Mechanisms and Structural Specificity of Hydrogen Peroxide Formation during Oxidation of Catechins

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Hydrogen peroxide (H2O2) formed in aqueous solutions of tea catechins such as epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) was measured by an HPLC method. The amount of H2O2 formed depended on their chemical structures, pH, temperature and incubation time. The gallyl moiety in the B-ring of the gallocatechins, namely EGC and EGCg, mainly contributed to the H2O2 formation. Superoxide dismutase inhibited oxidation of the gallocatechins and, consequently, the H2O2 formation. Cytotoxic effects were investigated by a colony formation assay with Chinese hamster V79 cells, and the effects of the gallocatechins were stronger than those of EC and ECg. The cytotoxic effects of the gallocatechins were inhibited completely by catalase and partially by superoxide dismutase. These results indicate that the gallocatechins were oxidized by superoxide, accompanied by the formation of H2O2, and their cytotoxic effects were ascribed to the H2O2 formation.

Keywords: superoxide anion, hydrogen peroxide, catechin, superoxide dismutase (SOD), epigallocatechin (EGC), epigallocatechin gallate (EGCg)

Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) are the major components of polyphenols in green tea infusions and are referred to as tea catechins. Activities of these catechins in growth inhibition of human lung cancer cell line, inactivation effects on human type-A influenza virus, inhibitory effects on the oxidative modification of low density lipoprotein, antibacterial activity against Clostridium botulinum, and lipoprotein-bound antioxidant activity, have been compared (for review see Hashimoto et al., 1999). These studies indicated that the activities of ECg and EGCg were higher than those of EC and EGC. We investigated the interaction of tea catechins with lipid bilayers with liposome systems and found that the order of affinity of the catechins was as follows: ECg > EGCg > EC > EGC (Hashimoto et al., 1999; Kajiya et al., 2001b). This means that the affinity of the gallic acid esters, namely ECg and EGCg, is higher than EC and EGC. Thus, the order of biological activities mentioned above partly reflected the order of the affinity.

In other cases, however, the biological activity of EGC was equal to or higher than the other catechins in spite of having the lowest affinity among the four catechins (Sae-ki et al. 2000). This implies that factors other than the affinity for lipid bilayers are attributable to the biological activities of EGC. EGC possesses a benzene ring with three hydroxyl groups referred to as a gallyl moiety, which has been ascribed to its antioxidant activity. In addition, EGC, ECg and compounds with a gallyl moiety have been reported to produce hydrogen peroxide (H2O2) during oxidation in aqueous media (Hoshino et al., 1999; Long et al., 2000; Yang et al., 2000). This suggests that H2O2 generation might be responsible for the biological activities.

The formation of H2O2 from (+)-catechin in aqueous solutions depended on pH, incubation time, and temperature (Nakayama et al., 1995). Superoxide dismutase (SOD) inhibited both oxidation of (+)-catechin and concomitant formation of H2O2. It was concluded that oxidation of (+)-catechin and the H2O2 formation proceeded by a chain reaction with superoxide as the chain carrier.

In this study, we investigated the effects of SOD on H2O2 formation during the oxidation of EGCg and EGC by an HPLC system and the effects of catalase and SOD on cytotoxicity of these compounds by a colony-formation assay with cultured mammalian cells. Its purpose was to clarify the mechanisms of H2O2 formation in aqueous solutions of catechins, and to show examples of results affected by the formation of superoxide and H2O2.

Materials and Methods

Reagents Sodium sulfate (Na2SO4), ethylenediamine-N,N,N′,N′-tetraacetic acid disodium salt (EDTA, 2Na) and catalase from bovine liver (5000–13000 units/mg) were purchased from Wako (Osaka). Copper/zinc superoxide dismutase from bovine erythrocytes (SOD) was obtained from Sigma Chemical Co. (St. Louis, MO). H2O2 (31% w/v) from Mitsubishi Gas Chemical Co., Ltd. (Tokyo) was freshly diluted prior to preparation of the standards. Sep-Pak Plus C18 cartridges (1 ml), short reverse-phase columns, were purchased from Millipore Corporation (Milford, MA), and were successively conditioned with 2 ml of acetonitrile and 4 ml of water before use. All other chemicals were of reagent grade and were used without further purification. All buffers were prepared with distilled water treated with a Milli-Q-Lab system from Japan Millipore, Ltd. (Tokyo).

Standard conditions of catechin incubation An aqueous catechin solution (500 μM) was prepared in 25 mM phosphate buffer (pH 7.0). Four milliliters of the solution in a lidless test tube was incubated in a water bath for 60 min at 37°C. After
immediate cooling to 0°C, the solution was passed through a Sep-Pak C18 cartridge. The first 1 ml of effluent was discarded and the rest of the effluent (3 ml) was collected in a test tube and preserved at 0°C until H₂O₂ measurement by HPLC.

**H₂O₂ measurement by HPLC** The concentration of H₂O₂ formed in aqueous solutions of catechins was determined by HPLC equipped with an electrochemical detector (ECD). A method for separation of H₂O₂ by HPLC and its detection by ECD was developed in our laboratory (Takahashi et al., 1999). Briefly, the system consisted of an HPLC column packed with a cation-exchange resin gel (Shodex Ionpak KS-801, 150 mm x 8 mm i.d.; Showa Denko, Tokyo), two HPLC pumps (Jasco PU-980; Japan Spectroscopic, Tokyo) and an ECD (BAS LC-4C, West Lafayette, Indiana). The electrolyte was prepared with Na₂SO₄ (50 mM) and EDTA, 2Na (10 mM). The flow rate of pure water from the first pump through a column was 1.0 ml/min and that of the electrolyte from the second pump was 0.25 ml/min. ECD was equipped with a Pt electrode as a working electrode and an Ag/AgCl electrode as a reference electrode. The peak areas were calculated with a Chromatocoder 21J (System Instruments, Tokyo). The amount of H₂O₂ was determined with the aid of a calibration curve prepared with standards of H₂O₂. These standards were prepared daily with the phosphate buffer. Four independent experiments at each measuring point with the same catechin solutions were carried out, and the results were expressed as the means and standard deviations (SD).

**Colony formation assay** Cytotoxic effects of catechins were assessed by a colony-formation assay. Chinese hamster lung fibroblast V79 cells were seeded in 60-mm petri dishes (100 cells/dish) and incubated in 5 ml of a minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ in air at 37°C for 3 h. After changing the medium to 5 ml of HEPES-buffered saline (HBS) containing the indicated concentration of each catechin, the cells were incubated for 30 min. After culture in MEM supplemented with 10% heat-inactivated FBS for 5 days, the colonies were fixed with methanol and stained with Giemsa’s solution and their number was counted. The survival rate (% of control) was calculated by dividing the number of colonies of the cells treated with a catechin by that of the untreated control cells. Each result is expressed as the means and SD of four separately treated cultures. The data were analyzed by Student’s t-test in some cases.

**Measurement of catechins by HPLC** The concentration of a catechin in the medium under the same conditions in the colony formation assay described above was measured by HPLC.

![Fig. 1. Structures of the four catechins and the related compounds.](image-url)
H2O2 Formation in Catechin Solutions

HBS (5 ml) containing each catechin (100 μM) was incubated in a dish at 37˚C for 30 min, after which the amount of catechin in the medium was measured by HPLC with a UV detector using a Capcell Pak C18 UG120 column (Shiseido Co., Ltd., Tokyo). The mobile phase was 10% acetonitrile for EGC, 15% acetonitrile for EC and EGCg, and 18% acetonitrile for ECg with water in the presence of 0.1% trifluoroacetic acid and detection wavelength was 280 nm.

Results

Relationship between the chemical structure and the amount of H2O2

The concentration of H2O2 formed in aqueous solutions of catechins and their related compounds depicted in Fig. 1 was determined by the HPLC system (Table 1). After incubation for 60 min at 37˚C, the concentration of H2O2 in the solution was measured by the HPLC-ECD system.

Table 1. Concentration of H2O2 formed in aqueous solutions of catechins and their related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration of H2O2 (μM)</th>
</tr>
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<tbody>
<tr>
<td>EC</td>
<td>21±3</td>
</tr>
<tr>
<td>ECg</td>
<td>0±0</td>
</tr>
<tr>
<td>EGC</td>
<td>179±5</td>
</tr>
<tr>
<td>EGCg</td>
<td>191±13</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>391±4</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>28±4</td>
</tr>
<tr>
<td>Gallic Acid Methyl Ester</td>
<td>4±1</td>
</tr>
</tbody>
</table>

An aqueous solution of each compound (500 μM) was prepared in 25 mM phosphate buffer (pH 7.0). After incubation for 60 min at 37˚C, the concentration of H2O2 in the solution was measured by the HPLC-ECD system.

The results of EC and EGC compared with the results of ECg and EGCg, respectively, implied that the presence of the galloyl moiety does not significantly contribute to the formation of H2O2. To confirm this concept, we measured the concentration of H2O2 formed from the model compounds under the same incubation conditions used for tea catechins (Table 1). Pyrogallol is the simplest model of the gallotyl moiety. Gallic acid and its ester, methyl gallate, are models of the galloyl moiety. Pyrogallol produced the highest amount of H2O2 among the compounds investigated in the present study, while the amounts produced by gallic acid and methyl gallate were lower. The low amount of

Fig. 2. Dose dependency of H2O2 formation in aqueous EGCg solutions. Aqueous solutions of EGCg in the indicated concentrations were prepared in 25 mM phosphate buffer (pH 7.0). After incubation for 60 min at 37˚C, the concentration of H2O2 in the solutions was measured by the HPLC-ECD system.

Fig. 3. Time dependency of H2O2 formation in aqueous EGCg solutions. Aqueous solutions of EGCg (500 μM) prepared in 25 mM phosphate buffer (pH 7.0). After incubation at 37˚C for 60 min, the concentration of H2O2 in the solutions was measured by the HPLC-ECD system.

Fig. 4. pH dependency of H2O2 formation in aqueous EGCg solutions. Aqueous solutions of EGCg (500 μM) were prepared in 25 mM phosphate buffer with the indicated pHs. After incubation at 37˚C for 60 min, the concentration of H2O2 in the solutions was measured by the HPLC-ECD system.
H$_2$O$_2$ in the aqueous solutions of ECg and methyl gallate indicates that the galloyl moiety in these compounds did not greatly contribute to the formation of H$_2$O$_2$.

**Effects of the incubation conditions** In the following experiments we investigated the formation of H$_2$O$_2$ during oxidation of EGCg, because the EGCg content in green tea infusions is usually the highest among the four catechins. The amount of H$_2$O$_2$ formed in the EGCg solution under the standard conditions varied in each experiment, but was around 180 to 220 µM. Figure 2 shows that the measured amount of H$_2$O$_2$ was almost 40% of the amount of EGCg added at these concentrations. H$_2$O$_2$ increased almost linearly with the incubation time until 60 min and then increased gradually (Fig. 3). Figure 4 shows the pH dependency of H$_2$O$_2$ formation in 25 mM sodium phosphate buffer. H$_2$O$_2$ was detected above pH 5.8 and its concentration steeply increased with pH up to pH 8.0. The effect of incubation temperature on the amount of H$_2$O$_2$ was investigated. After incubation of EGCg in 25 mM sodium phosphate buffer (pH 7.0) for 8 min, the amount of H$_2$O$_2$ was 16 µM at 37°C, and increased with rising temperature up to 77°C (Fig. 5), which is close to the temperature of tea infusions. We next investigated the effects of SOD on the amount of H$_2$O$_2$. SOD dose-dependently inhibited H$_2$O$_2$ formation in the solution of both EGCg and EGC (Fig. 6). Inactivated SOD showed no inhibitory effects (data not shown), indicating that the inhibitory effects of SOD were due to its enzymatic activity.

**Cytotoxic effects of catechins** Figure 7 shows the effects of various doses of the catechins on the survival rate of V79 cells. At both 100 µM and 500 µM, the order of toxicity was EGC ≈ EGCg ≫ ECg; EC showed no cytotoxicity at these concentrations. These results indicate that the gallocatechins showed higher cytotoxicity than the catechol-type catechins. To verify that the difference of the cytotoxic activities between the gallocatechins and the catechol-type catechins is ascribable to the H$_2$O$_2$ formation, we investigated the effects of catalase and SOD in cytotoxicity of the gallocatechins. The results of experiment 1 (Table 2) show that catalase almost abolished the cytotoxic effects of EGCg and EGC. This confirms that the cytotoxic effects were ascribable to the formation of H$_2$O$_2$. The result of experiment 2...
H2O2 was time-dependently generated in various cell-culture systems in the presence of Cu (II). Long-lasting cytotoxicity was ascribable to H2O2 production in the cells. These results indicate that Cu (II) resulted in the formation of H2O2 in the EC and (+)-catechin solutions at 37˚C. Miura et al. (1998) detected H2O2 in the media after incubation of (+)-catechin, EGC, EGCg and gallic acid. Among these compounds ECg and EGCg produced a relatively high amount of H2O2. Yang et al. (2000) reported that apo-SOD inhibited the cytotoxicity of EGCg and EGC. Under the same conditions, the galloca-techins were more unstable than the catechol-type catechins and SOD inhibited decomposition of the galloca-techins (Table 3).

Discussion
Formation of H2O2 during oxidation of tea catechins in aqueous solutions has been previously reported. H2O2 was formed in aqueous (+)-catechin solutions during the aerobic heating at 80˚C (Nakayama et al., 1995). The effects of pH and incubation time on the amount of H2O2 were similar to our present results with EGCg solutions at 37˚C. Miura et al. (1998) detected H2O2 in EC and (+)-catechin solutions incubated at 21˚C. Hoshino et al. (1999) reported that H2O2 was formed in a buffer containing EGCG in the presence of Cu (II). Long et al. (2000) found that H2O2 was time-dependently generated in various cell-culture media after incubation of (+)-catechin, EGC, EGCG and gallic acid. Among these compounds EGCG and EGC produced a relatively high amount of H2O2. Yang et al. (2000) reported that apoptosis of a human bronchial cell line induced by EGCG and EGCG was ascribable to H2O2 production in the cells. These results indicate that a relatively high amount of H2O2 was formed in the aqueous solutions of the galloca-techins such as EGCG and EGCG. However, they did not refer to the role of superoxide anion in the formation of H2O2.

We have established a method to separate H2O2 by HPLC and to quantify its amount by ECD (Takahashi et al., 1999). With this highly selective method, we succeeded in the measurement of H2O2 in coffee and aqueous ascorbic acid solutions. In the present study, we confirmed the formation of H2O2 in aqueous solutions of various catechins by this HPLC-ECD system and elucidated the relationship between the structure and the amount of H2O2 as follows. Tea catechins possess two kinds of benzene rings with three hydroxyl groups. The first one is a gallayl moiety in the B-ring, which is generally represented as “G” in the abbreviations of catechins. The second one is a galloyl moiety, which is represented as “g” in the abbreviations of gallic acid esters of catechins. Both moieties are thought to have reducing ability and antioxidant activity. Thus, it is reasonable to suppose that they are easily oxidized and that H2O2 is formed during oxidation of these compounds, but there has been no report on which moiety has the greater role in the H2O2 formation. The concentration of H2O2 formed in the aqueous solutions of the four catechins listed in Table 1 is as follows: EC>ECg, EGCg>EGCg, EGC>EC. EGCG > ECg. This indicates that the gallayl moiety in EGCG and EGCG contributed significantly to the formation of H2O2, but that the galloyl moiety in EC and EGCG did not. The much higher amount of H2O2 formed in the aqueous solution of pyrogallol than in the solution of methyl gallate (Table 1) supports the idea that the addition of an ester structure to the pyrogallol ring lowers the ability to produce H2O2. The substituents such as carboxylic acid and its esters are electron-withdrawing groups. Reduction of electron density of the benzene ring in the esters might result in less electron-donating ability of the galloyl moiety than that of the gallayl moiety.

SOD dose-dependently inhibited H2O2 formation in the aqueous solutions of EGCG and EGCG (Fig. 6). This result seems contradictory to the function of SOD, because H2O2 is a product of its enzyme reaction. We solved this problem by postulating a chain reaction with O2− as the chain carrier (Fig. 8), as already proposed for the oxidation of (+)-catechin (Nakayama et al., 1995). In this reaction, O2− instead of O2 initiates the oxidation of a gallayl moiety, resulting in the formation of a semiquinone-type radical and H2O2 (reaction (1)). The formed radical rapidly reacts with O2− resulting in the formation of various oxidation products and O2− (reaction (2)). The produced O2−, in turn, oxidizes another molecule with the gallayl moiety; thus, the chain reaction continues. The sum of reactions (1) and (2) is the observed overall reaction where no O2− appears. The rate constant of the spontaneous dismutation of superoxide is 5×104 M−1 s−1, while the rate constant of enzymatic dismutation of superoxide by SOD is 2×106 M−1 s−1 (Ogawa & Asada, 1996). This means that the latter reaction is almost diffusion controlled and the concentration of O2− can be almost 105 times lower than that in the absence of SOD. Thus, SOD inhibits reaction (1) by lowering the concentration of O2− and consequently inhibits the formation of H2O2 in the overall reaction by reducing the turnover number of the chain reaction. Inhibition of oxidation of the galloca-techins (Table 3) by SOD supports this mechanisms. Inhibition of H2O2 formation by SOD during the oxidation of various compounds has been experimentally proved (Cadenas et al., 1988; Nakayama et al., 1989; Bandy et al., 1990; Nakayama et al., 1992a; Nakayama et al., 1997; Hiramoto et al., 2001). Furthermore, inhibition of pyrogallol oxidation by SOD has been used as an assay for SOD (Marklund & Marklund, 1974). Thus, inhibition of H2O2 formation by SOD during oxidation is not peculiar to the

| Table 2. Effects of catalase and SOD on the survival rate of Chinese hamster lung V79 cells treated with EGCG or EGC. |
|--------------------------|--------------------------|
| Survival of cells (% of control) |                |
| Exp.1 | +catalase | –catalase |
| EGCg | 102±37* | 8±3 |
| EGC | 91±15* | 1±1 |
| Exp.2 | +SOD | –SOD |
| EGCG | 62±17* | 21±4 |
| EGC | 74±9* | 11±3 |

V79 cells (100 cells/dish) were incubated in 5 ml of HBS with each catechin (100 μM) for 30 min in the presence or absence of catalase (2 μg/dish) in experiment 1 and SOD (50 units/dish) in experiment 2. These experiments were carried out on separate days. The number of colonies was counted after culturing in MEM supplemented with 10% FBS for 5 days. The survival rate (% of control) was calculated by dividing the number of colonies in each catechin medium by that in the control medium. Results are expressed as the mean and standard deviation of four separately treated cultures. *Significantly different from the corresponding value in the absence of the respective enzyme at p<0.05 (Students’ t-test).

| Table 3. Effects of SOD on the amount of catechin in HBS after incubation. |
|--------------------------|--------------------------|
| Concentration of catechin (μM) |                |
| SOD | EC | EGC | EGCG |
| 15.0±1.5 | 59.4±5.7 |
| 13.8±3.5 | 58.7±4.8 |
| 70.2±0.7 | 64.±2.6 |
| 80.7±2.0 | 69.2±5.0 |

HBS (5 ml) containing each catechin (100 μM) was incubated in the absence or presence of SOD (50 unit) at 37˚C for 30 min. After incubation, the amount of catechin in HBS was measured by HPLC. The results are expressed as the mean and standard deviation of four separately treated samples.
gallopectechins.

The order of the affinity of the catechins for lipid bilayers was as follows: ECg>EGCg>EC>EGC (Hashimoto et al., 1999; Kajiya et al., 2001b). The orders of biological activities studied by in vitro experiments reflected this order to some degree in many cases. Figure 7, however, shows that the order of the cytotoxic activity under our experimental conditions with V79 cells was EGC=Egc>Eg>EC, which was different from the order of the affinity for lipid bilayers described above. The higher cytotoxicity of the gallocatechins than the catechol-type catechins suggested that the cytotoxic activity was partly ascribed to the formation of H$_2$O$_2$. This idea was confirmed by the inhibitory effects of catalase on the cytotoxicity (Table 2). The amount of H$_2$O$_2$ formed during incubation of ECg (100 μM) for 30 min was nearly equal to that of H$_2$O$_2$ formed during incubation of EC (50 μM) for 60 min (Fig. 2 and Table 1). Cytotoxicity of EGCG (100 μM) was, however, much higher than that of EC (500 μM) (Fig. 7). In addition, no H$_2$O$_2$ was formed during incubation of ECg (500 μM) for 60 min (Table 1), yet EGCG showed higher cytotoxicity than EC (Fig. 7). These results suggest that H$_2$O$_2$ formation is not the only factor governing the cytotoxicity of catechins. As described above, higher affinity of ECg and EGCG for the lipid bilayers than of EC might also contribute to their cytotoxicity to some degree. The inhibitory effect of SOD does not indicate that O$_2$·- directly participates in the cytotoxicity of the gallocatechins, because O$_2$·- formed in a medium with an O$_2$·- producing system such as hypoxanthine-xanthine oxidase does not enter cells, while not SOD but catalase in the medium inhibits the cytotoxicity. Since SOD inhibited decomposition of the gallocatechins (Table 3) and lowered the amount of H$_2$O$_2$ formed in their aqueous solutions (Fig. 6), the inhibitory effects of SOD on the cytotoxicity of the gallocatechins should be ascribed to the inhibition of H$_2$O$_2$ formation during their oxidation process.

Long et al. (2000) measured H$_2$O$_2$ formed in various cell culture media containing polyphenols and claimed that the effects of H$_2$O$_2$ should be considered when examining the biological activities of polyphenols by in vitro experiments. The results of the present study and those reported by Yang et al. (2000) show that formation of H$_2$O$_2$ in aqueous solutions can affect the results of the in vitro experiments with tea catechins. The cytotoxicity of the four flavonols determined by a colony-formation assay was in the order of myricetin>galangin>kaempferol>quercetin (Kajiya et al., 2001a). Apart from myricetin, the order of cytotoxicity was the same as that of lipophilicity. Only the cytotoxicity of myricetin was inhibited by catalase. EGCG, EGCG and myricetin possess a common gallyl moiety. Thus, participation of H$_2$O$_2$ in the biological activities studied in vitro seems to be characteristic of the compounds with a gallyl moiety. A small amount of H$_2$O$_2$ was detected in aqueous solutions of polyphenols with a catechol moiety or a gallyl moiety, e.g. (−)-catechin, quercetin and methyl gallate in this and previous papers (Long et al., 2000; Nakayama et al., 1995; Miura et al., 1998; Kajiya et al., 2001), but these compounds inhibited the cytotoxic effects of H$_2$O$_2$ in the cells (Nakayama et al., 1992b; Nakayama et al., 1993). We assume that higher amounts of these compounds were incorporated into the cells during incubation and less H$_2$O$_2$ was produced in the medium than the compounds with a gallyl moiety. Consequently, the antioxidant effects of the former compounds were supposed to overcome the pro-oxidant effects of H$_2$O$_2$. H$_2$O$_2$ would not be formed from tea catechins in our body. Some tea infusions, however, contained appreciable amounts of H$_2$O$_2$ (data not shown). The results of the present study indicate that the amount of H$_2$O$_2$ in tea infusions depends on various factors such as the composition of catechins, standing time, pH, and temperature. Consideration of the effects of H$_2$O$_2$ might be necessary to estimate the biological activities of tea infusions, especially in our mouth and digestive tract.

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References


