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Original Research

Protective effect of ferulic acid against 2,2′-azobis(2-amidinopropane) dihydrochloride-induced oxidative stress in PC12 cells

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Abstract: Oxidative stress is closely related to the pathogenesis of neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. However, the underlying antioxidant mechanisms of ferulic acid (FA) aganist oxidantive stress are poorly understood. We evaluated the potential protective effects of FA against 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced damage in PC12 cells. Our results indicated that pretreatment with FA prior to AAPH exposure significantly increased PC12 cell survival, and also increased catalase and superoxide dismutase activity. Furthermore, FA treatment reduced cellular lactate dehydrogenase release and malondialdehyde levels. It attenuated AAPH-induced apoptosis in PC12 cells, as determined by flow cytometric detection of annexin V. Reductions in mitochondrial membrane potential and accumulation of intracellular Ca^{2+} were also inhibited by FA treatment. These findings suggested that FA protected PC12 cells against AAPH-induced oxidative stress, and may be a neuroprotective agent.

Key words: Oxidative stress, ferulic acid, PC12 cells, antioxidant activity.

Introduction

Oxidative stress is caused by an imbalance between the production of various free radicals such as hydroxyl radical and superoxide anion and the scavenging ability of the antioxidant system. Excessively high levels of reactive oxygen species (ROS) can damage biological macromolecules, trigger chemical chain reactions, such as lipid peroxidation or DNA and protein oxidation, and induce cell death (1). Thus, oxidative stress is considered as an important etiological factor in chronic diseases, including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders such as Alzheimer's disease (2-6). Antioxidants are widely used to protect cells from ROS-induced damages. Thus, the identification of potential compounds with antioxidant properties, particularly polyphenols, has become a key strategy in efforts to delay or inhibit oxidative damage (7).

Ferulic acid (FA) is a caffeic acid derivative found in vegetables (8), fruits (9), grains (10), and beverages (11), including coffee (12) and beer (13). Moreover, extracts of herbs, spices, and coffee represent a major source of FA intake in Western countries (12). FA exhibits a wide range of therapeutic effects that are attributed to its potent antioxidant capacity(14). Furthermore, FA has been approved as an antioxidant additive and food preservative in Japan. Sodium ferulate, a salt of FA, is used in China for the treatment of cardiovascular and cerebrovascular diseases (15). Furthermore, multidrug resistance in cancer has been considered as one of the major threaten to the effective treatment of cancer diseases (16-19), which is mainly caused by the overexpression of multidrug resistance proteins on the cell membrane. Recent studies proved that FA were able to reverse the multidrug resistance phenotype of human breast carcinoma (20).Thus, FA may be beneficial in the prevention and treatment of oxidative stress-associated diseases, including Alzheimer's disease, diabetes, cancer, hypertension, and atherosclerosis (15).

FA can scavenge free radicals through providing electron, thereby exerting its antioxidant activity (12, 21, 22). Increasing evidence indicates that natural antioxidants may protect against hydrogen peroxide-induced oxidative stress in PC12 cells (23, 24). In cell model, AAPH is a soluble chemical that generates peroxyl radicals in the culture medium. oxidative stress can be induced by these peroxyl radicals (25). Thus, an AAPH-induced oxidative stress can visually reveal biological antioxidant ability by measured by ROS and malondialdehyde (MDA) levels as well as antioxidant enzyme activities (superoxide dismutase, SOD; catalase CAT; glutathione peroxidase, Gpx). Furthermore, FA can reverse late-stage diabetes in obese rats (26). However, the effects of FA on AAPH-induced oxidative stress and the resultant apoptotic cell death in PC12 cells have not been explored. In the present study, PC12 cells exposed to AAPH were used as a model of oxidative stress in order to investigate the protective effects of FA against AAPH-induced cytotoxicity and apoptosis, and its mechanisms of action.

Materials and Methods

Reagents

PC12 cells were obtained from the Institute of Bio-

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chemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). The lactate dehydrogenase (LDH), MDA, SOD, and catalase (CAT) assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The cell cycle and apoptosis analysis kit, annexin V-FITC apoptosis detection kit, mitochondrial membrane potential (MMP) assay kit with JC-1, Hoechst 33258 kit, and fluo 3/AM were purchased from the Beyotime Institute of Biotechnology (Haimen, China). All other chemicals were of analytical grade.

Cell culture and treatment

PC12 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37°C. Cells were cultured in DMEM with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100μg/mL streptomycin, and 2.5μg/mL amphotericin B. All experiments were carried out 24 h after the cells were seeded in microplates or in cell culture flasks. AAPH(150 mmol/L) was used to induce oxidative stress for 2 h and was freshly prepared prior to each experiment. PC12 cells were pretreated with various concentrations of FA (25-100 μg/mL) for 2 h, followed by FA treatment (150 mM) for 2 h. Trolox (20 μg/mL) was used as the positive control.

Cell viability and LDH assay

Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (23, 27). Briefly, PC12 cells were seeded in a 96-well plate (2×10^5 cells/mL). MTT (100 μL of 5 mg/mL in phosphate-buffered saline (PBS) was added to each well and incubated for 4 h at 37°C. The medium was then carefully removed, and 150μL of dimethyl sulfoxide was added to each well. The plate was shaken for 15 min, and the absorbance was determined at 570 nm using a Spectra-max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of the viability observed in the control cultures.

The release of LDH into culture medium due to cell membrane damage was measured as an *in vitro* indicator of cellular toxicity. LDH activity was measured using a commercial assay kit, according to the manufacturer's instructions. Cells were seeded in 6-well plates (4.5×10^5 cells/mL) and incubated for 24 h, followed by FA treatment (25-100μg/mL) for 2 h. Cells were then incubated with AAPH (150mM) for 2 h. After treatment, the medium was harvested for spectrophotometric determination of LDH release. Sample absorbance was read at 490 nm, and LDH release rate was calculated as the percent LDH in the culture medium versus total LDH in the cells, as follows:

LDH release rate (%) = LDH activity in culture medium / (LDH activity in culture medium + LDH activity in cells) × 100%.

Determination of ROS production

Cellular ROS were quantified by a dichlorofluorescein assay using a microplate reader, as described previously, with some modifications (28). Briefly, PC12 cells were seeded in a 96-well plate (2.85×10^4 cells/well) in medium containing fetal bovine serum. The following day, the medium was replaced with serum-free medium. After 24 h, 25-μM dichlorodihydrofluorescein diacetate (DCFH-DA) was added to the wells for 30 min at 37°C. Cells were then washed twice with PBS and placed in fresh serum-free medium containing various FA concentrations for 2 h. AAPH (150μM) was then added to all wells except the controls for 2 h. Multi-well plates were immediately measured (time 0) on a Spectra-max M5 microplate reader (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The results were expressed as a percent of the control, which was set at 100%. The assay was run in triplicate, and the results were expressed as the mean ± standard deviation (SD).

Determination of MDA, CAT, and SOD activity

PC12 cells (4.5×10^5 cells/mL) in 4 mL culture medium were plated in 6-well plates for 24 h and exposed to FA or Trolox for 2 h. Cells were then incubated with 150mM AAPH for 2 h. Cells were washed with ice-cold PBS, harvested by centrifugation at 1,000 g for 5 min, pooled in 0.5 mL PBS, and lysed by sonication. The homogenates were centrifuged at 8,000 *g* for 15 min, and the supernatant was collected for MDA, SOD, and CAT analysis. Protein content was measured using the Bradford method, with bovine serum albumin standards.

MDA was measured spectrophotometrically using a commercial assay kit, to provide an index of lipid peroxidation. The assay was based on the ability of MDA and thiobarbituric acid to conjugate and form a red product, which has a maximum absorbance at 532 nm. MDA levels were measured following the manufacturer's instructions. The activities of CAT and SOD were determined using commercially available kits, as previously described (29).

Nuclear staining with Hoechst 33258

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33258. Briefly, PC12 cells were pre-incubated with FA for 2 h, followed by incubation with AAPH (150 mM) for 2 h. Cells were collected and stained with Hoechst 33258 for 15 min. The stained cells were observed under an inverted phase fluorescence microscope.

Cellular apoptosis assessment

Cellular apoptosis was evaluated using an annexin V-FITC apoptosis detection kit. Briefly, PC12 cells were pre-incubated with FA for 2 h, followed by 2 h incubation with AAPH (150mM). Cells were trypsinized, harvested, washed in PBS, and incubated at 20–25°C for 10 min in the dark with 5μL of annexin V-FITC. The cells were harvested, gently, suspended, and incubated at 4°C for 10 min in the dark with 10μL of propidium iodide. Cells were analyzed on a flow cytometer (BD FACSCalibur, San Jose, CA, USA). Early apoptotic cells were estimated as the percentage of cells that stained positive for annexin V-FITC while remaining impermeable to PI (AV+/PI−). This method also distinguished viable cells (AV−/PI−) and cells undergoing advanced apoptosis (AV+/PI+).

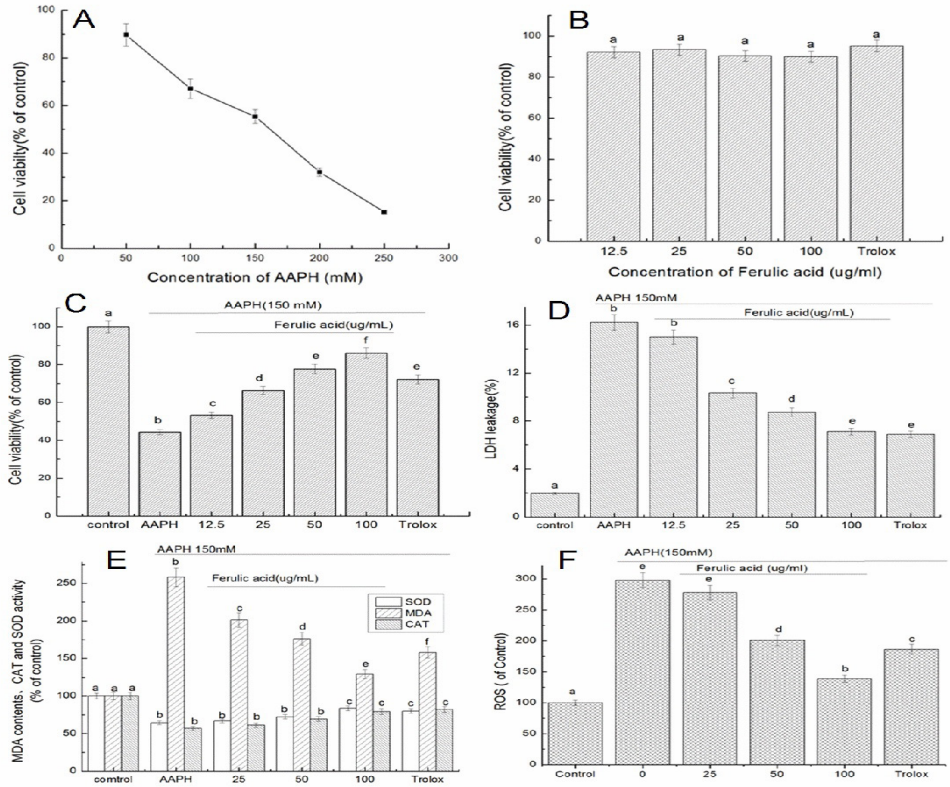

Figure1. Effect of FA on antioxidant defense system in PC12 cells. **A** and **B**, effect of AAPH and FA on cell viability in PC12 cells. **C**, effect of FA on AAPH-induced cytotoxicity in PC12cells. **D-F**, effect of FA on LDH leakage, MDA, CAT, SOD and ROS levels in PC12 cells injured by AAPH. Means with different superscript letters are significantly different. ($p<0.05$).

Measurement of intracellular Ca^{2+} levels

The concentration of intracellular Ca^{2+} was monitored by flow cytometry, as described previously(23, 30). Briefly, cells cultured with or without FA and AAPH were stained with fluo 3/AM (5μg/mL) for 30 min at 37°C. The cells were then washed and harvested with PBS prior to analysis of their mean fluorescent intensity (at 488-nm excitation and 530-nm emission wavelengths) by flow cytometry. Relative intracellular Ca^{2+} levels were calculated from the distribution histogram, with cell counts on the vertical axis and fluorescent intensity (log scale) on the horizontal axis. Data were collected from at least 10000 events.

Determination of MMP

MMP was measured using the fluorescent dye, JC-1 (Molecular Probes)(31). PC12 cells were seeded in 6-well culture dishes (10^6 cells/well) and incubated with FA and AAPH, as described in section 2.3. The cells were incubated with JC-1 for 30 min and then washed twice with PBS. The washed cells were analyzed by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 525 nm (BD FACSCalibur). The mean fluorescence intensity values were then calculated.

Statistical analysis

All experiments were performed in triplicate. Data from each experiment are presented as the mean ± SD (n=3). The significant differences between the means of parameters were calculated by Duncan's multiple-range test ($p < 0.05$) using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

FA protects against AAPH-induced cytotoxicity in PC12 cells

AAPH is an azo radical initiator used as an oxidant source in many previous studies (32-34). In the PC12 oxidative stress model, AAPH generates an alkyl radical after decomposition at 37°C. In the presence of oxygen, these alkyl radicals are converted to peroxyl radicals, which can cause lipid peroxidation. MTT assay data (Fig. 1-A) showed that AAPH reduced PC12 cell viability in a dose-dependent manner, and analysis using the SPSS 16.0 revealed that the IC_{50} value was 155.16 mM. Therefore, in the subsequent experiments, 150 mM AAPH was used to evaluate the protective effect of FA against oxidative stress in PC12 cells. This is similar to Liao's study that oxidative stress was induced by 200 mM AAPH for 2 h in HepG2 cell(35).

Exposure to FA alone (12.5-100μg/mL) did not significantly affect the viability of PC12 cells (Fig. 1-B). However, the data presented in Figure 1-C indicated that the AAPH-induced reduction in PC12 cell viability was significantly ($P < 0.05$) attenuated in the presence of FA at concentrations of 25μg/mL ($P < 0.05$) or higher ($P < 0.01$)(Fig.1-C).

LDH is a stable cytoplasmic enzyme present in all cells, and its release can be used to evaluate the escape of cellular contents into the plasma, reflecting cell membrane damage. The LDH levels in cell culture media were determined in order to evaluate the level of cell injury. As shown in Figure 1-D, the level of LDH in media conditioned by cells exposed to AAPH (150mM) was approximately eight times higher than that observed in media conditioned by control. PC12 cells pretreatment with FA (12.5, 25, 50, or 100μg/mL) decreased the le-

vel of LDH in the media in a dose-dependent manner. These data indicated that pretreatment with FA reduced AAPH-induced PC12 cell death and protected against AAPH-induced cell membrane damage.

Effect of FA pretreatment on MDA, CAT, and SOD levels in PC12 cells

Lipid peroxidation is one of the earliest recognized and most extensively studied manifestations of oxidative damage. MDA is a product of lipid peroxidation, and was evaluated to assess the degree of lipid peroxidation in cells. As shown in Figure 1-E, MDA levels increased significantly after AAPH treatment and were approximately 2.5-fold higher than those present in control cells. However, pretreatment with 25, 50, or 100μg/mL FA reduced this AAPH-induced increase in MDA levels in a dose-dependent manner; pretreatment with 20μg/mL Trolox also attenuated the increase of MDA levels (Fig. 1-E).

As illustrated in Figure 1-E, exposure of PC12 cells to AAPH significantly reduced CAT and SOD activity by 56.98% and 64.22%, respectively, as compared to the control group. Pretreatment with FA and Trolox significantly attenuated these AAPH-induced decreases in CAT and SOD activity. FA (100 μg/mL) restored CAT and SOD activity to 79.04% and 83.77% of the activity observed in control cells, respectively. These results were somewhat in accordance with a previous report (36) showing that 800-μM (155μg/mL) FA decreased MDA content and LDH activity, and increased SOD activity, as compared to PC12 cells treated with hydrogen peroxide alone.

Effect of FA on ROS levels in PC12 cells

ROS generation in PC12 cells was measured using DCF-DA dye and fluorescence microscopy (37). As shown in Figure 1-F, PC12 cells exposed to AAPH produced approximately 3-fold more ROS than did control cells. This elevation in the ROS level was significantly reduced by pretreatment with 50 and 100 μg/mL FA (Fig. 1-F). Although treatment with FA (100 μg/mL) attenuated the AAPH-induced increase in ROS most effectively, the levels remained higher than those observed in control cells ($P < 0.05$). These results indicated that co-incubation with FA effectively prevented AAPH-induced ROS production.

Effect of FA on PC12 cell apoptosis

In order to further investigate the effects of different treatments on DNA and nuclear structure in PC12 cells, we used the fluorescent nuclear stain, Hoechst 33258. This DNA dye stains normal nuclear chromatin blue pale, with an organized structure, while apoptotic cells can be identified by the presence of chromatin condensation and intact nuclear boundaries, and show bright blue condensed chromatin (38). As illustrated in Figure 2(1), cells treated with AAPH alone contained numerous bright blue dots, representing chromatin condensation or nuclear fragmentation. However, pretreatment with FA (100 μg/mL) or Trolox (20 μg/mL) significantly decreased nuclear condensation and fragmentation (Fig. 2(1) E-F). Furthermore, most cell nuclei maintained their normal shape and size.

The effect of AAPH treatment on apoptosis was investigated using flow cytometry. Positive staining with annexin V-FITC correlates with a loss of membrane polarity, and the complete loss of membrane integrity leads to apoptosis or necrosis. In contrast, PI can only enter cells after the loss of membrane integrity. Thus, dual staining with annexin V-FITC and PI allows for clear discrimination between unaffected and apoptotic cells. Our results showed that treatment with 150-mM AAPH significantly increased the levels of apoptotic cells by 839.66% compared with control group. Pretreatment with FA (25, 50, and 100μg/mL) or Trolox (20μg/mL) significantly attenuated this AAPH-induced apoptosis in a dose-dependent manner (Fig. 2). These results suggested that FA could protect against AAPH-induced apoptosis.

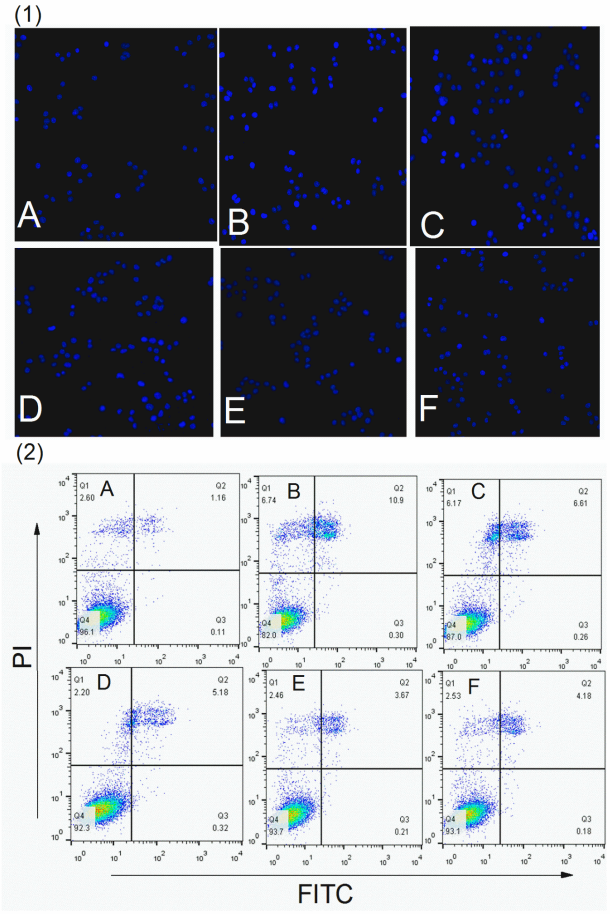

Figure 2. Inhibitory effect of FA on AAPH-induced apoptosis in PC12 cells using **(1)** DNA fluorescent dye Hoechst 33258 and **(2)** AV-FITC/PI. **A**, control; **B**,150mM AAPH treated; **C**, 25μg/ml FA+150mM AAPH treated; **D**, 50μg/ml FA+150mM AAPH treated; **E**, 100μg/ml FA+150mM AAPH treated; **F**, 20μg/ml Trolox +150mM AAPH treated.

Effect of FA on Ca^{2+} levels in AAPH-treated PC12 cells

Intracellular Ca^{2+} is one of the most important signaling molecules, regulating many aspects of cellular function. It plays a significant role in the induction of apoptosis and regulates apoptotic signaling pathways. Excessive intracellular Ca^{2+} accumulation is a major factor in cell injury and death in response to oxidative stress(39). It is widely accepted that intracellular Ca^{2+} levels are elevated by AAPH. The Ca^{2+}-sensitive fluorescent probe, fluo 3/AM, was employed to test altera-

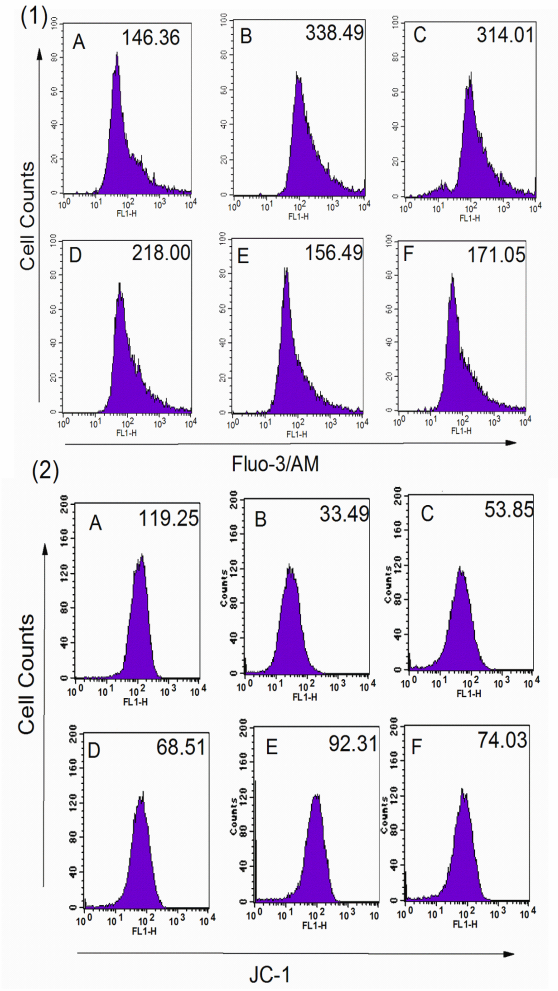

Figure 3. Effect of FA on (1) Ca^{2+} levels and (2) MMP in AAPH-induced PC12 cells. **A**, control; **B**,150mM AAPH treated; **C**, 25μg/ml FA+150mM AAPH treated; D 50μg/ml FA+150mM AAPH treated; **E**, 100μg/ml FA+150mM AAPH treated; **F**, 20μg/ml Trolox +150mM AAPH treated.

tions in intracellular Ca^{2+} using flow cytometry.

As illustrated in Fig. 3(1), after exposure to AAPH, fluo 3/AM fluorescence increased 2.31-fold, as compared to control cells. However, pretreatment with FA reduced this elevation in a dose-dependent manner; pretreatment with Trolox also reduced the AAPH-induced increase in intracellular Ca^{2+} concentration. Thus, FA protected PC12 cells against AAPH-induced Ca^{2+} accumulation.

Effect of FA on MMP in AAPH-treated PC12 cells

Maintenance of the MMP is essential for cell survival, particularly when the cell is under oxidative stress (40, 41). A decrease in MMP indicates impaired mitochondrial function, which is closely associated with ROS generation and cellular apoptosis. As shown in Figure 3(2), the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide) assay data indicated that treating cells with 150mM AAPH decreased the MMP to 28.08% of the value observed in control cells. However, pretreatment with FA dose-dependently ameliorated this AAPH-induced reduction in MMP. These results suggested that FA protected against AAPH-induced cell injury by inhibiting MMP dissipation.

Discussion

The oxidative-stress-induced tissue damage has been implicated in a number of disease processes, including cancer, diabetes, and neurodegenerative disorders (2, 42-44). Free radicals, such as the superoxide anion, and the hydroxyl radical, are able to produce many detrimental effects, including lipid peroxidation of cellular membranes, alteration of the lipid-protein interaction, enzyme inactivation, and DNA breakage and, in the end, to cause cell injury, necrosis, or apoptosis (45). Although antioxidant defense systems can help to protect the body against free radicals, they might become overwhelmed during periods of chronic oxidative stresses.

Therefore, in recent years, phenolic acids has generated considerable interests as potential therapeutic agents against a wide variety of chronic diseases. In the present study, we have demonstrated that FA exhibited cytoprotective effects against AAPH-induced damage and cell death in PC12 cells.

PC12 cells, a rat pheochromocytoma cell line, have been extensively used as a model system to study neurotrophic factors and neuronal differentiation (46). AAPH can induce apoptosis in many cell types, and has been extensively used to induce oxidative stress *in vitro* (35).

Generation of the superoxide anion under oxidative stress causes oxidative damage and results in lipid peroxidation of the plasma membrane. However, there are some cellular antioxidant enzymes, including CAT and SOD, that scavenge ROS and prevent cellular damage (47). SOD is able to transform intracellular superoxide anions to H_2O_2, which is subsequently scavenged by reactions catalyzed by CAT and glutathione peroxidase. In this study, AAPH significantly inhibited CAT and SOD activity, and increased the formation of MDA, a lipid peroxidation product, in PC12 cells. In contrast, FA pretreatment reversed these changes, suggesting that the cytoprotective effect of FA against AAPH-induced damage and cell death was related to its antioxidant activity.

There are some other sources of antioxidant materials that has potent antioxidant activity against AAPH-induced peroxyl radicals, such as catecholestrogens (48), sugarcane molasses (49), phenolic compounds (50) and uric acid (51). Numerous *in vitro* studies have indicated that AAPH treatment significantly increased ROS production (35). This overproduction damages major macromolecules in cells, including proteins, lipids, and DNA, causing neuronal dysfunction and depression (52, 53). Consistent with previous studies, we found that treating PC12 cells with AAPH not only led to ROS overproduction, but also increased MDA levels. Pretreating cells with FA mitigated these changes in a dose-dependent manner, suggesting that the antioxidant activity of FA may make it useful for attenuating and preventing apoptosis in Alzheimer's disease.

Many studies have confirmed that apoptosis is generally accompanied by increased intracellular Ca^{2+} levels. Upon initiation of apoptosis, the intracellular Ca^{2+} level rises rapidly and cytochrome c is released to the cytosol, leading to apoptosis. Our results were consistent with these observations (31). FA decreased intracellular Ca^{2+} levels, reflecting its inhibition of oxidative damage in the early stages of apoptosis. Previous work (54) found

that whey protein hydrolysates (200 μg/mL) significantly inhibited the H_2O_2-induced elevation in intracellular Ca^{2+} levels in PC12 cells.

The protective effect of FA was also supported by a higher MMP. Mitochondria serve as final arbiters of life and death of cells, because these organelles are not only required to generate ATP but can also trigger apoptosis or necrosis (55). Oxidative damage causes depletion of the materials that are necessary to produce high energy and strongly affects the electron transport chain in mitochondrial. MMP reflects the performance of the electron transport chain and can indicate a pathological disorder of this system. A higher MMP average is related to higher viability and vice versa. Mitochondrial membrane depolarization can cause cytochrome c to be released from the inner membrane, leading to activation of the caspase-3 cascade and apoptosis (56, 57). We found that AAPH led to mitochondrial membrane depolarization, while FA pretreatment significantly prevented the loss of MMP . The antioxidant property of FA can be attributed to the preservation of MMP.

In conclusion, pretreatment with FA effectively alleviated AAPH-induced oxidative stress and apoptosis in PC12 cells, suggesting that FA may provide a useful intervention for the treatment of chronic disease. FA produced antioxidant and anti-apoptotic effects by restoring CAT and SOD activity, elevating the MMP, and diminishing the level of MDA, ROS content, and LDH release. Therefore, the present study suggested that FA was a potent cytoprotective agent, capable of ameliorating AAPH-induced damage and cell death. Further work is being conducted to investigate the effect of FA on the antioxidant signaling pathway for cell protection.

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