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Nanoemulsified green tea extract shows improved hypocholesterolemic effects in C57BL/6 mice

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Abstract

Nanoemulsification of nutrients could improve bioavailability by enhancing intestinal uptake. We investigated the antioxidant and hypolipidemic effects of nanoemulsified green tea extract (NGTE). Antioxidant effect was measured by 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay and dichlorofluorescein diacetate (DCFH-DA) assay. C57BL/6 mice were fed a control high-fat diet, green tea extract (GTE), or NGTE diet for 4 weeks. In composition analysis, GTE and NGTE contained similar total catechin concentrations. The antioxidative effect of GTE was comparable with that of NGTE. In the ABTS assay, GTE had a marked effect, although NGTE was more effective than GTE in the DCFH-DA assay. In the mouse feeding experiment, total and low-density lipoprotein (LDL) cholesterol concentrations were significantly reduced after NGTE treatment in comparison with GTE treatment in high-fat-fed C57BL/6J mice over the course of 4 weeks. The hypocholesterolemic effects were greater in the NGTE group compared with the GTE group (24% vs. 15.4% LDL cholesterol reduction compared with the control). Expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase was significantly down-regulated. Protein expression of LDL receptor was significantly increased in the livers of both the GTE- and NGTE-treated groups (+234.1%, $P<.01$ and +274.7%, $P<.001$), with a greater effect in the NGTE than in the GTE group. Cholesterol 7α-hydroxylase gene expression was similarly increased in both the GTE and NGTE groups. These results suggest that nanoemulsification significantly increased hypocholesterolemic effects of GTE *in vivo* due to increased bioavailability.
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Keywords: Antioxidant; Bioavailability; Green tea; Hypolipidemia; Nanoemulsification

1. Introduction

Green tea has been considered a healthy beverage since ancient times. Traditional East Asian medicine recommends green tea for headaches, body aches and pains, digestion, depression and detoxification and as an energizer. In general, green tea is believed to prolong life [1]. A major component of green tea, (−)-epigallocatechin-3-gallate (EGCG), has several cellular and molecular effects related to the health-promoting actions of tea catechins. Over the last few decades, green tea has been subjected to many scientific and medical studies to determine its potential health benefits, including the possibility of extending the human life span. These studies showed that green tea drinkers are less likely to die from coronary heart disease [2] or stroke [3]. However, the data have been conflicting and inconsistent. In a recent study, consuming six capsules of green tea polyphenols (GTP; 714 mg/day) per day for 3 weeks did not significantly affect cardiovascular risk biomarkers [4]. In addition, no effects were observed on low-density lipoprotein (LDL) oxidation, a major step in atherogenesis, *ex vivo* after intake of green tea or green tea polyphenol isolate [5]. The conflicting results regarding the efficacy of green tea may be due to low bioavailability, as green tea catechins are not readily absorbed by the intestine and a limited amount becomes available to the human body.

One possible way of improving intestinal uptake is to introduce a nanoemulsified capsule. Recently, much attention has focused on developing a new delivery system to improve the bioavailability of compounds, which could result in greater efficacy [6,7]. Nanoemulsions promote enhanced gastrointestinal absorption and reduced inter- and intraindividual variability for a variety of drugs. Additionally, nanoemulsions exhibit excellent drug-release properties due to their very large interfacial area. Furthermore, nanoemulsions may offer a certain degree of protection against degradation or may improve difficult organoleptic properties of the active components [8].

To improve the efficacy of green tea extract (GTE), we produced nanoemulsified green tea extract (NGTE) using niosome technology and compared the antioxidative and hypolipidemic effects of the NGTE with regular GTE. We also investigated the effects of green tea on the expression of key genes involved in cholesterol metabolism, such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase,

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cholesterol 7α-hydroxylase (CYP7A1), LDL receptor and sterol regulatory element binding protein 2 (SREBP-2).

2. Materials and methods

2.1. Preparation of nanosome encapsulated green tea catechins and characterization of the catechin components

Nanosize emulsification of green tea was prepared as follows. Heated mixture A (cholesterol 2.5%, phytosterol 2.5%, Cetech-3 2.0%, Cetech-5 2.0%) and heated mixture B (cetyl phosphate 0.4%, glycerine 7.0%, water 92.6%) were mixed and homogenized at 1500 rpm for 5 min, followed by cooling at 45°C. Then, 10% green tea extract (GTE) and 15% medium chain fatty acid triglyceride (MCT) were added to the mixture before it was sprayed through a high-pressure microemulsifier at 13,500 psi. The niosome sample was analyzed by dynamic light scattering (ELS-Z2; OTSUKA, Tokyo, Japan) at a laser wavelength of 638 nm and a scattering angle of 165°, and field emission scanning electron microscope (LEO supra 55; Carl Zeiss, Oberkochen, Germany) at 1 kV.

Catechin contents of the green tea and niosome samples were analyzed using a high-performance liquid chromatography system equipped with two-solvent delivery systems (515; Waters, Milford, MA, USA), an autosampler (Waters 717) and a photodiode array detector (Waters 2996). A reverse-phase column was used for separation (25 cm×4.6 mm ID CAPCELL PAK C18, 5 μm; Shiseido Fine Chemicals, Tokyo, Japan). Mobile phase A was accomplished with 0.1% acetic acid, mobile phase B was achieved with acetonitrile and gradient elution was performed by varying A and B at 1.0 ml/min of total flow. Catechin and gallic acid were detected at UV 280 nm using 10 μl of each sample. The identification and quantification of compounds were carried out by comparing retention time and peak area from the calibration curves obtained from EGCG, EGC, ECG, EC, caffeine, and gallic acid. All used solutions were filtered through 0.45-μm membranes, and the mobile phase was degassed.

2.2. Measurement of total phenolic compounds and total flavonoid content

The amount of total phenolic compounds in extracts was determined according to the Folin–Ciocalteu procedure [9]. Samples were placed into test tubes, followed by the addition of 1.0 ml of 2 N Folin–Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate. The tubes were mixed and allowed to stand for 30 min. Absorption was measured at 765 nm (UV mini-1240; Shimadzu, Kyoto, Japan). Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material. Flavonoid concentration was determined as follows: the samples (50 μl) were diluted in 80% aqueous ethanol (450 μl), and a 0.5-ml aliquot was added to test tubes containing 0.1 ml of 0.1% aluminum nitrite, 0.1 ml of 1 M potassium acetate, and 4.3 ml of 80% ethanol. After 40 min at room temperature, the absorbance was determined with a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA) at 425 nm. Total flavonoid concentration was calculated using a quercetin standard [10].

2.3. The 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity

Antioxidant activity was measured by a modified 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay described by van den Berg et al. [11]. AAPH 2,2'-azobis[2-amidinopropane]dihydrochloride; (1.0 mM) and ABTS (2.5 mM) were dissolved in 100 mM potassium phosphate-buffered solution (PBS, pH 7.4). The $ABTS^{\cdot-}$ radical solution was heated at 70°C for 30 min and then cooled. After filtration through a 0.45-μm syringe filter, the $ABTS^{\cdot-}$ solution was diluted to an absorbance of 0.65±0.02 at 734 nm. $ABTS^{\cdot-}$ solution (0.98 ml) was mixed with 0.02 ml of sample solution, and the absorbance was measured at 734 nm after 20 min. The PBS solution was used as a blank, and the control consisted of 0.98 ml $ABTS^{\cdot-}$ radical solution and 0.02 ml water.

2.4. Cell culture

HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic–antimycotic and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were subcultured when the cultures were 80%–90% confluent (split ratio 1:6) by trypsinization with 0.05% trypsin–EDTA in a 100-mm tissue culture dish. The medium was refreshed every 2 days.

2.5. Preparation of chemicals and green tea sample solutions

A 25-mM dichlorofluorescein diacetate (DCFH-DA) solution was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. A 40-mM hydrogen peroxide (H_2O_2) stock solution was prepared in sterile water and stored at 4°C. Green tea extract (1–5 mg/ml) was prepared in treatment medium before treatment. Final DMSO concentration in treatment medium was <1%; HepG2 cytotoxicity was not observed at this solvent concentration. All solutions were stored as aliquots prior to use in experiments were and were heated to 37°C before use.

2.6. DCFH-DA assay

Cellular antioxidant activity of green tea samples was determined by DCFH-DA assay [12]. HepG2 cells were seeded into 96-well plates at a density of 3×10^4 cells per well in 100 μl of growth medium and incubated for 24 h. Triplicate wells were treated for 12 h with various concentrations of green tea samples in 120 μl of growth medium. HepG2 cells were then treated with 100 μM H_2O_2 or vehicle for 2 h. Then, cells were treated with 50 μM DCFH-DA for 50 min, and dichlorofluorescein (DCF) formation was quantified at 37°C using a Victor^3 multiplate reader (485 nm excitation, 535 nm emission; PerkinElmer, Waltham, MA, USA). The amount of DCF in control and blank wells was also measured; control wells contained DCFH-DA plus H_2O_2 without sample, and blank wells contained DCFH-DA only.

2.7. Measurement of antioxidant activity

Intercellular antioxidant activity of green tea samples was measured by ABTS and DCFH-DA assays as described above. Sample concentration was adjusted to 18 μmol of GAE and 9 μmol of GAE, respectively. The GTE and NGTE (100 mg) contained 179.1 and 8.6 μmol of GAE, respectively. The antioxidant activity was evaluated by measuring the free radical scavenging activity of GTE, and the absorbance was recorded on a UV/VIS spectrophotometer (model UV 2100, Shimadzu). Sample stock solutions and vitamin C were diluted in distilled water. The free radical solution was prepared before the experiment. The percentage free radical scavenging activity of GTE and vitamin C, a positive control, was calculated, and calibration curves were obtained.

$$\text{Antioxidant activity } (\%) = (100 - \text{As}/\text{Ac}) \times 100,$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

The antioxidant capacity was expressed as mg/100 ml sample solution of vitamin C equivalent antioxidant capacity and was calculated using vitamin C standard curves [13].

2.8. Animals and dietary conditions

Six-week-old male C57BL/6 mice were purchased from Samtako (Osan, Korea) and fed a high-fat diet containing 60% fat (D12492; DooYeol Biotech, Seoul, Korea) for 4 weeks. The mice were then fed a control diet, regular GTE-containing, or NGTE-containing diets for 4 weeks. The compositions of the control and test diets are shown in Supplemental Table 1. Calories from protein, carbohydrate and fat are 20%, 20% and 60%, respectively. Both GTE and NGTE diets replaced 1% of cellulose with either GTE or NGTE, respectively. All mice were maintained on a constant light/dark cycle with food and water ad libitum. All animal procedures were performed according to protocols approved by the Korea University Animal Experimentation and Ethics Committee.

2.9. Analysis of plasma lipid levels

Blood samples were collected retroorbitally at time zero and 4 weeks and analyzed for total cholesterol, high-density lipoprotein (HDL), LDL and triglyceride with an automatic analyzer (Cobas C111; Roche, Basel, Switzerland).

2.10. Isolation of total RNA and real-time polymerase chain reaction

The total RNA was isolated from liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For complementary DNA (cDNA) synthesis, 2 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Mbiotech, Seoul, Korea). Real-time polymerase chain reaction (PCR) was performed after reverse transcription. The primers were designed using published nucleotide sequences for CYP7A1 (F, 5′-CCTTGGACGTTTTCTCGCT and R, 5′-GCGCTCTTTGATTTAGGAAG), HMG-CoA reductase (F, 5′-GTTCTTTCCGTGCTGTGTTCTGGA and R, 5′-CTGATATCTTTAGTGCAGAGTGTGG-CAC) and SREBP2 (F, 5′- TGGGAGAGTTCCCTGATTTG and R, 5′-GATAATGGGACCTGGCT-GAA). β-Actin (F, 5′-TGCTGTC CCTGTATGCCTCT and R, 5′-AGGTCTTTACGGATGTCAACG) transcripts were used as internal controls. Real-time PCR was performed with 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 0.5 μl of each primer (15 μM), 1 μl of cDNA and 10.5 μl sterile water. All real-time PCR reactions were performed in iCyber iQ (Bio-Rad). Data were collected and viewed using the iCyber iQ optical system software (version 3.1; Bio-Rad).

2.11. Western blotting

Liver tissue was lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100 and protease inhibitor cocktail at 4°C. The lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4°C. Protein concentration was determined using a Bio-Rad protein kit with bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard. Equal amounts of protein were boiled in sample buffer (5% β-mercaptoethanol) for 5 min. Samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (0.45 μM Protran Nitrocellulose Transfer Membrane; Schleicher & Schuell BioScience, Dassel, Germany). Nonspecific protein binding sites were blocked by incubating samples in PBS (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. To examine LDL receptor, HMG-CoA reductase levels, and

monoclonal anti-α tubulin expression, the samples were incubated with an anti-LDL receptor, anti-HMG-CoA reductase (rabbit polyclonal IgG) and monoclonal anti-α tubulin (mouse immunoglobulin) antibodies, respectively (1:2000). After washing several times with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with goat antirabbit IgG and goat antimouse IgG (Calbiochem, San Diego, CA, USA), followed by a secondary antibody with H and L chain-specific peroxide conjugate (Sigma). Immunoreactive bands were detected by ECL Western blotting reagents (Amersham Pharmacia, Seoul, Korea) and imaged using ChemiDoc XRS (Bio-Rad). The intensity of protein bands was quantified using Quantity One software (Bio-Rad).

2.12. Statistical analysis

All data are expressed as the mean±SE. Two groups were compared using Student's *t* test. $P<.05$ was considered statistically significant.

3. Results

3.1. Preparation of nanoemulsion particles and composition analysis

A nanosized emulsion belongs to the class of stable emulsions composed of surfactant and oil suspended in water with a particle diameter typically less than 500 nm. It has been suggested that emulsion systems offer an appealing substitute for the formulation of poorly soluble drugs or active compounds [14]. The average size of a nanoemulsion tested in this study was approximately 300 nm in diameter (Fig. 1). Total phenolic content was 104.7 and 87.5 mg/ml for GTE and NGTE, respectively, calculated using a gallic acid standard curve (R^2=0.9748; Table 1). In addition, using the standard curve generated by quercetin (R^2=0.9992), the total flavonoid content of GTEs varied, with means of 38.48±0.01 and 42.98±0.08 mg/ml for GTE and NGTE, respectively (Table 1). Although total phenolic and flavonoid content was lower in NGTE compared with GTE, total catechin content was similar between NGTE and GTE. (−)-Epigallocatechin-3-gallate was most abundant, followed by EGC and GCG, in both types of green tea samples, and no significant differences in the total catechin content and distribution of catechins were found between GTE and NGTE (Table 2).

3.2. Antioxidant activity of GTEs

When the antioxidant activity of two different forms of GTE was measured by ABTS assay, the radical scavenging activities of both GTE and NGTE correlated with the concentrations of phenolic compounds (r^2>0.99). Several studies have detailed the correlation between polyphenol content and antioxidant activity [15,16]. Although the treated samples contained the same amount of GAE, the antioxidant activity of NGTE was lower than that of GTE in the ABTS assay (Fig. 2). However, NGTE significantly inhibited cellular oxidative stress to a greater degree than GTE in the DCFH-DA assay (Fig. 3). Dose-dependent effects on cellular antioxidant activity using the HepG2 cell line were observed up to 5 mg/ml.

Fig. 1. Scanning electron microscopy of nanoemulsified green tea extract.

Table 1
Total phenolic compound and flavonoid contents in GTE and NGTE

	Total phenolic compound (mg/ml)	Total flavonoid (mg/ml)
GTE	104.70±0.02	38.48±0.01
NGTE	87.50±0.06	42.98±0.08

Data are expressed as the mean±S.D. (n=3).

3.3. Plasma lipid levels in C57BL/6 mice

The *in vivo* hypolipidemic effects of both GTE and NGTE were studied in C57BL/6 mice. After 4 weeks of NGTE feeding, the mice had lower plasma total and LDL cholesterol levels compared with the levels at 0 week, and the reduction in NGTE group was significantly greater than the levels in GTE group (Fig. 4A). Total cholesterol was reduced by 10.91% and 25.84% for the GTE-fed and NGTE-fed groups, respectively, compared with control group at 4 weeks. The LDL cholesterol level showed a similar trend; the reduction in NGTE-treated group was significant after 4 weeks (Fig. 4B). Although triglyceride and HDL cholesterol levels were slightly reduced, the difference was not significant between the NGTE and control groups (Fig. 4C, D). The HDL/LDL cholesterol ratio decreased in the GTE group, but the ratio in the NGTE group was significantly higher than that of the GTE and slightly higher than that of the control group (Fig. 4E; 3.65, 2.82 and 3.84 for the control, GTE and NGTE, respectively). The HDL/total cholesterol ratio was similar between these groups.

3.4. Effects of GTE and NGTE on the expression of genes related to key cholesterol metabolism

To determine the effects of nanoencapsulated green tea extract, we further analyzed cholesterol-related gene expression in mouse liver after administration of GTEs for 4 weeks. Liver HMG-CoA reductase messenger RNA (mRNA) and protein levels were significantly lower in both GTE-treated groups than in the control group (44.36% and 86.54% for GTE and NGTE, respectively), with a greater reduction observed in the NGTE-fed animals than in the GTE-fed group (Fig. 5). The transcriptional and translational levels of CYP7A1 were similar between NGTE- and GTE-treated animals; however, CYP7A1 up-regulation was observed in the NGTE-treated group as compared with the GTE group. CYP7A1 gene expression increased to 71.06% and 76.26% in the GTE and NGTE groups, respectively, and the respective protein levels increased to 85.16% and 94.50%. We also investigated GTE effects on LDL receptor protein levels using α-tubulin as a reference. Western blotting revealed that the expression of LDL receptors in the GTE- and NGTE-treated groups were up-

Table 2
Catechin contents in GTE and NGTE

Catechin composition (mg/100 mg extract)	GTE	NGTE
Catechin	0.88±0.05	0.79±0.08
Epicatechin	0.76±0.03	0.72± 0.02
Epigallocatechin gallate	5.54±0.33	5.43±0.57
Gallocatechin gallate	3.32±0.13	3.27±0.20
Epicatechin gallate	1.33±0.13	1.24±0.09
Epigallocatechin	3.63±0.14	3.58±0.15
Total catechin	15.46	15.03

Data are expressed as the mean±S.D. (n=3).

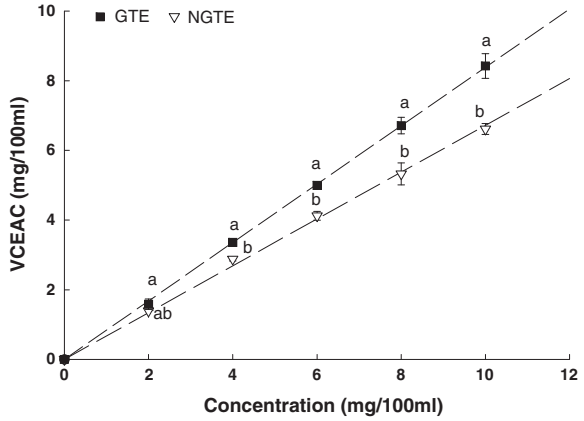

Fig. 2. Vitamin C equivalent antioxidant capacity of NGTE compared with GTE. Error bar=standard deviation (n=3); ▽ NGTE (r^2=0.998); ■ GTE (r^2=0.9998). Data with different letters at the same concentration were statistically different at P<.05.

regulated compared with the control group (i.e., 2.34- and 2.74-fold increase in the GTE and NGTE groups, respectively). Moreover, SREBP2 mRNA expression was also significantly suppressed following the intake of both GTEs (Fig. 6). SREBP2 mRNA expression was suppressed to 62.92% and 92.83% of the control level by GTE and NGTE administration, respectively.

4. Discussion

Green tea is a widely consumed beverage that is frequently used as an Asian traditional medicine. However, its limited absorption in the small intestine results in low bioavailability of the physiologically active compounds. Thus, we developed nanoencapsulated green tea extract with the expectation of enhanced bioavailability compared with normal GTE. The average size of the nanoemulsion tested in this study was approximately 300 nm in diameter, which is well within the range of nanoparticles that may increase bioavailability through enhanced intestinal uptake *in vivo*. Nanoscale emulsification may change the composition of GTE, which could influence its antioxidant activity and related lipid metabolism. Therefore, we compared the composition of GTE and NGTE. The concentration of catechin, the major active compound in GTE, was similar between the GTE and NGTE groups, indicating

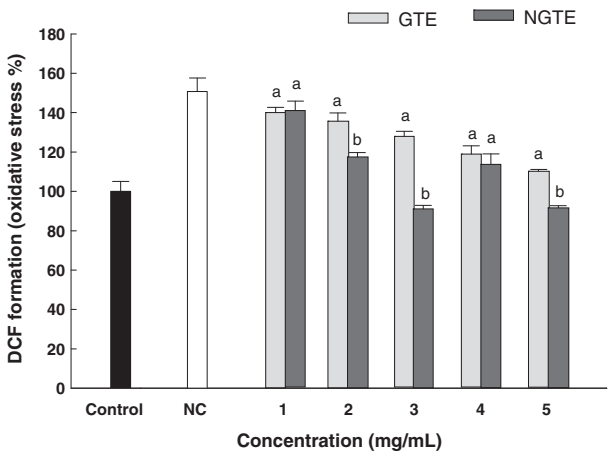

Fig. 3. Comparison of the cellular antioxidant activity of NGTE with GTE. Data are presented as the mean±standard deviation (n=3). Means followed by different superscripts are statistically different in the same treatment dose (P<.05). DCF, dichlorofluorescein.

that the nanoemulsion successfully captured the major active compounds during the emulsification process. Conversely, phenolic compounds and total flavonoid content were lower in NGTE than the levels in GTE.

In the ABTS assay, the antioxidant activity of NGTE was lower than that of GTE. It was speculated that components used in the encapsulation of green tea constituents affected their antioxidant activity. The lower antioxidant activity of NGTE might be due to the slow efflux of green tea constituents across the nanocapsule in the assay condition. However, the cellular antioxidant capacity of NGTE was higher than that of GTE. Sun et al. [17] and Kreuter [18] reported that the poly-DL-lactide nanoparticle was transported into the cell via endocytosis and transcytosis. Greater efficacy of NGTE in the cellular antioxidant assay compared with the ABTS assay might have been due to more efficient endocytosis of encapsulated particles, especially for flavonoids. Effective efflux of green tea ingredients into the cytosol may be possible after endocytosis of green tea nanoemulsions, but the exact mechanism for the nanoemulsion transport through the cell membrane should be further elucidated.

The oxidation of LDL particles in the subendothelial space is one of the major mechanisms for atherosclerotic lesion formation [19]. Oxidized LDL particles have low affinity for the LDL receptor and have remarkably increased affinity for macrophage scavenger receptors, such as MSR-A and CD36, and thus could accelerate foam-cell formation in major blood vessels [20]. The oxidized lipids in LDLs that are trapped in the extracellular matrix of the subendothelial space could activate NFκB-like transcription factors and induce the expression of genes containing NFκB binding sites. The protein products of these genes initiate an inflammatory response that initially leads to the progression of the lesion, which is associated with arterial calcification [21]. The prevention of LDL oxidation by antioxidative nutrients, such as GTE and NGTE, may contribute to reducing LDL oxidation and thus prevent atherogenesis in the major blood vessels.

Furthermore, green tea catechins may improve cholesterol metabolism by altering gene expression associated with the metabolism of hepatic cholesterol. Thus, we further evaluated the molecular basis of the hypolipidemic effects of GTE and NGTE on C57BL/6 mice at the level of gene and protein expression. Plasma cholesterol reduction could be achieved as follows: (a) reduction of dietary cholesterol in the small intestine, (b) increased uptake of plasma LDL by the hepatic LDL receptor, (c) reduction of hepatic cholesterol biosynthesis and (d) induction of hepatic cholesterol degradation by up-regulation of bile acid synthesis pathways where CYP7A1 encodes cholesterol 7 α-hydroxylase, the rate-limiting enzyme in the classic pathway of bile acid synthesis. Thus, we assessed the expression of three key genes in mouse liver, as well as quantified plasma lipid levels.

Indeed, there were multiple significant alterations in plasma lipid levels. Total and LDL cholesterol levels were significantly reduced in both the GTE and NGTE groups, with a greater reduction in the NGTE group (P<.05). This strongly suggests that nanoencapsulation of green tea extract led to more potent hypocholesterolemic effects compared with nascent GTE. Accordingly, we further investigated gene and protein expression in hepatic cholesterol metabolism. Interestingly, HMG-CoA reductase and SREBP2 were significantly down-regulated, while CYP7A1 was up-regulated, in the NGTE-fed group compared with the GTE group. Parallel changes in protein level of HMG-CoA reductase and CYP7A1 were observed in the Western blot analysis.

The reduction in HMG-CoA reductase expression at both the mRNA and protein levels may have lowered hepatic cholesterol biosynthesis, which decreased the level of total cholesterol and LDL cholesterol in plasma after NGTE consumption. 3-Hydroxy-3-methylglutaryl coenzyme A reductase is mainly regulated by three

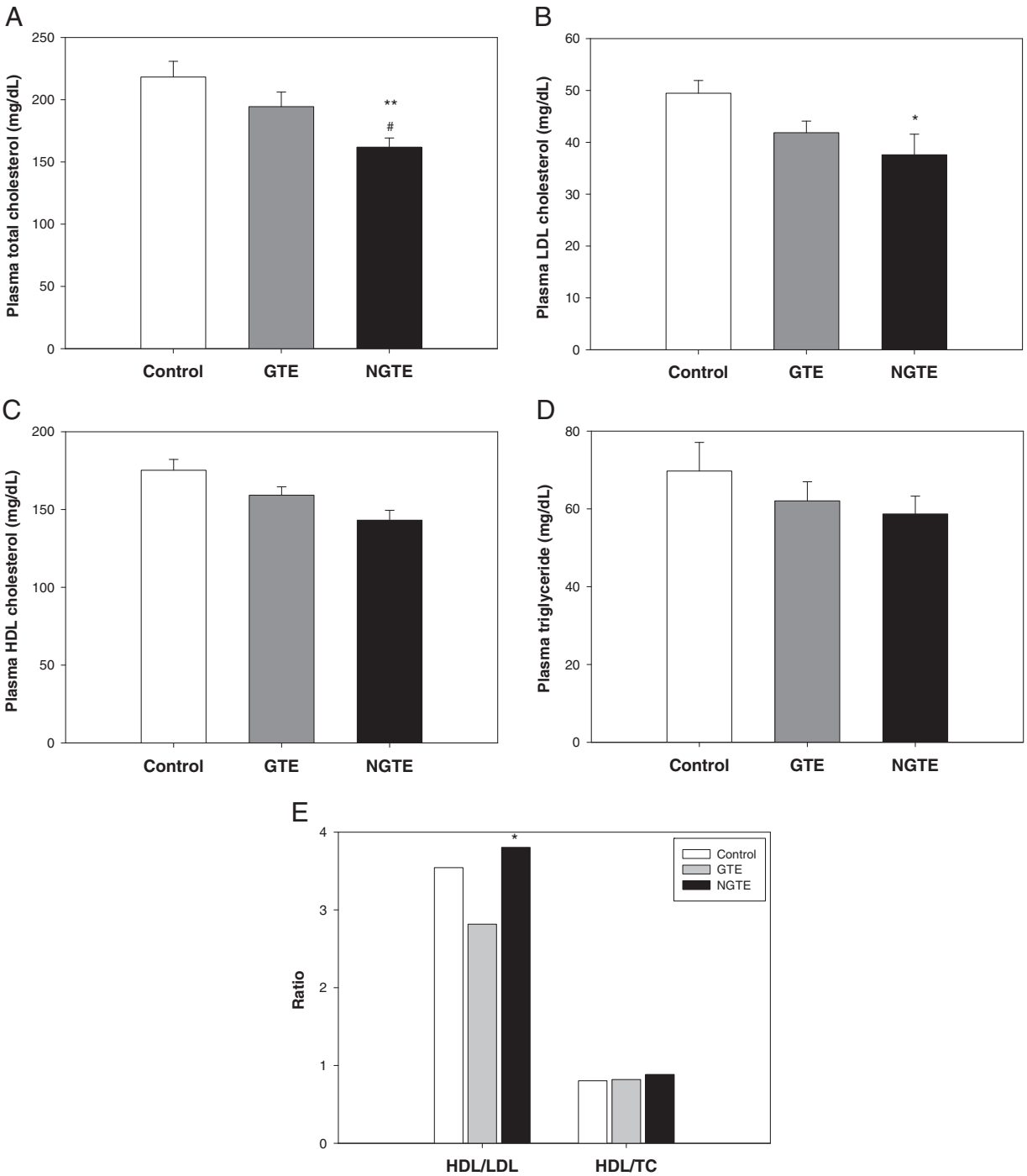

Fig. 4. Effects of GTE and its encapsulation on plasma lipid levels in C57BL/6 mice at baseline and 4 weeks after GTE and NGTE feeding. (A) Total cholesterol, (B) HDL cholesterol, (C) LDL cholesterol, (D) triglyceride levels in plasma. (E) HDL/LDL cholesterol and HDL/total cholesterol ratio. Data are expressed as the mean±standard error (n=5–7). *P<.05 vs. 0 week; **P<.01 vs. 0 week; #P<.05 vs. GTE. TC, total cholesterol.

mechanisms. First, transcriptional regulation is operated by a well-known mechanism with SREBPs. Sterol regulatory element binding protein 2 is a major positive regulator for cellular cholesterol homeostasis, regulating the expression of HMG-CoA reductase gene. Thus, SREBP-2 expression often accompanies that of its target gene, HMG-CoA reductase. Second, HMG-CoA reductase is also regulated posttranscriptionally. Insig-1, an ER membrane protein, has long been known to complex with HMG-CoA reductase and to facilitate ubiquitination of HMG-CoA. This ubiquitin-dependent proteolysis of HMG-CoA reductase is mainly accelerated in response to cellular sterol concentration. Finally, the HMG-CoA reductase activity could be inhibited by PKA-dependent phosphorylation. Our results suggest that green tea catechins dramatically reduced HMG-CoA expression at the transcription level, but reduction of the protein level was ameliorated potentially due to decreased ubiquitin-dependent degradation. In fact, one study showed no significant change in HMG-CoA reductase enzyme activity in rat livers after feeding EGCG [22], suggesting marginal alteration in the protein levels. The mechanism

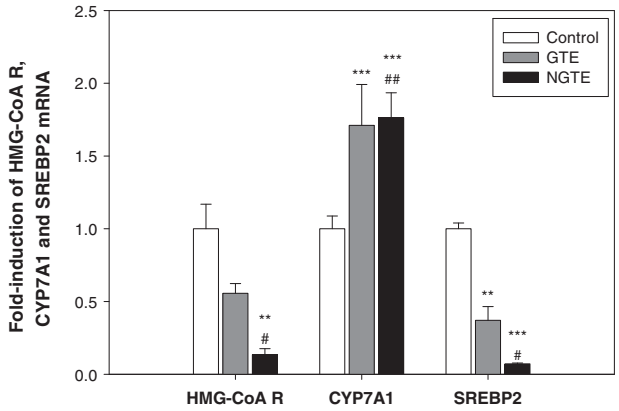

Fig. 5. CYP7A1, HMG-CoA reductase and SREBP2 mRNA expression. Gene expression levels were quantified with quantitative PCR. Data are expressed as the mean±standard deviation (n=3). *P<.05 vs. 0 week; **P<.01 vs. 0 week; ***P<.001 vs. 0 week ; $^{\#}P$<.05 vs. GTE; $^{\#\#}P$<.01 vs. GTE.

behind this finding will be explored in the future. The induction of LDL receptor and cholesterol 7-α hydroxylase may have additional effects.

Moreover, improved hypocholesterolemic effects and improved cholesterol gene and protein expression may be due to enhanced bioavailability of nanoemulsified particles with small micellar size, which is advantageous for intestinal uptake. Partitioning of the GTE particle into the aqueous phase is more likely due to a maximized interfacial area of NGTE. As reported in a previous study [23], smaller particle size seems to play a critical role in maximizing the intestinal area of encapsulated compound into the aqueous phase. These data are in accordance with the evidence reported in the literature [24,25].

In conclusion, we found that the antioxidant capacity of NGTE improved compared with GTE when assessed in cellular antioxidant assays with DCFH-DA, and more importantly, we showed that nanoemulsification significantly improved hypocholesterolemic effects in mice. Total and LDL cholesterol reductions were greater in NGTE-fed mice than in GTE-fed mice. The expression level of LDL receptor and CYP7A1 markedly increased, whereas the expression of HMG-CoA reductase decreased. Future studies are needed to verify the effects of nanoencapsulated green tea extract in humans.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2010.11.015.

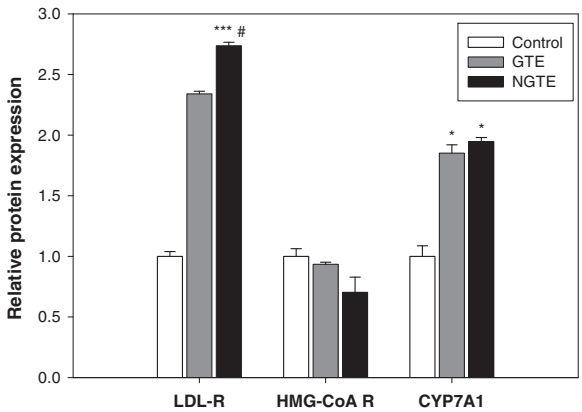

Fig. 6. Protein expression of CYP7A1, HMG-CoA reductase and LDL receptor. Protein expression levels were assessed by immunoblot analysis as described in the Materials and methods section. Data are expressed as the mean±standard deviation (n=3). *P<.05 vs. 0 week; **P<.01 vs. 0 week; $^{\#}P$<.05 vs. GTE.

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