Protective Effect of Lipoic Acid against Acrolein-Induced Cytotoxicity in IMR-90 Human Fibroblasts

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(Received August 14, 2008)

Summary Acrolein is a highly reactive unsaturated hazardous air pollutant of human health concern, particularly as a component of cigarette smoke. In this study, the effects of acrolein on mitochondrial damage in IMR-90 (a human lung fibroblast cell line), and the reduction of this damage by R-α-lipoic acid were examined. Our results show that acute acrolein exposure exceeding 100 μM (24 h) in IMR-90 cells caused serious cytotoxicity, including decreases in cell viability, mitochondrial membrane potential, SOD activity, GSH, and ATP levels, and acute exposure also increased in ROS levels. Pretreatment with R-α-lipoic acid effectively protected IMR-90 cells from acrolein toxicity. The results show that acrolein is a mitochondrial toxin in IMR-90 cells and that acrolein-induced oxidative mitochondrial dysfunction is reduced by R-α-lipoic acid. These experiments imply R-α-lipoic acid may be an effective antioxidant for reducing or preventing chronic oxidant-induced lung cells degeneration in vivo from a variety of sources, including cigarette smoke.

Key Words lipoic acid, acrolein, cytotoxicity, oxidative stress

Acrolein is a highly reactive unsaturated hazardous air pollutant of human health concern, particularly as a component of cigarette smoke. Smoking one cigarette per m² air of room-space in 10–13 min (10 puffs) generates acrolein levels up to 0.84 mg/m³ (1). Moreover, acrolein is estimated to reach 80 μM in the respiratory tract fluid in smokers (2). Recently, it has been suggested that acrolein is a major etiologic agent for tobacco smoking-related lung cancer (3).

Investigation suggests that acrolein may exert its detrimental effect by ROS generation and lipid peroxidation (4). Acrolein has been shown to impair the function of the respiratory chain in mitochondria isolated from heart (5), brain tissue (6) and retinal pigment epithelial cells (7). Acrolein could induce apoptosis in human bronchial epithelial cells, which may be an important pathogenic mechanism of acrolein-related respiratory tract toxicity (4).

Alpha-lipoic acid (LA) is a sulfur-containing compound found naturally in plants and animals. It is present in mitochondria as an essential cofactor for pyruvate dehydrogenase and a-ketoglutarate dehydrogenase (8). The antioxidant properties of LA have recently received great attention. LA can scavenge hydroxyl radicals, singlet oxygen, peroxynitrite, and nitric oxide (8–10). In addition, it is also able to chelate a number of transition metal ions (8–10). Due largely to its antioxidant properties, LA has recently been reported to afford protection against oxidative injury in various disease processes, including neurodegenerative disorders and diabetic syndrome (11–13). LA can inhibit airway inflammation and hyperresponsiveness in a mouse model of asthma (14).

Lung tissue is a primary target for acrolein toxicity in smokers. Do cigarette smoke components such as acrolein cause injury, especially mitochondrial dysfunction, to the lung cell, as in other cellular and tissue models? Does lipoic acid protect lung cells from acrolein-caused injury and mitochondrial dysfunction?

In the present study, the human lung fibroblast cell line IMR-90 (IMR-90) was treated with acrolein, and the effects on cellular toxicity and mitochondrial function were examined. Preparations were used to study the protective effects against acrolein-induced toxicity of LA. We hypothesize that oxidative damage by acrolein, especially mitochondrial dysfunction to lung cells, may be a major cause in promoting the onset and progress of smoking-related lung diseases, and LA may protect IMR-90 cells against acrolein-induced cytotoxicity and mitochondrial dysfunction.

MATERIALS AND METHODS

Materials. Acrolein, R-alpha-lipoic acid, MTT, 2’7’-dichlorofluorescein (DCF) and Trypsin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). DMEM medium and fetal bovine serum were purchased from Grand Island Biological Company (GIBCO).

Cell culture. The IMR-90 cell was obtained from the University of California, Berkeley cell culture facility in the US. The IMR-90 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 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

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changed every 3 to 4 d. IMR-90 cells were used within 10 generations.

Acrolein exposure and LA supplementation. All experiments were performed with an 80% confluence monolayer grown in 96-well plates or 6 well plates. α-Lipoic acid tris salt was dissolved in PBS, and acrolein was dissolved in PBS each time immediately before an experiment. For the acute toxicity study, cells were exposed to acrolein for 24 h. The protective effects of LA were studied with the acute toxicity model by pretreating cells with LA for 48 h.

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 using a microplate spectrophotometer (Bio-Tek Instruments, Inc., USA). Absorbance values were normalized with untreated cells to calculate the changes in cell viability.

JC-1 assay for mitochondrial membrane potential. Mitochondrial potential change (ΔΨ) was assessed in live IMR-90 cells using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodine (JC-1) (15). 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 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).

Intracellular adenosine 5′-triphosphate (ATP). Cells were cultured in six-well plates. After various treatments, cells were lysed by 0.5% Triton X-100 in 100 mM glycine buffer, pH 7.4. Intracellular ATP levels were assayed with an ATP bioluminescence assay kit (Sigma) based on the luciferase-catalyzed oxidation of d-luciferin (16).

Determination of intracellular reactive oxygen species (ROS) levels. The generation of intracellular ROS was determined by the formation of fluorescent 2′,7′-dichlorofluorescein (DCF) on oxidation of the nonfluorescent, reduced DCFH (17). 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 fluorescence per μg of protein (bicinchoninic acid (BCA) method).

Superoxide dismutase (SOD) measurement. Intracellular SOD activity was measured by a Superoxide Dismutase Assay Kit (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 (catalog no. 703002; Cayman Chemical) that is based on a thiol-specific reagent, dithionitrobenzoic acid (DTNB), and the adduct was measured spectrophotometrically at 412 nm.

Statistical analysis. The results are presented as mean±SD, as specified in the figure legends. Statistical significance was calculated (SPSS 10.0 software) with one-way ANOVA. p<0.05 was considered significant.

RESULTS

Effects of acrolein on cell viability

Figure 1 shows IMR-90 cells were acutely exposed to 25, 50, 100 and 200 μM acrolein for 24 h. There was no effect on IMR-90 cell viability when acrolein was 25 μM, but the viability of cells was gradually decreased to 83, 46 and 9% compared with control when acrolein was at 50, 100 and 200 μM respectively, and there was a dose-response relationship.

Effects of acrolein on mitochondrial membrane potential

The results of the JC-1 assay for mitochondrial membrane potential in IMR-90 cells are shown in Fig. 2. Acrolein below 50 μM had no effect on mitochondrial membrane potential in IMR-90 cells, but at 100 and 200 μM caused significant decreases (41.2% and 74.6% of control respectively).

Protective effect of LA on acrolein-induced decrease in cell viability

The IMR-90 cells were seeded at 4×10⁴ per well in a 96 well plate. Cells were pretreated with different levels of LA for 48 h when cells were 80% confluent and then treated with 100 μM acrolein for 24 h. Figure 3 shows that 100 μM LA itself had no apparent effect on cell viability. The pretreatments of IMR-90 cells with 50 and 100 μM LA resulted in a significant protection against 100 μM acrolein-induced toxicity. The protective effect on IMR-90 cells was almost 100% of the control for 50 and 100 μM LA. But there was no protective effect against acrolein-induced reduction in cells viability
when LA was at 10 μM.

**Protective effect of LA on acrolein-induced decrease in mitochondrial membrane potential**

Figure 4 shows that 100 μM LA itself had no apparent effect on mitochondrial membrane potential in IMR-90 cells. Treatment with 100 μM acrolein decreased the 590 : 530 fluorescence ratio of JC-1 dye by 55.3% in IMR-90 cells. Pretreatment with 50 and 100 μM LA resulted in about equal 27.0% increases in the red-green JC-1 fluorescence ratio relative to 100 μM acrolein-treated the cells, while pretreatment with 10 μM LA showed no protective effect against acrolein-induced decreases in cell mitochondrial membrane potential.

**Protective effect of LA on acrolein-induced decrease in intracellular ATP levels**

Figure 5 shows that acrolein treatment significantly decreased intracellular ATP levels to 30.5% of the untreated control level. Pretreatment with 100 μM LA significantly inhibited the acrolein-induced decrease in ATP level (72.9% of control).

**Inhibition by LA of acrolein-induced generation of intracellular ROS**

The data summarized in Table 1 show that treatment of IMR-90 cells with 100 μM acrolein significantly increased the levels of intracellular ROS 1.4 times compared with the control (p<0.01, n=4). Pretreatments with 100 μM LA significantly reduced the level of ROS in IMR-90 cells relative to acrolein treatment by 30% (p<0.05, n=4). This protection may be partly due to the effect of LA on inhibiting the basal level of ROS, because LA alone at a concentration of 100 μM caused an inhibition of ROS generation in untreated normal IMR-90 cells.

**LA modulates the acrolein-induced decrease in intracellular SOD and GSH**

Table 1 shows that SOD activity and GSH levels were decreased by 47.6% and 51.8% (p<0.01, n=4) in IMR-90 cells respectively after treatment with 100 μM acrolein. Pretreatments with 100 μM LA significantly increased intracellular SOD activity and GSH levels by

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**Table 1.** Acute acrolein exposure (24 h)-induced changes in intracellular ROS, SOD and GSH in IMR-90 cells.

| LA (μM) | Acrolein (μM) | ROS (fluorescence/μg protein) | SOD (U/μg protein) | GSH (nmol/mg protein) |
|---------|---------------|-------------------------------|--------------------|-----------------------|
| 0       | 0             | 46536.50±3606.58              | 7.17±0.12          | 46.82±3.74            |
| 0       | 100           | 63242.80±4251.34**            | 3.76±0.18**        | 22.56±4.19**          |
| 10      | 100           | 59768.45±1812.56              | 4.16±0.45          | —                     |
| 100     | 100           | 44427.72±3235.52^              | 5.43±0.45^         | 36.93±8.22^            |
| 100     | 0             | 36379.37±2267.13*              | 7.87±0.89          | 63.51±2.97^            |

The results for each group are expressed as mean±SD of data from two separate experiments; each experiment was performed in duplicate. ROS (fluorescence/μg protein), SOD (U/μg protein), GSH (nmol/mg protein). *p<0.05, **p<0.01 versus LA 0+acrolein 0. ^p<0.05 versus LA 0+acrolein 100. — represents no treatment.
addition product (intermediate product and then isomerize into a 1,2-Michael addition mechanism to form a 1,4-addition directly react with antioxidants such as GSH by a metabolites; by biotransformation of allyl alcohol; and acrolein had no apparent effect (Fig. 1). Our doses of saturated fatty acids during lipid peroxidation, both in 100 SOD activity. Pretreatment of the IMR-90 cells with level of ROS, and significant decreases in GSH levels and oxidants by different pathways (Fig. 5). All of these results show that LA is an effective mitochondrial antioxidant.

In this study, we found that concentrations of LA greater than 50 \( \mu M \) are necessary for this protection, and there was no difference between 50 and 100 \( \mu M \) LA in protection of IMR-90 cells against 100 \( \mu M \) acrolein-induced cytotoxicity (Fig. 1). Nardini et al. indicated that 25 \( \mu M \) \( \alpha \)-tocopherol and 100 \( \mu M \) ascorbic acid can strongly inhibit 25 \( \mu M \) acrolein-induced apoptosis and prevent an increase in the generation of intracellular oxidants in cultures human bronchial epithelial cells (4). These results suggested that a certain concentration of antioxidants in respiratory tract and lung cells may benefit smokers.

In summary, the present study demonstrates that acrolein, one major component in the gas phase of cigarette smoking, is an oxidant and mitochondrial toxin in IMR-90 cells. The protective effects of LA suggest that administering mitochondria-targeted antioxidants may be an effective strategy for reducing or preventing chronic oxidant-induced lung cell degeneration in vivo from a variety of sources including cigarette smoke.

Acknowledgments
The authors thanks Dr Jiankang Liu (now working at the Institute for Brain Aging and Dementia, University of California, 1261 Gillespie Neuroscience Research Facility, CA) for instruction and support.
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