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Journal of Nutritional Biochemistry 21 (2010) 1089–1098
Journal of Nutritional Biochemistry

Hydroxytyrosol protects against oxidative damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying enzyme systems in retinal pigment epithelial cells☆

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Received 18 March 2009; received in revised form 4 September 2009; accepted 14 September 2009

Abstract

Studies in this laboratory have previously shown that hydroxytyrosol, the major antioxidant polyphenol in olives, protects ARPE-19 human retinal pigment epithelial cells from oxidative damage induced by acrolein, an environmental toxin and endogenous end product of lipid oxidation, that occurs at increased levels in age-related macular degeneration lesions. A proposed mechanism for this is that protection by hydroxytyrosol against oxidative stress is conferred by the simultaneous activation of two critically important pathways, viz., induction of phase II detoxifying enzymes and stimulation of mitochondrial biogenesis. Cultured ARPE-19 cells were pretreated with hydroxytyrosol and challenged with acrolein. The protective effects of hydroxytyrosol on key factors of mitochondrial biogenesis and phase II detoxifying enzyme systems were examined. Hydroxytyrosol treatment simultaneously protected against acrolein-induced inhibition of nuclear factor-E2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1α) in ARPE-19 cells. The activation of Nrf2 led to activation of phase II detoxifying enzymes, including γ-glutamyl-cysteinyl-ligase, NADPH (nicotinamide adenine dinucleotide phosphate)-quinone-oxidoreductase 1, heme-oxygenase-1, superoxide dismutase, peroxiredoxin and thioredoxin as well as other antioxidant enzymes, while the activation of PPARGC1α led to increased protein expression of mitochondrial transcription factor A, uncoupling protein 2 and mitochondrial complexes. These results suggest that hydroxytyrosol is a potent inducer of phase II detoxifying enzymes and an enhancer of mitochondrial biogenesis. Dietary supplementation of hydroxytyrosol may contribute to eye health by preventing the degeneration of retinal pigment epithelial cells induced by oxidative stress.

Keywords: Hydroxytyrosol; RPE cells; Acrolein; AMD; Mitochondrial biogenesis; Phase II enzymes

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of vision loss in the Western world among people over 65 y of age [1], and worldwide, it is the third most common cause of blindness [2]. AMD is characterized by an age-related degeneration of retinal pigment epithelium (RPE) and the photoreceptors in the macular area of the retina. The underlying cause of the disease is unknown, but oxidative stress is involved [3], suggesting that consumption of diets rich in antioxidants may be of benefit.

The Mediterranean diet has been associated with a lower incidence of not only certain cancers, but also cardiovascular disease, which is the most common and serious complication of diabetes [4–6], all conditions associated with oxidative stress. Olives and olive oil are considered an important part of the Mediterranean diet. Evidence has accumulated recently that in addition to olive lipids, which are rich in monounsaturated fatty acids, antioxidant polyphenols such as hydroxytyrosol also contribute to the health effects of olives [7–10].

Work in our laboratory has led to the proposal that oxidative damage to mitochondria in RPE cells may contribute to the retinal

☆ Supported by National Eye Institute, NIH grant EY0160101, 5R01 CA119028-05, R01 CA116697, R01 ES015518, R01 ES015375, a UC Davis Center for Human and Nutrition Pilot Award (CHNR08-318), and DSM Nutritional Products.
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doi:10.1016/j.jnutbio.2009.09.006

Table 1
Primers and annealing temperatures

Gene	Annealing temp. (°C)	Forward primer	Reverse primer
18SrRNA	55	CATTCGAACGTCTGCCCTATC	CCTGCTGCCTTCCTTGGA
Cu/ZnSOD	55	CGGAGGCTTTGAAGGTGTGG	CTCCAACATGCCTCTCTTCATCC
MnSOD	55	AGGTTAGATTTAGCCTTATTCCAC	TTACTTTTTGCAAGCCATGTATCTTTC
UCP-2	55	TACAAAGCCGGATTCCGGCAGC	CTCCTTGGATCTGTAACCGGAC
PRDX3	60	CCTTTGGATTTCACCTTTGTGTG	CAAACCACCATTCTTTCTTGGTG
PRDX5	60	CCAATCAAGGTGGGAGATGCC	GCAGGTGTGTCTTGGAACATC
TRX2	60	GTCCACACCACTGTGCGTGG	TTGCAGGGAGATGGCTCAGCG

degeneration observed in AMD and the compounds that protect mitochondrial function may prevent or alleviate this damage. Our work also showed that acrolein, a lipid oxidation end product and mitochondrial toxin [11], causes oxidative mitochondrial damage in RPE cells; moreover, hydroxytyrosol protects RPE cells against this acrolein-induced oxidative stress [12]. To investigate the underlying mechanisms, the same acrolein model is now used to study the effects of hydroxytyrosol on the induction of phase II detoxifying enzymes and stimulation of mitochondrial biogenesis, two of the most important pathways for cells to fight against oxidative stress.

When cells are subjected to a variety of oxidative environmental stresses, they typically respond by inducing a coordinated expression of genes encoding the set of phase II detoxifying enzymes (Fig. 13) , principally mediated by activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [15,16]. Nrf2 controls the orchestrated expression of phase II enzymes and genes involved in oxidative defense, although normally Nrf2 protein is kept inactive in the cytoplasm by complexing with its cytosolic inhibitor keap-1. Upon activation and release from keap-1, Nrf2 protein translocates to the nucleus, where it binds to promoters containing antioxidant response elements, resulting in the transactivation of the respective genes for phase II detoxifying enzymes. Key phase II detoxifying enzymes include glutathione (GSH) S-transferase (GST), heme oxygenase-1 (HO-1), NAD(P)H quinone oxido-reductase-1 and γ-glutamyl cysteine ligase (GCL), enhanced expression of which leads to an increase in levels of endogenous antioxidants such as the major thiol antioxidant GSH and reduced quinones [13,15,17].

Phase II enzymes perform a variety of vital cellular functions important for protecting against oxidative damage. GCL controls the production of GSH, the major endogenous antioxidant thiol. GSH reductase (GR) catalyses the NADP-dependent reduction of GSSG (oxidized GSH) to GSH to maintain a high cytoplasmic GSH:GSSG ratio. GSH peroxidase (GPx), an enzyme widely present in many tissues, is thought to be an important cellular H_2O_2 detoxifier in neurons [18] and mice lacking GPx develop cataracts at a young age [19]. NAD(P)H:quinone oxidoreductase (NQO1) 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 [20]. Therefore, NQO1 is likely to play a crucial role in the protection of cells against oxidative damage. HO-1 produces the antioxidant bilirubin; it is typically associated with an increased production of ferritin, which results in reduced amounts of free iron [21], the main catalyst of the Fenton reaction. HO-1 expression is ubiquitous and its activity is increased by many types of agents, particularly those involved in oxidative stress such as heme, metalloporphyrins and transition metals.

The cytoplasmic antioxidant system [including NQO1, GST, GCL and Cu/Zn superoxide dismutase (SOD)] is mainly controlled by Nrf2. In contrast, the mitochondrial antioxidant system [thioredoxin-2, peroxiredoxin (Prdx)3, Prdx5 and Mn SOD] is modulated through the transcription factor FOXO3a.

Inducing phase II enzymes and stimulating mitochondrial biogenesis may also enhance other antioxidant defense systems, such as the antioxidant enzyme catalase. The functions of catalase include catalyzing the decomposition of hydrogen peroxide to water and oxygen to remove free radicals and protect cells from oxidative damage.

The role of mitochondrial dysfunction in the aging process [22] and in the development of chronic degenerative diseases, such as Type 2 diabetes [23] and neurodegenerative diseases [24], is being increasingly acknowledged. One underlying mechanism of mitochondrial dysfunction is the loss of mitochondria. For example, mitochondrial loss in adipose tissue is correlated with the development of Type 2 diabetes [25].

Thus, an effective strategy for preventing and treating mitochondrial dysfunction-related disease should be the effective stimulation of mitochondrial biogenesis. This may be achieved by activation of the key factor promoting mitochondrial biogenesis, peroxisome proliferator-activated receptor coactivator 1 alpha (PPARGC1α) [26]. PPARGC1α possesses dual activities — stimulation of mitochondrial electron transport while enforcing suppression of reactive oxygen species (ROS) — and may serve as an adaptive set-point regulator, capable of providing an accurate balance between metabolic requirements and cytotoxic protection [27]. Therefore, its dual activities of inducing mitochondrial

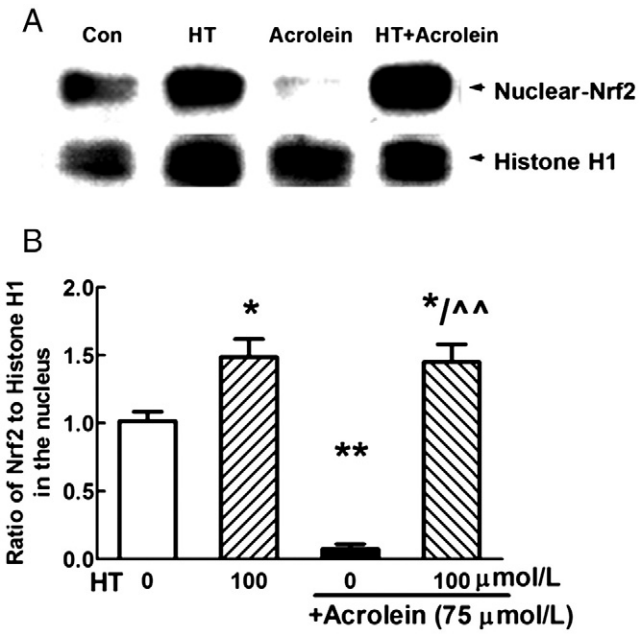

Fig. 1. Hydroxytyrosol increased nuclear Nrf2 levels both in untreated and in acrolein-challenged ARPE-19 cells. Hydroxytyrosol pre-treatment (100 μmol/L, 48 h) and acrolein exposure (75 μmol/L, 24 h): (A) Western blot image and (B) Quantification of Western blots from four separate experiments. *P<.05, **P<.01 vs. control cells (no acrolein, no hydroxytyrosol) and ^^P<.01 vs. acrolein-treated cells.

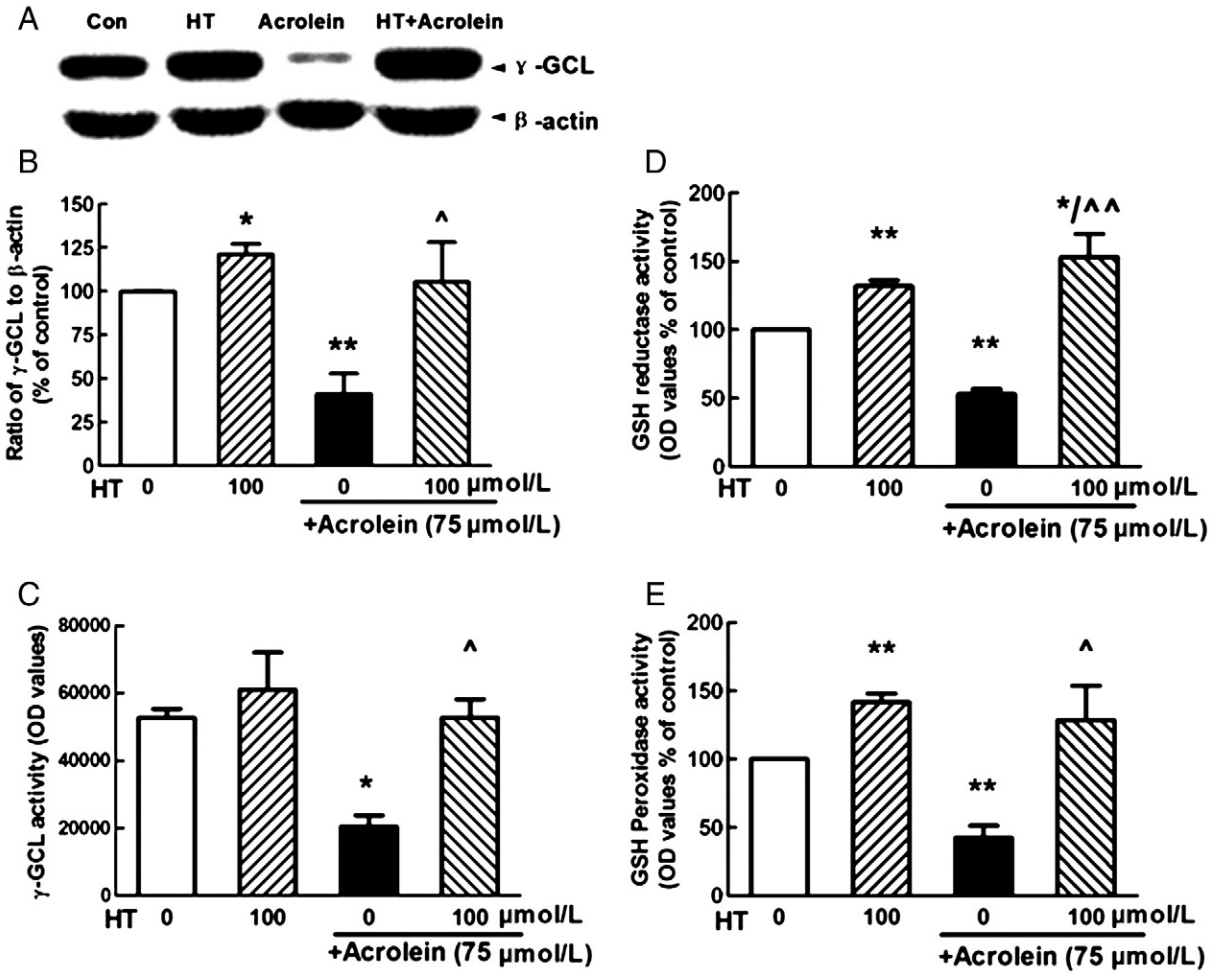

Fig. 2. The induction of γ-GCL by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein challenge (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot of GCL protein expression. Values are means±S.E. of four separate experiments. (C) GCL activity assay. Values are means±S.E. of one representative experiment chosen from three separate experimental repeats, all of which had the same trend. (D) GR activity and (E) GPx activity. Values are means±S.E of data from three separate experiments, each experiment performed in triplicate. *P<.05, **P<.01 vs. control (hydroxytyrosol 0 μmol/L) and ^P<.05, ^^P<.01 vs. acrolein-treated.

biogenesis and suppressing ROS make PPARGC1α an almost ideal target protein for the control or limiting of damage associated with mitochondrial dysfunction.

Signalling molecules upstream of PPARGC1, such as adenosine monophosphate kinase (AMPK) [28], nitric oxide [29], and calcium [30], can also promote mitochondrial biogenesis. Of these, AMPK also regulates other metabolic pathways, including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of glucose transporters [31]. The enzyme nitric oxide synthase (NOS) produces NO, and one isoform, endothelial cell NOS (eNOS), is an upstream regulating factor for mitochondrial biogenesis [32].

Finally, uncoupling protein 2 (UCP2), a mitochondrial factor controlled by PPARGC1α, is involved in maintaining acceptable ROS levels and is neuroprotective during ischemia/reperfusion [33]; it therefore may play a role in preventing and correcting mitochondrial dysfunction.

Polyphenols have been shown to protect RPE from oxidative-stress-induced death [34] and to induce phase II detoxifying enzymes [35]. Hydroxytyrosol is the major antioxidant polyphenol in olives and has been shown to have beneficial effects on human health. Results from our previous experiments have shown that hydroxytyrosol exhibits protective effects against acrolein-induced toxicity in the human retinal pigment epithelial cell line ARPE-19 [12]. Pretreatment with hydroxytyrosol dose-and time-dependently protected the ARPE-19 cells from acrolein-induced oxidative damage and mitochondrial dysfunction. A short-term pretreatment (48 h) with over 75 μmol/L hydroxytyrosol was required for protection while a long-term pretreatment (7 days) showed protective effects with as little as 5 μmol/L or more, suggesting that lower long-term doses of hydroxytyrosol treatment can achieve similar protective effects as the higher short-term doses. These results suggest that hydroxytyrosol may be a mitochondrial protecting nutrient even at a relatively low concentration when given for an extended period of time. Our hypothesis is that the mechanism behind hydroxytyrosol's protective effects against acrolein-induced RPE damage may be related to its capability to activate simultaneously both mitochondrial biogenesis and phase II detoxifying enzyme systems. In the present study, these pathways are investigated using acrolein-challenged ARPE-19 cells.

2. Materials and methods

2.1. Reagents

Acrolein was purchased from Sinopharm Chemical Reagent (Shanghai, China). Pure (>99%), synthetic hydroxytyrosol was a gift from DSM Nutritional Products, Kaiseraugst, Switzerland. The reverse transcription system kit was purchased from Promega (Mannheim, Germany) and HotStarTaq from Takara (Otsu, Shiga, Japan). Primers were synthesized by Bioasia Biotech (Shanghai, China). TRIzol and reagents for cell culture were from Invitrogen. Anti-oxphos complexes I, II, III and V were from Invitrogen (Carlsbad, CA, USA); nuclear-encoded subunits of the mitochondrial oxphos

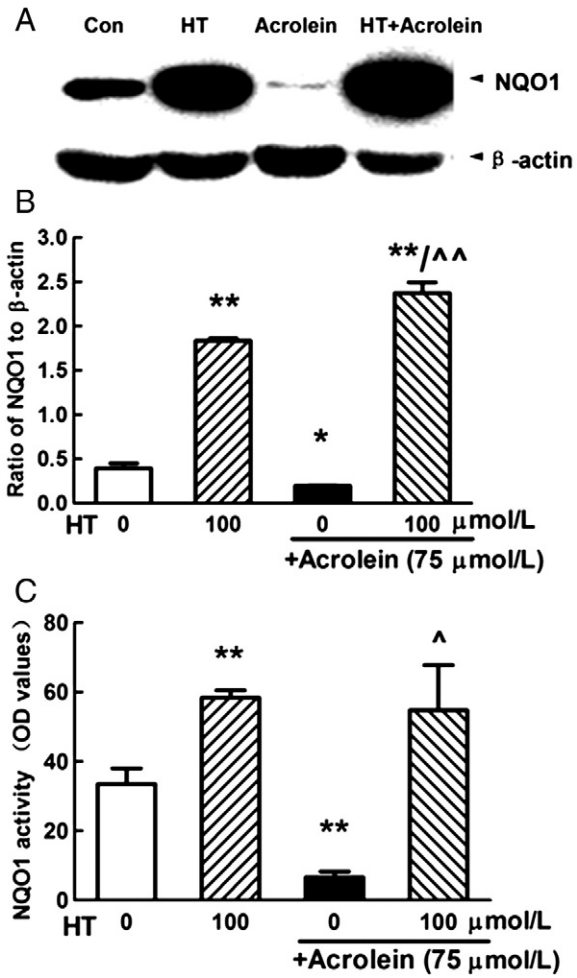

Fig. 3. The induction of NQO1 by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot. Values are means±S.E. of three separate experiments. (C) NQO1 activity. Values are means±S.E of data from three separate experiments; each experiment performed in triplicate. *P<.05 and **P<.01 vs. control cells (hydroxytyrosol 0 μmol/L); ^P<.05 and ^^P<.01 vs. acrolein-treated.

complexes detected were: for complex I, the 39-kDa protein (alpha subunit 9); for complex II, the 30 kDa subunit B protein (iron-sulfur protein); for complex III, the core I protein; and for complex V, the alpha-subunit (F1 complex). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human ARPE-19 cell line was obtained from Dr. Nancy J. Philp and was cultured according to her methods [36]. The ARPE-19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 2 mmol/L 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 hydroxytyrosol supplementation

All experiments were performed with an 80% confluent monolayer. Hydroxytyrosol was dissolved in dimethylsulfoxide (DMSO) (final DMSO concentration ≤0.025%). Acrolein was dissolved in DMEM-F12 medium immediately before each experiment and was incubated with cells for 24 h as an acute toxicity model [12,37]. The protective effects of hydroxytyrosol were studied with the acute toxicity model by pre-treating cells with hydroxytyrosol for 48 h followed by 24 h acrolein treatment in the absence of hydroxytyrosol.

2.4. Western blot analysis

After pre-treatment with hydroxytyrosol and following acrolein treatment, cells were washed twice with ice-cold phosphate-buffered saline, lysed in sample buffer [62.5 mmol/L Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 5 mmol/L dithiothreitol] at room temperature and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000×g, 10 min at 4°C). Protein concentrations were determined using a protein assay kit (Bio-Rad DC; Hercules, CA, USA). The soluble lysates (10 μg per lane) were subjected to 10% (w/v) SDS-polyacrylamide gel electrophoresis; proteins were then transferred to nitrocellulose membranes and blocked with 5% (w/v) nonfat milk/Tris-buffered saline Tween 20 (TBST) solution for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies directed against anti-β-actin (1:5,000), anti-γ-GCL (1:1,000), anti-NQO1 (1, 1:2000), anti-HO-1 (1:2,000), anti-catalase (1:1000), anti-PPARGC1α (1:1000), anti-total AMPK, anti-phosphor AMPK (1:1000), anti-eNOS (1:1000), anti-Tfam (1:1000), anti-Complex I (1:2000), anti-Complex II (1:2000), anti-Complex III (1:2000) and anti-Complex V (1:2000) in 5% (w/v) milk/TBST. After washing the membranes with TBST three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using electrochemiluminescence (Roche, Mannheim, Germany) and quantified by scanning densitometry [38].

2.5. Nuclear Nrf2 analysis

Total protein and nuclear protein were isolated for Western blot analysis of Nrf2 levels. Nrf2 was probed with anti-Nrf2 antibodies (Santa Cruz) at 1:1000 in the nuclear protein fraction. Histone H1 was used as loading control for nuclear proteins. Anti-histone H1 antibody (Sigma) was used at 1:2000. Chemiluminescence detection was done with an ECL Western blotting detection kit from Amersham Pharmacia [39].

2.6. Enzyme activity measurements

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; reduction was measured [40]. GCL activity was assayed with the NDA method described previously [41]. Cellular GR activity was measured by the nicotinamide adenine dinucleotide phosphate (NADPH) method described previously [42]. Cellular GPx activity was measured by the NADPH method described previously [43]. Catalase activity was assayed by the method of Aebi [42].

2.7. Quantitative reverse transcriptase-polymerase chain reaction

Real-time PCR was used to measure gene expression levels of Cu/Zn superoxide dismutase (Cu/Zn SOD, SOD1), Mn SOD (SOD2), uncoupling protein 2 (UCP2), peroxiredoxin 3 (*PRDX3*), peroxiredoxin 5 (*PRDX5*) and thioredoxin-2 (*TRX2*). Total RNA (1 μg), isolated by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) from cells cultured in 6-well plates, was reverse transcribed using Revertra Ace (Toyobo, Japan)

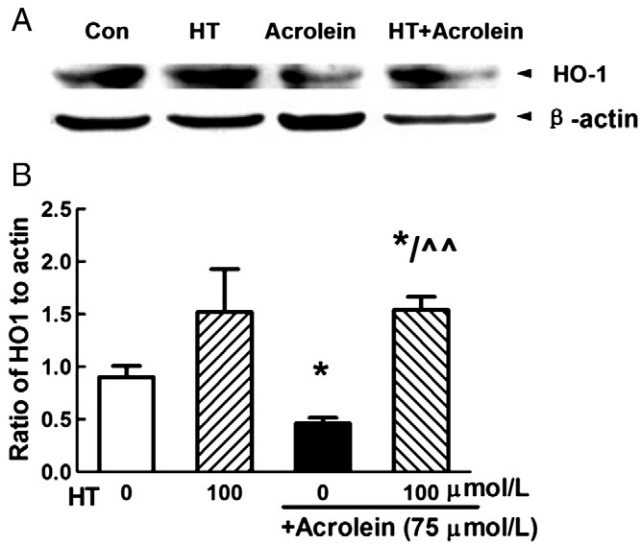

Fig. 4. The induction of heme oxygenase-1 (HO-1) protein by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Western blot of HO-1 from three separate experiments. *P<.05 vs. control cells (hydroxytyrosol 0 μmol/L) and ^^P<.01 vs. acrolein-treated.

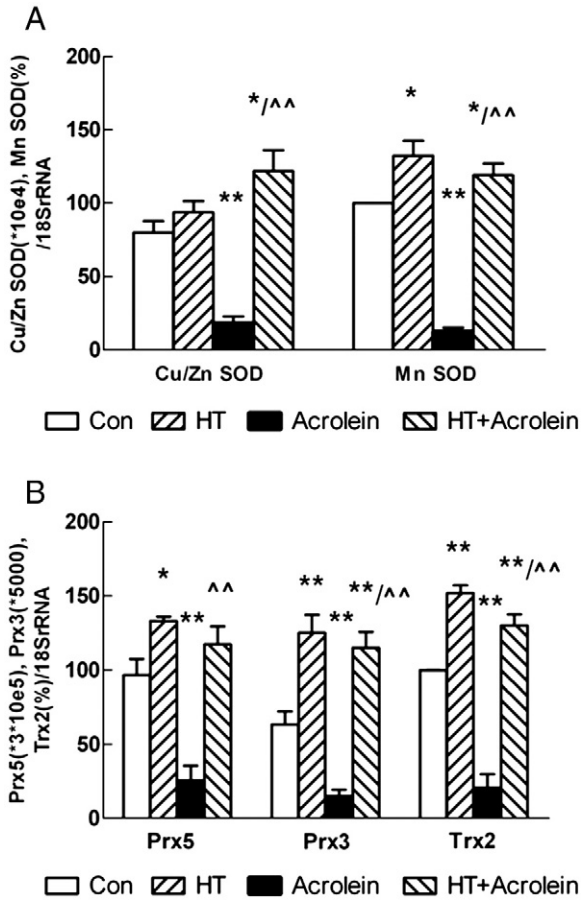

Fig. 5. Hydroxytyrosol increased the transcription levels of cytoplasmic and mitochondrial antioxidant defense system genes both in untreated and in acrolein-challenged ARPE-19 cells pretreated with hydroxytyrosol (100 μmol/L; 48 h) before acrolein exposure (75 μmol/L; 24 h). RNA was isolated, reverse-transcribed to cDNA and analyzed by real time PCR to measure expression levels of the target genes as ratios to 18SrRNA. mRNA levels of (A) *SOD1* (Cu/Zn SOD) and *SOD2* (Mn SOD); and (B) *PRDX5* (Prx5), *PRDX3* (Prx3), and *TRX2*. Values are means±S.E.M. from four separate experiments for *Cu/Zn SOD*, *PRDX5*, *TRX2*, and five separate experiments for *Mn SOD* and *PRDX3*. *P<.05, and **P<.01, vs. control cells; ^P<.05 and ^^P<.01 vs. acrolein group.

following the supplier's instructions. Primers were designed using Premier Primer 5 software (Palo Alto, CA, USA). Triplicate PCR reactions were carried out with real-time PCR Master Mix (Toyobo, Japan). PCR was performed on a Multiplex Quantitative PCR System Mx3000P (Stratagene, Cedar Creek, TX, USA) as follows: after an initial step of 10 min at 95°C, the samples were subjected to 40 cycles of 30s denaturizing at 95°C, 1 min annealing at 55–60°C and 30 s extension at 72°C. Melting curves were assessed over the range 55°C-99°C to ensure specific DNA amplification. The cycle number at which the various transcripts were detected (Ct) was compared with that of 18S-RNA, referred to as ΔCt. The relative gene level is expressed as $2^{(-\Delta\Delta Ct)}$, in which ΔΔCt equals ΔCt of the detected gene minus ΔCt of 18SrRNA ([29]). The nucleotide sequences and annealing temperatures of primers used for real-time PCR or cDNA probe construction (5′ to 3′) are shown in Table 1.

2.8. Statistical analysis

Results are presented as means±S.E. Group comparisons were made by one-way analysis of variance, followed by determination of significant differences using post hoc comparisons with a Tukey HSD test. P<.05 was considered significant.

3. Results

3.1. Hydroxytyrosol increases nuclear Nrf2

The central factor controlling phase II detoxifying enzyme activation is the activation of Nrf2. Acrolein treatment (75 μmol/L, 24 h) significantly suppressed nuclear Nrf2 levels to about 15% of control cell levels. Hydroxytyrosol pretreatment (100 μmol/L for 48 h for all experiments, same as in our previous study [12]) abolished the acrolein effect and further enhanced nuclear Nrf2 protein levels (Fig. 1). In addition, hydroxytyrosol significantly increased (to 34% above control cell values) nuclear translocation of Nrf2 in cells not challenged with acrolein (Fig. 1).

3.2. Hydroxytyrosol boosts phase II detoxifying enzymes

GCL expression and activity were significantly decreased by acrolein to approximately 50% of control levels, and hydroxytyrosol pretreatment effectively protected cells against the effects of acrolein (Fig. 2A-C). Hydroxytyrosol also significantly enhanced GCL protein expression in unchallenged cells (Fig. 2A and B).

GSH reductase (GR) activity was reduced by approximately 55% by acrolein, and again, hydroxytyrosol pretreatment abolished the acrolein effect (Fig. 2D). Hydroxytyrosol alone also significantly stimulated GR activity in unchallenged cells (Fig. 2D).

GSH peroxidase (GPx) activity dropped by 45% after acrolein treatment (Fig. 2E). Hydroxytyrosol pretreatment completely protected against the acrolein-induced GPx activity decrease. Hydroxytyrosol treatment alone significantly increased GPx activity (Fig. 2E).

Acrolein induced a significant decrease in both expression (Fig. 3A and B) and activity (Fig. 3C) of NQO1, and this decrease was prevented by hydroxytyrosol pretreatment. Moreover, hydroxytyrosol pretreatment significantly induced NQO1 protein expression (about 4 fold) and activity (about 2 fold) in unchallenged cells.

Acrolein also significantly decreased HO-1 protein expression. Hydroxytyrosol pretreatment protected cells against this decrease and also enhanced HO-1 expression in cells not challenged with acrolein (Fig. 4).

3.3. Hydroxytyrosol increases antioxidant gene transcription

Acrolein treatment significantly repressed the gene expression of Cu/Zn SOD, Mn SOD, PRDX5, PRDX3 and TRX2 in ARPE-19 cells by 75–88% (Fig. 5). Hydroxytyrosol pretreatment restored all these transcripts

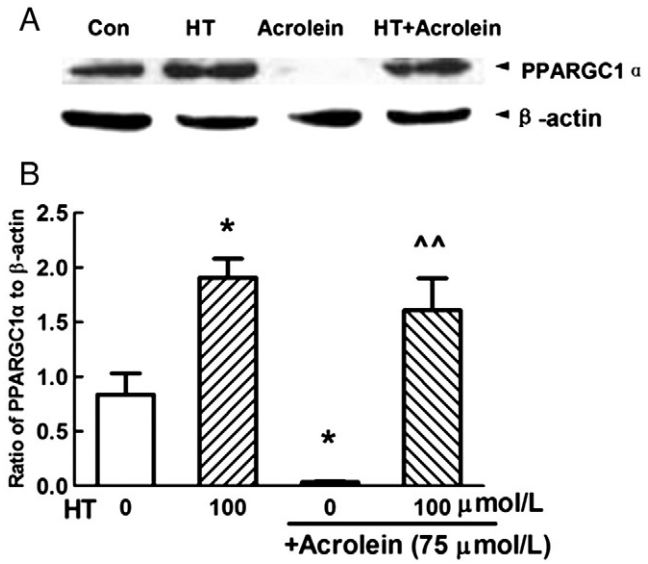

Fig. 6. Hydroxytyrosol increased PPARGC1α protein expression both in untreated and in ARPE-19 cells pretreated with hydroxytyrosol (100 μmol/L; 48 h) before acrolein exposure (75 μmol/L; 24 h). (A) Representative Western blot image and (B) quantification of PPARGC1α Western blots. Values are means±S.E. of three separate experiments. *P<.05 vs. control cells (hydroxytyrosol 0 μmol/L), and ^^P<.01 vs. acrolein-treated.

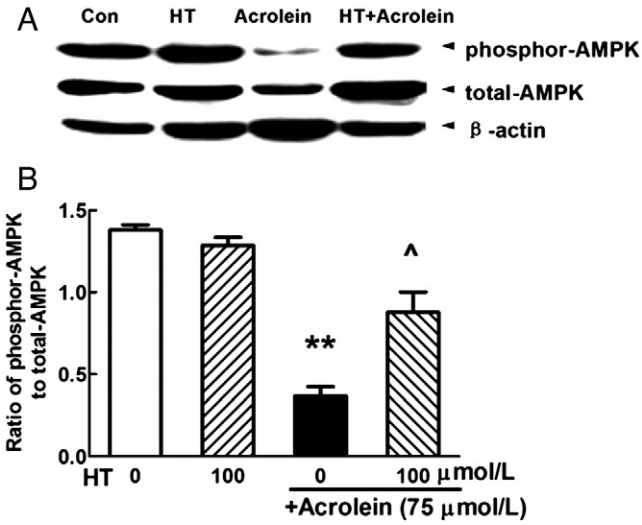

Fig. 7. Hydroxytyrosol prevented acrolein-induced suppression of the PPARGC1α-activating signal AMPK. Stimulation of AMPK phosphorylation by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μmol/L, 24 h). (A) Representative Western blot images and (B) quantification of AMPK phosphorylation Western blots. Values are means±S.E. of three separate experiments. $^{**}P<.01$ vs. control (hydroxytyrosol 0 μmol/L) and $^{\wedge}P<.05$ vs. acrolein-treated.

to above their control levels, by significantly increasing their expression 4.5-fold to 10-fold over the acrolein-depressed levels (Fig. 5). Moreover, hydroxytyrosol significantly up-regulated transcript levels of all but Cu/Zn SOD in cells not challenged with acrolein (by 30–98%; Fig. 5).

3.4. Hydroxytyrosol increases PPARGC1α expression

PPARGC1α protein expression was strongly suppressed in ARPE-19 cells after acrolein challenge (Fig. 6), while being markedly

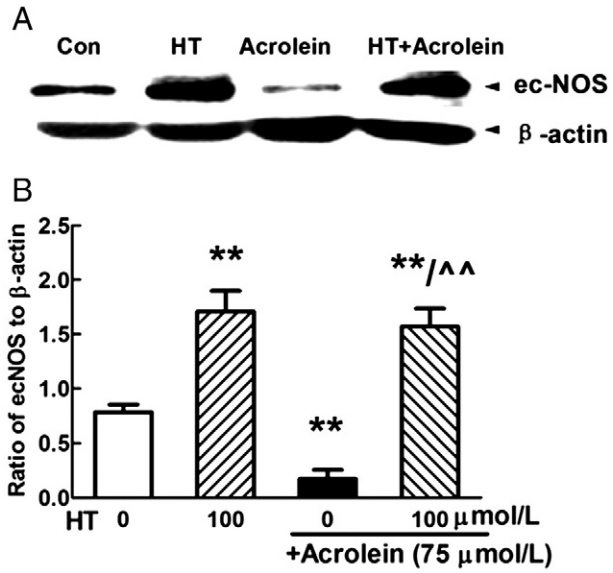

Fig. 8. Hydroxytyrosol prevented acrolein-induced suppression of the PPARGC1α-activating signal eNOS (ecNOS). Stimulation of eNOS signaling by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein (75 μmol/L, 24 h). (A) Representative Western blot images and (B) quantification of eNOS Western blots. Values are means±S.E. of four separate experiments. $^{**}P<.01$ vs. control (hydroxytyrosol 0 μmol/L) and $^{\wedge\wedge}P<.01$ vs. acrolein-treated.

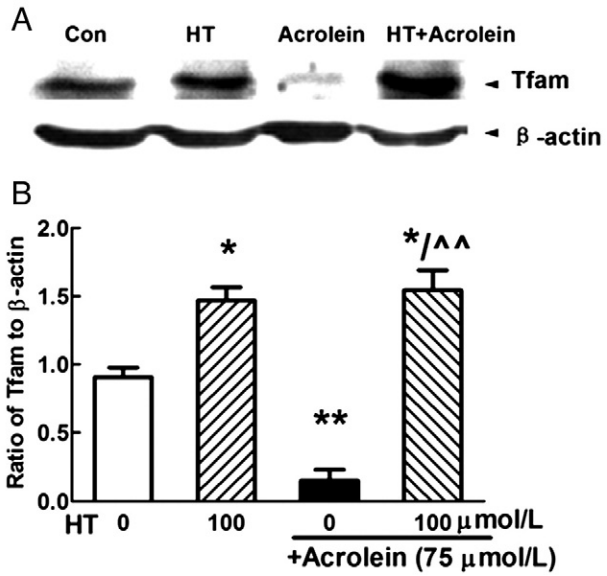

Fig. 9. Stimulation of the PPARGC1α-regulated mitochondrial marker Tfam by hydroxytyrosol (100 μmol/L, 48 h) in ARPE-19 cells without and with exposure to acrolein challenge (75 μmol/L, 24 h). (A) Representative Western blot image and (B) quantification of Tfam Western blots. Values are means±S.E. of three separate experiments. $^{*}P<.05$, $^{**}P<.01$ vs. control (hydroxytyrosol 0 μmol/L) and $^{\wedge\wedge}P<.01$ vs. acrolein-treated.

stimulated by hydroxytyrosol. Hydroxytyrosol pretreatment not only prevented the acrolein-induced suppression of PPARGC1α but nearly doubled its control cell levels (Fig. 6).

3.5. Hydroxytyrosol prevents acrolein-induced suppression of AMPK and eNOS

Acrolein treatment caused a significant inhibition of the PPARGC1α-activating signal AMPK phosphorylation and hydroxytyrosol pretreatment prevented this decrease (Fig. 7). However, hydroxytyrosol did not influence AMPK phosphorylation in non-acrolein-challenged cells. Just as with AMPK phosphorylation, acrolein treatment severely decreased eNOS protein expression, and the decrease of this PPARGC1α-activating signal was prevented by hydroxytyrosol pretreatment (Fig. 8). Moreover, hydroxytyrosol also increased eNOS protein expression in non-acrolein-challenged cells.

3.6. Hydroxytyrosol increases PPARGC1α-regulated mitochondrial markers

Protein expression levels of Tfam (Fig. 9) and the mitochondrial electron transport complexes I, II, III and V (Fig. 10) were significantly decreased by acrolein treatment and rescued by hydroxytyrosol pretreatment. Hydroxytyrosol significantly up-regulated Tfam and the mitochondrial complex proteins in cells not challenged by acrolein (Figs. 9,10). UCP2 gene expression was significantly decreased by acrolein and the acrolein effect was abolished by hydroxytyrosol (Fig. 11). Hydroxytyrosol also induced basal UCP2 expression.

3.7. Hydroxytyrosol increases catalase

Catalase protein expression and activity were significantly suppressed by acrolein (Fig. 12A-C). Hydroxytyrosol pretreatment not only abolished the acrolein-induced decreases in both these measures, but raised catalase expression above basal level as well. In

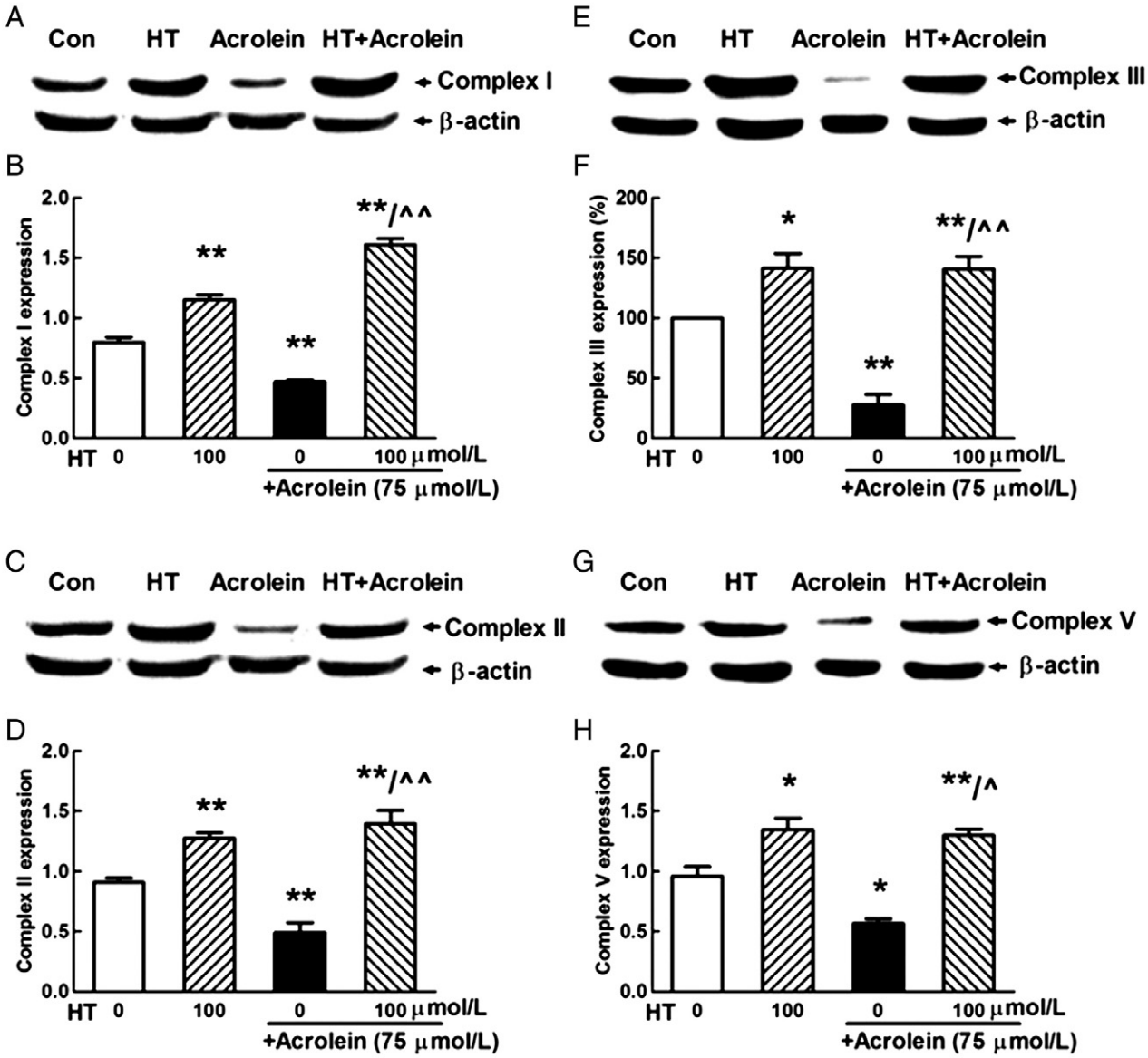

Fig. 10. Hydroxytyrosol protected ARPE-19 cells from acrolein challenge and increased protein expression of electron transfer complexes I, II, III, and V in both basal and challenged cells. Following pretreatment of cells with hydroxytyrosol (100 μmol/L, 48 h) and treatment or not with acrolein (75 μmol/L, 24 h), protein expression of the electron transfer complexes was detected by Western blotting. (A, C, E and G) show representative images and (B, D, F and H) display protein expression ratios relative to beta actin for complexes I, II, III and V, respectively. Values are means±S.E. of three to five experiments. *P<.05 and **P<.01 vs. control (no acrolein, no hydroxytyrosol); ^P<.05 and ^^P<.01 vs. acrolein-treated.

addition, hydroxytyrosol significantly increased basal catalase protein expression and activity in unchallenged cells (Fig. 12B and C).

4. Discussion

Mechanistic studies of hydroxytyrosol action so far have focused on its antioxidant activity [7,44,45]. Our previous study demonstrated that hydroxytyrosol protects RPE cells from acrolein-induced oxidative damage and mitochondrial dysfunction [12]. The present study further explores the underlying mechanisms of hydroxytyrosol's protective effects in RPE cells. Our study has focused mainly on the following two pathways:

1. Stimulation of endogenous antioxidant systems, including assessment of phase II detoxifying enzymes, mitochondrial antioxidants and catalase and
2. Stimulation of mitochondrial biogenesis.

Our results may be summarized in a mechanistic model of how hydroxytyrosol protects RPE damage by simultaneously activating mitochondrial biogenesis and phase II detoxifying enzyme systems (Fig. 13).

4.1. Phase II enzyme induction by hydroxytyrosol

The role of the activation of the transcription factor Nrf2 and its repression by Keap1 in the control of phase II gene expression (Fig. 13, right hand side) has been well established during the last decade. Recently, there has been great interest in finding natural phase II detoxifying enzyme inducers to enhance antioxidant response systems for health maintenance. Sulforaphane [46], lipoic acid [20,47,48] and lipoamide [49] are some examples. The present study investigated whether hydroxytyrosol may activate Nrf2 expression to promote the expression of phase II detoxifying enzymes [15]. As clearly shown, hydroxytyrosol indeed activated Nrf2 and increased the protein expression and activities of GCL, NQO1 and HO-1. These

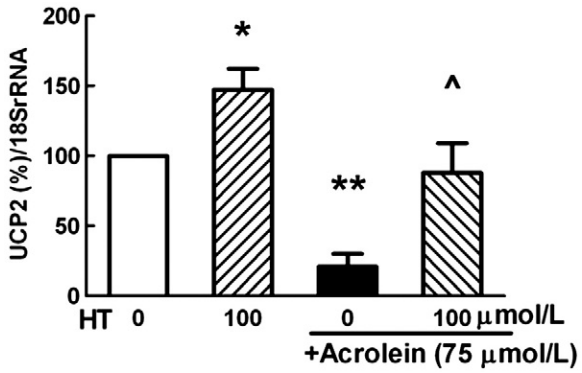

Fig. 11. Hydroxytyrosol treatment increased the gene expression of UCP2 and protected this gene from decrease by acrolein challenge (75 µmol/L, 24 h). ARPE-19 cells were treated with 100 µmol/L hydroxytyrosol for 48 hours; then RNA was isolated, reverse-transcribed to cDNA, and UCP2 gene expression relative to 18SrRNA was determined by real time PCR. Data are means±S.E. from five separate experiments. *P<.05, and **P<.01vs. control; ^P<.05 vs. acrolein-treated.

results strongly suggest that hydroxytyrosol confers additional antioxidant protection in addition to its direct antioxidant activities, such as free radical scavenging and iron chelation. Our previous work has demonstrated that hydroxytyrosol protects against the acrolein-induced decrease in Nrf2 expression on the total protein level [12,17]. Because Nrf2 causes transcriptional activation of antioxidant response elements in the nucleus, the increase in nuclear Nrf2 level should be the key index for phase II detoxifying enzyme activation, resulting in enhancement of the antioxidant defense system. Phase II detoxifying enzymes are part of an elaborate system for protection against the toxicity of xenobiotics and reactive oxygen and nitrogen species that are constant dangers to the integrity of mammalian DNA [46] and lipids. Induction of phase II detoxifying enzymes is also an effective means for achieving protection against a variety of carcinogens and other oxidative damage in animals and humans [50].

4.2. Stimulation of mitochondrial biogenesis by hydroxytyrosol

Mitochondrial function is related to mitochondrial content, which can be affected by exercise and environmental factors [28] (Fig. 13, left). The key regulators of mitochondrial biogenesis include PPARGC1α and its upstream signals, including AMPK, calcium/calmodulin-dependent protein kinase IV and nitric oxide [26,28–31]. Stimulation of PPARGC1α can suppress neurodegeneration [27], while repression of PPARGC1α can lead to mitochondrial dysfunction and neurodegeneration [51]. Work in our laboratory with two mitochondrial nutrients, lipoic acid and/or acetyl-L-carnitine in cellular and animal models of diabetes and Parkinson's disease has shown that lipoic acid and/or acetyl-L-carnitine are able to stimulate mitochondrial biogenesis in neurons [52], adipocytes [53] and muscle [54]. The present study was carried out to determine whether stimulation of mitochondrial biogenesis is a mechanism by which hydroxytyrosol can improve mitochondrial function, and thus reduce oxidative damage in acrolein-induced RPE toxicity. The present study demonstrates that hydroxytyrosol stimulated the expression of PPARGC1α, the key factor for mitochondrial biogenesis and the protein expression of Tfam, a key transcription factor involved in mitochondrial biogenesis and target gene of PPARGC1α. In line with this, hydroxytyrosol also increased the protein levels of mitochondrial complexes I, II, III and V. Moreover, hydroxytyrosol up-regulated gene expression of UCP2, also a PPARGC1α target gene involved in mitochondrial function. These results suggest that hydroxytyrosol stimulates mitochondrial biogenesis and function.

The dual activities of mitochondrial biogenesis and antioxidant defense mediated by PPARGC1α are closely coupled. However, based on our results on the activation of the upstream signals for regulating PPARGC1α, viz., phosphorylation of AMPK and expression of eNOS, the induction of phase II detoxifying enzymes by hydroxytyrosol seems to constitute a pathway independent of the dual activities of PPARGC1α. The detailed molecular mechanisms for this warrant further study.

Aldehydes, including acrolein, are important oxidative stress biomarkers of lipid peroxidation, and have been shown to increase during aging and in diseases [55]. Intravitreous injection of paraquat provides a new model of oxidative damage-induced retinal degeneration. Intraocular injection of paraquat caused condensation of chromatin and thinning of the inner and outer nuclear layers indicating cell death, and terminal deoxynucleotidyl transferase-mediated dUTP-biotinide end-labeling demonstrated that one mechanism of cell death was apoptosis. The cell death and apoptosis were accompanied by increases in acrolein, superoxide radicals and carbonyl adducts in the retina and retinal pigmented epithelium [56]. Acrolein toxicity was also demonstrated in retinal ganglion cell

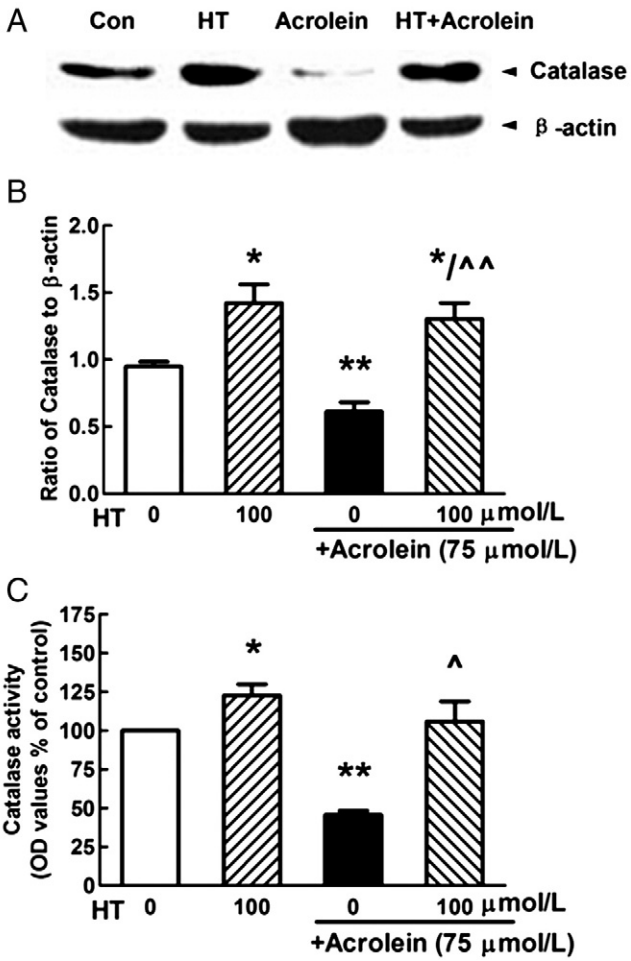

Fig. 12. Hydroxytyrosol increased catalase both in untreated and in acrolein-challenged ARPE-19 cells. Hydroxytyrosol (100 µmol/L, 48 h) up-regulated catalase protein and activity levels in untreated ARPE-19 cells, and prevented their suppression by acrolein (75 µmol/L, 24 h). Catalase protein expression is shown as (A) a representative Western blot image and (B) as quantification of Western blots. Values represent the arithmetic means±S.E. of data from three separate experiments. (C) Catalase activity. Values are means±S.E. of data from three separate experiments, each experiment performed in triplicate. *P<.05 and **P<.01 vs. control (hydroxytyrosol 0 µmol/L); ^P<.05 and ^^P<.01 vs. acrolein-treated.

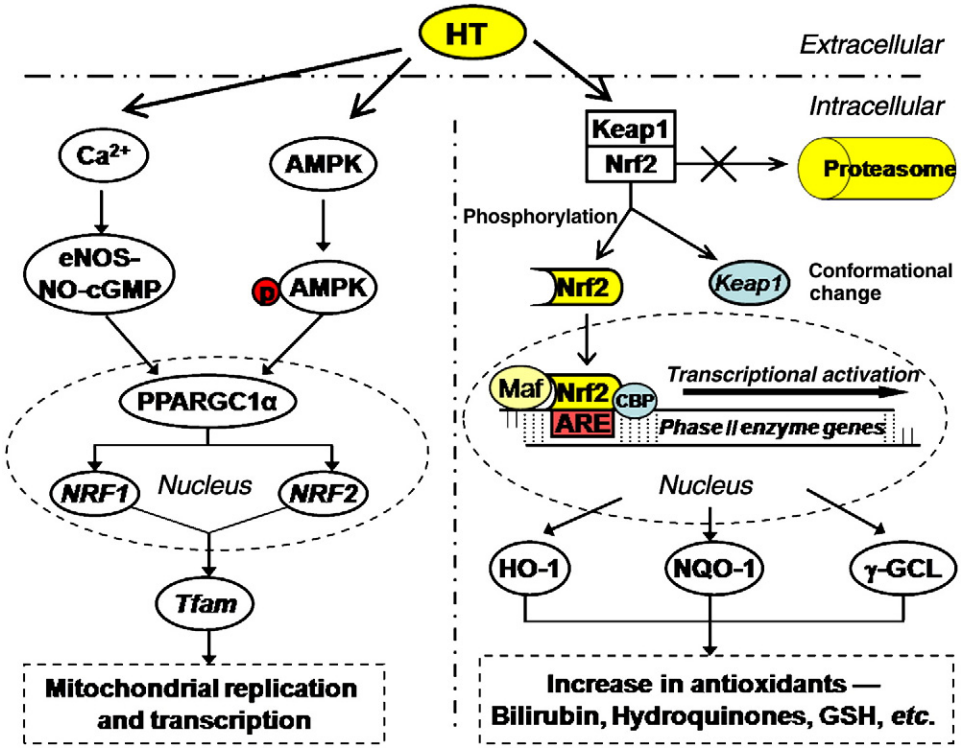

Fig. 13. Schematic illustration of the possible mechanisms of hydroxytyrosol protection against acrolein-induced oxidative damage and mitochondrial dysfunction. There are two pathways related to hydroxytyrosol protection of oxidative injury. The first one is the stimulation of mitochondrial biogenesis by up-regulating AMPK, eNOS and the PPARGC1α signaling pathway; the second is the induction of phase II detoxifying enzymes by up-regulating the Keap1/Nrf2 pathway. The interactions shown are based on information derived from our past and recent studies [13,14]. Lines terminating in arrows represent positive regulation. This scheme is adapted from *Toxicology* 246 2008 24–33 and *Neurochemical research* 33 2008194–203.

(E1A-NR.3) cultures [57]. Although the level of acrolein in retina is not known yet, in our estimation, it may be similar to 4-hydroxynonenal levels because both compounds have been used as indices of oxidative damage in cone cell death in retinitis pigmentosa [58]. Therefore, use of acrolein and hydroxytyrosol in model systems may have practical impact for elucidating the mechanisms of retinal degeneration and finding strategies for its prevention and treatment. It should be pointed out that primary human fetal RPE cells generally are considered a better model than the ARPE-19 cell line for use in RPE studies. However, work in our laboratory [37] has previously demonstrated that primary human fetal RPE cells are comparable to the ARPE-19 cells in sensitivity to acrolein toxicity and lipoic acid protection. Thus, all our experiments have been performed with the ARPE cell line. Of course, further studies with human fetal RPE and animal models are warranted.

In conclusion, hydroxytyrosol has been shown to be a potent inducer of phase II detoxifying enzymes, and a stimulator of mitochondrial biogenesis in retinal pigment epithelial cells. These results provide new mechanistic insights into how hydroxytyrosol may contribute to eye health.

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