Tea Catechins Protect against Lead-Induced Cytotoxicity, Lipid Peroxidation, and Membrane Fluidity in HepG2 Cells

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Recent studies have shown that lead causes oxidative stress by inducing the generation of reactive oxygen species (ROS) and reducing the antioxidant defense system of cells. This suggests that antioxidants may play an important role in the treatment of lead poisoning as a kind of excellent scavenger of free radicals and chelator of heavy metal. Whether tea catechins have protective effects against oxidative stress after lead treatment in cell systems remains unclear. The present study was designed to elucidate if tea catechins have any protective effects on lipid peroxidation damage in lead-exposed HepG2 cells. Exposure of HepG2 cells to Pb2+ decreased cell viability and stimulated lipid peroxidation of cell membranes as measured by the thiobarbituric acid reaction. Electron spin resonance (ESR) spin-labeling studies indicated that lead exposure could decrease the fluidity in the polar surface of cell membranes. Tea catechin treatment significantly increased cell viability, decreased lipid peroxidation levels, and protected cell membrane fluidity in lead-exposed HepG2 cells in a concentration-dependent manner. The galloylated catechins showed a stronger effect than nongalloylated catechins. Cotreatment with (-)epigallocatechin gallate (EGCG) and (-)epicatechin (EC), (-)epicatechin gallate (ECG), and (-)epigallocatechin gallate (EGCG) showed a synergistically protective effect. The results suggest that tea catechin supplementation may have a role in modulating oxidative stress in lead-exposed HepG2 cells.

Key Words: tea; catechins; lead toxicity; lipid peroxidation; antioxidants; oxidative stress; ESR spin labeling; membrane fluidity.

Lead (Pb) is one of the first discovered and most widely used metals in human history and is, therefore, one of the metals most commonly encountered in the environment (Shotyk et al., 1998). Its continued release into the environment as an exhaust emission product, as well as its widespread industrial use, has made lead a serious threat to human health (Juberg et al., 1997). Pregnant women, children, and inhabitants of large cities are at risk of lead intoxication, and lead poisoning is a serious occupational disease in some industries. Exposure to low levels of lead has been associated with behavioral abnormalities, learning impairment, decreased hearing, and impaired cognitive functions in humans and in experimental animals (Cory-Slechta et al., 1995).

A growing amount of evidence indicates that cellular damage mediated by reactive oxygen species (ROS) may be involved in the pathology associated with lead intoxication (Bechara et al., 1993; Hermes-Lima et al., 1991). The malondialdehyde levels in blood were strongly correlated with lead concentration in the blood of exposed workers (Jiun and Hsien, 1994). In erythrocytes from the workers exposed occupationally to lead, the activities of the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase, were remarkably higher than that in nonexposed workers (Monteiro et al., 1985). Gurer et al. demonstrated that lead increased the prooxidant/antioxidant ratio in a concentration-dependent manner in lead-treated CHO cells and in rats (Gurer et al., 1999). The results suggest that antioxidants might play an important role in the treatment of lead poisoning.

Tea, including black, green, and oolong tea, is one of the most widely consumed beverages in the world. During the last decade, numerous in vitro and in vivo studies had suggested that tea and tea polyphenols had strong antioxidant activity (Guo et al., 1996, 1999; Shen et al., 1993), and had numerous potentially beneficial medicinal properties including inhibition of carcinogenesis, tumorogenesis, and mutagenesis, as well as the inhibition of tumor growth and metastasis (Yang et al., 1993). The major polyphenolic compounds in tea are catechins. The four most abundant naturally occurring tea catechins, (-)epicatechin (EC), (-)epigallocatechin (EGC), (-)epicatechin gallate (ECG), and (-)epigallocatechin gallate (EGCG) are shown in Figure 1.

The antioxidant activities of tea catechins have been examined by various methods in vitro and in vivo. Using the chemiluminescence method and the ESR spin-trapping technique, our previous data indicated that green tea polyphenols had higher antioxidant activity than that of vitamins C and E (Zhao et al., 1989). In the lipoprotein oxidation model, which simulates the oxidation of low-density lipoproteins responsible for atherosclerosis, tea catechins also exhibited powerful antioxidant activity: 20 times more potent than vitamin C (Craig, 1999; Vinson et al., 1995). It also has been found that the presence of at least an ortho-dihydroxyl group in the B ring and a galloyl moiety at the 3 position were important in maintain-
ing the effectiveness of their radical scavenging ability (Hayakawa et al., 1999; Guo et al., 1999). However, catechins in high concentration, or in the presence of Cu^{2+}, induced DNA cleavage and accelerated the peroxidative property of unsaturated fatty acid, showing prooxidant (Hayakawa et al., 1997; Shen et al., 1992). Therefore, it is important to elucidate the relationship between their structures and functions, concentration, and effect in a cell system.

Tea is a kind of excellent scavenger of free radicals and chelator of heavy metal (Guo et al., 1991; Kumamoto et al., 2001), but whether tea catechins have protective effects on oxidative stress after lead treatment remains unclear. The present study showed tea catechins could reduce the toxicity of lead in HepG2 cells by examination of the effect of lead on cell viability, malondialdehyde (MDA) levels, and cell-membrane fluidity. The order of membrane fluidity characteristics were estimated from the line width and shape of the ESR spectra. Lower order and faster motion means higher membrane fluidity. The order of membrane hydrocarbon chains is described by the order parameter (S) and their motion is described by the rotational correlation time (τc). They are defined as follows (Juntao et al., 2001):

\[
S = \frac{A_{0} - A_{L}}{A_{0} - 0.5(A_{xx} + A_{yy})}
\]

\[
\tau_{c} = 6.51 \times 10^{-10} \times \Delta H(0)[\sqrt{h(0)/h(-1)} + \sqrt{h(0)/h(1)} - 2]
\]

where h(0), h(1), and h(−1) are the peak height of the center, low, and high field lines, respectively; ΔH(0) is the width of the central line; and A0 and AL are parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively, as shown in Figure 2.

**Data analysis.** Throughout the text, data were expressed as mean ± SE of triplicate determinations, from at least three independent experiments. Statistical analysis was determined using a 1-way analysis of variance (ANOVA) with p < 0.05 considered significant.

**RESULTS**

**The maximum non-cytotoxic concentrations of tea catechins.** To select appropriate concentrations of tea catechins to be used in this study, the maximum non-cytotoxic concentrations were determined. After the initial 24-h attachment period, cells were exposed to different concentrations of catechins for an additional 24 h, and the cytotoxicity was measured by the MTT method. As shown in Figure 3, the maximum non-cytotoxic concentrations of EGCg, ECG, EGC and EC were about 15, 5, 80, and 20 μM, respectively.

**Protective effects of tea catechins on lead-exposed HepG2 cell viability.** Using the MTT method, it was found that the toxicity of Pb^{2+} was both time- and concentration-dependent at 570 nm with a Bio-RAD 3350 microplate reader, and the percentage viability was calculated.

**Measurement of TBARS.** Lipid peroxidation was assayed by determining the production rate of thiobarbituric acid reactive substances (TBARS) and was expressed as malondialdehyde (MDA) equivalents. In brief, cells grown on 6-well plates were washed with 0.01 M PBS, scraped, and resuspended in 1 ml PBS. An aliquot was taken out for a protein assay, and 0.5 ml TBA reagent (100 mg trichloroacetic acid, 3.35 mg thiobarbituric acid) was added to each tube and vortexed. The reaction mixture was heated at 90°C for 20 min and stopped on ice. After cooling to room temperature, TBARS were extracted with 1.0 ml n-butanol and separated at 3000 x g centrifugation for 5 min. The absorbency of the total TBARS was measured at 532 nm. Tetraethoxypropane in absolute ethanol was used to prepare MDA standards. The measurements were performed in triplicate and the results were expressed as nmol equivalent of MDA/mg protein.

**Spin labeling the cells with 5-doxyl or 16-doxyl.** Fatty acid spin-labels of 5-doxyl and 16-doxyl, which have a stable nitroxide radical ring at the C-5 and C-16 positions, respectively, were used as a lipid probe in the cell membrane. They are well dissolved in lipids and their ordering and dynamics reflect the motion of the surrounding phospholipid hydrocarbon chains. In brief, 100 μl HepG2 cell suspension (10^7 cells/ml) was mixed with 5 μl of 5-doxyl or 16-doxyl (1.0 mM) spin label, incubated at 37°C for 60 min, then the free labels washed out by 0.01 M PBS from the cell system until there were no ESR signals in the supernatant. ESR measurement condition: microwave power 20 mW, modulation amplitude 0.2 mT, X-band, modulation frequency 100 kHz, sweep width 10 mT, and temperature 298K.

**Membrane fluidity calculation.** The membrane fluidity characteristics were estimated from the line width and shape of the ESR spectra. Lower order and faster motion means higher membrane fluidity. The order of membrane hydrocarbon chains is described by the order parameter (S) and their motion is described by the rotational correlation time (τc). They are defined as follows (Juntao et al., 2001):

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where h(0), h(1), and h(−1) are the peak height of the center, low, and high field lines, respectively; ΔH(0) is the width of the central line; and A0 and AL are parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively, as shown in Figure 2.

**Data analysis.** Throughout the text, data were expressed as mean ± SE of triplicate determinations, from at least three independent experiments. Statistical analysis was determined using a 1-way analysis of variance (ANOVA) with p < 0.05 considered significant.
in HepG2 cells, as shown in Figure 4 when the concentration exceeded to 1.0 μM, Pb⁺⁺ significantly decreased HepG2 cell viability. It was evident that increasing exposure times showed steeper concentration-response curves.

In order to test the protective effects of tea catechins, HepG2 cells were simultaneously exposed to 100 μM Pb⁺⁺ and tea catechins. As shown in Figure 5, the results indicated that tea catechins significantly increased viability of lead-exposed HepG2 cells in the used concentration. The effective protective concentration for EC EGC, ECG, and EGCG were about 15–20 μM, 40–50 μM, 3–5 μM, and 8–15 μM, respectively. As far as EC and EGCG were concerned, in the range of nontoxic concentration, the higher the concentration was, the stronger the protective effect became. It was surprising to find that 80 μM EGC synergistically promoted lead-induced cell toxicity.

Furthermore, the synergistic protective effect of tea catechins against Pb⁺⁺-induced cell toxicity was studied. As shown in Figure 6, simultaneous treatment by two kinds of catechins showed better protective effect on cell viability than one catechin, among which EC + EGCG was the best one.

*Inhibitory effect of tea catechins on TBARS formation in HepG2 cell exposed to lead.* In recent studies, the toxic effects of lead have been attributed to lead-induced oxidative stress and -stimulated lipid peroxidation of membrane lipids.

FIG. 2. Typical ESR spectra of the 5-doxyl (A) and 16-doxyl 1 (B) labeled HepG2 cell. The detailed experimental conditions were described in Materials and Methods.

FIG. 3. Effect of different concentrations of tea catechins on HepG2 cell viability. HepG2 cells were cultured with different catechins for 24 h and cell viability was determined by the MTT method. Data are expressed as a percentage of the untreated control ± SE, n = 7. *Significant difference from control by ANOVA, p < 0.05.

FIG. 4. Effect of Pb⁺⁺ on HepG2 cell viability. HepG2 cells were cultured with various concentrations of Pb⁺⁺ for different times, and cell viability was determined by MTT assay. Data are expressed as a percentage of the untreated control ± SE, n = 7. *Significant difference from control by ANOVA; p < 0.05.
This process results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation products (MDA and other aldehydes), which are extremely toxic for the cells. As shown in Figure 7, 0.01 \( \mu M \) \( Pb^{++} \) had no effect on TBARS formation, but when the concentration of \( Pb^{++} \) exceeded 0.1 \( \mu M \), \( Pb^{++} \) significantly promoted TBARS formation in HepG2 cells in a concentration-dependent manner.

In order to determine the structure-activity relation of different catechins, the effects of tea catechins on TBARS formation in HepG2 cells treated by \( Pb^{++} \) were studied. Compared with the control, only treated by 100 \( \mu M \) \( Pb^{++} \), all catechins significantly decreased TBARS formation in the range of the maximum non-cytotoxic concentrations, except that 80 \( \mu M \) EGC showed notably prooxidant (Fig. 8). The synergistic inhibitory effect of tea catechins on TBARS formation in HepG2 cells exposed to lead is shown in Figure 9. It was found that co-treatment with EC and EGC, EGCG and EC, and EGCG had a synergistic inhibitory effect against TBARS formation.

Protective effect of tea catechins on membrane fluidity in HepG2 cells exposed to lead. The order parameter (S) calculated from the spectra is shown in Figure 10. From the data labeled with 5-doxyl, it was found that \( Pb^{++} \) increased the order parameter (S) in a concentration-dependent manner, and the difference was significant when its concentration reached 100 \( \mu M \) (\( p < 0.05 \)). In addition, from the data labeled with 16-doxyl, it was found that 100 \( \mu M \) \( Pb^{++} \) also lightly increased the rotational correlation time (\( \tau_c \)), but the difference was not significant, even if the concentration of \( Pb^{++} \) reached 500 \( \mu M \) (data not shown). The results suggested that 100 \( \mu M \) \( Pb^{++} \) exposure decreased the fluidity near the polar surface of the cell membrane, but the membrane fluidity in the hydrophobic region was not affected significantly.

Tea catechins decreased the order parameter (S) of \( Pb^{++} \)-exposed HepG2 cell membrane in a concentration-dependent manner, as shown in Figure 11. The order parameter (S) treated by both 5 \( \mu M \) ECG and 15 \( \mu M \) EGCG showed little difference from the control. As shown in Figure 12, both EGCG + ECG and EC + EGCG showed synergistic effects on order parameter (S), which is similar to those of both cell viability and TBARS formation.

DISCUSSION

Many pieces of evidence suggest that cellular damage mediated by oxidative stress may be involved in some of the pathologies associated with lead toxicity (Adonaylo et al., 1999; Sandhir et al., 1995). Lead stimulated oxidative hemolysis of erythrocytes, decreased erythrocyte SOD activity and accelerated conversion of oxyhemoglobin to methemoglobin (Gurer et al., 2000). An inverse relationship was observed between blood-lead concentration and serum levels of \( \alpha \)-tocopherol and ascorbic acid in pregnant women (West et al., 1994). Therefore, it is reasonable to believe that antioxidants should be considered as a component of an effective treatment for lead poisoning.

Tea catechins are strong scavengers against superoxide, hydrogen peroxide, hydroxy radicals, and nitric oxide produced by various chemicals. They also could chelate with metals because of the catechol structure (Rice-Evans et al., 1997). These characteristics make tea catechins ideal candidates for treatment of lead toxicity. The data from our studies of HepG2 cells indicated that the higher concentration of lead treatment decreased cell viabilities and increased lipid peroxidation levels. Treatment by tea catechins increased cell viability and reversed the effects of lead on oxidative stress parameters in a concentration-dependent manner. The galloylated catechins showed stronger protective effect against oxidative damage than that of nongalloylated catechins, which is similar to the result of scavenging ability on free radicals (Guo et al., 1996, 1999). Galloylated catechins containing more phenolic hydroxyl groups had stronger chelating ability with metal ions than nongalloylated catechins (Guo et al., 1991). Therefore, the protective effect of tea catechins on oxidative damage in HepG2 cells exposed to lead might be related to both their ability to scavenge free radicals and to chelate metal ions.

ESR spin labeling technique is a sensitive and reliable method to study the physical state of cell membranes. Order parameter (S) and rotational correlation time (\( \tau_c \)) represent the degree of hydrocarbon chains’ long-range alignments along the membrane and the motion state of these chains. As shown in Figure 7, the increase of lipid peroxidation levels indicated that
lead caused oxidative damage to hepatic cell membranes. The peroxidation of hepatic cell membrane phospholipids and accumulation of lipid peroxides are expected to modulate the membrane fluidity and consequently the membrane function. The observed changes in the rotational correlation time ($\tau_c$) and order parameter ($S$) (Fig. 10) indicated that the fluidity near the surface of the membrane was decreased after 100 $\mu$M-lead treatment, but the fluidity in the hydrophobic core of the membrane was not affected after the treatment. Lead induced arachidonic acid augmentation (Lawton et al., 1991) and bound strongly to phosphatidylcholine membranes in vitro (Shafigur-Rehman et al., 1993), which could result in altered membrane integrity, permeability, and fluidity. These might be connected with the enhanced lipid peroxidation in HepG2 cells.

Tea catechins are mainly composed of 50–60% EGCG, 8–12% EGC, 15–20% ECG, and 4–7% EC. As reported previously, tea catechins scavenged free radicals in the order: EGCG ≈ ECG > EGC > EC (Guo et al., 1996, 1999). Okabe also reported the similar order in inhibiting growth of human lung cancer cell line PC-9 (Okabe et al., 1997). Because of its high activity and content, EGCG seems to be the most effective antioxidant in all the components of green tea catechins. However, several researches showed that the tea catechin complex had a stronger effect than EGCG in the scavenging capacity of free-radical and anticarcinogenic activities (Shen et al., 1993). This allows us to think that the constituents of tea catechin complex together have synergistic or additive effects on scavenging free-radical and cancer-preventive activity. Support for this activity was obtained from Suganuma’s study that ($^3$H) EGCG incorporation into PC-9 cells was significantly enhanced by EC. Also, co-treatment with EGCG, EC, ECG, EC,
EGC, and EC synergistically induced apoptosis of PC-9 cells and inhibited tumor necrosis factor-α release from BALB/c-3T3 cells (Okabe et al., 1997; Suganuma et al., 1999). Our previous research also demonstrated that various catechins in tea polyphenols constituted an antioxidant cycle, in accordance with the decreasing order of their first reductive potentials, and produced a coordinating, strengthening effect (Shen et al., 1993). As shown in Figures 6, 9, and 12, the current data indicated that both EC and ECG significantly promoted the protective effect of EGCG. The mechanisms of action of ECG and EC are thought to be different because ECG did not stimulate EGCG incorporation into cells, whereas EC did (Suganuma et al., 1999). Although the co-treatment with ECG and EGCG produced interesting results, the mechanisms of the action have not been well identified. Hashimoto et al. (1999) found that ECG had the highest affinity for the lipid bilayer in membrane, followed by EGCG, EC, and EGC, with the partition coefficients of ECG in n-octanol/PBS being highest. Our former research suggested that the closer the first reductive potentials were, the more significant the coordinating and strengthening effects became (Shen et al., 1993). Therefore, it is reasonable to deduce that the closer first reductive potential of EGCG and ECG, as well as their stronger affinity for lipid bilayer, allows them to easily enter the cell membrane and to show synergic effect. But the mechanism should be further investigated.

Recently, much attention has been paid to the prooxidant quality of natural products. It has been reported that, in the presence of the copper (II) ion under aerobic conditions, tea catechins induced DNA cleavage, accelerated the peroxidation of unsaturated fatty acid (Hayakawa et al., 1997), and killed Escherichia coli. (Kimura et al., 1998). These effects were apparently due to the prooxidant property of catechins. Our previous research also showed that both tea catechins complex and EGCG produced superoxide anion radical and semiquinone anion radicals in alkaline solution in vitro (Shen et al., 1992). The results in this paper showed that even in the range of maximum nontoxic concentration, EGC demonstrated significant prooxidant signs, as shown in Figures 5 and 8. These might be correlated with the toxicological effect of tea catechins. The investigation also placed catechins, under certain conditions, into radical-generating toxicological agents. Therefore, much consideration for safety should be required when tea catechins are used as therapeutic reagents or nutrition supplements.

Tea catechins are strong metal ion chelators because of the catechol structure (Guo et al., 1991; Kumamoto et al., 2001; Rice-Evans et al., 1997). Though they have been shown to form stable complexes with Fe²⁺, Ca²⁺, Al³⁺, Mn²⁺, Cr³⁺, and

**FIG. 7.** Effects of different concentrations of Pb²⁺ on TBARS formation in HepG2 cells. HepG2 cells were cultured with various concentrations of Pb²⁺ for 24 h and the lipid peroxidation level was determined as described in Materials and Methods. *Significant difference from control by ANOVA; p < 0.05.

**FIG. 8.** Effect of tea catechins on TBARS formation in HepG2 cells treated by Pb²⁺. HepG2 cells were cultured with various concentrations of tea catechins and 100 μM Pb²⁺ for 24 h and the lipid peroxidation level was determined as described in Materials and Methods. *Significant difference from control in the presence of 100 μM Pb²⁺ by ANOVA; p < 0.05.
Pb$^{2+}$ (Guo et al., 1991; Kumamoto et al., 2001), further investigation is needed as to whether tea catechins are capable of removing lead from the bloodstream and target organs.

The present study was designed to elucidate whether tea catechins resulted in decreased lipid peroxidation in HepG2 cells treated by lead. The hypothesis was evidenced in tea catechin-treated HepG2 cells exposed to lead. Therefore it can

![FIG. 9. Synergistic effect of tea catechins on TBARS formation in HepG2 cells exposed to Pb$^{2+}$. After the initial 24-h attachment period, HepG2 cells were exposed to both 100 μM Pb$^{2+}$ and different tea catechins for an additional 24 h, and the lipid peroxidation level was determined as described in Materials and Methods. The concentrations of EC, ECG, EGC, and EGCG were 10 μM, 3 μM, 10 μM, and 10 μM, respectively. *, #, &, and $: Significat differences from EC; ECG; EGCG, and EGC, respectively; p < 0.05.]

![FIG. 10. Effect of different concentrations of Pb$^{2+}$ on order parameters (S) of HepG2 cells. HepG2 cells were cultured with various concentrations of Pb$^{2+}$ and order parameter was determined by ESR spin-trapping technique. *Significant difference from control by ANOVA, p < 0.05.]

![FIG. 11. Effects of tea catechins on order parameters (S) of HepG2 cells exposed to Pb$^{2+}$. HepG2 cells were cultured with various concentrations of tea catechins and 100 μM Pb$^{2+}$ for 24 h, and the order parameter (S) was determined by ESR spin-labeling technique. *Significant difference from control in the presence of 100 μM Pb$^{2+}$ by ANOVA, p < 0.05.]

![FIG. 12. Synergistic effects of tea catechins on order parameter of HepG2 cells exposed to Pb$^{2+}$. After the initial 24-h attachment period, HepG2 cells were exposed to both Pb$^{2+}$ (100 μM) and different tea catechins for an additional 24 h, and order parameter was determined by ESR spin-labeling technique. The concentrations of EC, ECG, EGC, and EGCG were 10 μM, 3 μM, 10 μM, and 10 μM, respectively. *, #, and &: Significant difference from EC, ECG, and EGCG, respectively; p < 0.05.]

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be deduced that the increased cell viability in tea catechin-treated cells, along with improved lipid peroxidation levels, reflects the antioxidant action of tea catechins in lead-treated cells. Results from the study of cell membrane fluidity suggest that the beneficial effects of tea catechins on lipid peroxidation are related to its ability to protect cell membrane against damage by lead.

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