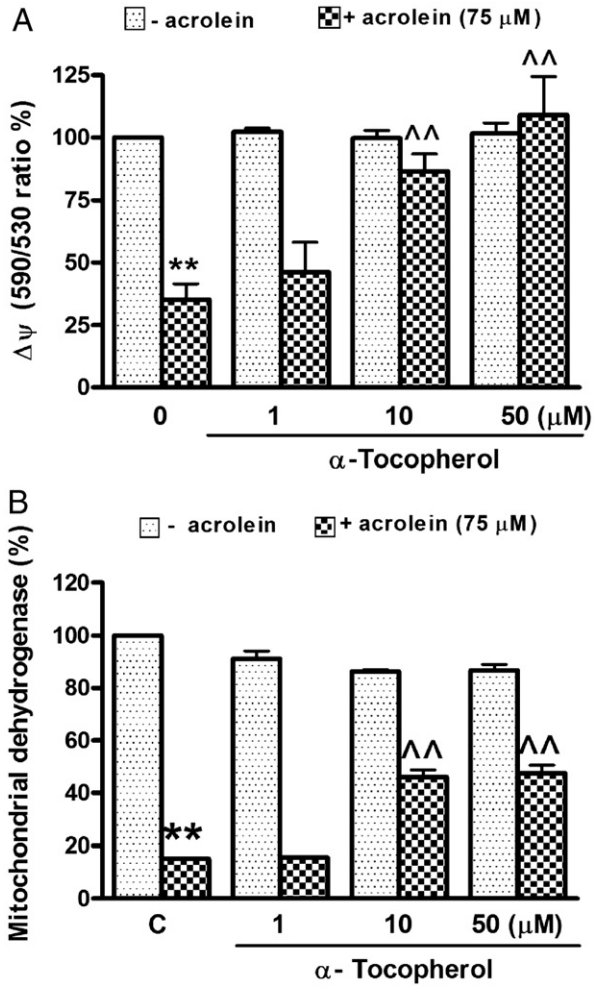

Fig. 2. Effects of α-tocopherol (α-T) on acrolein-induced decreases in MMP and mitochondrial dehydrogenases. ARPE-19 cells were pretreated with α-tocopherol for 48 h and then exposed to an acrolein challenge (75 μM, 24 h). (A) Mitochondrial membrane potential was assayed by JC-1 staining. Data are red/green (590/530 nm) fluorescence ratios. (B) Mitochondrial dehydrogenases assayed with MTT assay; expressed as milli-OD. Results are mean±S.E.M. of three independent experiments, in eight wells each. **$P<.01$ vs. control (no acrolein, no α-T); ^^$P<.01$ vs. acrolein.

3.2. Effect on mitochondrial function of α-tocopherol pretreatment prior to acrolein exposure

Mitochondrial membrane potential (MMP) is an important index of mitochondrial function, which is closely related to ATP production. Acrolein (75 μM, 24 h) caused a decrease in MMP of about 60%. α-Tocopherol pretreatment at 1 μM showed no protection. Both 10 and 50 μM α-tocopherol pretreatments showed significant protection (Fig. 2A).

Since the MTT assay depends on mitochondrial dehydrogenase activity for MTT conversion and these enzymes would possibly be damaged by acrolein, we have used the MTT assay as an index of mitochondrial function. Similar to the JC-1 assay for MMP, 1 μM α-tocopherol pretreatment showed no protection, 10 μM pretreatment began to show protection and 50 μM showed an effect similar to 10 μM pretreatment (Fig. 2B). Since the crystal violet, MTT and JC-1 assays all showed significant protection at α-tocopherol concentrations of 10 and 50 μM, we have chosen these two concentrations (primarily 10 μM) for use in subsequent experiments.

To further investigate the effects of acrolein and α-tocopherol on mitochondrial function, we assessed the activities of the mitochondrial respiratory chain enzyme complexes. Activities of Complex I, Complex II, Complex III, and Complex V were measured. Acrolein induced a significant loss in activity of Complexes I, II and V ($P<.01$), and 50 μM α-tocopherol pretreatment efficiently prevented these acrolein-induced activity losses ($P<.01$, $P<.05$) (Fig. 3A, B, D). Unlike the activities of Complexes I, II and V, the activity of Complex III was unaffected by acrolein treatment (Fig. 3C). However, 50 μM α-tocopherol pretreatment caused a significant increase in the activities of both Complex II (30%, Fig. 3B) and Complex III (70%, Fig. 3C).

3.3. Effects of α-tocopherol pretreatment on acrolein-induced ROS production and protein oxidation

Acrolein treatment induced ROS generation significantly ($P<.01$). Ten-micromolar α-tocopherol pretreatment effectively inhibited the acrolein-induced ROS generation ($P<.01$) (Fig. 4A).

Similar to the case of ROS production, acrolein caused a significant increase in protein carbonyls ($P<.01$), which was also significantly reduced by 10 μM α-tocopherol pretreatment ($P<.01$) (Fig. 4B and C).

3.4. Effects on total antioxidant capacity and GSH of acrolein exposure and α-tocopherol pretreatment

Acrolein treatment greatly reduced total antioxidant capacity (T-AOC), compared to control ($P<.01$). α-Tocopherol pretreatment showed no statistically significant effect on T-AOC, but after acrolein treatment, compared with both control and acrolein-only conditions, α-tocopherol pretreatment significantly increased T-AOC ($P<.01$) (Fig. 5A).

GSH is the most important endogenous small molecule antioxidant and its production is controlled by GCL, one of the Phase II enzymes. Similar to the results of T-AOC, acrolein exposure caused a

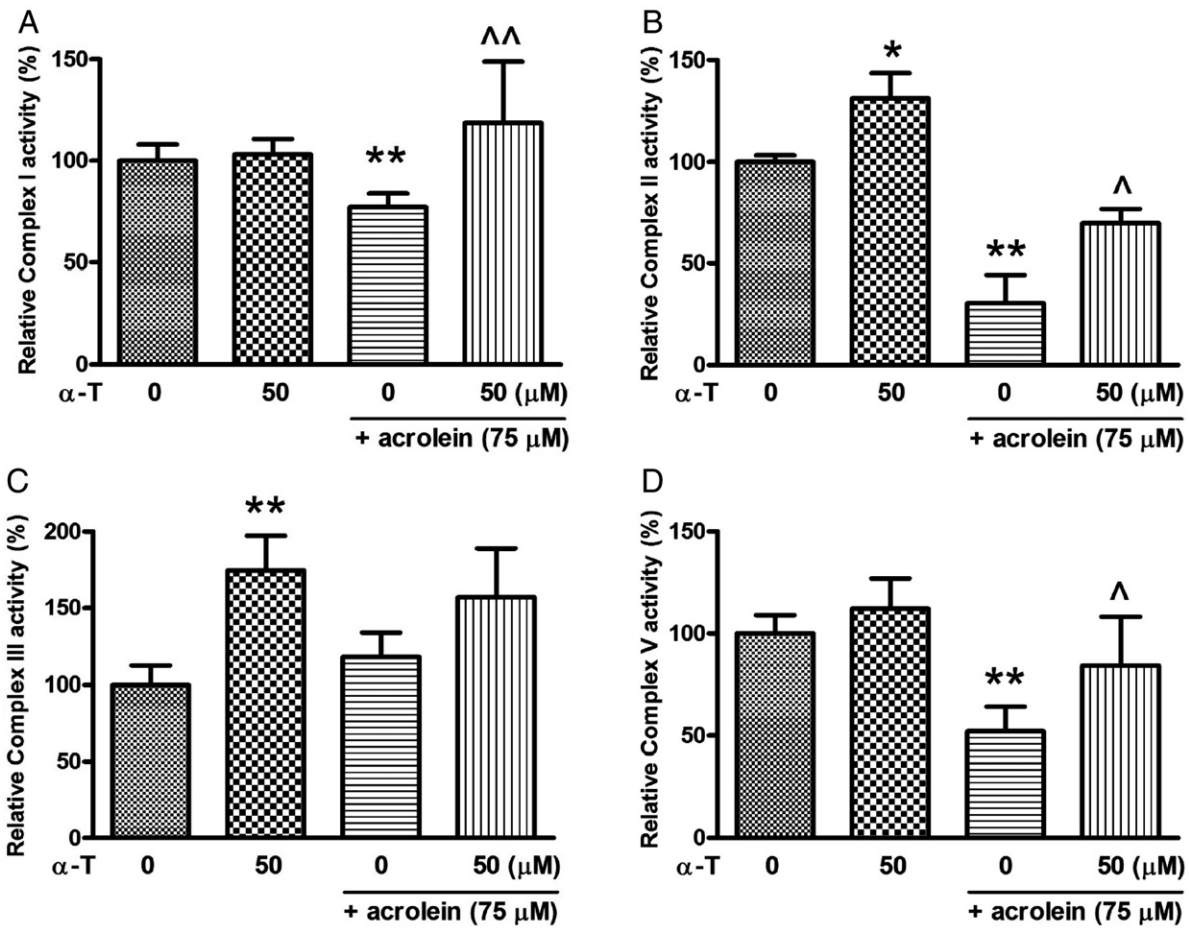

Fig. 3. Effects of α-tocopherol (α-T) on acrolein-induced changes in mitochondrial enzyme complex activities. ARPE-19 cells were pretreated with α-tocopherol (50 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Complex I, (B) Complex II, (C) Complex III, (D) Complex V. Values are mean±S.E.M. of data from three separate experiments; each experiment was performed in triplicate. *$P<.05$ and **$P<.01$ vs. control (no acrolein, no α-T). ^$P<.05$ and ^^$P<.01$ vs. acrolein.

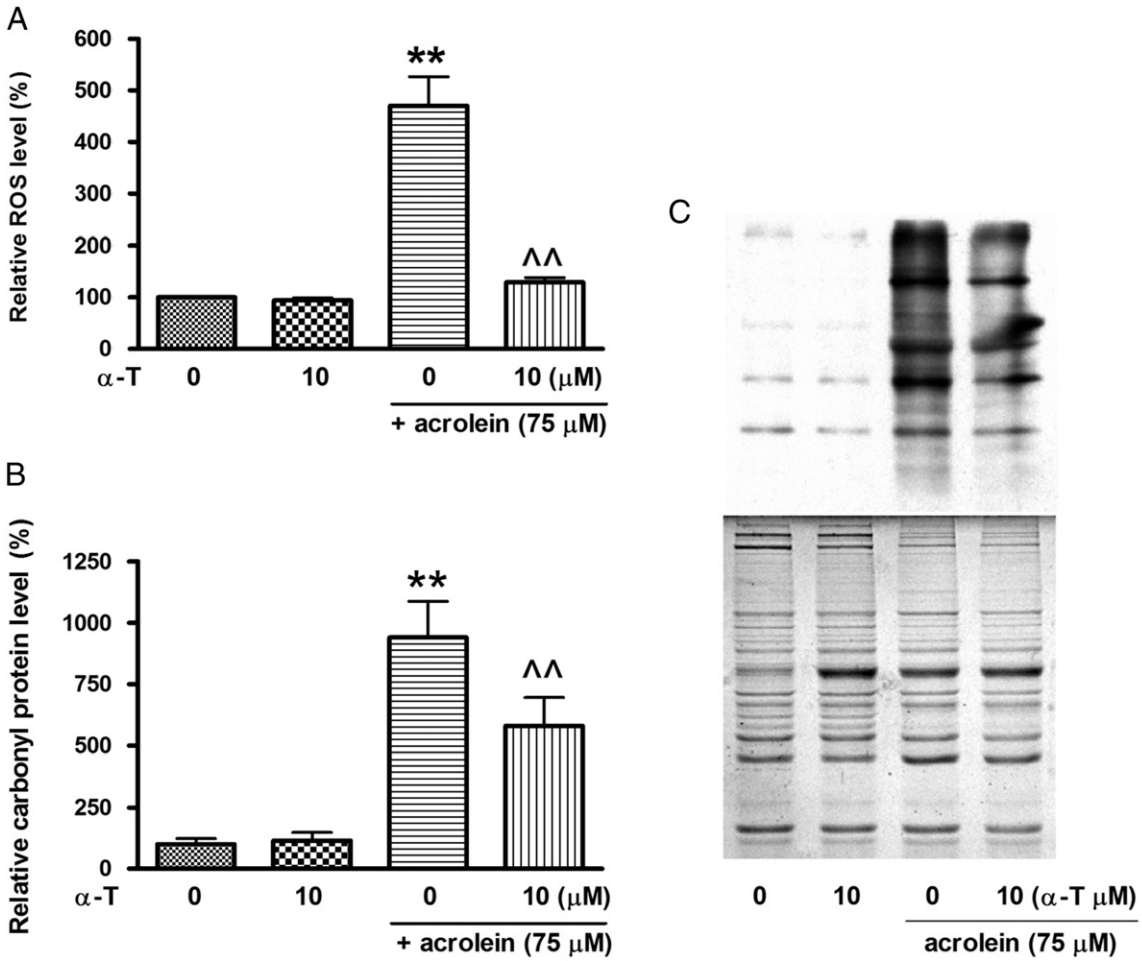

Fig. 4. Effects of α-tocopherol (α-T) on acrolein-induced increases in ROS and protein carbonyl levels. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) ROS generation tested with DCF-DA staining expressed as ratio relative to controls. Values are mean±S.E.M. of five independent experiments. ^^P<.01 vs. control (no acrolein, no α-T); **P<.01 vs. acrolein. (B) Protein carbonyl quantitative optical density results. Values are mean±S.E.M. of three independent experiments. ^^P<.01 vs. control (no acrolein, no α-T); **P<.01 vs. acrolein. (C) Western blot of protein carbonyls (upper panel) and total protein as a loading control (lower panel) stained with Coomassie Blue.

significant decrease in GSH levels (P<.05) and compared with both control and acrolein-only conditions, α-tocopherol pretreatment significantly increased GSH levels (P<.01) (Fig. 5B).

3.5. Effects of α-tocopherol pretreatment and acrolein exposure on Nrf2 expression and nuclear Nrf2 translocation

We have detected the Nrf2 expression both in whole cell homogenate (total Nrf2) and in cell nuclear fraction (nuclear Nrf2). It should be pointed out that the molecular weight of Nrf2 is 67 kDa; however, Nrf2 is easily polyubiquitin conjugated, leading to the apparent molecular weight in SDS-PAGE which ranges from 67 to 110 kDa in cell nucleus. In addition, one of the activation mechanisms of Nrf2 is phosphorylation, which also causes change of molecular weight of Nrf2 [28]. Nevertheless, most antibodies recognize a cluster of bands at ~100 kDa [29]. These might be the reasons for the more than one band in the Western blotting image (Fig. 6B).

Acrolein treatment significantly decreased total Nrf2 expression by 70% (P<.01), while α-tocopherol treatment in the absence of acrolein increased Nrf2 expression by about 40% (P<.01). Furthermore, α-tocopherol pretreatment effectively protected Nrf2 expression after acrolein treatment (P<.05) (Fig. 6A and C).

Similar to Nrf2 expression in whole cells, acrolein exposure significantly decreased nuclear Nrf2 levels, and α-tocopherol pretreatment apparently maintained high levels of nuclear Nrf2 (Fig. 6B).

3.6. Effects of α-tocopherol pretreatment and acrolein exposure on GCL expression

Using the Western blot method, we detected the expression of GCLc, which is the heavy chain of glutamate cysteine ligase. α-Tocopherol significantly induced GCLc expression compared to control (P<.01). Acrolein exposure greatly decreased GCLc expression (P<.01), while α-tocopherol pretreatment effectively protected the GCLc expression, compared to acrolein treatment alone (P<.05) (Fig. 7).

3.7. Effects of α-tocopherol pretreatment and acrolein exposure on NQO1 expression

NQO1 expression was significantly decreased after acrolein treatment (P<.01). α-Tocopherol pretreatment alone increased NQO1 expression by about 30% (P<.05). Compared with acrolein-

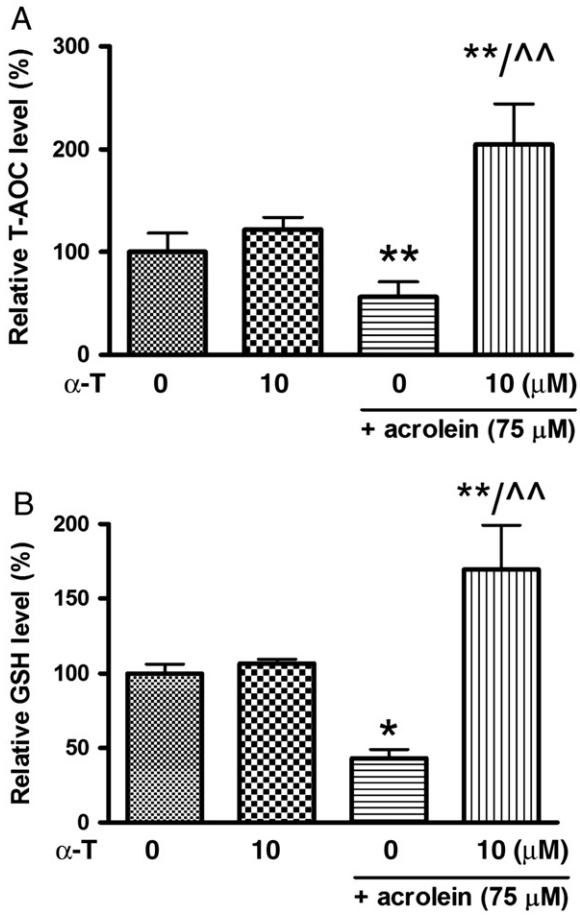

Fig. 5. Effects of α-tocopherol (α-T) on acrolein-induced changes in T-AOC and GSH. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Total-antioxidant capacity (T-AOC) and (B) GSH levels. Values are mean±S.E.M. of data from six separate experiments; each experiment was performed in triplicate. *P<.05 and **P<.01 vs. control (no acrolein, no α-T), and ^^P<.01 vs. acrolein.

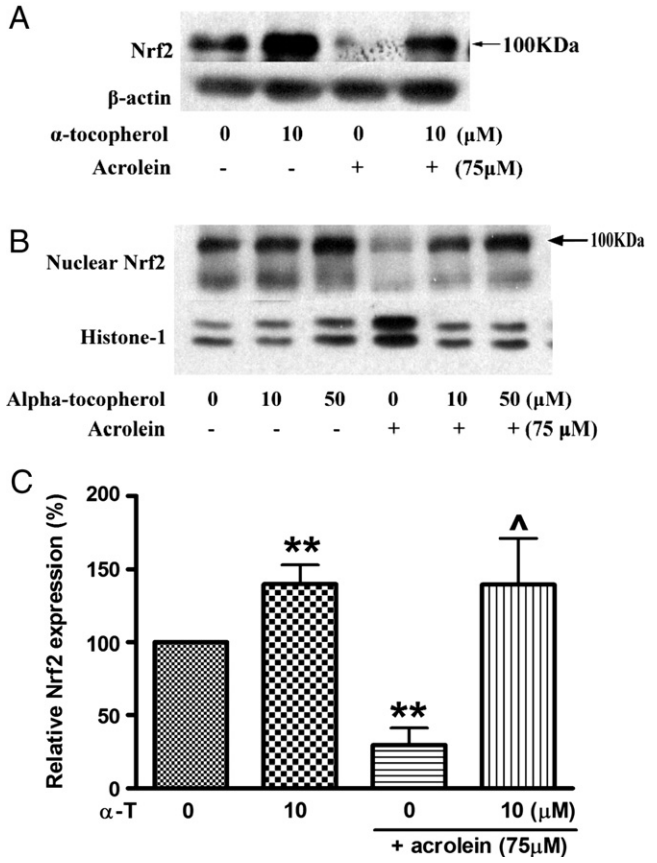

Fig. 6. Effects of α-tocopherol (α-T) on total and nuclear Nrf2 expression. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Representative Western blots for Nrf2 expression for whole-cell protein using β-actin as control, and (B) representative Western blots for Nrf2 expression for nuclear protein using histone-1 as control. (C) Optical density was analyzed by Quantity One software and normalized with β-actin for whole-cell protein. Values are mean±S.E.M. of four independent Western blots. **P<.01 vs. control (no acrolein, no α-T); ^P<.05 vs. acrolein.

only treatment, α-tocopherol pretreatment showed significant protection of NQO1 expression (P<.05) (Fig. 8).

3.8. Effects of α-tocopherol pretreatment and acrolein exposure on HO-1 expression

α-Tocopherol pretreatment greatly induced HO-1 expression by nearly 100% (P<.05) compared to control, and acrolein exposure significantly decreased HO-1 expression (P<.01). The acrolein-induced decrease in HO-1 expression was effectively protected by α-tocopherol pretreatment (P<.05) (Fig. 9).

3.9. Effects of α-tocopherol pretreatment and acrolein exposure on activities of Phase II enzymes (GST, NQO1 and SOD)

GSH is a critical molecule for detoxification and toxification mechanisms that would usually involve the conjugation of reduced GSH with numerous substrates by glutathione-S-transferase [30]. Acrolein exposure decreased GST activity by about 90% (P<.01) compared to control; α-tocopherol pretreatment had no effect on GST activity compared to control but showed a significant protective effect on acrolein-induced decreases in GST activity compared to acrolein exposure alone (P<.01) (Fig. 10A).

NQO1 activity was significantly decreased after acrolein exposure (P<.01). α-Tocopherol pretreatment itself did not affect NQO1 activity. Compared to the acrolein control, α-tocopherol pretreatment showed significant protection of NQO1 activity (P<.01) (Fig. 10B).

Acrolein exposure decreased SOD activity significantly (P<.05) compared to control; α-tocopherol pretreatment had no effect on SOD activity compared to control; however, compared to the acrolein-alone treatment, α-tocopherol pretreatment significantly protected SOD activity (P<.05) (Fig. 10C).

3.10. Effects of α-tocopherol pretreatment and acrolein exposure on pAKT/AKT, pERK1/2 and p38MAPK expression

Acrolein exposure had no effect on total AKT expression, but greatly increased the expression of pAKT, pERK1/2 and p38MAPK. In the absence of acrolein exposure, α-tocopherol pretreatment had no obvious effect on levels of pAKT/AKT, pERK1/2 and p38MAPK; however, compared to acrolein-alone treatment, α-tocopherol pretreatment greatly inhibited the expressions of pAKT, pERK and p38MAPK induced by acrolein exposure (Fig. 11).

4. Discussion

Phase II enzymes are categorized by the ways these enzymes metabolize drugs, phytoalexins, carcinogens and other plant metabolites and environmental pollutants. Phase II enzymes act on the oxygenated intermediates, usually by conjugating them with various endogenous moieties to produce extremely hydrophilic products that

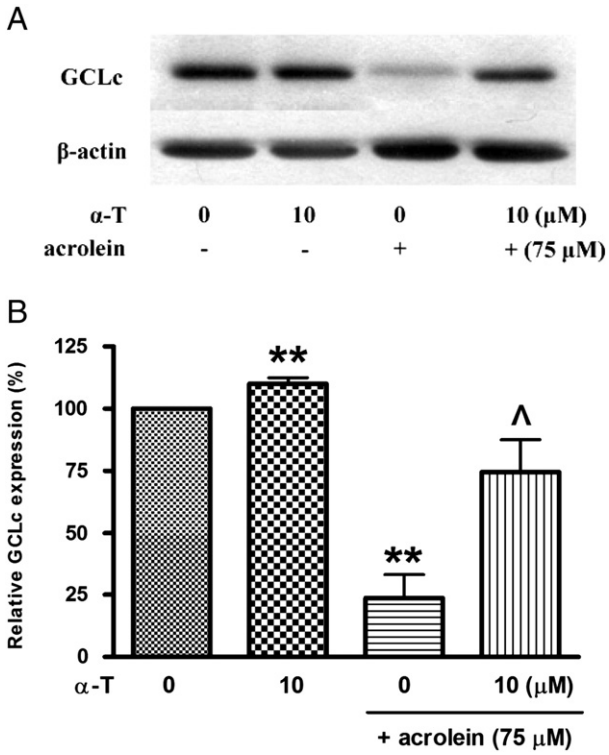

Fig. 7. Effects of α-tocopherol (α-T) on acrolein-induced decreases in GCL expression. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Representative Western blot images of GCL expression. (B) Quantification of Western blots from four separate experiments. **P<.01 vs. control (no acrolein, no α-T). ^P<.05 vs. acrolein.

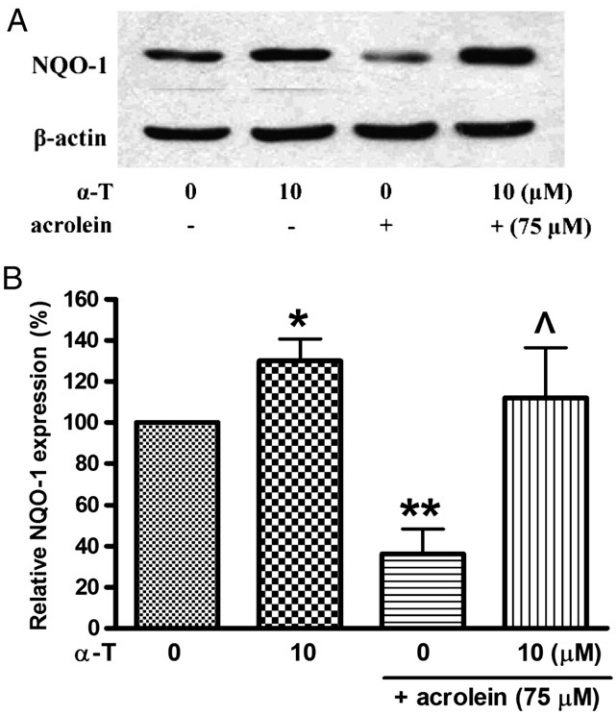

Fig. 8. Effects of α-tocopherol (α-T) on acrolein-induced decreases in NQO1 expression. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Representative Western blot images of NQO1 expression. (B) Quantification of Western blots from three separate experiments. Values are mean±S.E.M. *P<.05 and **P<.01 vs. control (no acrolein, no α-T). ^P<.05 vs. acrolein.

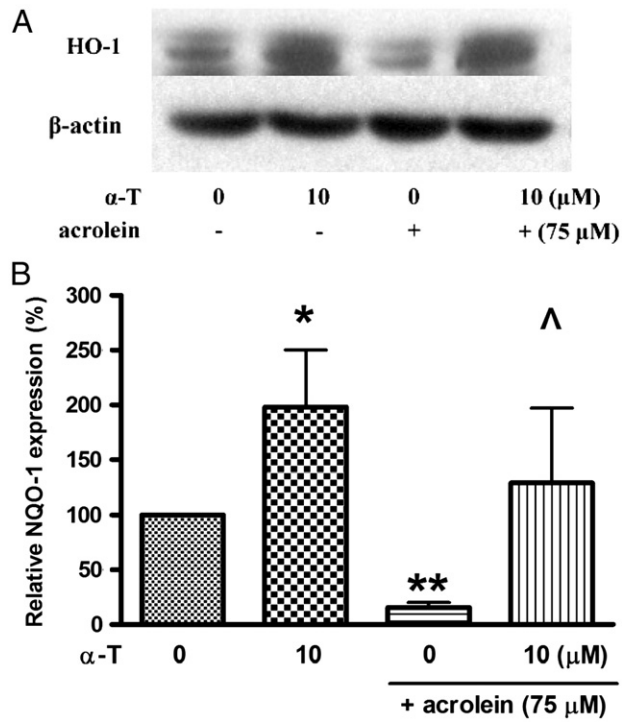

Fig. 9. Effects of α-tocopherol (α-T) on acrolein-induced decreases in HO-1 expression. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) Representative Western blot image of HO-1 expression. (B) Quantification of Western blot from three separate experiments. Values are mean±S.E.M. of three independent Western blots. *P<.05 and **P<.01 vs. control (no acrolein, no α-T). ^P<.05 vs. acrolein.

are easily excreted from the cell [31]. Phase II enzymes are regulated by a common upstream promoter regulatory element called the antioxidant-response element (ARE). Compounds that induce Phase II enzymes bring about activation of the ARE by activating an obligate transcription factor, Nrf2, which is a member of the cap 'n' collar family of basic leucine zipper (bZIP) transcription factors [32–34].

The role of Nrf2 transcription factor activation and its repression by Keap1 in the control of Phase II gene expression has been well established during the last decade. Activated Nrf2 is translocated to the nucleus and binds to the ARE to activate the transcription of Phase II enzymes and, consequently, to enhance the cell's antioxidant defense system. Recently, there has been great interest in finding natural Phase II enzyme inducers to enhance antioxidant response systems for health maintenance. Sulforaphane [35], lipoic acid [22,36,37] and lipoamide [15] are some examples. In the present study, we investigated whether α-tocopherol, a well-known chain-breaking antioxidant, could activate Nrf2 expression to promote the expression of Phase II enzymes. As we have shown clearly, α-tocopherol activated Nrf2 and its translocation to the nucleus (Fig. 6). This suggests that α-tocopherol is a possible Phase II enzyme inducer.

SOD, γ-GCL [38], GST [39], NAD(P)H NQO1 and HO-1 [40] are well-known Phase II enzymes. Nrf2 controls the orchestrated expression of Phase II enzymes and genes involved in cellular oxidative defense. For example, GCL controls the production of GSH, the major endogenous antioxidant thiol. NQO1, another Phase II enzyme, reduces quinones *via* a two-electron reduction and converts the dopamine quinones into less toxic hydroquinones that may be further detoxified *via* conjugation to sulfate or glucuronic acid; it is therefore likely to play a crucial role in the protection of cells against oxidative damage [22]. HO-1 produces the antioxidant bilirubin and stimulates the production of ferritin to reduce the amount of free iron, the main catalyst in the Fenton reaction [41]. In our study, we

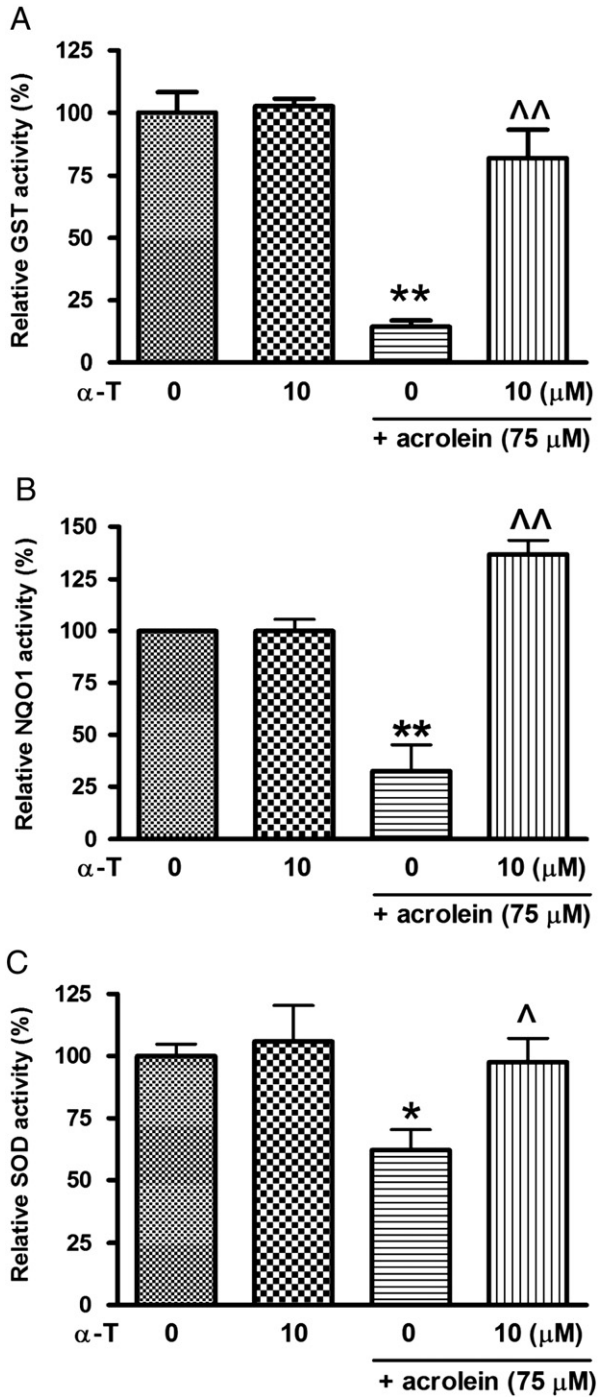

Fig. 10. Effects of α-tocopherol (α-T) on acrolein-induced changes in GST, NQO1 and SOD. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h). (A) GST activity values are mean±S.E.M. of data from six separate experiments. (B) NQO1 activity values are mean±S.E.M. of data from three separate experiments. (C) SOD activity values are mean±S.E.M. of data from four separate experiments; each experiment was performed in triplicate. *P<.05 and **P<.01 vs. control (no acrolein, no α-T). ^P<.05 and ^^P<.01 vs. acrolein.

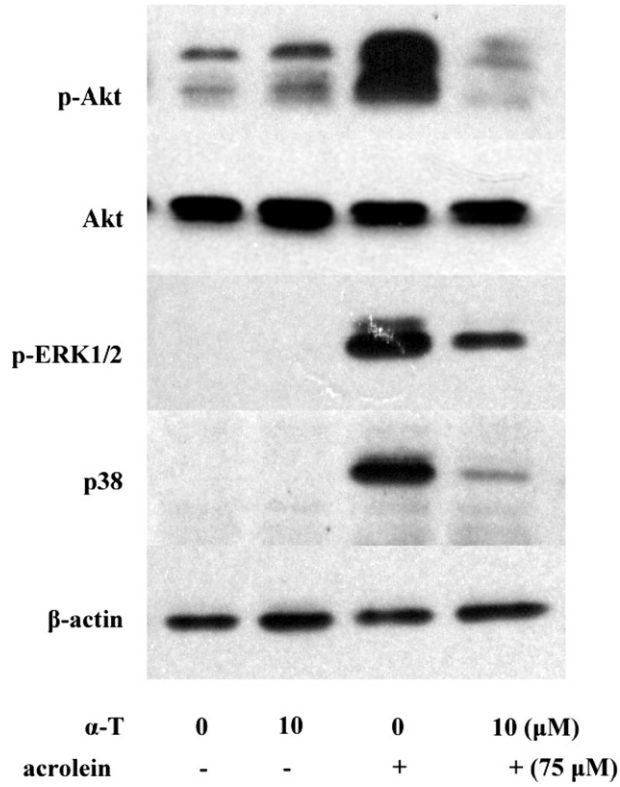

Fig. 11. Effects of α-tocopherol (α-T) on acrolein-induced increases in expression of pAKT/AKT, pERK1/2 and p38MAPK. ARPE-19 cells were pretreated with α-tocopherol (10 μM, 48 h) and then exposed to an acrolein challenge (75 μM, 24 h), and the protein expressions were assayed by Western blot.

have shown that Nrf2 activation consequently increased the expression and/or activity of enzymes regulated by Nrf2, including GCL (Fig. 7), NQO1 (Fig. 8), HO-1 (Fig. 9), GST (Fig. 5) and SOD (Fig. 5), and also small molecular antioxidants such as GSH (Fig. 5).

Phase II enzymes are part of an elaborate system for protection against the toxicity of xenobiotics, reactive oxygen and nitrogen species that are damaging to biomolecules (lipids, proteins and nucleic acids). As shown in our results, α-tocopherol pretreatment effectively protected ARPE-19 cells from an acrolein-induced decrease in cell viability (Fig. 1), inhibited the activation of an apoptosis signaling pathway (Fig. 10) and inhibited ROS and protein oxidation (Fig. 4). All of these protective functions apparently arise from the enhancement of the Phase II antioxidant enzyme system. Of course, some direct effect of α-tocopherol on oxidative damage can be involved. For example, lipid peroxidation occurs in mitochondria and the addition of α-tocopherol to the cells decreases lipid peroxidation and thus decreases potential signaling molecules [42].

Mitochondria are one of the primary targets of oxidant-induced RPE injury and may have a central role in RPE cell survival. As shown in our results, α-tocopherol pretreatment activated the Nrf2 antioxidant system and also protected the mitochondria from losing membrane potential (Fig. 2) and complex activities (Fig. 3). These results provide evidence to support the proposal that α-tocopherol is an effective mitochondria-targeting nutrient [43].

Two mechanisms have been proposed for the activation of the Nrf2-Keap1 pathway: (1) the oxidative modification of cysteine residues within Keap1 and (2) the phosphorylation of Nrf2 [28]. α-Tocopherol may activate Nrf2 through both pathways. First, it is known that α-tocopherol acting as antioxidant is oxidatively degraded to α-tocopheryl quinine [44], an oxidant which can oxidize the Keap1 thiol group. Second, Numakawa et al. [45] have demonstrated that vitamin E analogs including α-tocopherol, α-tocotrienol, γ-tocopherol and γ-tocotrienol for 24 h prevented the cultured cortical neurons from cell death in oxidative stress stimulated by H_2O_2. They further showed that α-tocopherol exposure induced the activation of both the MAP kinase (MAPK) and PI3 kinase (PI3K) pathways and that the α-tocopherol-dependent survival effect was blocked by an inhibitor, U0126 (an MAPK pathway inhibitor) or LY294002 (a PI3K pathway inhibitor). These results provide strong

evidence that α-tocopherol may cause Keap1 phosphorylization through the PI3K/Akt pathway. Further study on the PI3K/Akt pathway in RPE cells is warranted.

A population-based study of the risk factors for cataracts and AMD involving 2584 inhabitants of Sete, France, found that plasma α-tocopherol levels showed a weak negative association with late AMD (P=.07), while lipid-standardized plasma α-tocopherol levels showed a significant negative association with late AMD (P=.003). The risk of late AMD was reduced by 82% in the highest quintile compared with the lowest. Similarly, lipid-standardized plasma α-tocopherol levels were inversely associated with early signs of AMD; no associations were found with plasma retinol and ascorbic acid levels or with red blood cell glutathione values [46]. A small study involving 25 AMD patients and 15 AMD-free controls found statistically significant lower serum levels of vitamin E and Zn in the AMD subjects than in the AMD-free subjects, and a negative correlation between AMD grading of both the patients' eyes and serum vitamin E levels [47]. On the other hand, supplementing antioxidants such as vitamin E, the carotenoids, vitamin C, glutathione, SOD and catalase may be useful in preventing RPE damage and AMD [17,48,49]. AREDS was a large multicenter study of the natural course and clinical prognosis of age-related cataract and AMD. This study found a statistically significant reduction (by as much as 25%, P<.01) in the development of advanced AMD with antioxidants (vitamin C, 500 mg; vitamin E, 400 IU; and beta carotene, 15 mg), plus zinc (80 mg, as zinc oxide). The AREDS study suggests that if people at high risk for advanced AMD were to take these supplements, the potential impact on public health in the United States would be considerable during the next 5 years [50]. Our results support the conclusions of the AREDS study and also provide a possible mechanism for its results.

It is known that increased ROS can lead to activation of AKT, extracellular signal-regulated kinase (ERK1/2), p38, *etc*. For example, angiotensin II induces cardiac hypertrophy and increases in ROS and oxidative damage; it also induces phosphorylation of PKC, Erk1/2, JNK and p38 in cardiomyocytes. In contrast, isorhapontigenin, a new resveratrol analog, could inhibit cardiac hypertrophy by blocking oxidative stress and oxidative stress-mediated signaling pathways [51]. Epigallocatechin-3-gallate (EGCG) also inhibits these signaling activations [52]. In our study, we showed that α-tocopherol pretreatment significantly inhibited the acrolein-induced phosphorylation of AKT and ERK and expression of p38. Because it is known that EGCG [15] and resveratrol [53,54] are Phase II enzyme inducers, our results suggest that reduction of the acrolein-induced activation of these signaling pathways is due to the reduction of ROS by α-tocopherol *via* its induction of the Phase II enzyme system. This results in the enhancement of antioxidant defenses by α-tocopherol.

It should be pointed out that the T-AOC assays are mainly for *in vitro* assay for antioxidant capacity and different assays have different mechanisms and give different results [55]. The analysis kit used produced interesting data, but may still not have the capability of evaluating the effect of α-tocopherol antioxidant activity. As shown in Fig. 5, the T-AOC did not change with the addition of 10 μM α-tocopherol, but the GSH was increased when both acrolein and α-tocopherol were added, suggesting that the acrolein up-regulated GSH production, and the GSH was protected by the addition of α-tocopherol, thereby resulting in an increase in T-AOC.

These results strongly suggest that besides its direct antioxidant activities, such as free radical scavenging and iron chelation, another mechanism of the protective effects of α-tocopherol — just as for the thiol-reactive compounds sulforaphane [35], lipoic acid [22,36,37] and resveratrol [53,54] — proceeds by induction of the Phase II enzyme response *via* the transcription factor Nrf2.

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