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Inhibitory Effect of Tea Flavonoids on the Ability of Cells to Oxidize Low Density Lipoprotein

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ABSTRACT. Dietary flavonoid intake has been reported to be inversely related to mortality from coronary heart disease, and the anti-atherosclerotic effect of flavonoids is considered to be due probably to their antioxidant properties. Oxidation of low density lipoprotein (LDL) has been reported to be induced by the constituent cells of the arterial wall. Accordingly, we examined the effect of pretreatment with tea flavonoids, such as theaflavin digallate, on the ability of cells to oxidize LDL. Theaflavin digallate pretreatment of macrophages or endothelial cells reduced cell-mediated LDL oxidation in a concentration- (0–400 μM) and time- (0–4 hr) dependent manner. This inhibitory effect of flavonoids on cell-mediated LDL oxidation was in the order of theaflavin digallate > theaflavin ≥ epigallocatechin gallate > epigallocatechin > gallic acid. Further, we investigated the mechanisms by which flavonoids inhibited cell-mediated LDL oxidation using macrophages and theaflavin digallate. Theaflavin digallate pretreatment decreased superoxide production of macrophages and chelated iron ions significantly. These results suggest that tea flavonoids attenuate the ability of the cell to oxidize LDL, probably by reducing superoxide production in cells and chelating iron ions. BIOCHEM PHARMACOL **58**;11:1695–1703, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. flavonoid; theaflavin; catechin; cell-mediated LDL oxidation; macrophages; superoxide

Flavonoid intake has been reported to be inversely related to mortality from coronary heart disease and to show an inverse relationship to the incidence of myocardial infarction [1, 2]. This anti-atherosclerotic effect of flavonoids may be derived from their antioxidant properties, but that relationship remains unclear.

Flavonoids are polyphenolic antioxidants naturally occurring in vegetables, fruits, and beverages such as tea and wine. Regular drinking of red wine may explain the "French paradox": the incidence of coronary heart disease is low in France in spite of a high fat intake, which usually is correlated with high mortality from coronary heart disease [3]. Frankel *et al.* [4] reported that non-alcoholic, phenolic substances, such as flavonoids of red wine, have potent antioxidant properties toward oxidation of human LDL§.

Tea is a major source of daily flavonoid intake. Catechins and theaflavins are important groups of tea flavonoids. As principal components, green tea leaves contain large amounts of catechins, such as epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. Black tea leaves contain theaflavin, theaflavin monogallate, and theaflavin digallate as well as catechins, in addition to thearubigens. Theaflavins are dimers of catechins formed by enzymatic oxidation in the process of black tea manufacturing [5]. Several groups using different flavonoids, such as quercetin, catechin, morin, rutin, fisetin, and gossypetin, have shown that flavonoids *in vitro* inhibit copper-catalyzed and macrophage-mediated LDL oxidation [6–10]. Flavonoids are radical scavengers and can sequester metal ions through ligand binding [11–16]. Miller *et al.* [15] have reported that theaflavin digallate can dramatically chelate metal ions and scavenge radicals. However, the mechanisms by which flavonoids inhibit LDL oxidation remain to be elucidated. Oxidized LDL has been reported to play an important role in atherogenesis. Oxidative modification of LDL could be a prerequisite for macrophage uptake and cellular accumulation of cholesterol, leading to formation of early atherosclerotic lesions [17, 18]. Therefore, studies directed at examining antioxidative effects and their mechanisms are important in establishing a role for flavonoids as antioxidants in strategies of atherosclerosis prevention.

In tea, theaflavins are major flavonoid components

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§ *Abbreviations*: LDL, low density lipoprotein; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco's modified Eagle's medium; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; FBS, fetal bovine serum; MDA, malondialdehyde; and LPO, lipid peroxides.

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similar to catechins. The effect of theaflavins on LDL oxidation has not yet been established. We investigated the effect of theaflavins on the ability of cells—macrophages and endothelial cells—to oxidize LDL.

MATERIALS AND METHODS

Materials

Theaflavin, theaflavin digallate, epigallocatechin, and epigallocatechin gallate were gifts from Dr. Hara. BSA and DMSO were purchased from the Sigma Chemical Co. Basic fibroblast growth factor, collagenase, EDTA, gallic acid monohydrate, SOD, cytochrome *c*, neocuproine hydrochloride, and ascorbate were purchased from Wako Chemicals. 2-Thiobarbituric acid was purchased from Merck. [^{3}H]Thymidine was purchased from Amersham Pharmacia Biotech. (Tokyo). F-10 nutrient mixture (Ham's F10), M199 medium, FBS, and penicillin G–streptomycin sodium–amphotericin B were purchased from Life Technologies, GIBCO BRL. DMEM was purchased from ICN Biomedicals.

Lipoproteins

LDL (density 1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation from human plasma with EDTA (1 mg/mL) prepared from fasting, normolipidemic individuals [19]. The LDL for oxidation experiments was dialyzed at 4° for 24 hr against PBS (pH 7.4) to remove EDTA. LDL protein was determined according to the procedure of Lowry *et al.* [20] using BSA as a standard.

Cell Culture of Mouse Peritoneal Macrophages and Endothelial Cells

Mouse peritoneal resident macrophages were harvested from CD-1 (ICR) male mice, 5–7 weeks of age, purchased from Charles River Japan Inc. Mouse peritoneal macrophages were prepared as described previously [21, 22]. Mice were anesthetized by exposure to ether. Three milliliters of ice-cold, sterile PBS was injected into the peritoneal cavity. After a few gentle strokes, the saline solution was recovered and centrifuged at 600 *g* for 10 min at 5°. The cell pellet was washed twice in PBS and then resuspended in DMEM containing 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were seeded into 24-well plates in 1 mL of DMEM as described above at a density of 3 million cells per well. After incubation for 3 hr at 37° in an incubator that had been equilibrated with 95% air plus 5% CO_2, non-adherent cells were removed by washing twice with Ham's F-10 medium. All oxidation experiments were performed in Ham's F-10 medium.

HUVEC were prepared by following a method described previously [23, 24]. Human umbilical cords were obtained at normal deliveries. The umbilical vein was cannulated and perfused with 50–100 mL of sterile PBS to remove all traces of blood. Then the vein was filled with 10–20 mL of 0.1% collagenase dissolved in M199 medium containing 100 U/mL of penicillin and 100 μg/mL of streptomycin, and was incubated for 20 min at 37°. The collagenase solution was collected in a sterile tube, and the remaining cells were harvested by flushing gently with an equal volume of sterile PBS. Cells were spun down to pellets by centrifugation at 200 *g* and 10° for 5 min, were washed twice with M199 medium, were seeded into a 75-mL culture flask in M199 medium supplemented with 20% heat-inactivated FBS and 10 μg/mL of basic fibroblast growth factor, and were grown to confluence. The cells were harvested from the culture flask with trypsin–EDTA solution at confluence, and were seeded in 24-well plates in supplemented M199 medium. The medium was changed to Ham's F-10 medium before oxidation experiments.

Cell-Mediated LDL Oxidation

Mouse peritoneal macrophages or HUVEC were preincubated for 0–8 hr in the presence or absence of flavonoids or DMSO in Ham's F10 medium. The flavonoids were dissolved in 10% DMSO in distilled water and added to the medium. The cells were incubated in Ham's F-10 medium containing a final concentration of 0.5% DMSO (v/v). The medium was removed, and cells were washed twice with PBS. LDL (100 μg/mL) was incubated for 20 hr with the cells in Ham's F-10 medium. After this incubation, the medium was analyzed for the extent of LDL oxidation as described below.

Measurements of LDL Oxidation

TBARS. MDA generated in medium including LDL was measured by using the TBARS assay as described by Buege and Aust [25]. The sample absorbance was measured at 535 nm in a UV spectrophotometer (U-2000, HITACHI). Results are expressed as MDA equivalent content (nanomoles of MDA per milligram of LDL protein) calculated using the extinction coefficient for MDA ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) as previously described [25].

TOTAL LPO. LPO in medium that contained LDL were measured using a commercially available reagent (Determiner LPO), which is based on a colorimetric method that measures the reaction of a leucomethylene blue derivative with lipid hydroperoxides in the presence of heme compounds [26]. The sample was measured spectrophotometrically at 680 nm, and results are expressed as nanomoles of LPO per milligram of LDL protein.

AGAROSE GEL ELECTROPHORESIS. LDL was incubated for 20 hr in Ham's F-10 medium with macrophages after pretreating with or without theaflavin digallate at the indicated concentrations or 0.5% DMSO for the indicated times. The media including LDL were collected, and they were subjected to agarose gel electrophoresis. Electrophoresis was performed at 90 V for 25 min in a buffer consisting of 50 mM sodium barbital, 1 mM EDTA, and 0.1% sodium

azide, pH 8.6, by using the TITAN GEL lipoprotein electrophoresis system (Helena Laboratories). After the electrophoresis, the plates were stained with Fat Red 7B.

Superoxide Anion Production

The production of superoxide anion (O_2^-) was measured by following methods previously described [27]. Briefly, mouse peritoneal macrophages were incubated for 4 hr with or without flavonoids at the indicated concentrations in Ham's F-10 medium. After the medium was discarded and the cells were washed twice with PBS, cells were incubated for a subsequent 1-hr period with cytochrome *c* in the presence or absence of 150 U/mL of SOD in Ham's F-10 medium. The cell supernatant was collected, and the SOD-inhibitable reduction of cytochrome *c* was determined by measuring the change in absorbance at 550 nm and expressed as nanomoles of O_2^- per milliliter. The following equation was used to determine the nanomoles of O_2^- produced: nanomoles of O_2^- per milliliter = 47.6 [OD_{550} (in the absence of SOD) − OD_{550} (in the presence of SOD)], which is derived from the report of Cathcart *et al.* [27].

Measurement of Iron Release from Cells

Mouse peritoneal macrophages were incubated for 4 hr with or without flavonoids at the indicated concentrations in Ham's F-10 medium. After the medium was discarded and the cells were washed twice with PBS, the cells were incubated subsequently for 2 hr in 1 mL of Ham's F-10 medium.

Iron release was detected using ferrozine, a chelator of Fe^{2+} [28, 29]. Two hundred microliters of incubation medium was mixed with 200 μL of 56.8 mM ascorbate dissolved in 100 mL of 0.6 M sodium chloride with 0.1% Triton X-100 containing 0.3 M hydrochloric acid and 3.8 mM neocuproine hydrochloride. The formation of the iron complex was monitored spectrophotometrically at E (562 nm), equivalent to 27,900 M^{-1} cm^{-1}.

Assessment of Cytotoxicity and Cellular Injury

Cytotoxicity or cellular injury was evaluated by measuring DNA synthesis of cells and the amount of LDH released from the cells into the medium [30, 31]. To measure DNA synthesis, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 6 hr, washed twice with PBS, and fixed for 20 min with ice-cold 10% trichloroacetic acid (TCA). The radioactivity incorporated into TCA-insoluble material was determined in a liquid scintillation counter. The LDH activity in the medium was determined by spectrophotometric analysis of NADH oxidative consumption over time.

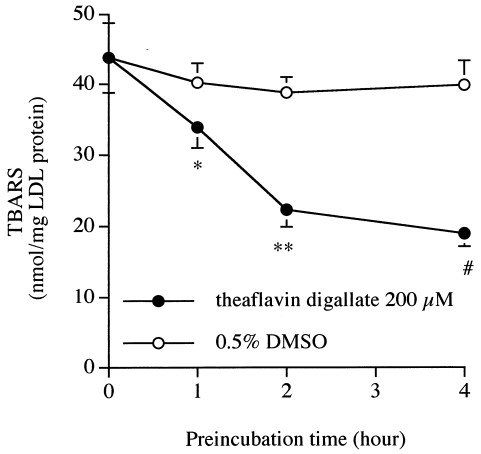

FIG. 1. Changes in TBARS formation in macrophage-mediated LDL oxidation in response to increasing theaflavin digallate pretreatment time. Mouse peritoneal macrophages were preincubated with or without 200 μM theaflavin digallate or 0.5% DMSO in Ham's F-10 medium for the indicated times, and then the cells were washed twice with PBS. LDL was incubated for 20 hr with macrophages in Ham's F-10 medium. The lipid oxidation products were assessed by the TBARS assay. All values are means ± SD, N = 4. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (#) $P < 0.001$, as compared with control (no preincubation).

Statistics

Each experiment was performed three times, each time in quadruplicate. Results are expressed as means ± SD. Multivariate ANOVA for repeated measurements was used to compare data in experiments with multiple groups or time–course experiments. Differences between means were tested by ANOVA with Student's unpaired two-tailed *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS

Effect of Theaflavin Digallate Pretreatment of Macrophages on Their Ability to Oxidize LDL

Pretreatment of macrophages with 200 μM theaflavin digallate significantly reduced TBARS formation in macrophage-mediated LDL oxidation in a time-dependent manner (Fig. 1). This inhibiting effect of pretreatment with theaflavin on macrophage-mediated LDL oxidation seemed to reach a plateau at 4 hr. Thereafter we adopted 4 hr as the pretreatment time for macrophages with theaflavin.

Table 1 shows that pretreatment with theaflavin digallate for 4 hr significantly inhibited macrophage-mediated LDL oxidation in a concentration-dependent manner. Pretreatment with 0.5% DMSO (control), which was the solvent for theaflavin digallate, as described in Materials and Methods, had no major effect on macrophage-mediated LDL oxidation. LDL also was incubated under identical conditions in the absence of macrophages (no-cell control). TBARS and LPO of the no-cell control without preincubation with theaflavin digallate were 4.9 ± 0.7 and 43.8 ±

TABLE 1. Cell-mediated LDL oxidation by mouse peritoneal macrophages pretreated with theaflavin digallate

Treatment	Concentrations (μM)	TBARS (nmol/mg LDL protein)	LPO (nmol/mg LDL protein)
Control		27.5 ± 4.1	349.8 ± 33.7
0.5% DMSO		24.6 ± 1.9	355.7 ± 33.9
Theaflavin digallate	100	19.6 ± 2.3*	233.8 ± 49.1*
	200	17.7 ± 1.9†	143.6 ± 20.2‡
	400	12.8 ± 0.9‡	73.2 ± 11.7‡

Mouse peritoneal macrophages were preincubated in Ham's F10 medium with or without theaflavin digallate at the indicated concentrations or with 0.5% DMSO in Ham's F-10 medium for 4 hr, and were washed twice with PBS; then LDL was added and incubated for 20 hr. The lipid oxidation products were assessed by both the TBARS assay and the LPO assay. All values are means + SD, $N = 4$.

*-‡ Significantly different from control (no addition): *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

4.2 nmol/mg of LDL protein, respectively. TBARS and LPO of the no-cell control preincubated with 400 μM theaflavin digallate for 4 hr in a cell-free system were 4.6 ± 0.8 and 46.1 ± 4.4, respectively. There were no significant differences between the presence and absence of preincubation with theaflavin digallate in a cell-free system.

Figure 2 shows the time- and concentration-dependent inhibitory effect of theaflavin digallate pretreatment on the increases in electrophoretic mobility of LDL induced by macrophage-mediated LDL oxidation.

Effect of Theaflavin Digallate Pretreatment of Endothelial Cells on Their Ability to Oxidize LDL

We also examined the effect of theaflavin digallate on cell-mediated LDL oxidation with HUVEC. Pretreatment of HUVEC with 200 μM theaflavin digallate significantly reduced TBARS formation in endothelial cell-mediated LDL oxidation in a time-dependent manner (Fig. 3). This inhibiting effect of pretreatment with theaflavin digallate on endothelial cell-mediated LDL oxidation seemed to reach a plateau at 4 hr. Thereafter we adopted 4 hr as the pretreatment time for endothelial cells with theaflavin.

Table 2 shows that pretreatment with theaflavin digallate for 4 hr significantly inhibited endothelial cell-mediated LDL oxidation in a concentration-dependent manner. Pretreatment with 0.5% DMSO (control) had no major effect on endothelial cell-mediated LDL oxidation. LDL also was incubated under identical conditions in the absence of HUVEC (no-cell control). TBARS and LPO of the no-cell control without preincubation with theaflavin digallate were 4.3 ± 0.5 and 47.2 ± 3.8 nmol/mg of LDL protein, respectively. TBARS and LPO of the no-cell control preincubated with 400 μM theaflavin digallate for 4 hr in a cell-free system were 4.2 ± 0.7 and 45.8 ± 4.5 nmol/mg of LDL protein, respectively. There were no significant differences between the presence and absence of preincubation with theaflavin digallate in a cell-free system.

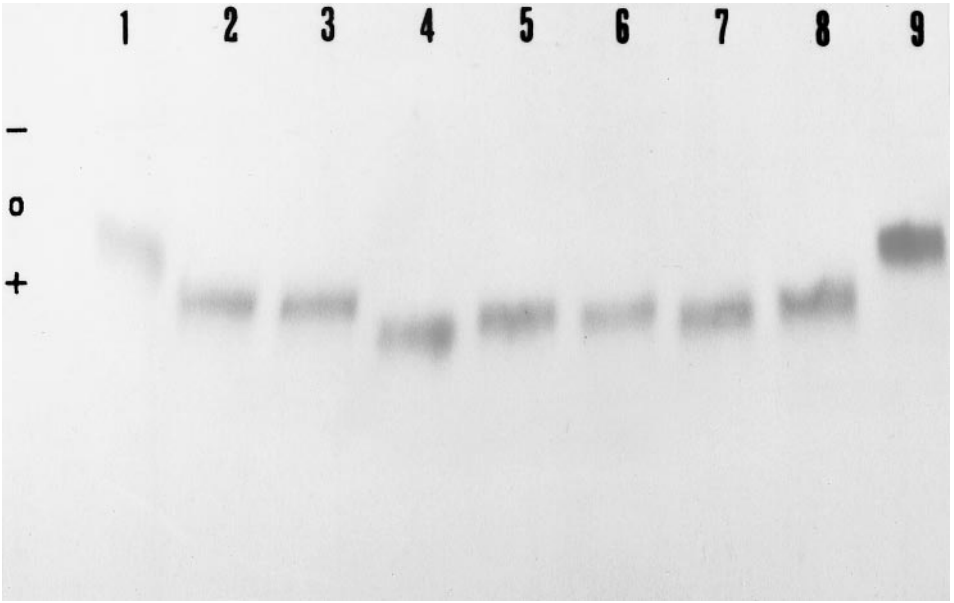

FIG. 2. Electrophoretic mobility of LDL oxidized by macrophages pretreated with theaflavin digallate. LDL was incubated for 20 hr in Ham's F-10 medium with mouse peritoneal macrophages after pretreating for the indicated times with or without theaflavin digallate at the indicated concentrations or 0.5% DMSO. The media were collected and were subjected to agarose gel electrophoresis; lane 1: LDL oxidized by macrophages pretreated with 400 μM theaflavin digallate for 4 hr; lane 2: LDL oxidized by macrophages pretreated with 200 μM theaflavin digallate for 4 hr; lane 3: LDL oxidized by macrophages pretreated with 100 μM theaflavin digallate for 4 hr; lane 4: LDL oxidized by macrophages without theaflavin digallate and 0.5% DMSO pretreatment; lane 5: LDL oxidized by macrophages pretreated with 0.5% DMSO for 4 hr; lane 6: LDL oxidized by macrophages pretreated with 200 μM theaflavin digallate for 1 hr; lane 7: LDL oxidized by macrophages pretreated with 200 μM theaflavin digallate for 2 hr; lane 8: LDL oxidized by macrophages pretreated with 200 μM theaflavin digallate for 4 hr; and lane 9: native LDL.

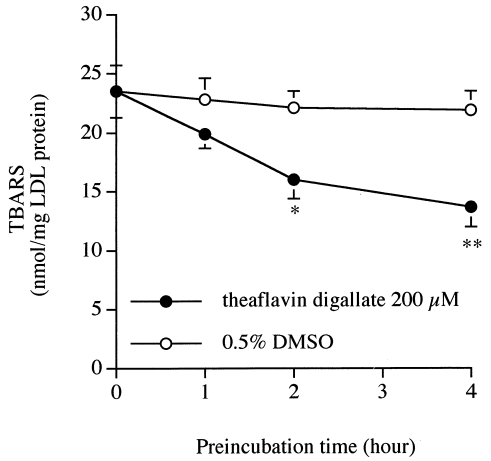

FIG. 3. Changes in TBARS formation in endothelial cell-mediated LDL oxidation in response to increasing theaflavin digallate pretreatment time. HUVEC were preincubated with or without 200 μM theaflavin digallate or 0.5% DMSO in Ham's F-10 medium for the indicated times, and then cells were washed twice with PBS. LDL was incubated for 20 hr with HUVEC in Ham's F-10 medium. The lipid oxidation products were assessed by the TBARS assay. All values are means ± SD, N = 4. Key: (*) $P < 0.05$, and (**) $P < 0.01$, as compared with control (no preincubation).

Effect of Various Tea Flavonoids on the Ability of Macrophages to Oxidize LDL

We compared the inhibitory effect of various theaflavins and catechins on the ability of macrophages to oxidize LDL. The concentrations of flavonoids were 100 and 400 μM. Taking the TBARS value in macrophage-mediated LDL oxidation without flavonoids as 100% (control), we determined peroxidation values under pretreatment with theaflavins, catechins, and gallic acid. As shown in Table 3, theaflavin digallate demonstrated a stronger trend in inhibitory activity against LDL oxidation than theaflavin, epigallocatechin, epigallocatechin gallate, or gallic acid. In addition, the inhibitory effect of theaflavin digallate on LDL oxidation was significantly stronger than that of epigallocatechin.

TABLE 2. Cell-medicated LDL oxidation by cultured endothelial cells pretreated with theaflavin digallate

Treatment	Concentrations (μM)	TBARS (nmol/mg LDL protein)	LPO (nmol/mg LDL protein)
Control		22.6 ± 1.0	417.7 ± 12.7
0.5% DMSO		23.1 ± 1.9	413.0 ± 19.1
Theaflavin digallate	100	19.0 ± 1.3†	356.7 ± 36.8*
	200	14.4 ± 2.3‡	205.4 ± 49.1†
	400	11.5 ± 1.3‡	102.0 ± 24.7‡

HUVEC were preincubated with or without theaflavin digallate at the indicated concentrations or with 0.5% DMSO in Ham's F-10 medium for 4 hr, and were washed twice with PBS; then LDL was added and incubated for 20 hr. The lipid oxidation products were assessed by both the TBARS assay and the LPO assay. All values are means ± SD, N =4.

*†‡ Significantly different from control (no addition): *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

TABLE 3. Inhibitory effect of pretreatment with various tea flavonoids on macrophage-mediated LDL oxidation

Flavonoids	% of Control TBARS value: Incubation concentrations 100 μM	400 μM
Epigallocatechin	89.3	64.1†
Epigallocatechin gallate	74.8*	53.8‡
Theaflavin	73.2*	56.5‡
Theaflavin digallate	71.4*§	46.6‡§
Gallic acid	99.3	74.7‡

Mouse peritoneal macrophages were preincubated with or without tea flavonoids at the indicated concentrations (100 and 400 μM) in Ham's F-10 medium for 4 hr. and were washed twice with PBS; then LDL was incubated for 20 hr with macrophages in Ham's F-10 medium. The lipid oxidation products were assessed by the TBARS assay. Data are expressed as mean percent of mean TBARS value for control (no addition). The mean TBARS value of the no-addition control was 32.8 ± 3.1 nmol/mg LDL protein. All values are means ± SD, N = 4.

*†‡ Significantly different from control (no addition): *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$.

§ Significantly different from epigallocatechin, $P < 0.05$.

Effect of Theaflavin Digallate Pretreatment of Macrophages on Superoxide Anion Production and Iron Release from Macrophages

SUPEROXIDE ANION PRODUCTION FROM MACROPHAGES. The results described above indicate that theaflavin digallate pretreatment could reduce cell-mediated LDL oxidation (by macrophages or endothelial cells) and had a stronger inhibitory activity against LDL oxidation than other tea flavonoids, as mentioned above. We conducted further studies to investigate the mechanisms by which flavonoids inhibited cell-mediated LDL oxidation, using macrophages and theaflavin digallate. We examined the effect of theaflavin digallate pretreatment on superoxide anion production by macrophages. As shown in Fig. 4, theaflavin digallate pretreatment inhibited superoxide anion production by macrophages in a time-dependent manner as compared with 0.5% DMSO pretreatment (control). In addition, theaflavin digallate pretreatment inhibited superoxide anion production by macrophages in a concentration-dependent manner, but the inhibitory effect of theaflavin digallate pretreatment at 50 μM on superoxide anion production by macrophages was not significant (Fig. 5).

IRON RELEASE FROM MACROPHAGES. As shown in Table 4, theaflavin digallate pretreatment at 400 μM significantly reduced iron release into the medium. Table 4 shows that iron release was reduced to 82 and 76% of control (no addition) by theaflavin digallate pretreatment at 100 and 200 μM, respectively, but this reduction was not significant.

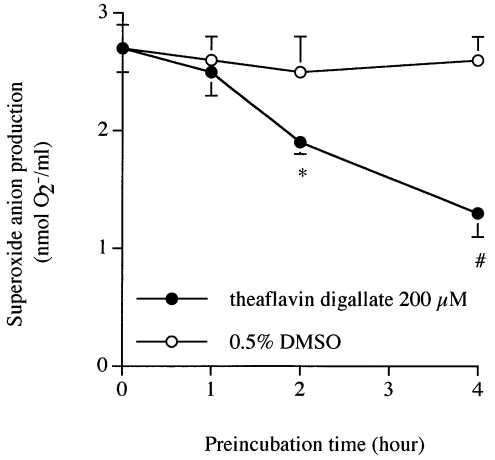

FIG. 4. Changes in superoxide anion production from macrophages in response to increasing theaflavin digallate pretreatment time. Mouse peritoneal macrophages were incubated with or without 200 μM theaflavin digallate or 0.5% DMSO in Ham's F-10 medium for the indicated times, and then macrophages were washed twice with PBS. Cells were incubated subsequently for 1 hr with 1 mM cytochrome *c* in the presence or absence of 150 U/mL of SOD in Ham's F-10 medium, and the SOD-inhibitable reduction of cytochrome *c* was determined by measuring the changes in absorbance at 550 nm and expressed as nanomoles O_2^- per milliliter. All values are means ± SD, N = 4. Key: (*) $P < 0.05$, and (#) $P < 0.001$, as compared with control (no preincubation).

Cell Viability

The effect of theaflavins and catechins on cell viability was evaluated in terms of the amount of LDH released from cells and in terms of [^{3}H]thymidine incorporation into cellular DNA. Neither theaflavins nor catechins had a major effect on cell viability at concentrations up to 400 μM for 4 hr (Table 5).

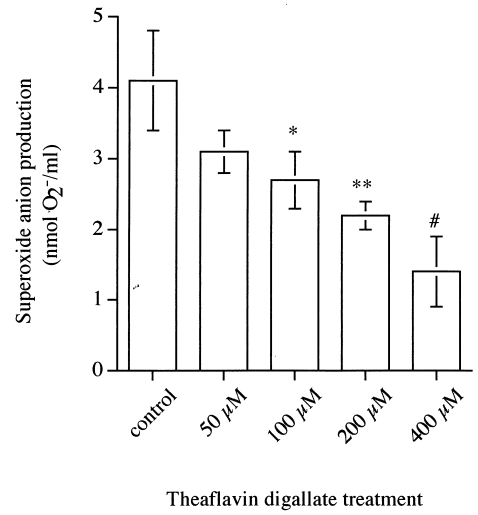

FIG. 5. Effect of theaflavin digallate pretreatment on superoxide anion production from macrophages. Mouse peritoneal macrophages were incubated with or without theaflavin digallate at the indicated concentrations for 4 hr, and then macrophages were washed twice with PBS. Cells were incubated subsequently for 1 hr with 1 mM cytochrome *c* in the presence or absence of 150 U/mL of SOD in Ham's F-10 medium, and the SOD-inhibitable reduction of cytochrome *c* was determined by measuring the changes in absorbance at 550 nm and expressed as nanomoles O_2^- per milliliter. All values are means ± SD, N = 4. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (#) $P < 0.001$, as compared with control (no addition).

TABLE 4. Effect of theaflavin pretreatment on iron release from mouse peritoneal macrophages

Treatment	Concentrations (μM)	Ferrozine-iron comples (μM)
Control		10.3 ± 3.6
0.5% DMSO		9.6 ± 2.9
Theaflavin digallate	100	8.4 ± 1.8
	200	7.8 ± 2.3
	400	6.3 ± 1.2*

Mouse peritoneal macrophages were preincubated with or without theaflavin digallate at the indicated concentrations or with 0.5% DMSO in Ham's F-10 medium for 4 hr, and were washed twice with PBS; then cells were further incubated for 2 hr in Ham's F-10 medium. The iron release from cells was evaluated by measuring ferrozine-iron complex spectrophotometrically at 562 nm. All values are means ± SD, N = 4.

* $P < 0.05$, as compared with control (no addition).

DISCUSSION

The present study demonstrated that pretreatment with flavonoids inhibits cell-mediated LDL oxidation and that theaflavin digallate pretreatment can decrease the ability of macrophages and endothelial cells to oxidize LDL in a concentration- and time-dependent manner without cell damage (Figs. 1–3, Tables 1–3 and 5). In addition, flavonoids reduced cell-mediated LDL oxidation substantially, with no major effect on LDL oxidation in a cell-free system.

TABLE 5. Effect of tea flavonoid treatment on macrophage viability

Treatment	LDH activity in medium (U/L)	[^{3}H]Thymidine incorporation into DNA (cpm/mg cell protein)
Control	24 ± 4	351 ± 47
Epigallocatechin (100 μM)	26 ± 5	403 ± 53
Epigallocatechin (400 μM)	27 ± 2	344 ± 26
Epigallocatechin gallate (100 μM)	26 ± 5	385 ± 45
Epigallocatechin gallate (400 μM)	28 ± 3	357 ± 35
Theaflavin (100 μM)	28 ± 5	329 ± 48
Theaflavin (400 μM)	30 ± 4	377 ± 36
Theaflavin digallate (100 μM)	27 ± 3	339 ± 51
Theaflavin digallate (400 μM)	31 ± 6	390 ± 44

Mouse peritoneal resident macrophages were incubated with tea flavonoids at the indicated concentrations for 4 hr at 37°C in Ham's F10 medium, and the media were harvested to measure LDH activity, which was examined as described in Materials and Methods. Subsequently, cells were washed three times with PBS and incubated for 6 hr with [^{3}H]thymidine (1 μCi/mL) in DMEM containing 2% FBS; [^{3}H]thymidine incorporation into DNA was measured as described in Materials and Methods. All values are means ± SD, N =4.

Flavonoids, derivatives of the phenylchromone ring, are a large group of naturally occurring antioxidant compounds. Some flavonoids, including catechins and theaflavins, have many phenolic hydroxy groups in their chemical structure, which are expected to exert antioxidant activities. Basically, not only the number of hydroxy groups but also their positioning and arrangement influence the antioxidant activity of flavonoids. Several previous reports demonstrated that flavonoids can protect LDL from oxidation induced by macrophages, but in all those studies the flavonoid was added just at the beginning of the incubation along with the LDL [7, 8, 22]. The cell-mediated LDL oxidation experiments in the present study were carried out after removing the flavonoid-supplemented medium and washing the cells with PBS. Thus, flavonoids in this study were present mainly intracellularly. Parthasarathy [32] reported that pretreatment of mouse peritoneal macrophages with probucol inhibits the subsequent ability of the cell to oxidize LDL. In our experiments, it is possible that flavonoid pretreatment of macrophages reduced the ability of the cell to oxidize LDL, as did probucol. These results suggest that antioxidant enrichment of cells might afford additional protection for LDL against cell-mediated oxidation. Table 3 shows that theaflavin digallate had stronger inhibitory activity against LDL oxidation than did free theaflavin without gallic acid, catechin, catechin gallate, or gallic acid. Theaflavins have more hydroxyl (OH) groups, which are considered to be necessary for exerting radical scavenging activity [11–15], than do catechins, since theaflavins are dimers of catechins. In addition, theaflavin digallate has two gallic acid moieties, and the gallic acid moiety has been reported to be important for theaflavins to exert antioxidant activity [15, 33, 34].

The proposed mechanisms by which flavonoid pretreatment inhibits the ability of the cell to oxidize LDL include the following: residual flavonoids may be released into the medium and/or transferred from the cell surface into the LDL, and thus protect LDL against cell-mediated oxidation. The investigation of the mechanism needs further study of uptake and release of radiolabeled flavonoids by cells, as reported by Parthasarathy [32]. We could not evaluate the concentrations of flavonoids in LDL in the present study, probably because of the very small amounts of flavonoids in LDL. It is probable that flavonoids could enter the cells because they are also mildly lipophilic on account of their amphipathic nature. In addition, we made sure that there was no evidence of cell damage by incubation with theaflavin digallate for 4 hr at 100 and 400 μM by measuring LDH release into the medium and DNA synthesis in the cells. Therefore, the observed inhibitory effect of flavonoids on cell-mediated LDL oxidation was unlikely to be due to cell damage induced by flavonoids.

To investigate the mechanisms by which tea flavonoid supplementation of macrophages induces the reduction in macrophage-mediated LDL oxidation, we examined the effect of flavonoid pretreatment on superoxide production by macrophages. Theaflavin digallate pretreatment decreased the production of superoxide by macrophages in a concentration- and time-dependent manner (Figs. 4 and 5). Superoxide has been implicated in smooth muscle cell and macrophage-mediated LDL oxidation, but the source of superoxide is uncertain [35]. Depending upon the cell type, superoxide anions involved in cell-mediated LDL oxidation are generated via the following radical generating systems: NADPH oxidase, xanthine oxidase, nitric oxide synthase, and thiol-mediated radical generation [36, 37]. It remains to be elucidated whether flavonoids inhibit the generation of superoxide anion or scavenge free radicals, resulting in reduction of superoxide anion.

The cellular oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition metal ions in the incubation medium [31, 38–42]. Ham's F-10 medium used in this study was formulated authentically to contain 3 μM $FeSO_4$. Leake and Rankin [41] reported that little or no modification of LDL occurs when it is incubated with macrophages in Ham's F-10 medium specially formulated to be deficient in heavy metal ions, and adding micromolar amounts of $FeSO_4$ in the medium increases the LDL oxidation by macrophages. Flavonoids have been reported to chelate iron and copper [14, 15, 43], and thereby they suppress the superoxide-driven Fenton reaction, which currently is considered the most important route to generate active oxygen radicals. It is possible that this metal-chelating effect may contribute to the antioxidant effect of flavonoids by reducing free radical formation mediated by iron ions in the incubation medium. Therefore, we examined the effect of theaflavin digallate pretreatment of macrophages on iron concentrations (iron release from macrophages) in the incubation medium. Table 4 shows that theaflavin digallate pretreatment significantly decreased iron concentrations in the medium only at 400 μM, although the pretreatment significantly reduced cell-mediated LDL oxidation and superoxide production at concentrations lower than 400 μM. It may be that theaflavin digallate concentrations greater than or equal to 400 μM inhibit the release of iron from macrophages into the medium by chelating iron ions intracellularly. Therefore, the reduced effect of theaflavin digallate on iron release from macrophages seems not to be primary in inhibiting the ability of the cell to oxidize LDL.

In the present study, tea flavonoids inhibited the ability of the cells to modify LDL oxidatively. The potential benefit of flavonoids in humans is highly dependent on their metabolism *in vivo*, i.e. digestive absorption, transport in the bloodstream, metabolic transformation, and excretion, but the pharmacokinetics of flavonoids is still not well known [44–46]. We have reported that in humans, black tea consumption (750 mL/day for 4 weeks) prolongs the lag time of LDL oxidation induced by copper [47]. The important question is whether sufficient amounts of flavonoids can be absorbed by the body to act as effective antioxidants. Lee *et al.* [46] analyzed plasma and urinary tea polyphenols in healthy men after ingestion of 1.2 g of decaffeinated green tea. Plasma samples that were collected 1 hr after

ingestion contained 98–569 nM epigallocatechin and 254–640 nM epigallocatechin. In our preliminary data as well, the concentrations of catechins in plasma increased to similar concentrations after ingestion of 400 mg of a tea flavonoid supplement. Miura *et al.* [9] reported that the direct addition of 0.5 μM epigallocatechin gallate or theaflavin to LDL inhibits copper-mediated oxidation. In our experimental design, flavonoids were added in the medium at concentrations of 100 μM and up, and incubated with cells. Subsequently, the medium was removed, and the cells were washed with PBS. Accordingly, the concentrations of flavonoids on the cell surface and within cells could have been lower than the concentrations during flavonoid incubation because we could not measure the concentrations of flavonoids in the cells. We used 100–400 μM as the experimental concentrations of flavonoids for incubation, following the reported dosages [48]. These concentrations are not easily achievable in humans with normal tea consumption. However, there are several possibilities for flavonoid action *in vivo*. In the actual *in vivo* condition, not only theaflavin digallate but other theaflavins and catechins in plasma may work together to prevent LDL oxidation. Although tea flavonoids may be metabolized quickly after entering the circulation, it is possible that these metabolites also exert preventive effects on LDL oxidation. In addition, repeated exposure of LDL particles to tea flavonoids over a long period of time may enrich LDL particles with flavonoids and their metabolites sufficiently to make LDL particles less susceptible to oxidative modification.

Tea flavonoids may have important roles as one of the major dietary antioxidants in preventing atherosclerosis by suppressing LDL oxidation. The clinical therapeutic and prophylactic significance of flavonoids may be much more than that of synthetic antioxidants such as butylhydroxytoluene, because flavonoids are naturally occurring and non-toxic at the usual levels of intake. The *in vivo* metabolism of flavonoids in humans needs to be investigated further.

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