Acrolein, a Toxicant in Cigarette Smoke, Causes Oxidative Damage and Mitochondrial Dysfunction in RPE Cells: Protection by (R)-α-Lipoic Acid

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PURPOSE. To understand better the cell and molecular basis for the epidemiologic association between cigarette smoke, oxidant injury, and age-associated macular degeneration, the authors examined the effects of acrolein, a major toxicant in cigarette smoke, on oxidative mitochondrial damage in retinal pigment epithelial (RPE) cells and the reduction of this damage by lipoic acid.

METHODS. Cultured human ARPE19 cells and primary cultures of human fetal (h)RPE were treated with acrolein. The toxicity of acrolein and the protective effects of R-lipoic acid were examined with a variety of previously described techniques.

RESULTS. Acrolein exposure exceeding 50 µM (24 hours) in ARPR19 cells caused toxicity, including decreases in cell viability, mitochondrial potential, GSH, antioxidant capacity, Nrf2 expression, enzyme activity (mitochondrial complexes I, II, III; superoxide dismutase; and glutathione peroxidase). Acute exposure also increased oxidant levels, protein carbonyls, and calcium. Continuous acrolein exposure over 8 or 32 days caused similar toxicity but from 10- to 100-fold lower doses (0.1–5 µM). Pretreatment with R-α-lipoic acid effectively protected ARPE-19 cells from acrolein toxicity. Primary hRPE cells were comparable to the ARPE-19 cells in sensitivity to acrolein toxicity and lipoic acid protection.

CONCLUSIONS. These results show that acrolein is a mitochondrial toxicant in RPE cells and that acrolein-induced oxidative mitochondrial dysfunction is reduced by lipoic acid. The similar sensitivity of the ARPE-19 and hRPE cells suggests that both models are useful for studying RPE toxicity and protection. These experiments indicate that mitochondria-targeted antioxidants such as lipoic acid may be an effective strategy for reducing or preventing chronic oxidant-induced RPE degeneration in vivo from a variety of sources, including cigarette smoke. (Invest Ophthalmol Vis Sci. 2007;48:339–348) DOI: 10.1167/iovs.06-0248

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moking is a primary risk factor associated with the prevalence and the incidence of neovascular macular degeneration and geographic atrophy. 1-2 This link between smoking and age-associated macular degeneration (AMD) has recently been strengthened by three large epidemiologic studies, including the AREDS (Age-Related Eye Disease Study). 3-5 Smoking is a cause of severe oxidative stress, due to the high concentrations of aldehydes and NOx in cigarette smoke, which markedly deplete ascorbic acid levels and protein sulfhydryl concentrations and cause oxidation of lipids and proteins.6-8

Six toxicants present in cigarette smoke are of particular concern as health risks: acrolein, acetaldehyde, acryonitrile, benzene, 1,3-butadiene, and formaldehyde. 9 Acrolein has a high hazard index and causes oxidative stress by reacting with sulfhydryl groups. 10 It is more toxic (10–1000 times) than formaldehyde, acetaldehyde, and 4-hydroxynonenal 11 and can reach 80 µM in the respiratory tract fluid in smokers. 12

Although the pathogenesis of AMD includes different clinical signs, the degeneration of RPE cells is often observed at early stages of the disease. Initial AMD pathogenesis includes abnormal RPE morphology and pigmentation, accumulation of lipofuscin in RPE cells, and accumulation of drusen between RPE and the underlying Bruch's membrane. Electron microscopic and morphometric studies reveal qualitative and quantitative alterations of mitochondria in human RPE from AMD and from age- and sex-matched control subjects. 13

The strong epidemiologic evidence linking smoking to AMD raises several questions that should be addressed: (1) What are the cellular and molecular mechanisms that underlie this link? (2) Do cigarette smoke components such as acrolein cause injury, especially mitochondrial dysfunction, to RPE cells, as in other cellular and tissue models? (3) Does lipoic acid, a potent inducer of phase-2 antioxidant and sulfhydryl protective enzymes, 14-15 protect RPE cells from smoke/acrolein-caused injury and mitochondrial dysfunction? (4) Are there different responses to acrolein toxicity in a human RPE cell line and primary human fetal RPE cells?

In the present study, the ARPE19 cell line, was treated with acrolein, a major toxicant in tobacco smoke, and the effects on cellular toxicity and mitochondrial function were examined. Acrolein-induced toxicity was also studied using primary cultures of hRPE, which are similar to native hRPE. 16 Both preparations were used to study the protective effects against acrolein-induced toxicity of α-lipoic acid (LA), which is a mitochondria-targeted antioxidant and mitochondrial nutrient. 17 We hypothesize that smoking may cause oxidative mitochondrial damage in RPE cells and that the mitochondrial dysfunction may be a major cause in promoting the onset and progress of age-related macular degeneration.
Acrolein was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). (R)-o-LA Tris salt was a gift from Klaus Wessel (Viatis, Germany) and was used for all experiments. R-ω-LA, 5,5'-dioxy acid, and R,5,5'-dioxy acid used in one comparative experiment were gifts from Asta Medica, Frankfurt, Germany. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Chemical Co.

**Cell Culture**

The human ARPE-19 cell line was obtained from Nancy J. Philp (Thomas Jefferson University, Philadelphia, PA) and was cultured according to her methods. The ARPE cells were maintained in DMEM-F12 supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. The medium was changed every 3 to 4 days. ARPE-19 cells were used within 10 generations. hRPE cells were obtained and cultured as previously reported.

**Acrolein Exposure and LA Supplementation**

All experiments were performed with an 80% confluent monolayer grown in 96- or 6-well plates. (R)-ω-LA Tris salt was dissolved in PBS, and other forms of LA (R-LA, S-LA, and R,S-LA) were dissolved in 1 N NaOH solution, and the pH of the solutions were adjusted to 7.4 with hydrochloric acid. Acrolein was dissolved in PBS each time immediately before an experiment. For the acute toxicity study, cells were exposed to acrolein for 24 hours while for the chronic toxicity study, cells were exposed to acrolein for 8 or 32 days. The protective effects of LA were studied with the acute toxicity model by pretreating cells 1 day before an experiment. For the acute toxicity study, cells were exposed to acrolein for 8 or 32 days. The protective effects of LA were studied with the acute toxicity model by pretreating cells with LA for 48 hours or 8 days.

**MTT Assay for Cell Viability**

The MTT reduction assay was used as a qualitative index of cell viability. The optical densities were read at 555 nm with a microplate spectrophotometer (Spectra Max 340; Molecular Devices, Sunnyvale, CA). Absorbance values were normalized with untreated cells to calculate the changes in cell viability.

**JC-1 Assay for Mitochondrial Membrane Potential**

Mitochondrial potential change (∆Ψ) was assessed in live ARPE-19 cells by using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carboxycyanine iodine (JC-1). For quantitative fluorescence measurement, cells were rinsed once after JC-1 staining and scanned with a multilabel counter (Wallac 1420; PerkinElmer Life Sciences, Wellesley, MA) at 485-nm excitation and 530- 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 an approximate beam area of 1 mm² (bottom scanning). For microscopic observation of JC-1 staining of ARPE-19 cells, images were collected with FITC and TRITC fluorescence filter cubes on a microscope (Axiovert25; Carl Zeiss Meditec, Inc., Thornwood, NY) equipped with a charge-coupled device (CCD) digital camera (Diagnostic Instruments, Sterling Heights, MI), and processed with image-management software (Photoshop, ver. 7.0; Adobe Systems, Mountain View, CA).

**Determination of Oxidant Generation**

The generation of intracellular oxidants was determined by the formation of fluorescent 2',7'-dichlorofluorescein (DCF) on oxidation of the nonfluorescent, reduced DCFH. The fluorescence intensity of the supernatant was measured with a plate reader (Wallac; PerkinElmer) at 485 nm excitation and 535 nm emission. Cellular oxidant level was expressed as relative DCF fluorescence per microgram of protein (bicinchoninic acid [BCA] method).

**Total Antioxidant Power**

Intracellular total antioxidant power of ARPE-19 cells was assayed by the a colorimetric microplate assay kit (total antioxidant power product no. TA 01; Oxford Biomedical Research, Oxford, MI) according to the kit instructions.

**Glutathione Peroxidase and Superoxide Dismutase Measurement**

Intracellular glutathione peroxidase (G-Px) and superoxide dismutase (SOD) activity was measured with respective assay kits (G-Px, catalog no. 703102; and SOD catalog no. 706002; Cayman Chemical), according to the manufacturer’s instructions.

**Assay for GSH Levels**

The GSH level was assayed with a commercially available assay kit (Jiancheng Biochemical Inc., Nanjing, China) that is based on a thiol-specific reagent, dithionitrobenzoic acid (DTNB), and the adduct was measured spectrophotometrically at 412 nm.

**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 a protein oxidation detection kit (Oxyblot; Cell Biolabs, San Diego, CA).

**Total and Nuclear Levels of Nuclear Factor-E2–Related Factor 2**

Cells were grown on 100-mm plates and were homogenized (1:10) in RIPA buffer (150 mM PBS containing 1% [vol/vol] Igepal CA630, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, and 5 μg/μL protease inhibitor mixture), pH 7.4, and 50 μg of protein was used for Western blot analysis of total and nuclear nuclear factor-e2-related factor 2 (Nfr2) levels and probed with anti-Nfr2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5000 titer. Chemiluminescent detection was performed with a Western Blot analysis kit (ECL; GE Healthcare, Piscataway, NJ).

**Intracellular Calcium Assay**

Intracellular Ca²⁺ levels were determined by a calcium assay kit (DICA-500, Quantichrom; BioAssay Systems, Hayward, CA), according to the manufacturer’s instructions.

**Assays for Activities of Mitochondrial Complex I, II, and III**

ARPE-19 cells were cultured in 100-mm plates, washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris-HCl [pH 7.5] and 0.1 mM phenylmethylsulfonyl fluoride), and homogenized. Mitochondria were isolated by differential centrifugation of the cell homogenates. NADH-CoQ oxido-reductase (Complex I), succinate-CoQ oxido-reductase (complex II), CoQ-cytochrome c reductase (complex III) were assayed spectrophotometrically using conventional assays, with minor modifications.

**Statistical Analysis**

Data are presented as the mean ± SD of results of two or three separate experiments, as specified in the figure legends. Statistical significance was calculated (Prism software, ver. 4.0a; GraphPad, San Diego, CA) with one-way ANOVA. P < 0.05 was considered significant.
RESULTS

Effects of Acrolein on Cell Viability in ARPE-19 and hfRPE Cells

ARPE-19 cells were acutely exposed to 1.0, 10, 25, 50, and 100 μM acrolein for 24 hours. Figure 1A shows that there was no effect on ARPE-19 cell viability when acrolein was 1 to 25 μM, but the viability of the cells was sharply decreased to 21% and 13% compared with the control when acrolein was at 50 and 100 μM. Figure 1B shows that, similar to ARPE-19 cells, acrolein below 25 μM had no effect on hfRPE cell viability but at 50 and 100 μM caused significant decreases (11% and 12% of control, respectively).

In the chronic toxicity assay, ARPE-19 cells were exposed to various concentrations of acrolein for 8 or 32 days, respectively. Acrolein caused a significant decrease in cell viability at concentrations of 5 and 10 μM with the 8-day exposure (Fig 1C) and of 0.1 and 1 μM with the 32-day exposure (Fig 1D).

Effects of Acrolein on Mitochondrial Membrane Potential in ARPE-19 and hfRPE Cells

Figure 2A shows the results of the JC-1 assay for mitochondrial membrane potential in ARPE-19 cells. Similar to the cell viability results shown in Figure 1A, acrolein below 25 μM had no effect on mitochondrial membrane potential in ARPE-19 cells but at 50 and 100 μM caused significant decreases (34.4% and 31.3% of control, respectively). Figure 2B shows the results in hfRPE cells. There was no effect below 25 μM acrolein, but significant decreases (29.6% and 28.2% of control) were seen at 50 and 100 μM acrolein concentrations.

Prolonged exposure of acrolein to ARPE cells caused a significant decrease in mitochondrial membrane potential. Ac-
Acrolein caused a decrease in mitochondrial potential at 5 and 10 μM in cells exposed for 8 days (Fig. 2C) and at 0.1 and 1 μM when exposed for 32 days (Fig. 2D).

**Protective Effect of LA on the Acrolein-Induced Decrease in Cell Viability in ARPE-19 and hfRPE Cells**

The ARPE-19 and hfRPE cells were seeded at 4 × 10^4 per well in a 96-well plate. Cells were pretreated with different levels of LA for 48 hours when they were 80% confluent and then were treated with 50 μM acrolein for 24 hours. LA itself had no apparent effect on cell viability in the concentrations used (1–300 μM LA in ARPE-19 and 10–100 μM in hfRPE cells; Fig 3). The pretreatments of ARPE-19 and hfRPE cells with LA resulted in a significant protection against 50 μM acrolein-induced toxicity when the concentration of LA was greater than 100 μM. The protection was 83% and 87% of the control for 100 and 300 μM LA in ARPE-19 cells (Fig. 3A) and 64% and 78% of the control for 50 and 100 μM LA in hfRPE cells (Fig. 3B), respectively. In the 10- to 50-μM range, LA protected against an acute acrolein-induced decrease in cell viability. LA at 50 μM completely abolished acrolein toxicity when ARPE-19 cells were pretreated for 8 days (Fig. 3C).

**Protective Effect of LA on the Acrolein-Induced Decrease in Mitochondrial Membrane Potential in ARPE-19 and hfRPE Cells**

Similar to the cell viability results, LA itself had no apparent effect on mitochondrial membrane potential in both ARPE-19 and hfRPE cells at the concentrations used (1–300 μM LA in

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/)

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/)
ARPE-19 and 10–100 μM in hfRPE cells). Treatment with 50 μM acrolein decreased the 590:530 fluorescence ratio of JC-1 dye by 74% and 80% in ARPE-19 and hfRPE cells, respectively. Pretreatment with LA resulted in 2.6-fold (100 μM LA) and 2.7-fold (300 μM LA) increases in the red-green JC-1 fluorescence ratio relative to 50 μM acrolein-treated cells, respectively, in ARPE-19 cells (Fig. 4A), and 2.3-fold (50 μM LA) and 3.6-fold (100 μM) increases respectively, in hfRPE cells (Fig. 4B). LA at concentrations lower than 10 μM showed no protective effect against acrolein-induced cell toxicity in both ARPE-19 and hfRPE cells. Similar to the protection of cell viability, an 8-day pretreatment enhanced the protective effect of LA. As shown in Figure 4C, 10, 20, and 50 μM LA significantly protected the acute acrolein-induced decrease in mitochondrial membrane potential.

Figure 5 shows representative images of ARPE-19 cells without (Fig. 5A) and with JC-1 staining (Fig. 5B). Acrolein treatment (50 μM) resulted in a significant decrease in cell numbers and a decrease in red J-aggregate fluorescence, reflecting an acrolein-induced decrease in cell viability and mitochondrial potential. Pretreatment with 100 μM LA prevented the acrolein-induced decrease in ARPE-19 cell viability and mitochondrial membrane potential, as demonstrated by the increased number and normal shape of the cells (Fig. 5A) and red J-aggregate staining of LA-pretreated cells (Fig. 5B). Similar results were obtained with hfRPE cells as reported previously on
protection by LA on the t-butylhydroperoxide-induced decrease in mitochondrial membrane potential in hRPE cells.20

Inhibition by LA of Acrolein-Induced Generation of Intracellular Oxidants in ARPE-19 and hRPE Cells

Acrolein at 50 μM induced a significant increase in intracellular oxidants in ARPE-19 and hRPE cells (2.9 and 2.2 times, respectively) compared with the control. Pretreatment with 100 and 300 μM LA caused significant inhibitions of oxidants (52% and 76% reduction, respectively) in ARPE-19 cells (Table 1). Pretreatment with 50 and 100 μM LA also caused significant inhibition of oxidants (50% and 54%, respectively) in hRPE cells (Table 2). This protection may be partly due to the effect of LA on inhibiting the basal level of oxidants, because LA alone at concentrations higher than 100 μM caused an inhibition of oxidant generation in untreated normal ARPE-19 and hRPE cells: 17% inhibition at 100 μM LA in ARPE-19 cells (Table 1) and 18% inhibition at 100 μM LA in hRPE cells (Table 2).

Modulation of the Acrolein-Induced Decrease in Intracellular G-Px in ARPE-19 and hRPE Cells

Treatment with 50 μM acrolein induced a significant decrease in intracellular G-Px activity in ARPE-19 cells (24%; P < 0.01; Table 1). Pretreatment with 1, 10, and 100 μM LA resulted in a significant protection against G-Px decrease by 39%, 35%, and 37% increase relative to acrolein treatment (Table 1). However, this protection may be due to an effect of LA on stimulating G-Px activity, because treatment with 100 μM LA without acrolein increased G-Px activity by 42% relative to the control (Table 1).

Treatment with 50 μM acrolein decreased intracellular G-Px activity by 36% (P < 0.01) in hRPE, and pretreatment with 50 and 100 μM LA resulted in a significant protection of G-Px activity (1.4- and 1.9-fold relative to acrolein treatment; Table 2). Treatment with 100 μM LA without acrolein also caused an increase in intracellular G-Px activity in hRPE cells as in ARPE-19 cells (Table 2).

Modulation of the Acrolein-Induced Decrease in Intracellular SOD in ARPE Cells

Treatment with 50 μM acrolein caused a significant decrease in intracellular SOD activity by 35% in ARPE-19 cells (Table 1). LA pretreatment at 100 μM prevented the decrease in SOD activity. The SOD activities were 1.5-fold for LA 100 μM and 1.9-fold for LA 300 μM group to that of acrolein treated, respectively. Similar to the results of G-Px activity, treatment with 100 μM LA without acrolein increased intracellular SOD activity by 23% relative to control in untreated normal ARPE-19 cells (Table 1).

Modulation of the Acrolein-Induced Decrease in Intracellular Total Antioxidant Power in ARPE-19 Cells

Acrolein at 50 μM decreased intracellular antioxidant power in ARPE-19 cells 30% relative to control (P < 0.01). Pretreatment with 100 μM LA prevented the cells from an acrolein-induced decrease completely (Table 1). Again as on G-Px and SOD activity, this protection may be due to the antioxidant activity of LA itself, since LA at 100 μM without acrolein elevated the intracellular total antioxidant power by 29% compared with control (P < 0.05; Table 1).

Modulation of the Intracellular Ca2+ Increase Caused by Acrolein in ARPE-19 Cells

Mitochondrial dysfunction usually results in an increase in cytoplasmic Ca2+ level, which is a biomarker of oxidative stress and mitochondrial dysfunction. Treatment of ARPE-19 cells with 50 μM acrolein caused a significant increase in the intracellular Ca2+ level by 10.9%, compared with the control (P < 0.01; Table 1). Pretreatment with 100 or 300 μM LA before 50 μM acrolein significantly inhibited the increase in Ca2+ by 4.6% and 8.8% (P < 0.05 and P < 0.01), respectively. LA at 100 μM without acrolein did not significantly change the intracellular Ca2+ level in ARPE-19 cells.

LA Inhibition of Acrolein-Induced Decreases in GSH Level in ARPE-19 Cells

Pretreatment of ARPE-19 cells with LA for 48 hours showed a trend in increasing the GSH level (Fig. 6A). Acrolein at 75 μM for 24 hours caused a significant decrease in the GSH level and LA at 100 μM for 48 hours provided full protection of the GSH level (Fig. 6A).

Inhibition of Acrolein-Induced Increase in Protein Carbonyls in ARPE-19 Cells

Acrolein at 75 μM for 24 hours caused a significant increase in protein carbonyls, an index of protein oxidation (Figs. 6B, 6C). Pretreatment with 100 μM LA for 48 hours showed significant inhibition of the acrolein-induced increase in protein carbonyls (Figs. 6B, 6C).

Modulation of the Acrolein-Induced Decrease in Total and Nuclear Nrf2 Expression in ARPE-19 Cells

Acrolein at 75 μM for 24 hours caused a significant decrease in both total and nuclear Nrf2 expression in ARPE-19 cells, and pretreatment with LA at 100 μM for 48 hours significantly

Table 1. Acute Acrolein Exposure-Induced Changes in Intracellular ROS, G-Px, SOD, Antioxidant Power, and Ca2+ Levels and Modulation by LA Pretreatment in ARPE-19 Cells

<table>
<thead>
<tr>
<th>LA (μM)</th>
<th>Acrolein (μM)</th>
<th>ROS</th>
<th>G-Px</th>
<th>SOD</th>
<th>Antioxidant Power</th>
<th>Ca2+</th>
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<tr>
<td>0</td>
<td>0</td>
<td>16205.4 ± 1312.9</td>
<td>63.85 ± 9.54</td>
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<td>100.51 ± 19.91</td>
<td>2.55 ± 0.02</td>
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<tr>
<td>0</td>
<td>50</td>
<td>46743.4 ± 429.5**</td>
<td>48.66 ± 3.86**</td>
<td>5.57 ± 0.13**</td>
<td>69.73 ± 9.01**</td>
<td>2.83 ± 0.07**</td>
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<tr>
<td>1</td>
<td>50</td>
<td>43454.1 ± 1572.8</td>
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<td>4.91 ± 0.67**</td>
<td>—</td>
<td>2.77 ± 0.04</td>
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<tr>
<td>10</td>
<td>50</td>
<td>43561.1 ± 1622.4</td>
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<td>5.52 ± 1.01**</td>
<td>63.57 ± 14.90**</td>
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<td>50</td>
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<td>—</td>
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<td>13450.5 ± 562.65*</td>
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<td>10.52 ± 0.29**</td>
<td>129.41 ± 11.24*</td>
<td>2.51 ± 0.06</td>
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Data are the mean ± SD. Exposure was for 24 hours, and pretreatment was for 48 hours. ROS (fluorescence/μg protein), G-Px (nmol/min per milligram protein), SOD (U/mg protein), Antioxidant power (copper reducing equivalences), and Ca2+ (mg/g protein). — no test.

* P < 0.05, ** P < 0.01 versus LA 0 + acrolein 0.
† P < 0.05, †† P < 0.01 versus LA 0 + acrolein 50.
formed in triplicate. **separate experiments, each protein carbonyl assays.

acrolein exposure for 24 hours in the LA pretreatment for 48 hours and yls from three separate experiments.

Quantitative data of protein carbonyls, (CBlacrolein without LA. (Fig. 6).

**FIGURE 6.** We compared the protective effects of three forms of LA (R-LA, S-LA, and R,S-LA) on the acrolein-induced decrease in cell viability in ARPE-19 Cells.

Acrolein at 75 μM for 24 hours caused a significant decrease in the activity of mitochondrial complex I, II, and III in ARPE-19 cells by 68%, 54% and 41% respectively relative to control (Fig. 8). Pretreatment with 100 μM LA showed significant protection of complexes I (Fig. 8A), II (Fig. 8B), and III (Fig. 8C) by 3.4-, 1.7-, and 1.5-fold, respectively, compared with 75 μM acrolein without LA.

**Comparison of the Effect of R-LA, S-LA, and R,S-LA on the Acrolein-Induced Decrease in Cell Viability in ARPE-19 Cells**

We compared the protective effects of three forms of LA (R-LA, S-LA, and R,S-LA) on the acrolein-induced decrease in cell viability in ARPE-19 cells and found that the protective effects of R-LA, the natural form, and S-LA are similar. They had no effect at concentrations lower than 100 μM and showed full protection at concentrations higher than 300 μM (Fig. 9). R,S-LA afforded weaker protection than R- or S-LA, though at higher concentrations, it also provided full protection (Fig. 9).

![Figure 6](https://iovs.arvojournals.org/)

**DISCUSSION**

We first investigated the effect of acrolein on cell viability in a 1-day (acute) study by MTT assay. Cells lost 79% and 87% of viability after ARPE-19 cells were exposed to 50 and 100 μM acrolein for 24 hours, whereas lower concentrations (1–25 μM) of acrolein had no apparent effect (Fig. 1). Our doses of acrolein are somewhat higher, but still comparable, to those in a study of human bronchial epithelial cells with an LD₅₀ (median lethal dose) of approximately 20 μM. This result suggests that acrolein, a particularly toxic cigarette smoke component, can cause RPE injury. It is estimated that acrolein is present in the gas phase of cigarette smoke in amounts of approximately 25 to 140 μg/cigarette. Acrolein reaches a concentration of approximately 80 μM in respiratory tract lining fluids as a result of smoking. Although the level of acrolein in the retina of smokers is unknown, this result suggests that 50 to 75 μM of acrolein may be higher than physiologcal levels in the retina. Because smoking is a habitual practice and the concentration of acrolein during smoking is less than that shown in the acute exposure, we exposed RPE cells to low doses of acrolein for 8 days or 32 days and found that long-term exposure to low doses of acrolein (as low as 0.1 μM) induced similar toxicity (demonstrated by cell viability and mitochondrial membrane potential) in the RPE cells. The toxic effect of acrolein, especially, at less than 1 μM during

**TABLE 2.** Acute Acrolein Exposure-Induced Changes in Intracellular ROS, G-Px, SOD, Antioxidant Power, and Ca²⁺ Levels and Modulation by LA Pretreatment in hRPE Cells

<table>
<thead>
<tr>
<th>LA (μM)</th>
<th>Acrolein (μM)</th>
<th>ROS</th>
<th>G-Px</th>
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<td>0</td>
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<td>254.46 ± 56.12</td>
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<tr>
<td>0</td>
<td>50</td>
<td>539.39 ± 61.79*</td>
<td>33.52 ± 5.24**</td>
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<td>10</td>
<td>50</td>
<td>493.71 ± 17.24**</td>
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<tr>
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<td>50</td>
<td>268.86 ± 68.34††</td>
<td>46.73 ± 8.26††</td>
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<td>50</td>
<td>248.81 ± 10.46††</td>
<td>64.48 ± 16.35††</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>209.33 ± 13.21*</td>
<td>61.13 ± 14.41*</td>
</tr>
</tbody>
</table>

Data are the mean ± SD. Exposure was for 24 hours, and pretreatment was for 48 hours. ROS (fluorescence/mg protein) and G-Px (nmol/min per milligram protein). *P < 0.05, **P < 0.01 versus LA0 + acrolein 0.
†P < 0.05, ††P < 0.01 versus LA0 + acrolein 50.

Prevented the cells from acrolein-induced decrease in both total Nrf2 (Fig. 7A) and nuclear Nrf2 (Fig. 7B).

**FIGURE 7.** (A) Western blot image of protein carbonyls, (B) Changes in GSH levels and protective effect of LA in ARPE cells. Data are the mean ± SD of results of three separate experiments, each performed in triplicate. **P < 0.01 versus control, **P < 0.05 versus 75 μM acrolein without LA. (B) Western blot image of protein carbonyls from three separate experiments. LA pretreatment for 48 hours and acrolein exposure for 24 hours in the experiments for both GSH and protein carbonyl assays.

**FIGURE 8.** Pretreatment with 100 μM LA showed significant protection of complexes I (Fig. 8A), II (Fig. 8B), and III (Fig. 8C) by 3.4-, 1.7-, and 1.5-fold, respectively, compared with 75 μM acrolein without LA.

**DISCUSSION**

We first investigated the effect of acrolein on cell viability in a 1-day (acute) study by MTT assay. Cells lost 79% and 87% of viability after ARPE-19 cells were exposed to 50 and 100 μM acrolein for 24 hours, whereas lower concentrations (1–25 μM) of acrolein had no apparent effect (Fig. 1). Our doses of acrolein are somewhat higher, but still comparable, to those in a study of human bronchial epithelial cells with an LD₅₀ (median lethal dose) of approximately 20 μM. This result suggests that acrolein, a particularly toxic cigarette smoke component, can cause RPE injury. It is estimated that acrolein is present in the gas phase of cigarette smoke in amounts of approximately 25 to 140 μg/cigarette. Acrolein reaches a concentration of approximately 80 μM in respiratory tract lining fluids as a result of smoking. Although the level of acrolein in the retina of smokers is unknown, this result suggests that 50 to 75 μM of acrolein may be higher than physiological levels in the retina. Because smoking is a habitual practice and the concentration of acrolein during smoking is less than that shown in the acute exposure, we exposed RPE cells to low doses of acrolein for 8 days or 32 days and found that long-term exposure to low doses of acrolein (as low as 0.1 μM) induced similar toxicity (demonstrated by cell viability and mitochondrial membrane potential) in the RPE cells. The toxic effect of acrolein, especially, at less than 1 μM during
prolonged exposure, strongly suggests that acrolein is a plausible RPE toxicant and that it may be a contributing factor to RPE degeneration due to smoking. The oxidant effect of the high NOx concentration in cigarette smoke is another plausible contributor. Acrolein, besides being a smoking component and a ubiquitous pollutant in the environment, can also be generated in several other ways: by polyunsaturated fatty acids during lipid peroxidation, both in vitro and in vivo; by enzymatic oxidation of polyamine metabolites; by biotransformation of allyl alcohol; and by the widely used anticancer drug cyclophosphamide.

ARPE-19 is a human diploid RPE cell line, which displays many differentiated properties typical of RPE in vivo; therefore, the ARPE-19 cell line has been the most commonly used RPE cell in eye research. However, hRPE primary cells from fetal eyes, may more closely mimic the morphology, biochemistry, and physiology of native human and adult RPE. We compared the ARPE-19 cells and hRPE cells by testing the following parameters: cell viability, mitochondrial potential, oxidant generation, and G-Px activity. We found that both ARPE-19 and hRPE cells have similar sensitivity to acrolein toxicity on all parameters tested, and LA, showed similar protective effects in both cell systems. These results suggest that the effects of acrolein and LA shown in the present study may be applicable to clinical eye problems, and that ARPE-19 cells may be suitable for studying the biochemical mechanisms of RPE toxicants and protective agents.

Oxidative damage to RPE cells and photoreceptors has been implicated in the pathogenesis of AMD, and RPE protein oxidation could be induced by different types of oxidative stress, such as hyperoxia, H$_2$O$_2$, and paraquat. Both in vitro and in vivo studies show that acrolein toxicity is mediated by increased oxidants and oxidative damage and that acrolein may act as a generator of oxidants by different pathways. Acrolein can directly react with antioxidants such as GSH by a Michael addition mechanism to form a 1,4-addition intermediate product and then isomerize into a 1,2-addition product. Exposure of the ARPE-19 cells to micromolar levels of acrolein resulted in significant increases in the levels of oxidants, in protein oxidation, and in significant decreases in GSH, in total antioxidant power, and in antioxidant enzymes (SOD and G-Px). Pretreatment...
Protective Effect of Lipoic Acid against Acrolein Toxicity in RPE

Figure 9. Different forms of LA (R-LA, S-LA, and R,S-LA) protect against a 50 μM acrolein-induced decrease in cell viability in ARPE-19 cells. Data are the mean ± SD of results of two separate experiments, and each experiment was performed in duplicate. **P < 0.01 versus control, and *P < 0.05 and **P < 0.01 versus acrolein at 50 μM without LA.

Mitochondrial dysfunction may be a key mechanism for acrolein toxicity. Mitochondria are the main generation sites of oxidants and are the targets of oxidants because they are particularly sensitive to oxidative insults. Acrolein is a cytotoxic and genotoxic environmental pollutant. The mechanisms of the toxicity have been suggested to be linked to mitochondrial dysfunction and tested in mitochondria isolated from rat brain and heart; however, no studies have been performed with RPE systems. To demonstrate the involvement of mitochondrial dysfunction in acrolein toxicity, we assessed the effects of acrolein on mitochondrial membrane potential. Pretreatment with LA at 100 μM or higher before exposure to acrolein significantly protected against this acrolein-induced decrease in membrane potential. These results suggest that acrolein acts as a mitochondrial toxicant to cause mitochondrial dysfunction in RPE cells. The protection of LA against an acrolein-induced decrease in mitochondrial membrane potential in ARPE-19 cells suggests that LA is a potent mitochondrial antioxidant, perhaps by inducing GSH synthesis of enzymes and other phase-2 antioxidant protective enzymes.

Increased cytosolic Ca²⁺ as a consequence of mitochondrial dysfunction may play an important role in the development of AMD because gene expression can be changed due to increasing cytosolic Ca²⁺, and these gene expression changes contribute to some of the manifestations in AMD, such as RPE cell migration, angiogenesis, and lipid deposition. Acrolein at 50 μM increased intracellular Ca²⁺ significantly compared with control and a pretreatment with 100 and 300 μM LA before acrolein protected the acrolein-induced decrease in intracellular Ca²⁺.

Decreased activity of the inner membrane complexes is another index of oxidative mitochondrial dysfunction. We measured the effects of acrolein treatment on mitochondrial electron transfer chain complex I, II, and III, and found that acrolein caused significant decreases in complex I, II, and III. Pretreatment with LA effectively protected ARPE cells from acrolein-induced damage to these mitochondrial enzymes. These results, together with the intracellular Ca changes, support the involvement of mitochondrial dysfunction in acrolein toxicity due to oxidative stress. Because smoking components cause damage to the choroid and Bruch’s membrane, then RPE, it is possible that the direct RPE toxicity could be further amplified by their effects on choroid/Bruch’s membrane during the development of AMD.

The natural endogenous mitochondrial form of LA is R-LA, but synthetic LA is an R,S mixture (50% R/50% S), which is used for dietary supplements. By comparing their protective effects on acrolein-induced decrease in cell viability in ARPE-19 cells, we found that the protective effects of R-LA and S-LA are similar, but the R,S-LA showed a weaker protection than R- or S-LA. These results are consistent with that obtained by Wolz and Kriegstein in primary cultures of neurons from chick embryo telencephalons, in an in vivo study in rats, with subcutaneous injection, and also in a HT22 mouse neuronal cell line, but different from that in an ex vivo study with isolated rat hepatocytes.

In summary, the present study demonstrated that acrolein, one major component in the gas phase of cigarette smoking, is an oxidant and mitochondrial toxicant in RPE and provided strong evidence for the role of mitochondrial dysfunction in RPE oxidative damage. The protection effects of LA suggest that administering mitochondria-targeted nutrients may be an effective strategy for reducing or preventing chronic oxidant-induced RPE degeneration in vivo from a variety of sources including cigarette smoke.
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