School of Life Sciences[1], Lanzhou University, Laboratory Center for Medical Science[2], Lanzhou Medical College, Department of Biochemistry and Molecular Biology[3], Lanzhou Medical College, Lanzhou, P. R. China

Antiapoptotic and antioxidant effects of rosmarinic acid in astrocytes

L. P. Gao[1, 3], H. L. Wei[2], H. S. Zhao[2], S. Y. Xiao[3], R. L. Zheng[1]

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Prof. Rong-liang Zheng, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730000, P. R. China
zhengrl@lzu.edu.cn

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The protective effects of rosmarinic acid (RA), a polyphenolic compound, on apoptosis induced by hydrogen peroxide in astrocytes were studied. Pretreating cells with RA significantly increased cell viability and decreased apoptosis rate induced by H_2O_2. The antiapoptotic effect of RA was further confirmed by increase of mitochondrial membrane potential and inhibition of caspase-3 activity. RA also attenuated cellular oxidative stress by decreasing the amount of reactive oxygen species and malondialdehyde. Results clearly show that RA was able to attenuate H_2O_2-induced cell injury by its antiapoptotic and antioxidant activity.

1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($HO^{\cdot}$) play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease (Coyle and Puttfarcken 1993; Markesbery 1997; Chan 2001) and many neurological events, such as ischemia, anoxia and reperfusion injury (Kitagawa et al. 1990; Flamm et al. 1978; Kontos 2001). Previous results from our laboratory showed that excessive production of ROS, especially H_2O_2 may lead to cell apoptosis (Ren et al. 1998; Li et al. 2000; Huang et al. 2000). The most valuable exogenous ROS generator is H_2O_2, although not being a radical for itself. However, H_2O_2 is a potential source for $HO^{\cdot}$, one of the most dangerous radicals, through the Fenton reaction in the presence of transition metal ions. Recently, attention has been focused on a wide array of natural antioxidants that are able to scavenge free radicals and protect cells from oxidative damage (Ishige et al. 2001; Sagara et al. 1999).

Glial cells including astrocytes and microglial cells provide mechanical and metabolic support for neurons. Moreover astrocytes play an essential role in maintaining the function of neurons by producing high concentrations of reduced glutatione and antioxidant enzymes to prevent neuronal oxidative injuries and to recover neuronal functions in various pathologic conditions (Cardile et al. 2000).

Rosmarinic acid (RA), a polyphenolic compound, distributed in many medicinal plants exhibits strong free radical scavenging activity (Zheng and Wang 2001; Liu et al. 1992) including a potent antioxidant effect on low-density lipoprotein (LDL) oxidation (Lin et al. 2002). Several studies demonstrated neuroprotective of antiodidants in astrocytes (Wei et al. 2003; Martin et al. 2002; Flier et al. 2002). In the present study, we reported the antiapoptotic and antioxidant effects of RA in astrocytes.

2. Investigations, results and discussion

After exposure to H_2O_2 250 μM for 24 h, astrocytes viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Fig. 1 shows that H_2O_2 250 μM alone markedly decreased cell viability to 32% compared to control, while pretreatment with RA at concentrations of 10 and 40 μM increased cell viability to 40% and 42% respectively ($P < 0.01$). However RA at the above concentrations alone had not any effect on the viability of astrocytes ($P > 0.05$). H_2O_2 was reported to trigger apoptosis in various cell types (Ren et al. 1998; Li et al. 2000; Huang et al. 2000).

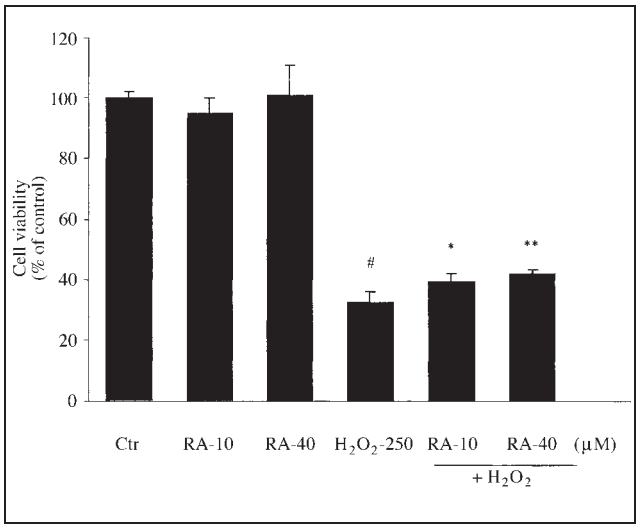

Fig. 1: Enhancement of rosmarinic acid (RA) on the viability of astrocytes. Cell viability was assessed by the MTT method as described in Experimental. Cells pretreated with RA 10 and 40 μM for 30 min followed by exposure to H_2O_2 250 μM for additional 24 h. Values are means ± S.D. n = 4. $^{\#}P < 0.05$ in comparison with control group. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with H_2O_2 group

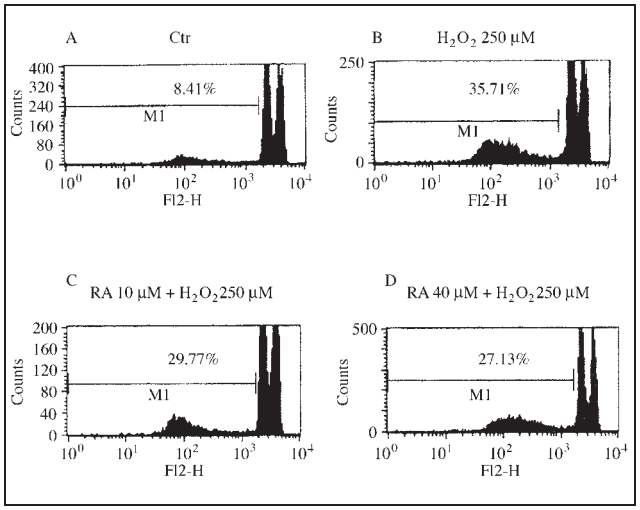

Fig. 2: Rosmarinic acid (RA) prevents apoptosis in astrocytes induced by H_2O_2. Cells were preincubated with RA 10 and 40 μM for 30 min prior to the expose of H_2O_2 250 μM for additional 24 h. DNA fragmentation was determined by flow cytometry after propidium iodide staining. The M_1 marker shows the sub-G1 cell fraction that correspond the apoptotic cells

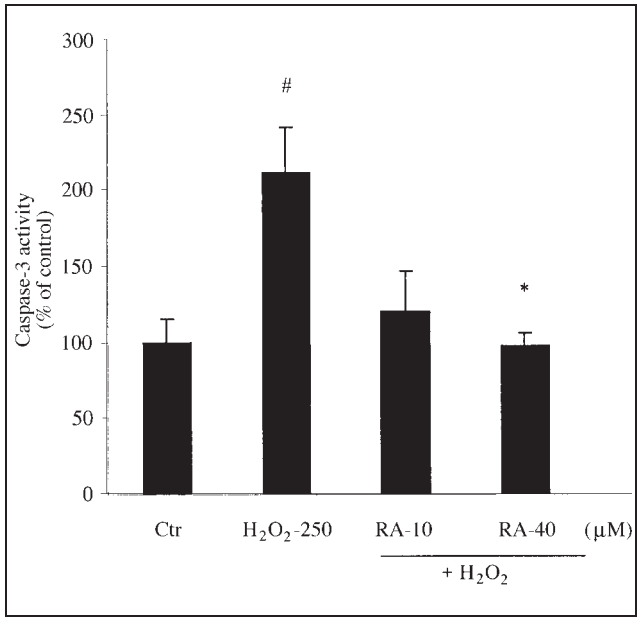

Fig. 4: Inhibition of rosmarinic acid (RA) on caspase-3 activity. RA 10 and 40 μM was added 30 min prior to H_2O_2 stimulation. Values are means ± S. D. n = 3. $^{\#}P < 0.05$ in comparison with control group. $^{*}P < 0.05$ in comparison with H_2O_2 group

Apoptosis is the physiological process that removes superflous and damaged neurons during nervous system maturation. However, the triggering of unregulated apoptosis has been implicated in the development of several neurodegenerative diseases and stroke (Bredesen 1995). The apoptosis rate was defined as the percentage of cells with subdiploid DNA content (DNA fragmentation) determined with flow cytometry. When treated with H_2O_2 250 μM alone, the percentage of apoptotic cells with fractional DNA was 35.7% and a large amount of debris was observed, indicating a pronounced degree of cell disruption. Pretreatment with RA at concentrations of 10 and 40 μM, the percentage of apoptotic cells was significantly reduced to 29.8% and 27.1% respectively (Fig. 2).

Astocytes apoptosis *in vitro* is induced by several signals, including oxidative stress (Fauconneau et al. 2002; Dare et al. 2001). The factors determining the apoptotic cell death is the change in mitochondrial membrane permeability as a result of the occurrence of mitochondrial permeability transition pore (MPT) (Zoratti and Szabo 1995). Under certain conditions, MPT opens and shorts circuits of the inner mitochondrial membrane to H^+. Then the H^+ electrochemical gradient collapses, and the mitochondrial membrane potential drops, causes Ca^{2+} release, increases production of ROS, and precipitates the release of proapoptotic factors such as cytochrome c, caspase 9 and apoptosis-inducing factor (AIF) (Susin et al. 2000). The mitochondrial membrane potentials were measured by flow cytometry using Rhodamine 123 (Rh123) as a mitochondrial energization-sensitive fluorescence probe (Emaus et al. 1986). The intensity of Rh123 fluorescence is directly related to the mitochondrial membran potentials, an indirect indicator of the energetic status of mitochondria (Mark et al. 1997). Exposure of cells to 250 μM H_2O_2 alone for 24 h caused a significant decrease in Rh123 fluorescence to 62.2% campared with control.

Pretreatment with RA at concentrations of 10 and 40 μM significantly increased Rh123 fluorescence to 97.2% and 96.3% respectively (Fig. 3). The result suggested that H_2O_2 caused a dysfunction of mitochondria. Pretreating cells with the RA effectively prevented the mitochondria from undergoing dysfunction.

Another characteristics of apoptosis are the activation of caspase-3, a cysteine aspartate protease and mammalian homologue of *ced* 3 from *Caenorhabditis elegans* (Kumar 1997). The inactive 32-kDa caspase-3 precursor protein CPP32 is cleaved into an active 17-kDa and an inactive 20-kDa fragment. Caspases are the molecular machinery that drive apoptosis, and are responsible for the morphologic and biochemical characteristics of apoptotic cells. Following 24 h treatment of astrocytes with 250 μM H_2O_2 alone, an expected 211.2% increase of caspase-3-like activity compared with the control cells was detected. When astrocytes were pretreated with RA 10 and 40 μM, the caspase-3 activities were decreased to 120.5% and 96.9% respectively (Fig. 4). The results showed that RA inhibited caspase-3 activity activated by H_2O_2.

Mitochondria are the major source of intracellular ROS production. Even under resting conditions 2 to 5% of molecular oxygen consumed by mitochondria is partially reduced by the electron transport chain to form superoxide

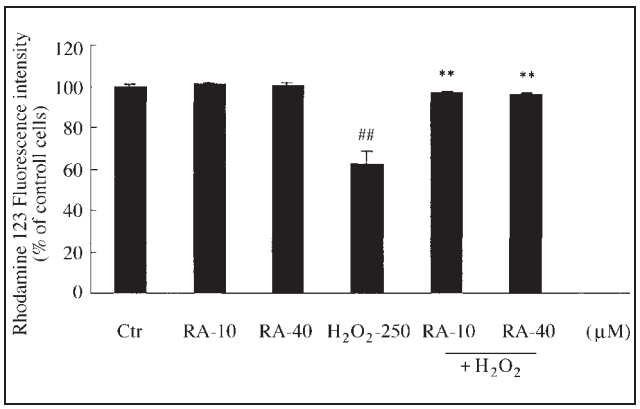

Fig. 3: Enhancement of rosmarinic acid (RA) on mitochondrial membrane potential. Mitochondrial membrane potential was assessed by flow cytometry as described in Experimental. Cells pretreated with RA for 30 min followed by exposure to 250 μM H_2O_2 for additional 24 h. Values are means ± S. D. n = 4. $^{\#\#}P < 0.01$ in comparison with control group. $^{**}P < 0.01$ in comparison with H_2O_2 group

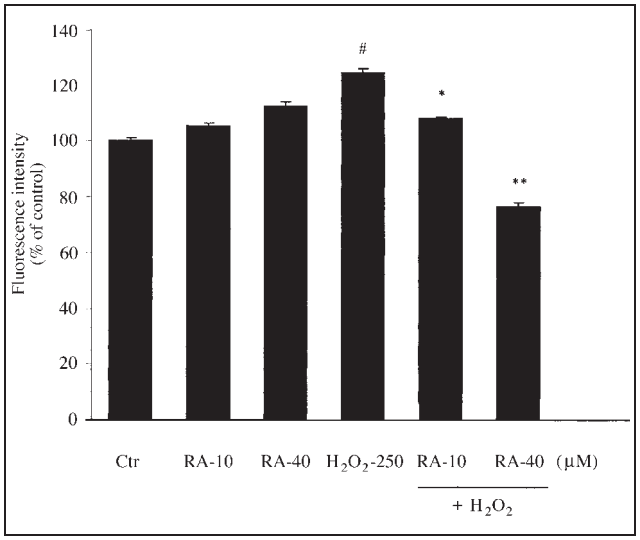

Fig. 5: Inhibition of rosmarinic acid (RA) on ROS production. ROS was assessed by flow cytometry as described under Experimental. Cells were pretreated with RA 10 and 40 μM for 30 min, and then added H_2O_2 for another 24 h. Values are means ± S.D. n = 4. $^{\#}P < 0.05$ in comparison with control group, $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with H_2O_2 group

and subsequently H_2O_2. Furthermore, mitochondrial ROS may inhibit one or more of the components of the respiratory chain, further accelerating the rate of superoxide formation (Kumar 1997). Increased generation of ROS leads to lipid peroxidation and consequent disruption of membranes. In order to elucidate the mechanism of RA protection, the intracellular ROS and the product of lipid peroxidation, malondialdehyde (MDA) were further investigated in this study. ROS fomation was estimated by 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to fluorescent DCF in the presence of ROS (Shen et al. 1996). The intensity of fluorescence is parallel to the levels of intracellular ROS. H_2O_2 alone significantly triggered ROS formation, resulting in a large elevation of the flurorescence emission. A 124.6% increase of fluorescence intensity compared with control cells was detected. Pretreatment with RA 0 and 40 μM resulted in a significant reduction of the fluorescence to 108.3% and 76.6% respectively (Fig. 5). H_2O_2 250 μM alone markedly increased MDA to 146.8% compared with the control cells. However, pretreatment with RA 10 and 40 μM decreased MDA to 106.9% and 100.8% respectively (Fig. 6). The results show that RA attenuated cellular oxidative stress by decreased production of ROS and MDA.

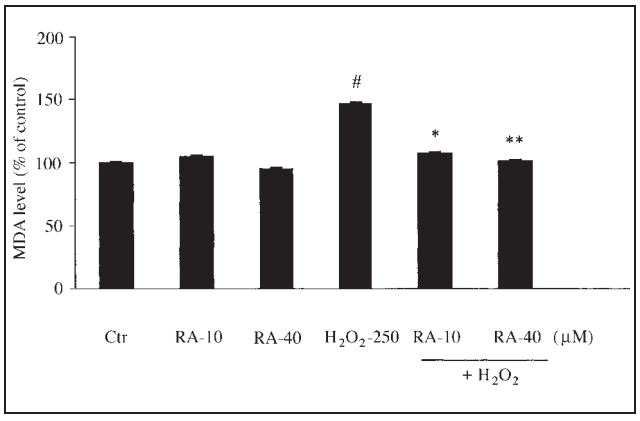

Fig. 6: Inhibition of rosmarinic acid (RA) on MDA in astrocytes. MDA level was assessed by spectrophotometric methods as described in Experimental. Cells were pretreated with RA 10 and 40 μM for 30 min, and then added H_2O_2 for another 24 h. Values are means ± S. D. n = 4. $^{\#}P < 0.05$ in comparison with control group. $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison with H_2O_2 group

In our study, RA had a significant neuroprotective effect against H_2O_2-induced apoptotic death and significantly protected against oxidative stress. It is possible that the protective effect of RA in astrocytes may at least partly result from its antiapoptotic and antioxidant properties.

3. Experimental

3.1. Materials

Rosmarinic acid (RA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide were puchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum and RNase A were products of Gibco BRL (USA). Rhodamine 123 and 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) were puchased from Molecular Probes (Eugene, OR, USA). Caspase-3 colorimetric assay (R and D System, UK), all other reagents or drugs were of analytical grade.

3.2. Cell culture and treatment

Astrocytes were isolated from 1-day-old Wistar rat pups and cultured as described previously (McCarthy and DeVellis 1980). Briefly, cortices free meninges and vessels were dissociated by passing them through a nylon mesh and further triturated with a pipette. Cells were washed and resuspended in DMEM, containing 10% fetal bovine serum and penicillin-streptomycin. Cells were then seeded in 75 cm^2 flasks and incubated at 37 °C in a humidified 5% CO_2 atmosphere. By changing culture medium 24 h following initial plating, the adherent astrocytes were preserved, while the less adherent neurons and oligodendrocytes were removed. The culture medium was changed twice a week. The astrocytes were subcultured and their purity was examined using a specific marker of anti-glial fibrillary acidic protein. In all experiments, cells were preincubated with 10 and 40 μM RA for 30 min and then H_2O_2 250 μM was added to the medium (Jang and Surh 2001).

3.3. Cell survival assay

The cell viability was determined using a modified MTT assay as described previously (Mosmann 1983). Briefly, astrocytes cells were seeded in 96-well plates at a density of 2×10^4 cells per well and pretreated with RA 10 and 40 μM for 30 min, and then 250 μM H_2O_2 was added in the cell culture for another 24 h. After that, cells were washed with phosphate-buffered saline (PBS, pH 7.4) twice and grown in MTT solution 0.5 mg/ml at 37 °C. Four hours later, the supernatant was carefully removed, dimethylsulfoxide was added to dissolve the insoluble crystals, and the absorbance at 570 nm was measured with a model 550-microplate reader (Bio-Rad)

3.4. Apoptotic analysis by flow cytometry

After treatment with 250 μM H_2O_2 alone or pretreatment with RA 10 and 40 μM for 24 h, the cells were harvested and washed with PBS. Cell pellets were fixed in cold 70% ethanol. After centrifugation at 200 × g for 5 min, cells were resuspended in 0.5 ml DNA staining reagent containing 50 μg/ml propdidium iodide, 50 μg/ml RNase A, 0.1% Triton X-100 and 0.1 mM EDTA (pH 7.4). The samples were read on a Becton Dickson FACStar Plus cytometer, and the results were analysed with CellQuest 3.3 software (Zhou and Tang 2002).

3.5. Measurement of mitochondrial membrane potential

Following RA or H_2O_2 treatments, cells were treated with 0.02% trypsin, washed twice with PBS solution, and stained with Rh123 in PBS solution at 37 °C for 30 min. After washed twice with PBS solution, propidium iodide was added to discard non-viable cells. Changes in Rh123 were measured by a flow cytometer (Beckman FACS) with 488 nm exitation and 520 nm emission (Sureda et al. 1999).

3.6. Measurement of caspase-3 activity

Enzymatic activity of caspase-3 was determinded by caspase-3 colorimetric assay, according to the manufacturer's instructions. This assay is based on the release of the fluorochrome p-nitroaniline (*p*-NA) combined with a caspase-3 specific peptide substrate (DEVD–*p*-NA). Peptide cleavage through an active caspase-3 releases the chromophor *p*-NA, which can be quantified with a colormetric plate reader (405 nm). Briefly, cultured astocytes were lysed and cell extracts were centrifuged to eliminate cellular debris. Aliquots (50 μl of cell extracts) were incubated for 2 h at 37 °C in

the presence of the substrate. The caspase-3 activity, proportional to the color reaction intensity was expressed as a percentage of control (Gabryel et al. 2002).

3.7. Assessment of the ROS formation in astrocytes

Cells were treated with H_2O_2 for 24 h and then incubated with 50 μM DCFH-DA for additional 30 min at 37 °C. After chilling on ice, cells were washed with ice-cold PBS, scraped from the plate, and resuspended in PBS containing 10 mM EDTA. The fluorenscence intensities of 2,7-dichlorofluorescein formed by the reaction of DCFH-DA with ROS of more than 5000 viable cells from each sample were analyzed by recording FL-1 fluorescence by a flow cytometer. Prior to data collection, propidium iodide was added to the sample for gating out dead cells (Ciriolo et al. 2000).

3.8. Malondialdehyde (MDA) determination

MDA has been used as a biochemical marker for lipid peroxidation and was measured by a method previously descriped (Wei et al. 1999). Briefly, 2 ml of each sample treated with trichloroacetic acid (15% w/v) containing 1 mM EDTA was centrifuged at 1000 × g for 10 min. The supernatant was heated at 100 °C with an equal volume of thiobarbituric acid (0.7% w/v) for 20 min. After cooling, the absorbance at 532 nm was monitored.

3.9. Statistical analysis

The data are expressed as means ± S. D. Statistical comparisons were made by Student's t-test. $P < 0.05$ was considered significant.

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