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Acrolein-induced cell death in PC12 cells: Role of mitochondria-mediated oxidative stress

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Abstract

Oxidative stress has been implicated in acrolein cytotoxicity in various cell types, including mammalian spinal cord tissue. In this study we report that acrolein also decreases PC12 cell viability in a reactive oxygen species (ROS)-dependent manner. Specifically, acrolein-induced cell death, mainly necrosis, is accompanied by the accumulation of cellular ROS. Elevating ROS scavengers can alleviate acrolein-induced cell death. Furthermore, we show that exposure to acrolein leads to mitochondrial dysfunction, denoted by the loss of mitochondrial transmembrane potential, reduction of cellular oxygen consumption, and decrease of ATP level. This raises the possibility that the cellular accumulation of ROS could result from the increased production of ROS in the mitochondria of PC12 cells as a result of exposure to acrolein. The acrolein-induced significant decrease of ATP production in mitochondria may also explain why necrosis, not apoptosis, is the dominant type of cell death. In conclusion, our data suggest that one possible mechanism of acrolein-induced cell death could be through mitochondria as its initial target. The subsequent increase of ROS then inflicts cell death and further worsens mitochondria function. Such mechanism may play an important role in CNS trauma and neurodegenerative diseases.

Keywords: PC12 cells; Acrolein; Oxidative stress; Reactive oxygen species; Mitochondrial membrane potential; Oxygen consumption

1. Introductory statement

Oxidative stress has been implicated in various neurodegenerative conditions and disorders including ischemia-reperfusion, traumatic injury, Parkinson's disease (PD) and Alzheimer's disease (AD) (Coyle and Puttfarcken, 1993). Recent evidence suggests the products of lipid peroxidation, such as 4-hydroxynonenal (HNE) and 2-propenal (acrolein), may mediate oxidative stress related tissue damage (Esterbauer et al., 1991; Luo and Shi, 2004). Consistent with such notion, tissue levels of acrolein and HNE are elevated in neurodegenerative diseases, such as Alzheimer's disease (Markesbery and Lovell, 1998; Calingasan et al., 1999; Lovell et al., 2001), as well as after CNS injury (Springer et al., 1997; Baldwin et al., 1998; Luo et al., 2003).

Acrolein is the strongest electrophile among the unsaturated aldehydes (Dennis and Shibamoto, 1990; Esterbauer et al., 1991; Lovell et al., 2000). Acrolein formed in vivo exhibits facile reactivity with various biomolecules including proteins, DNA and phospholipids, and thus has the potential to disrupt the function of these molecules (Esterbauer et al., 1991; Kehrer and Biswal, 2000). Acrolein can react with sulfhydryl groups of cysteine, histidine, and lysine residues of proteins (Kehrer and Biswal, 2000). Acrolein has been shown to rapidly incorporate into proteins and generate carbonyl derivatives (Uchida, 1999). Acrolein can also react with

Abbreviations: ROS, reactive oxygen species; PD, Parkinson's disease; AD, Alzheimer's disease; HNE, 4-hydroxynonenal; NAC, *N*-acetylcysteine; CsA, cyclosporin A; $DCFH_2$-DA, dichlorofluorescein-diacetate; Rh123, Rhdamine 123; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GSH, glutathione; PHPA, *p*-hydroxyphenylacetate; PEG, polyethylene glycol; MPTP, mitochondrial permeability transition pore

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nucleophilic sites in DNA and modify DNA bases with the formation of exocyclic adducts. Available data indicates that, compared to other alkenals such as HNE, acrolein may play a particularly important role in inflicting damage to cells, especially in CNS trauma and neurodegeneration. Although it is clear that acrolein is capable of damaging neuronal tissues (Shi et al., 2002; Luo and Shi, 2004), the mechanisms of how acrolein inflicts tissue damage are not fully established. Existing data suggests that acrolein may inflict neuronal tissue damage via oxidative stress (Luo and Shi, 2004). There are two lines of evidence that support such hypothesis. First, acrolein causes significant depletion of glutathione (Kehrer and Biswal, 2000) and increased generation of reactive oxygen species (ROS) (Luo and Shi, 2004), both of which lead to oxidative injury. Second, acrolein-induced neuronal membrane damage in spinal cord tissue can be lessened by antioxidants (Luo and Shi, 2004). Since mitochondria are the main cellular organelles that produce ROS in physiological conditions (Balaban et al., 2005), we hypothesize that mitochondria may also be the main source of ROS over-production in pathological conditions in the presence of acrolein.

The overall objective of the present study is to further explore the role of oxidative stress in acrolein toxicity. Particularly, we plan to examine the role of mitochondrial dysfunction in acrolein-induced oxidative stress and subsequent cell death. We chose to carry out this study in a well-established neuronal PC12 cell tissue culture where the oxidative stress, mitochondria function, and cell viability can be assessed at the same time and therefore can be correlated in the same sample. We have found that acrolein-induced PC12 cell death was accompanied by severe oxidative stress and mitochondrial dysfunction. Our data is consistent with the hypothesis that acrolein induces cell death through stimulating the production of mitochondrial ROS and inflicting mitochondrial dysfunction.

2. Experimental procedures

2.1. Chemicals

Acrolein (product number: 48501, solvent: neat), *N*-acetylcysteine (NAC) and cyclosporin A (CsA), polyethylene glycol (PEG, MW ~2000) and Adenosine 5′-triphosphate (ATP) Bioluminescent Assay Kit were obtained from Sigma-Aldrich (St. Louis, MO). Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Dichlorofluorescein-diacetate ($DCFH_2$-DA) and Rhodamine 123 (Rh123) were purchased from Molecular Probes (Eugene, OR). Other routine laboratory reagents were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD). PC12 cells were grown in DMEM with 12.5% horse serum, 5% fetal bovine serum and 1% Pen–Strep (penicillin–streptomycin). PC12 cells were placed in 12-well cell culture dishes at 37 °C under a humidified atmospheric condition of 5% CO_2 and 95% air, and 3–6-day-old cells were used for the experiments. When used, cells were either suspended in Krebs-Ringer solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 6 mM glucose, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.4 mM $CaCl_2$, pH 7.4) at different concentrations or maintained in 12-well culture dishes depending on the treatment paradigms.

2.3. Treatment paradigms

PC12 cells were exposed to acrolein (final concentrations: 1, 10 and 100 μM) freshly prepared in distilled water everyday before use. These concentrations equaled to 0.35, 3.5 and 35 nmol/mg protein, respectively, calculated based on the average protein concentration of the cells. The in vivo acrolein concentrations in Alzheimer's disease brain are 2.5 nmol/mg in amygdale and 5.0 nmol/mg in parahippocampal gyrus (Lovell et al., 2001). Therefore, the concentrations used in this study were pathologically relevant. Two treatment paradigms were used. For cell viability and ATP content assays, the cells were maintained in the cell culture dishes and exposed to acrolein. Pretreatment with *N*-acetylcysteine, cyclosporin A and polyethylene glycol (PEG 2000) were performed 30 min before acrolein exposure. Twenty-four hours after exposure, the cells were collected and assays were performed according to the following protocols. For all other experiments, the cells were suspended in Krebs-Ringer solution at the 2×10^6 cells/ml and acrolein was added before the beginning of each assay.

2.4. MTT assay of cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (Monner, 1988; Varming et al., 1996). The assay was initiated by removing the culture medium and adding MTT (0.3 mg/ml). Following 1 h incubation at 37 °C, the medium was aspirated, and 0.3 ml of isopropanol was added to lyse the cells and dissolve the formazan crystals. Aliquots (100 μl) of this solution were pipetted into 96-well microplates and the absorbance was recorded at 570 nm (with background subtraction at 620 nm) in a microplate reader. Cell viability was expressed as a percentage of the absorption in control cultures (100%).

2.5. Determination of necrosis and apoptosis

Necrotic and apoptotic cell death was determined as previously described (Delgado-Esteban et al., 2000; Delgado-Esteban et al., 2002). Necrosis was assessed by

examination of trypan blue-stained cells (Koh and Choi, 1988). In brief, 24 h after acrolein exposure, the PC12 cultures were washed with warm (37 °C) Krebs-Ringer solution and incubated with 0.2% trypan blue for 2 min at room temperature. Microphotographs (20× magnification) were taken for each experimental condition and viable plus necrotic (stained) cells were counted. Apoptosis was assessed by staining the nuclei of the cells with propidium iodide, a fluorescent dye that binds DNA and allows quantification of apoptotic neurons, i.e. neurons displaying fragmented or condensed nuclei. Briefly, PC12 cultures were washed with warm Krebs-Ringer solution (37 °C) and fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature. After being washed with Krebs-Ringer solution, the cells were exposed to 1 μg/ml propidium iodide for 10 min at room temperature in the dark and were then washed twice with PBS. Cells were scored for chromatin condensation by fluorescence microscopy (20× magnification). Total and apoptotic nuclei were counted.

2.6. ROS assay by flow cytometry

Dichlorofluorescein-diacetate was used to measure ROS (H_2O_2) by flow cytometry assay using a standard protocol (Robinson, 1993). $DCFH_2$-DA was chosen because acrolein itself does not interfere with the fluorescence of $DCFH_2$-DA (Nardini et al., 2002). Briefly, the cells were harvested and washed in Krebs-Ringer solution, resuspended at 2×10^6 cells/ml, and loaded with $DCFH_2$-DA (15 min, 37 °C in dark). Cells were pelleted by centrifugation and then resuspended in 2 ml of Krebs-Ringer solution. Fluorescence intensity was then monitored using Coulter Epics XL flow cytometer (Coulter Corporation, Miami, FL) at 488/525 nm. Data were analyzed by System II Software (Coulter Corporation, Miami, FL) and expressed as a percentage of the control (without acrolein exposure).

2.7. Analysis of mitochondrial membrane potential

The changes in mitochondrial membrane potential ($\Delta\psi_m$) were estimated using the fluorescent cationic dye Rh123 (Zamzami et al., 2001), which accumulates in mitochondria as a direct function of the membrane potential and is released upon membrane depolarization (Palmeira et al., 1996). Cells were harvested and incubated for 15 min at 37 °C with Rh123 (5 μM), in an atmosphere of 95% air and 5% CO_2. Cells were then washed with Krebs solution and allowed to stand in regular medium for 30 min. After medium change, cells were transferred to a fluorometer cuvette and treated with different concentrations of acrolein. The fluorescence was determined at excitation and emission wavelengths of 490 and 535 nm, respectively, at 37 °C. After a baseline recording (usually 2 min), acrolein was added and the decrease of $\Delta\psi_m$ was recorded as the increase of Rh123 fluorescence for 400 s. Finally, FCCP (final concentration: 5 μM) was added and the fluorescence was continuously recorded for another 60 s. This concentration of FCCP (5 μM) has previously been shown to cause complete depolarization of mitochondrial membrane potential (Palmeira et al., 1996). The mitochondrial membrane potential loss caused by different concentrations of acrolein was expressed as the percentage of that caused by FCCP. To ensure the accuracy of the measurement, two controls, i.e. PC12 cells and Rh123 only (without acrolein) and Rh123 and acrolein only (without PC12 cells) were observed under similar conditions.

2.8. ATP determination

The ATP measurement (Li et al., 2003) was performed using an ATP bioluminescent assay kit purchased from Sigma-Aldrich. Briefly, PC12 cells were treated with various concentrations of acrolein for 24 h and then collected in 1-ml tubes. After being washed with ice-cold PBS, cells were lysed and the ATP assay was carried out according to the manufacturer's instructions. The bioluminescence was assessed with a luminometer. The ATP content was determined by running an internal standard and expressed as percentages of untreated cells (control).

2.9. Measurement of hydrogen peroxide production by intact mitochondria

Intact mitochondria were isolated from cultured PC12 cells according to a previously described procedure (Li et al., 2003) with modifications. Briefly, PC12 cells (1×10^9 cells) were collected by centrifugation after exposure to various concentrations of acrolein. The cells were washed with ice-cold $Ca^{2+}$and Mg^{2+}-free PBS and were resuspended in 2 ml of isolation buffer containing 250 mM sucrose, 10 mM HEPES (pH 7.4), and 1 mM EDTA. The cells were homogenized with a Dounce homogenizer for 20 strokes. The disrupted cells were centrifuged for 10 min at $600 \times g$ at 4 °C. The supernatant was collected and centrifuged at $15{,}000 \times g$ at 4 °C for 10 min. The resulting pellet was considered as crude mitochondria. The crude mitochondria were further washed twice with the same isolation buffer. The resulting mitochondria pellet was resuspended in isolation buffer without EDTA. Mitochondrial protein was determined using the bicinchoninic acid method (Pierce), using bovine serum albumin as a standard.

Mitochondrial hydrogen peroxide (H_2O_2) production was measured from the increase of oxidized *p*-hydroxyphenylacetate (PHPA) fluorescence by horseradish peroxidase (Li et al., 2003). Mitochondria (0.5 mg of protein) were added to 4 ml of medium containing (mM) 300 mannitol, 10 potassium HEPES (pH 7.4), 5 potassium phosphate (pH 7.4), 1 $MgCl_2$, plus 10 μg/ml PHPA, 10 units of horseradish peroxidase and Glutamate (5 mM) plus malate (5 mM). Fluorescence of oxidized PHPA was measured by a fluorometer (Perkin Elmer 3B) with excitation 320 nm and emission 400 nm. Mitochondrial hydrogen peroxide

production was calculated from the standard curve generated by hydrogen peroxide standard.

2.10. Measurement of oxygen consumption

Oxygen consumption was measured with a Clark electrode (model 203, Instech, Plymouth Meeting, PA) as described before (Li et al., 2003) with modifications. All experiments were performed in a respiration medium containing 0.3 M mannitol, 10 mM potassium HEPES (pH 7.4), 5 mM potassium phosphate (pH 7.4), and 1 mM $MgCl_2$, with Glutamate (5 mM) plus malate (5 mM) as substrates, at 37 °C in a sealed chamber (600 μl) equipped with a magnetic stirrer. 1×10^6 cells were injected into the chamber and respiration was then monitored. The rate of oxygen consumption by PC12 cell was calculated before and after the addition of acrolein, and expressed as percentage of control.

2.11. Data analysis and statistics

Data are expressed as mean ± S.D., and statistical significance was determined by analysis of variance with one-way ANOVA using the statistical program SPSS (version 12, SPSS, Chicago, IL, USA), followed by post hoc least-significance difference test; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Acrolein induces cell death on PC12 cells

We first investigated the effects of acrolein on cell viability. PC12 cells were exposed to 1–100 μM acrolein and cell viability was measured by MTT assay. Fig. 1A shows the relationship between acrolein concentration and percentage of cell survival relative to the control. Exposure of PC12 cells to various concentrations of acrolein resulted in a concentration-dependent decrease in cell viability. Specifically, after a 24-h exposure with 100 μM acrolein, about 92% of the cells died; while in the cells exposed to 1 and 10 μM acrolein, the percentage of dead cells was 25 and 58%, respectively (Fig. 1A, $P < 0.01$ for all comparisons between acrolein and control). Treatment with *N*-acetylcysteine reduced acrolein-induced cell death. NAC is a scavenger of reactive oxygen species and a precursor for the endogenous antioxidant glutathione (GSH) (Kelly, 1998). In addition, NAC has a free thiol that can directly scavenge acrolein through a reaction with the C3 carbon of acrolein (Kelly, 1998). Pretreatment with NAC (20 mM) significantly increased cell survival. Specifically, the percentage of dead cells was reduced to 4, 20 and 54% in groups exposed to 1, 10 and 100 μM acrolein, respectively (Fig. 1A). All were significantly different from untreated groups (exposed to acrolein only) ($P < 0.01$ for all comparisons). Incubation of NAC alone did not affect the cell viability. These data suggest that oxidative stress is involved in acrolein-induced cell death. However, pretreatment with two inhibitors of mitochondrial permeability transition, cyclosporine A and polyethylene glycol, failed to

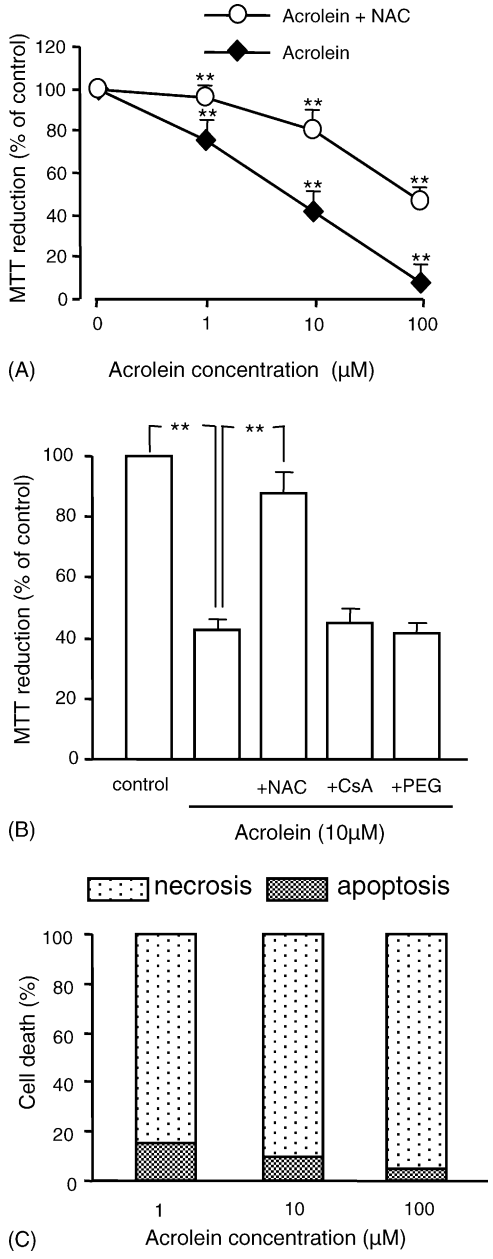

Fig. 1. Cell death in PC12 cells after a 24-h acrolein exposure. (A) Acrolein caused a dose-dependent cell death. Cell death was assayed by MTT test. NAC was added 30 min before acrolein exposure. Treatment with NAC significantly reduced cell death. $^{**}P < 0.01$ of comparisons between control (acrolein at 0 μM) and acrolein, and acrolein and acrolein + NAC. (B) Effects of NAC, CsA and PEG on acrolein-induced cell death. Cells were preincubated with NAC (20 mM), CsA (4 μM) or PEG (10.2 mM) for 30 min before exposure to 10 μM acrolein. $^{**}P < 0.01$. (C) Characteristic of acrolein-induced cell death. Necrotic and apoptotic cell death was determined by trypan blue or PI (see Section 2 for details). Acrolein-induced cell death was mostly necrotic. With higher acrolein levels, a lower percentage of apoptosis occurred.

show the ability to attenuate acrolein toxicity in all three of the concentrations tested. The effects of NAC, CsA and PEG on 10-μM acrolein-induced cell death were shown in Fig. 1B. Neither CsA (4 μM) nor PEG (10.2 mM) significantly reduced acrolein-induced cell death. This suggests that mitochondrial permeability transition is not involved in the process of acrolein-induced cell death.

We also characterized acrolein-induced cell death. Interestingly, we found that acrolein-induced cell death was mostly necrosis. As shown in Fig. 1C, the percentage of necrosis was 86, 90 and 94% in groups exposed to 1, 10 and 100 μM acrolein, respectively.

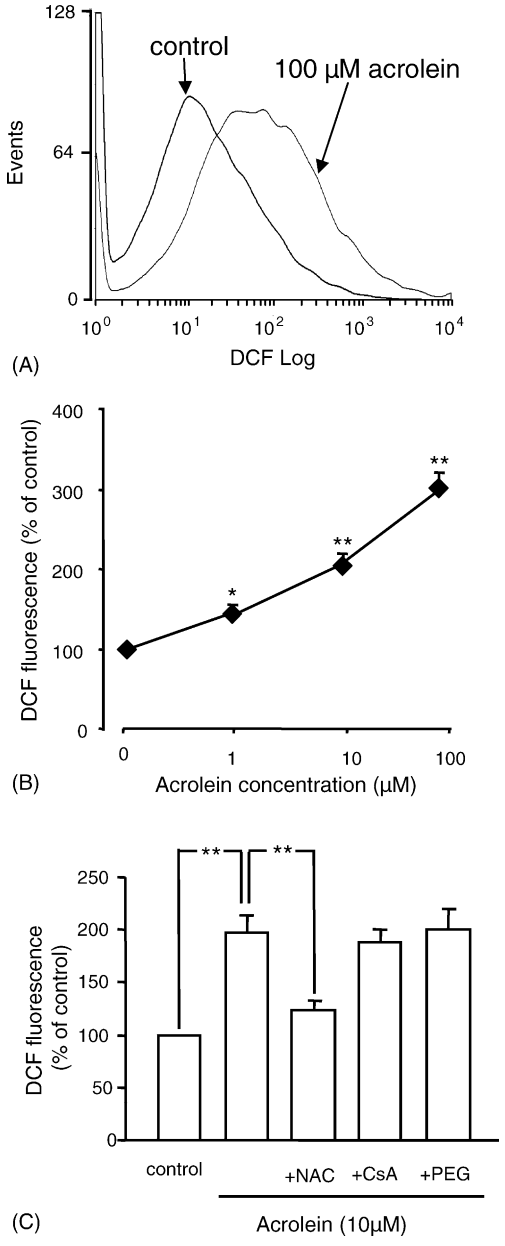

Fig. 2. Hydrogen peroxide generation in PC12 cells after acrolein exposure. H_2O_2 was measured by 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA). (A) A 15-min exposure of acrolein caused a dramatic increase of DCF fluorescence. The peak shift induced by acrolein indicates a dramatic increase of DCF fluorescence, which is proportional to the cellular H_2O_2 level. (B) A summary of five different experiments with various concentrations of acrolein. Average DCF fluorescence intensity was obtained and expressed as percentage of control (non-acrolein exposure). Acrolein at all three concentrations (1, 10 and 100 μM) significantly increased DCF fluorescence $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to control (acrolein at 0 μM). (C) Effects of NAC, CsA and PEG on acrolein-induced ROS production. Cells were preincubated with NAC (20 mM), CsA (4 μM) or PEG (10.2 mM) for 30 min before exposed to 10 μM acrolein. $^{**}P < 0.01$.

3.2. Acrolein induces oxidative stress on PC12 cells

To further demonstrate that oxidative stress is involved in acrolein toxicity, we tested the effects of acrolein on ROS generation in PC12 cells. Enhanced generation of ROS was observed in PC12 cells after acrolein exposure as measured by the ROS-detecting fluorochrome $DCFH_2$-DA (Fig. 2). The DCF fluorescence, the peroxidized product of $DCFH_2$-DA, increased in an acrolein concentration-dependent manner. An example of 100-μM acrolein with 15 min stimulation is shown in Fig. 2A. The peak shift induced by acrolein indicates a dramatic increase of DCF fluorescence, which is proportional to the cellular ROS level. A summary of five individual experiments is shown in Fig. 2B; 1, 10 and 100 μM acrolein caused significant increase of ROS production, i.e. ROS was increased to 146, 198 and 289% of control, respectively. These results demonstrate that acrolein causes a dose-dependent increase of ROS generation in PC12 cells. Treatment with NAC significantly reduced the cellular ROS level while CsA and PEG did not in all of the tests with three concentrations. The results of 10-μM acrolein and treatment are shown in Fig. 2C. NAC significantly reduced the cellular ROS level in the cells exposed to 10-μM acrolein ($P < 0.01$). Treatment with CsA or PEG did not display any significant effect on ROS production.

To investigate the source of acrolein-induced cellular oxidative stress, we isolated mitochondria from PC12 cells exposed to acrolein. The level of mitochondrial hydrogen peroxide was then estimated. As shown in Fig. 3, exposure of acrolein to PC12 cells caused dose-dependent production of hydrogen peroxide in the mitochondria isolated from the cells. All of the three concentrations (1–100 μM) caused significantly increased production of hydrogen peroxide in the mitochondria ($P < 0.01$). These results suggest that the mitochondria-derived ROS might contribute to, at least in part, the overall cellular oxidative stress observed after acrolein stimulation (Fig. 2).

3.3. Acrolein exposure leads to mitochondrial functional impairment in PC12 cells

To further demonstrate the involvement of mitochondria in acrolein toxicity, we designed a series of experiments estimating mitochondrial function. First, we assessed the effects of acrolein on mitochondrial membrane potential. The mitochondrial membrane potential ($\Delta\psi$) is an important

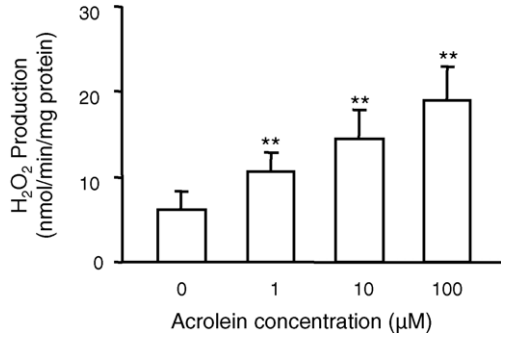

Fig. 3. Hydrogen peroxide level in mitochondria isolated from PC12 cells exposed to acrolein. PC12 cells were exposed to acrolein for 15 min and intact mitochondria were isolated from the cells. Mitochondrial hydrogen peroxide production was measured by the oxidation of *p*-hydroxyphenylacetate and estimated from a standard curve. Acrolein at all three concentrations (1, 10 and 100 μM) significantly increased mitochondrial hydrogen peroxide level. $^{**}P < 0.01$ with respect to control (acrolein at 0 μM).

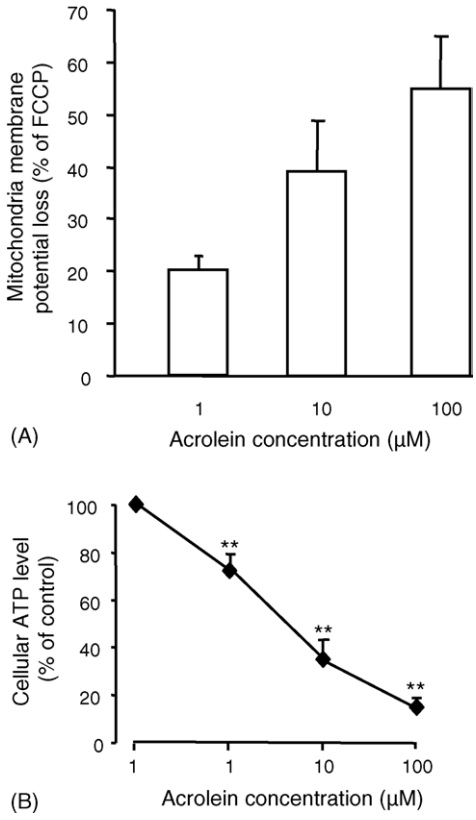

Fig. 4. Mitochondrial membrane potential ($\Delta\psi_m$) changes and ATP levels in PC12 cells after acrolein exposure. (A) Cells were incubated with Rh123 (5 μM) for 15 min. After a baseline recording, acrolein was added and the decrease of $\Delta\psi_m$ was recorded as the increase of Rh123 fluorescence. The decrease of $\Delta\psi_m$ was expressed as the percentage of that caused by FCCP. (B) Cellular ATP levels after acrolein exposure. ATP was estimated using an ATP bioluminescent assay kit. Acrolein at all three concentrations (1, 10 and 100 μM) caused significant reduction of cellular ATP levels. $^{**}P < 0.01$ with respect to control (acrolein at 0 μM).

mitochondrial parameter controlling key cellular processes. The decrease of the mitochondrial membrane potential induced by acrolein was evaluated by determining the cellular retention of Rh123, which is known to be concentrated in the mitochondria and quenched at a high $\Delta\psi_m$ level. As $\Delta\psi_m$ decreases, Rh123 is released, causing dequenching and an increase in Rh123 fluorescence (Zamzami et al., 2001). As shown in Fig. 4A, addition of acrolein (1, 10 and 100 μM) caused a dose-dependent loss of mitochondria membrane potential after a 400 s period of incubation. The PC12 cells without acrolein treatment did not show significant changes of Rh123 fluorescence during the observation. Acrolein itself did not affect Rh123 fluorescence measurement, either.

We then estimated the intracellular ATP levels of PC12 cells, after exposure to various concentrations of acrolein. It was observed that acrolein, at the concentrations tested (1–100 μM), caused a dose-dependent decrease of intracellular ATP. As shown in Fig. 4B, exposure of 1, 10 and 100 μM significantly reduced the ATP levels ($P < 0.01$), which were approximately 72, 30 and 15% of control values, respectively. These data clearly demonstrate that mitochondrial function is severely impaired by acrolein.

Finally, we tested the effects of acrolein on cellular oxygen consumption, another indicator of mitochondrial function. The extent of the oxygen consumption recorded during the addition of acrolein is shown in Fig. 5. When 1–100 μM acrolein was introduced into the chamber, continuous oxygen consumption was still observed; however, the rate of oxygen consumption displayed a dose-dependent decrease in the cells exposed to acrolein (Fig. 5A). A summary of three independent experiments (Fig. 5B) demonstrated that the oxygen consumption rate of the cells exposed to 1, 10 and 100 μM was 68.8, 31.4 and 16.4%, respectively; all were significantly lower than that of control ($P < 0.01$).

4. Discussion

4.1. Acrolein induces cell death in PC12 cells

In this study, we demonstrated that acrolein is toxic to PC12 cells in a concentration- and time-dependent manner, as determined by acrolein-induced cell death assessed by the MTT reduction assay (Fig. 1). We also demonstrated that exposure of the cells to acrolein resulted in significant generation of ROS (Fig. 2) and antioxidants were protective against acrolein-induced cell death (Fig. 1B). These data suggest that oxidative stress is, at least in part, responsible for acrolein's cytotoxic activity. Our data also suggested that the ability of acrolein to induce cell death is closely related to mitochondrial ROS production (Fig. 3), and that mitochondria-derived ROS upon acrolein stimulation may play a major role in acrolein toxicity. It is also apparent that the mitochondrial permeability transition pore (MPTP) is not involved in this process, since the inhibition of MPTP by CsA and PEG was not effective in reducing cell death. Overall, these results are consistent with our previous

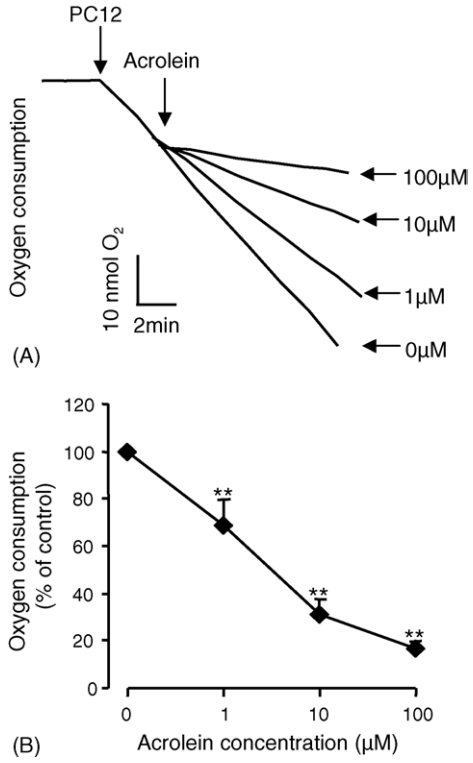

Fig. 5. Acrolein inhibits cellular respiration. Cellular respiration was estimated by oxygen consumption using a Clarke electrode. (A) A typical experiment showing the effects of different concentrations of acrolein on oxygen consumption. Note the dose-dependent response of the rate of oxygen consumption after the addition of acrolein. (B) A summary of three independent experiments with various concentrations of acrolein. Respiration rate was obtained and expressed as percentages of control (non-acrolein exposure). $^{**}P < 0.01$ with respect to control.

hypothesis that oxidative stress is involved in acrolein toxicity (Luo and Shi, 2004).

Oxidative stress has been implicated in acrolein related cytotoxicity in different experimental situations from whole animal to cell culture. Systemic exposure of rats to acrolein resulted in a significant decrease of glutathione and enhanced lipid peroxidation (Arumugam et al., 1999a; Arumugam et al., 1999b). Incubation with acrolein produced a concentration-dependent oxidative stress and tissue damage in lung slices (Monteil et al., 1999) and spinal cord segments (Luo and Shi, 2004), which was reduced by treatment with antioxidants (Luo and Shi, 2004). The involvement of oxidative stress in acrolein toxicity has also been suggested in other cell types such as pneumocytes (Monteil et al., 1999) and epithelial cells (Nardini et al., 2002), in addition to PC12 cells. In the current study, the peroxide-sensitive dye $DCFH_2$-DA, which has the advantage of providing insight into total cytosolic ROS levels, was used to document that acrolein induced the accumulation of ROS in PC12 cells. A significant increase in hydrogen peroxide accumulation, evaluated as an increased rate of DCF formation, could be seen 30 min after acrolein treatment (Fig. 2). The observed protection afforded against acrolein-induced cell death by NAC, an antioxidant and precursor of GSH (Kelly, 1998), further suggests the significance of ROS in acrolein toxicity in the PC12 cell line.

Acrolein has been reported to cause death in a variety of cell lines (Kern and Kehrer, 2002; Nardini et al., 2002). Whether this cytotoxicity may lead to apoptosis (Nardini et al., 2002) or necrosis (Kern and Kehrer, 2002) possibly depends upon cell type. Our results showed that, in PC12 cells, acrolein-induced cell death mainly through a necrotic mechanism (Fig. 1C). A possible mechanism might be that acrolein inhibits the cellular respiratory function (Fig. 5), decreasing the cellular ATP level (Fig. 4). It has already been well established that ATP can act as a switch between apoptosis and necrosis (Leist et al., 1997; Stefanelli et al., 1997). A depleted cellular ATP level has been shown to inhibit apoptosis (Leist et al., 1997; Stefanelli et al., 1997). Therefore, in PC12 cells, the drastically decreased cellular ATP level after acrolein treatment could encourage necrotic over apoptotic cell death. The mechanism of acrolein-induced ATP reduction may be related to direct inhibition of mitochondrial respiration, since acrolein has been shown to be able to directly inhibit respiration in isolated brain mitochondria (Picklo and Montine, 2001). Similarly, using an MTT test (Cohen et al., 1997), we recently found that acrolein impairs mitochondrial electron transport function in mitochondria isolated from the guinea pig brain (Luo and Shi, 2005). Another possible mechanism of acrolein-induced necrosis could be due to the inhibition of apoptosis pathways, since acrolein has been shown to be able to reduce caspase activities needed for apoptosis induction (Kern and Kehrer, 2002).

4.2. Role of mitochondria in acrolein cytotoxicity

The results presented in the current study show that mitochondria may play a critical role in acrolein-induced cytotoxicity in the following ways: (1) Significant decreases of the mitochondrial transmembrane potential, measured by a reduction of mitochondrial retention of Rh123 (Fig. 4A), were observed after acrolein exposure. This indicates that acrolein induces mitochondrial dysfunction in PC12 cells. (2) In the mitochondria isolated from acrolein-treated cells, the level of hydrogen peroxide is significantly increased (Fig. 3). (3) The exposure of acrolein resulted in a significant impairment of cellular respiration, as shown by the decreased oxygen consumption (Fig. 5). (4) The significant decrease of the intracellular ATP levels in PC12 cells that were incubated for 24 h in the presence of acrolein (Fig. 4B) also supported the notion that the mitochondrial function is severely impaired by acrolein.

In the mitochondria isolated from acrolein-exposed cells, significantly higher levels of ROS production were observed (Fig. 3). These results suggest that mitochondria, a major source of intracellular ROS under normal physiological conditions, may also be an important source of ROS over-production upon acrolein stimulation. This notion was supported by our recent investigations using isolated brain

mitochondria. We found that acrolein can directly stimulate ROS generation in mitochondria isolated from guinea pig brain (Luo and Shi, 2005). These results imply that the mitochondria-derived ROS may significantly contribute to the overall cellular oxidative stress observed after acrolein exposure.

The results of the current study also indicate that the loss of the mitochondrial transmembrane potential, induced by acrolein, may be not the cause of the increase of ROS accumulation after acrolein stimulation. The most direct clue comes from the fact that CsA, a known protector of mitochondrial transmembrane potential, and to a lesser extent, PEG, did not rescue PC12 cells. This suggests that the loss of the mitochondrial transmembrane potential caused by acrolein is more likely not an early event after acrolein attack leading to mitochondrial dysfunction and subsequent cell death, but an end sign of cell death. Consistent with our conclusion, impairment of mitochondrial membrane potential could result from oxidative stress, caused by the increased generation of ROS (Castilho et al., 1995; Vercesi et al., 1997).

4.3. The possible role of acrolein in neurodegenerative disease and CNS trauma

Along with 4-oxo-2-nonenal (Oe et al., 2003) and isoketals (Roberts et al., 2005), acrolein is one of the most reactive products of lipid peroxidation (Esterbauer et al., 1991). While numerous possible mediators of oxidative damage exist in vivo, acrolein may represent an important one, particularly in neurodegenerative disease and CNS trauma. Such hypothesis is based on following observations. First, acrolein is increased in neurodegenerative diseases (Calingasan et al., 1999; Lovell et al., 2001), and following spinal cord traumatic injury (Luo et al., 2003). Second, we have shown that acrolein can inflict structural and functional damage to isolated spinal cord tissue (Shi et al., 2002; Luo and Shi, 2004), as well as PC12 cells in culture (current study) at concentrations that are pathologically relevant. Third, it is estimated from aqueous environments that acrolein has a half-life of 7–10 days (Ghilarducci and Tjeerdema, 1995). Therefore, acrolein is significantly more stable, compared with short-lived ROS which have a half-life of 10^{-12} s. Fourth, the CNS may be more vulnerable to acrolein compared with other organs such as liver and heart. For example, in rat brain mitochondria, IC_{50} of State III respiration is approximately 40 μM (Picklo and Montine, 2001), while in the mitochondria from rat heart this value was 20 times higher (800 μM) (Biagini et al., 1990).

In summary, the results of the current study demonstrate that acrolein induces cell death in PC12 cells and that acrolein may exert its toxic effects through mitochondria as one possible target. Specifically, we suggest that acrolein stimulates the overproduction of ROS in mitochondria, which can further impair mitochondrial function, and eventually lead to cell death.

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References

Arumugam, N., Thanislass, J., Ragunath, K., Niranjali Devaraj, S., Devaraj, H., 1999a. Acrolein-induced toxicity—defective mitochondrial function as a possible mechanism. Arch. Environ. Contam. Toxicol. 36, 373–376.

Arumugam, N., Sivakumar, V., Thanislass, J., Pillai, K.S., Devaraj, S.N., Devaraj, H., 1999b. Acute pulmonary toxicity of acrolein in rats—underlying mechanism. Toxicol. Lett. 104, 189–194.

Balaban, R.S., Nemoto, S., Finkel, T., 2005. Mitochondria, oxidants, and aging. Cell 120, 483–495.

Baldwin, S.A., Broderick, R., Osbourne, D., Waeg, G., Blades, D.A., Scheff, S.W., 1998. The presence of 4-hydroxynonenal/protein complex as an indicator of oxidative stress after experimental spinal cord contusion in a rat model. J. Neurosurg. 88, 874–883.

Biagini, R.E., Toraason, M.A., Lynch, D.W., Winston, G.W., 1990. Inhibition of rat heart mitochondrial electron transport in vitro: implications for the cardiotoxic action of allylamine or its primary metabolite, acrolein. Toxicology 62, 95–106.

Calingasan, N.Y., Uchida, K., Gibson, G.E., 1999. Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. J. Neurochem. 72, 751–756.

Castilho, R.F., Kowaltowski, A.J., Meinicke, A.R., Bechara, E.J., Vercesi, A.E., 1995. Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. Free Radic. Biol. Med. 18, 479–486.

Cohen, G., Farooqui, R., Kesler, N., 1997. Parkinson disease: a new link between monoamine oxidase and mitochondrial electron flow. Proc. Natl. Acad. Sci. USA 94, 4890–4894.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689–695.

Delgado-Esteban, M., Almeida, A., Bolanos, J.P., 2000. D-Glucose prevents glutathione oxidation and mitochondrial damage after glutamate receptor stimulation in rat cortical primary neurons. J. Neurochem. 75, 1618–1624.

Delgado-Esteban, M., Almeida, A., Medina, J.M., 2002. Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia. J. Neurochem. 82, 1148–1159.

Dennis, K.J., Shibamoto, T., 1990. Gas chromatographic analysis of reactive carbonyl compounds formed from lipids upon UV-irradiation. Lipids 25, 460–464.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 81–128.

Ghilarducci, D.P., Tjeerdema, R.S., 1995. Fate and effects of acrolein. Rev. Environ. Contam. Toxicol. 144, 95–146.

Kehrer, J.P., Biswal, S.S., 2000. The molecular effects of acrolein. Toxicol. Sci. 57, 6–15.

Kelly, G.S., 1998. Clinical applications of *N*-acetylcysteine. Altern. Med. Rev. 3, 114–127.

Kern, J.C., Kehrer, J.P., 2002. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. Chem. Biol. Interact. 139, 79–95.

Koh, J.Y., Choi, D.W., 1988. Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. J. Neurosci. 8, 2153–2163.

Leist, M., Single, B., Castoldi, A.F., Kuhnle, S., Nicotera, P., 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. 185, 1481–1486.

Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J.A., Robinson, J.P., 2003. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J. Biol. Chem. 278, 8516–8525.

Lovell, M.A., Xie, C., Markesbery, W.R., 2000. Acrolein, a product of lipid peroxidation, inhibits glucose and glutamate uptake in primary neuronal cultures. Free Radic. Biol. Med. 29, 714–720.

Lovell, M.A., Xie, C., Markesbery, W.R., 2001. Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiol. Aging 22, 187–194.

Luo, J., Shi, R., 2005. Acrolein induces oxidative stress in brain mitochondria. Neurochem. Int. 46, 243–252.

Luo, J., Shi, R., 2004. Acrolein induces axolemmal disruption, oxidative stress, and mitochondrial impairment in spinal cord tissue. Neurochem. Int. 44, 475–486.

Luo, J., Uchida, K., Shi, R., 2003. Lipid peroxidation products, acrolein and 4-hydroxynonenal, are increased following traumatic spinal cord injury in guinea pig. In: Proceedings of the 33rd Society for Neuroscience Annual Meeting Neurobiology of Lipids Sessions. Neurobiol. Lipids 2(3) (2003). Published online Oct. 1, 2003. Available at: http://neurobiologyoflipids.org/content/2/3/.

Markesbery, W.R., Lovell, M.A., 1998. Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. Neurobiol. Aging 19, 33–36.

Monner, D.A., 1988. An assay for growth of mouse bone marrow cells in microtiter liquid culture using the tetrazolium salt MTT, and its application to studies of myelopoiesis. Immunol. Lett. 19, 261–268.

Monteil, C., Le Prieur, E., Buisson, S., Morin, J.P., Guerbet, M., Jouany, J.M., 1999. Acrolein toxicity: comparative in vitro study with lung slices and pneumocytes type II cell line from rats. Toxicology 133, 129–138.

Nardini, M., Finkelstein, E.I., Reddy, S., Valacchi, G., Traber, M., Cross, C.E., Van der Vliet, A., 2002. Acrolein-induced cytotoxicity in cultured human bronchial epithelial cells. Modulation by alpha-tocopherol and ascorbic acid. Toxicology. 170, 173–185.

Oe, T., Arora, J.S., Lee, S.H., Blair, I.A., 2003. A novel lipid hydroperoxide-derived cyclic covalent modification to histone H4. J. Biol. Chem. 278, 42098–42105.

Palmeira, C.M., Moreno, A.J., Madeira, V.M., Wallace, K.B., 1996. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. J. Pharmacol. Toxicol. Methods 35, 35–43.

Picklo, M.J., Montine, T.J., 2001. Acrolein inhibits respiration in isolated brain mitochondria. Biochim. Biophys. Acta 1535, 145–152.

Roberts, L.J., Fessel, J.P., Davies, S.S., 2005. The biochemistry of the isoprostane, neuroprostane, and isofuran. Pathways of lipid peroxidation. Brain Pathol. 15, 143–148.

Robinson, J.P., 1993. Handbook of Flow Cytometry Methods. Wiley-Liss, New York.

Shi, R., Luo, J., Peasley, M.A., 2002. Acrolein inflicts axonal membrane disruption and conduction loss in isolated guinea pig spinal cord. Neuroscience 115, 337–340.

Springer, J.E., Azbill, R.D., Mark, R.J., Begley, J.G., Waeg, G., Mattson, M.P., 1997. 4-Hydroxynonenal, a lipid peroxidation product, rapidly accumulates following traumatic spinal cord injury and inhibits glutamate uptake. J. Neurochem. 68, 2469–2476.

Stefanelli, C., Bonavita, F., Stanic, I., Farruggia, G., Falcieri, E., Robuffo, I., Pignatti, C., Muscari, C., Rossoni, C., Guarnieri, C., Caldarera, C.M., 1997. ATP depletion inhibits glucocorticoid-induced thymocyte apoptosis. Biochem. J. 322 (Pt 3), 909–917.

Uchida, K., 1999. Current status of acrolein as a lipid peroxidation product. Trends Cardiovasc. Med. 9, 109–113.

Varming, T., Drejer, J., Frandsen, A., Schousboe, A., 1996. Characterization of a chemical anoxia model in cerebellar granule neurons using sodium azide: protection by nifedipine and MK-801. J. Neurosci. Res. 44, 40–46.

Vercesi, A.E., Kowaltowski, A.J., Grijalba, M.T., Meinicke, A.R., Castilho, R.F., 1997. The role of reactive oxygen species in mitochondrial permeability transition. Biosci. Rep. 17, 43–52.

Zamzami, N., Maisse, C., Metivier, D., Kroemer, G., 2001. Measurement of membrane permeability and permeability transition of mitochondria. Methods Cell Biol. 65, 147–158.