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Hydroxytyrosol protects retinal pigment epithelial cells from acrolein-induced oxidative stress and mitochondrial dysfunction

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Abstract

Hydroxytyrosol (HTS) is a natural polyphenol abundant in olive oil. Increasing evidence indicates HTS has beneficial effect on human health for preventing various diseases. In the present study, we investigated the protective effects of HTS on acrolein-induced toxicity in human retinal pigment epithelial cell line, ARPE-19, a cellular model of smoking- and age-related macular degeneration. Acrolein, a major component of the gas phase cigarette smoke and also a product of lipid peroxidation *in vivo*, at 75 μmol/L for 24 h caused significant loss of cell viability, oxidative damage (increase in oxidant generation and oxidative damage to proteins and DNA, decrease in antioxidants and antioxidant enzymes, and also inactivation of the Keap1/Nrf2 pathway), and mitochondrial dysfunction (decrease in membrane potential, activities of mitochondrial complexes, viable mitochondria, oxygen consumption, and factors for mitochondrial biogenesis, and increase in calcium). Pre-treatment with HTS dose dependently and also time dependently protected the ARPE-19 cells from acrolein-induced oxidative damage and mitochondrial dysfunction. A short-term pre-treatment with HTS (48 h) required >75 μmol/L for showing protection while a long-term pre-treatment (7 days) showed protective effect from 5 μmol/L on. The protective effect of HTS in this model was as potent as that of established mitochondria-targeting antioxidant nutrients. These results suggest that HTS is also a mitochondrial-targeting antioxidant nutrient and that dietary administration of HTS may be an effective measure in reducing and or preventing cigarette smoke-induced or age-related retinal pigment epithelial degeneration, such as age-associated macular degeneration.

Keywords: DNA damage, hydroxytyrosol, macular degeneration, mitochondrial membrane potential, nuclear factor-E2-related factor 2, protein carbonyl.
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Cigarette smoke is now considered a strong risk factor for the development of age-related macular degeneration (Thornton *et al.* 2005; Khan *et al.* 2006). The particular concern as health risks in cigarette smoke are the following six toxins: acrolein, acetaldehyde, acrylonitrile, benzene, 1,3-butadiene, and formaldehyde (Nazaroff and Singer 2004). Among them, acrolein has a high hazard index and causes oxidative stress by reacting with sulfhydryl groups (Esterbauer *et al.* 1991) and is more toxic (about 10–1000 times) than formaldehyde, acetaldehyde and 4-hydroxynonenal (Nguyen and Picklo 2003). It is estimated that acrolein is present in the gas phase of cigarette smoke in amounts of about 124–468 μg/cigarette (Shibamoto 2006) and can reach concentration of up to 80 μmol/L in respiratory tract lining fluids as a result of smoking (Eiserich *et al.* 1995). Acrolein, besides being a smoking component and an ubiquitous pollutant in environment, can also be formed from polyunsaturated fatty acids during lipid peroxidation, both *in vitro* and *in vivo* as well as enzymatic oxidation of polyamine metabolites (Esterbauer *et al.* 1991). Being an important oxidative stress biomarkers, lipid peroxidation has been shown to increase during aging and in diseases (Esterbauer *et al.* 1991; Liu *et al.* 1997). Although the level of acrolein in retina is not known yet, we estimate it may be at similar level as 4-hydroxynonenal because both of them have been used similarly as indices of oxidative damage in retinal degeneration using immunohistochemical staining (Shen *et al.* 2005; Cingolani *et al.* 2006).

We have hypothesized that acrolein, whether from smoking or as an *in vivo* by-product of lipid peroxidation, may cause oxidative mitochondrial damage in retinal pigment epithelial (RPE) cells and that the mitochondrial dysfunction

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Abbreviations used: DCFH, 2′,7′-dichlorofluorescein; GSH, glutathione; GST, glutathione *S*-transferase; HTS, hydroxytyrosol; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodine; mtTFA, mitochondrial transcription factor A; Nrf2, nuclear factor-E2-related factor 2; RPE, retinal pigment epithelial; SOD, superoxide dismutase.

may be a major cause to the onset and progress of age-related macular degeneration. In our previous study, we have treated human fetal RPE cells, or ARPE-19 cells, an established cell line with many of the characteristics of RPE cells, with acrolein and found that exposure to acrolein caused cytotoxicity, including decreases in cell viability, mitochondrial potential, glutathione (GSH), antioxidant capacity, nuclear factor-E2-related factor 2 (Nrf2) expression, activities of mitochondrial enzymes, and increases in the levels of oxidants, protein carbonyls, and calcium. In addition, we have shown that pre-treatment with R-α-lipoic acid, a well-known mitochondrial targeting antioxidant nutrient, effectively protected RPE cells from acrolein toxicity (Jia *et al.* 2007). Therefore, the acrolein-induced oxidative mitochondrial dysfunction in RPE cells may be a useful model to study the mechanisms of smoking-induced RPE degeneration and subsequently to search mitochondria-targeting antioxidant nutrients and drugs for preventing smoking-caused RPE degeneration.

Epidemiologic studies demonstrated that the people of the Mediterranean basin enjoy a healthy lifestyle with decreased incidence of heart disease (Covas *et al.* 2006), prostate and colon cancer (Tuck and Hayball 2002; Owen *et al.* 2004), and rheumatoid arthritis (Wahle *et al.* 2004). One of the possible reasons is that Mediterranean people have a high intake of olive and olive oil. Among all known natural antioxidants, olive phenols contain the highest antioxidant activity. One of them, hydroxytyrosol (HTS), is abundant in olives (especially those that have not been subjected to the Spanish brining process), and virgin olive oil, which has been widely used in European, especially Mediterranean diets. Studies have shown that HTS, which is absorbed in the intestine after oral administration is an effective antioxidant including in the post-prandial phase (Manna *et al.* 2000). HTS exerts various health effects (Tuck and Hayball 2002), e.g. olive water extract with high percentage of HTS, increased plasma antioxidant capacity and reduced the effect of free radicals (Manna *et al.* 1999; Visoli *et al.* 1998, 2002; Stupans *et al.* 2002). There are studies on the protective effect of HTS on cytotoxicity induced by various oxidants (hydrogen peroxide, cyclosporine) in various cellular systems, such as in rat renal tubular cells (Galletti *et al.* 2005), human erythrocytes (Manna *et al.* 1999), and Jurkat cells (Nousis *et al.* 2005). However, no study has been reported which studied the effect of HTS on acrolein-induced RPE damage and age-related macular degeneration. In the present study, we have tested the protective effects of HTS on oxidative stress and mitochondrial dysfunction in the acrolein-induced RPE cellular model.

Materials and methods

Reagents

Acrolein was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HTS was a gift from DSM Nutritional Products Ltd, Switzerland and used for all experiments.

Cell culture

The human ARPE-19 cell line was obtained from Dr Nancy J. Philp and was cultured according to her methods (Philp *et al.* 2003). The ARPE cells were maintained in Dulbecco's modified Eagle's medium-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.

Acrolein exposure and HTS supplementation

All experiments were performed with an 80% confluence monolayer. HTS was dissolved in dimethyl sulfoxide (final dimethyl sulfoxide concentration ≤ 0.025%). Acrolein was dissolved in Dulbecco's modified Eagle's medium-F12 medium right before each experiment (Jia *et al.* 2007). For the toxicity experiment, cells were exposed to acrolein for 24 h (Jia *et al.* 2007). The protective effects of HTS were studied with the acute toxicity model by pre-treating cells with HTS for 48 h or 7 days.

MTT assay for cell viability

The ARPE-19 cells were seeded at 4×10^4 per well in a 96-well plate. Cells were pre-treated with different concentration of HTS for either 48 h or 7 days upon 80% confluence and then treated with 75 μmol/L acrolein for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay reduction assay was used as a qualitative index of cell viability. The optical densities were read at 555 nm using a microplate spectrophotometer (Spectra Max 340; Molecular Dabices, Sunnyvale, CA, USA).

JC-1 assay for mitochondrial membrane potential

Mitochondrial potential change ($\Delta\Psi$) was assessed in live ARPE-19 cells using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodine (JC-1) (Smiley *et al.* 1991). For quantitative fluorescence measurement, cells were rinsed once after JC-1 staining and scanned with a multilabel counter (Wallac 1420; Perkin-Elmer Life Sciences, Wellesley, MA, USA) at 485 nm excitation and 535 nm and 590 nm emission, to measure green and red JC-1 fluorescence respectively. Each well was scanned at 25 areas rectangularly arranged in 5 × 5 pattern with 1 mm intervals and approximate beam area of 1 mm^2 (bottom scanning). For microscopic observation of JC-1 staining ARPE-19, images were collected with FITC and TRITC fluorescence filter cubes on a microscope (Axiover25; Carl Zeiss Meditec, Inc., Thornwood, NY, USA) equipped with a charge-coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI, USA), and processed with image-management software (Photoshop ver. 7.0; Adobe Systems, Mountain View, CA, USA).

Determination of oxidant generation

The generation of intracellular oxidants was determined by the formation of a fluorescent 2′, 7′-dichlorofluorescein (DCFH) on oxidation of the non-fluorescent, reduced, DCFH (LeBel *et al.* 1992). The fluorescence intensity of the supernatant was measured with a

plate reader (Wallac; Perkin-Elmer) at 485 nm excitation and 535 nm emission. Cellular oxidant level was expressed as relative 2′, 7′-dichlorofluorescein per μg of protein (bicinchoninic acid method).

Total antioxidant power

The total antioxidant capability was assayed with a commercially available assay kit (Jiancheng Biochemical Inc., Nanjing, China) The principle of the test is to measure the change of colors of the reduction of Fe^{3+} to Fe^{2+} by the reducing components in the samples. The reducing components may include enzymatic and non-enzymatic molecules such as lipid-soluble antioxidant vitamin E and water-soluble antioxidants vitamin C, uric acid, bilirubin, thiols, and gluthathione, etc. The optical density was measured at 520 nm by a microplate reader.

Superoxide dismutase measurement

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

Assay for GSH levels

The GSH level was assayed with a commercially available assay kit (Jiancheng Biochemical Inc.) using an assay based on a thiol-specific reagent, dithionitrobenzoic acid. The adduct was measured spectrophotometrically at 412 nm.

Glutathione *S*-transferase activity assay

Cells were cultured in 6-well plates. After treatments, cells were lysed by ultrasonic in 10 mmol/L sodium phosphate buffer, pH 6.5. The total protein contents of cell lysate were quantified by bicinchoninic acid method. The activity was measured with 5 mg protein, 1 mmol/L GSH, 1 mmol/L chloro-2, 4-dinitrobenzene, 3 mg/ml bovine serum albumin in 10 mmol/L sodium phosphate buffer. The mixture was scanned at 340 nm for 5 min at 25°C as previously described (Pabst *et al.* 1974).

Detection of protein carbonyls

For determination of protein carbonyls, a measure of protein oxidation, cells were grown on 100 mm plates. 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 method.

Comet assay for DNA damage test

DNA damage was detected by Comet assay (Tice *et al.* 2000). Cells were imaged using Olympus BX61 microscope with a ×60 oil immersion objective (numerical aperture = 1.25), which is linked to an Olympus DP70 microcomputer imaging device. Nuclei stained with 4,6-diamidino 2-phenylindole dihydrochloride were excited with a UV laser (380 nm). Nuclear with tail was considered as being damaged. Forty-six images were randomly selected from each sample and the comet tail density (a product of the DNA fraction in the tail) was measured. The ratio of nuclear material migrated in the comet head (head DNA) and out of the comet head into the comet tail (tail DNA, comet intensity) were considered. Each data point of the initial DNA damage in the graphic presentation represents the mean ± SE of three individual experiments.

Total levels of nuclear factor-E2-related factor 2

Cells were grown on 100 mm plates and homogenized (1 : 10) in cell lysis buffer for western and IP (Beyotime, Jiangsu, China). Thirty micrograms of protein was used for western analysis of total Nrf2 levels and probed with anti-Nrf2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1 : 500 titer. Chemiluminescent detection was performed by an ECL western blotting Detection kit from Amersham Pharmacia (Buckinghamshire, UK) (Suh *et al.* 2004).

Intracellular calcium assay

The intracellular calcium level was measured by a commercially available assay kit (Jiancheng Biochemical Inc.). Calcium reacted with methyl thymol blue, and formed blue complex. Optical density was measured at 610 nm by a microplate reader.

Viable mitochondria

The fluorescent probe Mito-Tracker Green FM (Molecular Probes, Carlsbad, CA, USA) was used to determine the viable mitochondria. Cells were gown in 6-well plates until confluent. The cells were then incubated with Mito-Tracker Green FM (100 nM) for 30 min at 37°C in 5% CO_2. At the end of incubation the cells were washed three times in phosphate-buffered saline and analyzed by flow cytometry (FACSAria™, Becton-Dickinson, Franklin Lakes, NJ, USA).

Oxygen consumption

The cell was pre-treated with HTS for 48 h and acrolein for 24 h about 1×10^6 cells are incubated in a medium without serum. BD™ Oxygen Biosensor System plate (BD Biosciences, San Jose, CA, USA) is employed to detect cell oxygen consumption. Plates were sealed and scanned by a fluorescence spectrometer (Flex StationII 384; Molecular Devices, Sunnyvale, CA, USA) at 1-min intervals for 60 min at an excitation wavelength of 485 nm and emission wavelength of 630 nm (Wilson-Fritch *et al.* 2004).

Quantitative reverse transcriptase-polymerase chain reaction

Real-time PCR was used to measure levels of Nrf1 and mitochondrial transcription factor A (mtTFA). 1 μg of total RNA, isolated by TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) from the cells cultured in 6-well plates, was reverse transcribed using Revertra Ace (Toyobo, Japan) following the supplier's instruction. 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: initial step of 10 min at 95°C, then 40 cycles of 30-s denaturation at 95°C, 30-s annealing at 65°C for Nrf1 or 60°C for mtTFA and 30-s extension at 72°C. The cycle time value was automatically detected. The cycle number at which the various transcripts were detectable (CT) was compared with that of 18S-RNA, referred to as ΔCT. The gene relative level was expressed as 2-(ΔΔCT), in which ΔΔCT equals ΔCT of Nrf1 minus ΔCT of 18S-RNA (Nisoli *et al.* 2003).

Mitochondrial DNA level

Mitochondrial DNA expression was determined by detecting D-Loop quantity with real-time PCR (described as previous). Genome DNA was isolated by H. Q. & Q. Tissue DNA Kit (An Hui University-Gene Biotechnology Co., Ltd, Jixi, Anhui, China). The reaction was performed following as described above, except 1 min annealing at 55°C. The Primers for real-time PCR or cDNA probe construction (5′ to 3′) were:

Gene	Forward primer	Reverse primer
18S RNA	CATTCGAACGTCTG-CCCTATC	CCTGCTGCCTTCCTT-GGA
Nrf1	GCCGTCGGAGCAC-TTACT	CTGTTCCAATGTCAC-CACC
MtTFA	ATTCCAAGAAGCTA-AGGGTG	CTTCCCAAGACTTCA-TTTC
D-Loop	CCCACTAGGATACC-AACAAAC	CCAGATGTCGGATAC-AGTTCA

Assays for activities of mitochondrial complexes

ARPE-19 cells were cultured in 100 mm plates, washed in phosphate-buffered saline, resuspended in an appropriate isotonic buffer (0.25 mol/L sucrose, 5 mmol/L Tris–HCl, pH 7.5, and 0.1 mmol/L phenylmethylsulfonyl fluoride), and homogenized. Mitochondria were isolated by differential centrifugation of the cell homogenates. NADH-CoQ oxidoreductase (complex I), succinate-CoQ oxidoreductase (complex II), were assayed spectrometrically using the conventional assays (Humphries and Szweda 1998; Humphries *et al.* 1998; Picklo and Montine 2001) with minor modifications. Complex V activity was measured as oligomycin-sensitive, Mg^{2+}-ATPase activity (Picklo and Montine 2001).

Statistical analysis

Data were presented as mean ± SE. Statistical significance was calculated using Prism software (version 4.0a; Irvine, CA, USA) using one-way ANOVA, and p-value < 0.05 was considered significant.

Results

Effect on cell viability and in mitochondrial membrane potential

Hydroxytyrosol itself had no apparent effect on cell viability at the concentrations tested (10–100 μmol/L HTS). Acrolein (75 μmol/L, 24 h) induced a significant decrease in cell viability by 49%. The pre-treatments of ARPE-19 cells with HTS for 48 h resulted in a significant protection against acrolein-induced toxicity at the concentrations of at 75 and 100 μmol/L, with respectively 93% and almost 100% of the control (Fig. 1a).

The protective effect of HTS was greatly enhanced by a longer pre-treatment. As shown in Fig. 1b a pre-treatment with HTS for 7 days protected against the acrolein-induced cell viability decrease significantly from 5 μmol/L on, resulting in a 15-fold decrease than the shorter-term (48 h) pre-treatment on the lowest effective concentration and a complete protection at 20 μmol/L.

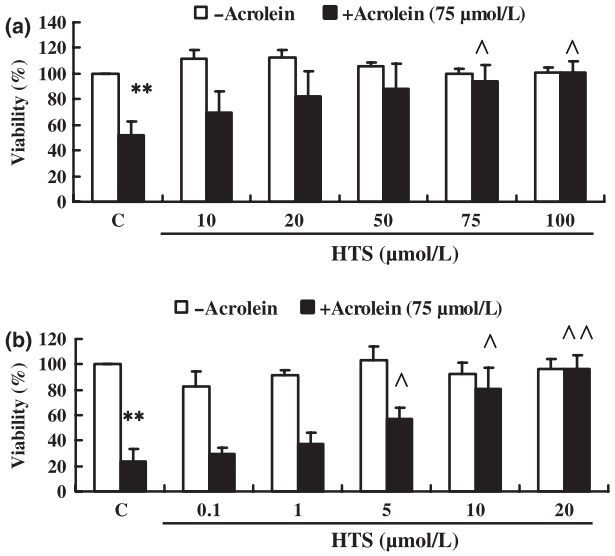

Fig. 1 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease in cell viability measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (a) ARPE-19 cells with 48 h-HTS pre-treatment. Values are percentage to the control (no acrolein, no HTS) and are mean ± SE from four separate experiments; each experiment was performed in triplicate. $^{**}p < 0.01$ versus control (no acrolein, no HTS). $^{\wedge}p < 0.05$ versus acrolein 75 μmol/L without HTS. (b) ARPE-19 cells with 7 days-HTS pre-treatment. Values are mean ± SEM of data from three separate experiments, each experiment performed in triplicate. $^{**}p < 0.01$ versus control (no acrolein, no HTS). $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ versus acrolein without HTS.

Hydroxytyrosol itself had no apparent effect on mitochondrial membrane potential in ARPE-19 cells at concentrations < 20 μmol/L, however, a decrease of 12%, 14%, and 18% was found at the concentrations of 50, 75, and 100 μmol/L, respectively (Fig. 2a). Acrolein (75 μmol/L, 24 h) caused a 64% decrease in mitochondrial membrane potential, expressed as the red–green JC-1 fluorescence ratio. Short-term (48 h) treatment with HTS at concentrations lower than 50 μmol/L showed no protective effect against acrolein-induced decrease in membrane potential; however, 75 and 100 μmol/L HTS pre-treatment for 48 h resulted in 2.3- and 2.2-fold increases compared with the level of acrolein treatment (Fig. 2a).

Similar to the protection on cell viability, a 7-day pre-treatment enhanced the protective effect of HTS. As shown in Fig. 2b, HTS at 10 μmol/L significantly protected the acrolein-induced decrease in mitochondrial membrane potential, and 20 μmol/L HTS showed a complete reverse to the control level.

Figure 3 shows representative images of ARPE-19 cells without (Fig. 3a) and with JC-1 staining (Fig. 3b). Acrolein treatment (75 μmol/L) resulted in a significant decrease in cell numbers and a decrease in red J-aggregate fluorescence,

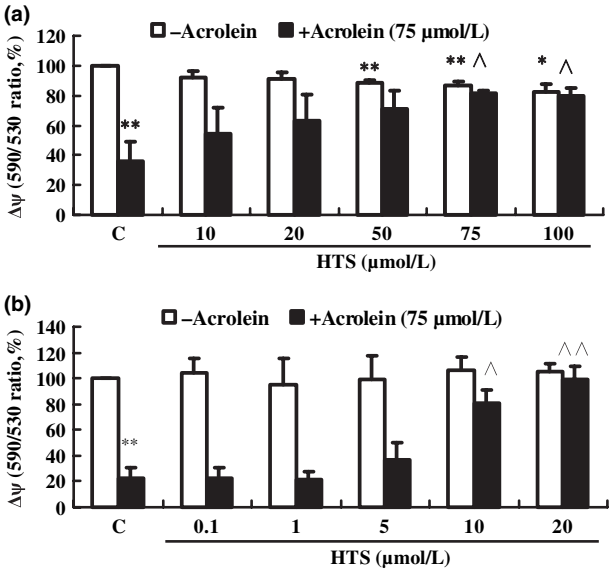

Fig. 2 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease acrolein-induced decrease mitochondrial membrane potential measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodine (JC-1) assay. (a) ARPE-19 cells with 48 h-HTS pre-treatment. Values are percentage to the control (no acrolein, no HTS) and are mean ± SE of data from four separate experiments; each experiment was performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus HTS 0. ^$p < 0.05$ versus HTS 0 + acrolein 75 μmol/L. (b) ARPE-19 cells with 7 day-HTS pre-treatment. Values are mean ± SE of data from one representative of three experiments, performed in triplicate.**$p < 0.01$ versus control (no acrolein, no HTS), ^$p < 0.05$ and ^^$p < 0.01$ versus acrolein without HTS.

reflecting an acrolein-induced decrease in cell viability and mitochondrial potential. Pre-treatment with 100 μmol/L HTS prevented the acrolein-induced decrease in ARPE-19 cell viability and mitochondrial membrane potential as demonstrated by increased cell numbers and cells in normal shapes (Fig. 3a) and red J-aggregate staining of HTS-pre-treated cells (Fig. 3b).

Effects on mitochondrial biogenesis-related factors: mitochondrial DNA synthesis and expressions of Nrf1 and mtTFA

Treatment with acrolein at 75 μmol/L for 24 h caused a significant decrease in the expression of Nrf1, mtTFA, and D-Loop in ARPE-19 cells by 50%, 47%, and 65%, respectively (Fig. 4). Pre-treatment with 100 μmol/L HTS showed significant protections on Nrf1, mtTFA, and D-Loop (Fig. 4) by 2.9-, 2.3-, and 7.4-fold compared with 75 μmol/L acrolein without HTS.

Effect of HTS on acrolein-induced decrease in viable mitochondria

Treatment with 75 μmol/L acrolein caused a significant decrease in viable mitochondria by 42% in ARPE-19 cells (Fig. 5). HTS pre-treatment at 100 μmol/L prevented the decrease in viable mitochondria by an increase of 170% (Fig. 5).

Effect on oxygen consumption

Acrolein at 75 μmol/L decreased oxygen consumption in ARPE-19 cells to 86% relative to control ($p < 0.01$). The oxygen consumption was increased to 19-fold by HTS treatment at 100 μmol/L (Fig. 6).

Effects on mitochondrial complexes

Treatment with acrolein at 75 μmol/L for 24 h caused a significant decrease in the activity of mitochondrial complex I, II, and V by 50%, 58%, and 60%, respectively (Fig. 7). Pre-treatment with 100 μmol/L HTS (48 h) showed significant protections on complex I, complex II, and complex V by 1.8-, 3.1-, and 2.8-fold increase, compared with acrolein treatment alone (Fig. 7).

Effect on intracellular oxidants and Ca^{2+}

Treatment with acrolein at 75 μmol/L for 24 h induced a significant increase in intracellular oxidants approximately 3.3 times compared with control. Pre-treatment with 100 μmol/L HTS for 48 h caused a significant inhibition (77% reduction) (Fig. 8). This protection may be partly because of the free radical scavenging effect of HTS as seen HTS alone at 100 μmol/L also caused an inhibition (22%) of oxidant level in the ARPE-19 cells.

Mitochondrial dysfunction usually results in an increase in cytoplasmic Ca^{2+} level, which is a biomarker of oxidative stress and mitochondrial dysfunction. Acrolein treatment (75 μmol/L) caused a significant increase (22.9%), in intracellular Ca^{2+} level, compared with control ($p < 0.01$) (Fig. 8). Pre-treatment with 100 μmol/L HTS for 48 h before acrolein treatment significantly inhibited the increase in Ca^{2+} by 19.6% ($p < 0.05$).

Effect on protein oxidation

Treatment with acrolein at 75 μmol/L for 24 h caused a significant increase in protein carbonyls, an index of protein oxidation. Pre-treatment with 100 μmol/L HTS for 48 h significantly inhibited the acrolein-induced protein carbonyl increase by 48% (Fig. 9).

Effect on DNA damage

Comet assay demonstrated that acrolein treatment (75 μmol/L for 24 h) caused a 98% nuclear DNA fragmentation. HTS pre-treatment showed a complete protection (Fig. 10).

Effects on intracellular total antioxidant power and antioxidant GSH level

Treatment with acrolein at 75 μmol/L for 24 h decreased intracellular antioxidant power to 23% relative to control ($p < 0.05$). Pre-treatment with 100 μmol/L HTS prevented

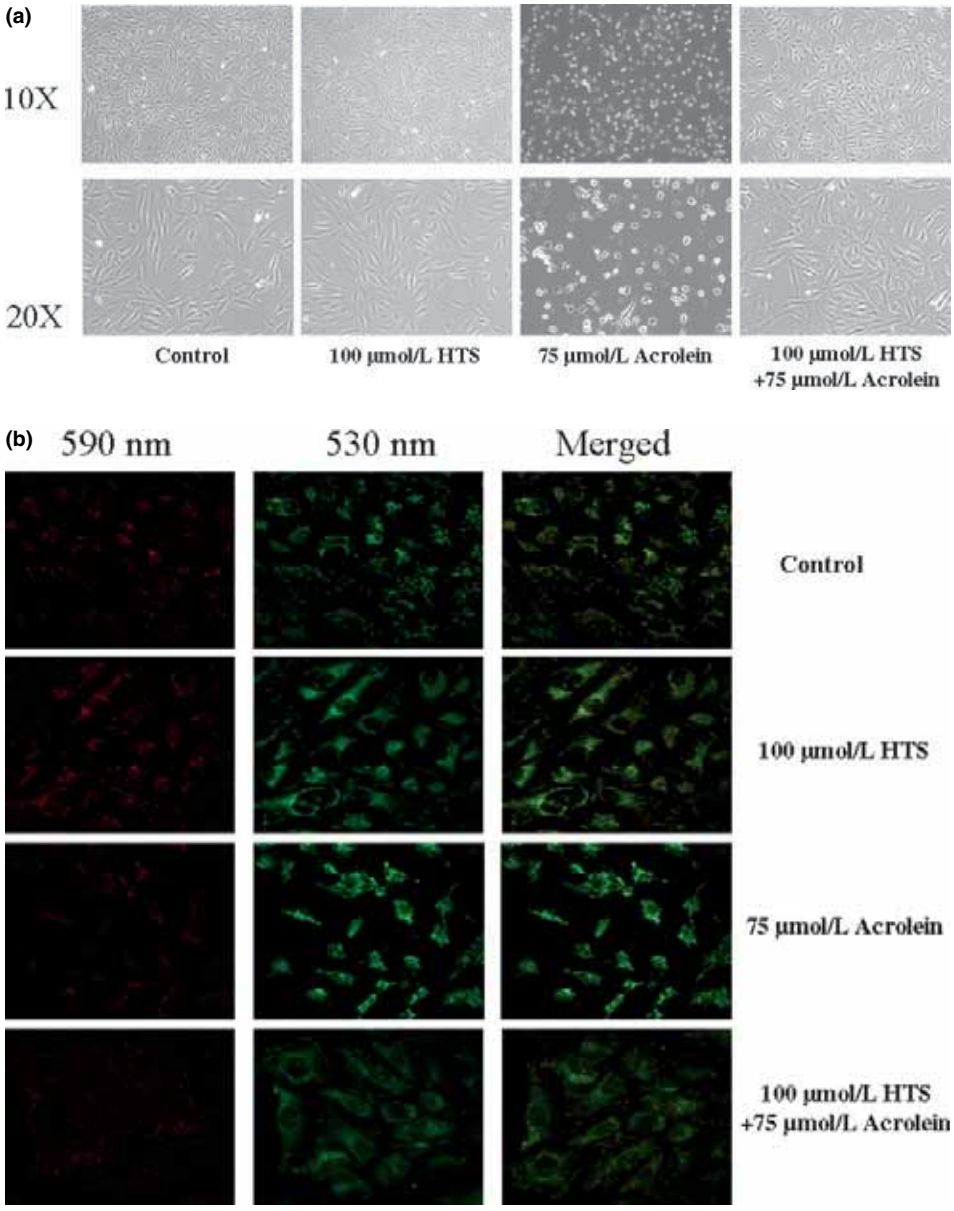

Fig. 3 Representative images of ARPE-19 cell viability and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodine (JC-1) staining of cells pre-treated with hydroxytyrosol (HTS) for 48 h and followed by acrolein exposure for 24 h. (a) Cells were observed under light microscopy with magnification at 10X and 20X. (b) Cells were stained with JC-1 and observed under fluorescent microscopy: left-hand panel shows the mitochondrial membrane potential at 590 nm, the middle panel shows the living cells at 530 mm, and the right-hand panel is the merged image. Each panel is representative of two separate experiments, performed in duplicate.

the cells from this decrease by 32% (Fig. 11). Acrolein treatment also caused a significant decrease in the level of endogenous antioxidant GSH, and HTS pre-treatment (100 µmol/L for 48 h) provided a full protection (Fig. 11).

Effects on the activity of SOD and GST

Treatment with acrolein (75 µmol/L for 24 h) caused a significant decrease (15%) in intracellular total SOD activity by in ARPE-19 cells, and HTS pre-treatment at 100 µmol/L for 48 h prevented the SOD decrease significantly (Fig. 12).

Acrolein treatment also caused a significant decrease (83%) in the activity of glutathione *S*-transferase (GST) and the HTS pre-treatment showed a significant protection by sixfold increase compared with level of the acrolein treatment (Fig. 12).

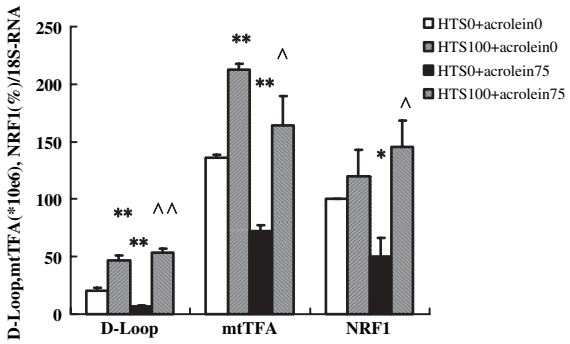

Fig. 4 Protection by hydroxytyrosol (HTS) of the acrolein-induced decrease in DNA and RNA level of mitochondrial biogenesis in ARPE-19 cells D-Loop of mitochondrial DNA, mtTFA, and nuclear factor-E2-related factor 1 (NRF1). ARPE-19 cells were pre-treated with different concentrations of HTS and then treated with 75 μmol/L acrolein. Values are mean ± SEM of data from four separate experiments for mtTFA, and three separate experiments for D-Loop and Nrf1, and each experiment was performed in duplicate.*$p < 0.05$, **$p < 0.01$ versus control (no acrolein, no HTS), ^$p < 0.05$, ^^$p < 0.01$ versus 75 μmol/L acrolein without HTS.

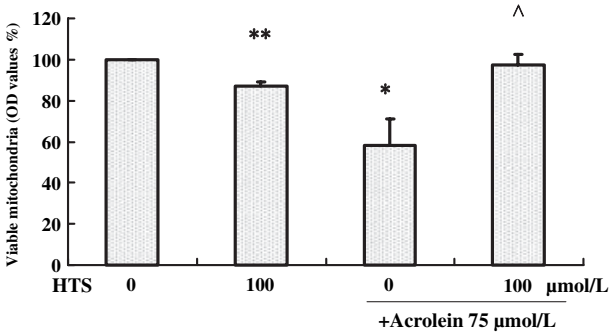

Fig. 5 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease viable mitochondria. Values are mean ± SEM of data from four separate experiments; each experiment was performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus control (no acrolein, no HTS). ^$p < 0.05$ versus HTS 0 + acrolein 75 μmol/L.

Effect on total Nrf2 expression

Acrolein at 75 μmol/L for 24 h caused a significant decrease in the expression of total Nrf2, a key regulator of phase II enzymes. The pre-treatment with HTS at 100 μmol/L for 48 h significantly prevented the cells from acrolein-induced decrease in total Nrf2 level as shown in Fig. 13a (representative western blot image) and Fig. 13b (quantitative results).

Discussion

A number of preventative strategies are under consideration for age-associated macular degeneration (Chakravarthy and Hart 2007). One of the most promising preventions is the NIH-sponsored Age-Related Eye Disease Study, a multicenter clinical trial to evaluate the effects of antioxidant vitamins E and C and beta-carotene with and without zinc (AREDS 2001). In the present study, we have demonstrated that HTS, a natural polyphenol and a rich component in olive oil, significantly protected acrolein-induced cellular toxicity in ARPE-19 cells, a cellular model for smoking- and age-related macular degeneration. The protective ability is quite potent at the μmol/L levels and comparable with the well-known mitochondrial targeting antioxidant nutrient alpha-lipoic acid (Packer *et al.* 1997; Liu and Ames 2005), suggesting HTS may protect the acrolein-induced RPE toxicity by acting as a mitochondrial-targeting antioxidant nutrient.

We have defined mitochondrial nutrients as those which protect mitochondria from oxidative damage and improve mitochondrial function, including those that can: (i) prevent or repair mitochondrial damage, (ii) inhibit oxidant produc-

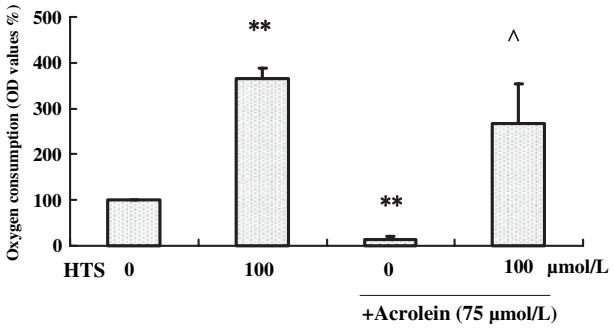

Fig. 6 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease in oxygen consumption. Values are mean ± SEM of data from three separate experiments; each experiment was performed in triplicate. **$p < 0.01$ versus control (no acrolein, no HTS). ^$p < 0.05$ versus acrolein 75 μmol/L without HTS.

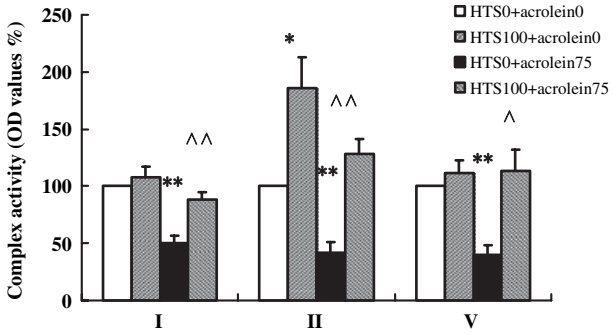

Fig. 7 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease in mitochondrial complex I, II, and V in ARPE-19 cells pre-treated with HTS (100 μmol/L, 48 h) and then exposed to acrolein challenge (75 μmol/L, 24 h). Values are mean ± SEM of data from three separate experiments; each experiment was performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus control (no acrolein, no HTS). ^$p < 0.05$ and ^^$p < 0.01$ versus acrolein.

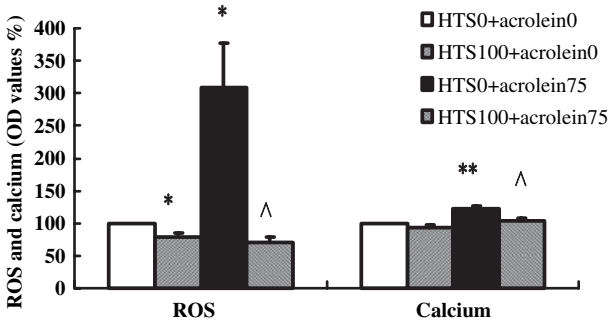

Fig. 8 Protective effects of hydroxytyrosol (HTS) on acrolein-induced increase in reactive oxygen species (ROS) and Ca^{2+} in ARPE-19 cells pre-treated with HTS (100 μmol/L, 48 h) and then exposed to acrolein challenge (75 μmol/L, 24 h). Values are mean ± SEM of data from three separate experiments; each experiment was performed in triplicate. *$p < 0.05$ and **$p < 0.01$ versus control (no acrolein, no HTS). ^$p < 0.05$ versus acrolein.

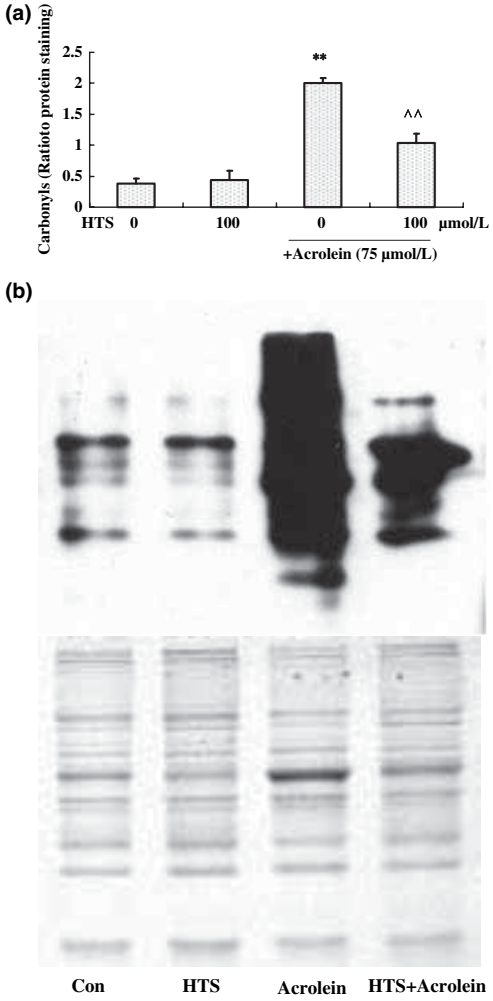

Fig. 9 Acrolein-induced changes in protein carbonyls and protective effect of hydroxytyrosol (HTS) in ARPE cells assayed by western blotting. HTS pre-treatment for 48 h and acrolein exposure for 24 h. (a) Representative western blot image. (b) Quantification of protein carbonyls from four separate experiments. **$p < 0.01$ versus control (no acrolein, no HTS). ^^$p < 0.01$ versus acrolein.

tion in mitochondria, inactivate oxidants, or enhance antioxidant defense, and (iii) repair oxidative damage and enhance antioxidant defense either by induction of phase 2 enzymes through the Nrf2 pathway or by stimulating mitochondrial biogenesis through the activation of peroxisome proliferation activator receptor γ-coactivator 1α pathway, and (iv) act as cofactors/substrates to protect mitochondrial enzymes and/or stimulate enzyme activity (Liu and Ames 2005; Liu 2007). In the present study, we investigated whether HTS acts as a mitochondrial nutrient to protect mitochondria from oxidative damage, thus improve mitochondrial function.

First, we have focused on the effects of HTS on improving mitochondrial function. In the acrolein RPE model, acrolein induced a significant decrease in mitochondrial membrane potential, viable mitochondria, oxygen consumption, and activity of mitochondrial complexes, and increased significantly the calcium ion level. All of these mitochondrial dysfunctions were prevented by the pre-treatment with HTS. These results clearly demonstrate that HTS can protect mitochondria against oxidative insult and can improve mitochondrial function under oxidative challenges.

Second, we examined the effect of HTS on protecting RPE cells from oxidative damage. In the acrolein RPE model, acrolein induced a significant increase in oxidants, and oxidative damage to proteins and DNA, and decreased the total antioxidant power, endogenous antioxidant GSH level, and the activities of antioxidant enzymes SOD and GST. The HTS pre-treatment effectively inhibited the generation of oxidants reduced the oxidative damage to proteins and DNA, increased antioxidant power and GSH level, and also elevated the activities of SOD and GST. Aldehydes in environment pollution and from *in vivo* lipid peroxidation play important role in aging and age-related diseases because aldehydes themselves cross-link proteins/enzymes and also generate other oxidants to cause the inactivation of enzymes by losing binding affinity for coenzymes and substrates (Ames *et al.* 2006). The acrolein-induced loss of GST and SOD activity may be attributed to the covalent binding to the active enzyme sites to form different adducts, such as acrolein–lysine adduct (Uchida *et al.* 1998a,b). Similar to the loss of mitochondrial complexes in the ARPE cells (Fig. 7), we have previously shown that acrolein could also cause the loss of activity of mitochondrial complexes and pyruvate/alpha-ketoglutarate dehydrogenases in rat liver mitochondria (Sun *et al.* 2006). In addition, both *in vitro* and *in vivo* studies showed that acrolein toxicity is mediated by increased oxidants (Fig. 8) and oxidative damage (Luo and Shi 2004), suggesting that acrolein, acts not only as a direct oxidant, but also as a generator of oxidants (Adams and Klaidman 1993). The generated oxidants can further cause

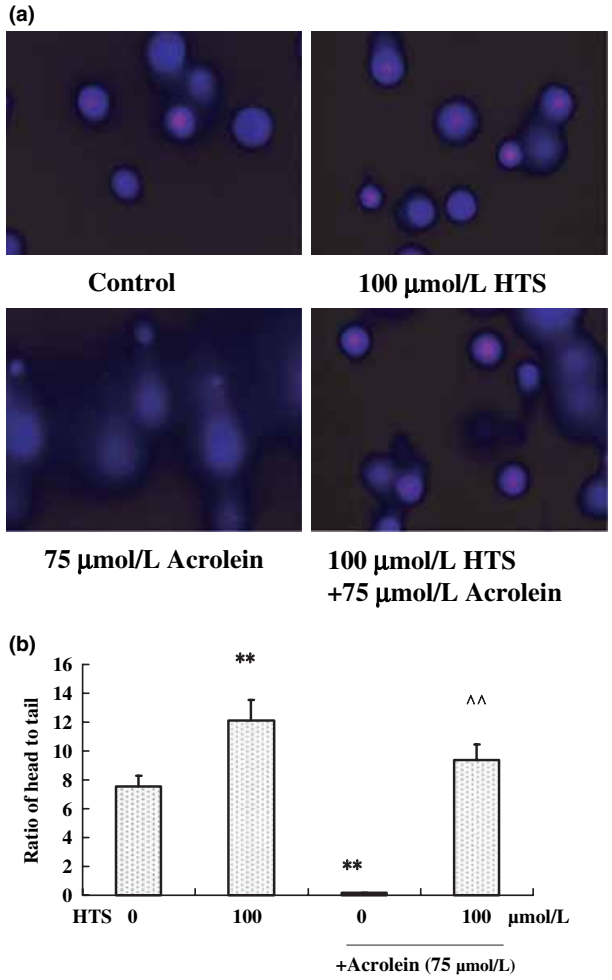

Fig. 10 Effects of pre-treatment with hydroxytyrosol (HTS) on acrolein-induced increase in DNA damage. (a) Representative images of comet assay. (b) Statistic analysis of representative images was performed with three independent experiments. More than 45 images were analyzed in each group in each experiment. Values are mean ± SEM. $^{**}p < 0.01$ versus control (no acrolein, no HTS); $^{\wedge\wedge}p < 0.01$ versus acrolein.

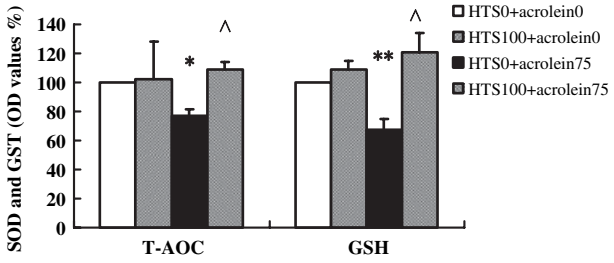

Fig. 11 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease in total-antioxidant power (T-AOP) and glutathione (GSH) in ARPE-19 cells pre-treated with HTS (100 μmol/L, 48 h) and then exposed to acrolein challenge (75 μmol/L, 24 h). Values are mean ± SE of data from three separate experiments; each experiment was performed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control (no acrolein, no HTS). $^{\wedge}p < 0.05$ versus acrolein.

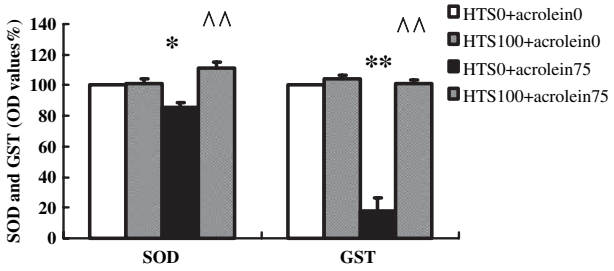

Fig. 12 Protective effects of hydroxytyrosol (HTS) on acrolein-induced decrease in the activity of superoxide dismutase (SOD) and glutathione *S*-transferase (GST) in ARPE-19 cells pre-treated with HTS (100 μmol/L, 48 h) and then exposed to acrolein challenge (75 μmol/L, 24 h). Values are mean ± SEM of data from three separate experiments; each experiment was performed in triplicate. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control (no acrolein, no HTS). $^{\wedge\wedge}p < 0.01$ versus acrolein.

loss of enzyme activities. Therefore, the inhibition of oxidants may be from the direct inactivation of acrolein as an aldehyde across-link breaker or as a scavenger of the oxidants generated by acrolein. Whatever the actions are, the result will be a reduction of the oxidative damage to proteins, DNA and enzyme activities in the organism. These protective effects of HTS strongly suggest that HTS plays a powerful antioxidant role to modulate acrolein-induced oxidative RPE toxicity.

One possible protective mechanism for mitochondrial nutrients is to enhance the antioxidant defense by the activation of the Keap1/Nrf2 pathway (Liu 2007). Nrf2 is known as a key regulator of antioxidant response element-mediated gene expression and the induction of phase 2 detoxifying enzymes and antioxidant enzymes such as SOD and GST. Sulforaphane (Gao and Talalay 2004) and α-lipoic acid (Suh *et al.* 2004; Jia *et al.* 2007), have been shown to be natural phase 2 enzyme inducers, and thus exert health effect. In the present study, acrolein treatment caused significant decrease in nuclear Nrf2 expression and HTS pre-treatment significantly protected the cells from acrolein-induced Nrf2 decrease and elevated the level of antioxidant GSH and total antioxidant power. These results suggest that HTS, like sulforaphane and lipoic acid, prevents acrolein-induced oxidative damage by activating the Keap1/Nrf2 pathway as a phase 2 enzyme inducer.

Another mechanism of the protection of HTS may be the protection or stimulation of mitochondrial biogenesis like α-lipoic acid (Liu 2007). Promoting mitochondrial synthesis by up-regulation of the peroxisome proliferation activator receptor γ-coactivator 1α pathway has been suggested as a strategy for preventing and reversing insulin resistance, obesity, and diabetes (McCarty 2005), such as metformin and 5-aminoimidazole-4-carboxamide ribonucleoside (Kukidome

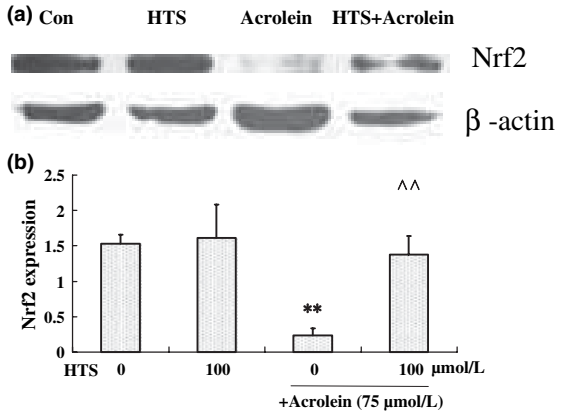

Fig. 13 Acrolein-induced changes total nuclear factor-E2-related factor 2 (Nrf2) expressions and protective effect of hydroxytyrosol (HTS) in ARPE cells assayed by western blotting. HTS pre-treatment for 48 h and acrolein exposure for 24 h. (a) Western blot image and (b) quantification of western blot from four separate experiments. $^{**}p < 0.01$ versus control (no acrolein, no HTS). $^{\wedge\wedge}p < 0.01$ versus acrolein.

et al. 2006), thiazolidenediones such as pioglitazone (Wilson-Fritch *et al.* 2003, 2004; Bogacka *et al.* 2005). Feeding high dose of alpha-lipoic acid to rats increased mitochondrial biogenesis (Kujoth *et al.* 2006). We have demonstrated that the pre-treatment with α-lipoic acid and acetyl-L-carnitine in 3T3L1 adipocytes increased viable mitochondria, mtDNA (measured as the D-loop), complex I expression, and decreasing oxidant level (unpublished). Acrolein induced a significant decrease in the mitochondrial function because of its toxicity on decreasing viable mitochondria. Therefore, an effective protection should be the protection of mitochondrial biogenesis. As we have shown clearly, HTS significantly protected ARPE cells from decrease in mitochondrial DNA synthesis and transcription factors of Nrf1 and mtTFA, suggesting HTS is an effective mitochondrial biogenesis protector or stimulator, like metformin, pioglitazone, or α-lipoic acid.

In conclusion, our studies demonstrate that HTS protected acrolein-induced RPE oxidative damage and mitochondrial dysfunction by multiple mechanisms. The effects on reducing oxidative damage and improving mitochondrial function suggest that HTS is a powerful mitochondrial nutrient like α-lipoic acid and that dietary administration of HTS or its related products may be an effective strategy for reducing and/or preventing cigarette smoke-induced or age-related RPE degeneration, such as age-related macular degeneration.

Acknowledgements

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