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Neuroprotective Effects of Resveratrol on MPTP-Induced Neuron Loss Mediated by Free Radical Scavenging

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Resveratrol is a natural polyphenol and possesses many biological functions such as anti-inflammatory activity and protection against atherosclerosis and myocardial infraction. Parkinson's disease is a common progressive neurodegenerative disease. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the most useful neurotoxin to induce Parkinsonism. The present study was carried out to elucidate the neuroprotective effect and possible mechanism of resveratrol on MPTP-induced striatal neuron loss. Sixty adult Balb/c mice were divided into four groups: sham operation, MPTP treatment (30 mg/kg, ip), MPTP combined with resveratrol administration (20 mg/kg, iv), and resveratrol treatment alone. Microdialysis and high-performance liquid chromatography were used to analyze dihydroxybenzoic acid (DHBA) that reflected the hydroxyl radical level. In the present study, we found MPTP chronic administration significantly induced motor coordination impairment in mice. After MPTP administration, the hydroxyl radical levels in substantia nigra were also significantly elevated and animals displayed severe neuronal loss. Resveratrol administration significantly protected mice from MPTP-induced motor coordination impairment, hydroxyl radical overloading, and neuronal loss. Our results demonstrated that resveratrol could elicit neuroprotective effects on MPTP-induced Parkinsonism through free radical scavenging.

KEYWORDS: Resveratrol; Parkinson's disease; MPTP; free radicals; neuronal damage

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring polyphenol which belongs to the phytoalexin family. It has been found in the seeds of various plant species including grapes and peanuts and constitutes one of the components of red wine (*1, 2*). Resveratrol exhibits many biological functions such as anti-inflammatory activity attributed to inhibition of cyclooxygenase, estrogenic activity, and antiplatelet activity (*3, 4*). Recently, resveratrol was found to have a beneficial effect in treatment of ischemia (*5*) and neurodegenerative disease (*6, 7*).

Parkinson's disease (PD) is a common progressive neurodegenerative disease which is characterized by muscle rigidity, akinesia, and resting tremor. The characteristic pathological and biochemical changes are severe loss of dopamine (DA) cell bodies in the substantia nigra (SN) and a severe decrease of DA concentration in the striatum (nigrostriatal pathway). Accumulating evidence indicates that multiple factors, including genetic and environmental ones, contribute to acceleration of dopaminergic neurodegeneration in this neurological disorder (*8*). Free radical damage has been shown to have a significant impact on the pathogenesis of PD. Dopamine is a relatively unstable molecule, subject to hydroxyl radical attack, that can induce free radicals both from within the cell as well as from outside the cell. At present, the most useful neurotoxin used to induce Parkinsonism is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (*9, 10*). MPTP is metabolically converted to 1-methyl-4-phenyl pyridinium (MPP^+) which interferes with mitochondrial respiration via inhibition of mitochondrial complex I, thereby triggering dopaminergic neurodegeneration that leads to Parkinsonism.

A growing body of evidence supports that the chronic administration of resveratrol could protect a variety of tissues against ischemic injury by reducing the free radical production (*5, 11, 12*). The present study is aimed to determine the possible

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neuroprotective effects of resveratrol on MPTP-induced Parkinsonism and to elucidate its possible mechanisms.

MATERIALS AND METHODS

Animals. Adult male Balb/C mice (20−25 g, $n = 60$) were purchased from the Animal Center of the National Science council (Taipei, Taiwan). The animals were housed five in a group at an ambient temperature of 22 ± 1 °C, with a 12 h light/dark cycle. Pelleted rat chow and tap water were available ad libitum. The phytoestrogen levels of rat chows are normal. All the procedures were approved by the Animal Care and Use Committee. The care and handling of the animals were in accord with internationally recognized guidelines for ethical animal research. At the end of the experiments, mice were killed with an overdose of pentobarbital (100 mg/kg, ip). All efforts were made to minimize the animal numbers, which are required to produce meaningful experimental data.

Drugs. MPTP was purchased from Sigma (U.S.A.) and dissolved in saline. Resveratrol (20 mg/kg, iv, Sigma-Aldrich, Chemical Co., St. Louis, MO) was dissolved in 20% ethanol. Drugs were prepared freshly right before using. Mice were randomly assigned to four different groups of 60 animals. They received one of the following treatments: sham operation (vehicle; 20% ethanol), MPTP treatment (30 mg/kg, ip) (vehicle + MPTP), MPTP combined with resveratrol administration (20 mg/kg, iv) (resveratrol + MPTP), and resveratrol alone (resveratrol). All animals received a single combination of drug injection per day for a total of 7 days.

Rota-Rod Accelerating Test. Animals were placed in the rota-rod apparatus (Ugo Basile, Varese, Italy) and received the accelerating test. Animals were placed on the rod while it is rotating at its slowest speed (4 rpm). The rotational speed of the rod then was automatically increased to its maximum of 40 rpm over the next 300 s. Animals remaining on the rod at this speed were tested for up to an additional 600 s. The animal's score for the week is the average number of seconds it remained on the rod per trial. Change of the rota-rod performance represents impairment of the motor coordination (*13, 14*).

Grasp Strength Analysis. To assess the animal's grasp strength, the animal was held gently by the examiner and its front paws were placed on the holding bar of the grasp strength analyzer which was assembled by us. The grasp strength (in g) was recorded and analyzed. Each session included six trials. The animal's score for the week is the average of the six trials.

Measurement of Extracellular Free Radicals. A 2 mm burr hole was made in the right parietal bone over the SN for the placement of the microdialysis probes. The dura mater was removed, and the microdialysis probe with a 4 mm long dialysis membrane was implanted vertically into the right SN with the following coordinates: anterior, −0.9 mm; lateral, 2.8 mm; horizontal, 4.7 mm from the top of the skull. Hydroxyl radical concentrations were measured by the production of dihydroxybenzoic acid (DHBA) (Sigma-Aldrich, Chemical Co., St. Louis, MO), which resulted from the hydroxylation of sodium salicylate by hydroxyl radicals. The microdialysis probe was perfused with aCSF (149 mM NaCl, 2.8 mM KCl, 1.3 mM $CaCl_2$, 0.125 mM ascorbic acid, and 5.4 mM D-glucose, pH 7.2−7.4) which contains 2 mM sodium salicylate at a constant flow rate of 1.5 μL/min by means of a microinjection pump (CMA 102; CMA Microdialysis Inc., Stockholm, Sweden). Of each dialysate, 25 μL was injected into a high-performance liquid chromatography system (Bioanalytical System Inc., West Lafayette, IN) equipped with an electrochemical detector (Bioanalytical System Inc., West Lafayette, IN) and a phase II ODS-3 column (Alltech Asso. Inc., IL). Elution of DHBA was accomplished with a 100 mM monochloroacetic acid buffer with 0.5 mM EDTA added 1% methanol and 1% tetrahydrofuran.

Histology Evaluation. At the end of the experiments, mice were sacrificed with an overdose of pentobarbitol (100 mg/kg sodium pentobarbital, ip) and were then perfused transcardically with 0.9% NaCl and 10% neutral formalin. After formalin perfusion, the mice were decapitated and their brains were removed from the skulls and embedded in paraffin blocks. Coronal sections (15 μm) through the brain were stained with hematoxylin and eosin (H&E) for microscopic evaluation. Each hemisphere was evaluated independently without the examiner knowing the experimental conditions.

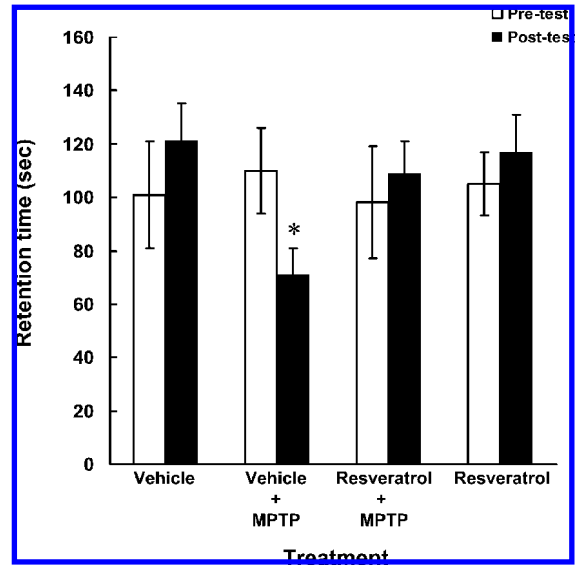

Figure 1. Effects of MPTP and resveratrol administration on the retention time of rota-rod in the mice. Bars represent mean ± SEM values ($n = 12$). *, $p < 0.05$, significantly different from control values; ANOVA.

Statistical Analysis. Data from experiments were expressed as mean ± SEM. Statistical analysis was conducted by ANOVA with Student's t test. A p-value <0.05 was considered statistically significant.

RESULTS

The first experiment evaluated the effect on motor coordination among groups. In the present study, we found MPTP administration significantly decreased the retention time from 120 ± 15 to 70 ± 20 s, which suggests MPTP administration significantly induced the impairment of motor coordination. In comparison with the MPTP alone group, resveratrol administration significantly increase the retention time from 70 ± 20 to 100 ± 12 s. This result suggests that resveratrol could partially recover the motor coordination impairment which is induced by MPTP administration (**Figure 1**).

Motor function impairment is one characteristic of PD. The grasp strength test is used to evaluate the motor function. The effect of MPTP and MPTP combined with resveratrol administration on grasp strength is shown in **Figure 2**. In comparison with the control group, MPTP significantly increased the grasp strength from 100 ± 15 to 135 ± 12 g which is due to the increasing of muscle rigidity. Resveratrol administration significantly reduces the MPTP-induced muscle rigidity in the animals and decreases the grasp strength to the basal level.

In this study, we found resveratrol could significantly protect animals from MPTP-induced motor coordination and function impairment. Our previous study indicates the resveratrol administration significantly attenuates the cerebral ischemia-induced free radical overloading. A similar mechanism may also take place here. To test this hypothesis, the third experiment was aimed to test the effect of MPTP and resveratrol on extracellular DHBA concentration in the SN, and the results are shown in **Figure 3**. The DHBA value could reflect the levels of free radicals. It was evidenced that DHBA increased about 1.4-fold after MPTP administration and resveratrol administration could significantly attenuate the DHBA overloading in the SN.

The histological photographs of neuronal damage are shown in **Figure 4**. In comparison with the control mice, mice subjected

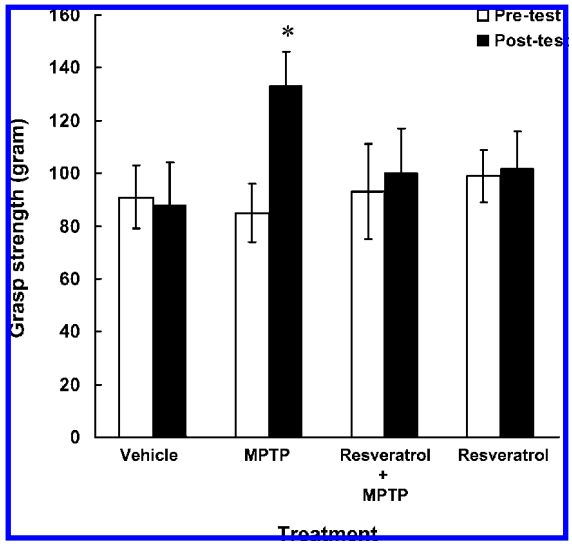

Figure 2. Effects of MPTP and resveratrol administration on the grasp strength in the mice. Bars represent mean ± SEM values ($n = 12$). ∗, $p < 0.05$, significantly different from control values; ANOVA.

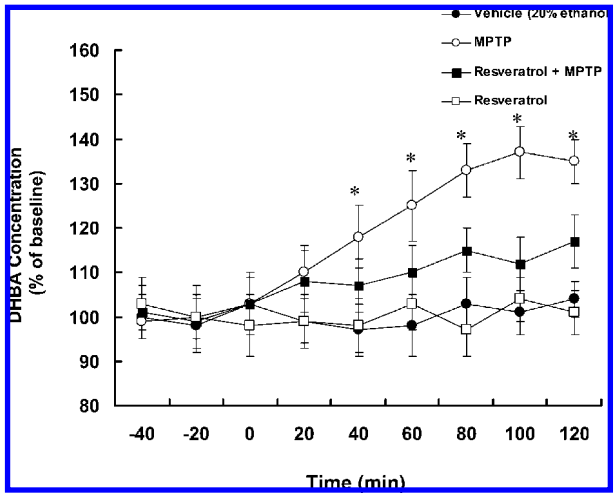

Figure 3. Effects of resveratrol administration on DHBA concentration in the substantia nigra (SN). Solid circles indicate values in vehicle controls. Open circles indicate values in MPTP-treated animals. Solid squares indicate animals treated with MPTP and resveratrol, and open squares indicate animals treated with resveratrol alone. Points represent mean ± SEM values. ∗, $p < 0.05$, significantly different from control values.

to MPTP injection display severe neuronal damage in the SN in which it appeared that more neurons are shrunk and damaged. Administration of resveratrol significantly reduced the neuronal damage in the SN, which appeared to be consistent with the improvement of motor function.

DISCUSSION

In the present study, we provide the evidence that resveratrol administration attenuated the MPTP-induced Parkinsonism, which included motor coordination and function impairment. Resveratrol administration also decreased the MPTP-induced neuronal damage and free radical overloading in the SN. We suggested that resveratrol may elicit neuroprotective effects on MPTP-induced Parkinsonism through free radical scavenging.

MPTP selectively damages dopaminergic neurons in the SN and has been proposed as an experimental animal model to study PD (*15, 16*) for many decades. The catecholamine-degrading enzyme monoamine oxidase B transforms MPTP into toxic

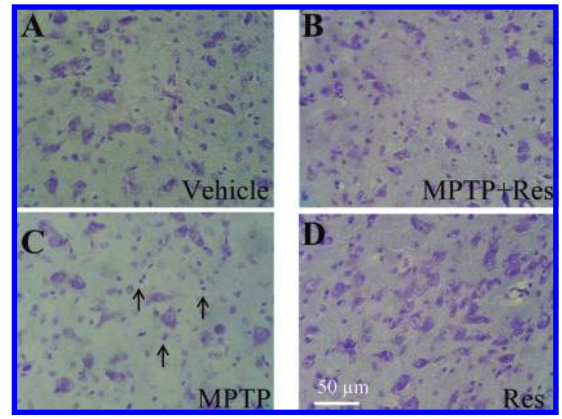

Figure 4. Photomicrographs showing the morphology of the substantia nigra (SN) neurons in vehicle control mice (**A**), mice treated with MPTP (**C**), or mice treated with MPTP and resveratrol (20 mg/kg, iv) (**B**), and mice treated with resveratrol alone (**D**). As compared to the vehicle control group, MPTP administration significantly induced neuronal shrinkage and damage (indicated as arrows) and administration of resveratrol significantly attenuated this MPTP-induced neuronal loss.

metabolite, MPP^+. Dopaminergic neurons are the primary targets of MPP^+. MPP^+ accumulates in mitochondria and interrupts the complex I of the respiratory chain. MPP^+ interaction with complex I not only blocks ATP production but also promotes oxygen free radical formation (*17*). Oxygen free radicals could damage mitochondria and other cellular components and ultimately cause dopaminergic neuron death. In the present study, we found DHBA concentration significantly increased in 40 min after MPTP administration. Resveratrol administration significantly decreased the MPTP-induced free radical overloading. This result provides in vivo evidence that resveratrol protects animals from MPTP-induced neuronal damage via free radical scavenging. However, MPP^+ not only induced free radical release but also enhanced DA release (*18*). Accumulating evidence suggests that enzymatic breakdown by monoamine oxidase or autoxidation of excess cytosolic DA triggers neurotoxic reactive oxygen species formation (*19*), which has been implicated in the pathogenesis of PD. The oxidation of DA can be catalyzed by itself and could enhance the MPP^+ redox cycling which results in the local increase in free radicals (*20*). We demonstrated that resveratrol administration decreases the MPTP-induced free radicals release which may be partially induced by DA autoxidation.

Recent studies have evidenced a possible neuroprotective role for estrogens in neurodegenerative disease, including PD (*21*). Estrogen decreases the striatal DA depletion in MPTP-intoxicated mice and in 6-OHDA-lesioned rats (*22, 23*). Estrogen use has been associated with lower symptom severity in women with early onset of PD (*24*). It is also found that hormonal replacement therapy significantly improved dyskinesia without worsening motor disability (*25*). Although evidence suggests the beneficial effects of estrogen in PD, many women turn to phytoestrogen (plant-derived nonsteroidal estrogen) because of the latter's undesirable side effects, such as increased risk of breast and endometrial cancer (*26*). Resveratrol is a natural polyphenol which is present in high concentrations in peanut and grape. Resveratrol has been studied for its potential beneficial effects such as anti-inflammatory activity and protection against atherosclerosis and myocardial infraction (*27*). Our previous studies also suggested that a single infusion of resveratrol could elicit neuroprotective effects on cerebral ischemia-induced neuron damage through free radical scavenging and cerebral blood elevation due to NO release (*5*). Recently,

it is demonstrated that resveratrol has neuroprotective effects on dopaminergic neurons in the MPTP-induced PD in mice that may be attributed to enhanced Bcl-2 gene expression (*28*), restored DA transporter expression (*29*), or increased heme oxygenase activity (*30*). The present studies suggest that resveratrol not only scavenges the MPTP-induced free radical overloading but also decreases the neuronal damage and motor function impairment. This finding may open the door toward the rational design of a neuroprotective antioxidant with decreased hormonal side effects.

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