Eugenol [2-Methoxy-4-(2-propenyl)phenol] Prevents 6-Hydroxydopamine-Induced Dopamine Depression and Lipid Peroxidation Inductivity in Mouse Striatum

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As superoxide (·O2−) and hydroxyl radical (·OH) have been implicated in the pathogenesis of Parkinson disease, free radical scavenging and antioxidants have attracted attention as way to prevent progression of this disease. We examined the effects of eugenol, an essential oil extracted from cloves, on 6-hydroxydopamine (6-OHDA)-induced dopamine (DA) depression in the mouse striatum. Eugenol administration 3 d before and 7 more days following one intracebroventricular 6-OHDA injection prevented the reduction of striatal DA and its metabolites. Eugenol administration for 3 d reduced the increase of thiobarbituric acid-reactive substances (an indicator of lipid peroxidation) induced by ferric ion and increased glutathione (GSH) and l-ascorbate (Asc) in the striatum. Eugenol did not change the levels of catalase, glutathione peroxidase, or superoxide dismutase-like activities. Eugenol is known to have ·O2− and ·OH scavenging activities in vitro. These results suggest that eugenol prevents 6-OHDA-induced DA depression by preventing lipid peroxidation directly and indirectly (via stimulation of GSH and Asc generating systems). Furthermore, increased GSH may protect cell death by conjugating with p-quinone produced in 6-OHDA auto-oxidation. The effects of eugenol treatment in this model suggest its possible usefulness for the treatment of Parkinson disease.

Key words Parkinson disease; eugenol; antioxidant; dopamine; lipid peroxidation; glutathione

Parkinson disease (PD) is characterized by progressive degeneration of dopaminergic neurons of the nigrostriatal system and dopamine (DA) depletion in the striatum. While the pathogenesis of PD is not clear, damage of dopaminergic neurons by oxygen derived free radicals is considered to be an important contributing mechanism. 1) 6-Hydroxydopamine (6-OHDA) is used to produce an animal model of PD, 2) and is considered to be an endogenous toxin, having been found in urine from parkinsonian patients. 3) The toxicity of 6-OHDA is thought to be related to its ability to produce free radicals and to cause oxidative stress. 4,5) 6-OHDA is susceptible to auto-oxidation, resulting in the formation of 6-OHDA quinone and hydrogen peroxide (H2O2), superoxide radical (·O2−), and hydroxyl radical (·OH). 4–7) These active oxygen species are neurotoxic because of their strong oxidizing potential. Various studies have shown that eugenol is an antioxidant and can prevent 6-OHDA-induced neurotoxicity. 5) We also reported that zingerone, an alkaloid extracted from ginger root, protected against 6-OHDA-induced depletion of striatal DA by increasing SOD activity. 9) Eugenol (4-allyl-2-methoxyphenol), a naturally occurring phenol extracted from cloves, is known to be an antioxidant 10–12) and a monoamine oxidase (MAO) inhibitor, 13) and to have neuroprotective effects. 14)

In this study, we examined the effects of eugenol on 6-OHDA-induced DA depletion in the mouse striatum, and on the activities of catalase and GPx as well as superoxide scavenging activity (SOSA) in the mouse striatum. We also examined the levels of antioxidants such as GSH and Asc, and the lipid peroxidation-inhibiting activity of eugenol.

MATERIALS AND METHODS

Effect of Eugenol on Oxidation of Mouse Brain Homogenate in Vitro  Mouse brains were homogenized in 10 volume of ice-cold Tris–HCl buffer (pH 7.6, 50 mm). After eugenol in 1% ethanol/saline solution (100 µl; final concentration: 0.1—1000 µM) and ferric chloride in Tris–HCl buffer solution (20 µl; final concentrations: 5 µM) was added to homogenate (100 µl), the mixture was incubated at 37 °C for 20 min to induce oxidation. Lipid peroxides produced were monitored by measuring concentrations of thiobarbituric acid reactive substance (TBARS).

Effect of Eugenol on SOD-Like Activity of Mouse Brain Homogenate in Vitro  Mouse brains were homogenized with 10 volume of ice-cold phosphate buffer (pH 8.0, 10 mm) and centrifuged at 10000×g for 10 min at 4 °C. After eugenol in 1% ethanol/saline solution (100 µl; final concentrations: 0.1—1000 µM) was added to the supernatants, SOD-like activity of the mixture was measured using a SOD assay kit (SOD Test Wako: Wako Pure Chemical, Japan).

Animals  Male ICR mice (Charles River, Japan Inc., Yokohama, Japan) weighing 30 to 35 g were used in all experiments. They were provided with water and standard laboratory diet containing 24% protein (MF; Oriental Yeast, Tokyo, Japan) ad libitum, and housed at 25 °C with a 12-h light and 12-h dark cycle (7 a.m. to 7 p.m., lights on). New mice were used for every assay. The experimental protocol was approved by the Ethics Review Committee for the Animal Experimentation of the Kagawa Prefectural College of...
Health Sciences.

Effect of Treatment with Eugenol on Activity of Striatal Active Oxygen Scavenging Enzymes, Lipid Peroxidation Level, and GSH and Asp Levels in Mouse Striatum

Eugenol was dissolved with ethanol and diluted with 99% volume of saline, and was injected subcutaneously at a dose of 1.0 or 0.1 μmol/kg body weight. The same volume of 1% ethanol/saline (5 ml/kg body weight) was injected as control. After 3 days' administration (once daily) of eugenol, mice were killed 30 min after the last administration under sodium pentobarburate anesthesia, and the striatum was removed on an ice plate and stored at -80 °C until analysis.

Effect of Treatment with Eugenol against 6-OHDA Toxicity

Eugenol was injected subcutaneously at a dose of 1.0 or 0.1 μmol/kg body weight. After 3 days' administration (once daily) of eugenol, 6-OHDA was injected into right lateral ventricle at a dose of 60 μg in 2 μl of saline 30 min after the last administration of eugenol. Two microliters of saline was injected as control. Eugenol was injected at the same dose once daily for 7 d after 6-OHDA injection. Seven days after 6-OHDA injection, mice were killed under sodium pentobarburate anesthesia and the right striatum was removed on an ice plate and stored at -80 °C until analysis.

DA, 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Homovanillic Acid (HVA) Levels

Right striatal tissues were homogenized with 500 μl of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (10000×g for 10 min at 4 °C), the supernatant was filtered (pore size, 0.45 μm) and then injected directly into a HPLC (Shimadzu, Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode vs. Ag/AgCl reference electrode) was set at 700 mV. The analytic column was TSKgel Super-ODS (4.6 mm I.D.×100 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate–sodium acetate buffer (pH 3.9) containing methanol (18%, v/v), disodium EDTA (4 mg/l), and sodium octanesulfonate (0.8 mm).

Superoxide Scavenging Activity (SOSA)

SOSA was assayed by measuring · O$_2^-$ using electron spin resonance (ESR) spectrometry of 5,5’-dimethyl-1-pyrroline N-oxide (DMPO)–· O$_2^-$ adducts after the · O$_2^-$ produced in the hypoxanthine (HPX)/xanthine oxidase (XOD) system was trapped by DMPO. Striatal tissues were homogenized with 10 volumes of ice-cold phosphate buffer (10 mM, pH 8.0) and centrifuged at 12000×g for 10 min at 4 °C. A mixture of 50 μl of 2.0 mM HPX, 35 μl of 11 mM diethylenetriaminepentaacetic acid (DETAPAC), 50 μl of sample (supernatant after homogenate centrifugation, or serum), 20 μl of DMPO–water solution (DMPO : water, 1 : 1), and 50 μl of XOD was freshly prepared and transferred to the ESR spectrometry cell. DMPO–· O$_2^-$ spin adducts was quantified 60 s after addition of XOD. The signal intensities were evaluated by the peak height of the first signal of the DMPO–· O$_2^-$ spin adducts. SOD from bovine erythrocytes (Sigma, St. Louis, MO, U.S.A.) was used as a standard.

Catalase Activity

Catalase activity was assayed according to the method described by Aebi.$^{15}$ In brief, the striatum was homogenized with 20 volumes of ice-cold RIPA buffer [0.1 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.01% digitonin, and 0.25% sodium cholate] and centrifuged at 10000×g for 30 min at 4 °C. Phosphate buffer (50 mM, pH 7.0) containing EDTA (5 mM) and H$_2$O$_2$ (10 mM) preincubated for 10 min at 37 °C was added to the supernatant, and the decomposition of H$_2$O$_2$ was assayed directly by measuring the decrease in absorbance at 240 nm for 2 min. Catalase from bovine liver (Wako Pure Chemical, Osaka, Japan) was used as a standard.

GPx Activity

GPx activity was assayed using a Bioxytech GPx-340 kit (OXIS International, Portland, OR, U.S.A.). The striatum was homogenized with 10 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM 2-mercaptoethanol, and centrifuged at 10000×g for 10 min at 4 °C. Supernatant or serum was used for the analysis. GPx from bovine erythrocytes (Sigma, St. Louis, MO, U.S.A.) was used as a standard.

TBARS Levels

The concentration of TBARS was measured according to the method described by Buege and Aust$^{16}$ with a slight modification. In brief, tissues were homogenized with 20 volumes of ice cold Tris–HCl buffer (20 mM, pH 7.4) containing 2 mM EDTA. Five hundred microliters of TBA reagent (0.375% thiobarbituric acid, 15% trichloroacetic acid, 0.25% HCl, 0.1 mM EDTA) were added to 250 μl of homogenate and the mixture was heated at 100 °C for 15 min. The reaction was terminated by placing the sample into ice-cold water for 10 min. Finally, the sample was centrifuged at 10000×g for 10 min, and its absorbance was measured at 535 nm. Malonaldehyde was used as a standard.

Lipid Peroxidation Susceptibility

Each striatum was homogenized in 10 volumes of ice-cold Tris–HCl buffer (20 mM, pH 7.4). After ferric chloride in Tris–HCl buffer solution (20 μl, final concentrations: 5 μM) was added to the homogenate (200 μl), the mixture was incubated at 37 °C for 20 min to induce oxidation. · OH was generated by a Fenton-like reaction. Lipid peroxides produced were monitored by measuring the concentration of TBARS.

GSH and Asc Determination

Striatal tissues were homogenized with 10 volumes of ice-cold 5% metaphosphate containing 20 mM deeroxamine mesylate. After centrifugation (10000×g for 10 min at 4 °C), the supernatant was diluted tenfold and filtered (pore size, 0.45 μm) and then injected directly into a HPLC (Shimadzu, Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (gold electrode vs. Ag/AgCl reference electrode) was set at 600 mV. The analytic column was TSKgel Super-ODS (4.6 mm I.D.×100 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate–sodium acetate buffer (pH 3.9) containing methanol (18%, v/v), disodium EDTA (4 mg/l), and sodium octanesulfonate (0.8 mm).

Protein Level

The protein assay was performed by the method of Lowry et al.$^{17}$ using bovine serum albumin as standard.

Chemicals

All chemicals and reagents were of the highest quality available and obtained either from Wako Pure Chemical Industries (Osaka, Japan) or Sigma (St. Louis, MO, U.S.A.).

Statistical Analysis

All data are expressed as the mean±S.D. Differences between groups were examined for statistical significance using one-way analysis of variance. If F values showed significance, the Scheffe’s F test was used to compare groups. A p value less than 0.05 was considered to indicate statistical significance.
RESULTS

Effect of Eugenol on Oxidation of Mouse Brain Homogenate in Vitro

TBARS were determined after incubation of mouse brain homogenates with ferric ion for 20 min at 37 °C in the presence of eugenol, at concentrations from 0 to 1000 μM. The concentrations between 0 to 1 μM did not reduce lipid peroxidation in mouse brain homogenate in terms of blunting the increase in TBARS. Only higher concentrations of eugenol (10 to 1000 μM) suppressed oxidation significantly (Fig. 1, left panel).

Effect of Eugenol on SOD-Like Activity of Supernatant of Mouse Brain Homogenate in Vitro

SOD-like activity of the supernatant was determined in the presence of eugenol at concentrations from 0 to 1000 μM. None of these concentrations reduced SOD-like activity in mouse brain supernatant (Fig. 1, right panel).

Effect of Eugenol Administration for 3 d before and 7 More Days Following One icv 6-OHDA Injection on the Levels of DA, DOPAC, and HVA in Mouse Striatum

The mean tissue concentrations of DA and its metabolites (DOPAC and HVA) in the mouse striatum are shown in Fig. 2. DA, DOPAC, and HVA were significantly decreased by 6-OHDA injection compared with the levels in the control striatum. Eugenol treatment blunted the 6-OHDA-induced DA, DOPAC and HVA reduction in a dose-dependent manner. DA recovered to control levels when 1.0 μmol/kg of eugenol was injected once daily for 3 d before and 7 d after 6-OHDA injection. Eugenol alone had no effect on the levels of striatal DA or its metabolites at this time point.

Effect of Eugenol Administration for 3 d before and 7 More Days Following One icv 6-OHDA Injection on the Levels of TBARS (an Index of Lipid Peroxide) in Mouse Striatum

The mean tissue concentrations of TBARS in the mouse striatum are shown in Fig. 3. The level of TBARS was significantly increased by 6-OHDA injection compared with that in the control striatum. Eugenol treatment inhibited the 6-OHDA-induced TBARS increase. Eugenol alone had no effect on the striatal TBARS level.

Effect of Eugenol Administration for 3 d on the Activities of SOSA, Catalase and GPx in Mouse Striatum

The mean±S.D. of SOSA, catalase activity, and GPx activity in the mouse striatum 30 min after the last eugenol administration are shown in Table 1. No significant changes in SOSA, catalase, or GPx activity were induced in the striatum by eugenol administration. However, SOSA had a tendency to increase in a dose-dependent manner.

Effect of Eugenol Administration for 3 d on the Level of TBARS and Lipid Peroxidation Susceptibility in

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Eugenol 0.1 μmol/kg</th>
<th>Eugenol 1.0 μmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOSA (U/mg protein)</td>
<td>11.4±1.4</td>
<td>12.4±2.94</td>
<td>13.6±3.2</td>
</tr>
<tr>
<td>Catalase (U/mg protein)</td>
<td>17.1±1.7</td>
<td>16.6±1.8</td>
<td>16.0±3.0</td>
</tr>
<tr>
<td>GPx (mU/mg protein)</td>
<td>64.5±9.2</td>
<td>65.4±12.3</td>
<td>61.5±7.8</td>
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</tbody>
</table>

Data are given as the mean±S.D. (n=6 to 8). No significant changes were observed.
Mouse Striatum  The mean tissue concentrations of TBARS in the mouse striatum are shown in Fig. 4. No significant change was induced in the striatum by eugenol administration (left panel). However, the treatment prevented the increase of TBARS after incubation with ferric ion in a dose-dependent manner (right panel).

Effect of Eugenol Administration for 3 d on the Levels of GSH and Asc in Mouse Striatum  The mean tissue concentrations of GSH (left panel) and Asc (right panel) in the mouse striatum are shown in Fig. 5. Eugenol treatment increased the levels of GSH and Asc in a dose-dependent manner.

DISCUSSION

Increased oxidative stress, a decrease of endogenous antioxidants such as GSH, and decreased activities of free radical-scavenging enzymes such as GPx and catalase have been reported in the brains of patients with PD. Accordingly, free radicals and oxidative stress are thought to be important contributors to the pathogenesis of PD. The toxin 6-OHDA has been used to develop parkinsonian models, and the mouse model used here is considered to be an excellent one. Endogenously produced 6-OHDA is thought to act via oxidative mechanisms.

Various antioxidants can prevent 6-OHDA-induced neurotoxicity. Eugenol is an essential oil constituent extracted from cloves and used as a food flavouring agent, and was reported to have antioxidant activity.

In this study, we examined the effect of eugenol against 6-OHDA neurotoxicity by measuring decreases of DA, DOPAC, and HVA as markers of dopaminergic neuronal injury. Treatment with eugenol (for 3 d before and 7 d after 6-OHDA injection) inhibited 6-OHDA-induced neurotoxicity, blunting the reductions of striatal tissue DA and its metabolites. Eugenol is reported to be a MAO inhibitor. Its K_i value against MAO-B is 221 μM. The amount of eugenol we injected into mice (1.0 μmol/kg daily) was not sufficient to inhibit DA metabolisms. Therefore, the levels of DA, DOPAC, and HVA were not affected by eugenol only injection. It also inhibited the 6-OHDA-induced increase of TBARS (an indicator of lipid peroxidation). These results suggest that eugenol protects dopaminergic neurons by inhibiting lipid peroxidation. However, our in vitro results showed that the amount of eugenol injected into mice was not sufficient to directly inhibit lipid peroxidation. Therefore, we think that eugenol might enhance endogenous antioxidant mechanisms of the mouse. As 6-OHDA is susceptible to auto-oxidation, resulting in the formation of 6-OHDA qui-
of brain homogenate with iron ions induces lipid peroxidation and protects cell from 6-OHDA toxicity. Increased GSH can also play a role in reducing lipid peroxide. 6-OHDA is known to produce active oxygen species and also to be a substrate of GPx. It plays a very important role in reducing lipid peroxide. GSH conjugates with $\cdot$OH through a series of reactions called $\cdot$OH, protecting dopaminergic neurons from 6-OHDA neurotoxicity in a dose-dependent manner. The increases of GSH and Asc may decrease the susceptibility to 6-OHDA toxicity. The eugenol-treated mouse striatum homogenate was lower than that of the control. This result shows that lipid peroxidation susceptibility was decreased by eugenol treatment. The increases of GSH and Asc may decrease the susceptibility to lipid peroxidation induced by free radical exposure.

Increases of GSH, Asc, and, to a lesser extent, eugenol itself, together may enhance anti-oxidative stress activity and protect dopaminergic neurons against 6-OHDA toxicity in the mouse striatum.

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**REFERENCES**