Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 467 (2003) 41–47
www.elsevier.com/locate/ejphar

Iron-induced oxidative damage and apoptosis in cerebellar granule cells: attenuation by tetramethylpyrazine and ferulic acid

Zhaohui Zhang[a], Taotao Wei[b,*], Jingwu Hou[b], Gengshan Li[a], Shaozu Yu[a], Wenjuan Xin[b]

[a] *Department of Neurology, Renmin Hospital, Wuhan University, Wuhan 430060, PR China*
[b] *Center for Molecular Biology, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, PR China*

Received 4 January 2003; received in revised form 4 March 2003; accepted 7 March 2003

Abstract

Tetramethylpyrazine and ferulic acid are two active ingredients of a Chinese herbal medicine *Ligusticum wallichi Franchat*. In the present investigation, iron-induced oxidative neuronal damage and the protective effects of tetramethylpyrazine and ferulic acid against this induction were studied in primary cultures of rat cerebellar granule cells. When neurons were treated with 200 μM of $FeSO_4$ for 1 h, lipid peroxidation in neurons increased time dependently, as measured with the thiobarbituric acid assay. Thirty-six hours after iron treatment, the cell viability decreased to 43.6% and the percentage of apoptotic cells increased to 50.6%. Transmission electron microscopic examination showed a disrupted nuclear envelope and condensed chromatin in iron-treated neurons. Analysis of DNA extracted from iron-treated cells by agarose gel electrophoresis showed the typical ''ladder pattern'', which indicated the formation of mono- and oligonucleosomes. After iron treatment, caspase 3 activity increased significantly, as measured in a fluoregenic assay. The results above suggested that iron treatment triggered oxidative stress and apoptosis in neurons. Western blot revealed that iron treatment up-regulated the apoptosis-related gene p53 as well as its effector gene $p21^{waf1/cip1}$. Pretreatment of the cells with 100 μM of tetramethylpyrazine or ferulic acid effectively decreased the activation of caspase 3 as well as the expression of p53 and $p21^{waf1/cip1}$, and attenuated iron-induced oxidative damage and apoptosis. The results suggest that tetramethylpyrazine and ferulic acid might be used as preventive agents against neuronal diseases associated with oxidative stress.

Keywords: Cerebellar granule cell; Fe ion; Apoptosis; Tetramethylpyrazine; Ferulic acid

1. Introduction

Iron is the most abundant transition metal in the brain and is the key element of enzymes that regulate cellular energy metabolism (Beard et al., 1993). Iron also plays a central role in myelin formation. In the brain of neonatal rats, the uptake of iron is at its highest during the period of rapid brain growth (Taylor and Morgan, 1990; Roskams and Connor, 1994). Iron is vital in life because it is an important component of molecules that undergoes redox reactions in cells. However, this property also makes iron potentially toxic, since redox reactions may generate reactive oxygen species. In fact, reactive oxygen species are known to damage almost all cellular components including proteins, lipids and nucleic acids. Recently, it has been proposed that a disturbed iron metabolism may cause certain neurodegenerative disorders such as Parkinson's, Alzheimer's, Huntington's, Hallervorden-Spatz's and Friedreich's diseases, as well as stroke and epilepsy (Shoham and Youdim, 2000), while oxidative stress induced by iron play an important role in the pathogenesis of iron-related diseases (De Freitas and Meneghini, 2001). Thus effective antioxidants that can attenuate iron-induced oxidative damage might be potential neuroprotectors against these disorders.

Apoptosis is a certain type of cell death in multicellular organisms and involves a cascade of closely regulated intracellular events leading to cell suicide. In the last decade, more and more evidence has suggested that reactive oxygen species play an important role in the neuronal apoptosis associated with neuronal diseases (Jacobson, 1996). In this paper, primary culture of rat cerebellar granule cells was used as the experimental model. The cytotoxic effect of oxidative stress induced by iron and the protective effect of tetramethylpyrazine and ferulic acid, two active ingredients of a Chinese herbal medicine named Chuan Xiong (*Ligu-*

* Corresponding author. Tel.: +86-10-6488-8576; fax: +86-10-6487-1293.
E-mail address: weitt@moon.ibp.ac.cn (T. Wei).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01597-8

usticum wallichi Franchat), on neuronal cells were examined. The results will help us to understand the process of iron-induced neuronal apoptosis and neurodegeneration, which will contribute to the search for selective methods to control them.

2. Materials and methods

2.1. Materials

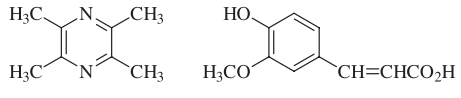
Seven-day-old Wistar rats were purchased from Beijing Experimental Animal Center. Cell culture plastic ware was purchased from Corning Costar. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, cell culture supplements, proteinase K, RNase A, and trypsin (1:250) were products of Invitrogen. Tetramethylpyrazine was a generous gift from Beijing Fourth Pharmaceutical Factory and ferulic acid was a generous gift from Chendu First Pharmaceutical Factory, the structures of which are shown in Fig. 1. Monoclonal antibodies against p53 or $p21^{waf1/cip1}$ and the caspase 3 substrate acetyl-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) were purchased from Pharmingen. Other reagents were purchased from Sigma.

2.2. Cell culture

Primary cultures of rat cerebellar granule cells were prepared from 7-day-old Wistar rats following procedures described previously (Resink et al., 1994). By this method, a relatively homogenous and well-characterized culture system of cerebellar neurons can be obtained (Dudek et al., 1997). Briefly, cerebella were dissected out, rinsed with Hanks' balanced salt solution (HBSS), and dissociated by mild trysinization. Cells were plated on 6-well multidishes (2×10^6 cells/ml, 2 ml/well) or 96-well multidishes (1.0×10^6 cells/ml, 0.1 ml/well) previously coated with poly-L-lysine. Culture medium consisted of DMEM supplemented with KCl (19.6 mM), glutamine (2 mM), HEPES (10 mM) and fetal bovine serum (10%, v/v). Cells were maintained at 37 °C in a humidified 5% CO_2–95% air atmosphere. Experiments were carried out 48 h after plating.

2.3. Treating cells with iron

Two hundred micromolar $FeSO_4$ was added to cells from freshly prepared stock solutions and cells were cultured for 1 h. Then the medium was removed, fresh medium was added, and cells were cultured for the indicated time. The concentration of $FeSO_4$ (200 μM) was chosen after preliminary experiments. Lower concentrations could not induce notable oxidative stress in neurons, whilst higher concentrations caused necrotic rather than apoptotic cell death. The antioxidants tetramethylpyrazine or ferulic acid were added to cells from freshly prepared stock solutions 15 min before the treatment with $FeSO_4$.



Tetramethylpyrazine Ferulic Acid

Fig. 1. Structure of tetramethylpyrazine and ferulic acid.

2.4. Assessment of cell injury

Cell viability was assessed with the thiazolyl blue assay (Wei et al., 1998). Thiazolyl blue (0.5 mg/ml final concentration) was added to cells cultured in 96-well multidishes and incubated at 37 °C for 30 min. Then 100 μl of lysis solution [10% sodium dodecyl sulfate (SDS), 25% *N*,*N*-dimethylformamide, pH 3.5] was added to each well and the optical density at 570 nm was measured by a microplate reader.

2.5. Measurement of lipid peroxidation

Lipid peroxidation in cells was measured with the thiobarbituric acid assay as described previously (Salgo and Pryor, 1996) with minor modifications. Briefly, 1.2×10^7 cells were pelletted, rinsed with cold phosphate-buffered saline (PBS), mixed with 0.4 ml of 2.8% trichloroacetic acid, 1 ml of 0.67% thiobarbituric acid, 0.1 ml of 0.3% butylated hydroxytoluene and then heated at 95 °C for 1 h. After extraction with 1 ml of *n*-butanol, the optical density of the organic layer was determined spectrophotometrically. The thiobarbituric acid-reactive substrates were quantified using 1,1,3,3-tetraethoxypropane as the standard. The protein concentration was determined by the Bradford method.

2.6. Morphological study

The ultrastructure of cells was observed by transmission electron microscopy (Watt et al., 1994). Briefly, cells were pelletted and fixed with 2.5% glutaraldehyde at 4 °C for 1 h and post-fixed with 1% OsO_4 at 4 °C for 1 h, dehydrated through a series of graded ethanol solutions, and embedded in resin. Ultrathin sections of samples were stained with uranyl acetate/lead citrate and then observed with a transmission electron microscope.

2.7. Detection of DNA fragmentation

The "ladder pattern" of DNA fragmentation was detected by agarose gel electrophoresis (Didier et al., 1996). Briefly, 1.2×10^7 cells were pelletted and lysed in buffer [10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, pH 7.5] by incubation at 4 °C for 15 min. After centrifugation at $12{,}000 \times g$ for 10 min, the supernatant (containing fragmented DNA) was treated with 0.5 mg/ml Proteinase K and 0.1 mg/ml RNase A at 50 °C for 3 h.

After phenol extraction and chloroform extraction, DNA was precipitated with 0.1 volume of 10 M ammonium acetate and 2 volume of ethanol at −20 °C overnight, and harvested by centrifugation at 12,000 × *g* for 15 min. After being washed with 70% ethanol, DNA was dissolved in Tris–EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 8.0) and subjected to electrophoresis in 1.5% agarose gel. After staining with ethidium bromide (0.5 μg/ml), DNA bands were visualized by UV transillumination.

2.8. Analysis of apoptosis by flow cytometry

Flow cytometry analysis of DNA was performed in order to evaluate the percentage of apoptotic cells, whose DNA content was lower than that of diploid cells. As generally accepted, apoptotic cells can be recognized by their diminished stainability with DNA-specific fluorochromes, which is due to DNA degradation and subsequent leakage from the cell. The method for DNA labeling was performed as previously described (Nicoletti et al., 1991) with minor modification. Briefly, 2×10^6 cells were prepared as a single cell suspension in 500 μl PBS and fixed with 2 ml of ice-cold 70% ethanol at 4 °C overnight. Then cells were harvested after being centrifuged at 200 × *g* for 10 min, resuspended in 500 μl PBS supplemented with RNase A (100 μg/ml), incubated at 37 °C for 30 min, and stained with 50 μg/ml propidium iodide at 4 °C for 30 min. The red fluorescence of individual cells was measured with FACScan (Becton Dickinson, Mountain View, CA) and the number of apoptotic cells was counted by software provided by the manufacturer.

2.9. Western blot

In neurons, p53 has been associated with apoptotic cell death following ischemia, excitotoxicity and irradiation. One of its effector genes is $p21^{waf1/cip1}$, which is expressed in p53-dependent apoptosis. Generally, p53 is activated in response to cellular stress, most notably DNA damage (Kastan et al., 1991). Because p53 and $p21^{waf1/cip1}$ genes are expressed during the early stage of apoptosis, the expression of p53 and $p21^{waf1/cip1}$ was determined by Western blot 12 h rather than 36 h after iron treatment. Briefly, 1.2×10^7 cells were lysed with 500 μl of lysis buffer [1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethyleneglyco-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′ -tetraacetic acid (EGTA), 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin] at 4 °C for 30 min. After centrifugation at 12,000 × *g* for 15 min, the supernatant was collected and protein concentrations were determined by the Bradford method. Each sample containing 60 μg of cellular protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-p53 antibody (1:2500 dilution), anti-$p21^{waf1/cip1}$ antibody (1:5000 dilution) or anti-β-Actin antibody (1:2500 dilution), respectively, and then incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:5000 dilution). The target protein was detected by chemiluminescence using ECL Plus Western blotting detection kit (Amersham Pharmacia) and exposed to Kodak X-ray autoradiography films.

2.10. Measurement of caspase 3-like activity

The activity of caspase 3, the main execution caspase, was measured by fluoregenic assay according to methods described previously using acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (DEVD-MCA) as the specific substrate (Chen et al., 1998; Dare et al., 2002). Protein extracts were prepared from 1.2×10^7 cells by Dounce homogenization on ice. Homogenization buffer contained 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) pH 7.5, 5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 10 μg/ml each of pepstatin, leupeptin, aprotinin and phenylmethanesulfonyl fluoride. After centrifugation at 12,000 × *g* for 15 min, the supernatants were collected and protein concentrations were determined by the Bradford method. Volumes of protein extracts containing 100 μg of protein were incubated for 1 h at 37 °C with the reaction buffer (25 mM HEPES pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonil]-1-propane sulfonate, 5 mM dithiothreitol, and 5 mM EDTA) in a total volume of 150 μl containing 25 μM of the substrate DEVD-MCA. Enzyme-catalyzed release of 4-methylcoumaryl-7-amide was measured by a fluorescence microplate reader at excitation 355 nm and emission 460 nm. Fluorescent units were converted to pmol of 4-methyl-

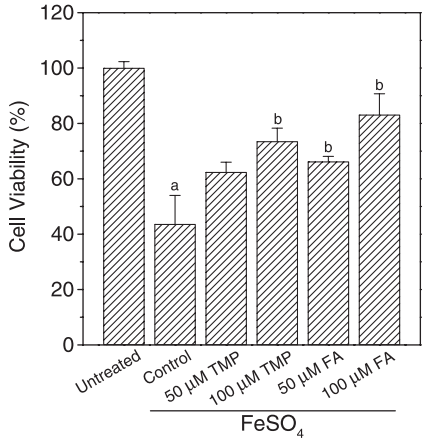


Fig. 2. Iron-induced cell death in cerebellar granule cells and the protective effect of tetramethylpyrazine and ferulic acid. Cells were treated with 200 μM of $FeSO_4$ for 1 h and cultured in fresh medium for 36 h. Then the viability of cells was measured with the thiazolyl blue assay. The antioxidant tetramethylpyrazine or ferulic acid (50 or 100 μM) was added to cells 15 min before the treatment with $FeSO_4$. Data are means ± S.D. of 12 samples. $^aP<0.05$ in comparison with untreated cells; $^bP<0.05$ in comparison with cells treated with $FeSO_4$.

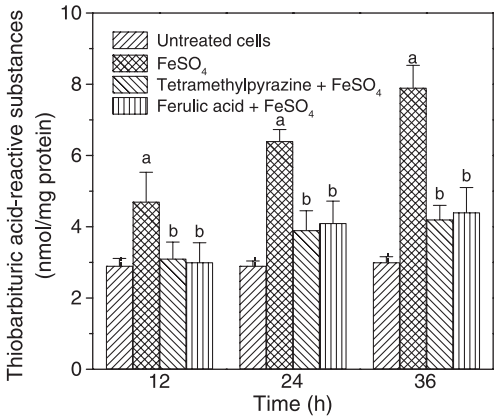


Fig. 3. Lipid peroxidation in cells. Cells were treated with 200 μM of $FeSO_4$ for 1 h and cultured in fresh medium for the indicated time. Lipid peroxidation in cells was measured with the thiobarbituric acid assay. The antioxidant tetramethylpyrazine or ferulic acid (100 μM) was added to cells 15 min before the treatment with $FeSO_4$. Data are means ± S.D. of four samples. $^{a}P<0.05$ in comparison with untreated cells; $^{b}P<0.05$ in comparison with cells treated with $FeSO_4$.

coumaryl-7-amide released using a standard curve generated with 4-methylcoumaryl-7-amide. One unit of caspase 3-like activity was defined as releasing 1 pmol of 4-methylcoumaryl-7-amide per min at 37 °C at saturating substrate concentrations.

2.11. Statistical analysis

Each experiment was performed at least three times and the results are presented as means ± S.D. The data were analyzed by one-way analysis of variance (ANOVA). A level of $P<0.05$ was considered significant.

3. Results

3.1. Iron-induced cell death

Iron treatment induced cell death in cultures of cerebellar granule cells, as measured by the decrease in thiazolyl blue reduction (Fig. 2). In cells treated with 200 μM $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h, cell viability decreased to 43.6%. Pre-treatment with tetramethylpyrazine or ferulic acid dose dependently attenuated cell death induced by iron. Thirty-six hours after iron treatment, the viability of cells pretreated with 100 μM of tetramethylpyrazine or ferulic acid was 76.5% and 83.1%, respectively.

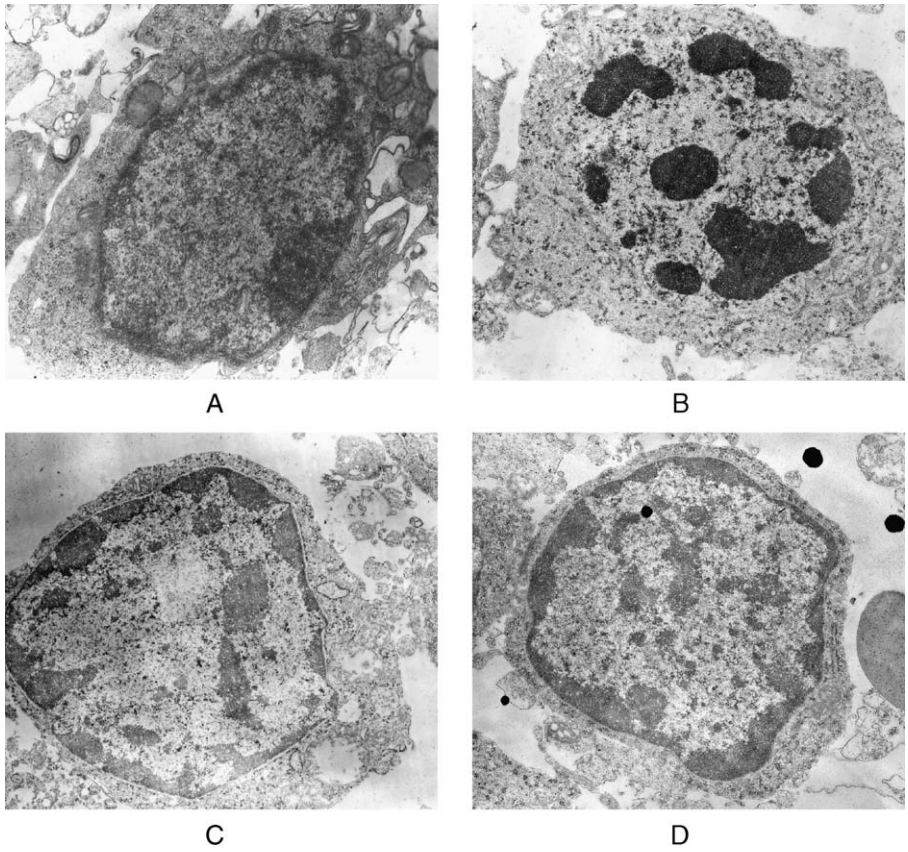


Fig. 4. Ultrastructure of the nucleus of a cerebellar granule cell. Cerebellar granule cells were exposed to 200 μM of $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h. The antioxidant tetramethylpyrazine or ferulic acid (100 μM) was added to cells 15 min before the treatment with $FeSO_4$. (A) Untreated cell; (B) cell treated with 200 μM of $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h; (C) cell pretreated with 100 μM of tetramethylpyrazine before exposure to $FeSO_4$; (D) cell pretreated with 100 μM of ferulic acid before exposure to $FeSO_4$. Magnification: 6000 ×.

3.2. Lipid peroxidation in cells

Exposure to 200 μM $FeSO_4$ induced time-dependent lipid peroxidation in cerebellar granule cells, as indicated by the significant increase in thiobarbituric acid-reactive substrate levels (Fig. 3). In cells exposed to 200 μM of $FeSO_4$ for 1 h and cultured in fresh medium for 36 h, the thiobarbituric acid-reactive substrate level was significantly higher than that of untreated cells (7.9 versus 3.0 nmol/mg protein; $P<0.05$ compared with untreated cells). In cells pretreated with tetramethylpyrazine or ferulic acid, the formation of thiobarbituric acid-reactive substrate was markedly suppressed ($P<0.05$ compared with iron-treated cells). Thirty-six hours after iron treatment, the thiobarbituric acid-reactive substrate level of tetramethylpyrazine- or ferulic acid-pretreated cells was 4.2 and 4.4 nmol/mg protein, respectively.

3.3. Characteristics of apoptosis

After exposure to 200 μM $FeSO_4$ for 1 h, cerebellar granule cells underwent apoptosis gradually, which was characterized morphologically by chromatin condensation and nuclei fragmentation, as shown in Fig. 4B. Agarose gel electrophoresis of DNA extracted from iron-treated cells showed a ladder pattern (Fig. 5), which indicated the formation of mono- and oligonucleosomes. The formation of mono- and olignucleosomes is a well-accepted biochemical characteristic of apoptosis. No apparent morphological alterations occurred in cells pre-treated with tetramethylpyrazine or ferulic acid (Fig. 4C,D). Pretreatment of cells with tetramethylpyrazine or ferulic acid also prevented DNA from fragmentation.

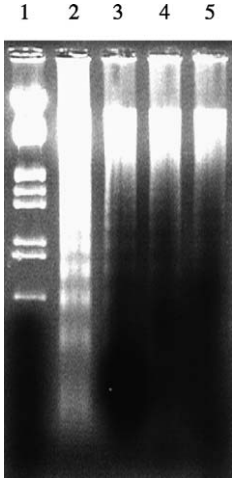


Fig. 5. Agarose gel electrophoresis analysis of DNA fragmentation. Cerebellar granule cells were exposed to 200 μM of $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h. Lane 1, DNA marker (λDNA/*Eco*RI + *Hin*dIII); Lane 2, cells exposed to 200 μM of $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h; Lane 3 and 4, cells pretreated with 100 μM of tetramethylpyrazine or ferulic acid, respectively, and then exposed to $FeSO_4$; Lane 5, untreated cells.

Table 1
Quantitative determination of apoptosis by flow cytometry

Group	Untreated cells	Control	Tetramethylpyrazine	Ferulic acid
Apoptotic cells (%)	3.4 ± 3.3	50.6 ± 7.4[a]	13.0 ± 2.8[b]	9.9 ± 4.6[b]

Data are means ± S.D. of four samples.
[a] $P<0.05$ in comparison with untreated cells.
[b] $P<0.05$ in comparison with cells treated with $FeSO_4$.

3.4. Quantitative measurement of apoptosis

The percentages of normal (diploid DNA content) and apoptotic cells (subdiploid DNA content) were calculated according to the DNA content and are shown in Table 1. In untreated cells, fewer than 3.4% of total cells underwent apoptosis. In control cells treated with 200 μM $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 36 h, about 50.6% cells underwent apoptosis. When cells were pretreated with tetramethylpyrazine or ferulic acid and then exposed to $FeSO_4$, the percentage of

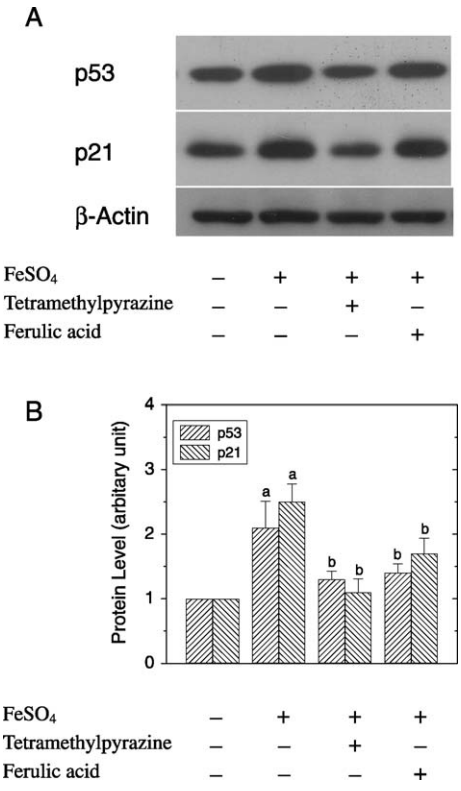


Fig. 6. Western blot analysis of p53 and $p21^{waf1/cip1}$ expression in cerebellar granule cells. After exposure to 200 μM of $FeSO_4$ for 1 h and subsequent culture in fresh medium for 12 h, the expression of p53 and $p21^{waf1/cip1}$ was analyzed by Western blot. The antioxidant tetramethylpyrazine or ferulic acid (100 μM) was added to cells 15 min before the treatment with $FeSO_4$. (A) Typical results of Western blot. (B) Quantitative analysis of the expression of p53 and $p21^{waf1/cip1}$. Data are means ± S.D. of three samples. [a]$P<0.05$ in comparison with untreated cells; [b]$P<0.05$ in comparison with cells treated with $FeSO_4$.

apoptotic cells was 13.0% and 9.9%, respectively, which was significantly lower than that of control cells ($P<0.01$).

3.5. Activation of caspase 3

Activation of caspases is a typical feature of apoptosis. To confirm the activation of caspases in iron-treated cerebellar granule cells, the caspase 3-like activity was determined. The results revealed that iron treatment induced a significant increase in caspase 3-like activity, as shown in Fig. 7. In cells treated with 200 μM $FeSO_4$ for 1 h and subsequently cultured in fresh medium for 24 h, the caspase 3-like activity was 16.5 units/mg protein, which was more than 10-fold higher than that of untreated cells (1.2 ± 0.2 units/mg protein; $P<0.01$). Pretreatment with tetramethylpyrazine or ferulic acid significantly lowered the caspase 3-like activity in comparison with that in control cells ($P<0.01$).

3.6. Expression of p53 and $p21^{waf1/cip1}$ genes

Fig. 6(A) shows the typical result of the expression of p53 and $p21^{waf1/cip1}$ measured by Western blot. Iron treatment up-regulated the expression of p53 as well as its effector gene $p21^{waf1/cip1}$ in cerebellar neurons. Quantitative analysis revealed that in iron-treated cells, the p53 protein level increased to 2.1-fold that of untreated cells, while the $p21^{waf1/cip1}$ protein level increased to 2.5-fold that of untreated cells (Fig. 6(B)). Both tetramethylpyrazine and ferulic acid inhibited the up-regulation of p53 and $p21^{waf1/cip1}$ genes significantly.

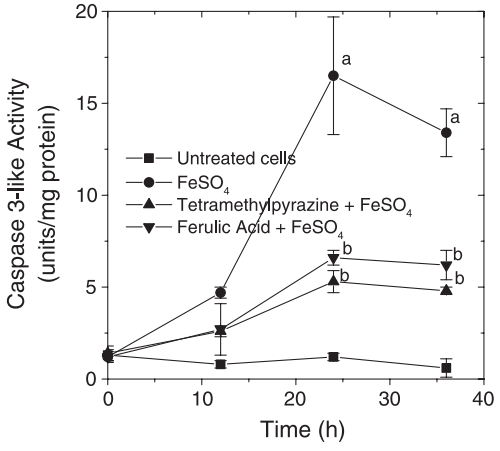


Fig. 7. Fluoregenic determination of caspase 3 activation in cerebellar granule cells. After exposure to 200 μM of $FeSO_4$ for 1 h and subsequent culture in fresh medium for the indicated time, caspase 3-like activity was determined by fluoregenic methods using DEVD-MCA as the specific substrate. The antioxidant tetramethylpyrazine or ferulic acid (100 μM) was added to cells 15 min before the treatment with $FeSO_4$. Data are means ± S.D. of eight samples. $^aP<0.05$ in comparison with untreated cells; $^bP<0.05$ in comparison with cells treated with $FeSO_4$.

4. Discussion

Under certain pathological conditions when the antioxidant capacity of the tissue is surpassed, the over-generation of free radicals may lead to apoptosis. Antioxidant agents suppress apoptosis induced by a variety of stimuli (Stoian et al., 1996). Studies have shown that down-regulation of superoxide dismutase leads to apoptosis (Troy et al., 1996) while the up-regulation of superoxide dismutase delays apoptosis in neuronal cells (Greenlund et al., 1995). Accordingly, it is possible that apoptosis can be regulated by modulation of the cellular redox status.

In this paper, iron-induced oxidative stress and apoptosis in cultured cerebellar neurons was used as a model for iron- and reactive oxygen species-related neuronal disorders. The protective effect of tetramethylpyrazine and ferulic acid, two active ingredients of a Chinese herbal medicine named Chuan Xiong (*L. wallichi Franchat*), on neuronal cells was also examined. It is well documented that iron can induce the generation of free radicals, which attack important biomolecules including proteins, deoxynucleic acids, and lipid membranes and thus cause oxidative stress. After exposure to $FeSO_4$, the lipid peroxidation level in cerebellar granule cells increased time dependently. Lipid peroxidation may alter the fluidity of the cell membrane and damage receptors and ion channels on the cell membrane, which may cause more serious consequences including calcium influx, and may ultimately result in cell death (Xin et al., 2000; Mattson, 1998). In the present investigation, the cytotoxic effects of iron on neurons were evaluated by different methods. In cerebellar granule cells exposed to 200 μM of $FeSO_4$, morphological alterations that characterized apoptosis could be observed. DNA underwent internucleosomal cleavage after iron treatment, a well-accepted criterion of apoptosis, as shown by agarose gel electrophoresis. However, transition metal ions may induce DNA internucleosomal fragmentation directly (Tsang et al., 1996). To further confirm whether iron treatment triggered apoptosis in neuronal cells, we measured the activation of caspases and analyzed the expression of apoptosis-related p53 and $p21^{waf1/cip1}$ genes. Measurement of cytosolic caspase 3-like activity revealed the activation of caspase 3 after iron treatment in cerebellar granule cells. Iron treatment also significantly up-regulated the p53 gene as well as the $p21^{waf1/cip1}$ gene. Upon exposure to iron, the expression of p53 increased to 2.1-fold that of untreated cells, while $p21^{waf1/cip1}$ protein level increased to 2.5-fold that of untreated cells. This might be due to the DNA damage induced by iron. The results above suggested that iron-induced neuronal apoptosis is mediated by p53-related pathways.

In cerebellar granule cells pretreated with 100 μM of tetramethylpyrazine or ferulic acid, iron-induced activation of caspase 3 and the expression of p53 and $p21^{waf1/cip1}$ were suppressed significantly, and apoptosis was attenuated effectively. It has been reported that tetramethylpyrazine, an

alkaloid, exhibits strong scavenging effects on cytotoxic oxygen radicals (superoxide anion, lipid peroxyl radical and hydroxyl radical) and is almost as effective as vitamin E (Zhang et al., 1994). Ferulic acid, as other polyphenol compounds, is also a potent scavenger of oxygen radicals, as reported previously (Ju et al., 1990). Despite the direct scavenging of reactive oxygen species, both tetramethylpyrazine and ferulic acid can chelate the ferrous ion and decrease the formation of hydroxyl radicals via inhibition of the iron-dependent Fenton reaction. The protective effect of tetramethylpyrazine and ferulic acid against apoptosis may be the overall effect of both scavenging of reactive oxygen species directly and chelation of iron to inhibit the Fenton reaction indirectly.

In conclusion, both tetramethylpyrazine and ferulic acid effectively prevented oxidative stress and apoptosis induced by iron, suggesting that tetramethylpyrazine and ferulic acid may be used as potential protective agents for diseases associated with disturbed iron metabolism.

Acknowledgements

This work is supported by a grant from the National Natural Science Foundation of China (No. 30000033).

References

Beard, J.L., Connor, J.R., Jones, B.C., 1993. Iron in the brain. Nutr. Rev. 51, 157–170.

Chen, J., Nagayama, T., Jin, K., Stetler, R.A., Zhu, R.L., Graham, S.H., Simon, R.P., 1998. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J. Neurosci. 18, 4914–4928.

Dare, E., Tofighi, R., Vettori, M.V., Momoi, T., Poli, D., Saido, T.C., Mutti, A., Ceccatelli, S., 2002. Styrene 7,8-oxide induces caspase activation and regular DNA fragmentation in neuronal cells. Brain Res. 933, 12–22.

De Freitas, J.M., Meneghini, R., 2001. Iron and its sensitive balance in the cell. Mutat. Res. 475, 153–159.

Didier, M., Bursztajn, S., Adamec, E., Passani, L., Nixon, R.A., Coyle, J.T., Wei, J.Y., Berman, S.A., 1996. DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures. J. Neurosci. 16, 2238–2250.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., Greenberg, M.E., 1997. Regulation of neuronal survival by the serine–threonine protein kinase Akt. Science 275, 661–665.

Greenlund, L.J., Deckwerth, T.L., Johnson Jr., E.M., 1995. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron 14, 303–315.

Jacobson, M.D., 1996. Reactive oxygen species and programmed cell death. Trends Biochem. Sci. 21, 83–86.

Ju, H.S., Li, X.J., Zhao, B.L., Hou, J.W., Han, Z.W., Xin, W.J., 1990. Scavenging effects of sodium ferulate and 18β-glycyrrhetic acid on oxygen free radicals. Acta Pharmacol. Sin. 11, 466–470.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., Craig, R.W., 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51, 6304–6311.

Mattson, M.P., 1998. Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. Trends Neurosci. 21, 53–57.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., Riccardi, C., 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods 139, 271–279.

Resink, A., Hack, N., Boer, G.J., Balazs, R., 1994. Growth conditions differentially modulate the vulnerability of developing cerebellar granule cells to excitatory amino acids. Brain Res. 655, 222–232.

Roskams, J.A., Connor, J.R., 1994. Iron, transferrin and ferrin in the rat brain during development and aging. J. Neurochem. 63, 709–716.

Salgo, M.G., Pryor, W.A., 1996. Trolox inhibits peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes. Arch. Biochem. Biophys. 333, 482–488.

Shoham, S., Youdim, M.B., 2000. Iron involvement in neural damage and microgliosis in models of neurodegenerative diseases. Cell. Mol. Biol. 46, 743–760.

Stoian, I., Oros, A., Moldoveanu, E., 1996. Apoptosis and free radicals. Biochem. Mol. Med. 59, 93–97.

Taylor, E.M., Morgan, E.H., 1990. Developmental changes in transferrin and iron uptake by the brain in the rat. Dev. Brain Res. 55, 35–42.

Troy, C.M., Derossi, D., Prochiantz, A., Greene, L.A., Shelanski, M.L., 1996. Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide–peroxynitrite pathway. J. Neurosci. 16, 253–261.

Tsang, S.Y., Tam, S.C., Bremner, I., Burkitt, M.J., 1996. Copper-1,10-phenanthroline induces internucleosomal DNA fragmentation in HepG2 cells, resulting from direct oxidation by the hydroxyl radical. Biochem. J. 317, 13–16.

Watt, J.A., Pike, C.J., Walencewicz-Wasserman, A.J., Cotman, C.W., 1994. Ultrastructural analysis of β-amyloid-induced apoptosis in cultured hippocampal neurons. Brain Res. 661, 147–156.

Wei, T.T., Chen, C., Zhao, B.L., Xin, W.J., Mori, A., 1998. EPC-K1 attenuates peroxynitrite-induced apoptosis in cerebellar granule cells. Biochem. Mol. Biol. Int. 46, 89–97.

Xin, W.J., Wei, T.T., Chen, C., Ni, Y.C., Zhao, B.L., Hou, J.W., 2000. Mechanisms of apoptosis in rat cerebellar granule cells induced by hydroxyl radicals and the effects of EGb761 and its constituents. Toxicology 148, 103–110.

Zhang, Z.H., Yu, S.Z., Wang, Z.T., Zhao, B.L., Hou, J.W., Yang, F.J., Xin, W.J., 1994. Scavenging effects of tetramethylpyrazine on active oxygen free radicals. Acta Pharmacol. Sin. 15, 229–231.