

# Differences in Antioxidative Efficiency of Catechins in Various Metal-Induced Lipid Peroxidations in Cultured Hepatocytes

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The effects of tea catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechingallate (ECg) or (–)-epigallocatechingallate (EGCg) on the lipid peroxidation induced by either ferrous (Fe), copper (Cu) or vanadium (V) ions were examined in normal and  $\alpha$ -linolenic acid-loaded (LNA-loaded) cultured rat hepatocytes. The order of antioxidative activity was almost similar and ranked as follows: EGCg > ECg > EGC > EC against the three metal-induced lipid peroxidations in both groups of cultured hepatocytes. However, each catechin displayed a marked variation in its antioxidative potency depending on the added metal ion species, whereas dibutyl hydroxy toluene (BHT), a typical lipid radical scavenger, exhibited a similar antioxidative potency with all metal ions. The half inhibition concentration ( $IC_{50}$ ) values of the catechins for Fe ion-induced lipid peroxidation were 2 to 3 times and 5 to 14 times higher than those for V ion and Cu ion, respectively in normal hepatocytes. Only in LNA-loaded hepatocytes, was EC antioxidative at 20  $\mu$ M Fe concentration but it became prooxidative above 50  $\mu$ M Fe concentration. Catechins such as (+)-catechin, (+)-epicatechin and ( $\pm$ )-catechin also acted as prooxidants at high Fe concentrations in LNA-loaded hepatocytes. The antioxidative efficiency of EGC, ECg or EGCg decreased with the increase in Fe ion concentrations from 20  $\mu$ M up to 500  $\mu$ M in LNA-loaded hepatocytes. These findings suggest that the metal-chelating property of catechins may play a major role in determining antioxidative activity in cultured hepatocytes.

**Key words** — lipid peroxidation, metal ion, catechin, cultured rat hepatocyte, free radical

## INTRODUCTION

Flavonoid compounds which are widely distributed in fruits and vegetables have many positive health effects through their antioxidative properties.<sup>1–3</sup> The antioxidant activity of flavonoids is considered to be exerted by a combination of the reaction with free radicals and the metal ion chelating through phenolic hydroxyl groups in the flavonoid nucleus, which consists of A, B and C-rings (Fig. 1). There is much discussion in the literature regarding the relative contribution of these two modes of action to the antioxidative activity of flavonoids.<sup>4–7</sup> Radical scavenging ability resides in the availability of free hydroxyls on the B-ring for hydrogen atom-donation.<sup>8–11</sup> On the other hand, metal chelat-

ing ability is derived from three structural arrangements: 1) the 3',4'-o-dihydroxycatechol on the B-ring; 2) the 3-hydroxyl in conjugation with a 4-oxo function on the C-ring; and 3) the 5-hydroxyl on the A-ring in conjugation with a 4-oxo function on the C-ring.<sup>12,13</sup> Flavonols including quercetin satisfy all these structural features (Fig. 1). There are conflicting reports regarding the metal chelating domain of flavonols. Paganga *et al.*<sup>14</sup> reported that the chelation property of flavonoids for Fe and Cu ions are derived primarily from the presence of the 3-hydroxy-4-keto group and secondarily from the presence of the 5-hydroxy-4-keto group on the C-ring rather than the 3',4'-dihydroxy group on the B-ring. On the other hand, Brown *et al.*<sup>15</sup> proposed that the major site for Cu chelation in flavonoids is the catechol moiety rather than the 4-carbonyl group with its adjacent hydroxyl groups. Therefore, the contributions of these domains of flavonoids to their metal chelating ability is a subject of considerable debate.

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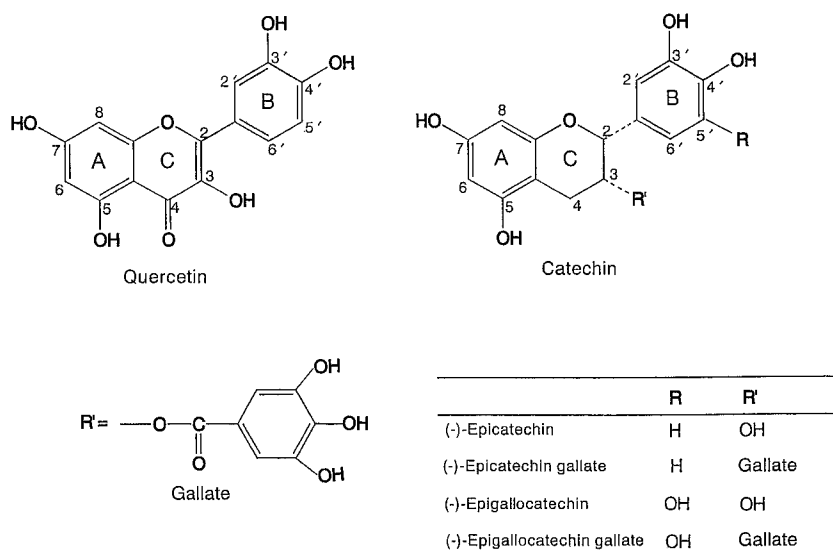


Fig. 1. Structures of Flavonoids

Green tea contains a flavanol group of polyphenols, so-called catechins, which have a peculiar backbone structure (Fig. 1). In comparison with quercetin, catechins are devoid of a C2-C3 double bond and a C4 carbonyl group on the C-ring. Therefore, catechins lack the structural advantage of flavonols in regard to metal chelating ability. In this study, we compared the antioxidative activity of four major components of green tea catechins, namely (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECg) and (-)-epigallocatechingallate (EGCg) against lipid peroxidation caused by either ferrous (Fe), copper (Cu) or vanadium (V) ions in cultured hepatocytes. Our working hypothesis is that if catechins exert antioxidative action by virtue of chelating metal ion, catechins may exhibit different behaviors in preventing metal ion-induced lipid peroxidation depending on the metal ion species.

## MATERIALS AND METHODS

**Materials** — Material and chemical reagents were obtained from the following companies: plastic culture dishes from Falcon, Becton Dickinson Co. (Japan); Williams' medium E from Gibco BRL, Life Technologies, Inc. (U.S.A.);  $\alpha$ -linolenic acid (LNA) and dimethyl sulfoxide from Nacalai Tesque, Inc. (Kyoto, Japan); Catechins from Funakoshi Co. (Tokyo, Japan); Ferrous sulfate heptahydrate, copper sulfate pentahydrate and vanadium chloride from

Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were of the highest purity available.

**Preparation of Hepatocytes** — Male Wistar rats weighing 180–220 g were provided with standard rat chow and water *ad libitum*. Hepatocytes were isolated by the collagenase perfusion method of Berry and Friend.<sup>16)</sup> The isolated cells were subjected to fractionation on Percoll density gradients to obtain more than 98% viability as verified by the trypan blue exclusion test. These cells were resuspended in Williams' medium E containing dexamethasone (1  $\mu$ M), insulin (0.1  $\mu$ M), triiodothyronine (1  $\mu$ g/ml),  $\delta$ -aminolevulinic acid hydrochloride (0.2 mM) and 7% fetal calf serum (referred to as complete Williams' medium E). The cells were seeded in 35 mm plastic culture dishes at a density of  $1 \times 10^6$  cells/dish, and placed in an incubator in an atmosphere of 5% CO<sub>2</sub> - 95% air at 37°C. After a 9 hr incubation, the monolayers of hepatocytes were cultured for an additional 12 hr in complete Williams' medium E containing 1.0 mM LNA-BSA (LNA-loaded cells). More than 70% of added LNA was taken by the cultured cells after the incubation.<sup>17)</sup> The control hepatocytes were maintained in culture in complete Williams' medium E without LNA. The amount of cell protein was determined by the method of Lowry *et al.*<sup>18)</sup>

**Hepatocyte Incubation** — Hepatocytes were washed twice with Hanks' medium and incubated in 2 ml of fresh complete Williams' medium E. Each flavonoid dissolved in dimethyl sulfoxide was added to the cultures at the concentrations indicated in the

text 10 min before the addition of metallic salts dissolved in saline. After incubation for 6 hr, the medium was separated. Malondialdehyde (MDA) in the medium was assessed by a modification of the Uchiyama and Mihara method.<sup>19)</sup> Briefly, to 0.1 ml of the medium in a 12 ml glass tube, 3 ml of 1% phosphoric acid and 1 ml of 0.67% thiobarbituric acid was added. The tube was capped with a screw cap and heated at 100°C for 45 min. After cooling in ice water, 4 ml of *n*-butanol was added. The mixture was then shaken and centrifuged to separate the organic layer. The fluorescence intensities in the butanol layer were measured at the excitation and emission wavelengths of 515 and 553 nm, respectively. The artifactual production of MDA due to the autooxidation of fatty acid in the medium during the assay procedure was approximately 0.3 nmol and was treated as a blank. Dimethyl sulfoxide (20  $\mu$ l) was always diluted in 2000  $\mu$ l of the culture medium, including control cultures which were exposed to metal ions without flavonoids. The final concentration of 1% dimethyl sulfoxide had no detectable effect on lipid peroxidation in cultured hepatocytes.

**Preparation of LNA-BSA Complex** — LNA was adsorbed to bovine serum albumin (BSA) as described previously.<sup>20)</sup> 1 mmol of LNA was dissolved in 10 ml of 0.1 N NaOH solution. This solution was added dropwise to 240 ml of complete Williams' medium E containing 1 mM BSA, which had a fatty acid/albumin molar ratio of 4. The resulting fatty acid-BSA complex was filter-sterilized with a 0.2  $\mu$ m Millipore filter.

**Statistical Analysis** — Multiple comparisons were performed among the three metal groups for each flavonoid by analysis of variance (ANOVA) followed by Fishers' protected least significant different test using Stat View (Abacus Concepts, Inc. CA, U.S.A.). A *p* value of < 0.05 was considered significant.

## RESULTS

Normal and LNA-loaded hepatocytes were incubated for 6 hr with either ferrous (Fe), vanadium (V) or copper (Cu) ions at a concentration of 100  $\mu$ M. MDA accumulation in the medium, an index of lipid peroxidation, increased linearly with the incubation time up to 6 hr after the addition of metal ions. The amount of MDA accumulation for Fe, V and Cu ions was 2.3, 2.4 and 2.1 nmol/mg protein/6 hr in normal hepatocytes, and 6.1, 6.8 and 12.4 nmol/mg protein/6 hr in LNA-loaded cells, respectively.<sup>21)</sup> The accumulation of MDA was only slightly influenced by the cellular metabolism of MDA.<sup>22)</sup> The effect of tea catechins such as EC, EGC, ECg and EGCg on the metal-induced lipid peroxidation in normal and LNA-loaded hepatocytes were examined. The half inhibition concentration (IC<sub>50</sub>) of four catechins and dibutyl hydroxy toluene (BHT) against lipid peroxidation in both groups of hepatocytes are summarized in Tables 1 and 2, respectively.

Fe-induced lipid peroxidation in normal hepatocytes was suppressed less effectively by the catechins than V- and Cu-induced lipid peroxidation. Additionally, the antioxidative efficiency of the four catechins was similar. Catechins were effective in suppressing V-induced lipid peroxidation in normal cells in the following decreasing order EGCg > ECg > EC > EGC. Catechins were most effective for Cu-induced lipid peroxidation in normal hepatocytes, in the efficiency order of EGCg > ECg > EGC > EC. Although the degree of lipid peroxidation by the three metals were similar, IC<sub>50</sub> values of catechins for Fe ion were higher than those for V and Cu ions by 2 to 3 and 5 to 14 times, respectively, in normal hepatocytes. BHT as a lipid radical chain oxidation-breaking agent,<sup>23)</sup> was antioxidative to a similar extent against Fe-, V- and Cu-induced lipid peroxidation in normal hepatocytes.

**Table 1.** Inhibiting Activities of Various Catechins on FeSO<sub>4</sub>, VCl<sub>3</sub> or CuSO<sub>4</sub> -Induced Lipid Peroxidation in Normal Hepatocytes

	EC	EGC	ECg	EGCg	BHT
FeSO <sub>4</sub>	25.7 ± 2.56	24.9 ± 0.93	24.6 ± 1.36	19.0 ± 2.89	7.66 ± 1.86
VCl <sub>3</sub>	9.33 ± 0.62 <sup>a)</sup>	12.5 ± 0.58 <sup>a)</sup>	7.20 ± 0.88 <sup>a)</sup>	6.68 ± 0.35 <sup>a)</sup>	6.90 ± 0.82
CuSO <sub>4</sub>	5.24 ± 0.38 <sup>ab)</sup>	3.87 ± 0.07 <sup>ab)</sup>	2.57 ± 0.20 <sup>ab)</sup>	1.34 ± 0.28 <sup>ab)</sup>	3.89 ± 0.99

EC, (–)-Epicatechin; EGC, (–)-Epigallocatechin; ECg, (–)-Epicatechingallate; EGCg, (–)-Epigallocatechingallate; BHT, Dibutyl hydroxy toluene. Normal hepatocytes, were incubated with 100  $\mu$ M metal ions in the presence of various concentrations of catechins. Values are the concentrations that inhibit lipid peroxidation by 50% (IC<sub>50</sub>,  $\mu$ M). IC<sub>50</sub> is calculated from the concentration-activity curves. Data are expressed as the mean ± S.E. of four experiments. Statistical analysis was performed among metal groups for each catechin. a) *p* < 0.05 significantly different from the value of Fe, b) *p* < 0.05 vs V.

**Table 2.** Inhibiting Activities of Various Catechins on FeSO<sub>4</sub>, VCl<sub>3</sub> or CuSO<sub>4</sub>-Induced Lipid Peroxidation in LNA-Loaded Hepatocytes

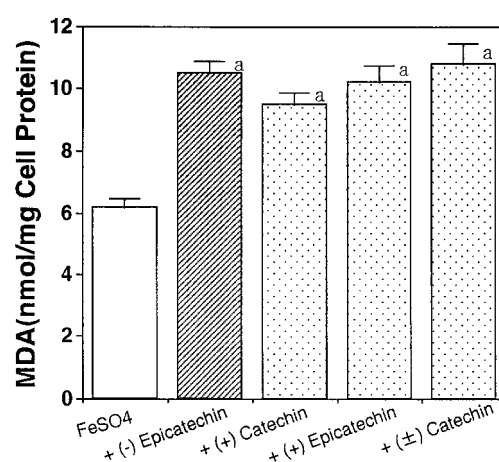
	EC	EGC	ECg	EGCg	BHT
FeSO <sub>4</sub>	prooxidant	128.4 ± 5.9	51.6 ± 2.6	21.6 ± 1.6	16.1 ± 1.2
VCl <sub>3</sub>	21.6 ± 1.8	13.5 ± 1.2 <sup>a</sup>	9.9 ± 0.7 <sup>a</sup>	9.4 ± 1.2 <sup>a</sup>	14.4 ± 1.4
CuSO <sub>4</sub>	19.8 ± 0.9	18.6 ± 1.3 <sup>a</sup>	9.4 ± 0.8 <sup>a</sup>	9.0 ± 0.8 <sup>a</sup>	13.5 ± 0.8

EC, (-)-Epicatechin; EGC, (-)-Epigallocatechin; ECg, (-)-Epicatechingallate; EGCg, (-)-Epigallocatechingallate; BHT, Dibutyl hydroxy toluene. LNA-loaded hepatocytes, were incubated with 100 μM metal ions in the presence of various concentrations of catechins. Values are the concentrations that inhibit lipid peroxidation by 50% (IC<sub>50</sub>, μM). IC<sub>50</sub> is calculated from the concentration-activity curves. Data are expressed as the mean ± S.E. of four experiments. Statistical analysis was performed among metal groups for each catechin. *a*) *p* < 0.05 significantly different from the value of Fe.

EGC, ECg and EGCg were also less effective in suppressing Fe-induced lipid peroxidation in LNA-loaded hepatocytes. EC, however, acted prooxidatively in the presence of 100 μM Fe ion. The antioxidative efficiency of catechins decreased in the order of EGCg > ECg > EGC against Fe-induced lipid peroxidation in LNA-loaded hepatocytes. The catechins similarly suppressed V- and Cu-induced lipid peroxidation in LNA-loaded hepatocytes. The rank order of antioxidative efficiency was EGCg = ECg > EGC > EC and EGCg = ECg > EGC = EC for V and Cu ions, respectively. The catechin gallates were always more effective than the regular catechins especially in LNA-loaded hepatocytes as compared with normal cells. BHT also had a similar degree of suppressive efficiency against Fe-, V- and Cu-induced lipid peroxidation in LNA-loaded hepatocytes. In contrast, the catechins were about 2–10 times more active for V and Cu ions than for Fe ion in LNA-loaded hepatocytes.

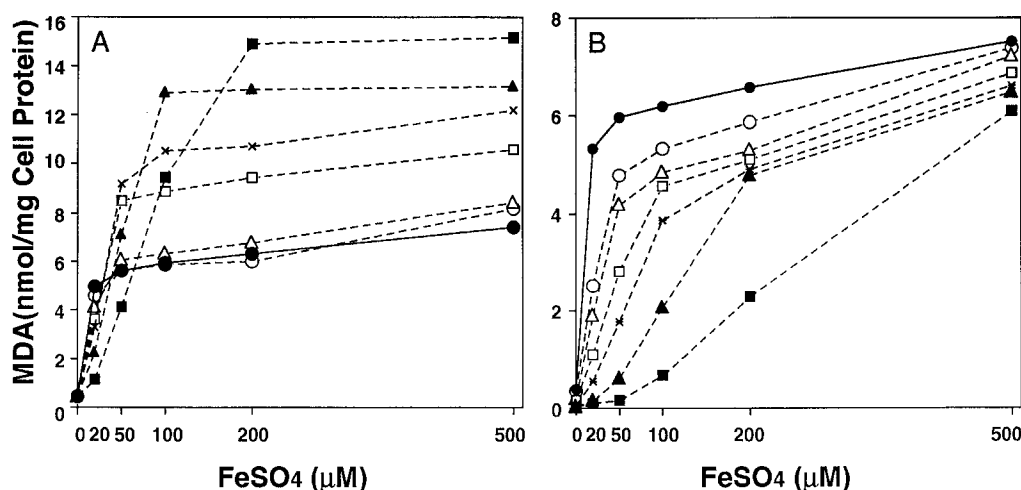
Catechins such as (+)-catechin, (+)-epicatechin and (±)-catechin were examined for their effects on Fe-induced lipid peroxidation in LNA-loaded hepatocytes. As shown in Fig. 2, all those catechins of them acted prooxidatively in the presence of 100 μM Fe ion in LNA-loaded hepatocytes. There was no significant difference in prooxidative efficiency among them. These catechins acted antioxidative against V- and Cu-induced lipid peroxidation in LNA-loaded hepatocytes (data not shown).

The effects of EC and EGC at concentrations of 10 to 500 μM on Fe-induced lipid peroxidation were examined after 6 hr incubation with LNA-loaded cells in the presence of Fe ion at various concentrations (Fig. 3). The increase in MDA accumulation was dose-saturated above 50 μM Fe ion. EC at concentrations below 200 μM suppressed lipid peroxidation induced by 20 μM Fe ion but promoted

**Fig. 2.** Prooxidative Effects of Catechin Enantiomers on FeSO<sub>4</sub>-Induced Lipid Peroxidation in LNA-Loaded Hepatocytes

The medium of LNA-loaded hepatocytes was replaced by fresh complete Williams' medium E without LNA-BSA prior to the addition of catechins. Each catechin dissolved in dimethyl sulfoxide was added to the cultures at a concentration of 100 μM 10 min before the addition of Fe salts (100 μM) dissolved in saline. After incubation for 6 hr, MDA in the medium was assayed. *a*: significantly different from the value of FeSO<sub>4</sub> only, *p* < 0.05.

oxidation induced by Fe ion above 50 μM. 500 μM EC was antioxidative at 50 μM Fe ion but was most prooxidative above 200 μM Fe ion. Thus, the anti- and prooxidative effects of EC were concentration-dependent. On the other hand, EGC inhibited lipid peroxidation in a concentration-dependent manner at all Fe concentrations ranging from 20 to 500 μM. The inhibitory efficiency of EGC decreased with increasing Fe ion concentrations, indicating that the chelating ability of EGC is involved in its antioxidative activity. The antioxidative efficiency of ECg and EGCg also decreased with the decrease in the molar ratio of catechins to Fe ion (data not shown).



**Fig. 3.** Effects of Varying Concentrations of EC (A) and EGC (B) on Lipid Peroxidation Induced by FeSO<sub>4</sub> at Various Concentrations in LNA-Loaded Hepatocytes

Hepatocytes in culture were incubated for 12 hr with 1.0 mM LNA-BSA. The medium was replaced by fresh complete Williams' medium E without LNA-BSA prior to the addition of EC and EGC. LNA-loaded hepatocytes were incubated with EC and EGC at concentrations of 0 (●), 10 (○), 20 (△), 50 (□), 100 (×), 200 (▲) or 500 (■) μM 10 min before the addition of Fe salts dissolved in saline. After incubation for 6 hr, the medium was separated and MDA in the medium was assayed. The values shown represent the typical results of at least four separate experiments.

## DISCUSSION

Flavonoids are generally considered to act as antioxidants via scavenging free radicals generated during lipid peroxidation rather than metal chelating.<sup>5-7</sup> Radical scavenging occurs by reducing the active oxygen species and/or lipid peroxy radicals by means of hydrogen atom donation from the free hydroxyls on the B-ring, which subsequently gives phenoxy radicals.<sup>8-11</sup> Bors *et al.*<sup>24</sup> proposed that the C2-C3 double bond in conjugation with the C4 carbonyl group on the C-ring strengthens radical scavenging by stabilizing phenoxy radicals via an electron delocalization from phenoxy radicals on the B-ring to the C-ring. Therefore, they reported that quercetin with the most favorable structural features for scavenging free radicals exhibited the most effective antiradical activity. Catechin groups with a saturated heterocyclic ring lack the essential structural aspect required for effective radical scavenging. Nevertheless, in contrast to Bors' view, tea catechins such as EGC, ECG and EGCg inhibited lipid peroxidation in cultured hepatocytes just as or more effectively than flavonols such as myricetin, quercetin and fisetin, which satisfy all aspects of structural advantage.<sup>22</sup>

Another possible mechanism for the antioxidative activity of flavonoids is via metal chelating by phenolic OH groups. There are three

possible metal-complexing domains which can interact with metal ions by hydrogen bonding, 1) between the 3',4'-dihydroxy group on the B-ring, 2) between the 3-hydroxy and 4-carbonyl group on the C-ring, and 3) between the 5-hydroxy and 4-carbonyl group on the C-ring. In comparison with quercetin, the catechin group such as catechin, EC and EGC has only the first domain. The metal chelating ability of flavonoids can be estimated by the bathochromic shifts of their absorption peak. Paganga *et al.*<sup>14</sup> suggested that the interaction between catechin and iron and copper ions was less extensive than that of quercetin when judged by the changes in spectroscopic properties. In contrast, Morel *et al.*<sup>25</sup> reported that catechin was more effective than quercetin in removing Fe ion inside the hepatocytes. In the present investigation, we investigated the metal chelating potency of the 3',4'-dihydroxy catechol on the B-ring by comparing the antioxidative ability of tea catechins to inhibit various metal ion-induced lipid peroxidation in cultured hepatocytes. Our working hypothesis was that if the antioxidative action of catechins was not via metal chelating but mostly via scavenging free radicals, each catechin should have the same preventive efficiency against all metal-induced lipid peroxidation irrespective of the species of metal ion. However, depending on the metal ions, each catechin showed significantly different degrees of efficiency in pre-

venting lipid peroxidation. In addition, the antioxidative activity of EGC, ECg and EGCg became less effective with the increase in Fe ion concentration up to 500  $\mu\text{M}$ , whereas the potency of Fe ion to induce lipid peroxidation reached a plateau at about 50  $\mu\text{M}$  Fe concentration (Fig. 3). These results suggest that the metal chelating ability of the 3',4'-dihydroxy catechol configuration on the B-ring may make an essential contribution to the antioxidative activity of catechins.

Transition metals are strongly implicated in the generation of highly reactive hydroxyl radicals by the superoxide-driven Fenton reaction and in the direct reductive decomposition of lipid hydroperoxides (LOOH) to provide alkoxy and lipid peroxy radicals as propagation radicals.<sup>26,27)</sup> Thus, metal chelating by flavonoids could be considered to prevent lipid peroxidation either by sequestering metal ions into inert complexes unable to decompose  $\text{H}_2\text{O}_2$ , or by restricting the access of metal ions toward LOOH which are consistently produced in living cells.<sup>28,29)</sup> The question always arises as to why flavonoids can inhibit lipid peroxidation at concentrations too low to chelate the supplemented metal ions. There is one possible explanation, namely that flavonoids are highly accessible toward LOOH because of their hydrophobic property. However, catechin and EC unexpectedly promoted Fe-induced lipid peroxidation in LNA-loaded hepatocytes susceptible to lipid peroxidation by metal ions. The prooxidative behavior of flavonoids in the presence of metal ions has been observed when deoxyribose, DNA and rarely lipids were used as oxidative substrates.<sup>30-32)</sup> Quercetin and myricetin stimulate Fe-dependent oxidative degradation of deoxyribose and DNA in the presence of EDTA and bleomycin, respectively.<sup>30,31)</sup> In liposomes oxidized at 37°C with 10  $\mu\text{M}$  cupric ion, EGC and EGCg promoted lipid peroxidation.<sup>32)</sup> The mechanism underlying the prooxidative action of these flavonoids is assumed to be ascribed to the reduction of the ferric and cupric ions to a lower valence state, ferrous and cuprous ions which cause the generation of reactive oxygen species, and the propagation of lipid peroxidation. Flavonoids including catechins are considered to have the capacity to reduce metal ions due to their relatively low redox potential.<sup>33)</sup> However, the capability to reduce metal ions are common to flavonoids including the catechin group, and EC is a rather weak hydrogen donor. Furthermore, flavonols such as quercetin and myricetin, which have the lowest oxidative potential, acted consis-

tently as antioxidants in LNA-loaded cells.<sup>22)</sup> These findings suggest that the prooxidative effect of catechin and EC do not directly reflect the strength of their metal reducing capacity. A few reports state that the redox potential of metal ions is strongly affected by the presence and nature of chelators.<sup>34,35)</sup> Yoshida *et al.*<sup>35)</sup> previously studied the effect of various chemically synthetic chelators on metal-induced lipid peroxidation in relation to the change in the redox potential of metal-chelator complexes. They reported that the change in the redox potential of metal ions influences the rate of lipid peroxidation. It is plausible that the chelation of metal ions by flavonoids decrease the redox potential of metal-flavonoid complexes. The induction of lipid peroxidation in cells would require the close access of metal ions in the low valence state toward LOOH. To participate effectively in the reductive decomposition of LOOH, metal ions need to be reduced from the high valence state to the low valence state by cellular reducing compounds. One possible explanation of the antioxidative action of flavonoids is that the redox potential of their metal complexes becomes too low to allow metal reduction for redox-cycling. On the other hand, the prooxidative action of catechin and EC may be ascribed to the appropriate degree of alteration of the redox potential of the metal-catechin complexes for redox-cycling. We have, at present, no explanation for the antioxidative action of catechin and EC in normal hepatocytes in contrast to their prooxidative action in LNA-loaded cells. Further studies to explain this query are being carried out in our laboratory.

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