Protective Effects of Chlorogenic Acid on Paraquat-induced Oxidative Stress in Rats

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The protective effects of chlorogenic acid on paraquat-induced oxidative stress were examined in rats. The activities of erythrocytes and liver glutathione peroxidase, and of both liver catalase and glutathione reductase, which were increased by feeding paraquat, declined to the levels in the control rats by supplementing chlorogenic acid to the paraquat diet. The activity of superoxide dismutase was not changed by dietary paraquat or by supplementing chlorogenic acid to the paraquat diet. Paraquat in the diet markedly decreased the liver triacylglycerol and phospholipid concentrations, as well as the food intake and body weight gain, while chlorogenic acid protected against these decreases. These in vivo results and the in vitro superoxide anion scavenging activity of chlorogenic acid suggest that chlorogenic acid acted preventively against paraquat-induced oxidative stress.

Key words: chlorogenic acid; dietary antioxidant; paraquat toxicity; hepatic lipid

Chlorogenic acid is one of the phenolic phytochemicals contained in coffee beans and in many kinds of fruits and vegetables. In relation to the abundant presence of chlorogenic acid in food products, the physiological functions of chlorogenic acid and its analogues compounds have recently received strong attention and been widely reported. Chlorogenic acid is already known to suppress the elevation of serum cholesterol level by alcoholism, and to prevent carcinogenesis, to stimulate bile acid secretion, and to inhibit lipid peroxidation in liver mitochondria and microsomes. Some of these activities of chlorogenic acid have been considered due to the antioxidative activity of chlorogenic acid; however, the antioxidative activity of chlorogenic acid against paraquat-induced oxidative stress has not yet been examined in vivo, in spite of some reports dealing with the antioxidative effects of vitamin E and ß-carotene in cultured cells exposed to paraquat.

It is well known that paraquat undergoes a redox-cycling reaction which can lead to the production of reactive oxygen species such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical, resulting in oxidative damage to membrane lipids. In this report, the superoxide anion scavenging activity of chlorogenic acid in vivo was first investigated. Subsequently, the antioxidative activity of dietary chlorogenic acid in vivo was determined in rats given paraquat as a pro-oxidant by measuring the effects of chlorogenic acid on erythrocyte and liver antioxidative enzyme activities, and on thiobarbituric acid-reactive substances (TBARS).

Materials and Methods

Chlorogenic acid. Chlorogenic acid was isolated from unroasted coffee beans according to the method of Sondheimer et al. for use in this experiment. The chemical structure of the isolated chlorogenic acid was confirmed by measuring its 1H- and 13C-NMR spectra.

Scavenging activity. To measure the superoxide anion scavenging activity of chlorogenic acid in vitro, a mixture of 2.4 ml of a 50 mM sodium carbonate buffer (pH 10.2), 0.1 ml of a 3 mM xanthine solution, 0.1 ml of a 3 mM EDTA solution, 0.1 ml of a 0.15% bovine serum albumin solution, and 0.1 ml of a xanthine oxidase (XOD) solution, which had been prepared by diluting commercial XOD (Wako Pure Chemical Ind., Osaka, Japan) 20 fold with H2O, was pre-incubated for 1 min at 25 C. The maximum generation of the superoxide anion was obtained 1 min after this preincubation. The mixture was then supplemented with 20 µl of dimethyl sulfoxide containing 0.03, 0.045, or 0.09 µmol of chlorogenic acid. After a further incubation for 1 min at 25 C, 0.1 ml of a 0.75 mM nitro blue tetrazolium (NBT) solution was added, and the mixture incubated for 1 min at 25 C. Then, 0.1 ml of a 6 mM CuCl2 solution was added to the mixture to stop the reaction, and the absorbance of the mixture was measured at 560 nm. The scavenging ratio (%) was calculated from the difference in O.D. between the reaction mixture with and without chlorogenic acid. The activities of vitamin E, ß-carotene and 3-tert-butyl-4-hydroxyanisole (BHA) were also measured in the same manner.

Animals and diets. Five-week-old male Wistar rats (Japan SL, Hamamatsu, Japan), each weighing about 70 g, were divided into 3 groups of 5 rats each. Each rat was housed in a stainless-steel cage with a screen bottom and kept under controlled conditions with a 12-h light and 12-h dark cycle (06:00-18:00), a temperature range of 22-24 C, and relative humidity of about 55%.

The basal diet (20% casein diet) contained (by weight) 20% casein, 41.9% corn starch, 29.9% sucrose, 10% corn oil, 4% Harpre's mineral mixture (Orinental Yeast Co., Tokyo, Japan), 1% Harpre's vitamin mixture (Oriental Yeast Co., 2% cellulose, 0.1% sodium chloride. Four rats from each group were given paraquat or paraquat plus chlorogenic acid diet (20% + PQ, 20% casein diet with paraquat; 20% + PQ + CG, 20% casein diet with both paraquat and chlorogenic acid) were made up by adding 0.02% paraquat or both 0.02% paraquat and 0.2% chlorogenic acid to the basal diet at the expense of a mixture of cornstarch and sucrose (2:1 ratio of cornstarch to sucrose). Food and water were provided ad libitum for 10 days.

Blood and liver. Blood was collected by cardiac puncture from rats anesthetized with Nembutal (50 mg/kg) Dainippon Pharmaceutical Co., Osaka, Japan) after 12h of starvation at the end of the feeding period. A 0.1 ml aliquot of the blood was added to 1.9 ml of physiological saline by gently shaking, and the mixture centrifuged at 1000 x g for 10 min to obtain the serum for measuring the serum TBARS concentration and to provide erythrocytes. The erythrocytes were lysed with H2O for measuring the antioxidative enzyme activities. A section of the right lobe in the liver which had been excised from each of the rats was used to measure the liver glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) activities. The other parts of the liver were stored at -20 C until needed for measuring the superoxide dismutase (SOD) and catalase activities and liver lipids. The serum for measuring the lipids was separated by centrifuging the blood at 1000 x g for 15 min.

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Preparation of subcellular fractions. To determine the liver SOD activity, 1 g of frozen liver (a section from the main lobe) was homogenized with 5 ml of 0.2% Triton X-100(v/v) at 4°C in a glass-glass homogenizer and then centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was then treated with 0.25 vol. of ethyl alcohol and 0.15 vol. of chloroform, before being centrifuged at 5000 x g for 20 min at 4°C. The supernatant obtained was used for an assay of SOD activity. To determine the liver catalase activity, frozen liver tissue (0.6 g, a section from the left lobe) was homogenized in 4 ml of a buffer solution containing 0.32 mol sucrose and 1 mol EDTA per liter of 10 mm Tris HCl (pH 7.4) in a Teflon-glass homogenizer, according to the method of Rao et al. The supernatant obtained by centrifuging the homogenate at 13,600 x g for 30 min at 4°C was used for an assay of catalase activity.

An enzyme solution for determining liver GSH-Px and GSSG-R activities was prepared according to the method of Rowe et al. Liver tissue (1 g, a section from the right lobe) excised from the rats was immediately homogenized in 5 ml of ice-cold 0.1 M Tris at pH 8.1 in a homogenizer with a Teflon pestle, the homogenate then being sonically treated (Brown sonifier, 30W) for 2 min in a centrifuge tube embedded in ice and centrifuged at 600 x g for 30 min at 4°C. The supernatant obtained was further centrifuged at 105,000 x g for 60 min at 4°C, and the obtained supernatant was dialyzed against three changes of 100 volumes of 0.1 M Tris at pH 8.1 for 3 h at 4°C. The dialyzed solution was used for measuring the enzyme activities.

Measurement of enzyme activities. The SOD activity was measured by the xanthine XOD-NBT system, and the catalase and GSH-Px activities were measured by the methods of Tomita et al. and Lawrence et al. respectively. One unit of SOD and catalase activity is defined as the amount of enzyme required to inhibit the rate of diormal formation from NBT with 50% per mg of hemoglobin or protein, and the amount required to decompose 1 μmol of H<sub>2</sub>O<sub>2</sub> in 1 min per mg of hemoglobin or protein, respectively. One unit of GSH-Px activity, which was measured by using tert-butyl-4-hydroperoxide as a substrate, is defined as the amount of enzyme required to oxidize 1 μmol of NADPH in 1 min per mg of hemoglobin or protein. The GSSG-R activity was measured according to the methods of He et al. One unit of GSSG-R activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH in 1 min per mg of protein.

The hemoglobin and protein contents were measured by using a commercial hemoglobin test kit (Wako Pure Chemical Ind.) and by using the method of Lowry et al. respectively.

Measurement of TBARS. The serum TBARS concentration was determined by the method of Yagi, and is expressed as nmol of malondialdehyde per ml of blood. The liver TBARS concentration was measured by the method of Uchiyama et al. using the homogenate which had been obtained by homogenizing 1 g of frozen liver (a section from the main lobe) with 9 ml of 1.15% KCl. TBARS concentration is expressed as nmol per g of liver or as nmol per mg of total liver lipid, this latter being the sum of total cholesterol, triacylglycerol and phospholipid.

Lipid analyses. The liver lipids were extracted and purified by the method of Folch et al. Total cholesterol, triacylglycerol and phospholipid in the extracts and in the serum were enzymatically measured by using commercial kits (Cholesterol E-test, Triglyceride E-test, and Phospholipid E-test, respectively, Wako Pure Chemical Ind., Osaka, Japan).

Statistical analyses. The data for each of the three groups were statistically analyzed by Duncan's multiple-range test after an analysis of variance (ANOVA), and significant differences in the means were inspected at p<0.05.

Results and Discussion

Superoxide anion scavenging activity of chlorogenic acid in vitro

As shown in Table I, the superoxide anion scavenging activity of chlorogenic acid in vitro increased with increasing amount of chlorogenic acid. It was stronger than that of β-carotene and BHA, and weaker than that of vitamin E. These superoxide anion scavenging activities of chlorogenic acid in vitro suggest the possibility that chlorogenic acid would scavenge the superoxide anion generated by the action of paraquat in vivo if chlorogenic acid is absorbed from the gastrointestinal tract, as suggested by Czok et al.

Food intake and body weight gain

As shown in Fig., the food intake and body weight gain had markedly decreased after 7 days with the rats fed on the paraquat diet (20C + PQ group) when compared with the figures in the control rats (20C group). The supplement of chlorogenic acid to the paraquat diet relieved these decreases in food intake and body weight. This relief by supplementing chlorogenic acid to the paraquat diet indicate the possibility that chlorogenic acid acted preventively against paraquat intoxication which causes oxidative damage to such organs as the lungs, liver, kidneys and heart.

Antioxidative enzyme activities

The antioxidative enzyme activities in the rats fed with the paraquat diet and those in the rats fed with the diet supplemented with chlorogenic acid are shown in Table II. The activities of SOD and catalase in the erythrocytes of the rats fed with the paraquat diet were not different from those of the control rats, although the erythrocyte GSH-Px activity was significantly increased in the rats fed on the paraquat diet when compared to that in the control rats. Furthermore, this increased erythrocyte GSH-Px activity was almost unchanged by supplementing chlorogenic acid to the paraquat diet. Liver SOD activity was not different.

### Table I. Comparison of the Superoxide Anion Scavenging Activities of Chlorogenic Acid and Other Authentic Antioxidants

<table>
<thead>
<tr>
<th>Compound</th>
<th>Added amount (μmol)</th>
<th>Scavenging ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>0.03</td>
<td>0.045</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>55</td>
<td>62</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>BHA*</td>
<td>42</td>
<td>51</td>
</tr>
</tbody>
</table>

Values are the means from three measurements.

* BHA, 3-tert-Butyl-4-hydroxyanisole.
Table II. Effect of Chlorogenic acid on the Antioxidative Enzyme Activities in Rats Fed on the Paraquat-added Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>20C</th>
<th>20C + PQ</th>
<th>20C + PQ + CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>In erythrocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD activity (U/mg of Hb)</td>
<td>3.61 ± 0.09*</td>
<td>3.77 ± 0.14*</td>
<td>3.78 ± 0.10*</td>
</tr>
<tr>
<td>Catalase activity (U/mg of Hb)</td>
<td>53.7 ± 1.3*</td>
<td>51.0 ± 2.7*</td>
<td>58.2 ± 4.3*</td>
</tr>
<tr>
<td>GSH-Px activity (U/mg of Hb)</td>
<td>38.3 ± 3.9*</td>
<td>61.8 ± 6.1*</td>
<td>66.2 ± 8.5*</td>
</tr>
<tr>
<td>In liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD activity (U/mg of protein)</td>
<td>2.48 ± 0.07*</td>
<td>2.29 ± 0.11*</td>
<td>2.42 ± 0.10*</td>
</tr>
<tr>
<td>Catalase activity (U/mg of protein)</td>
<td>154.9 ± 9*</td>
<td>205 ± 7*</td>
<td>186 ± 17*</td>
</tr>
<tr>
<td>GSH-Px activity (U/mg of protein)</td>
<td>84.4 ± 5.0*</td>
<td>107.6 ± 6.5*</td>
<td>90.0 ± 2.8*</td>
</tr>
<tr>
<td>GSSG-R activity (U/mg of protein)</td>
<td>18.9 ± 0.5*</td>
<td>22.1 ± 0.8*</td>
<td>20.5 ± 0.3*</td>
</tr>
</tbody>
</table>

Values are the means ± SE for 5 rats per group. Values within the same row and not sharing a common superscript letter are significantly different at p < 0.05. 20C, 20% casein; 20C + PQ, 20% casein + 0.02% paraquat; 20C + PQ + CG, 20% casein + 0.02% paraquat + 0.2% chlorogenic acid.

Table III. Effect of Chlorogenic acid on the Serum and Liver Lipid Concentrations in Rats Fed on the Paraquat-added Diet

<table>
<thead>
<tr>
<th>Group</th>
<th>20C</th>
<th>20C + PQ</th>
<th>20C + PQ + CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg dl)</td>
<td>86.2 ± 3.0*</td>
<td>76.3 ± 3.7*</td>
<td>79.9 ± 5.7*</td>
</tr>
<tr>
<td>Triacylglycerol (mg dl)</td>
<td>26.1 ± 0.9*</td>
<td>22.4 ± 1.5*</td>
<td>25.8 ± 5.2*</td>
</tr>
<tr>
<td>Phospholipid (mg dl)</td>
<td>126 ± 3*</td>
<td>108 ± 5*</td>
<td>121 ± 7*</td>
</tr>
<tr>
<td>TBARS* (nmol/mL of blood)</td>
<td>2.14 ± 0.17*</td>
<td>1.61 ± 0.31*</td>
<td>1.88 ± 0.30*</td>
</tr>
<tr>
<td>Liver lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg g of liver)</td>
<td>2.89 ± 0.05*</td>
<td>2.68 ± 0.16*</td>
<td>2.88 ± 0.13*</td>
</tr>
<tr>
<td>Triacylglycerol (mg g of liver)</td>
<td>13.5 ± 1.2*</td>
<td>13.9 ± 0.3*</td>
<td>15.1 ± 0.8*</td>
</tr>
<tr>
<td>Phospholipid (mg g of liver)</td>
<td>16.4 ± 0.6*</td>
<td>13.9 ± 0.3*</td>
<td>15.1 ± 0.8*</td>
</tr>
<tr>
<td>Total lipid** (mg g of liver)</td>
<td>32.9 ± 1.6*</td>
<td>34.7 ± 1.2*</td>
<td>42.2 ± 2.8*</td>
</tr>
<tr>
<td>TBARS* (nmol/g of liver)</td>
<td>43.6 ± 4.7*</td>
<td>34.7 ± 1.2*</td>
<td>42.2 ± 2.8*</td>
</tr>
<tr>
<td>TBARS/Total lipid (nmol/mg of total lipid)</td>
<td>1.28 ± 0.20*</td>
<td>1.30 ± 0.09*</td>
<td>1.88 ± 0.31*</td>
</tr>
</tbody>
</table>

Values are the means ± SE for 5 rats per group. Values within the same row and not sharing a common superscript letter are significantly different at p < 0.05. 20C, 20% casein; 20C + PQ, 20% casein + 0.02% paraquat; 20C + PQ + CG, 20% casein + 0.02% paraquat + 0.2% chlorogenic acid.
* The amount of TBARS is expressed as that of malondialdehyde (nmol).
** The amount is expressed as the sum of total cholesterol, triacylglycerol, and phospholipid.

among the three groups, similar to the case of erythrocyte SOD activity. In contrast to the erythrocyte catalase activity, liver catalase activity was markedly increased in the rats fed on the paraquat diet, but this increased activity was mitigated to the level in the control rats by supplementing chlorogenic acid to the paraquat diet. The liver GSH-Px and GSSG-R activities were also significantly increased in the rats fed on the paraquat diet, but these increased activities were mitigated to the levels in the control rats by supplementing chlorogenic acid to the paraquat diet. These results suggest that the antioxidative activity of chlorogenic acid in vivo may have been closely related to the activity of chlorogenic acid for preventing the increases in liver catalase, GSH-Px and GSSG-R activities caused by paraquat. The increases in liver catalase and GSH-Px activities in the paraquat-fed rats were similar to those in paraquat-administered mice, which has been previously reported by Matkovics et al.15.

Increases in the erythrocyte GSH-Px activity, and increases in the liver catalase, GSH-Px and GSSG-R activities in the rats fed on the paraquat diet when compared to those of the control rats indicate the possibility that paraquat increased the erythrocyte GSH-Px activity and liver catalase and GSH-Px activities to remove H2O2 and hydroperoxide which may have been produced from the superoxide anion in the rats given paraquat.180 and that the liver GSSG-R activity was increased to reduce oxidized glutathione which can be produced in the course of the action of GSH-Px.

It is well known that paraquat easily undergoes redox cycling by microsomal NADPH-cytochrome P-450 reductase, resulting in the generation of the superoxide anion and other active oxygen species.181–183 Therefore, when considering the superoxide anion scavenging activity of chlorogenic acid in vitro, the lower liver GSH-Px, catalase and GSSG-R activities in the rats fed with the paraquat + chlorogenic acid diet, in comparison to those in the paraquat-fed rats, may in part have been caused by the superoxide anion scavenging activity of chlorogenic acid in vitro. However, the precise mechanism of action of chlorogenic acid for lowering these enzyme activities that had been increased in the paraquat-fed rats remains to be further clarified.

TBARS and lipid levels
As shown in Table III, the serum TBARS level tended to be decreased in the rats fed on the paraquat and paraquat + chlorogenic acid diets, when compared to that in the control rats, despite paraquat being generally known as an agent to increase serum and liver TBARS.14,34 The liver TBARS value expressed as nmol per g of liver showed a slightly lower value in the paraquat- and paraquat +
chlorogenic acid-fed rats than in the control rats. However, when the liver TBARS concentration is expressed in nmol per mg of total liver lipid, the values are significantly higher in both paraquat- and paraquat + chlorogenic acid-fed rats than in the control rats. The supplement of chlorogenic acid to the paraquat diet had hardly any influence on the liver TBARS concentration.

The concentrations of triacylglycerol and phospholipid in the serum were not different among the three groups (20C, 20C + PQ, and 20C + PQ + CG groups), although the values tended to be decreased in paraquat-fed rats and to return to the levels in the control rats by supplementing chlorogenic acid to the paraquat diet. On the other hand, the concentrations of liver total lipids, triacylglycerol and phospholipid were significantly decreased by feeding the paraquat diet, although the supplement of chlorogenic acid to the paraquat diet slightly mitigated these decreases (Table III). Since the effect of paraquat on the liver lipid level, apart from its effect on the composition of liver phospholipid, has not previously been reported, it is noteworthy that dietary paraquat caused a decrease in the concentrations of liver total lipid, triacylglycerol and phospholipid, and that chlorogenic acid prevented these decreases.

Decreases in the liver triacylglycerol and phospholipid concentrations in the paraquat-fed rats, in comparison to the levels in the control rats, may have been due to the inhibition of lipid synthesis by paraquat and/or the use of these lipids as an energy source which may be necessary in the rats which can not retain body weight.

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References