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 減輕大花鼠梗塞及再灌注性傷害之機轉與抑制嗜中性白血球活性的關係

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計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC－89－2314－B－002－295

執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：蔡勝國

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：國立台灣大學醫學院

中　華　民　國　90 年　7　月　27　日

in ischemic region.

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 up-regulation of NO production and by inhibition of neutrophil infiltration. Resveratrol is therefore a potential agent for the treatment of focal cerebral ischemia.

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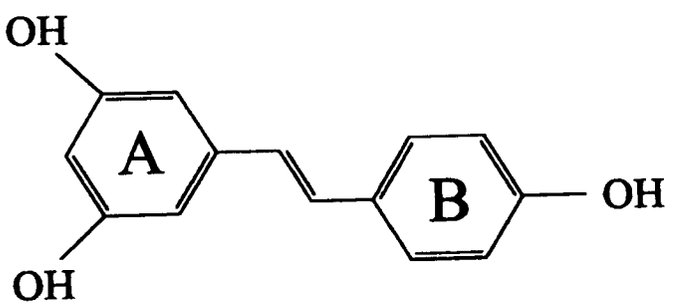
Legends for Figures

Fig. 1. Chemical structure of resveratrol.

Fig. 2. Changes in LDH activity in plasma after 1 hr MCA occlusion and 24 hr reperfusion. In pretreatment groups, resveratrol, at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg for 15 min before MCA occlusion. In treatment groups, resveratrol, at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg when the common carotid arteries clips were removed. Each experimental group is seven rats. Results are expressed as mean ± SEM. Statistical analysis was performed by unpaired Student's *t*-test (*: $p<0.05$ versus vehicle control.)

Fig. 3. Changes in NO (nitrate + nitrite) levels in plasma after of 1 hr MCA occlusion and 24 hr reperfusion. In pretreatment groups, resveratrol, at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg for 15 min before MCA occlusion. In treatment groups, FC_4S, at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg when the common carotid arteries clips were removed. Each experimental group is seven rats. Results are expressed as mean ± SEM. Statistical analysis was performed by unpaired Student's *t*-test (*: $p<0.05$ versus vehicle control.)

Fig. 4. Effect of resveratrol on focal cerebral ischemia induced neutrophil infiltration in the rat brain. In pretreatment groups, resveratrol at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg for 15 min before MCA occlusion. Neutrophil infiltration in the brain was determined by MPO activities and data were expressed as mean ± SEM. Each experimental group is seven rats. Statistical analysis was performed by unpaired Student's *t*-test (*: $p<0.05$ versus vehicle control.)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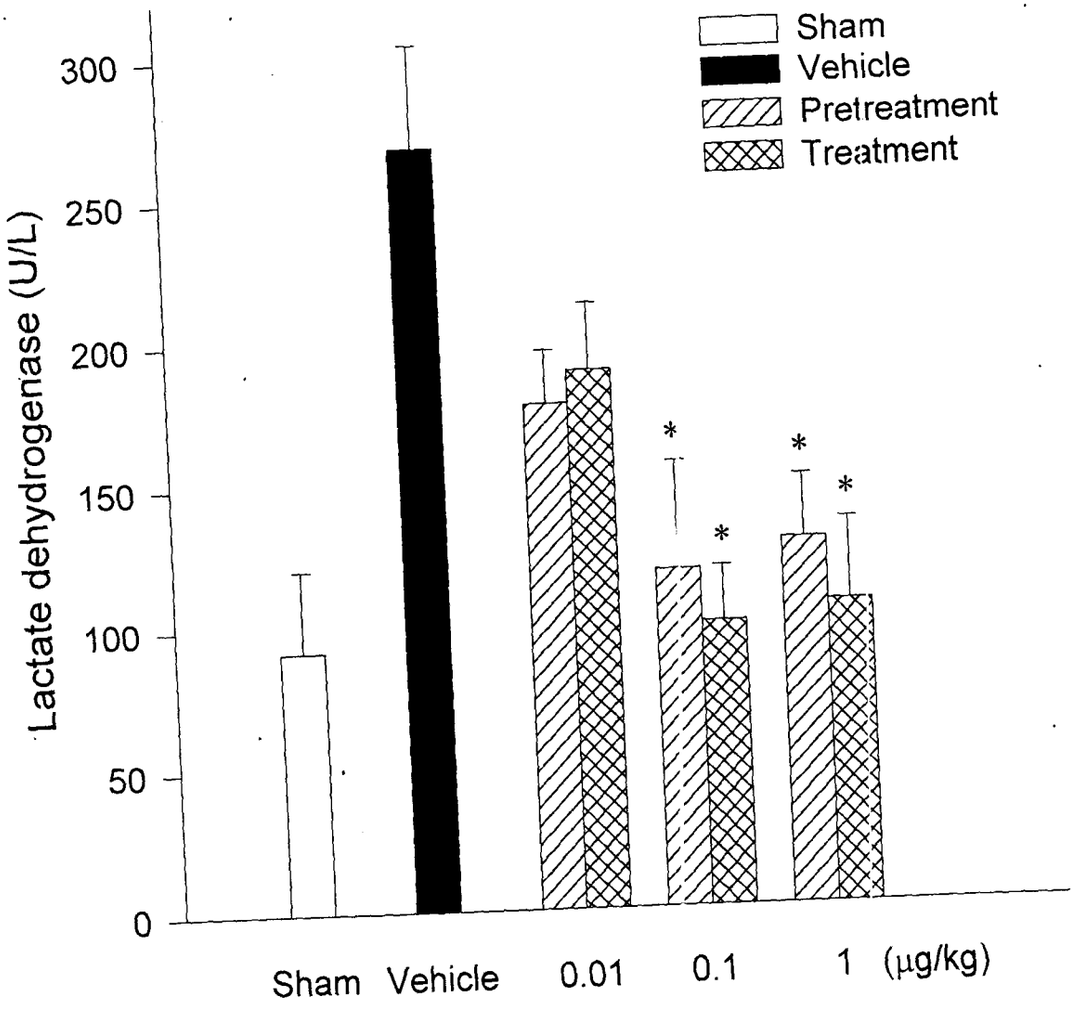
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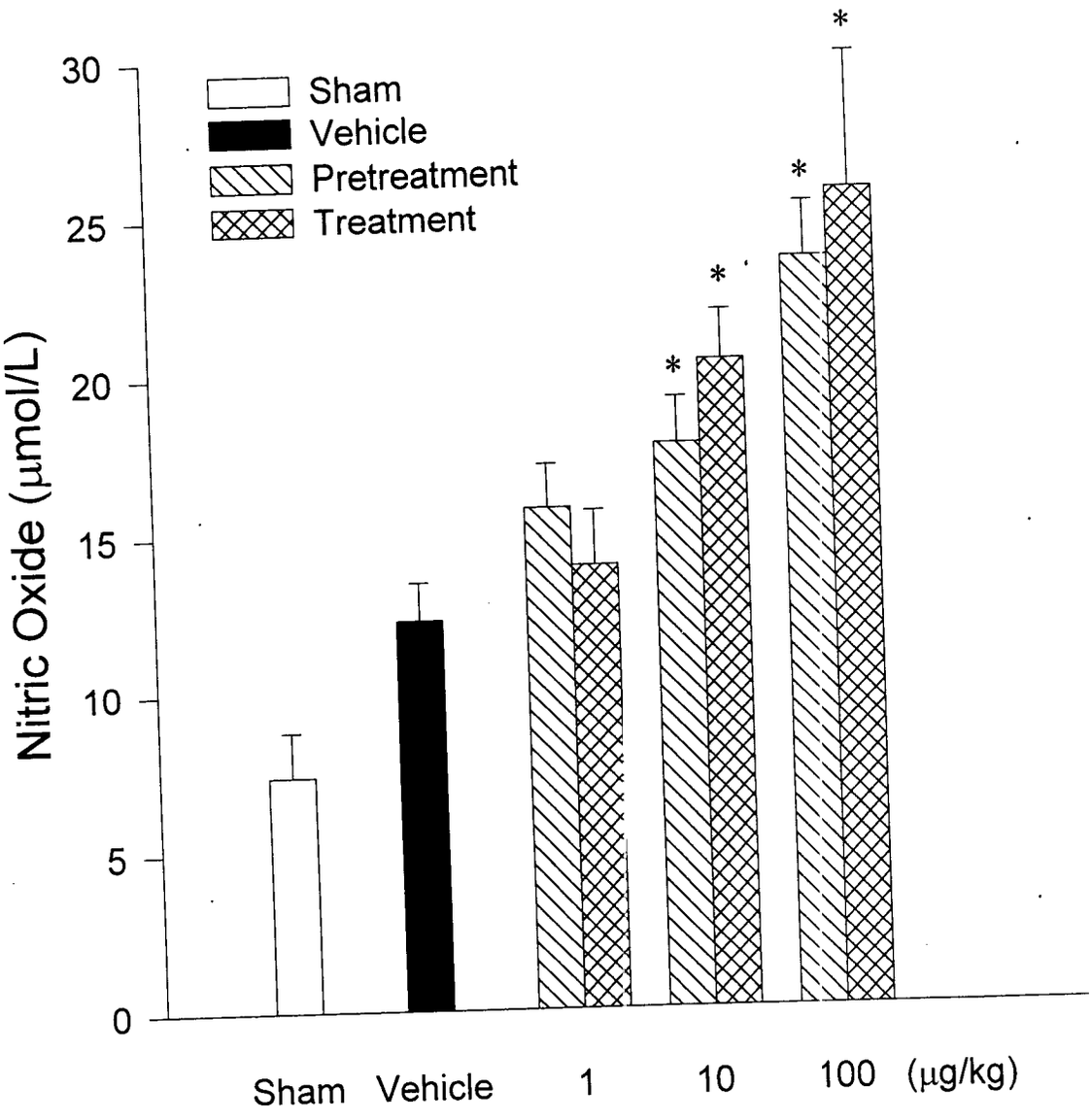
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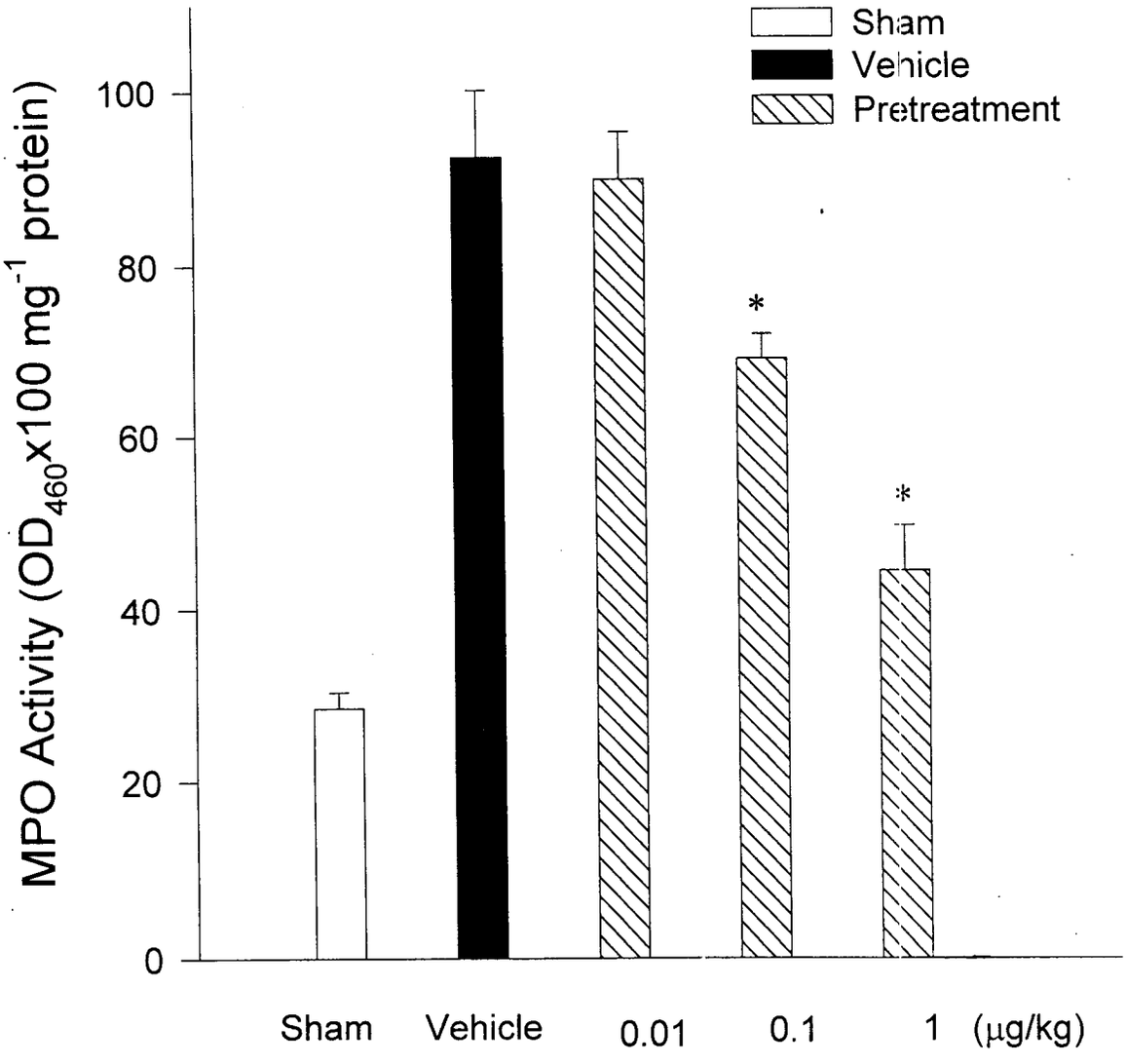
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1
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of differences in volume of infarcts between control and resveratrol treatment groups was carried by unpaired, two-tailed *t* tests, and by two-way analysis of variance (ANOVA) for combined data. Physiological measurements were compared among control and resveratrol-treated animals by unpaired, two-tailed *t* tests for each experiment using the Bonferroni correction for multiple pairwise comparisons. While the difference in plasma NO and LDH levels 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 release of NO

The effects of resveratrol on NO contents in both pretreated and treated groups were shown in Fig. 3. The NO content in sham-operated rats was 10.7±1.4 μmol/L. In the operated animals without resveratrol infusion, the NO content in plasma of rats was 11.2±1.1 μmol/L. In pretreatment group, resveratrol at a dose of 100 μg/kg, the plasma NO was increased to 17.5±1.6 μmol/L (n=7). In treatment group, at a resveratrol dose of 100 μg/kg, the plasma NO was increased to 19.8±2.7 μmol/L (n=7).

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. 4. 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 19.8±2.7 $OD_{460}\times100$ mg^{-1} of proteins (n=7). In resveratrol-treated animals, MPO activity was significantly diminished.

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 (14).

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. Compared with pre-drug control groups, it revealed that after resveratrol administration, the lactate dehydrogenase (LDH) levels in the plasma was decreased. The LDH activity was used as an indicator of intracellular oxidative stress (15). The decreasing of LDH activity after resveratrol administration suggested that resveratrol might decrease the neuronal damages elicited after ischemia and reperfusion. However, compared with pre-drug control groups, the nitric oxide (NO) content in the plasma was increased after resveratrol administration. There are several evidences in the literatures exhibiting a variety of physiological effects of NO including autoregulatory modulation of coronary blood flow [16, 17] and inhibition of neutrophil-endothelial interactions [18, 19] as well as platelet aggregation [20]. By

virtue of its free radical nature, NO can participate in a wide spectrum of biologically important reactions. No can direct biradical coupling with superoxide anions (21). NO can also direct attenuation of superoxide anion generation by neutrophils through inhibition of NADPH oxidase (22) and can inhibit neutrophil adherence to the endothelium (23). The neurotoxic and neuroprotective role of nitric oxide in experimental cerebral ischemia have generated considerable debate. Whereas, it has been proposed that interaction of NO with superoxide anion formed the potent oxidant peroxynitrite, thus, it will induce cellular injury [24, 25]. However, the free radical trapping activity of resveratrol may prevent the injury from the interaction of NO with superoxide anion which then will contribute to the increase of NO level.

Compared with control groups, 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.

Focal cerebral ischemia is associated with a massive production of intravascular free radicals [26, 27]. resveratrol act as a free radical remover. resveratrol may act through suppression of $O2^{-}$, thus protecting endothelium cell and leading to exert a dose-dependent enhancing NO production. NO is also known to inhibit platelet aggregation [20], which helped to prevent the blockage of microvascular blood flow

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 $OD_{460}\times100$ mg^{-1} of proteins. Protein concentration was determined with a BCA kit (Pierce, Rockford, USA).

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}, 10^{-8} and 10^{-9} 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 $\pm$ standard error of mean (SEM). Statistical analysis

of differences in volume of infarcts between control and resveratrol treatment groups was carried by unpaired, two-tailed *t* tests, and by two-way analysis of variance (ANOVA) for combined data. Physiological measurements were compared among control and resveratrol-treated animals by unpaired, two-tailed *t* tests for each experiment using the Bonferroni correction for multiple pairwise comparisons. While the difference in plasma NO and LDH levels were statistically evaluated by unpaired Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

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.

Plasma LDH and NO 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 (17), by

following the rate of conversion of NADH to NAD^+, at 340 nm.

The deproteinized plasma samples were frozen and kept until analysis. For measurement of NO we employed the NO/ozone chemiluminescence technique (280 NOA^{TM}, Sievers Instruments, Boulder, CO 80303, USA) (18). The detection of plasma NO level is based on its reaction with ozone, which leads to the emission of red light. The photons from this reaction are detected and transformed to an electrical signal by a photomultiplier tube (PMT). Due to the use of filters in front of the PMT, NO/O_3 chemiluminescence recorded with the Sievers NOA 280^{TM} is highly specific for NO. The current from the PMT is A/D converted and fed into a PC running the Asyst software (Sievers NO Analysis Liquid Program, USA). The amount of light produced by NO/O_3 chemiluminescence is proportional to the amount of NO sampled. Hence, the calculated area under the curve of the PMT current for each determination is proportional to the amount of NO. This was verified before each experiment by standard curves (1, 5, 10, 20, 40, 100 μmol/L) which were produced using freshly prepared solutions of sodium nitrite in distilled water, which was reduced to NO in an equimolar manner by the reducing agent. We chose to measure the level of nitrite or nitrate on blood sample, by using a reaction vessel containing a reducing system (Vanadium (III) dissolved in 1 M HCl), to which the sample was injected and NO was generated from nitrite or nitrate in an equimolar manner. A continuous stream of

Helium (99.999%) purged the resultant NO from the reaction vessel to the chemiluminescence chamber.

Measurement of myeloperoxidase (MPO) activity in brain

MPO activity has been used as a quantitative assessment of neutrophil infiltration into brain ischemic areas (11, 12). In this study, the method of quantifying the MPO activity in brain ischemic areas was performed according Barone et al., (1991) with modification. 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. (13). Briefly, 0.1 ml of the supernatant was mixed with 2.9 ml of PPB containing 0.19

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中文摘要

Resveratrol 廣泛的存在在許多種植物中，它不只在葡萄的種皮以及種子中具有豐富的含量，它也是存在紅酒中的一個主要的化合物。在本研究中，我們評估在麻醉的大花鼠經過一個小時的中大腦動脈結紮後再灌注二十四小時，嗜中性白血球浸潤至腦組織的情形，以及研究在大花鼠在一個小時的中大腦動脈結紮後再灌注二十四小時期間，血漿中一氧化氮產生的量以及 Lactate dehydrogenase (LDH) 的活性。研究顯示，和沒有給予 Resveratrol 的對照組比較，大花鼠在經過在一個小時的中大腦動脈結紮後再灌注二十四小時期間，給予 Resveratrol 的治療組可以有意義的減少血漿中 Lactate dehydrogenase (LDH)的活性以及增加血漿中一氧化氮產生的量。然而測量 MPO 的活性，MPO 的活性為觀察嗜中性白血球浸潤至組織中的一個生化監測標記，我們發現大花鼠在經過在一個小時的中大腦動脈結紮後再灌注二十四小時，其 MPO 的活性明顯的增加，而預先給予 Resveratrol 的治療組可以有意義的減少 MPO 的活性。由結果顯示對於大腦經過缺血的傷害，Resveratrol 是一個很有效的神經保護藥物，它的保護作用可能是藉由增加血漿中一氧化氮的產生以及減少組織中嗜中性白血球的浸潤而來，所以我們推定 Resveratrol 對於治療大腦缺血的傷害是一個很有潛力的藥物。

關鍵字: resveratrol，紅酒，中風，局部大腦缺血，梗塞

The Role of Nitric Oxide and Neutrophils on The Neuroprotective Effect of Resveratrol After Focal Cerebral Ischemia in Long-Evans Rats

Shen Kou Tsai and Shiang Suo Huang

The Role of Nitric Oxide and Neutrophils on The Neuroprotective Effect of Resveratrol After Focal Cerebral Ischemia in Long-Evans Rats

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Abstract

Resveratrol is found in a wide variety of plant species. It is present in the seeds and skin of grapes and constitutes one of the major components of red wine. This study was undertaken to evaluate the neutrophil infiltration in the brain and to study the NO production and the lactate dehydrogenase (LDH) activity in plasma during the middle cerebral artery was occluded for 1 hr and 24 hr reperfusion in anesthetized Long-Evans rats. Compared with pre-drug control groups, it revealed that after resveratrol administration, the lactate dehydrogenase (LDH) levels in the plasma was decreased and the nitric oxide (NO) content in the plasma was increased after resveratrol administration. However, compared with control groups, 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. Resveratrol is a potent neuroprotective agent in focal cerebral ischemia. Its beneficial effects may be part related to its up-regulation of NO production and by inhibition of neutrophil infiltration. Resveratrol is therefore a potential agent for the treatment of focal cerebral ischemia.

Key words: resveratrol, red wine, stroke, focal cerebral ischemia, infarct maturation

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. 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 hypothesize the neuroprotective effects of resveratrol may be related to its anti-platelet aggregation activity, vasodilating effect, antioxidant property or by all mechanisms together (9).

Resveratrol is a potent neuroprotective agent in focal cerebral ischemia. The purpose

of this study was to examine whether resveratrol protect brain subjected to focal cerebral ischemia through suppressing neutrophil function. Besides, whether nitric oxide (NO) was involving in the neuroprotective effect of resveratrol. The present experiment was undertaken to evaluate the neutrophil infiltration in the brain and to study the NO production and the lactate dehydrogenase (LDH) activity in plasma during the middle cerebral artery was occluded for 1 hr and 24 hr reperfusion in anesthetized Long-Evans rats.

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 Koketsu 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.

Plasma LDH and NO 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 (17), by