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Green Tea Extract Protects Leptin-Deficient, Spontaneously Obese Mice from Hepatic Steatosis and Injury[1,2]

Richard S. Bruno,[3]* Christine E. Dugan,[3] Joan A. Smyth,[4] Dana A. DiNatale,[3] and Sung I. Koo[3]

Departments of [3]Nutritional Sciences and [4]Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269-4017

Abstract

The incidence of nonalcoholic fatty liver disease (NAFLD) has risen along with the ongoing obesity epidemic. Green tea extract (GTE) inhibits intestinal lipid absorption and may regulate hepatic lipid accumulation. The objective of this study was to determine whether GTE protects against hepatic lipid accumulation during the development of NAFLD in an obese mouse model. Five-wk-old *ob/ob* (obese) mice and their lean littermates (8 mice·genotype^{-1}·dietary treatment^{-1}) were fed GTE at 0, 1, or 2% (wt:wt) for 6 wk. The body weights of obese mice and lean littermates fed diets containing GTE were 23–25% and 11–20% lower ($P < 0.05$) than their respective controls fed no GTE. Histologic evaluation showed a significant reduction in hepatic steatosis in GTE-fed obese mice only and histologic scores were correlated with hepatic lipid concentration ($r = 0.84$; $P < 0.05$), which was reduced dose dependently by GTE. GTE protected against hepatic injury as suggested by 30–41% and 22–33% lower serum alanine aminotransferase and aspartate aminotransferase activities, respectively. Hepatic α-tocopherol was 36% higher in obese mice than lean mice. GTE tended ($P = 0.06$) to lower hepatic α-tocopherol, which was not fully explained by the GTE-mediated reduction in hepatic lipid. Hepatic ascorbic acid was lower in obese mice than in lean mice ($P < 0.05$) and was unaltered by GTE. Obese mice had lower serum adiponectin than lean mice and this was not affected by GTE. The results suggest that GTE protects against NAFLD by limiting hepatic lipid accumulation and injury without affecting hepatic antioxidant status and adiponectin-mediated lipid metabolism. Further study is underway to define the events by which GTE protects against obesity-triggered NAFLD. J. Nutr. 138: 323–331, 2008.

Introduction

Hepatic steatosis is 1 of several liver diseases that are collectively termed nonalcoholic fatty liver disease (NAFLD)[5] (1). The incidence of NAFLD is associated with obesity and has risen dramatically over the past several decades, coincident with the obesity epidemic resulting in nearly 66% of Americans that are currently overweight or obese (2). An estimated 40 million Americans have some form of NAFLD (3) and various studies have indicated that 58–74% of obese adults and 23–53% of obese children are afflicted with NAFLD (4–8).

The mechanisms leading to the development of hepatic steatosis remain poorly understood, but hepatic steatosis is often characterized by excess hepatic lipid accumulation, hepatic injury, and dyslipidemia (9,10). These features comprise what is referred to as the "first-hit" and were initially thought to be relatively benign (10). However, studies in this area have suggested that fatty livers are highly vulnerable to secondary insults (i.e. second-hit), such as those mediated by oxidative stress, which could accelerate the progression of hepatic steatosis toward more debilitating and advanced stages of NAFLD. In turn, these events may enhance the risk for developing liver-related morbidity and mortality (9).

Hepatic steatosis is commonly asymptomatic and its presence is often suspected by unexplainable elevations in serum aminotransferases (3,11). However, invasive liver biopsy remains as the only reliable means to diagnose hepatic steatosis and assess its severity. At present, there are no well-established treatments for hepatic steatosis beyond weight management or comorbidity management. Because weight loss has a poor long-term success rate (12), complementary therapeutic strategies for NAFLD are needed. Therefore, the prevention of hepatic steatosis or limiting hepatic lipid accumulation and injury using unique dietary approaches may reduce the incidence and/or likelihood of progressing toward more severe forms of NAFLD.

Epidemiological data suggest that the consumption of green tea (*Camellia sinensis*) is associated with reduced mortality from all causes and from cardiovascular disease (13). The mechanisms

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[5] Abbreviations used: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DTPA, diethylenetriaminepentaacetic acid; EGCG, epigallocatechin gallate; GTE, green tea extract; NAFLD, nonalcoholic fatty liver disease; NE, norepinephrine; *ob/ob*, obese mice or leptin-deficient spontaneously obese mouse; Q12, 1-dodecyltriethyl-ammonium phosphate.
* To whom correspondence should be addressed. E-mail: richard.bruno@uconn.edu.

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by which green tea or its catechins protects against chronic disease remain unclear. However, considerable evidence from in vitro, animal, and human studies suggests the protective effect of green tea may be partly mediated through the antioxidant properties of its catechins (14–16). Additionally, experimental data from rodent models indicated that green tea or its catechins inhibit intestinal lipid absorption (17–19) and lower blood lipids (20–22). Moreover, acute oral or intraperitoneal administration of epigallocatechin gallate (EGCG), the principal green tea catechin, protects against ischemia-/reperfusion-induced hepatic steatosis and injury in obese (*ob/ob*) mice by decreasing hepatic lipid accumulation and serum alanine aminotransferase (ALT) activity (23). Thus, green tea may protect against the development of hepatic steatosis via multiple mechanisms yet to be fully defined.

This study was conducted to investigate whether dietary green tea extract (GTE) would attenuate the development of obesity-triggered hepatic steatosis and injury in spontaneously obese (*ob/ob*) mice, a commonly used model for studying the mechanisms leading to the development of hepatic steatosis (24). We used 5-wk-old *ob/ob* mice and their lean littermates fed diets containing 0, 1, or 2% GTE for 6 wk, which corresponds to the time period when *ob/ob* mice become obese and develop hepatic steatosis, hepatic injury, and dyslipidemia (23–25). We then assessed the GTE-mediated effects on hepatic lipid accumulation and injury, hepatic antioxidants, serum lipids, and serum adiponectin.

Materials and Methods

Animals and study design. The protocol for the care and use of animals was approved by the Institutional Care and Use Committee at the University of Connecticut. Male leptin-deficient (*ob/ob*) mice and their C57BL/6J lean littermates (4 wk of age; $n = 24$/genotype) were purchased from Jackson Laboratories. Mice were individually housed in a temperature- and humidity-controlled room with a 12-h-light/-dark cycle. All mice were acclimated for 1 wk prior to the experiment. Mice ($8 \cdot \text{genotype}^{-1} \cdot \text{dietary treatment}^{-1}$) were assigned randomly to 1 of 3 modified AIN-93G diets (26) for 6 wk: *1*) a control diet containing 0% GTE; *2*) the same diet but containing 1% GTE (wt:wt); or *3*) the same diet containing 2% GTE. The powdered diet was purchased commercially (Dyets no. 111568) and we substituted tocopherol-stripped soybean oil for soybean oil to control the dietary vitamin E concentration (provided as *all rac*-α-tocopherol acetate; 75 mg/kg diet) of the diet. Egg white was also substituted for casein as the protein source. GTE (powder; wt:wt) was kindly provided by Unilever BestFoods and was mixed homogenously into the powdered diet as appropriate for each treatment. The GTE contained 30% catechins (wt:wt) consisting of 48% EGCG, 31% epigallocatechin, 13% epicatechin gallate, and 8% epicatechin as verified by HPLC-UV (18). Mice had free access to the diets and water throughout the study. The GTE at 1% was chosen on the basis that it was equivalent to ~7 servings (~120 mL/serving) of green tea consumed per day in humans (estimated on the basis on energy intake), which is similar to the amounts consumed by Japanese adults who had reduced mortality from all causes and cardiovascular disease (13). GTE at 2% was chosen to reflect greater (>10 servings per day) tea consumption, which is common in certain parts of the world (27).

Body weights were recorded weekly and food intake was measured daily for each mouse by determining pre- and postweights of food jars. At the conclusion of the 6-wk dietary intervention, mice were starved for 4–6 h. Under isoflurane anesthesia, blood was collected from the retro-orbital sinus into anticoagulant free collection tubes. After blood collection, mice were killed by cervical dislocation under anesthesia. Serum was obtained by centrifugation (2000 × *g*; 15 min, 4°C; Brinkmann Instruments Model 5415R). Livers were excised, rinsed in ice-cold PBS, and blotted. A portion of the central lobe was removed, snap frozen in liquid nitrogen, and stored at −80°C until analysis. A small portion of the same lobe was processed for histologic examination and the remainder was processed for lipid analysis.

Materials

HPLC-grade solvents were purchased from Fisher Scientific as were the following chemicals: ascorbic acid, butylated hydroxytoluene, diethylenetriaminepentaacetic acid (DTPA), PBS, KOH, potassium phosphate, lithium perchlorate, perchloric acid, sodium acetate, sulfuric acid, and triton X-100. The HPLC pairing reagent, 1-dodecyltriethyl-ammonium phosphate (Q12), was purchased from Regis Technologies. Vitamin E standards (*RRR*-α- and γ-tocopherol) were purchased from Sigma Aldrich. Uric acid was purchased from Pointe Scientific.

Hepatic histology. The liver tissue was immediately fixed in 10% buffered formalin (Fisher Scientific) for pathologic analysis. Formalin-fixed livers were paraffin embedded and then sections of 4–5 μm were prepared and subsequently stained with hematoxylin and eosin. Histologic evaluation was performed twice by a pathologist (J.A.S.) unaware of the treatments on 2 separate occasions. A semiquantitative scoring system was used to assess the severity of hepatic steatosis and inflammatory cell infiltration in 10 microscopic fields examined at 200× as described previously (25). In brief, the following criteria were used for scoring hepatic steatosis: grade 0, no fat; grade 1, fatty hepatocytes occupying <33% of the hepatic parenchyma; grade 2, fatty hepatocytes occupying 33–66% of the hepatic parenchyma; grade 3, fatty hepatocytes occupying >66% of the hepatic parenchyma. Similarly, for inflammatory cell infiltration, scoring criteria were: grade 0, none; grade 1, <5 foci/field; and grade 2, ≥5 foci/field. Additionally, we specifically examined sections for evidence of fibrosis, Mallory bodies, Councilman bodies, biliary hyperplasia, degree of variation in nuclear size, and necrosis.

Serum and hepatic lipids. Serum total cholesterol (kit no. 2350–400H) and triglyceride (kit no. 2780–400H) were analyzed by standard clinical assays (Thermo Electron) and performed in accordance with the manufacturer's instructions. Hepatic total lipid was extracted with chloroform:methanol (28), determined gravimetrically, and subsequently analyzed for total cholesterol and triglyceride as described (29).

Serum hepatic enzymes and adiponectin. Serum ALT, aspartate aminotransferase (AST), and alkaline phosphate (ALP) activities were measured spectrophotometrically using commercially available kits (kit nos. TR71121, TR70121, and TR11110; Thermo Electron) and performed in accordance with the manufacturer's instructions. Serum adiponectin was measured using an ELISA kit (kit no. EZMADP-60K; Linco Research) and performed in accordance with the manufacturer's instructions.

Hepatic vitamin E. Vitamin E, as α- and γ-tocopherol, was extracted and measured as described with modifications (30–32). A portion of liver (~50 mg) was added to a screw-top test tube containing 2 mL of 1% ascorbic acid prepared in ethanol (wt:v). Purified water (1 mL) was then added, followed by 300 μL of saturated KOH (79.2 g KOH dissolved in 74.2 mL water). The sample was saponified (30 min, 70°C) and then immediately placed into an ice bath. To each sample, 1 mL of 1% ascorbic acid (wt:v) prepared in water and 20 μL of 4.5 mmol/L butylated hydroxytoluene prepared in ethanol was added. The sample was then extracted with 2 mL of hexane and a known volume was transferred, dried under nitrogen gas, and the residue dissolved in a known volume of 1:1 ethanol:methanol. The sample was injected onto a HPLC-Coularray system (ESA) consisting of 2 solvent delivery modules (Model 582), a refrigerated autosampler (Model 542) maintained at 4°C, and a 4-channel coulometric analytical cell (Model 6210). The injected sample was separated under isocratic conditions (0.6 mL/min) on a Luna C18(2) separation column (150 mm × 3 mm i.d., 3 μm; Phenomenex) and detected at potential settings of 150, 250, 350, and 450 mV. The filtered and degassed mobile phase consisted of 98:2 methanol:water containing 10 mmol/L lithium perchlorate. The lower limit of detection for the tocopherols was ~50 fmol injected onto the column. Identities of α- and γ-tocopherol were confirmed by matching peak retention times and multi-channel electrochemical responses with those of purified

standards. Concentrations of α- and γ-tocopherol standards dissolved in ethanol were determined spectrophotometrically using the following molar absorption coefficients (32): $\varepsilon^{292\ nm}$ = α-tocopherol, 3270 $L \cdot mol^{-1} \cdot cm^{-1}$; γ-tocopherol, $\varepsilon^{298\ nm} = 3810\ L \cdot mol^{-1} \cdot cm^{-1}$.

Hepatic vitamin C and uric acid. Immediately after the mice were killed, a portion of liver (~100 mg) was homogenized (PowerGen Model 1000; Fisher Scientific) in 10 volumes of ice-cold PBS containing 1 mmol/L DTPA. An aliquot of the homogenate was retained for protein determination (33) and the remainder was thoroughly mixed 1:1 with ice-cold 10% perchloric acid (v:v) containing 1 mmol/L DTPA. The sample was centrifuged (13,000 × *g*; 5 min, 4°C). The acidified supernatant was collected and stored at −80°C until analyzed. Ascorbic acid and uric acid were measured by HPLC-Coularray as described with minor modifications (34,35). The acidified supernatant (20 μL) was mixed with 20 μL 1 mmol/L DTPA prepared in PBS and 148 μL mobile phase [40 mmol/L sodium acetate, 7.5% methanol (v:v), 0.5 mmol/L DTPA, 0.5 mol/L Q12 and 12 μL 2.58 mol/L potassium phosphate buffer, pH 9.8]. The sample was injected onto the HPLC-Coularray and separated under isocratic conditions (1 mL/min) on a C_8 separation column (Supelco Supelcosil LC-8; 150-mm × 4.6-mm i.d. 3 μm) and detected with potential settings of 150, 275, 400, and 525 mV. Analyte identity was confirmed by matching peak retention times and multi-channel electrochemical responses with those of authentic standards. Lower limits of detection for ascorbic acid and uric acid were ~0.5 pmol, each detected off the column. Ascorbic acid standard was prepared fresh daily and the concentration was determined spectrophotometrically using the molar absorption coefficient $\varepsilon^{265\ nm} = 14{,}500\ L \cdot mol^{-1} \cdot cm^{-1}$.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (Version 4.03; GraphPad Software) on untransformed data. Data are expressed as means ± SD throughout the text and figures. Initial lean and obese mouse body weights were compared using the Student's *t* test. Two-way ANOVA was used to evaluate genotype, GTE, and their interaction for most statistical analyses. Bonferroni's post-test was used to evaluate pair-wise differences of the interaction or GTE effect, as appropriate. Specific GTE effects within a genotype are illustrated in the figures and tables using superscripts (a > b > c) as appropriate. For the evaluation of hepatic steatosis scores, the nonparametric Kruskal-Wallis test with Dunn's multiple comparison post-test was used to determine effects attributed to genotype and specific GTE treatment effects within a genotype. Regression analysis was used to evaluate associations between data variables. Results for all analyses were considered to significant at $P < 0.05$.

Results

Mouse body weights and dietary intakes. At study onset (d 0), 5-wk-old *ob/ob* mice weighed 34% more ($P < 0.05$) than their lean littermates, consistent with their phenotypic propensity to develop obesity at an early age (**Fig. 1**). As expected, obese mice (*ob/ob*) weighed more ($P < 0.05$) than lean mice at the conclusion of the study (**Table 1**). Lean and obese mice fed GTE weighed less ($P < 0.05$) than their respective controls. A significant interaction between diet and genotype ($P < 0.05$) was observed in which GTE affected the body weight of obese mice more substantially than lean littermates. Lean mice fed GTE at 1 and 2% weighed 11% ($P < 0.05$) and 20% ($P < 0.05$) less, respectively, compared with mice fed no GTE. Obese mice fed GTE at either dose weighed 23–25% less ($P < 0.05$) than obese control mice. Also, the body weight of obese mice fed 2% GTE did not differ from lean controls fed no GTE. Body weights did not differ between groups fed 1 and 2% GTE, regardless of the genotype. Likewise, adipose mass in obese mice was greater ($P < 0.05$) compared with lean mice and the 2 levels of GTE reduced ($P < 0.05$) adipose weight to a similar extent in obese mice only.

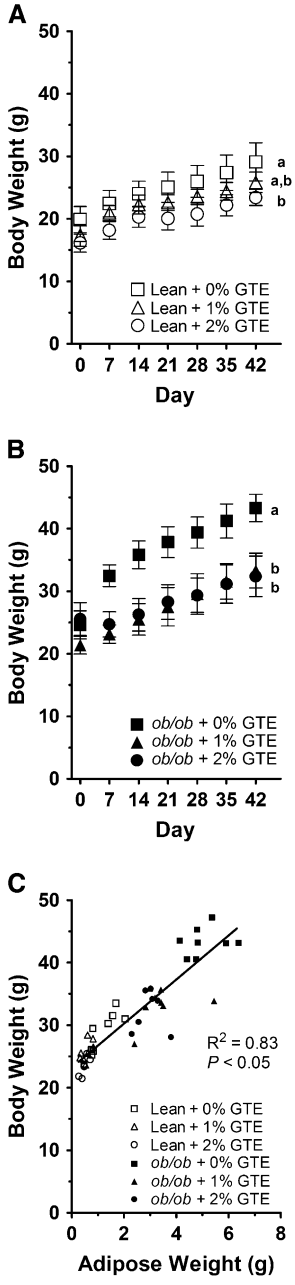


FIGURE 1 Weekly body weight of lean (*A*) and obese (*B*) mice fed 0, 1, or 2% GTE for 6 wk and the relationship between body and adipose weights in lean and obese mice (*C*). Values are means ± SD, $n = 8$. Final body weights were affected, $P < 0.05$, by genotype, GTE, and genotype × GTE. Within a genotype, labeled means without a common letter differ, $P < 0.05$.

Adipose and body weight were correlated ($R^2 = 0.83$; $P < 0.05$; Fig. 1C), suggesting that the GTE-mediated reduction in body weight was attributed, in large part, to the substantial reduction in adipose mass. Despite the GTE-mediated changes in body weight and adipose weight, food intakes were not affected by genotype or GTE treatment throughout the study period (Table 1).

Hepatic histology. Histologic evaluation is regarded as the "gold standard" approach to evaluate the presence and severity of NAFLD (36). Thus, we evaluated liver sections histologically to assess the extent to which GTE attenuated the development of

TABLE 1 Body composition, food intake, and serum adiponectin in lean and obese mice fed 0, 1, or 2% GTE for 6 wk[1]

	Lean			Obese			*P-value*		
	0% GTE	1% GTE	2% GTE	0% GTE	1% GTE	2% GTE	Genotype	GTE	Genotype × GTE
Final body weight, *g*	29.1 ± 3.1[a]	25.8 ± 1.6[a,b]	23.4 ± 1.3[b]	43.3 ± 2.2[a]	33.3 ± 2.8[b]	32.4 ± 3.3[b]	<0.05	<0.05	<0.05
Daily food intake[2], *g/d*	5.5 ± 1.5	5.9 ± 0.5	5.8 ± 0.3	5.5 ± 0.5	4.9 ± 0.7	5.4 ± 0.2	NS[3]	NS	NS
Total adipose[4], *g*	1.24 ± 0.51	0.57 ± 0.19	0.49 ± 0.13	5.07 ± 0.76[a]	3.40 ± 0.90[b]	2.98 ± 0.49[b]	<0.05	<0.05	<0.05
Epididymal adipose, *g*	0.96 ± 0.40	0.49 ± 0.16	0.42 ± 0.12	3.73 ± 0.75[a]	2.58 ± 0.95[b]	2.25 ± 0.57[b]	<0.05	<0.05	<0.05
Retroperitoneal adipose, *g*	0.28 ± 0.14	0.08 ± 0.05	0.07 ± 0.03	1.34 ± 0.52[a]	0.82 ± 0.22[b]	0.73 ± 0.22[b]	<0.05	<0.05	<0.05
Serum adiponectin, *mg/L*	13.1 ± 0.7	12.7 ± 0.8	13.2 ± 1.5	9.0 ± 0.8	8.4 ± 1.7	8.0 ± 0.7	<0.05	NS	NS

[1] Values are means ± SD, $n = 8$. Within a genotype, means in a row without a common letter differ, $P < 0.05$.
[2] Food intakes were compared using the overall mean daily food intake from each mouse throughout the investigation.
[3] NS, $P \geq 0.05$.
[4] Total adipose is the sum of epididymal and retroperitoneal adipose weights.

hepatic steatosis. Lean mice exhibited little or no histologic evidence of hepatic steatosis (**Fig. 2***A*; **Table 2**) and GTE had no noticeable effect on liver histology in the lean littermates. In contrast, severe steatosis was observed in obese mice fed no GTE (Fig. 2*B*; $P < 0.05$) compared with lean littermates. A marked reduction ($P < 0.05$) in the degree of steatosis was noted in the livers from obese mice fed GTE (Fig. 2*C*,*D*), but there was no difference between the groups fed the 2 levels of GTE (Table 2). Whereas hepatic steatosis scores were lower in the obese mice fed GTE at both levels, 4 of 16 obese mice responded maximally to GTE, resulting in grade 1 histologic scores and, in most of these, the effect was dramatic (Fig. 2*C*). Moreover, histologic steatosis scores and body weight were correlated ($R^2 = 0.65$; $P < 0.05$), suggesting the importance of body weight regulation on the development of hepatic steatosis. No histologic evidence of hepatotoxicity was observed in lean or obese mice. Scattered necrotic cells were observed in 2–5 mice/group, but there were no effects of genotype or GTE treatment. Likewise, hepatic inflammation was absent or minimal in all mice and there was no histologic evidence of fibrosis or any visible evidence of GTE altering any of these variables. For the other histologic parameters assessed, such as fibrosis, Mallory bodies, Councilman bodies, billiary hyperplasia, degree of variation in nuclear size, and necrosis, no recognizable effects of genotype or diet were found.

Hepatic and serum lipid concentrations. Consistent with histologic assessment, obese mice had greater ($P < 0.05$) hepatic total lipid concentrations than lean littermates (**Fig. 3***A*). GTE decreased the hepatic lipid of obese mice dose dependently ($P < 0.05$); the concentration was 22% ($P < 0.05$) and 40% ($P < 0.05$) lower in obese mice fed GTE at 1 and 2%, respectively, compared with the obese mice fed no GTE. Hepatic total lipid levels were correlated with body weight ($R^2 = 0.59$; $P < 0.05$) as well as histologic steatosis scores ($R^2 = 0.70$; $P < 0.05$), confirming the protective effect of GTE against the development of hepatic steatosis.

The hepatic triglyceride concentration was greater ($P < 0.05$) in obese mice than in lean littermates (Fig. 3*B*). It was 20–35% lower ($P < 0.05$) in obese mice fed GTE than in the obese controls but did not differ between mice fed the 2 levels of GTE. However, hepatic concentrations of total lipids and triglycerides were correlated ($R^2 = 0.88$; $P < 0.05$), as were hepatic triglycerides and hepatic steatosis scores ($R^2 = 0.73$; $P < 0.05$). Thus, the data suggest that hepatic triglyceride is predictive of hepatic lipid accumulation and the severity of hepatic steatosis. Hepatic cholesterol was significantly higher (Fig. 3*C*) in the livers of obese mice compared with lean littermates. However, GTE did not affect hepatic cholesterol in obese or lean mice.

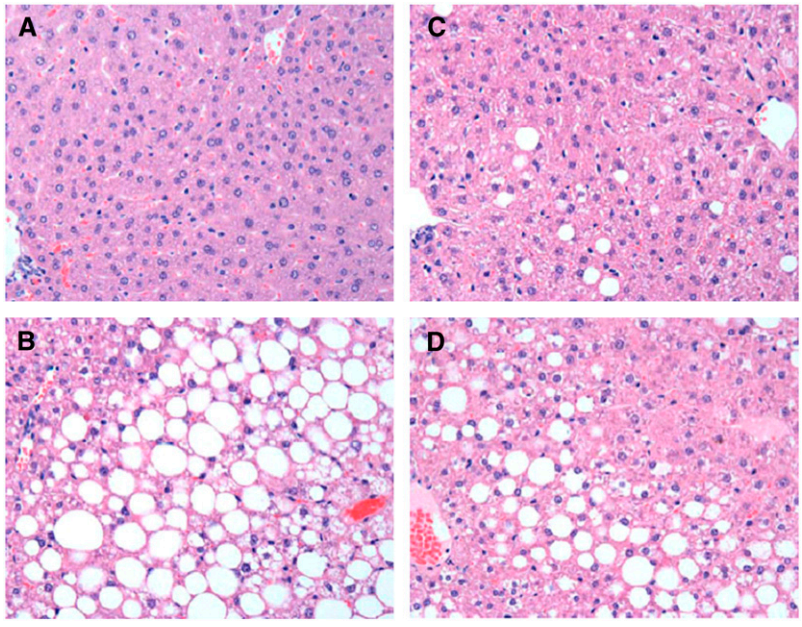


FIGURE 2 Histologic evaluation of hepatic steatosis in lean (*A*) and obese (*B–D*) mice fed 0, 1, or 2% GTE for 6 wk. Representative liver sections (original magnification ×200) from lean and obese mice receiving GTE are illustrated. (*A*) Liver section from a lean mouse fed no GTE. In the lean mice, GTE at 1 or 2% had no visible effect (not shown). (*B*) Obese mouse fed no GTE illustrates the severity of hepatic steatosis. (*C*) Maximal reduction in hepatic steatosis in obese mice fed GTE at 1 or 2%. (*D*) Liver section from an obese mouse fed 1% GTE illustrating the representative reduction in hepatic steatosis of obese mice when fed GTE at either level.

TABLE 2 Hepatic steatosis grading in obese and lean mice fed 0, 1, or 2% GTE for 6 wk[1]

	0% GTE	1% GTE	2% GTE	*P*-value
Lean	0.4 ± 0.5	0.3 ± 0.5	0.3 ± 0.7	NS[2]
Obese	3.0 ± 0.0[a]	2.0 ± 0.9[b]	2.1 ± 0.6[b]	<0.05

[1] Values are means ± SD, $n = 8$. Within a genotype, means in a row without a common letter differ, $P < 0.05$. Hepatic steatosis scores were affected, $P < 0.05$, by genotype.
[2] NS, $P \geq 0.05$.

The serum total cholesterol concentration of lean mice (2.53 ± 0.39 mmol/L) was unaffected by GTE, whereas obese mice fed GTE at 1 and 2% had 39–42% lower ($P < 0.05$) concentrations than the obese controls. The serum cholesterol concentration of obese mice fed no GTE (5.81 ± 1.58 mmol/L) was more than twice ($P < 0.05$) that of lean mice (2.53 ± 0.39 mmol/L). In obese mice, those fed GTE at 2% (0.49 ± 0.12 mmol/L), but not at 1%, had a lower ($P < 0.05$) serum triglyceride concentration than the obese controls fed no GTE (0.71 ± 0.09 mmol/L), whereas GTE did not affect serum triglyceride concentration in lean mice (0.52 ± 0.11 mmol/L). The serum triglyceride concentration in obese mice fed 2% GTE did not differ from that of lean mice, indicating that GTE at 2% normalized the serum level of triglyceride to the lean control level.

Serum markers of hepatic injury. Serum activities of hepatic enzymes are often elevated among obese individuals with NAFLD (37). Obese mice had significantly higher serum ALT, AST, and ALP activities than lean mice (**Fig. 4**). Among mice fed no GTE, obese mice had 3.6-times higher ALT, 2.3-times higher AST, and 1.6-times higher ALP activity than lean mice, consistent with the presence of severe steatosis (Fig. 2*B*; Table 2). In lean mice, these serum enzyme activities were unaffected by GTE. However, obese mice provided GTE had ALT and AST activities that were 30–41% ($P < 0.05$) and 22–33% ($P < 0.05$) lower, respectively, compared with obese controls. Serum ALT ($R^2 = 0.57$) and AST ($R^2 = 0.63$) activities were correlated ($P < 0.05$) with hepatic lipid concentrations, suggesting predictive value of these aminotransferases for hepatic steatosis severity in this experimental model of obesity-mediated fatty liver disease.

Hepatic antioxidants and serum adiponectin. We assessed hepatic vitamin E, ascorbic acid, and uric acid as well as serum adiponectin to better define the role of oxidative stress during the development of hepatic steatosis on these variables. Hepatic γ-tocopherol was below detection limits (<3:1 signal noise ratio), consistent with the omission of γ-tocopherol from the vitamin mix of the test diet and the use of tocopherol-stripped oil, which enabled a more precise regulation of the dietary vitamin E content. However, obesity strikingly increased ($P < 0.05$) the accumulation of hepatic α-tocopherol by >2-fold and 2% GTE decreased hepatic α-tocopherol by nearly 40% ($P < 0.05$) compared with obese controls (**Fig. 5***A*). Due to the substantial reduction in hepatic steatosis among obese mice fed GTE (Fig. 2; Table 2), hepatic α-tocopherol was normalized to hepatic lipid. Hepatic α-tocopherol remained significantly higher among obese mice compared with lean littermates (Fig. 5*B*). GTE tended ($P = 0.06$) to reduce hepatic α-tocopherol in lean and obese mice, suggesting that the GTE-mediated decline in hepatic α-tocopherol may not be entirely due to its lipid-lowering effect on the liver, because the lipid concentration of lean mice was unaffected by GTE (Fig. 3). Hepatic ascorbic acid was ~18% lower ($P < 0.05$) among obese mice (8.8 ± 1.8 nmol/mg protein) than in lean mice (10.5 ± 2.3 nmol/mg protein). GTE did not affect hepatic ascorbic acid in either lean or obese mice. Uric acid was not affected by obesity (lean, 4.1 ± 0.7 vs. obese, 4.5 ± 1.0 nmol/mg protein) or GTE (Fig. 5*D*). Serum adiponectin was lower ($P < 0.05$) in obese mice than in lean mice and was not affected by GTE in lean or obese mice (Table 1).

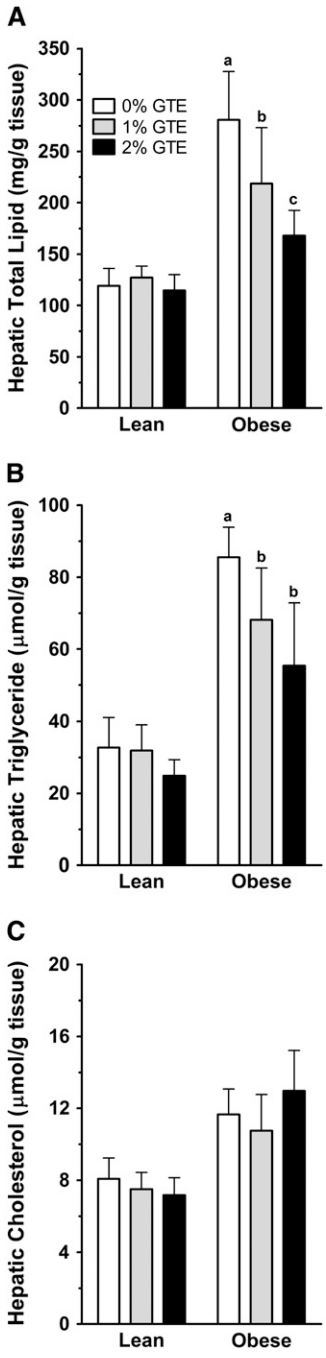
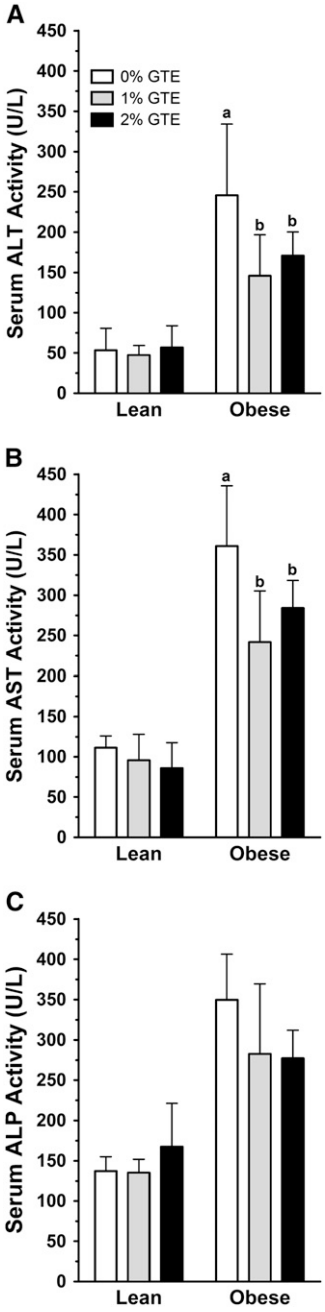


FIGURE 3 Concentrations of hepatic total lipid (*A*), triglyceride (*B*), and cholesterol (*C*) in lean and obese mice fed 0, 1, or 2% GTE for 6 wk. Values are means ± SD, n = 8. Hepatic total lipid and triglyceride were affected, $P < 0.05$, by genotype, GTE, and genotype × GTE. Hepatic cholesterol was affected, $P < 0.05$, by genotype only. Within a genotype, labeled means without a common letter differ, $P < 0.05$.



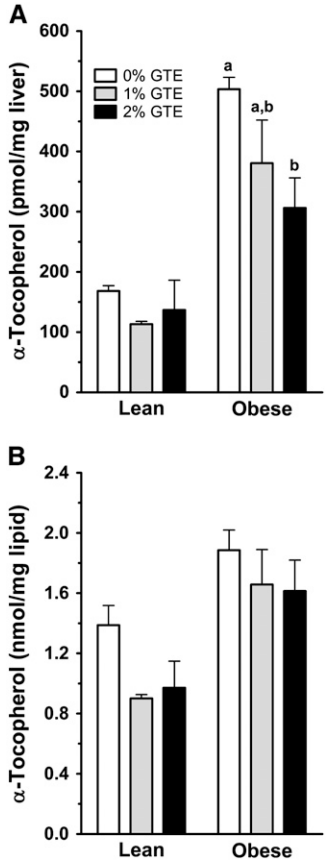


FIGURE 4 Serum activities of ALT (*A*), AST (*B*), and ALP (*C*) measured from nonhemolyzed serum obtained from lean and obese mice fed 0, 1, or 2% GTE for 6 wk. Values are means ± SD, $n = 6$–8. Serum ALT and AST were affected, $P < 0.05$, by genotype, GTE, and genotype × GTE. Serum ALP was affected, $P < 0.05$, by genotype only. Within a genotype, labeled means without a common letter differ, $P < 0.05$.

FIGURE 5 Hepatic α-tocopherol, normalized to liver weight (*A*) and hepatic lipid (*B*), from lean and obese mice fed 0, 1, or 2% GTE for 6 wk. Data are means ± SD, $n = 8$. Hepatic α-tocopherol, expressed per tissue weight, was affected, $P < 0.05$, by genotype and GTE. Hepatic α-tocopherol, expressed per hepatic lipid weight, was affected, $P < 0.05$, by genotype. Within a genotype, labeled means without a common letter differ, $P < 0.05$.

Discussion

This study provides evidence that dietary supplementation of GTE protects against the development of hepatic steatosis and injury in obese (*ob/ob*) mice. Histologic evidence clearly showed that 6-wk feeding of GTE at 1 and 2% reduced macrovesicular hepatic steatosis. Moreover, direct measurement of hepatic lipid and triglyceride indicated that obese mice fed GTE had significant reductions in these variables, which were correlated with histologic steatosis scores. Also, the reductions in hepatic lipid and triglyceride were accompanied by significant improvements in obesity-induced hepatic injury as assessed by serum aminotransferase activities. The GTE-mediated amelioration of hepatic steatosis and injury were not associated with hepatic antioxidants or serum adiponectin. Thus, the findings from this investigation suggest that the protective effect of GTE against hepatic steatosis and hepatic injury is likely due to its inhibitory effect on hepatic lipid accumulation but not to changes in hepatic antioxidants or serum adiponectin.

To the best of our knowledge, our study is the first to examine the protective effect of GTE on the development of hepatic steatosis in *ob/ob* mice. At present, however, the specific mechanism(s) by which GTE protects against hepatic steatosis and injury is yet to be defined. There are several likely possibilities based on evidence available from our work and other studies. Considerable evidence exists that green tea inhibits intestinal lipid absorption (17–19,38–40). Previously, green tea has been shown to lower the lymphatic recovery of lipids, including triglyceride, cholesterol, and α-tocopherol (18), after duodenal infusion of these lipids in mesenteric lymph-cannulated rats. The potential mechanism may involve the inhibition of pancreatic lipolytic enzymes such as lipase and phospholipase A_2 by green tea catechins, particularly EGCG (17). Thus, it is probable that the decreased hepatic lipid accumulation may be

associated partly with the decreased absorption of lipids at the intestinal level.

Enhanced de novo lipogenesis in the liver and adipose tissue has been well documented in *ob/ob* mice (41–43). Thus, a reduction in de novo lipogenesis is another possible explanation for the protective effects of GTE against the development of hepatic steatosis. In *ob/ob* mice, the expression of sterol regulatory binding protein-1c and its responsive genes, including fatty acid synthase and acetyl-CoA carboxylase, are increased (44,45). Fiorini et al. (23) showed that oral or intraperitoneal administration of EGCG in *ob/ob* mice decreased hepatic steatosis, which was accompanied by decreased hepatic de novo lipogenesis. Also, a recent study by Koo et al. (46) demonstrated that dietary GTE at 0.5 and 1% significantly lowered hepatic and plasma triglyceride and decreased hepatic sterol regulatory binding protein-1c, fatty acid synthase, stearoyl-CoA desaturase 1, and hydroxy-methylglutaryl-CoA reductase messenger RNA (mRNA) in ovariectomized rats fed a diet high in fructose, which is a diet-induced model of fatty liver.

Another line of evidence suggests that green tea increases energy expenditure (47,48), which would also support the protective effects of GTE on hepatic steatosis in this investigation. GTE increases sympathetic nervous system activity and enhances thermogenesis by stimulating the release of norepinephrine (NE), decreasing NE degradation through the inhibition of catechol-*O*-methyl-transferase, and reducing phosphodiesterase-mediated degradation of cAMP, which may be attributed to caffeine found in green tea (49,50). The thermogenic effect of green tea is supported by studies indicating that green tea catechins, particularly EGCG, inhibit catechol-*O*-methyl-transferase activity in liver cytosol (51). Also, an acute intervention in humans indicated that green tea increases 24-h energy expenditure, consistent with increased thermogenesis (52). Interestingly, *ob/ob* mice have decreased NE that inhibits adipocyte lipolysis and favors lipid storage (53). Although the impact of green tea or its catechins on NE in *ob/ob* mice has yet to be investigated, *ob/ob* mice treated with NE results in restored hepatic natural killer T cell population, decreased pro-inflammatory cytokines, and protects against lipopolysaccharide-induced steatohepatitis (54).

Evidence also suggests that green tea or its catechins stimulate fatty acid oxidation (47). In support of this, green tea catechins have been shown to increase hepatic β-oxidation in mice fed high-fat diets and this was accompanied by increased hepatic acyl-CoA oxidase and medium chain acyl-CoA dehydrogenase mRNA expression (55). Also, EGCG increases hepatic uncoupling protein-2 and skeletal muscle uncoupling protein-3 mRNA levels in mice fed high-fat diets (56), whereas Murase et al. (57) demonstrated that GTE increases skeletal muscle β-oxidation and fatty acid translocase mRNA levels in mice.

We have also considered the possibility that the effect of GTE may be mediated by affecting food intake, because a reduction in food intake would be expected to significantly affect body weight and hence hepatic steatosis. In this study, however, food intake did not differ between the groups. This provides additional support for the protective mechanisms of GTE against hepatic steatosis independent of food intake. Although unlikely, the use of powdered diets may have limited our ability to detect subtle differences in food intake due to potential differences in spillage among the groups. Nonetheless, future studies in this area should more precisely monitor food intake to potentially detect subtle changes in food intake and markers of hepatic steatosis.

Collectively, the above-cited observations indicate that green tea or its catechins decreases intestinal lipid absorption, lipogenesis, and fat storage while promoting fat utilization for energy. Thus, these potential mechanisms, acting individually or synergistically, may provide a possible explanation for the observed GTE-mediated reductions in hepatic steatosis, hepatic lipid, and body weight. It is likely that the reduction in hepatic injury, as assessed by serum aminotransferases, occurred as a result the lipid-lowering actions of GTE. This is supported by our observation that serum ALT and AST are significantly correlated with hepatic lipid. In this study, obese mice fed GTE had 23–25% lower body weight than the obese controls, which was accompanied by improved serum ALT and AST activities. Although these observations in themselves are significant, further studies are needed to more precisely define the requisite of weight loss and the reduction of hepatic lipid deposition on serum hepatic injury markers.

The polyphenolic catechins found in green tea, particularly EGCG, exhibit antioxidant activity (16). Because oxidative stress is implicated in the pathogenesis of NAFLD (10,58), we measured specific hepatic antioxidants to determine whether GTE regulates their concentrations during the development of hepatic steatosis. Contrary to our hypothesis, GTE did not improve hepatic ascorbic acid, uric acid, or α-tocopherol. In support of an antioxidant effect, EGCG supplementation in a diet-induced model of NAFLD increases hepatic glutathione with a simultaneous reduction in hepatic lipid peroxidation (59), suggesting that the antioxidant effects of GTE may be mediated partly by hepatic total glutathione status. Continued work in this area should consider assessing the extent to which GTE affects reduced and oxidized glutathione concentrations to more comprehensively investigate its impact on hepatic oxidative stress and lipid metabolism. Of particular interest from this study, obese mice had significantly greater hepatic α-tocopherol than lean littermates, suggesting that hepatic steatosis may "trap" α-tocopherol in hepatic lipid droplets, rendering it unavailable to protect cellular phospholipid membranes from free radical-mediated oxidation consistent with its antioxidant function (30,34). Also, GTE tended ($P = 0.06$) to decrease hepatic α-tocopherol in lean and obese mice, which could not be fully explained by the lipid-lowering effects of GTE. This suggests that GTE may decrease hepatic α-tocopherol by inhibiting its intestinal absorption (17–19) or by activating xenobiotic pathways such as tocopherol metabolism to its physiological metabolite, carboxyethyl-hydroxychroman (60,61).

Adiponectin, an adipokine involved in glucose and fatty acid metabolism as well as insulin resistance, is inversely related to inflammation and obesity (62). Thus, it was not surprising that obese mice had lower serum adiponectin than lean mice in this study. However, we expected that the GTE-mediated body weight reduction would have increased serum adiponectin. In our study, no such relationship was observed in lean or obese mice, suggesting that the effect of GTE is not mediated via a pathway involving adiponectin. Also, leptin is a likely prerequisite to restore plasma adiponectin as was demonstrated in *ob/ob* mice treated with leptin (63).

In conclusion, this study provides evidence that GTE protects against the development of hepatic steatosis and reduces hepatic injury in *ob/ob* mice, a model for NAFLD. This finding suggests that GTE may be used as a potential dietary strategy for preventing NAFLD. This is particularly important because weight loss, the primary treatment strategy for NAFLD, has a poor long-term success rate (12). Clearly, further investigation is warranted to define the mechanisms by which GTE protects against obesity-mediated hepatic steatosis and injury. Future studies should also consider the use of GTE as a dietary therapy

under conditions of preexisting hepatic steatosis as well as to evaluate the extent to which GTE can prevent the transition toward more debilitating forms of NAFLD.

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