

Ferulic acid protects PC12 neurons against hypoxia by inhibiting the p-MAPKs and COX-2 pathways

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ABSTRACT

Objective(s): Hypoxia induces cellular oxidative stress that is associated with neurodegenerative diseases. Here, the protective effects of ferulic acid (FA) on hypoxia-induced neurotoxicity in PC12 cells were evaluated.

Materials and Methods: We investigated the effect of FA on PC12 cells subjected to hypoxia stress, *in vitro*.

Results: FA increased cell viability, prevented membrane damage (LDH release), scavenged free radicals, increased superoxide dismutase (SOD) activity, and attenuated the elevation of intracellular free Ca²⁺, lipid peroxidation, apoptosis (evaluated by TUNEL staining) and PGE₂ production in hypoxia-stressed PC12 cells. MAPKs were activated during hypoxia. FA reduced p-p38 MAPK, caspase-3, and COX-2 activation which correlated well with diminished LDH release in PC12 cells under hypoxia. Furthermore, FA reduced lipid peroxidation in PC12 cells subjected to hypoxia.

Conclusion: Taken together, these results indicate that FA antioxidant effects could partly be involved in inhibition of p38 MAPK pathway and apoptosis through scavenging ROS in hypoxia-stressed PC12 cells.

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Introduction

Stroke is the third most important cause of death in Taiwan and the ischemic brain injury produced by stroke is a major cause of human neurological disability. Ferulic acid (FA) is a ubiquitous plant constituent that is resulted from the metabolism of phenylalanine and tyrosine. It is a phenolic compound present in seeds and leaves of vegetables, fruits, and cereals such as rice, bran, cabbage, celery and tomatoes. Extracts of these plants have anti-inflammatory properties and are rich in antioxidant compounds.

The aim of the present study was to investigate the neuroprotective effects of FA on hypoxia-stressed PC12 cell. Hypoxia is a pathophysiological condition that is characterized by an increase in reactive oxygen species (ROS) and a change in the intracellular redox level in cell (1). ROS can be scavenged by antioxidants such as sesamin, lycopene and resveratrol and antioxidant enzymes like superoxide dismutase (SOD). These provide the first line of defense against overproduction of ROS by mitochondria (2). Lipoic acid and vitamin E, as antioxidants have shown synergistic effects against lipid peroxidation induced by oxidant radicals in

several pathological conditions during brain ischemia (3, 4). FA is thought to play an important role in plant's defense against microorganisms (5). FA enhances carcinogens detoxification, has hepatoprotective effects, and protects against oxidative stress in mice (6, 7). The neuroprotection afforded by silymarin and sesamin has been attributed to the antioxidative properties of these compounds (8, 9). We were therefore interested in assessing the potential neuroprotective effects of ferulic acid.

Mitogen-activated protein kinases (MAPKs) have been implicated in regulating cell survival and cell death under numerous stressful conditions (10). ROS can activate the signal transduction pathway, thereby affecting MAPKs (8, 11). Since PC12 cells have long been accepted as a model of neuronal cells, they should be utilized in studies concerning the protective effects of ferulic acid on the central nervous system (12). Cytokine and ROS released from activated neuron may participate in neurodegenerative processes (9, 13). We were specifically interested in the effects of FA on neuronal cells under hypoxia. Their effects on hypoxia-induced MAPKs activation and apoptosis were also investigated.

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Materials and Methods

Reagents and cell culture

Ferulic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was obtained from Molecular Probes (Eugene, OR, USA). Rabbit anti-mouse antibodies were obtained from various sources: iNOS (Calbiochem, San Diego, CA, USA), β -actin (Rockland, Gilbertsville, PA, USA), caspase-3, COX-2 and antiphospho-MAPKs (Cell Signaling, Danvers, MA, USA). PC12 cells were maintained in Dulbecco's modified Eagle medium supplemented with 5 % (v/v) fetal bovine serum and 10 % horse serum at 37 °C in 5% CO₂. Experiments were carried out in glucose-free DMEM and gassed with 85% N₂, 10% H₂, and 5% CO₂ for 1 hr in the absence or presence of FA.

Cytotoxicity

Cytotoxicity was assessed by evaluation of the release of the cytosolic enzyme lactic dehydrogenase (LDH) from damaged cells using LDH diagnostic kit (Boehringer Mannheim). Absorbance values were recorded at 490 and 630 nm by SpectraMAX340 reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined using the blue formazan assay in which colorless 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is metabolized to a blue product by mitochondrial dehydrogenases. Absorbance was recorded at 540 nm using SpectraMAX 340 reader. Data were expressed as mean percentage of viable cells compared to control.

ROS diminishing

Reactive oxygen species (ROS) content was determined using H₂DCF-DA which accumulates within cells upon deacetylation. Cellular fluorescence was measured using Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland) with excitation at 485 nm and emission at 538 nm (8).

Western Blotting

Protein samples containing 40 μ g of protein were separated on 11.5 % (w/v) sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with rabbit anti-mouse beta-actin (ROCKLAND, Gilbertsville, PA, USA), caspase-3, or anti-phospho-MAPKs (Cell Signaling, Danvers, MA, USA). The membranes were subsequently incubated with conjugated goat anti-rabbit IgG (PerkinElmer, Boston, MA, USA). Caspase-3 and phosphorylated MAPK proteins were detected by chemiluminescence detection system (Western Lightning® Plus-ECL, PerkinElmer, Waltham, MA, USA) and quantified with a densitometric scanner (PDI, Huntington Station, NY, USA).

Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) in PC12 cells using lipid

peroxidation (LPO) assay kit (Cayman Chemical, Ann Arbor, MI, USA). MDA is a thiobarbituric acid-reacting substance (TBARS) used as an indicator of lipid peroxidation. The amount of MDA expressed in 5×10^5 cells was measured. Absorbance at 500 nm was determined using an ELISA reader (SpectraMAX 340).

Calcium release

PC12 cells treated with various concentrations of ferulic acid were subjected to hypoxia for 1 hr. The supernatant was used to measure the release of Ca²⁺. Briefly, 10 μ l of the supernatant was added to 1 ml Ca²⁺ reagent (Diagnostic Systems, Holzheim, Germany). Calcium concentration was determined by a microplate reader (absorbance at 620 nm) and quantified using 10 mg/ml Ca²⁺ standard solution.

SOD activity

Superoxide dismutase (SOD) activity was determined using a RANSOD kit (Randox Laboratories Limited Crumlin, UK). This method is based on the formation of red formazan from the reaction between 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl-tetrazolium chloride and superoxide radical. Absorbance was read at 505 nm.

PGE₂ assay

PGE₂ was measured using ELISA kit (R&D, Minneapolis, MN, USA). Absorbance at 450 nm was read by a microplate reader (SpectraMAX 340).

TUNEL staining

TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling) Staining Kit (Roche, Mannheim, Germany) enzymatically labels the free 3'OH ends of DNA in a template-independent manner. In the later stages of apoptosis, DNA cleavage results in fragments. Cells will be labeled with nucleotides attached to the 3'OH ends of DNA, followed by fluorescent markers and nucleotide polymers. Apoptosis was measured using fluorescence microscopy with excitation at 450–500 nm and emission at 515–565 nm.

Statistical analysis

All data are expressed as mean \pm SD. For single variable comparisons, Student's t-test was used. For multiple variable comparisons, data were analyzed by one-way ANOVA and Dunnett's test. *P*-values of less than 0.05 or 0.01 were considered significant.

Results

FA protected PC12 cells against hypoxia

FA was tested for its ability to protect PC12 cells from hypoxia-induced cell death. Lactate dehydrogenase (LDH) release was decreased by 22–29% by different concentrations of FA (Figure 1B). Hypoxia for 1 hr caused 40–50% cell death in PC12 cells. However, FA at 1 μ M significantly protected the

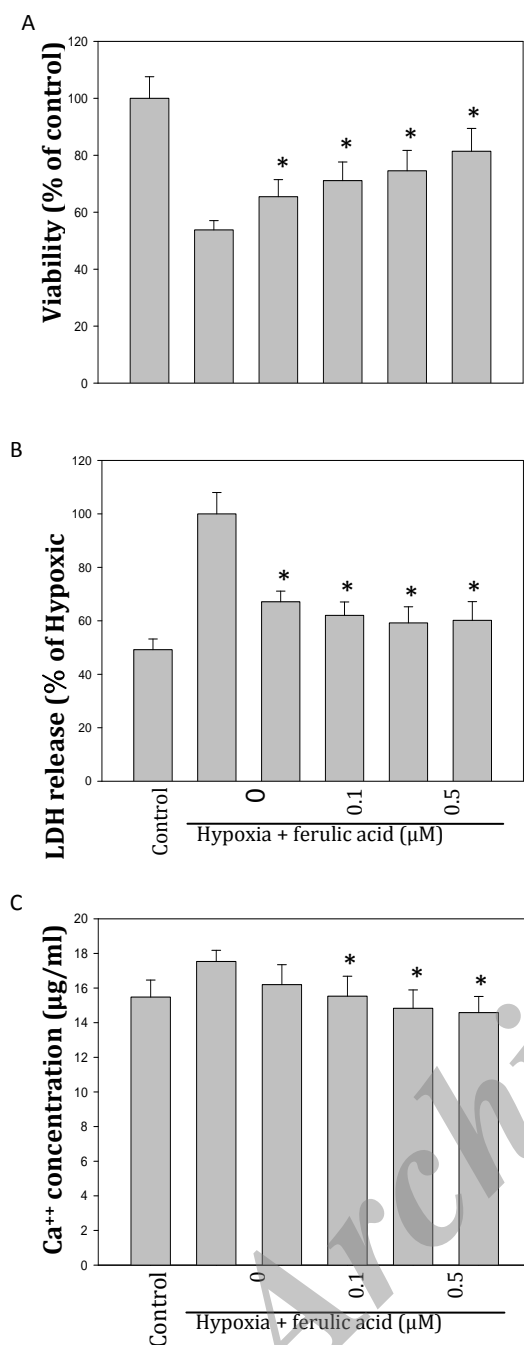


Figure 1. The effect of FA on the viability, cytotoxicity and Ca²⁺ release from hypoxic PC12 cells. FA dose-dependently improved cell viability based on the MTT assay (A), significantly inhibited LDH release (B) in hypoxic PC12 cells, and protected cells from hypoxic death. Cells were treated with hypoxia alone or with hypoxia plus various concentrations (0.1, 0.5, 1.0 μM) of FA for 1 hr. Treatment only with FA 0.5 or 1.0 μM but not 0.1 μM, reduced the release of Ca²⁺ under hypoxic stress (C). Data are expressed as mean±SD of five separate experiments. **P*<0.01 as compared to the hypoxic control

hypoxia-induced cell death 35±8% (MTT) and 29± 3% (LDH). Thus, FA significantly reduced hypoxia-induced LDH release and increased cells viability (MTT, Figure 1A). Incubation of cells with FA 1 μM reduced the release of Ca²⁺ under hypoxic stress by 15% (Figure

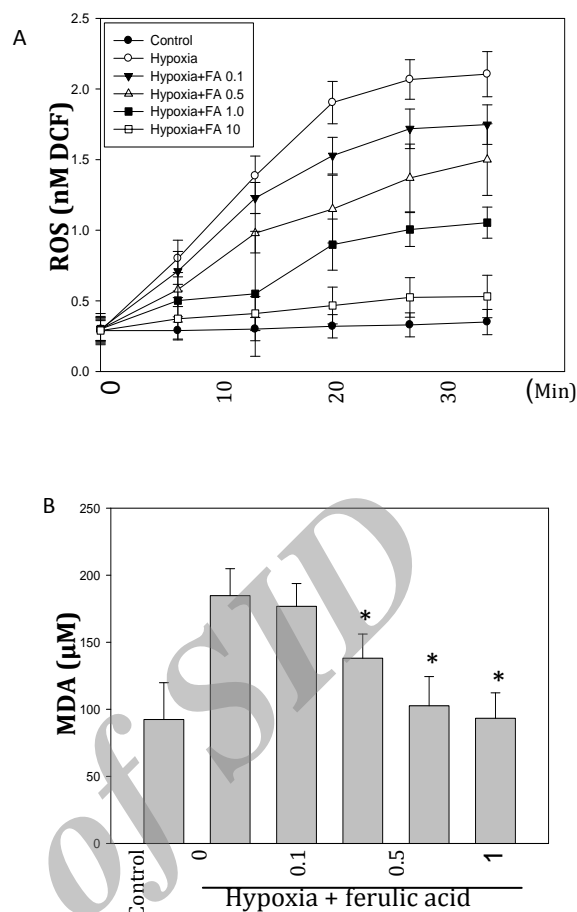


Figure 2. The effect of FA on ROS generation and lipid peroxidation in PC12 cells of hypoxia. In PC12 cells, FA (0.1, 0.5, 1.0 10 μM) diminished ROS induced by hypoxia (A). Lipid peroxidation (LPO) was determined using an LPO assay kit. The malondialdehyde (MDA) content of PC12 cells induced by 1 hr of hypoxia was reduced by FA (B). **P*< 0.01 as compared to the hypoxic control

1C). Hypoxia for 1 hr, resulted in an increase in Ca²⁺ release from PC12 cells, but FA protected PC12 cells from hypoxic damage and reduced the release of Ca²⁺.

FA diminished ROS generation and prevented hypoxia-induced lipid peroxidation in PC12 cells

Following 1 hr hypoxia, ROS were generated which were dose-dependently scavenged by FA (Figure 2A). PC12 cells exposure to hypoxia increased the concentration of ROS in the culture supernatant. FA was able to scavenge 38–63% of hypoxia-induced ROS in PC12 cells (Figure 2A). The increase in malondialdehyde (MDA) level in PC12 cells caused by 1 hr hypoxia was reduced by 44% by FA 1 μM (Figure 2B).

FA preserved SOD activity in Hypoxia-stressed PC12 cells

We studied whether hypoxia effects of FA influence antioxidant enzymes in PC12 cells. SOD activities were

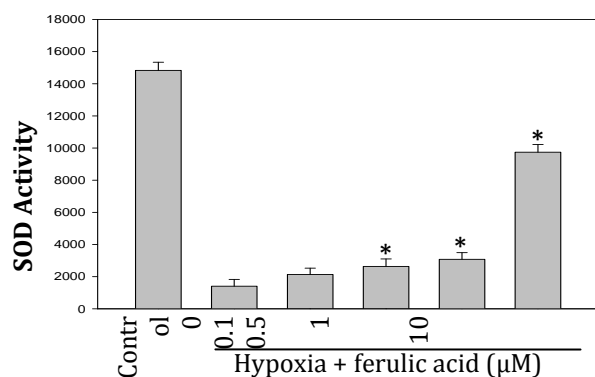


Figure 3. The effects of FA on SOD activity in hypoxia-stressed PC12 cells. FA (0.5, 1.0 10 μ M) dose-dependently maintained SOD activities in PC12 cells subjected to hypoxia-stress for 1 hr. Effect of FA on antioxidant enzyme in hypoxic PC12 cells. The cells were treated with hypoxia alone or with various concentrations (0.1, 0.5, 1.0, and 10 μ M) of FA for 1 hr. FA were able to increase SOD activity in hypoxia-stressed PC12 cells. * $P < 0.01$ as compared to hypoxia control

measured at different concentrations of FA in PC12 cells under hypoxia stress for 1 hr. FA dose-dependently maintained SOD activity in PC12 cells when they were subjected to hypoxia-stress for 1 hr. PC12 cells subjected to hypoxia lost 31% of their SOD activity, however FA prevented this reduction in SOD activity in hypoxic PC12 cells. The results demonstrated that FA at different concentrations (0.1, 0.5, 1, and 10 μ M) significantly maintained SOD activities of hypoxic PC12 cells for 1 hr (Figure 3).

FA reduced PGE2 production in PC12 cells

FA dose-dependently protected PC12 cells from hypoxia-induced PGE2 production. Incubation of cells with FA 1 μ M reduced the production of PGE2 under

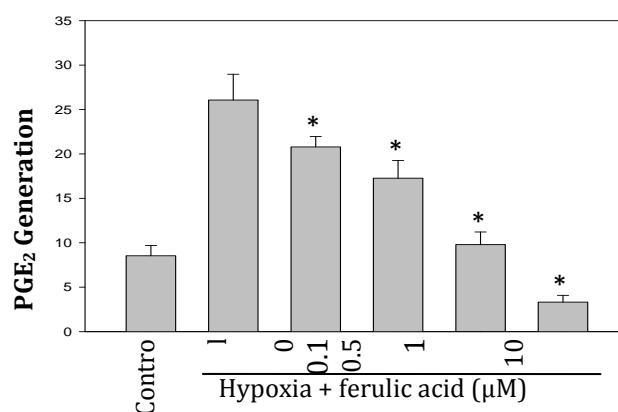


Figure 4. The effect of FA on PGE2 production in hypoxia-stressed PC12 cells. FA dose-dependently reduced hypoxia-induced PGE2 production in PC12 cells. PGE2 concentration was determined using an ELISA assay. * $P < 0.01$ as compared to the hypoxia control

hypoxic stress by 62% (Figure 4).

FA reduced apoptosis in hypoxic PC12 cells

Apoptosis is involved in ischemic neuronal damage. Chromatin condensation can be evaluated by TUNEL staining with a fluorescent microscope as an indicator of apoptotic cells. In the microscope field, fluorescent cells were counted per 100 cells PC12 cells after TUNEL staining. The numbers of apoptotic cells treated with FA 1 and 10 μ M were 33 ± 12 and 14 ± 6 , respectively relative to the hypoxic PC12 cells 76 ± 11 , and control 4 ± 3 . The number of TUNEL-stained PC12 cells was reduced significantly by FA after 1 hr of hypoxia. FA protected PC12 cells from hypoxia-induced apoptosis (Figure 5).

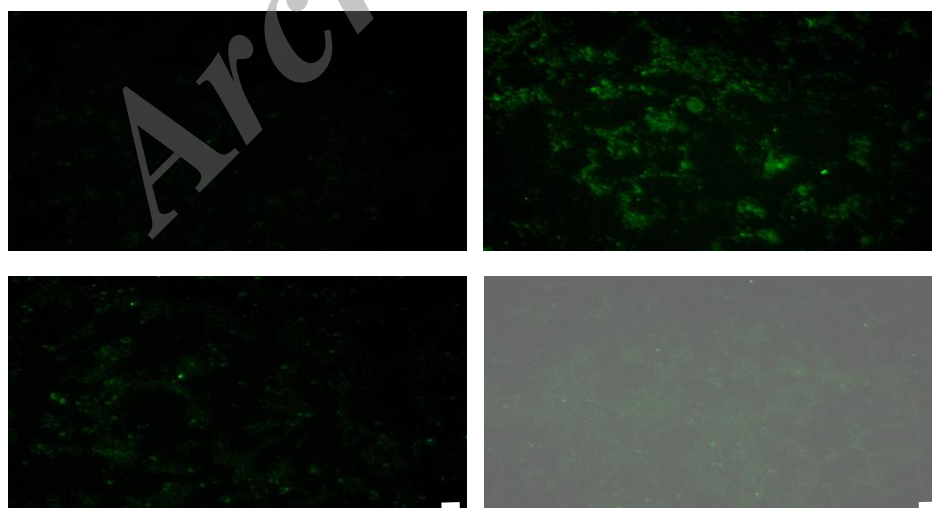


Figure 5. The effect of FA on apoptosis of hypoxic PC12 cells. FA significantly reduced cell apoptosis as measured using the TUNEL assay. (A) control. (B) 1 hr hypoxia. (C) 1 hr hypoxia with 1 μ M FA (D) 1 hr hypoxia with FA 10 μ M. The bar represents 50 μ M. (microscope with 200 \times field)

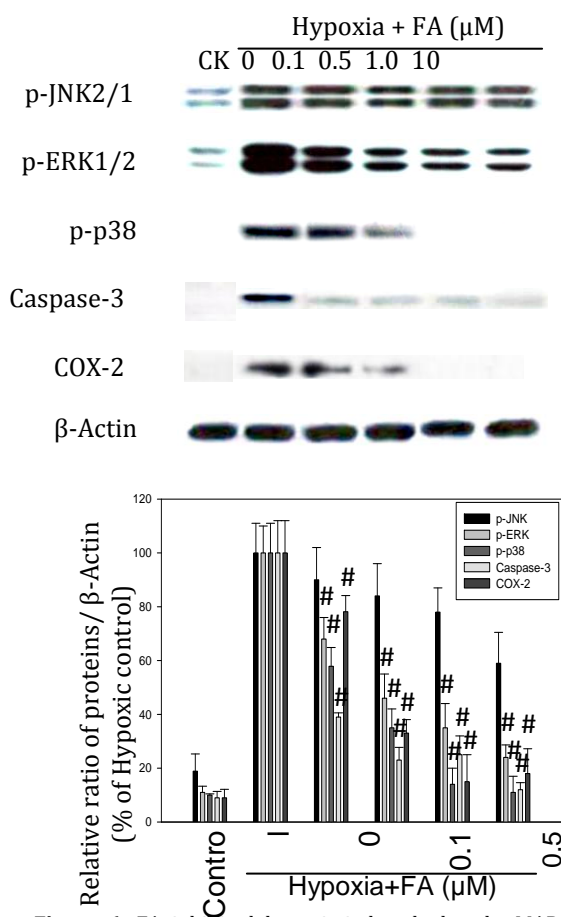


Figure 6. FA inhibited hypoxia-induced phospho-MAP kinases, caspase-3 and COX-2 expression in PC12 cells. Western blots showed that hypoxia increased phospho-MAP kinases, caspase-3 and COX-2 expression but FA (0.5, 1.0 10 μ M) decreased the hypoxia-induced phospho-ERK MAPK, phospho-p38, MAPK, caspase-3, and COX-2 expression of PC12 cells (A). Results of scanning densitometric analysis of the western blots are presented as value of proteins/beta-Actin. Values represent the mean of three independent experiments (lower panel, B). * $P < 0.05$, hypoxia alone vs hypoxia in the presence of FA

FA inhibited hypoxia-induced phospho-MAP kinases, caspase-3 and COX-2 expression of PC12 cells

Hypoxia-induced activation of phosphorylated MAPKs, caspase-3, and COX-2 cell signaling proteins were examined by means of Western blotting. Phosphorylated MAPKs, caspase-3 and COX-2 expression proteins raised as the duration of hypoxia increased. Following 10 min of hypoxia, FA 1 μ M reduced the expression ($P < 0.01$) of p-JNK ($22 \pm 10\%$), p-ERK ($65 \pm 8\%$), p-p38 ($85 \pm 7\%$) MAPKs, caspase-3 ($74 \pm 6\%$), and COX-2 ($84 \pm 9\%$), respectively relative to the hypoxic PC12 cells. However, after 30 and 60 min of hypoxia, the expression of none of the above mentioned proteins were suppressed by FA (data not shown). Western blot analysis showed that hypoxia increased phospho-MAP kinases, caspase-3 and COX-2 expression but FA (1.0 μ M) decreased hypoxia-induced phospho-ERK MAPK and COX-2 expression in PC12 cells (Figure 6).

Discussion

The main results of this study were the finding that FA significantly reduced hypoxia-induced cytotoxicity and cell damage, scavenged ROS production, increased cell viability and SOD activity, and attenuated the elevation of intracellular free Ca^{2+} in hypoxia-stressed PC12 cells. Furthermore, FA inhibited hypoxia-stimulated lipid peroxidation, PGE2 production and apoptosis in PC12 cells. Many published reports have shown that various antioxidants can inhibit the decrease of cell viability, scavenge ROS, increase antioxidative enzymes activity, and attenuate the elevation of intracellular free Ca^{2+} in oxygen-stressed neuronal cells (8, 14, 15). Excessive ROS production in the brain is widely believed to contribute to neurodegenerative processes (10, 16, 17) and antioxidants that inhibit the ROS production may have neuroprotective implication (8, 14, 17). LDH release was increased in neuronal cells subjected to hypoxia (18). We observed that FA protected the hypoxia-induced PC12 from cell death and significantly reduced Ca^{2+} release following hypoxia. Exposure of PC12 cells to hypoxia increased the generation of ROS in culture supernatants (Figure 2A). FA was able to decrease hypoxia-induced ROS generation and attenuate the generation of malondialdehyde which is the product of increased lipid peroxidation in PC12 cells (Figure 2B). Hypoxia reduces SOD activity in PC12 cells whereas our data showed that FA preserved SOD activities in PC12 cells subjected to hypoxia (Figure 3).

The inhibition of hypoxia-induced MAPKs and caspase-3 expression by FA correlated well with diminished LDH release due to hypoxia (Figure 1B and 6) suggesting that FA exerted its protective effects directly and indirectly through ROS scavenging. ROS levels have been found to increase markedly following hypoxia and ROS generation has been proposed to play a critical role in hypoxia-induced neuronal death (4). ROS damage the lipids, DNA, and proteins, and induce production of inflammatory proteins that contribute to the neuronal death (13, 14, 19). In brain ischemia, free radical scavengers can reduce brain damage (4). Antioxidants increase intracellular glutathione, lower ROS, and prevent calcium release in neuronal cells (8, 18). We found that FA diminished ROS generation under hypoxia and this might affect cellular signaling pathways. Neuronal cells are protected by antioxidants that scavenge peroxyl radicals and hydroxyl radicals (8, 17). Our results show that FA can scavenge ROS and protect PC12 cell from hypoxia-induced cell death. In addition, it appears that FA also preserves SOD activity in these cells when they are subjected to hypoxia. SOD is an important enzyme to eliminate free radicals and protect the cerebral tissues from ischemic injury (17) and global ischemia (20). Oxidative stress which is induced following CNS injury causes neural cell death through extrinsic and intrinsic pathways and cell death occurs by ROS-induced oxidative insult through a

caspase-3-independent apoptotic mechanism (21). We demonstrated that FA significantly reduced cell apoptosis (Figure 5). Following hypoxic injury, PC12 cells exhibited increased apoptosis and FA dose-dependently reduced apoptotic activity. At the same time, FA scavenged ROS, attenuated decreases of superoxide dismutase activity and decreased DNA damage in hypoxia-induced PC12 cells (Figure 5).

The present study showed that hypoxia activated MAPK signaling pathways by increasing phospho-JNK, ERK, p38 MAPKs, caspase-3 and COX-2 in PC12 cells. FA inhibited MAPKs cascades by inhibiting ERK and COX-2 expression in PC12 cells after 10 min hypoxia. These observations are consistent with those reported by other investigators who stated that hypoxia resulted in the activation of MAPKs in neuronal cells (8, 22) and that inhibition of the phospho-JNK, ERK, or p38 MAPK pathways reduces acute ischemic injury (8, 23). Other investigations have shown that inhibition of apoptotic markers (MAPKs and caspase-3) reduces hypoxia-induced neuron death (24). Therefore, the effects of FA might be mediated through repression of MAPK or caspase-3 activation. Hypoxia-induced PC12 cell death can be partially prevented by FA. The effect of FA on PC12 cell death was probably via attenuation of ROS generation during hypoxia and the concomitant down-regulation of MAPKs and caspase-3 expression. Regulation of ROS generation, MAPK cascades, and apoptosis by FA might lead to protection of neuronal cells from hypoxic insults.

In the present study, FA significantly protected PC12 cells from hypoxia damage. The results showed that following 1 hr hypoxia, while LDH release (Figure 1) and ROS levels (Figure 2) significantly increased, MTT and MDA generation showed a significant decrease, suggesting the role of ROS generation in hypoxia-induced cell death. The results suggest that the protective mechanism of FA in hypoxic neuronal cells might be through the suppression of ROS generation. In cultured neurons, increased ROS production under conditions such as hypoxia, induced neuronal cell death with morphological and biochemical characteristics of apoptosis. In addition, down-regulation of SOD or mutant SOD causes neuronal PC12 cell death and addition of SOD inhibits PC12 cell death. Therefore, the effect of FA might be due to its ROS-scavenging properties that spared SOD in hypoxia-stressed PC12 cells. Based on morphological and biochemical evidence, however, recent studies suggest that apoptosis is involved in ischemic neuronal damage. One major apoptotic pathway involves caspase-3 activation. In addition, chromatin condensation can be evaluated by TUNEL staining with a fluorescent microscope as an indicator of apoptotic cells. The number of TUNEL-stained cells was significantly reduced by FA in PC12 cells after 1 hr hypoxia

(Figure 5). The present study also showed that hypoxia activated MAPKs, increasing immunoactivity of MAP kinase, caspase-3, and COX-2 (Figure 6). FA inhibited hypoxia-induced MAPKs (mainly ERK, p38 and lightly JNK), caspase-3, and COX-2 expression in PC12 cells after 10 min hypoxia. FA prevented phosphorylation and activation of MAPKs, caspase-3, and COX-2, and reduced the level of LDH release after hypoxia. The results suggest that MAPKs activation happens in hypoxia-exposed PC12 cells.

Recently, studies have shown that cyclooxygenase-2 (COX-2) induces neuronal oxidative stress by mechanisms involving free radicals. Although oxygen radicals are not directly involved in the COX-2-catalytic cycle, superoxide anion radicals have been shown to play a critical role in COX-2-associated oxidative stress (25). Furthermore, the potent neuroprotective effect of antioxidants was found to be due to inhibition of COX-2 expression and PGE₂ production (26). This observation is in consistency with our findings that hypoxia activated COX-2 signaling pathways and increased PGE₂ production in PC12 cells. FA potentially inhibited COX-2 cascades, attenuated PGE₂ generation and prevented PC12 cells from hypoxic damage.

Conclusion

The present data showed that FA could reduce the oxidative stress induced by hypoxia in PC12 cell. The neuroprotective action of FA may be mediated by a reduction of oxidative stress due to its ROS scavenging properties. These observations expand our knowledge of the role of FA and its therapeutic potential in neuroprotection and warrants further molecular and mechanistic studies.

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Conflict of interest

The authors have no financial conflict of interest. The results described in this paper were part of student thesis.

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