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Inhibition of caspases and intracellular free Ca^{2+} concentrations are involved in resveratrol protection against apoptosis in rat primary neuron cultures[1]

Qi-hai GONG[2,3,4], Qian WANG[2,3], Jing-shan SHI[2,3], Xie-nan HUANG[2,3], Qiong LIU[2], Hu MA[2]

[2]Department of Pharmacology, Zunyi Medical College, Zunyi 563000, China; [3]the Key Laboratory of Basic Pharmacology of Guizhou Province, Zunyi Medical College, Zunyi 563000, China

Key words

resveratrol; oxygen-glucose deprivation/reperfusion; brain ischemia; apoptosis; neurons

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[4] Correspondence to Dr Qi-hai GONG.
Phn 86-852-860-9623.
E-mail gongqihai@yahoo.com.cn

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Abstract

Aim: To investigate the influence of resveratrol (Res), a nutritional antioxidant, on the inhibition of apoptosis in rat primary neuron cultures. **Methods:** The cultured cortical neurons of neonatal Sprague-Dawley rats were pretreated with Res (0.1, 1.0, and 10.0 μmol/L) and oxygen-glucose deprivation/reperfusion (OGD/RP) with oxygen and glucose were initiated at d 10 *in vitro*. Neuronal apoptosis was determined by flow cytometry, and morphological changes of neurons were observed by an electron microscope. For the mechanism studies, the intracellular free calcium concentration ($[Ca^{2+}]_i$) and the transcription of caspases-3 and -12 in neurons were detected by Fura 2/AM loading and real-time RT-PCR, respectively. **Results:** OGD/RP insult could induce an increase in the apoptotic rate of neurons (from 11.1% to 49.0%), and elicit an obvious morphological change in neurons; pretreatments with Res (0.1, 1.0, and 10.0 μmol/L, respectively) significantly reduced the elevated rate of apoptosis to 41.7%, 40.8%, and 37.4%, respectively, and ameliorated the neuronal morphological injury. Similarly, the OGD/RP insult obviously elicited the elevated levels of the $[Ca^{2+}]_i$ and the expressions of caspases-3 and -12 mRNA in neurons. Res pretreatments markedly depressed the neuronal abnormal elevation of $[Ca^{2+}]_i$ and the overexpression of caspases-3 and -12 mRNA in a concentration-dependent manner. **Conclusion:** Res can attenuate the rat cortical neuronal apoptosis induced by OGD/RP. The mechanisms are, at least partly, due to the inhibition of the calcium overload and the overexpression of caspases-3 and -12 mRNA.

Introduction

The brain is the most vulnerable organ to ischemic insult because of its high metabolic rate, relative low oxygen stores, and insufficient reserves of high-energy carbohydrates, which usually result in severe functional loss in the central nervous system. The re-establishment of blood flow is essential to repair ischemic brain tissue damage. However, ischemia-reperfusion injury is the most imperative problem during the course of repairing of ischemic brain tissue damage. Neuronal apoptosis has been implicated in the pathophysiology of brain ischemia and reperfusion injury[1–3]. Accordingly, protection from the abnormally increased neuronal apoptosis will be beneficial to the therapy.

The polyphenolic compound resveratrol (*trans*-3,4',5-trihydroxystilbene, Res) is a naturally occurring phytochemical which has been found in more than 70 plant species, including human food products like grapes, peanuts, berries, and some herbs. The physiological function of this polyphenol is thought to serve as a phytoalexin, protecting plants against environmental stress or pathogenic attack, and as a strong antioxidant to reduce the oxidation of lipoproteins in animals[4–6]. Notably, it can also protect the blood vessels from atherosclerosis and inhibit the platelet aggregation and cyclo-oxygenase[4,7,8]. Recent studies have demonstrated the ability of Res to exert protective effects against brain

injury induced by ischemia–reperfusion in gerbils[9], and epidemiological studies have also shown that the consumption of Res-enriched red wine is significantly correlated with a reduction in the incidence of age-related macular degeneration, Alzheimer's disease, and stroke[10–12]. Furthermore, our study concluded that Res had significant protective effects on injury induced by oxygen-glucose deprivation/reperfusion (OGD/RP) insult in primary cultured neurons of neonatal rats[13].

In this study, we examined the influence of Res on the neuronal apoptosis for further investigation of its effects on the injury induced by OGD/RP in primary cultured neurons of neonatal rats, and investigated the possible protective mechanisms.

Materials and methods

Animals and reagents Sprague-Dawley rats (200–250 g) purchased from the Animal Center of the Institute of Field Surgery of the Third Military Medical University in China (Chongqing, China; Specific-pathogen free Grade II, Certificate No scxk 20020003) were kept in a regulated environment (23±1 °C, 50%±2% humidity) with 12 h light/dark cycle (light on 8:00–20:00). Newborn Sprague-Dawley rats (less than 72 h) were incubated in the animal room. Res (purity ≥98% via HPLC) was provided by Hunan Huaguang Biological Products (Huaihua, China). The concentration of solvent (DMSO) in the final culture media was less than 0.05% (*v*/*v*), which was safe to the culture in the control group. Nimodipine (Nim, No 95060120, molecular weight: 418.4) was obtained from Zhengzhou Chemical Pharmaceutical Factory (Zhengzhou, China). Dulbecco's modified Eagle's medium (DMEM)/F12, and fetal bovine serum (FBS) were purchased from Hyclone (Logan City, Utah, USA). Hydroxyethyl piperazine ethanesulfonic acid (HEPES), Fura-2/AM, ethyleneglycol *bis* (2-aminoethyl ether)tetraacetic acid (EGTA), and Triton X-100 were from Sigma (St Louis, MO, USA). The Annexin V/ fluorescein isothiocyanate (FITC) kit was purchased from Jingmei Biotechnology (Shenzhen, China).

Primary cortical neuron culture[14] The cortices taken from 1–3 d old newborn Sprague-Dawley rats were collected into cold D-Hanks' solution and dissected free of meninges and blood vessels. The cerebral tissues were minced and incubated in 0.125% trypsin at 37 °C for 30 min. Then the DMEM/F12 medium with fetal bovine serum was added to terminate digestion. The whole cell suspension was filtered with a nylon mesh (200 mesh, hole width: 95 μm), the filtrate was centrifuged at 3 000×*g* for 10 min, then the sediment was resuspended with DMEM/F12 containing 20% FBS, 100 kU/L benzylpenicillin, and 100 mg/L streptomycin. The cells were adjusted to approximately 1×10^9/L and planted into 96-well plates or dishes, which were previously coated with 10 mg/L poly-*L*-lysine for 24 h, at 37 °C in an atmosphere of 5% CO_2 and 95% O_2. Arabinosylcytosine (5 μg/mL) was added on the third day after incubation to prevent the growth of non-neuronal cells. After 24 h, the culture was changed with the normal medium and refreshed every 2–3 d[15,16]. Under these conditions, the cultures typically contained more than 95% neurons as assessed by staining with an antibody directed against neuron-specific enolase.

OGD/RP and treatment OGD were performed on mature cultures at d 10 using the previously described methods[17,18]. Briefly, the cells exposed to glucose-free Earl's solution [116.4 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgSO_4$, 2.6 mmol/L NaH_2PO_4, 26.2 mmol/L $NaHCO_3$, and 20.1 mmol/L HEPES (pH 7.4)] containing different concentrations of Res (0.1, 1.0, and 10.0 μmol/L, respectively) or solvent were cultured at 37 °C in an incubator bubbled in an atmosphere of 5% CO_2 and 95% N_2 (OGD) for 2 h. Then the culture medium was changed into normal Earl's solution (with the addition of 5.6 mmol/L glucose into the glucose-free Earl's solution) bubbled in an atmosphere of 5% CO_2 and 95% O_2 (RP) for another 12 h.

Apoptosis assay[19] Neuronal apoptosis was assayed by flow cytometry with the Annexin V/FITC kit. In brief, 1×10^6 single cells per sample were collected after OGD for 2 h and the following RP for 12 h and washed twice with the buffer; Annexin V/FITC was then added. After incubation for 10 min at room temperature in the dark, the cells were washed and resuspended, propidium iodide was then added to a final concentration of 1 mg/L. The percentage of apoptosis was detected and 1×10^4 cells were counted for each sample by flow cytometry.

Morphological evaluation by electron microscope Morphological observation was conducted in 4 groups: control group, OGD/RP group, OGD/RP+Nim 1.0 μmol/L group and OGD/RP+1.0 μmol/L Res group. The cortical neurons of the newborn rats were incubated in a 6-well plate, in which glass slides were placed in advance. After being treated with OGD for 2 h and RP for 12 h according to the method previously described, the glass slides full of growing neurons were harvested from the 6-well plate, rinsed gently 3 times with cold phosphate-buffered saline (PBS), fixed for 1 h at 4 °C with 2.5% glutaral, then rinsed twice with 0.1 mol/L PBS, each time lasting more than 30 min. Then they were fixed for 1 h with 1% osmic acid, rinsed twice with 0.1 mol/L PBS, dehydrated with different concentrations (30%, 50%, 70%, 80%, 90%, and 100%) alcohol, replaced with isoamyl acetate, dried with

CO_2 at critical point, and sherardized in a vacuum. Finally, the morphological changes of the neurons were observed and photographed by transmission electron microscope (JEOL, Tokyo, Japan).

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) The cultured cells were incubated with 0.125% trypsin to obtain dispersed single cell suspension. The cell suspension was then incubated with Fura-2/AM at a concentration of 10 μmol/L in the dark for 50 min at 37 ºC. After rinsing twice with Hank's solution containing bovine sera albumin, the cells were resuspended in Hank's solution and used to measure the $[Ca^{2+}]_i$ with RF-5000 spectrometry. The fluorescent dye was measured with an emission wavelength of 500 nm and the maximum absorption at 340 nm when the calcium bound. The concentration of the $[Ca^{2+}]_i$ was represented as $K_d \times (F-F_{min})/(F_{max}-F)$[20], where K_d is 224 nmol/L (the K_d value of Fura-2/AM), F is the measured fluorescence intensity, while F_{max} and F_{min} refers to the maximum and minimum values at 340 nm after adding Triton X-100 (0.98 g/L) and EGTA (5 mmol/L), respectively.

Real-time RT-PCR analysis The transcription of caspases-3 and -12 were detected by real-time RT-PCR. The total RNA of primary cultured neurons was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the RNeasy mini kit (Qiagen, Palo Alto, CA, USA). RNA was spectrophotometrically quantified by measuring the optical density of samples at 260/280 nm, redissolved in 30–50 μL diethyl pyrocarbonate water (RNA concentration of each samples was 50 ng/μL), and stored at -80 ºC.

Primer preparations were devised according to the sequence searched on GenBank. The nucleotide sequence of the primers used in this experiment were as follows: (1) caspase-3 (GenBank Access No NM_012922.2): sense 5'-CAG AGC TGG ACT GCG GTA TTG A-3', antisense 5'-AGC ATG GCG CAA AGT GAC TG-3'; (2) caspase-12 (GenBank Access No NM_130422.1): sense 5'-CTG GCC CTC ATC ATC TGC AA-3', antisense 5'-TGG ACG GCC AGC AAA CTT-3'; and (3) β-actin (GenBank Access No V01217.1): sense 5'-TGA CAG GAT GCA GAA GGA GA-3', antisense 5'-TAG AGC CAC CAA TCC ACA CA-3'. The predicted length of the PCR product was 300 bp.

Total RNA was reverse transcribed with MuL V reverse transcriptase and oligo (dT) primers. The SYBR green DNA PCR kit (Applied Biosystems, Foster City, CA, USA) was used for the real-time PCR analysis. The relative differences in expression among the groups were expressed using cycle time (C_t) values as follows: the C_t values of the interested genes were first normalized with β-actin of the same sample, and then the relative difference between the control and each treatment group was calculated and expressed as a relative increase, setting the control at 100%.

Statistical analysis All the data were expressed as mean±SD. The data were analyzed statistically using the SPSS 11.5 for Windows statistical program (SPSS, Chicago, IL, USA) by one-way ANOVA or Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Effects of Res on apoptosis Neuronal apoptosis was assayed after OGD/RP injury. There was a very low level (11.1%) of neuronal apoptosis under normal conditions. After OGD/RP insult, the percentage of apoptosis significantly increased by 49.1% ($P<0.01$). Similar to Nim treatment, the addition of 0.1, 1.0, and 10.0 μmol/L Res during OGD/RP markedly reduced the percentage of cell apoptosis to 41.7%, 40.8%, and 37.4%, respectively. The anti-apoptotic effect of 10.0 μmol/L Res was more significant than that of 1.0 μmol/L Nim and 0.1 or 1.0 μmol/L Res ($P<0.05$). The vehicle had no effect on cell apoptosis induced by OGD/RP (Figure 1A–B).

Transmission electron microscope observation Electron microscope evidence showed that the nucleus of normal cortical neurons were big, round, or oval. The euchromatin distributed homogeneously, the structures of intracytoplasmic mitochondria and rough endoplasmic reticulum were clear, and the ribosomes were abounded (Figure 2A). On the contrary, the nucleus of cortical neurons subjected to OGD/RP were irregular, a chromatin mass formed, the perinuclear space obviously became thicker, mitochondria swelled and vacuolized, cristae arrangement became disordered, mitochondria membranes were ruptured, and the endocytoplasmic reticula were expanded. Some neurons were even obviously broken to pieces and the membrane dissolved (Figure 2B). However, pretreatment with 1.0 μmol/L Nim during the course of OGD/RP insult could obviously relieve the neuronal damage compared with those of OGD/RP alone. Most of the nuclear chromatins distributed homogeneously, the ultramicrostructure was similar to that of normal neurons with swollen mitochondria mitigated, lamellar cristae in mitochondria became clear, and the ribosomes became more abundant (Figure 2C). Pretreatment with 1.0 μmol/L Res during the course of OGD/RP insult relieved neuronal morphological damage compared with those of OGD/RP alone, and the ultramicrostructure was similar to that of the OGD/RP+1.0 μmol/L Nim group (Figure 2D).

Effects of resveratrol on $[Ca^{2+}]_i$ At the end of OGD/RP insult, the significant elevation of $[Ca^{2+}]_i$, which was nearly 3.5-fold of that under normal conditions, was observed in

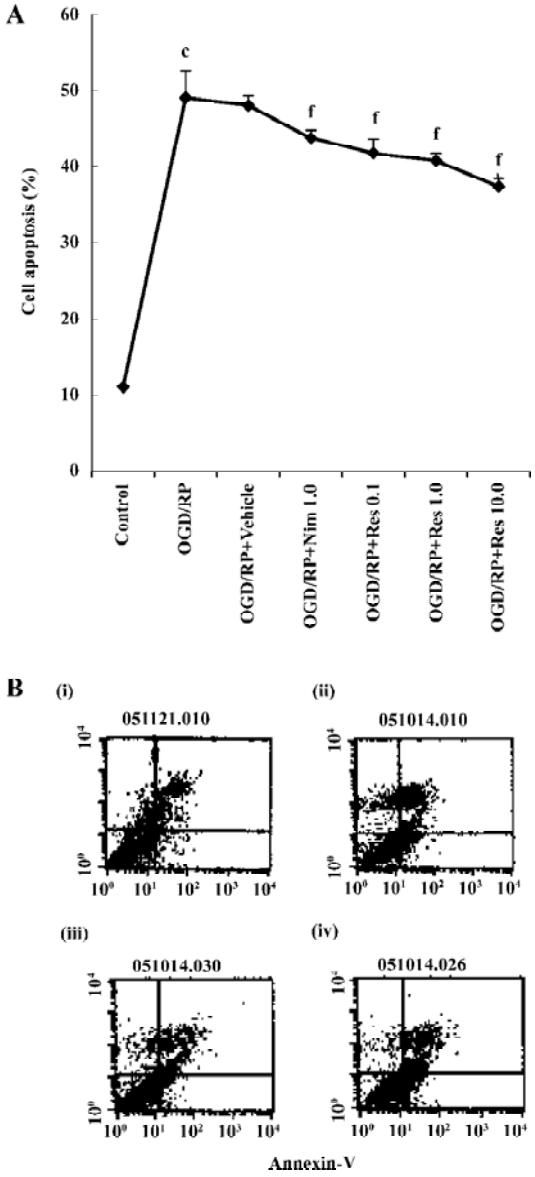

Figure 1. Effects of Res on cell apoptosis in the primary cultured neurons of neonatal rats subjected to OGD/RP injury. (A) Graph of apoptosis. Mean±SD. n=6. ^{c}P<0.01 *vs* control; ^{f}P<0.01 *vs* OGD/RP; ^{h}P<0.05 *vs* OGD/RP+1.0 μmol/L Nim. (B) Scatterplot of apoptosis (i) control; (ii) OGD/RP; (iii) OGD/RP+1.0 μmol/L Nim; (iv) OGD/RP+1.0 μmol/L Res.

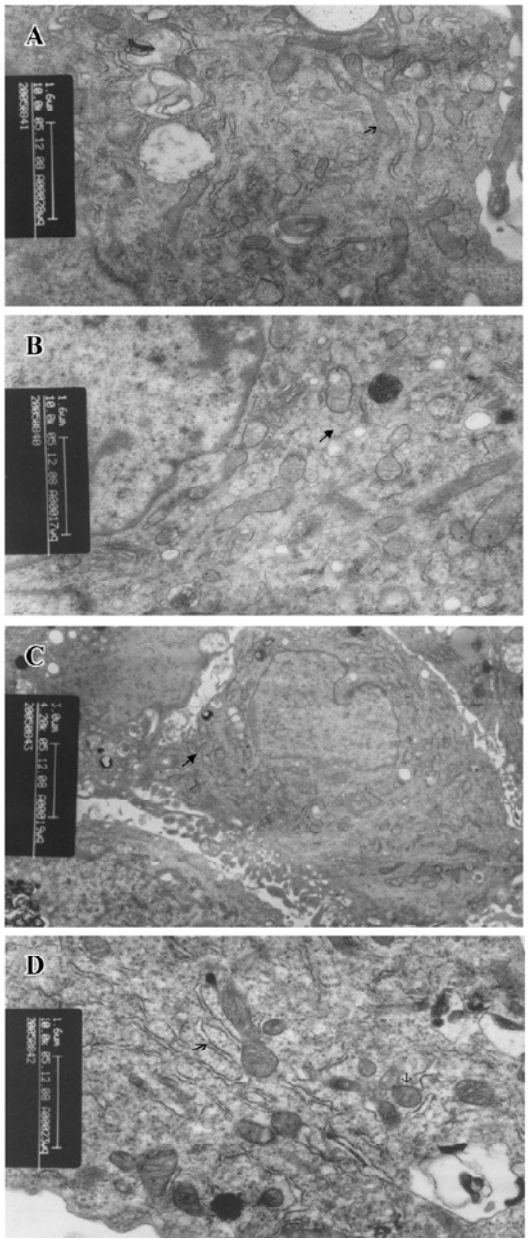

Figure 2. Morphological change of primary cultured neurons by transmission electron microscope (×10000) in the control group (A), OGD/RP group (B), OGD/RP+1.0 μmol/L Nim group (C), and OGD/RP+1.0 μmol/L Res group (D).

the insulted neurons. Nim, a Ca^{2+} blocker used to relax the cerebral vasculature, could to some extent depress the elevation of $[Ca^{2+}]_i$ in OGD/RP-insulted cells. Similarly, Res also obviously inhibited the elevation of $[Ca^{2+}]_i$ induced by OGD/RP in a concentration-dependent manner. Consistent with in the findings of the apoptosis experiment, the inhibitory effect of 10.0 μmol/L Res on the elevation of $[Ca^{2+}]_i$ was more significant than that of 1.0 μmol/L Nim and 0.1 or 1.0 μmol/L Res (Table 1).

Real-time RT-PCR analysis of selected gene expression

The real-time RT-PCR analysis showed that OGD/RP insult significantly caused an increase in the transcription of caspases-3 and -12 in cultured neurons, which were approximately 6-fold of that under normal conditions. The vehicle did not affect the selected gene transcriptions. Similar to

Table 1. Effects of OGD/RP-induced alteration of $[Ca^{2+}]_i$ in rat cortical neurons. Mean±SD. n=6. ^cP<0.01 *vs* control; ^eP<0.05, ^fP<0.01 *vs* OGD/RP; ^iP<0.01 *vs* OGD/RP+1.0 μmol/L Nim.

Group	$[Ca^{2+}]_i$ (nmol/L)
Control	163±5
OGD/RP	581±25^c
OGD/RP+Vehicle	573±49
OGD/RP+1.0 μmol/L Nim	389±36^f
OGD/RP+0.1 μmol/L Res	502±18^e
OGD/RP+1.0 μmol/L Res	400±45^f
OGD/RP+10.0 μmol/L Res	223±19^{fi}

that of the elevation of $[Ca^{2+}]_i$, Nim could also blunt the overexpression of the selected genes, and Res treatment also obviously depressed the overexpression of caspases-3 and -12 mRNA induced by OGD/RP in a concentration-dependent manner. The depressing effect of 10.0 μmol/L Res was also more remarkable than that of 1.0 μmol/L Nim (Table 2).

Table 2. Effects of Res on OGD/RP-induced transcription of caspases-3 and -12 in rat cortical neurons as determined by real-time RT-PCR. Data are mean±SD of 3 separate experiments. ^cP<0.01 *vs* control; ^eP<0.05, ^fP<0.01 *vs* OGD/RP; ^hP<0.05 *vs* OGD/RP+1.0 μmol/L Nim.

Group	Caspase-3	Caspase-12
Control	62±24	20±4
OGD/RP	367±55^c	122±27^c
OGD/RP+Vehicle	363±63	120±37
OGD/RP+1.0 μmol/L Nim	217±38^f	53±12^f
OGD/RP+0.1 μmol/LRes	258±41	55±4^e
OGD/RP+1.0 μmol/L Res	232±62^e	39±2^f
OGD/RP+10.0 μmol/L Res	175±24^{fh}	22±18^{fh}

Discussion

It has been reported that the neuroprotective effect of an antioxidant in ischemic brain injury is involved in neuronal apoptosis[1], and during early reperfusion, the apoptotic mechanisms are engaged in vulnerable neurons, such as the cortex and hippocampus[20]. Combined with our previous findings that Res decreases lactate dehydrogenase leakage and improves cell survival in the OGD/RP model of cultured neonatal rat cortical neurons[13], we further studied the protective effects of Res, including its anti-apoptotic effect on the same model, which imitated ischemia-reperfusion performance in the brain. We found that OGD/RP insult caused a remarkable increase in the neuronal apoptotic percentage, which was depressed by the addition of Res in a concentration-dependent manner and elicited an obvious abnormal neuronal morphological change, which was ameliorated by the addition of Res. All the facts indicated that the anti-apoptotic activity of Res contributed to the beneficial effect on neuronal injury in this model.

Abnormalities of $[Ca^{2+}]_i$ homeostasis, especially the $[Ca^{2+}]_i$ overload, have been linked to neuronal apoptosis induced by ischemia-reperfusion[21]. The increasing $[Ca^{2+}]_i$ leads to the activation of many pivotal cellular processes that can alter the cellular functions and even lead to cell injury and death, including neuronal apoptosis[22,23]. Our results showed that OGD/RP insult caused an elevated $[Ca^{2+}]_i$ in the subjected neurons, and pretreatment with Res inhibited cell apoptosis concentration-dependently, which was consistent with its anti-apoptotic effect in the same model. The results suggest that the effect of Res on neuronal apoptosis may be, at least partly, involved in its depressing activity in the abnormal elevated $[Ca^{2+}]_i$ of neurons. However, the mechanism of the inhibitory effects of Res on $[Ca^{2+}]_i$ remains to be elucidated. It has been reported that Res inhibits Ca^{2+} influx in isolated rat ventricle myocytes and thrombin-stimulated human platelets by inhibiting L-type Ca^{2+} channels or store-operated Ca^{2+} channels[24,25], respectively, indicating the possibility that Res may have a direct inhibitory effect on $[Ca^{2+}]_i$ in neurons by acting Ca^{2+} channels. On the other hand, Res is well known as an antioxidant depressing the production of reactive oxygen species[26]. It has been established that the Ca^{2+} overload can be elicited by oxidative stress in neurons subjected to ischemia–reperfusion injury[27]. The facts suggest another possibility: the depressing effect of Res on the $[Ca^{2+}]_i$ of neurons may indirectly originate from its antioxidant activity in our model. We deduce that the inhibitory effects of Res on Ca^{2+} overload originated from both the direct acting on the Ca^{2+} channel and its antioxidant activity.

It was interesting that the effects of Res (1.0 μmol/L) on apoptosis and the elevated $[Ca^{2+}]_i$ of neurons were equal to that of Nim, a high-lipid solubility Ca^{2+} blocker used to relax the cerebral vasculature[28], at the same concentration; more significant effects could be elicited by increasing the concentration of Res. Furthermore, a higher concentration of Res can be reached in the brain tissue after administration[9]. This result seems to indicate the clinical potential of Res for brain ischemic diseases.

The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances.

An activation of caspases, the aspartate-specific cysteine proteases and members of the interleukin-1 β-converting enzyme family, is a critical step in neuronal apoptosis[29]. Caspase-3 is one of several caspases that integrate various cell death signals and initiate the cleavage of key cellular substrates, such as gelsolin, fodrin, actin, focal adhesion kinase, and poly adenosine diphosphate ribose[30]. In the present study, the transcription of caspase-3 in neurons was increased by OGD/RP insult, consistent with previous reports that showed significant increases in the transcriptional activity of caspase-3 gene during neuronal apoptosis *in vitro*[31] and after traumatic brain injury *in vivo*[32] or transient cerebral ischemia[33] or brain ischemia–reperfusion injury[34]. Caspase-12 plays a key role in many nervous system diseases, including brain ischemia–reperfusion injury[34]. The activated caspase-12 then activates procaspase-9, and the activated caspase-9 in turn activates its downstream substrates, including procaspase-3, to elicit cell apoptosis[35]. Notably, our study also showed that OGD/RP insult could cause an increase in the expression of caspase-12 mRNA to the same extent of the overexpression in caspase-3 mRNA (about 6-fold), and pretreatment with Res could remarkably depress the overexpression of caspases-3 and -12 mRNA in a concentration-dependent manner, which were parallel with its effects on the elevated $[Ca^{2+}]_i$ and the elevated apoptosis of neurons induced by OGD/RP performance. The results suggest that the protective effects of Res on neuronal apoptosis induced by OGD/RP may be also related to its downregulation of caspases-3 and -12 mRNA.

In conclusion, the results of the present study demonstrated that Res could attenuate rat cortical neuronal apoptosis induced by OGD/RP. The mechanisms are, at least partly, due to the inhibition of the calcium overload and the overexpression of caspases-3 and -12 mRNA.

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