行政院國家科學委員會補助專題研究計畫成果報告

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※ Resveratrol 在減輕大花鼠腦梗塞及再灌注性害中抑 ※
※ 嗜中性白血球黏著之發炎現象的影響 ※
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共同主持人：

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□赴國外出差或研習心得報告一份

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□國際合作研究計畫國外研究報告書一份

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Abstract

Resveratrol, a natural phytoalexin (3,5,4'-trihydroxystilbene), is present in the seeds and skin of grapes and constitutes one of the major components of red wine. In previously study, we found that resveratrol is a potent neuroprotective agent on rats subjected to focal cerebral ischemia. The present study was undertaken to evaluate the lactate dehydrogenase (LDH) activity in plasma and to study the neutrophil infiltration in the brain after right middle cerebral artery was occluded for 1 hr and reperfusion for 24 hr in anesthetized Long-Evans rats. Compared with control groups, it revealed that pretreatment or treatment resveratrol groups, the LDH levels was decreased in plasma. In addition, compared with control groups, the change in cerebral myeloperoxidase (MPO) activity, a biochemical marker of neutrophil infiltration, which was considerably increased following focal cerebral ischemia and reduced by resveratrol pretreatment and treatment groups. We further examine whether on neutrophil resveratrol could prevent phorbol-12-myristae-13-acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) induced reactive oxygen species (ROS) production. Results demonstrated that PMA and fMLP induced rapid accumulation of H2O2 in neutrophils within 30 minutes. Resveratrol decreased the accumulation of the oxygen radical metabolites. These data suggest that resveratrol is a potent neuroprotective agent in focal cerebral ischemia. Its beneficial effects may be part related to its anti-inflammatory.

Key words

resveratrol, red wine, stroke, focal cerebral ischemia, neutrophil

Resveratrol 廣泛的存在許多植物中，其在葡萄的種子以及果皮中含量豐富，是紅酒中主要的抗氧化成分之一。我們先前的研究發現，在大花鼠進行右中大腦動脈結紮所造成的大腦局部缺血，投予 Resveratrol 可以有意義的減少大花鼠腦梗塞區域的體積，為一很有效之神經保護劑。而此研究我們評估在大花鼠進行右中大腦動脈結紮所造成的大腦局部缺血的實驗，Resveratrol 保護神經對抗局部大腦缺血的作用中，其血漿中 LDH 的活性，以及嗜中性白血球所扮演的角色。

研究方法是在麻醉的大花鼠將其中大腦動脈以及兩邊的頸總動脈結紮六十分鐘，而後再灌注二十四小時，造成大花鼠的局部大腦缺血以及再灌注傷害，預防組以及治療組分別是在中大腦動脈結紮前十五分鐘以及雙邊頸總動脈放鬆時分別投與 10^{-6}，10^{-7} 以及 10^{-8} g/kg 的 Resveratrol，實驗中測量血漿中 LDH 的活性來評估細胞損傷情形，而以 MPO 的活性則用來評估嗜中性白血球浸潤至缺血

再灌注區域組織的量，另以 PMA 以及 fMLP 引起嗜中性白血球引起反應氧化屬產生，測量 Resveratrol 之自由基清除能力。

研究結果發現實驗動物不論在預防組或是在治療組投與 Resveratrol，皆可以有效的降低血漿中其 LDH 的活性，也可以有意義的減少 MPO 的活性，即減少受缺血再灌注傷害區域嗜中性白血球的浸潤，此外也可以清除 PMA 以及 fMLP 引起嗜中性白血球產生之自由基。

先前的研究顯示 Resveratrol 可以減少中樞遭受缺血以及再灌注的傷害，是一個很有效的神經保護劑，而本研究指出其有效的作用機轉可能和其抗發炎的活性有關。

Introduction

Resveratrol (Fig. 1), a natural phytoalexin (3,5,4'-trihydroxystilbene) is found in a wide variety of plant species. It is abundantly present in the seeds and skin of grapes and constitutes one of the major components of red wine (1). Resveratrol has been reported to have several biologic effects such as, anti-inflammatory activity attributed to cyclooxygenase inhibition (2, 3), estrogenic activity (4), anti-platelet activity (5) and anti-lipid peroxidation effect (6, 7). Resveratrol was also found to stimulate nitric oxide (NO) production in endothelial cells and vasodilatory effect on blood vessels was demonstrated (8).

In previously study, we found that in anesthetized Long-Evans rats subjected to focal cerebral ischemia (9). In pretreatment or treatment groups, resveratrol (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg) did not produce any changes in pH, blood gases, heart rate or mean arterial blood pressure, but it significantly reduced the total infarct volume at the doses 10^{-6} and 10^{-7} g/kg. We suggested that resveratrol is a potent neuroprotective agent in rats subjected to focal cerebral ischemia.

The purpose of this study was to examine whether neutrophil were involving in the neuroprotective effect of resveratrol. We evaluate the LDH activity in plasma and study the change in MPO activity, a biochemical marker of neutrophil infiltration, in the brain after right middle cerebral artery was occluded for 1 hr and reperfusion for 24 hr in anesthetized Long-Evans rats. We further examine whether resveratrol could prevent

PMA and fMLP induced ROS production, as well as fMLP induced adhesion by rat neutrophils.

Materials and Methods

Animals

The present investigation conforms to the Guide for the Car and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Long-Evans rats (National Lab. Animal Breeding and Research Center) weighing 270-350g were used throughout this study. These animals were housed in a room with controlled temperature (24±1°C) and humidity (55±5%) under a 12:12 h light-dark cycle. They were allowed free access to food and water.

Surgical procedure

Our technique was a modification of the method of Huang et al. (10). In brief, each male Long-Evans rat was anesthetized with halothane (1% to 3.5% in a mixture of 70% N_2O and 30% O_2) with the use of a mask. Body temperature was maintained during surgery at 37±0.5°C with a heating pad servo-controlled by a rectal probe. The right femoral artery was cannulated with PE-50 polyethylene catheters for continuous monitoring of heart rate and mean arterial blood pressure (MABP) by Statham P23 XL transducer and displayed on a Gould RS-3400 physiological Recorder (Gould, Cleveland, OH, USA).

Focal ischemic infarcts were made in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA). Both common carotid arteries were exposed by midline anterior cervical incision. The animal was placed in a lateral position, and a skin incision was made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle was retracted, and a small (3-mm diameter) craniectomy was made at the junction of the zygoma and squamosal bone using a drill (Dremel Multipro+5395, Dremel com. USA) cooled with saline solution. Using a dissecting microscope (OPMI-1, ZISS, Germany), the dura was opened with fine

forceps, and the right MCA was ligated with 10-0 monofilament nylon ties. Both common carotid arteries were then occluded by microaneurysm clips for 1 hr. After removing the clips, return of flow was visualized in the arteries. The middle cerebral artery was occluded for 1 hr and reperfusion 24 hr in anesthetized Long-Evans rats.

Plasma LDH analysis

Cellular damage was evaluated by measuring the LDH in plasma. Samples of arterial blood were drawn from the carotid catheter at the end after 1hr MCA occlusion and 24 hr reperfusion, collected in heparinized tubes. The blood was kept at 4°C until it was centrifuged at 2000 × g for 15 min. The plasma was recovered and aliquots were used for determination of LDH activity. LDH activity was measured spectrophotometrically, according to the method of Bergmeyer and Brent (11), by following the rate of conversion of NADH to NAD^+, at 340 nm.

Measurement of myeloperoxidase (MPO) activity in brain

MPO activity has been used as a quantitative assessment of neutrophil infiltration into brain ischemic areas. In this study, the method of quantifying the MPO activity in brain ischemic areas was performed according the method of Barone et al. with modification (12, 13). Twenty-four hours after cerebral infarction, animals were anesthetized and killed by rapid decapitation. Brain were removed, inspected visually for the anatomy of the MCA and for signs of hemorrhage or infection, and stored at –80°C for later biochemical analysis. The frozen tissue specimens weighting approximately 100 mg were homogenized in 1.5 ml of potassium phosphate buffer (PPB, 50 mM, pH 6.0). One ml of homogenate was centrifuged at 10,000×g for 10 min, and the pellet was suspended in 1 ml of PPB containing 0.5% hexadecyltrimethylammonium bromide (Sigma) to negate the pseudoperoxidase activity of haemoglobulin and to solubilize membrane-bound MPO. The suspensions were treated with three cycles fo freezing and thawing, sonicated on ice for 10 s, and centrifuged at 12,000×g for 10 min. MPO activity in the supernatants was assayed as described by Shen et al. (14). Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of PPB

containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005% hydrogen peroxide as a substrate for MPO. Oxidized *o*-dianisidine forms a soluble chromophore absorbing at wavelength of 460 nm and absorbance (OD_{460}) was determined by spectrophotometry over 2 min. The values of tissue MPO activity were expressed as $OD_{460}\times1000$ mg^{-1} of proteins. Protein concentration was determined with a BCA kit (Pierce, Rockford, USA).

Preparation of rat neutrophils

Peripheral whole blood of anesthetized male Long-Evans rats (270-250 g) was drawn from the cannulated carotid artery into tubes containing heparin (10 unit/ml). Neutrophils were isolated by dextran sedimentation and Ficoll-Hypaque centrifugation, followed by hypotonic lysis of residual erythrocytes as previously described (15).

Flow cytometric analysis of intracellular ROS production

Intracellular accumulation of ROS by neutrophils was measured by a flow cytometer (FACSCalibur^{TM}; Becton Dickinson) according to Robinson's method (16). Briefly, neutrophils (1×10^6/ml) were incubated at 37°C for 10 min with 20 μM of 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc.; Eugene, OR). The acetate moieties of DCFH-DA were cleaved off intracellularly by esterases, liberating the membrane impermeable 2',7'-dichlorofluorescin (DCFH), which fluoresced when oxidized to 2',7'-dichlorofluorescein (DCF) by H_2O_2. After labeling, cells were treated with resveratrol for 10 min and stimulated with PMA (100 ng/ml) or fMLP (1 μM). Production of intracellular H_2O_2 was then determined 30 min later by a flow cytometer for measurements with emission at 525 nm (FL1) for DCF. Data were expressed as percentages (%) of control by calculating the mean channel fluorescence resulting from resveratrol treatments over that of PMA or fMLP alone.

Drug administration

Resveratrol (Sigma, USA) was dissolved in 40% (v/v) propylene glycol to the desired

concentrations in normal saline. Final concentration of propylene glycol in the infected resveratrol solution was 4×10^{-3} % (v/v). At this concentration, propylene glycol had no effect on the infarct size of focal cerebral ischemia. In pretreatment or treatment groups, resveratrol solution of 0.3 ml was administered at four different doses (10^{-6}, 10^{-7} and 10^{-8} g/kg) via intravenous injection 15 minutes before MCA occlusion or when the common carotid arteries clips were removed. Rats injected with 0.3 ml normal saline with 4×10^{-3} % (v/v) propylene glycol were used as controls. Animals were randomly allocated to each drug treatment and control groups.

Statistics

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis of differences in ROS production between control and resveratrol treatment groups was carried by analysis of variance (ANOVA) followed by post-hoc Dunnett's *t*-test for multiple comparisons. While the difference LDH levels in plasma and MPO activity in brain tissue were statistically evaluated by unpaired Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

Results

Effects of resveratrol on plasma LDH

The effects of resveratrol on LDH activity in both pretreated and treated groups were shown in Fig. 2. Low LDH activity was seen in sham-operated animals (58.2±7.4 U/L) before occlusion. In the operated animals without resveratrol infusion, the LDH activity was increased to 259.1±35.4 U/L. In pretreatment group, at a resveratrol dose of 100 μg/kg, the LDH activity was reduced to 121.9±34.4 U/L (n=7). In treatment group, at a resveratrol dose of 100 μg/kg, the LDH activity was reduced to 50.4±13.5 U/L (n=7). Administration of resveratrol attenuated of LDH release with a dose-dependent manner during 1 hr MCA occlusion and 24 hr reperfusion.

Effects of resveratrol on MPO activity

We sequentially measured the MPO activity in the brain as a marker of neutrophilic infiltration in parenchyma and of neutrophilic adherence to endothelium after 1 hr middle cerebral artery occlusion and 24 hr reperfusion.

The effects of resveratrol on MPO activity in both pretreated and treated groups were shown in Fig. 3. The MPO activity in sham-operated rats was 28.5±1.9 $OD_{460}\times100$ mg^{-1} of proteins. In the operated animals without resveratrol infusion, the MPO activity of rats was increased to 92.3±7.8 $OD_{460}\times100$ mg^{-1} of proteins. In pretreatment group, resveratrol at a dose of 1 μg/kg, the MPO activity was decreased to 44.4±5.2 $OD_{460}\times100$ mg^{-1} of proteins (n=7). In treatment group, at a resveratrol dose of 1 μg/kg, the MPO activity was decreased to 42.1±3.6 $OD_{460}\times100$ mg^{-1} of proteins (n=7). In resveratrol-pretreated and -treated animals, MPO activity were significantly diminished.

Effects of resveratrol on PMA-induced ROS production

The effects of resveratrol on PMA -induced ROS production was shown in Fig. 4. At 30 minutes, PMA enhanced the fluorescence of DCF from the control levels (10.3±0.3 %) to 55.8±4.2. Pretreatment the cells with resveratrol (0.01, 0.1 and 1 μM) dose-dependent suppressed the PMA induced H_2O_2 accumulation to 47.3±5.6, 41.0±3.6 and 40.3±5.2, respectively.

Effects of resveratrol on fMLP-induced ROS production

The effects of resveratrol on fMLP-induced ROS production was shown in Fig. 5. At 30 minutes, fMLP enhanced the fluorescence of DCF from the control levels (11.7±2.8%) to 35.7±1.5. Pretreatment the cells with resveratrol (0.01, 0.1 and 1 μM) dose-dependent suppressed the fMLP induced H_2O_2 accumulation to 30.0±7.5, 26.3±5.5 and 22.7±4.2, respectively.

Discussion

Resveratrol is found in a wide variety of plant species. It is present mostly in the seeds and skin of grapes and constitutes one of the major components of red wine (1). It is the active component of Kojokon prepared from roots of Polygonum species and used in Asian traditional medicine to treat several diseases (17).

In previously study, we founded that pretreatment or treatment with resveratrol

possesses robust neuroprotection properties during focal cerebral ischemia in Long-Evans rats. Resveratrol (10^{-6} and 10^{-7} g/kg) provides reduction of injury in this model (9). In this study, compared with control group, the LDH activity revealed a large increase in the plasma of focal ischemia and reperfusion rats and reduced the enzyme activity in both pretreatment and treatment resveratrol administration groups. The LDH activity was used as an indicator of intracellular oxidative stress (18). The decreasing of LDH activity after resveratrol administration suggested that resveratrol might decrease the neuronal damages elicited after ischemia and reperfusion. This finding paralleled with neutrophil infiltration. Compared with control group, the change in myocardial MPO activity, a biochemical marker of neutrophil infiltration, which was considerably increased following focal cerebral ischemia and reduced by resveratrol pretreatment and treatment.

Recently, there are several reports have demonstrated that neutrophil infiltration in the brain tissue via neutrophil-endothelial cell interactions play a significant role in the pathogenesis of focal cerebral ischemia injury (19, 20). In addition, Matsuo et al., using a new electron spin resonance method coupled with microdialysis reported that free radicals were mainly generated by neutrophil during reperfusion in rat subjected to cerebral ischemia-reperfusion injury (21). Resveratrol act as a free radical remover. In this study, resveratrol may act through suppression of H_2O_2 to prevent the injury of the free radicals generated from neutrophil during ischemia-reperfusion injury.

In conclusion, the previously report represents the evidence that resveratrol can reduce the size of cerebral infarction. In this study indicated that the beneficial effect of resveratrol on neuroprotection may be in part related to its anti-inflammatory.

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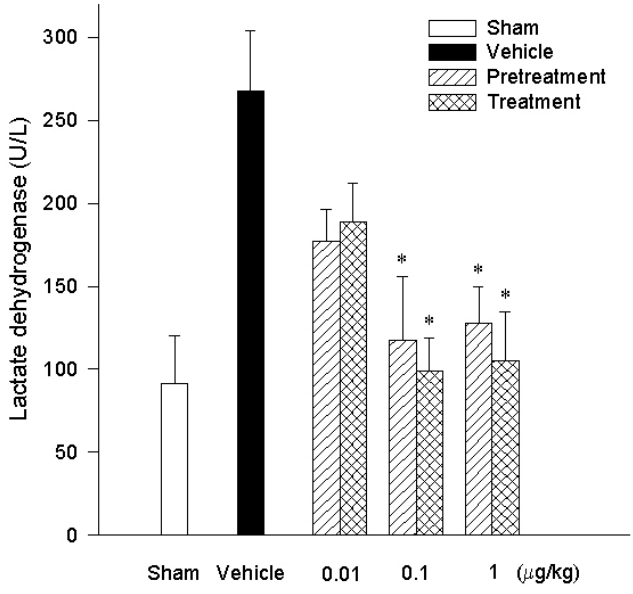

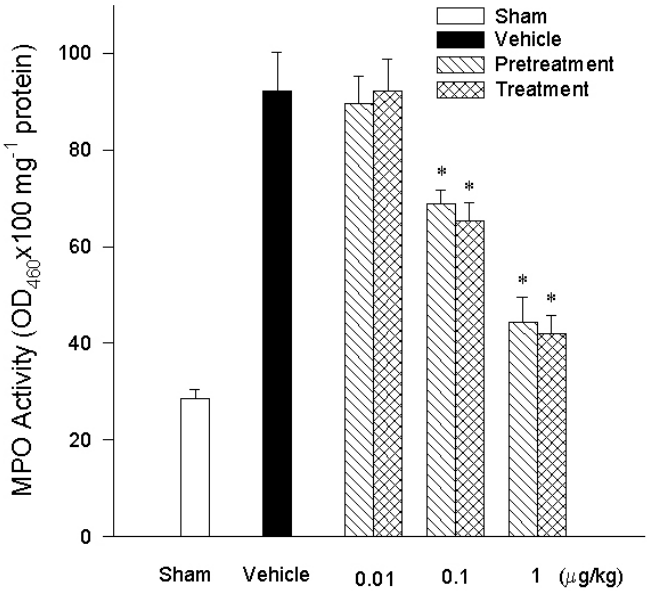
Sham
Vehicle
Pretreatment
Treatment
MPO Activity (OD460x100 mg-1 protein)
100
80
60
40
20
0
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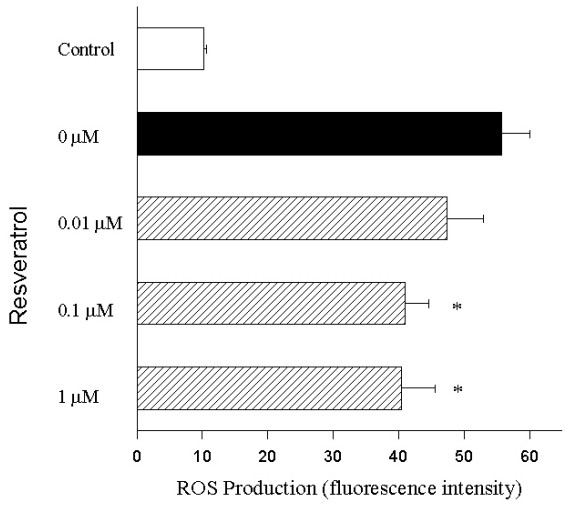
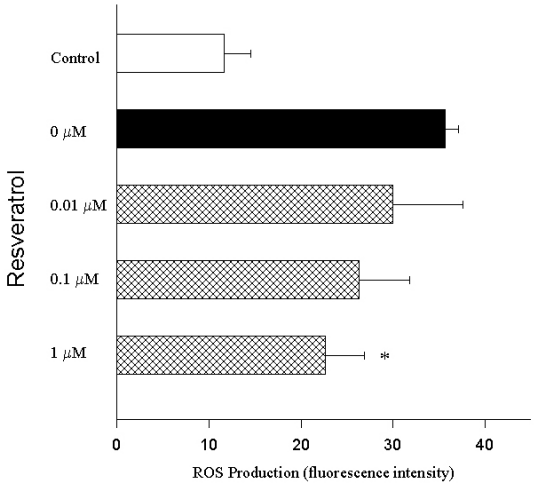
Sham
Vehicle
0.01
0.1
1 (μg/kg)


Control
0 μM
0.01 μM
0.1 μM
1 μM
Resveratrol
*
*
0
10
20
30
40
50
60
ROS Production (fluorescence intensity)


Control
0 μM
0.01 μM
0.1 μM
1 μM
Resveratrol
*
0
10
20
30
40
ROS Production (fluorescence intensity)