# Inhibition of Endothelial Cell Mediated Low-Density Lipoprotein Oxidation by Green Tea Extracts

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Plant phenolics present in fruits and vegetables and that are particularly rich in tea and wine have received considerable attention because of their potential antioxidant activity. Two types of commercial green tea from Japan and two active components of green tea, catechin and epicatechin, were assessed for their relative abilities to inhibit the oxidation of low-density lipoprotein (LDL) mediated by human aortic endothelial cells (HAEC). The tea extracts, catechin and epicatechin, were incubated with HAEC and LDL for 12 h. After incubation, conjugated dienes were measured by spectrophotometry at 234 nm as an index of hydroperoxide formation, and hexanal was measured by static headspace gas chromatography as an index of hydroperoxide decomposition. On the basis of conjugated dienes and hexanal, inhibition of LDL oxidation was dose dependent for all compounds tested. LDL oxidation was inhibited 3.9−98% at concentrations ranging from 0.08 to 5 ppm of the green tea extracts. Both catechin and epicatechin inhibited oxidation by 0.5−97% at concentrations ranging from 0.08 to 5.00 $\mu$M. The pure compounds and the two tea extracts tested inhibited the formation of early lipid peroxidation products as well as the end stage lipid peroxide decomposition products. The polyphenolic components of green tea may have nutritional benefits as inhibitors of LDL oxidation.

**Keywords:** *Antioxidants; green tea; catechin; epicatechin; plant phenolics; human aortic endothelial cells; low-density lipoprotein (LDL) oxidation*

## INTRODUCTION

Tea, particularly green tea, is a rich source of the flavan-3-ol class of flavonoids, also known as catechins. Recent research has suggested that dietary flavonoids, which possess antioxidant activity, may play a role in human health and disease, particularly in diseases believed to involve, in part, oxidation, such as coronary heart disease, inflammation and mutagenesis leading to carcinogenesis. The Dutch Zutphen elderly longitudinal study found an inverse relationship between flavonoid intake and mortality from heart disease (Hertog et al., 1993). Black tea was the major source of dietary flavonoids in this Dutch population. In an epidemiological study of Japanese men, an inverse relationship was reported between green tea consumption and both serum cholesterol levels and systolic blood pressure (Kono et al., 1992). In a population of Norwegian men and women a similar inverse relationship was found; although the type of tea was not specified, black tea is mainly consumed in Norway (Stensvold et al., 1992). Both elevated serum cholesterol and systolic blood pressure are known risk factors for heart disease.

Cholesterol-fed rats supplemented with crude green tea catechins had decreased plasma total cholesterol levels compared to nonsupplemented rats (Muramatsu et al., 1986). Rats fed high palm or perilla oil diets supplemented with an extract of green tea catechins had higher plasma and erythrocyte α-tocopherol levels as compared to nonsupplemented rats, suggesting an in vivo antioxidant function for the tea catechins (Nanjo et al., 1993). Research on the anticarcinogenic effects of green tea was reviewed by Yang and Wang (1993). Protective benefits from consumption of tea catechins was demonstrated in various animal tumorigenesis models. The growth of tumors was suppressed in a dose-dependent fashion in sarcoma-cell inoculated rats consuming diets containing crude tea catechins (Hara et al., 1989). Chemically induced tumorigenesis was delayed in mice fed crude tea catechins (Hara, 1994).

The animal and epidemiological studies cited above are further supported by in vitro studies that demonstrate antioxidant activity of tea catechins in protecting low-density lipoprotein (LDL). The oxidative modification of LDL is currently viewed as an important step in the pathogenesis of atherosclerosis (Steinberg et al., 1989; Esterbauer et al., 1992). Consequently protection of LDL against oxidation by various plasma antioxidants is considered to be important in the prevention or slowing of this disease process. Although the exact mechanism for initiation of oxidation is not known, LDL can be oxidized in vitro by several vascular cells, including endothelial cells (Henriksen et al., 1981; Morel et al., 1984; Steinbrecher et al., 1984), monocytes and macrophages (Morel et al., 1983; Parthasarathy et al., 1986). Modified LDL is taken up by macrophages via scavenger receptors, leading to the formation of lipid-engorged foam cells. Recognition and uptake of oxidized LDL is believed to require derivatization of amino acid residues in apoprotein B-100 (apo B) that is caused by aldehyde decomposition products of lipid hydroperoxides

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(Rosenfeld et al., 1990). Decomposition of lipid hydroperoxides is a necessary prerequisite for the formation of apo B epitopes that are recognizable by scavenger receptors (Jessup et al., 1990). Dietary flavonoids have been shown to inhibit the oxidation of LDL in various in vitro assays.

The monomers (+)-catechin, (−)-epicatechin, and their gallate esters (−)-epigallocatechin (EGC), (−)-epicatechingallate (ECG), and (−)-epigallocatechingallate (EGCG), found in green tea, inhibited copper-catalyzed LDL (Miura et al., 1994) or LDL/VLDL oxidation (Vinson et al., 1995) as measured by formation of conjugated dienes or thiobarbituric acid-reactive substances (TBARS). Conjugated dienes are an early marker of polyunsaturated fatty acid peroxidation, and TBARS are commonly used as a relatively nonspecific marker of lipid peroxidation. Chinese green tea polyphenols (Zhenhua et al., 1991) and (+)-catechin (Mangiapane et al., 1992) inhibited the oxidative modification of LDL by macrophages as measured by macrophage uptake. The antioxidant activities of green tea extracts were assessed in a variety of other lipid systems with varying results. Green tea extracts had antioxidant activity in vegetable oils and animal fats (Das et al., 1965; Matsuzaki and Hara 1985; Balentine 1992). Green tea extracts had antioxidant activity in bulk corn oil but had prooxidant activity in the corresponding oil-in-water emulsions (Frankel et al., 1997). These varying results highlight the need to evaluate potential antioxidants with different biologically relevant systems.

We previously reported on the antioxidant activity of green tea components and commercial green tea extracts in tocopherol-stripped bulk corn oil, in the corresponding oil-in-water emulsions, and in soybean lecithin liposomes (Frankel et al., 1997; Huang and Frankel, 1997). In the present study, two commercial green tea extracts were compared with the individual green tea components (±)-catechin and epicatechin in a human aortic endothelial cell culture system, which mediates the oxidation of LDL. Lipid oxidation was followed by determining conjugated diene lipid hydroperoxides spectrophotometrically and hexanal by static headspace gas chromatography.

## MATERIALS AND METHODS

**Materials.** Human aortic endothelial cells (HAEC) were obtained from Clonetics (San Diego, CA) at passage 3. Cells were grown in Clonetics microvascular endothelial cell growth medium containing 5% fetal bovine serum, epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL), bovine brain extract containing 10 ng/mL heparin, gentamicin (50 μg/mL), and amphotericin-B (50 ng/mL). Stock solutions of antioxidants were prepared in dimethyl sulfoxide (DMSO). Catechin and epicatechin were obtained from Sigma (St. Louis, MO). Nikken tea extract powder (NTEP) from Nikken Foods Co. Ltd. (Tokyo, Japan) and green tea catechin powder (GTCP), a spray-dried aqueous extract of Japanese Sencha green tea prepared according to EP 456 023 (Lunder, 1991), were a gift from T. L. Lunder Nestec Ltd. (Lausanne, Switzerland).

**HPLC Analyses of the Green Tea Extracts.** The compositions of the two commercial green tea extracts were determined by HPLC, as previously described (Frankel et al., 1997). As determined by HPLC, the composition of the NTEP extract, expressed as the percentage weight of the total sample weight, was as follows: EGC plus catechin, 3.8; caffeine, 8.1; epicatechin, 6.7; EGCG, 23.5; ECG, 5.2; and total catechins, 39.2. The GTCP extract contained EGC plus catechin, 5.3; caffeine, 4.4; epicatechin, 6.8; EGCG, 29.7; ECG, 5.3; and total catechins, 47.1.

**Table 1. Inhibition of Cell-Mediated LDL Oxidation by Green Tea Extracts[a]**

| | % inhibition of conjugated dienes[b] from given green tea extracts | |
|---|---|---|
| concn (ppm) | NTEP | GTCP |
| 0.08 | $32.4 \pm 2.6^{c,x}$ | $3.9 \pm 0.3^{d,y}$ |
| 0.16 | $51.1 \pm 3.5^{b,x}$ | $36.4 \pm 6.7^{c,x}$ |
| 0.31 | $93.7 \pm 6.3^{a,x}$ | $63.8 \pm 5.4^{b,y}$ |
| 0.63 | $93.8 \pm 4.5^{a,x}$ | $86.0 \pm 3.5^{a,x}$ |
| 1.25 | $98.0 \pm 0.6^{a,x}$ | $88.7 \pm 9.9^{a,x}$ |
| 2.50 | $94.9 \pm 5.3^{a,x}$ | $91.9 \pm 2.5^{a,x}$ |

[a] Human LDL was incubated with HAEC in Ham's F-10 medium in the presence or absence of the phenolic compounds. Absorbance readings at 234 nm were taken initially and at the end of a 12 h incubation period. [b] Mean % inhibition ± SD, $n = 2$. Percent inhibition of conjugated dienes = $[(C - S)/C \times 100]$, where $C$ = conjugated diene production in the presence of DMSO and $S$ = conjugated diene production in the presence of the test compound. Within columns, means with the same superscripts a−d are not significantly different. Within rows, means with the same superscripts x−y are not significantly different.

**Preparation of Human LDL.** Blood was obtained from healthy, nonsmoking male volunteers by venipuncture into EDTA-containing vacutainer tubes. LDL was obtained by sequential density ultracentrifugation, as previously described (Pearson et al., 1997).

**Cell Incubation.** HAEC between passages 5 and 7 were seeded onto 35 mm, six-well plates and used at confluence, as previously described (Pearson et al., 1997). Briefly, duplicate wells were incubated for 12 h in phenol red-free Ham's F-10 (Gibco, Gaithersburg, MD), containing 200 μg of LDL protein/mL and the test compounds. The extent of lipid oxidation was determined spectrophotometrically by measuring conjugated diene hydroperoxides at 234 nm.

For hexanal determination, the media from wells were pipetted into 6 mL headspace vials, capped, and allowed to equilibrate in a 37 °C shaker-water bath for 15 min. Vials were then injected into a Perkin-Elmer Sigma 3B gas chromatograph equipped with an HS 6 control static headspace sampler. Retention time was determined with a hexanal standard. The results for conjugated dienes and hexanal were calculated as percentage inhibition by the following equation: [(DMSO control − sample/DMSO control) × 100].

The Ham's F-10 medium used in the experiments did not contain fetal bovine serum, linoleic acid, or other polyunsaturated fatty acids. Thus, the measurements of conjugated dienes and hexanal, a decomposition product of ω-6 polyunsaturated fatty acid hydroperoxides, were specific markers for LDL oxidation in this system. In the absence of endothelial cells, minimal oxidation of LDL was observed. DMSO inhibited HAEC-mediated LDL oxidation by approximately 10%. Therefore, the levels of conjugated diene and hexanal produced in the presence of DMSO were used to calculate the percentage inhibition of conjugated dienes and hexanal for the various test compounds.

**Statistical Analyses.** The antioxidant effects of the green teas, catechin, and epicatechin were analyzed for significant differences between compounds and concentrations by two-way analysis of variance using the general linear model (GLM) procedures in the SAS statistical software package 6.10 (SAS Institute Inc., Cary, NC). Tukey's studentized range test ($p = 0.05$) was used to determine which combinations of compounds and concentrations were statistically different. Least-squares regression analysis was performed to examine relationships between the inhibition of conjugated dienes and hexanal.

## RESULTS

The two green tea extracts inhibited LDL oxidation in a dose-dependent manner at the lower concentrations tested as measured by conjugated dienes (Table 1).

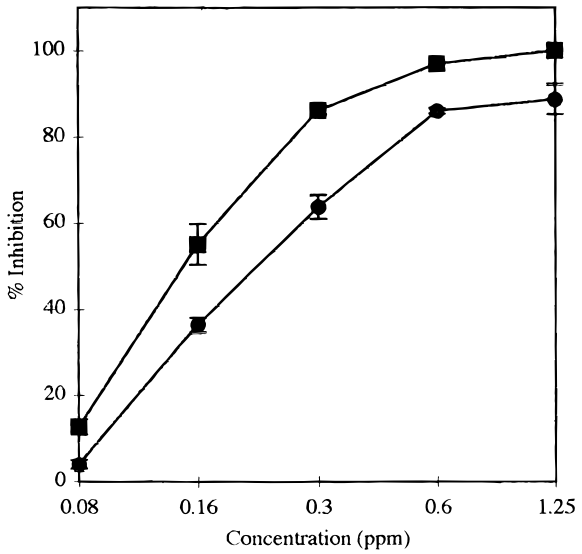


**Figure 1.** Inhibition of cell-mediated LDL oxidation by GTCP extract as measured by conjugated dienes and hexanal. Human LDL was incubated as described in Materials and Methods. Results are expressed as the mean % inhibition ± SD, $n = 2$. Percent inhibition of conjugated dienes (●) or hexanal (■) = $[(C - S)/C \times 100]$, where $C$ = conjugated dienes or hexanal production in the presence of DMSO and $S$ = conjugated dienes or hexanal production in the presence of the test compound.

From 0.08 to 0.31 ppm, the NTEP extract increased inhibition of LDL oxidation linearly, followed by a plateau between 0.31 and 2.5 ppm at approximately 94−95% inhibition. The GTCP extract inhibited LDL oxidation linearly between 0.08 and 0.63 ppm and plateaued between 0.63 and 2.5 ppm at approximately 86−92% inhibition (Table 1). Although there was a trend for the NTEP extract to inhibit oxidation more than the GTCP extract at each concentration tested, the difference was significant only at 0.08 and 0.31 ppm.

Possible cell toxicity by the green tea extracts was evaluated by incubating HAEC with 5 ppm NTEP or GTCP for 12 and 24 h. After incubation, the cells were trypsinized, and cell growth and viability, as indicated by trypan blue exclusion, were determined. No significant differences were observed between the cell growth or percentage viability of the HAEC grown in the absence of the tea extracts (control) and those grown in the presence of either extract.

The inhibition of hexanal production closely paralleled the inhibition of conjugated dienes for the GTCP extract (Figure 1). Inhibition of hexanal by the GTCP extract was linear between 0.08 and 0.3 ppm, followed by a plateau from 0.3 to 1.25 ppm at approximately 94% inhibition (Figure 1). Regression analysis indicated a significant relationship between the percentage inhibition of conjugated dienes and the percentage inhibition of hexanal production for the GTCP extract ($p < 0.01$; $r^2 = 0.98$).

Catechin and epicatechin inhibited LDL oxidation in a dose-dependent manner at concentrations ranging from 0.08 to 1.25 $\mu$M (Table 2). From 1.25 to 5.0 $\mu$M, there was no further increase in inhibition of LDL oxidation for either compound, which reached a maximum of approximately 93% inhibition. At each concentration tested, except 0.63 $\mu$M, the antioxidant activity of each compound was statistically equivalent for catechin and epicatechin (Table 2).

**Table 2. Inhibition of Cell-Mediated LDL Oxidation by Catechin and Epicatechin[a]**

| | % inhibition of conjugated dienes[b] | |
|---|---|---|
| concn ($\mu$M) | catechin | epicatechin |
| 0.08 | nd | $0.5 \pm 1.0^{d}$ |
| 0.16 | $0.9 \pm 5.2^{c,x}$ | $8.2 \pm 1.6^{cd,x}$ |
| 0.31 | $10.1 \pm 4.0^{c,x}$ | $16.3 \pm 2.8^{c,x}$ |
| 0.63 | $69.1 \pm 1.8^{b,x}$ | $33.6 \pm 0.6^{b,y}$ |
| 1.25 | $90.0 \pm 0.4^{a,x}$ | $94.7 \pm 3.5^{a,x}$ |
| 2.50 | $90.9 \pm 0.1^{a,x}$ | $95.5 \pm 0.4^{a,x}$ |
| 5.00 | $97.3 \pm 0.7^{a,x}$ | $94.8 \pm 6.6^{a,x}$ |

[a] See Table 1, footnote a. [b] See Table 1, footnote b. nd: not determined.

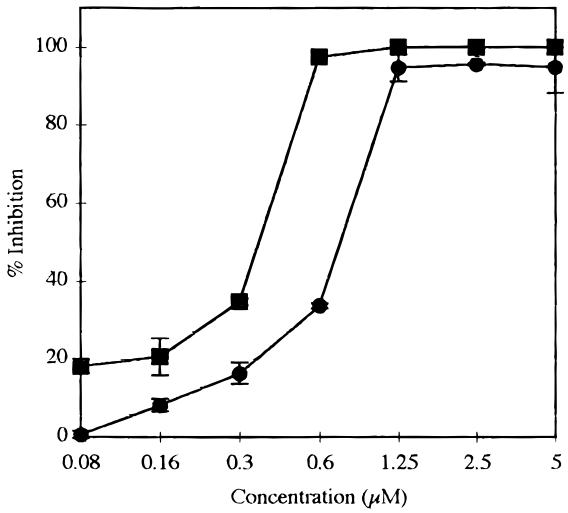


**Figure 2.** Inhibition of cell-mediated LDL oxidation by epicatechin as measured by conjugated dienes and hexanal. See Figure 1.

The inhibition of hexanal production closely paralleled the inhibition of conjugated dienes for each concentration of epicatechin tested, with the exception of 0.63 $\mu$M (Figure 2). The inhibition of hexanal production by epicatechin followed a sigmoidal pattern (Figure 2). There was no significant change in the inhibition of hexanal production from 0.08 to 0.16 $\mu$M. Between 0.16 and 0.60 $\mu$M there was a rapid increase in the inhibition of hexanal, followed by a plateau in inhibition between 0.6 and 5.0 $\mu$M at approximately 98% inhibition. Regression analysis indicated a significant relationship between the percentage inhibition of conjugated dienes and the percentage inhibition of hexanal production for epicatechin ($p < 0.01$; $r^2 = 0.78$).

## DISCUSSION

The green tea extracts both inhibited endothelial cell-mediated LDL oxidation in the range of concentrations tested (0.08−2.5 ppm). The NTEP extract was more active than the GTCP extract at each concentration tested, but the difference was significant only at 0.08 and 0.3 ppm. Although the NTEP extract contained less total catechins (39.2%) by weight than the GTCP extract (47.1%), the remaining unidentified components (53−61%) in the extracts may have contributed to the relative antioxidant activities observed. In a previous study, when these extracts were tested in different systems, there were striking differences in antioxidant activity (Frankel et al., 1997). Thus, in bulk corn oil triglycerides stripped of tocopherols, NTEP and GTCP had equivalent antioxidant activity after 8 days of

copper-catalyzed oxidation and inhibited hexanal production by 22% (Frankel et al., 1997). In contrast, using copper to catalyze the oxidation of corresponding oil-in-water emulsions, both tea extracts were prooxidants as measured by either conjugated dienes or hexanal formation. NTEP and GTCP extracts acted as antioxidants in soybean lecithin liposomes oxidized with copper at 37 °C as measured by conjugated dienes or hexanal. In contrast to these results, in the present study with human LDL, the tea extracts were very active as antioxidants, and the difference may be due to interaction with the apo B protein on LDL. This interaction may block copper sites on apo B and afford protection of LDL against oxidation.

In the present study, using endothelial cells to mediate the oxidation of LDL, catechin maximally inhibited oxidation between 1.25 and 5.0 $\mu$M. Mangiapane et al. (1992) reported that 68 $\mu$M catechin was required to completely inhibit transformed macrophage J774-mediated LDL oxidation and that 172 $\mu$M was required for human monocyte-derived macrophage-mediated LDL oxidation. The different cell types may explain the differences in the concentration of catechin needed to produce 90−100% inhibition. Mangiapane et al. (1992) also used TBARS as a marker to follow LDL oxidation, and the period of cell incubation with LDL was twice as long (24 h) as that used in the present study. Malondialdehyde is a product of cellular eicosanoid metabolism and is measured in the TBARS assay. Thus, the TBARS measurements may not be specific for $\omega$-6 polyunsaturated fatty acid oxidation in the cell system used by Mangiapane et al. (1992).

Catechins have a strong metal chelating capacity. Small amounts of transition metals, specifically copper ions, are necessary in the incubation media for the oxidation of LDL. The Ham's F-10 media used in the present study contains 0.01 $\mu$M copper sulfate. While metal chelation of the copper ions by the catechins added to the incubation media cannot be ruled out, it is clear that metal chelation is not the sole mechanism involved. Catechin and epicatechin inhibited oxidation by approximately 10−16% at a concentration of 0.3 $\mu$M, which is 30 times greater than the copper concentration in the media used for LDL oxidation. This difference strongly argues that other mechanisms operate in cell-mediated oxidation, such as endothelial lipoxygenase activity.

In the present study, catechin and epicatechin exhibited equivalent antioxidant activities at the concentrations tested. Catechin and epicatechin also had equivalent radical scavenging abilities against the artificial water-soluble phenothiazine radical cation and lipid peroxyl radicals generated in aqueous and lipophilic phases, respectively (Salah et al., 1995). These workers reported that epicatechin and catechin inhibited metmyoglobin-catalyzed LDL oxidation similarly, with $IC_{50}$s ranging from 0.25 to 0.38 $\mu$M. This range is similar to the 0.74 and 0.68 $\mu$M $IC_{50}$s (0.21 and 0.20 ppm) for epicatechin and catechin reported in the present study (Table 3). The two isomers were also equivalent in their abilities to spare $\alpha$-tocopherol during metmyoglobin-catalyzed LDL oxidation (Salah et al., 1995). Catechin and epicatechin similarly inhibited bovine brain phospholipid liposome peroxidation by an $FeCl_3$-ascorbate system (Scott et al., 1993). In contrast to these findings, epicatechin inhibited copper-catalyzed LDL oxidation to a greater extent than did catechin as measured by conjugated dienes, lipid hydroperoxides, and TBARS (Miura et al., 1994). In aqueous systems, catechin and epicatechin scavenged hydroxyl radicals and superoxide anions to different extents (Hanasaki et al., 1994). These differences highlight the need for using more than one system to evaluate the antioxidant or prooxidant activities of polyphenolic compounds.

**Table 3. Concentration for 50% Inhibition of LDL Oxidation by Green Tea Compounds**[a]

| test compd | $IC_{50}$[b] | test compd | $IC_{50}$[b] |
|---|---|---|---|
| catechin | 0.20 | NTEP | 0.15 |
| epicatechin | 0.21 | GTCP | 0.31 |

[a] See table 1; footnote *a*. [b] The concentration of phenolic that inhibited cell- mediated oxidation by 50% ($IC_{50}$) was determined by linear regression of percentage inhibition. The $IC_{50s}$ are expressed in parts per million.

A comparison of the antioxidant activities of the two tea extracts and the two pure compounds suggests that there may be synergistic effects between various components and contribution to the antioxidant activity by unidentified components in the extracts. Further research is required to identify each component, to define their contribution to and any synergistic effects on antioxidant activity.

Various biologically relevant assays have been used to evaluated catechins, with different results. Hypochlorous acid is produced at local sites of inflammation by activated neutrophils. Catechin and epicatechin differed in their ability to scavenge hypochlorous acid in an in vitro assay at the lower concentrations tested (0.025−0.25 mM) (Scott et al., 1993). In the bleomycin assay, which detects the ability of a compound to damage DNA, both catechin and epicatechin similarly promoted DNA damage, although they were much less damaging than ascorbate (Scott et al., 1993). The levels that stimulated DNA damage, between 0.05 and 0.5 mM, were 10−50 times greater than the levels that inhibited LDL oxidation by 93% in the present study. This suggests that their antioxidant activity could be attained in vivo at much lower concentrations to avoid adverse effects.

The present study supports a beneficial role for tea catechins in the diet for both human health and antioxidant protection of foods. However, there is a need for further testing using a variety of complementary in vivo and ex vivo biologically relevant assays to better evaluate their antioxidant and other functional activities.

## ABBREVIATIONS USED

Apo B, apoprotein B-100; DMSO, dimethyl sulfoxide; ECG, epicatechingallate; EGC, epigallocatechin; EGCG, epigallocatechingallate; GLM, general linear model; GTCP, green tea catechin powder; HAEC, human aortic endothelial cells; LDL, low-density lipoprotein; NTEP, Nikken tea extract powder; TBARS, thiobarbituric acid reactive substances.

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