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 effects 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 enzymatic oxidation of polyamine metabolites (Esterbauer 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 biomarker, 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 (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...
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 (Nouiss 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% CO2. 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 with HTS for 48 h or 7 days.

MTT assay for cell viability
The ARPE-19 cells were seeded at 4 × 104 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 Dabics, Sunnyvale, CA, USA).

JC-1 assay for mitochondrial membrane potential
Mitochondrial potential change (ΔΨm) was assessed in live ARPE-19 cells using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carboxyanine 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 mm2 (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 Fe³⁺ to Fe²⁺ 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 glutathione, 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 mM/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 mM/L GSH, 1 mM/L chloro-2, 4-dinitrobenzene, 3 mg/ml bovine serum albumin in 10 mM/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 oxidized, 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 ratio. Chemiluminescent detection was performed by an ECL western blotting Detection kit from Amersham Pharmacia (Buckinghamshire, UK) (Suh et al. 2004).

**Viable mitochondria**

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.

**Oxygen consumption**

The cell was pre-treated with HTS for 48 h and acrolein for 24 h about 1 × 10⁶ 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 Reverta 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 ACT. 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:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>18S RNA</td>
<td>CATTGGAACGTTCTG-</td>
<td>CCGTTGCGTTTCT-</td>
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<tr>
<td></td>
<td>CCGTATC</td>
<td>GGA</td>
</tr>
<tr>
<td>Nrf1</td>
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<td>CTCTCCAAAGCTTCA-</td>
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<td></td>
<td>AGGGTT</td>
<td>TTTT</td>
</tr>
<tr>
<td>D-Loop</td>
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<td>CCAGATGTCGGATACC-</td>
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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, Mg2+-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) indicated 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, 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 showed a complete reverse protection 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,
Effects 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 when 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 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 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 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 protein oxidation

Treatment with acrolein at 75 µmol/L for 24 h caused a significant increase in protein carbonyl, 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...
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. 3 Representative images of ARPE-19 cell viability and 5,5′,6′,6′-tetrachloro-1′,3′,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 nm, and the right-hand panel is the merged image. Each panel is representative of two separate experiments, performed in duplicate.
versus 75 µmol/L acrolein. Values are mean ± SEM of data from four separate experiments; each experiment was performed in triplicate. *p < 0.05, **p < 0.01 versus control (no acrolein, no HTS), ^p < 0.05 and ^^p < 0.01 versus 75 µmol/L acrolein without HTS.

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-
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...
loss of enzyme activities. Therefore, the inhibition of oxidants may be from the direct inactivation of acrolein as an aldehyde cross-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 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...
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 \( \alpha \)-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 acrolein-induced RPE oxidative damage and mitochondrial degeneration. This study was supported by National Eye Institute, NIH grant EY0160101, Macular Degeneration Research (MDR Grant 2005-038), Chinese Academy of Sciences grant 05PG14104, and DSM Nutritional Products Ltd.

**References**


