JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 1365–1369

Received March 9, 2005
Accepted May 16, 2005
DOI 10.1211/jpp.57.10.0017
ISSN 0022-3573

Oral administration of a decaffeinated green tea (*Camellia sinensis*) extract did not alter urinary 8-epi-prostaglandin $F_{2\alpha}$, a biomarker for in-vivo lipid peroxidation

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Funding: This work was made possible in part by Grant Number R21 AT00511 from the National Center for Complementary and Alternative Medicine (NCCAM), National Institutes of Health, along with Grant number M01 RR01070-18 from the National Institutes of Health, for support of the General Clinical Research Center at the Medical University of South Carolina.

Abstract

Oxidative stress is involved in the pathogenesis of numerous chronic human diseases. The objective of this study was to determine whether administration of a decaffeinated green tea extract providing 844 mg flavonoids daily reduced the urinary excretion of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), a product of lipid peroxidation in cellular membranes and of low-density lipoprotein (LDL). Nine healthy male and female subjects were studied at baseline and after 14 days of green tea supplementation. Analysis of urinary 8-epi-$PGF_{2\alpha}$ was performed using immunoaffinity extraction-gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS). Urinary 8-epi-$PGF_{2\alpha}$ concentrations were 0.286 ± 0.120 nmol (mmol creatinine)$^{-1}$ at baseline and 0.244 ± 0.177 nmol mmol^{-1} creatinine after green tea supplementation. There were no significant differences in the excretion of urinary 8-epi-$PGF_{2\alpha}$ after treatment with green tea. We conclude that 14 days of green tea supplementation did not significantly alter in-vivo lipid peroxidation.

Introduction

Green tea (*Camellia sinensis*) consumption has been suggested to have numerous beneficial health effects including prevention of cardiovascular disease and cancer (Williamson & Manach 2005). The purported health benefits of green tea consumption are thought to be due to catechins, a class of flavonoids that are potent antioxidants (Yang & Landau 2000; Rietveld & Wiseman 2003; Vinson et al 2004). A typical glass of green tea contains approximately 300 mg of catechins (Anonymous 2004). The major catechin present in green tea is (−)-epigallocatechin-3-gallate (EGCG). Green tea catechins have been detected in human plasma in their native forms and as glucuronide and sulfate conjugates shortly after consumption of green tea infusions and supplements (Yang et al 1998; Donovan et al 2004).

Oxidative stress and free radicals are involved in the pathogenesis of numerous chronic human diseases, including cardiovascular disease, cancer and neurodegenerative diseases (Rietveld & Wiseman 2003). Dietary antioxidants are likely important in reducing exposure to free radicals and reducing oxidative stress (Rietveld & Wiseman 2003). Recent research efforts have sought to identify more reliable biomarkers of in-vivo oxidation. One such non-invasive marker of in-vivo lipid peroxidation is the excretion of urinary F_2-isoprostanes. F_2-isoprostanes are formed from free-radical-catalysed peroxidation of arachidonic acid and appear to reflect oxidative damage to tissues and lipoproteins such as low-density lipoprotein (LDL) (Lawson et al 1999; Roberts & Morrow 2000; Fam & Morrow 2003). We chose to focus this study on the most commonly studied F_2-isoprostane isomer, 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$). 8-Epi-$PGF_{2\alpha}$ concentrations in plasma and urine have been shown to be dramatically increased in smokers (Morrow et al 1995) and in the brains of patients with Alzheimer's disease (Pratico et al 1998). Supplementation with the antioxidant

vitamin E decreased urinary 8-epi-$PGF_{2\alpha}$ excretion in a dose-dependent manner in hypercholesterolaemic subjects (Davi et al 1997). A diet enriched with soy isoflavones significantly reduced the plasma concentrations of 8-epi-$PGF_{2\alpha}$ in normal subjects (Wiseman et al 2000). At present, F_2-isoprostanes appear to be one of the best available biomarkers of in-vivo lipid peroxidation (Lawson et al 1999; Roberts & Morrow 2000; Hodgson et al 2002).

The objective of this pilot study was to determine whether a two-week intervention with a decaffeinated green tea extract altered the urinary excretion of the isoprostane, 8-epi-$PGF_{2\alpha}$. We previously reported that supplementation with green tea extract did not alter the activity of cytochrome P450 (CYP) 2D6 and CYP3A4 in healthy non-smoking subjects (Donovan et al 2004). We now report the excretion of urinary 8-epi-$PGF_{2\alpha}$ before (baseline) and after a 14-day exposure to a standardized green tea supplement in these same subjects.

Methods

Subjects and blood sampling

Nine healthy research subjects (5 male, 4 female), aged 20–48 (mean ± s.d.: age, 34.2 ± 11.3 years; weight, 67.8 ± 16.2 kg; body-mass index (BMI), 23.5 ± 4.5), participating in a green-tea–drug interaction study, provided written informed consent approved by the Medical University of South Carolina (MUSC) Office of Research Integrity. All subjects were determined to be healthy by medical history, physical examination, basic serum chemistry and electrocardiogram. Additionally, all were non-smokers, were taking no prescription or over-the-counter medications or supplements and abstained from caffeine during the study period.

The general study design, described elsewhere in detail (Donovan et al 2004), involved two discrete inpatient visits within the MUSC inpatient General Clinical Research Center (GCRC). Early morning blood and urine collection was performed on two occasions at identical time points following an overnight hospitalization visit. The initial samples, which served as baseline measures, were obtained during each subject's first GCRC study visit while receiving no medication or green tea supplement. The second blood and urine samples were obtained at an identical time during the second GCRC study day after being treated with a standardized decaffeinated green tea supplement twice daily for 14 days. Samples of urine from each visit were collected and stored at −70°C until analysis. Blood samples were collected 2 h after morning administration of the green tea (following the 14-day treatment) for analysis of plasma EGCG concentration. Heparinized blood collection tubes (Vacutainers; Becton Dickinson, Franklin Lakes, NJ, USA) were used for blood sampling and plasma was stored at −70°C until analysis.

Green tea extract and dosing

The decaffeinated green tea extract used in this study was purchased from Life Extension (Fort Lauderdale, FL, USA). Subjects were instructed to take 2 capsules twice daily (0800 h and 2000 h) for 14 days. Analysis of the flavonoid content of the green tea product was previously described (Donovan et al 2004). A total of 211 ± 25 mg of catechins were present in each green tea capsule and individual constituents, including catechin, epigallocatechin, epicatechin, EGCG and epicatechin gallate, were quantified (Table 1). Consistent with a decaffeinated product, the caffeine content in each green tea capsule was <1 mg.

Analysis

Urinary F_2-isoprostane analysis using immunoaffinity extraction-GC-NICI-MS

A modification of the immunoaffinity extraction-gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) method of Tsikas et al (2003) was used. Urine (1 mL) was fortified with 3,3,4,4-[$^2H_4$]-8-epi-prostaglandin $F_{2\alpha}$ (1 ng in 10 μL methyl acetate; Cayman Chem, Ann Arbor, MI, USA), centrifuged (5 min at 500 *g*), then applied to a commercial 8-epi-$PGF_{2\alpha}$ immunoaffinity column (Cayman Chem). After washing with eicosanoid affinity column buffer (2 mL; Cayman Chem), followed with water (2 mL), the 8-epi-$PGF_{2\alpha}$ isomer was eluted with 95% ethanol (2 mL). The sample was dried under nitrogen and the analyte was transferred to a silanized microvial insert in 50 μL ethanol. After drying under nitrogen, the analyte was esterified using 10% pentafluorobenzylbromide in acetonitrile (40 μL) and 10% diisopropylethylamine in acetonitrile (20 μL). The sample was heated at 40°C for 20 min and then evaporated under nitrogen. Silyl ethers were formed by treating the residue with bis(trimethylsilyl)trifluoroacetamide (50 μL) at 60°C for 15 min. The derivatized sample was analysed by GC-NICI-MS using an Agilent model 5973N. GC separations were on a 30 m × 0.25 μm film 5% phenylmethylpolysiloxane column (DB-5MS, J&W Scientific) with a helium linear velocity of 50 cm min^{-1}). The column was held at 190°C for 2 min after injection (2 μL; pulsed splitless), then ramped to 300°C at 20°C min^{-1} and held for 6 min. Methane was used as the reagent gas. Using selected ion monitoring, the debenzylated fragment ions m/z 569 for the analyte and m/z 573 for the deuterated internal

Table 1 Components of the decaffeinated green tea extract used in this study

Compound	Amount/capsule (mg)	Mean amount/day (mg)
Caffeine	0.9 ± 0.1	<4
Catechin	2 ± 0	8
Epicatechin	11 ± 2	44
Epigallocatechin	18 ± 4	72
Epigallocatechin gallate	126 ± 16	504
Catechin gallate	13 ± 2	52
Epicatechin gallate	41 ± 5	164
Total	211 ± 25	844

Data are expressed as mean ± s.d. of 3 separate analyses.

standard were detected at 8.10 and 8.02 min, respectively, after injection. Urinary 8-epi-$PGF_{2\alpha}$ concentrations were standardized to milligrams of urinary creatinine. Urine creatinine measurements were determined in the MUSC general clinical chemistry laboratory on a Synchron LX Clinical Chemistry analyzer (Beckman Coulter, Inc., Fullerton, CA, USA) using the kinetic Jaffe rate method (kinetic alkaline picrate).

Analysis of epigallocatechin gallate (EGCG) in plasma

The plasma concentration of EGCG was determined from a sample obtained 2 h after the first morning dose on the 14th day of the green tea supplementation. The EGCG concentrations were determined by HPLC with and without β-glucuronidase/sulfatase treatment. The extraction and analytical procedure is described elsewhere in detail (Donovan et al 2004).

Statistics and data analysis

Statistical analyses were performed using the Wilcoxon signed ranks test with SPSS for Windows version 12.0. All results are presented as mean ± s.d., and P values < 0.05 were considered statistically significant. We estimate based on the power analyses reported by Hodgson et al (2002) that with 9 subjects, our study had 80% power to detect a 30% difference in 8-epi-$PGF_{2\alpha}$ excretion.

Results

Urinary 8-epi-$PGF_{2\alpha}$ excretion

Extraction procedures from subjects receiving a supplement high in flavonoid components (i.e. green tea) resulted in excellent chromatograms and detection of urinary 8-epi-$PGF_{2\alpha}$ pre- and post-treatment. No interferences were evident on the chromatogram at baseline or after supplementation with green tea. Urinary 8-epi-$PGF_{2\alpha}$ values were normalized to urine creatinine concentration (Table 2). There were no significant differences in the excretion of urinary 8-epi-$PGF_{2\alpha}$ after treatment with green tea.

Plasma concentrations of EGCG

The plasma concentrations of EGCG for individual subjects are presented in Table 3. The mean plasma concentration of the total EGCG (i.e. conjugated plus unconjugated) was $1.4 \pm 1.9\ \mu mol\ L^{-1}$ 2 h after green tea administration. EGCG was present in plasma in both conjugated and unconjugated form. Of the total EGCG in plasma, 19 ± 17% was in the unconjugated form. There was no apparent relationship between the plasma concentration of EGCG (whether native or conjugated) and the change in urinary 8-epi-$PGF_{2\alpha}$ excretion (Figure 1). The two subjects with the highest plasma EGCG levels both had increased urinary 8-epi-$PGF_{2\alpha}$ concentrations.

Table 2 Urinary excretion of 8-epi-$PGF_{2\alpha}$ in 9 subjects at baseline and following a 14-day treatment period with green tea

Subject	Baseline	Post-green tea	Difference
1	0.274	0.366	+0.092
2	0.334	0.267	−0.067
3	0.426	0.043	−0.383
4	0.297	0.060	−0.237
5	0.229	0.271	+0.042
6	0.379	0.187	−0.192
7	0.096	0.153	+0.057
8	0.121	0.220	+0.099
9	0.418	0.629	+0.210
Mean ± s.d.	0.286 ± 0.120	0.244 ± 0.177	−0.042

Values are normalized to the concentration of creatinine and are expressed as nmol 8-epi-$PGF_{2\alpha}$/mnol creatinine.

Table 3 Plasma concentrations of EGCG in 9 subjects 2 h after administration of the final dose of green tea

Subject	Total EGCG ($\mu mol\ L^{-1}$)	Unconjugated EGCG ($\mu mol\ L^{-1}$)	% Unconjugated
1	0.16	0.02	11.4
2	0.24	0.04	15.0
3	0.80	0.03	4.2
4	0.77	0.29	37.3
5	0.86	0.04	4.1
6	0.77	0.04	5.2
7	6.18	2.46	39.8
8	2.27	1.01	44.7
9	0.19	0.02	11.1
Mean ± s.d.	1.4 ± 1.9	0.4 ± 0.8	19.2 ± 16.6

The total EGCG concentration is the amount after hydrolysis by β-glucuronidase and arylsulfatase. The unconjugated form is the concentration of EGCG determined without enzymatic hydrolysis.

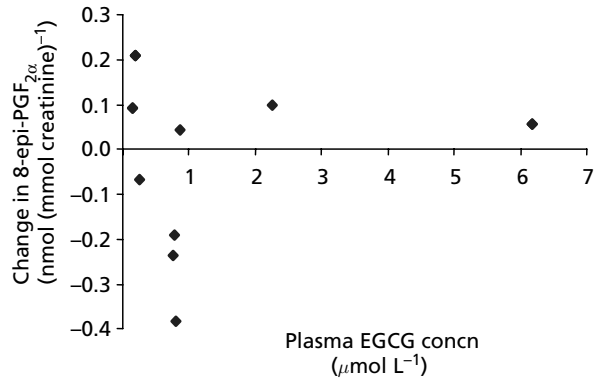

Figure 1 Apparent lack of relationship between the maximum plasma concentration of EGCG (both free and unconjugated) in 9 healthy subjects and the change in urinary excretion of 8-epi-$PGF_{2\alpha}$ at baseline and after supplementation with green tea.

Discussion

In this study we demonstrated that two weeks of supplementation with a decaffeinated green tea extract did not significantly alter the urinary excretion of 8-epi-$PGF_{2\alpha}$, suggesting that administration in this manner did not significantly affect in-vivo oxidative stress. We chose to perform analyses in urine rather than plasma to avoid artifactual formation of 8-epi-$PGF_{2\alpha}$ ex-vivo (Fam & Morrow 2003). The GC-NICI-MS used for this study is superior to commercially available enzyme-linked immunoassay (ELISA) test kits due to cross-reactivity and non-specificity with the ELISA (Proudfoot et al 1999). The analysis of 8-epi-$PGF_{2\alpha}$ using GC-NICI-MS is labour-intensive and has often been limited to more specialized laboratories. When performed in our laboratory, the methodology appeared robust and had sufficient selectivity and sensitivity to measure 8-epi-$PGF_{2\alpha}$ in all subjects both before and after supplementation with the decaffeinated green tea extract.

The time required for an intervention to induce measurable changes in urinary 8-epi-$PGF_{2\alpha}$ excretion in man varies in the literature. Refraining from cigarette smoking for 3 days resulted in a significant decrease in urinary 8-epi-$PGF_{2\alpha}$ excretion although the decrease continued for 4 weeks (Pilz et al 2000). A combination of high-dose vitamin C, vitamin E and β-carotene for a period of two weeks inhibited the formation of 8-epi-$PGF_{2\alpha}$ in plasma (Roberts & Morrow 1999). In addition, a two-week course of vitamin E (D,L-α-tocopherol acetate, 100–600 mg daily) decreased urinary 8-epi-$PGF_{2\alpha}$ excretion (measured by immunoassay) in a dose-dependent manner in hypercholesterolaemic subjects (Davi et al 1997). A subsequent study using the more robust GC-mass spectrometry method demonstrated that vitamin E administered in doses of 800 IU or greater significantly reduced F_2-isoprostane production only after 16 weeks (Roberts et al 2002).

The effect of decaffeinated green tea supplementation on urinary 8-epi-$PGF_{2\alpha}$ has not been previously investigated. However, numerous studies have shown that green tea consumption alters ex-vivo markers of antioxidant activity in as little as 1 h after consumption (see Rietveld & Wiseman 2003 for review). Consistent with the findings in this study, a cross-over study investigating 7-day treatment periods with caffeinated water or green or black tea infusions also did not observe a difference in the excretion of urinary 8-epi-$PGF_{2\alpha}$ (Hodgson et al 2002).

F_2-Isoprostanes are thought to be formed by free-radical-catalysed oxidation of arachidonic acid, which is esterified to phospholipids in cellular membranes or by the oxidation of arachidonic acid within circulating LDL. It is possible that the hydrophilic flavonoids in the green tea, such as EGCG, did not partition well into cellular membranes or into the LDL particle. Vitamin C, a hydrophilic antioxidant, did not alter plasma F_2-isoprostanes in mildly hypercholesterolaemic men (Kaikkonen et al 2001). Studies investigating the effects of chocolate consumption, another source of catechin-type flavonoids, did not observe any significant difference in the excretion of urinary F_2-isoprostanes in a period of 2 weeks (Wang et al 2000; Engler et al 2004). Alternatively, it is possible that dosing in this study was not appropriate for antioxidant effect. We must conclude that although studies utilizing other methodologies have reported increases in markers of plasma antioxidant activity 1–2 h after consumption of green tea, that systemic oxidant stress does not appear to be significantly affected by our method of green tea administration.

This pilot study was performed using healthy, non-smoking subjects. As mentioned, significant differences in 8-epi-$PGF_{2\alpha}$ plasma concentrations of urinary excretion were observed in previous dietary intervention studies in both normal subjects and those with clinical conditions such as hyperlipidaemia. In those studies, the observed decreases generally were in the range 10–40% (Fam & Morrow 2003). Smokers were able to reduce 8-epi-$PGF_{2\alpha}$ by 50% by cessation of smoking for 4 weeks (Pilz et al 2000). Our study was not adequately powered to detect modest changes in urinary 8-epi-$PGF_{2\alpha}$, although approximately half of the subjects showed an increase in urinary 8-epi-$PGF_{2\alpha}$ excretion while the other half showed a decrease. We conclude from these data that there is no significant or relevant effect of supplementation with green tea on 8-iso-prostaglandin $F_{2\alpha}$ excretion in healthy non-smoking subjects. A future study could address whether supplementation with green tea has any significant effects in smokers or patients with conditions associated with oxidative stress.

This study also found no evidence of a correlation between the maximum plasma concentration of the major flavonoid component, EGCG, and the urinary excretion of 8-epi-$PGF_{2\alpha}$. In fact, the two subjects with the highest plasma concentrations of EGCG, both had increases in the urinary excretion of 8-epi-$PGF_{2\alpha}$. This observation supports our conclusion that green tea supplementation, at the studied dose and duration of treatment, does not have significant effects on the urinary excretion of 8-epi-$PGF_{2\alpha}$. Although a more rigorous analysis could have been performed by comparing the area under the plasma EGCG concentration versus time curves (AUCs) with the urinary excretion of 8-epi-$PGF_{2\alpha}$, plasma samples to define the complete AUCs of EGCG were not available in this study. It is worth noting that the plasma EGCG concentrations observed in this study were in the same range as observed in previous studies that administered green tea extracts or infusions at similar doses (Lee et al 1995; Yang et al 1998; Chow et al 2001; Manach & Donovan 2004), suggesting normal absorption and pharmacokinetics of EGCG from this supplement.

Conclusion

This study demonstrates that green tea supplementation for 14 days did not significantly alter the urinary excretion of 8-epi-$PGF_{2\alpha}$, an in-vivo marker of lipid peroxidation, in healthy subjects. Studies may need to address whether green tea supplementation alters 8-epi-$PGF_{2\alpha}$ in those with a clinical condition associated with increased oxidative stress.

Future studies may also address whether other markers of in-vivo oxidation are altered by green tea supplementation

or if a longer time-course or higher dose has significant effects on this emerging biomarker.

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