

Acute effects of ingestion of black and green tea on lipoprotein oxidation¹⁻³

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ABSTRACT

Background: Tea has been associated with a reduced risk of cardiovascular disease. One proposed mechanism of this risk reduction involves inhibition of lipoprotein oxidation in vivo by antioxidant polyphenolic compounds derived from tea. However, controlled interventions uniformly failed to show that ingestion of tea can inhibit LDL oxidation ex vivo. The absence of effects in previous studies may be due to the isolation of LDL particles from polyphenolic compounds that are present in the aqueous phase of serum.

Objective: The objective of this study was to examine the acute effects of ingestion of black and green tea on ex vivo Cu²⁺-induced lipoprotein oxidation without prior isolation of lipoproteins from serum.

Design: The acute effects of 4 hot drinks—green tea and black tea (each at a dose equivalent to 4 standard cups), water matched to the teas for caffeine content, and water—were assessed in 20 healthy men by using a Latin-square design. The lag time to lipoprotein diene formation, slope of the propagation phase of the oxidation curve, and area under the oxidation curve were calculated. Urinary concentrations of 4-*O*-methylgallic acid were used as a marker of uptake and metabolism of polyphenolic compounds from tea.

Results: Significant increases in urinary 4-*O*-methylgallic acid for black and green tea ($P < 0.0001$) were observed. Caffeine did not significantly influence lipoprotein oxidation. Compared with the water control, there was a greater lag time for black tea (5.4 ± 2.9 min; $P = 0.05$) that was of borderline significance and a similar trend for green tea (4.4 ± 2.8 min; $P = 0.17$). Slope and area under the oxidation curve were not altered.

Conclusion: Black tea has a mild acute effect on ex vivo lipoprotein oxidation in human serum. *Am J Clin Nutr* 2000; 71:1103–7.

KEY WORDS Black tea, green tea, antioxidant, caffeine, flavonoid, polyphenolic compounds, lipoprotein, Australia

INTRODUCTION

Results of epidemiologic studies show that tea (1–3) and flavonoids derived from tea (1, 3–5) are associated with a reduced risk of cardiovascular disease. A potential mechanism for such an effect involves inhibition of lipoprotein oxidation by

flavonoids derived from tea (6). The development and progression of atherosclerosis is thought to be critically dependent on lipoprotein oxidation (7).

The antioxidant activity of tea has been assessed in vitro (8–10) and ex vivo after ingestion of tea (9–13). We showed previously that extracts of black and green tea inhibit lipoprotein oxidation in human serum to a similar extent in vitro (8). In addition, significant increases in the total antioxidant capacity of serum after tea ingestion have been reported (10, 11), but this has not been a consistent finding (9, 11). Although assessment of lipoprotein oxidizability in vitro may provide a useful guide as to the potential of tea to inhibit oxidation, it does not establish any in vivo effects.

Controlled interventions of tea drinking have consistently failed to show that tea ingestion can inhibit LDL oxidation ex vivo (9, 11–13). The absence of effects on LDL oxidizability ex vivo may be related to the method used to assess LDL oxidizability. The standard method involves isolation of the LDL particles from the aqueous phase of serum. Catechins, the major class of flavonoids found in tea, were measured in LDL but only at <10% of the total serum concentration (14). Most of the catechins were associated with the water-soluble fraction and it was concluded that there was insufficient accumulation of catechins in LDL to improve resistance to LDL oxidation ex vivo (14). It was suggested that flavonoids may have action in the aqueous phase (15, 16), perhaps at the surface of lipoprotein particles. Therefore, the isolation of LDL from antioxidant flavonoids in the water-soluble fraction of serum may be inappropriate. Increases in total antioxidant capacity of serum after ingestion of tea (10, 11) may be due to the presence of phenolic antioxidants in the aqueous phase.

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To assess possible *in vivo* effects of tea, we studied the acute effects of ingestion of black and green tea on *ex vivo* lipoprotein oxidation without prior isolation of lipoproteins from serum. Such effects have not been examined previously without isolation of lipoproteins from the aqueous phase of serum. The use of this method may resemble *in vivo* conditions more closely than previously. Total antioxidant capacity of serum was also assessed and urinary concentrations of 4-*O*-methylgallic acid, a major gallic acid metabolite (17, 18), were used as a marker of tea polyphenol uptake.

SUBJECTS AND METHODS

Subjects

Twenty healthy men aged 35–73 y were recruited from the general population in response to media advertisements. Potential volunteers were excluded after initial screening if they reported the use of any medication or dietary supplements; a history of major illness, including heart disease, diabetes mellitus, liver disease, and renal disease; current smoking or smoking cessation for <6 mo; a body mass index (BMI; in kg/m²) >33; an alcohol intake averaging >40 g/d; or regular tea or coffee intake averaging <1 cup/d. The Royal Perth Hospital Ethics Committee approved the study and all participants gave written, informed consent.

Experimental design

The acute effects on lipoprotein oxidation in serum of 4 hot drinks—black tea, green tea, water matched to the teas for caffeine content, and water—were assessed and compared by using a Latin-square design. The use of caffeine and water allowed determination of any effects of caffeine on lipoprotein oxidation.

A total of 4 clinic visits were conducted ≥1 wk apart and as close as possible to the same day of the week and at the same time of the day. Subjects consumed each of the 4 drinks over 30 min, 1 at each visit, in random order. The subjects were fasting and had avoided tea, coffee, and other caffeine-containing beverages for ≥12 h before each visit. The subjects were instructed to maintain their usual diets throughout the 4 wk within which the clinic visits occurred. A blood sample was taken just before the subject started drinking each drink and 60 min after the subject finished the drink. A urine sample was taken before the subject started the drink and again 90 min after he finished the drink.

Preparation of tea

Tea bags with a total of 7.6 g tea leaves were extracted, with constant stirring movement, for 4 min in 400 mL boiled water. The black tea was a blended black tea and the green tea was a Japanese-type “Sen-Cha” tea. The other 2 drinks were 400 mL water with a caffeine content matched to that of the teas and 400 mL water. The caffeine content of each tea and the caffeine drink was 180 mg. All drinks were consumed hot and were prepared and consumed without additives such as milk and sugar.

Lipoprotein oxidation in serum

We used a modification of the serum oxidation method described by Regnstrom et al (19), which was described previously in detail (20, 21). This method provides an indication of diene formation in lipoprotein fatty acids in serum exposed to Cu²⁺, assessed by measuring change in absorbance at 234 nm. Briefly, the fasting blood sample was drawn from the antecubital

fossa vein into evacuated glass tubes and the serum was separated by using standard procedures. Serum samples were then frozen at –80°C until assayed. Serum was thawed and then diluted to a final concentration of 0.67% in phosphate-buffered saline solution (pH 7.4). Oxidation was initiated by the addition of 12 μmol Cu²⁺/L (final concentration) added as CuSO₄·5H₂O. Oxidation kinetics were determined by measuring the absorbance at 234 nm with a DU650 ultraviolet-visible spectrophotometer (Beckman Instruments Inc, Fullerton, CA). Absorbance readings were taken every 20 min over 240 min at 37°C.

The variables used to describe any differences between oxidation curves were lag time to lipoprotein diene formation (lag time), the slope of the propagation phase of the oxidation curve (slope), and the area under the oxidation curve. Lag time was measured from the plot of absorbance against time and was defined as the intercept between the baseline and the tangent of the absorbance curve during the propagation phase. The lag time provides an indication of protection of lipoproteins against oxidation. Two blinded observers measured the lag times and the results were averaged. All comparisons were made on within-run assessments to limit between-run variability. The within-run CV for the assessment of lag time when this technique was used was 8.8%. Slope and area under the curve were calculated by using PRISM software (GraphPad, San Diego).

Total antioxidant activity in serum

The total antioxidant activity of serum was determined by a spectrophotometric assay for total peroxyl radical-trapping antioxidant potential in human serum, as described in detail by Valkonen and Kuusi (22). Briefly, serum was thawed and then diluted to 1.25% in phosphate-buffered saline solution (pH 7.4). The oxidation of the free radical probe dichlorofluorescein-diacetate (DCFH-DA) (14 μmol/L) was then monitored at room temperature after addition of the free radical generator 2,2'-diazobis(2-amidinopropane)dihydrochloride (AAPH) (56 mmol/L) by measuring absorbance at 504 nm. Oxidation of DCFH-DA begins once serum antioxidants have been used. Trolox (Aldrich Chemical Co, Milwaukee) (8.4 μmol/L) was used as an internal standard. Each Trolox molecule is able to neutralize 2 molecules of peroxyl radicals. Therefore, the total antioxidant activity could be quantified by using the following equation:

$$\text{Total antioxidant activity} = \left(\frac{T_{\text{serum}}}{T_{\text{Trolox}}} \right) \times 80(\text{serum dilution factor}) \times 2 \times 8.4 \mu\text{mol/L} \quad (1)$$

where T_{serum} is the lag time for serum and T_{Trolox} is the lag time for Trolox. Values are expressed as micromoles of peroxyl radicals trapped by 1 L serum. All comparisons were made on within-run assessments to limit between-run variability. The within-run CV for assessment of total antioxidant activity was 1.9%.

Measurement of total polyphenolic and gallic acid

The total polyphenolic concentration of the tea infusions was measured by using a method described previously (23). The gallic acid content of the tea infusions was measured as follows. Infusions of black and green tea were prepared as described above. Gallic acid esters were then hydrolyzed by adding 1 mL methanolic KOH (1 mol/L) to 1 mL of the tea infusion, mixing, and then incubating at 50°C for 16 h. Samples were acidified to a pH of 2 and extracted with ethyl acetate to remove gallic acid. Gallic acid concentrations were analyzed on a Hewlett-

TABLE 1

Serum lipoprotein oxidation–derived variables and total antioxidant activity of serum before and 60 min after and urinary 4-*O*-methylgallic acid concentrations before and 90 min after ingestion of water, caffeine, black tea, and green tea¹

	Water (<i>n</i> = 20)	Caffeine (<i>n</i> = 20)	Black tea (<i>n</i> = 20)	Green tea (<i>n</i> = 19)
Lag time to lipoprotein diene formation (min)				
Before	72.3 ± 4.9	76.7 ± 4.8	75.9 ± 5.7	73.9 ± 6.2
After	69.7 ± 4.7	72.6 ± 4.5	78.2 ± 5.1	75.6 ± 5.7
Slope of propagation phase of oxidation curve (absorbance units/min)				
Before	8.1 ± 0.5	9.1 ± 0.7	8.7 ± 0.9	7.9 ± 0.8
After	8.1 ± 0.4	8.4 ± 0.5	8.0 ± 0.5	7.6 ± 0.5
Area under the oxidation curve (absorbance units/min)				
Before	11.8 ± 1.2	11.9 ± 1.2	11.7 ± 1.3	11.6 ± 0.9
After	12.2 ± 1.4	12.3 ± 1.1	11.4 ± 1.2	11.3 ± 1.0
Total antioxidant activity of serum (μmol/L)				
Before	1711 ± 82	1651 ± 78	1658 ± 80	1672 ± 77
After	1719 ± 81	1683 ± 88	1703 ± 94	1740 ± 80
Urinary 4- <i>O</i> -methylgallic acid (nmol/L)				
Before	14.6 ± 6.2	12.2 ± 9.2	10.8 ± 3.6	10.5 ± 4.3
After	5.4 ± 2.6	5.9 ± 4.7	236.6 ± 23.9 ²	70.5 ± 8.0 ²

¹ $\bar{x} \pm \text{SEM}$.

²Change from baseline significantly different from change after ingestion of water, $P < 0.05$.

Packard HP 5890 gas chromatograph coupled to an HP 5970 mass spectrometer fitted with an HP-1 cross-linked methyl silicone column (25 m × 0.20 mm, 0.33-mm film thickness; Hewlett Packard, Palo Alto, CA) by using helium as the carrier gas. An inlet pressure of 30 kPa was used and injections were made in a splitless mode. The initial column temperature of 120°C was held for 0.5 min and then programmed to increase to 280°C at 15°C/min, at which it was held for 5 min. The mass spectrometer was operated in the electron impact mode (70 eV). The major characteristic ions for gallic acid ($m/z = 281$ and 458) were monitored in selected ion monitoring mode. Peak identification was based on retention time and mass spectra compared with authentic standards. Quantification was performed by using the external standard method.

Urinary 4-*O*-methylgallic acid

Urinary 4-*O*-methylgallic acid concentrations were measured by using a previously described method (18). Briefly, urine (2 mL) and 2-hydroxy-3-naphthoic acid (1 μg, internal standard) were acidified to pH 4.8 with dilute hydrochloric acid. β-Glucuronidase (30 μL, with 3000 units of activity, catalogue no. G707; Sigma Chemical Co, St Louis) was added, mixed, and then placed in a 37°C water bath for 24 h and mixed occasionally. Samples were extracted with ethyl acetate (2 mL) and centrifuged at 2000 × *g* for 10 min at 10°C; the organic phase was dried under nitrogen and then derivatized with *bis*(trimethylsilyl)-trifluoroacetamide (50 μL) and pyridine (50 μL) at 40°C for 30 min.

4-*O*-Methylgallic acid esters were analyzed on a Hewlett-Packard HP 5890 gas chromatograph coupled to an HP 5970 mass spectrometer by using the method described for measurement of gallic acid. The major characteristic ion for 4-*O*-methylgallic acid ester ($m/z = 370$) and the molecular ion minus a methyl group for identification of internal standard ($m/z - 15 = 317$) were monitored in selected ion-monitoring mode. Peak identification was based on retention time and mass spectra compared with

authentic standards. The authentic standard of 4-*O*-methylgallic acid was prepared according to procedures described in the literature (24). For quantification, calibration curves were established by measuring peak areas versus response compared with the internal standard. The intraassay variability was 5%.

Statistics

All statistical analyses were performed by using SPSS (SPSS Inc, Chicago). Results are presented as means ± SEMs; significance was set at $P < 0.05$. General linear models were used to examine differences in derived oxidation-related variables between treatments; P values were adjusted for multiple tests. Because of the possible dependence of change on initial values, postdrink values were compared after adjustment for initial or predrink values by using general linear models. Log transformations were performed on variables that were not normally distributed. This study had >80% power to detect a 12% (≈7 min) increase in lag time with ingestion of black or green tea.

RESULTS

The mean age of the men recruited to this study was 56.2 ± 1.1 y. The subjects were healthy nonsmokers with BMIs between 19.0 and 32.4 (\bar{x} : 25.6 ± 0.8). Variables derived from the serum lipoprotein oxidation curves and the total antioxidant activity values before and 60 min after ingestion of water, water matched to the teas for caffeine content, black tea, and green tea are shown in **Table 1**. There were no significant differences in lag time, slope, area under the curve, or total antioxidant activity in serum between caffeine and water ingestion. The effects of ingestion of black and green tea on oxidation-related variables were compared with those of water.

Baseline-adjusted differences in postdrink lag time, slope, and area under the curve and total antioxidant activity of serum are shown in **Table 2**. A greater lag time for black tea than for water



TABLE 2

Baseline-adjusted differences in 60-min postdrink assessments of serum lipoprotein oxidation–derived variables and total antioxidant activity of serum for black tea and green tea compared with water¹

	Black tea (n = 20)	Green tea (n = 19)
Lag time to lipoprotein diene formation (min)	5.4 ± 2.9 ²	4.4 ± 2.8
Slope of propagation phase of oxidation curve (absorbance units/min)	−0.38 ± 0.44	−0.41 ± 0.49
Area under the oxidation curve (absorbance units/min)	−0.85 ± 0.65	−0.81 ± 0.64
Total antioxidant activity (μmol/L)	37 ± 46	60 ± 32 ³

¹ $\bar{x} \pm \text{SEM}$.

²Significantly different (borderline) from water, $P = 0.05$.

³Trend toward increase compared with water, $P = 0.09$.

was of borderline significance. There were no other significant differences in other variables derived from curves of Cu^{2+} -induced lipoprotein oxidation in serum for black tea or green tea. A trend toward greater total antioxidant activity for green tea than for water was also observed.

The total polyphenol intakes from the tea infusions were 1.44 and 1.37 g for black tea and green tea, respectively. The total gallic acid intakes from the tea infusions were 103 and 102 mg for black tea and green tea, respectively. Changes in 4-*O*-methylgallic acid concentrations in urine with ingestion of each drink are shown in Table 1. Compared with water, black tea ($P < 0.0001$) and green tea ($P < 0.0001$) were associated with significantly greater 4-*O*-methylgallic acid concentrations. The increases were significantly larger for black tea than for green tea ($P < 0.001$).

DISCUSSION

The main objective of this study was to determine whether consumption of black or green tea had acute inhibitory effects on lipoprotein oxidation *ex vivo*. We found a greater lag time to lipoprotein oxidation in human serum after ingestion of black tea than after ingestion of the control drinks that was of borderline significance, and a similar trend for green tea that did not reach significance. However, the observed effects on variables derived from curves of Cu^{2+} -induced lipoprotein oxidation, and on total antioxidant activity, were generally small.

“Black” and “green” are the 2 main classes of tea. Black teas are produced by promoting enzymatic oxidation of flavonoids, whereas enzymes involved in flavonoid oxidation are inactivated during the manufacture of green tea. Flavonoids and other polyphenols with antioxidant activity constitute $\approx 40\%$ of the weight of the black and green tea leaves (25). The measurements of total polyphenols in the tea infusions used were 1.44 g (19%) for black tea and 1.37 g (18%) for green tea. These amounts correspond to $\approx 50\%$ recovery and are reasonable given that infusions of tea prepared with hot water will not extract all polyphenols from the tea leaf.

About 5% of the weights of black and green tea leaves are in the form of gallic acid esters or free gallic acid (25). The measured gallic acid contents of the black and green tea infusions were similar (103 and 102 mg, respectively). A major metabolite of gallic acid in humans is 4-*O*-methylgallic acid (17), which

appears in the urine soon after ingestion of tea. Therefore, urinary concentrations of 4-*O*-methylgallic acid were used as a marker of uptake and metabolism of polyphenolic compounds from the tea.

The increases in urinary 4-*O*-methylgallic acid concentrations were significantly larger after ingestion of black tea than of green tea. Although the total concentration of gallic acid is similar for black and green tea, the form in which the gallic acid is present differs as a result of oxidative changes in black tea during production. Free gallic acid is higher in black tea—estimated at 1% of dry weight (25)—and possibly is more available for uptake and metabolism.


Ingestion of tea can make a major contribution to the intake of polyphenolic compounds (1). The high concentration of antioxidant polyphenolics in tea and the proposed link between lipoprotein oxidation and cardiovascular risk has led to investigations of possible antioxidant effects of tea on lipoprotein oxidation *ex vivo*.

Controlled interventions have consistently failed to show that ingestion of tea can inhibit LDL oxidation *ex vivo* (9, 11–13). One study did show a significant prolongation of lag time after ingestion of black tea (12), but this was an uncontrolled comparison. We suggest that the lack of effects of tea on LDL oxidation *ex vivo* in previous controlled interventions (9, 11–13) may relate to the method used to assess LDL oxidizability. The isolation of LDL from serum for the LDL oxidation assay, and findings showing little incorporation of tea polyphenols into LDL (14), strengthen this explanation. The assay used in the present study to assess *ex vivo* lipoprotein oxidation does not involve isolation of lipoproteins (19, 20). The oxidation of lipoprotein fatty acids occurs in the presence of the aqueous phase of serum. Assessment of lipoprotein oxidation in serum therefore may more closely resemble subendothelial fluid, where lipoprotein oxidation is suggested to occur (26).

Our findings are generally consistent with the theory that ingestion of tea can inhibit lipoprotein oxidation *ex vivo*. In addition, our results agree with the suggestion that assays that measure lipoprotein oxidation in an environment more closely resembling *in vivo* conditions (26) may be more likely to show significant effects. However, observed effects on variables related to serum lipoprotein oxidation were small and generally not significant.

Small, nonsignificant increases in total antioxidant activity were observed in this study 60 min after ingestion of black or green tea. These increases were $\approx 3\%$ and 4% for black tea and green tea, respectively. Using a similar assay, Serifini et al (10) found a significant 40–50% increase in total antioxidant activity 30–50 min after ingestion of 1 cup of black or green tea. However, McAnlis et al (9) found no increase in total antioxidant activity from 30 to 180 min after ingestion of a single cup of strong tea. In addition, in studies of long-term tea consumption, increases in total antioxidant activity were small (3–10%) and generally not significant (9, 11).

The timing of blood sampling after tea ingestion is a factor that has the potential to influence the results obtained. In the present study, drinks were consumed over 30 min and blood samples were taken 60 min after the subjects finished the drink (90 min after they started). Data published before this study began suggested that antioxidant activity peaks within 90 min of ingestion of black or green tea (10). However, more recent findings suggest that the peak concentration of the main class of polyphenolic antioxidants in tea occurs ≈ 120 –140 min after ingestion of green tea (27). Therefore, the assessment of lipoprotein oxidation in serum and total antioxidant activity closer to 120 min after tea ingestion could have resulted in more significant effects.

In conclusion, polyphenolic compounds from tea were absorbed and excreted in the urine within 90 min of tea ingestion. We showed that black tea can have a mild acute effect on the oxidation of lipoproteins in serum *ex vivo*. Tea consumption had no significant acute effect on total antioxidant activity in serum. Overall, although effects on oxidation-related variables were generally consistent in direction with the proposed antioxidant activity of tea, they were small. The final conclusion as to whether tea consumption affects lipid peroxidation *in vivo* will have to await the outcome of long-term studies using markers that are more representative of oxidative stress *in vivo*. 

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