Research Article

The effects of green tea (–)-epigallocatechin-3-gallate on reactive oxygen species in 3T3-L1 preadipocytes and adipocytes depend on the glutathione and 67 kDa laminin receptor pathways

Chih-Ting Wang*, Hsin-Huei Chang*, Chiao-Hsin Hsiao, Meng-Jung Lee, Hui-Chen Ku, Yu-Jung Hu and Yung-Hsi Kao

Department of Life Science, College of Science, National Central University, Chung-Li City, Taoyuan, Taiwan

Green tea (–)-epigallocatechin-3-gallate (EGCG) is known as to regulate obesity and fat cell activity. However, little information is known about the effects of EGCG on oxidative reactive oxygen species (ROS) of fat cells. Using 3T3-L1 preadipocytes and adipocytes, we found that EGCG increased ROS production in dose- and time-dependent manners. The concentration of EGCG that increased ROS levels by 180 – 500% was approximately 50 μM for a range of 8 – 16 h of treatment. In contrast, EGCG dose- and time-dependently decreased the amount of intracellular glutathione (GSH) levels. EGCG was more effective than (–)-epicatechin, (–)-epicatechin-3-gallate, and (–)-epigallocatechin in changing ROS and GSH levels. This suggests a catechin-specific effect. To further examine the relation of GSH to ROS as altered by EGCG, we observed that exposure of preadipocytes and adipocytes to *N*-acetyl-L-cysteine (a GSH precursor) blocked the EGCG-induced increases in ROS levels and decreases in GSH levels. These observations suggest a GSH-dependent effect of EGCG on ROS production. While EGCG was demonstrated to alter levels of ROS and GSH, its signaling was altered by an EGCG receptor (the so-called 67 kDa laminin receptor(67LR)) antiserum, but not by normal rabbit serum. These data suggest that EGCG mediates GSH and ROS levels *via* the 67LR pathway.

Keywords: Epigallocatechin gallate / Fat cell / Glutathione / Laminin receptor / Reactive oxygen species

Received: January 13, 2008; revised: May 4, 2008; accepted: June 8, 2008

1 Introduction

Green tea catechins (GTCs) are polyphenolic flavonoids once called vitamin P [1]. Since the discoveries that they have unique chemical structures and are major ingredients of unfermented tea [2, 3], they have been found to possess widespread biological functions and health benefits [3 – 6]. *In vivo*, GTCs, especially (–)-epigallocatechin-3-gallate (EGCG), lower the incidence of cancers [3 – 6], collagen-induced arthritis [7], oxidative stress-induced neurodegenerative diseases [8, 9], and streptozotocin-induced diabetes [10]. Also, EGCG can reduce body weight and body fat [11 – 13]. This antiobese effect of EGCG was supported by other *in vivo* data that EGCG or EGCG-containing green tea extract reduces food uptake, lipid absorption, and blood triglyceride levels, as well as stimulating energy expenditure, fat oxidation, and fecal lipid excretion [11 – 14]. These *in vivo* observations may be explained by *in vitro* findings that EGCG and caffeine synergistically with norepinephrine stimulate the thermogenesis of brown adipose tissue [15], that EGCG regulates various enzymes related to lipid anabolism and catabolism, such as acetyl-CoA carboxylase, fatty acid synthase, pancreatic lipase, and lipoxygenase

Correspondence: Dr. Yung-Hsi Kao, Department of Life Science, College of Science, National Central University, Chung-Li City, Taoyuan 32054, Taiwan
E-mail: ykao@cc.ncu.edu.tw
Fax: +886-3-4228482

Abbreviations: BSO, L-buthionine-[*S*,*R*]-sulfoximine; **DCFDA,** 2′,7′-dichlorofluorescein diacetate; **EC,** (–)-epicatechin; **ECG,** (–)-epicatechin-3-gallate; **EGC,** (–)-epigallocatechin; **EGCG,** (–)-epigallocatechin-3-gallate; **GAPDH,** glyceraldehydes-3-phosphate dehydrogenase; **γ-GCS,** γ-glutamylcysteine synthetase; **GSH,** glutathione; **GTC,** green tea catechin; **LDH,** lactate dehydrogenase; **67LR,** 67 kDa laminin receptor; **NAC,** *N*-acetyl-L-cysteine; **NRS,** normal rabbit serum; **OPA,** *o*-phthaldialdehyde; **ROS,** reactive oxygen species

* Both the authors contributed equally to this work.

[11 – 13], that EGCG is a potent prooxidant and antioxidant [16, 17], and that EGCG reduced serum- or insulin-induced increases in cell numbers and the triacylglycerol content of 3T3-L1 adipocytes during a 9 days period of differentiation [11]. Decreases in the number of fat cells caused by EGCG may result from its inhibition of mitogenesis of preadipocytes [18] and from its stimulation of apoptosis of preadipocytes [19] and adipocytes [20]. Recent studies have indicated that EGCG downregulates the expression of adipokines [11, 12, 21] and inhibits the adipogenic differentiation [11, 12]. These *in vivo* and *in vitro* observations suggest that green tea EGCG can modulate the mitogenic, developmental, endocrine, and metabolic functions of fat cells.

Despite the importance of EGCG, little is known about the mechanisms of its action in regulating reactive oxygen species (ROS) production of preadipocytes and adipocytes. Although a number of reports have shown that oxidative stress in fat cells can be regulated by vitamin nutrients [22] and herbal products [23], results have not demonstrated whether EGCG regulates adipocyte oxidative stress. In other cell types, 50 μM EGCG induced oxidative stress and radical formation in neuroblastoma cells, while at 20 μM it acted as an antioxidant [16]. Whether high concentrations of EGCG induce oxidative stress production from fat cells is unknown. Because changes in intracellular ROS and glutathione (GSH) levels are used as indicators for oxidative stress [16, 17], an examination of ROS and GSH levels in preadipocytes and adipocytes should help clarify these observations.

Signaling of EGCG in stimulating antioxidant and prooxidant activity in cancer cells and nonfat cells has been extensively described [3 – 6, 8, 9]. Whether any of these actions of EGCG is dependent on its own receptor pathway was not determined in previous studies. An EGCG receptor was discovered in cancer cells [24] and found in normal cells, such as muscle cells, hepatocytes, and nerve cells [25]. This receptor regulates the antimitogenic effect of EGCG on cancer cell growth [24], but is unknown for its modulation of EGCG's action on normal cells. It is evident that certain types of laminin receptors and laminins are present in preadipocytes or adipocytes and are related to fat cell adhesion and differentiation [25 – 27]. However, the results did not identify the nucleotide sequence of the adipocyte 67 kDa laminin receptor (67LR) in fat cells. For this reason, the possible roles of the 67LR in mediating the actions of EGCG on preadipocytes and adipocytes are unknown.

In this study, we used 3T3-L1 preadipocytes and adipocytes to examine the influence and signaling of EGCG on ROS production. We investigated whether EGCG-regulated ROS levels are dependent on the GSH pathway. GSH was chosen because it has been reported to be a vital endogenous antioxidant role in the fat cells [28, 29]. Finally, we investigated whether the 67LR can mediate the effects of EGCG on ROS production.

2 Materials and methods

2.1 Chemical reagents

All materials (*e.g.*, BSA, insulin, GSH, and so forth) were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated. Green tea EGCG and other catechins (>98% pure) were isolated from green tea (*Camellia sinensis*) in our laboratory as described previously [18]. DMEM, calf serum, and FBS were purchased from GIBCO-BRL Life Technologies (New York, NY). A 100-bp DNA ladder marker was purchased from OneStar Biotechnology (Taipei, Taiwan). All serum (normal rabbit serum (NRS)) and antiserum (67LR, actin, and IgG-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 3′-RACE system, Trizol, and *Pfu* polymerase were purchased from Invitrogen Life Science Technologies (Carlsbad, CA, USA).

2.2 Cell culture

According to a published method [18], 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were grown in a DMEM (pH 7.4) containing a 10% calf serum, 100 U/mL of penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air-5% CO_2 at 37°C. Medium was replaced every 2 days. The 3T3-L1 adipocytes were obtained according to a published method by Liu *et al*. [21], in which 2 days postconfluent 3T3-L1 preadipocytes (3×10^6 cells in a 10 cm plate) were treated with DMEM containing a final concentration of 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/mL insulin, and 10% FBS for 48 h. The medium was then changed to DMEM containing FBS and insulin for additional 6 – 10 days. With this protocol, greater than 90% adipocyte differentiation was achieved, as indicated by the phenotypical appearance and triglyceride accumulation.

2.3 Experimental treatments

The 3T3-L1 cells were serum-starved for 16 h in DMEM and then, unless noted otherwise, incubated with or without GTCs (*e.g.*, (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and EGCG) at various concentrations for the indicated time periods. Tea catechins were dissolved in 0.1% DMSO and sterile medium for cell treatment. According to the literature [28, 29], which shows that *N*-acetyl-L-cysteine (NAC) and L-buthionine-[*S*,*R*]-sulfoximine (BSO) are respectively able to stimulate and inhibit GSH levels, we treated serum-starved 3T3-L1 cells with or without EGCG in the presence and absence of either NAC (5 – 10 mM) or BSO (40 μM) for 8 h. In other experiments, we followed the method of Tachibana *et al*. (24) and treated 3T3-L1 cells with either a rabbit polyclonal 67LR antibody (∼5 μg/mL) or preimmu-

nized NRS (as the control) for 1 h, and then stimulated them with or without EGCG (50 μM) for 8 h. After treatment, ROS and GSH levels were measured.

2.4 ROS

We followed the methods reported by Ferrer *et al*. [30] to perform our ROS assay. After the treatment, cells were washed with 10 mM PBS (pH 7.4), and then 30 μM of 2′,7′-dichlorofluorescein diacetate (DCFDA) was added. After a 30 min incubation in the dark at 37°C, cells were washed with PBS to remove free DCFDA from the medium. Dichlorofluorescein produced from the reaction of DCFDA with ROS was detected with the fluorescence emission using a fluorescence spectrophotometer (F-4500, Hitachi) under an excitation wavelength of 504 nm and an emission wavelength of 529 nm. Based on the absorbance value, ROS levels were normalized to the number of cells and then expressed as multoples relative to the control. Cells were trypsinized and counted using the 0.4% trypan blue exclusion method [18]. Only live cells were represented in this study.

2.5 GSH levels

GSH levels were measured essentially as reported by Cohn and Lyle [31]. Harvested cells were lysed in 1 mL buffer containing 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM Na_3VO_4, 0.2% Triton X-100, and 1 mM phenylmethylsulfoneyl fluoride (PMSF). The protein content of the lysates was determined in duplicate by a dye-binding method [32] using BSA (Sigma) as the standard. Assays were performed at 37°C for 20 min in the dark at a final volume of 2.62 mL *per* cuvette containing cell lysates (~10 μg protein), 20 mM sodium phosphate, and 0.004% w/v *o*-phthaldialdehyde (OPA). Because GSH reacts with OPA to produce the conjugated compound called GS–OPA, which can be stimulated with an excitation wavelength of 350 nm and an emission wavelength of 420 nm, GSH levels was determined by measuring the fluorescence emitted from GS–OPA [31]. The reproducible results were obtained in the range of the reduced form of the GSH standard from 0 to 400 ng *per* cuvette ($od_{420\ nm} = 7.7 + 2.4 \times$ ng/cuvette; $r^2 = 0.99$). Based on the standard value, the amount of GSH in a sample was determined and then divided by milligrams of protein. The data are expressed as multiples relative to the control.

2.6 γ-Glutamylcysteine synthetase (γ-GCS) activity

γ-GCS activity was measured as essentially reported by Sekura and Meister [33]. Assays were performed at 37°C for 30 min in a final volume of 80 μL *per* vial containing 30 μL of protein lysate (~50 μg protein) and 50 μL of substrate solution. The final substrate mixture *per* vial contained 10 mM sodium L-glutamate, 10 mM L-aminobutyrate, 20 mM $MgCl_2$, 5 mM Na_2ATP, 2 mM Na_2EDTA, 100 mM Tris-HCl buffer (pH 8.2), and 10 μg BSA. The reaction was terminated by adding 80 μL of 10% trichloroacetic acid. The inorganic phosphate formed was measured from the absorbance at 720 nm by the method of Taussky and Shorr [34]. Reproducible results were obtained in the range of the phosphate standard from 2 to 40 μm *per* well ($od_{720\ nm} = 0.214 + 0.052 \times$ μg/well; $r^2 = 0.96$). One unit of γ-GCS activity was expressed as 1 mg of inorganic phosphate formed *per* hour, and its specific activity was that unit divided by milligrams of protein.

2.7 RNA extraction, oligonucleotide primers, and RT-PCR

Using the RNA miniprep system kit (Viogene, Synnyvale, CA, USA), total RNA was isolated from 3T3-L1 preadipocytes and adipocytes [35]. For the 3'-RACE system, cDNA was synthesized from 5 μg of RNA (a quality ratio of 1.6–2.0 measured at $A_{260\ nm}/A_{280\ nm}$) and 0.1 μg of the 3′-RACE adapter primer (5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′) using M-MLV RT (Stratagene; 40 U) according to the protocol included with the SMART™ 3′-RACE kit (Invitrogen Life Science Technologies). PCR was performed by PfuUltrar^R II DNA polymerase under the following conditions: an initial denaturing cycle at 94°C for 5 min, followed by 25 cycles of amplification consisting of denaturation at 94°C for 40 s, annealing at 61°C for 45 s, and extension at 72°C for 45 s. A final extension at 72°C for 10 min was added after the last cycle. The PCR product was run using 1.5% w/v agarose gel electrophoresis with 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA, visualized using ethidium bromide (0.5 μg/mL) and UV transillumination, and quantified using a Molecular Imager (BioRad Laboratories, Hercules, CA, USA). Gene-specific oligonucleotide primers for amplification of adipocyte 67LR cDNAs were designed according to the conserved region of the human [36] and mouse [37] 67LRs. The forward and reverse primers were 5′-CACAATGTCCGGAGCCCTTGA-3′ and 5′-GCCTTCTCAGCAGCAGCC-3′ for 67LR (accession number NM_011029), and 5′-CCTCTGGAA AGCTGTGGCGT-3′ and 5′-TTGGCAGGTTTCTCAG GCG-3′ for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; accession no. M32599), respectively. The 67LR and GAPDH PCR products, respectively predicted to be 666 and 190 bp, were sequenced by Tri-I Biotech (Taipei, Taiwan). An almost-linear range for the number of PCR amplifications for 67LR was observed between 20 and 40 cycles when compared to the β-actin standard. Thus, 25 cycles of PCR amplification were subsequently used for all experiments. Based on the scanned values, 67LR cDNA levels in each sample were divided by levels of GAPDH to correct for the variance in the RNA amount in each sample

that was loaded on the gels. After correction, 67LR levels were expressed as a percent of the control.

2.8 Western blot analysis

The immunoblot analysis was modified as described by Hung *et al*. [18] to obtain whole-protein extracts from preadipocytes and adipocytes. A minor modification from this method was to use Wito's lysis buffer containing 50 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5% glycerol, 1 mM EDTA, 0.2 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 15 mM 2-mercaptoethanol, 0.25% NP-40, and protein inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). An aliquot of the supernatant (50 μg of protein) was used and then loaded on 12.5% SDS-PAGE. Immunoblot analyses with 67LR and β-actin antisera were performed. All primary antibodies were used at a dilution of 1:1000 (~0.2 μg/mL). Donkey antirabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody at a dilution of 1:2000 (~0.2 μg/mL). The immunoblots were visualized after the addition of a Western Lightning™ chemiluminescence reagent plus kit (Perkin-Elmer Life Science, Boston, MA, USA) for 3 min and exposure to Fuji film for 2~3 min. They were then arbitrarily quantified using the Molecular ImagerR FX Pro Plus (BioRad Laboratories). After normalization to β-actin protein, levels of the intracellular 67LR protein were expressed as a percent of the control, unless noted otherwise.

2.9 Cytotoxicity assay

We followed the methods reported by Harmon and Harp [38] to perform our cytotoxicity assay by measuring lactate dehydrogenase (LDH) release into the culture medium. Briefly, 3T3-L1 preadipocytes and adipocytes (15000–20000 cells/cm^2) were plated in triplicate wells of a 12-well plate. Vehicle and 5–100 μM EGCG were added to phenol red-free culture medium with or without cells at the time of plating. At 8 h after plating, all media were collected from the wells and stored for later analysis of the LDH activity. The protein amounts of the media and lysates were determined in duplicate by a dye-binding method [32] using BSA (Sigma) as the standard. Assays were performed at 25°C in a final volume of 250 μL *per* well containing 50 μL of protein lysate (~5 μg protein) and 200 μL of substrate solution. The final substrate mixture *per* well contained 0.3 mM sodium pyruvate, 0.25 mM β-NADH, and 20 mM Tris-HCl buffer (pH 7.4). The kinetic reaction was measured within 5 min from the absorbance at 340 nm. A unit of LDH activity was that which caused an initial rate of oxidation of one micromole of $NADH_2$ *per* min under the conditions specified at 25°C. Reproducible results were obtained in the range of the rabbit muscle LDH (Sigma) standard from 0.00098 to 0.098 μg *per* well ($od_{340\,nm}$ = 0.0098 + 0.003121 × log (μg protein); r^2 = 0.97). The LDH protein amounts of the media and lysates were determined in duplicate using rabbit muscle LDH (Sigma) as the standard. The percent LDH release was calculated as follows:%LDH release = (LDH in culture medium/LDH in culture medium + LDH in cell lysate) × 100 [38].

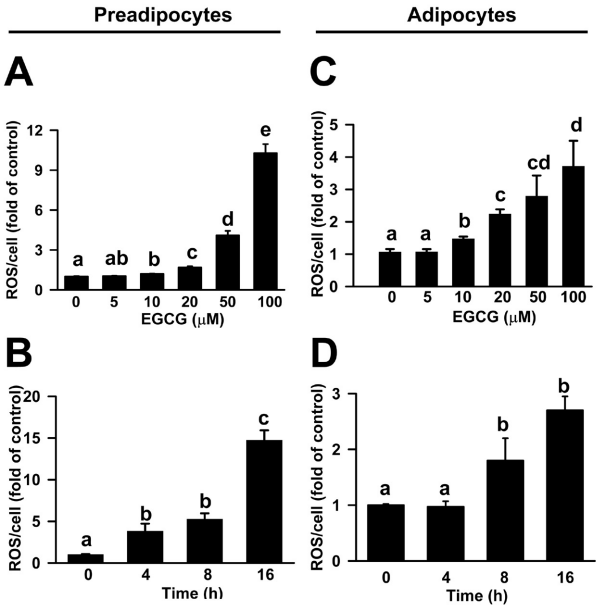

Figure 1. EGCG increased the production of ROS in 3T3-L1 preadipocytes (A and B) and adipocytes (C and D) in dose- and time-dependent manners. (A) and (C) show a dose-dependent effect of EGCG after 8 h of treatment. (B) and (D) show a time-dependent effect of EGCG after treatment with 50 μM EGCG. Data are expressed as the means ± SEM, n = 4. ROS levels are expressed as multiples of the control after normalization to the number of cells. * $p < 0.05$ *versus* control (A and C: without EGCG treatment; B and D: at time zero). a–e: groups with different letters are significantly different ($p < 0.05$) from each other.

2.10 Statistical analysis

Data are expressed as the mean ± SEM. Unpaired Student's *t*-test was used to examine differences between the control and EGCG-treated groups. One-way ANOVA followed by the Student–Newman–Keuls multiple-range test was used to examine differences among multiple groups. Differences were considered significant at $p < 0.05$. Statistics were performed using SigmaStat (Jandel Scientific, Palo Alto, CA, USA), and ROS data were logarithmically transformed.

3 Results

3.1 Effects of EGCG on ROS levels

EGCG increased the levels of ROS in concentration-dependent (Figs. 1A and C) and time-dependent (Figs. 1B

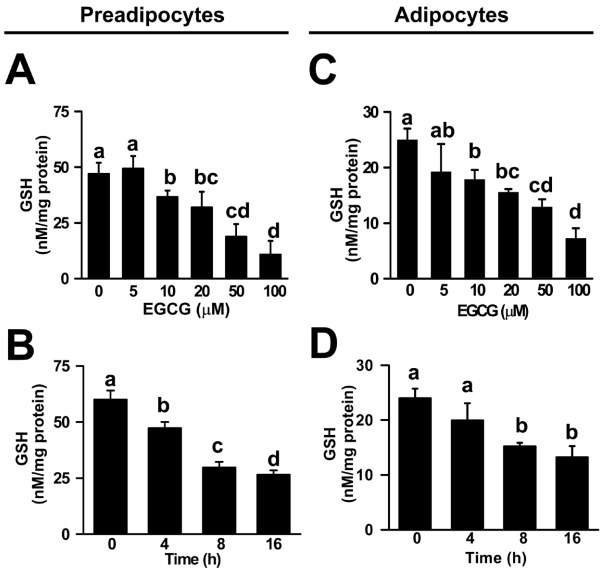

Figure 2. EGCG decreased the amounts of GSH in 3T3-L1 preadipocytes (A and B) and adipocytes (C and D) in dose- and time-dependent manners. (A) and (C) show a dose-dependent effect of EGCG after 8 h of treatment. (B) and (D) show a time-dependent effect of EGCG after treatment with 50 μM EGCG. Data are expressed as the means ± SEM, $n = 3$. * $p < 0.05$ *versus* the control (A and C: without EGCG treatment; B and D: at time zero). a – d: groups with different letters are significantly different ($p < 0.05$) from each other.

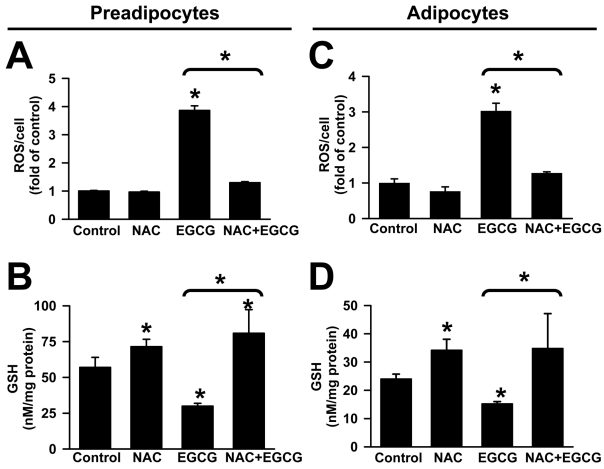

Figure 3. NAC reversed the EGCG-altered levels of ROS (A and C) and GSH (B and D) of 3T3-L1 preadipocytes (A and B) and adipocytes (C and D). Data are expressed as the means ± SEM, $n = 3$. * $p < 0.05$ *versus* control, or EGCG *versus* NAC + EGCG (brackets).

and D) manners. The activation concentration of EGCG to increase ROS levels of preadipocytes by 300 – 500% was approximately 50 μM after 4 – 8 h of treatment (Figs. 1A and B). While EGCG at 5 μM for 8 h had no significant effect on ROS levels of preadipocytes, doses of EGCG at 10, 20, 50, and 100 μM, respectively increased the ROS levels by 20, 68, 310, and 928% (Fig. 1A). At a given concentration of 50 μM, EGCG at 4, 8, and 16 h significantly increased ROS levels of preadipocytes (Fig. 1B). To investigate whether EGCG was also able to affect ROS levels of adipocytes, serum-starved 3T3-L1 adipocytes were exposed to different concentrations (0 – 100 μM) of EGCG for various time periods (Figs. 1C and D). We found that exposure of adipocytes to 50 μM EGCG increased the intracellular ROS levels by 180 – 300% at 8 h. In addition, the adipocyte ROS levels were significantly increased by 47, 124, 179, and 271%, respectively, 8 h after 10, 20, 50, and 100 μM of EGCG treatment (Fig. 1C). At a given concentration of 50 μM, EGCG at 8 and 16 h, but not 4 h, significantly increased ROS levels of adipocytes (Fig. 1D).

3.2 Effects of EGCG on GSH levels

To understand whether GSH is related to the EGCG-induced changes in ROS levels in 3T3-L1 preadipocytes, changes in GSH levels were assessed (Fig. 2). First, we found that EGCG decreased intracellular GSH levels in concentration-dependent (Figs. 2A and C) and time-dependent (Figs. 2B and D) manners. The concentration of EGCG that decreased preadipocyte GSH levels by 50% was approximately 50 μM with 8 h of treatment (Figs. 2A and B). A similar effect of EGCG on adipocyte GSH levels was observed (Figs. 2C and D). EGCG at 50 μM for 8 h decreased adipocyte GSH levels by 30 – 50%.

The possibility that the EGCG-induced stimulation of ROS levels resulted from the alteration in GSH levels was also examined (Fig. 3). The 3T3-L1 preadipocytes and adipocytes were treated with the GSH activator, NAC, in the presence and absence of 50 μM EGCG for 8 h. In preadipocytes, NAC alone increased the GSH, but not ROS, levels, while in the presence of EGCG, it suppressed the EGCG-increased levels of ROS (Fig. 3A) and EGCG-decreased levels of GSH (Fig. 3B). In adipocytes, NAC alone tended to decrease ROS levels and significantly increased the GSH levels; but in the presence of EGCG, it suppressed the EGCG-increased ROS levels (Fig. 3C) and the EGCG-decreased GSH levels (Fig. 3D).

3.3 Differences of EGCG with other specific GSH inhibitors

On the basis of the IC_{50} value of EGCG (50 μM) and BSO (40 μM; data not shown) for inhibiting preadipocyte GSH levels, we tested whether the stimulatory effects of EGCG on ROS and GSH levels of fat cells differed from those of BSO (Fig. 4). Preadipocyte ROS levels were significantly increased by 90 and 450% by respective treatments with BSO and EGCG (Fig. 4A). However, EGCG in combination with BSO induced a 600% increase in ROS production. Preadipocyte GSH levels were decreased by 50 and 55% by

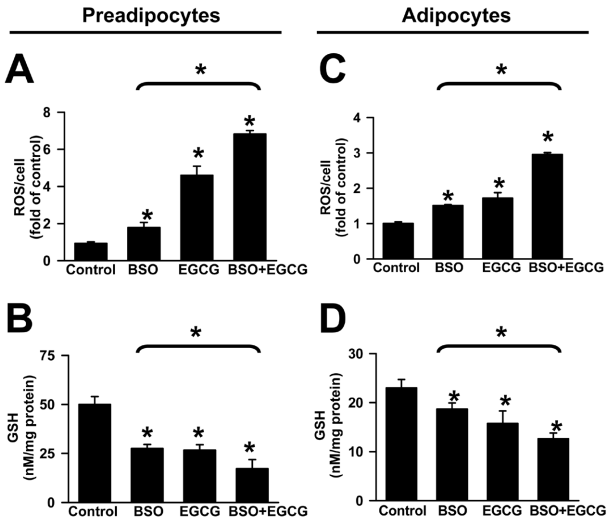

Figure 4. EGCG enhanced the levels of ROS (A and C) and GSH (B and D) of 3T3-L1 preadipocytes (A and B) and adipocytes (C and D) altered by BSO. Data are expressed as the means ± SEM, $n = 3$. * $p < 0.05$ *versus* control, or BSO *versus* BSO + EGCG (brackets).

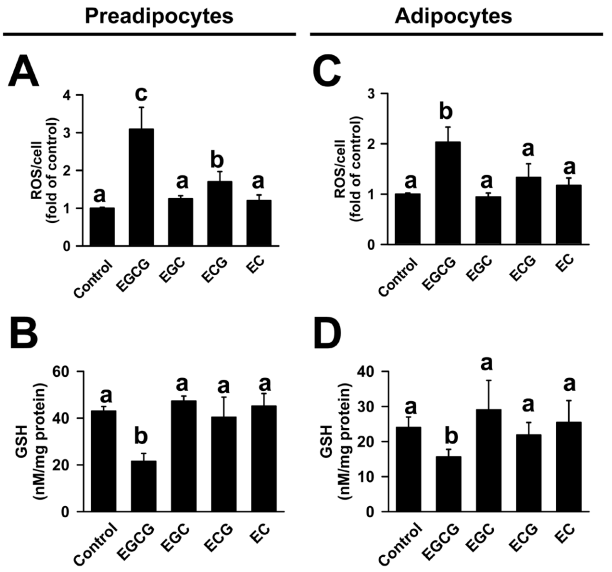

Figure 5. Catechin-specific effects of green tea on the levels of ROS (A and C) and GSH (B and D) of 3T3-L1 preadipocytes (A and B) and adipocytes (C and D). Serum-starved cells were treated with 50 μM of different catechins for 8 h. Data are expressed as the means ± SEM, $n = 3$. EGCG, EGC, ECG, and EC. * $p < 0.05$ *versus* control. a and b: groups with different letters are significantly different ($p < 0.05$) from each other.

treatment with BSO or EGCG alone, respectively, when compared to the control (Fig. 4B). A combination of EGCG with BSO decreased GSH levels in preadipocytes by 65%. In adipocytes, ROS levels increased by 50 and 80% by respective treatments with BSO and EGCG, while GSH levels decreased by 20 and 31% (Figs. 4C and D). Treatment with EGCG and BSO induced 290% increases in ROS production and 50% decreases in GSH amounts, respectively.

BSO was reported to specifically inhibit the activity of γ-GCS [29], which catalyzes the first step in the synthesis of GSH [33]. To examine whether EGCG can alter the activity of this enzyme in our cell culture system, we treated serum-starved 3T3-L1 preadipocytes and adipocytes with or without EGCG for 8 h, and then analyzed their γ-GCS activity. EGCG was observed to dose-dependently inhibit γ-GCS activity in preadipocytes after 8 h of treatment (data not shown). The concentrations of EGCG to inhibit 50% activity of preadipocyte γ-GCS were in the ranges of 20 – 50 μM as compared to the control (control: 4.3 ± 0.55 mg phosphate/mg protein *vs.* 50 μM EGCG: 1.65 ± 0.19 mg phosphate/mg protein). In adipocytes, 50 μM EGCG for 8 h inhibited 50% activity of γ-GCS (data not shown). These results might explain the effect of EGCG in reducing GSH levels of fat cells (Fig. 2).

3.4 EGCG-specific effects on ROS and GSH levels

When 50 μM of the four GTCs of EC, EGC, ECG, and EGCG were individually added to preadipocytes for 8 h, EGCG was the most effective in increasing the amounts of ROS (Fig. 5A). EC and EGC did not significantly alter the amounts of ROS. Preadipocyte GSH levels were significantly inhibited by EGCG (Fig. 5B). Neither EC, EGC, nor ECG significantly changed the amount of GSH. In adipocytes, EGCG, but not EC, EGC, or ECG, significantly increased ROS levels (Fig. 5C) and decreased GSH (Fig. 5D) levels.

3.5 The 67 kDa laminin receptor (67LR) antibody blocked the effects of EGCG

The 67LR is considered to be a receptor for green tea EGCG in human lung cancer cells [24]. Accordingly, the possibility that the 67LR mediates the EGCG-altered ROS and GSH levels in preadipocytes and adipocytes was examined (Figs. 6 and 7). We first found that 67LR mRNA (Fig. 6A) and protein (Fig. 6B) levels were changed during differentiation of 3T3-L1 preadipocytes to adipocytes. Second, we observed that pretreatment of preadipocytes with either NRS or 67LR antiserum alone did not alter the amounts of preadipocyte ROS (Fig. 7A) or GSH (Fig. 7B). In addition, NRS did not block EGCG-stimulated ROS and GSH levels. But, 67LR antiserum prevented the EGCG-altered ROS and GSH levels produced by preadipocytes. Treatment with both 67LR antiserum and EGCG induced a 43% increase in ROS production and a 51% decrease in GSH depletion from preadipocytes when compared to treatment with NRS and EGCG. In adipocytes, 67LR antiserum also prevented alterations in ROS (Fig. 7C) and GSH levels (Fig. 7D) caused by EGCG. Adipocytes treated with both 67LR antiserum and EGCG exhibited a 24% increase in

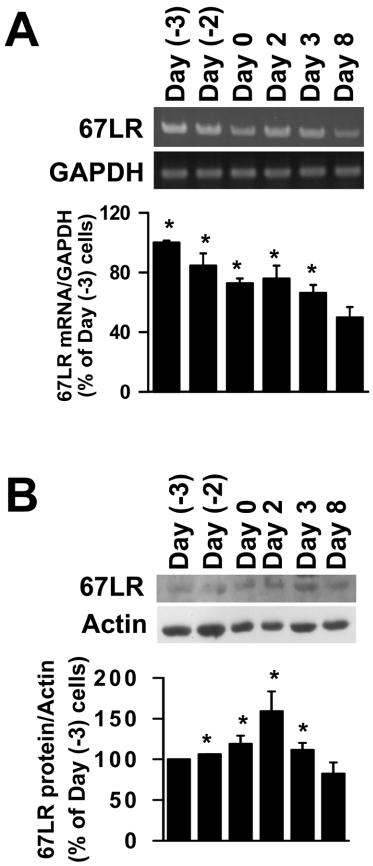

Figure 6. The 67LR gene expression changed when 3T3-L1 cells differentiated. (A) Shows 67LR mRNA levels as examined by RT-PCR GAPDH. (B) Shows 67LR protein levels as examined by Western blot analysis. Different phases (days 0 – 8 with day 0 being the day of cell differentiation when the differentiation medium was added; day (-3) and day (-2) cells, log-phase preadipocytes; day 0 cells, confluent preadipocytes; day 2 and day 3 cells, differentiating preadipocytes; day 8 cells, differentiated adipocytes.) are indicated. Data are expressed as the means ± SEM, $n = 3$~5. * $p < 0.05$ *versus* differentiated adipocytes.

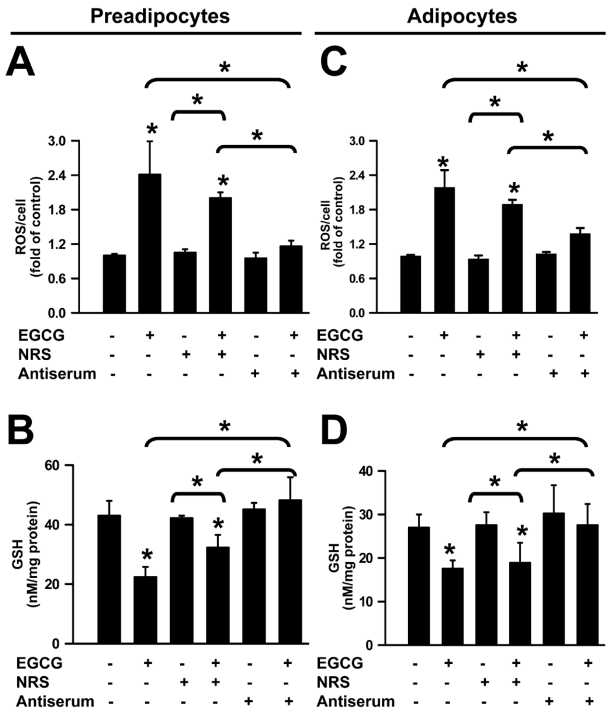

Figure 7. The 67LR antiserum altered the effects of EGCG on the levels of ROS (A and C) and GSH (B and D) of 3T3-L1 preadipocytes (A and B) and adipocytes (C and D). Data are expressed as the means ± SEM, $n = 3$. * $p < 0.05$ *versus* control, or EGCG + NRS *versus* EGCG + antiserum (brackets).

ROS production and a 45% decrease in GSH depletion when compared to those treated with both NRS and EGCG.

Further demonstrations indicated that EGCG did not significantly alter 67LR mRNA and protein levels of 3T3-L1 preadipocytes and adipocytes in the presence and absence of the 67LR antiserum (Fig. 8). But, EGCG alone stimulated the translocation of 67LR from cytosol to membrane, and the stimulation appeared to be inhibited by 67LR antiserum treatment (Fig. 9).

3.6 Cytotoxic effect of EGCG

To determine whether EGCG induced alterations in ROS and GSH levels through cytotoxic effects, we measured LDH release into the culture medium in response to 8 h of EGCG treatment (Fig. 10). First, we found that control 3T3-

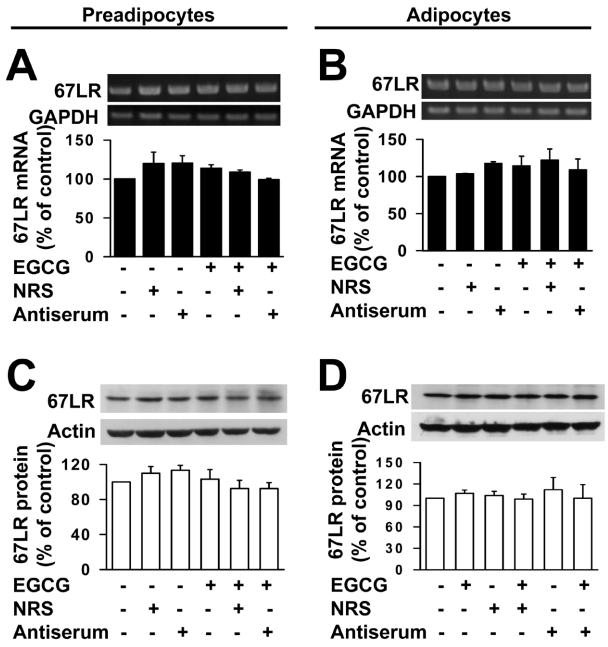

Figure 8. EGCG and the 67LR antiserum did not significantly alter the levels of 67LR mRNA (A and B) and protein (C and D) of 3T3-L1 preadipocytes (A and C) and adipocytes (B and D). Data are expressed as the means ± SEM, $n = 3$.

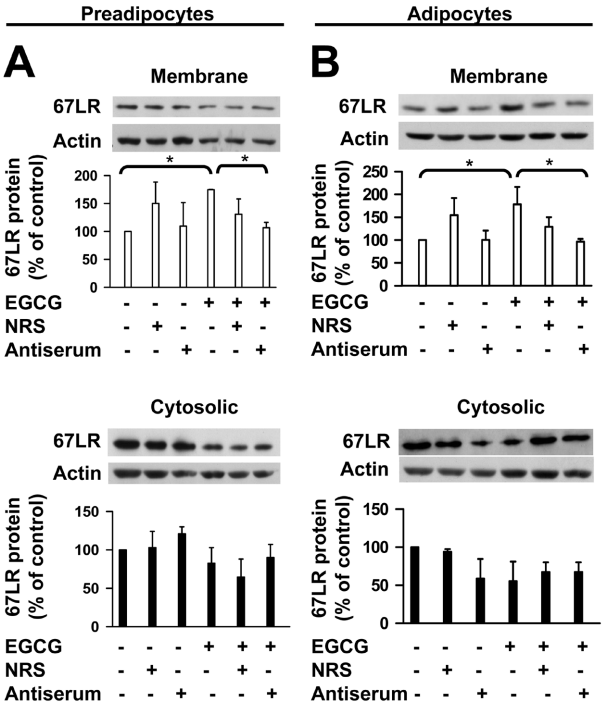

Figure 9. Effects of the 67LR antiserum on the EGCG-stimulated translocation of 67LR protein of 3T3-L1 preadipocytes (A) and adipocytes (B). The white and black bars in (A) and (B) represent the membrane and cytosolic 67LR, respectively. Data are expressed as the means ± SEM, $n = 3$. * $p < 0.05$ *versus* control, or EGCG *versus* EGCG + antiserum (brackets).

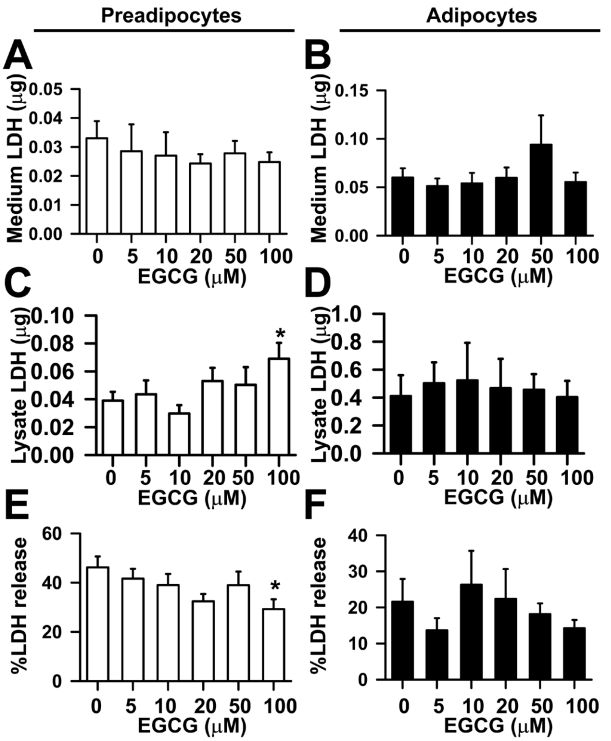

Figure 10. No cytotoxicity of EGCG on 3T3-L1 preadipocytes (A, C, and E) and adipocytes (B, D, and F). Cells were treated with 5 – 100 μM EGCG or vehicle at the 8 h of plating. LDH was measured in the culture medium (A and B) and cell lyates (C and D). Cytotoxicity was expressed as% LDH release (E and F). Data are expressed as the means ± SEM, $n = 6$. * $p <$ 0.05 *versus* the control (C and E).

L1 preadipocytes (Figs. 10A and C) exhibited a lower magnitude of LDH levels than did control 3T3-L1 adipocytes (Figs. 10B and D). Second, EGCG dose-dependently increased intracellular LDH levels in preadipocytes. Although 3T3-L1 preadipocytes and adipocytes released LDH over 8 h of incubation, roughly 14 – 46%, 5 – 100 μM of EGCG did not trigger any statistically significant LDH release than did the control.

4 Discussion

The present study demonstrates that green tea EGCG increases production of ROS in preadipocytes and adipocytes. The effects of EGCG were dose and time dependent. In general, concentrations exceeding 50 μM were more effective than low concentrations of <20 μM. These results are similar to those observed previously for its regulatory activities of neuroblastoma [17], hepatoma [16], bronchial cells [39], colon adenocarcinoma HT29 cells [40], and leukemia Jurkat cells [41]. It is likely that EGCG increases ROS levels of fat cells by decreasing GSH levels. This conclusion is supported by the findings that treatment with NAC prevented EGCG-increased ROS levels and the EGCG-decreased GSH levels. It is interesting in our findings that NAC alone slightly decreased ROS levels of preadipocytes and adipocytes by 10 and 25%, respectively, while it significantly increased GSH levels of preadipocytes and adipocytes by 25 and 42%. One of the possible explanations for the lesser effect of NAC on ROS than GSH production is that the distinct types of antioxidant enzymes (*i.e.*, catalase, NADPH oxidase, xanthine oxidase, and superoxide dismutase) and factors (*i.e.*, NFκB and adiponectin) [28, 42] may affect ROS and GSH production in 3T3-L1 cells at varying levels. This explanation is supported by the facts that NAC suppressed tumor necrosis factor α-decreased superoxide dismutase and catalase in 3T3-L1 adipocytes [28] and that NAC reversed the effects of H_2O_2 on the expression levels of adiponectin gene to normal levels in 3T3-L1 adipocytes [42].

The GSH-dependent effect of EGCG observed in 3T3-L1 cells is also strengthened by our findings that the GSH depleter, BSO, alone decreased GSH and increased ROS levels. However, it is interesting that EGCG used to treat preadipocytes and adipocytes within the IC_{50} range enhances BSO-altered levels of ROS and GSH. It appears that EGCG works differently from BSO in altering ROS and GSH levels. Since BSO was reported as specifically inhibit

the synthesis of GSH due to its inhibiting γ-GCS activity [29] and since EGCG was found in this study to inhibit γ-GCS activity in preadipocytes and adipocytes, the possibility that the synergistic effect of EGCG and BSO on GSH levels may be attributable to their having different binding sites with γ-GCS enzyme remains to be demonstrated. Since decreased GSH content could result from decreased GSH synthesis and increased GSH degradation, another possible explanation is that EGCG may regulate the degradation of GSH, in additional to the biosynthesis of GSH. But, this assumption requires further demonstrations. Differences in the synergistic effect of EGCG and BSO on ROS levels between preadipocytes and adipocytes may be also explained by the cell specificity. It is interesting in our findings that EGCG in combination with BSO induced a dramastic increase (~600%) in ROS production and a marginal decrease (~65%) in GSH levels. Possible explanations for this difference is that the distinct types of antioxidant enzymes and factors [28, 42] may be involved in the regulatory effect of EGCG or BSO on ROS and GSH productions in 3T3-L1 cells at varying levels. Accordingly, our study could not exclude the possibility that the other enzymatic mechanisms of action of EGCG [29] may help explain the differential effects of both EGCG and BSO on ROS and GSH levels.

Catechin-specific effects of green tea have been related on biological systems and cell functions based on a variety of laboratory studies [3 – 6]. We found that the four major GTCs increased preadipocyte ROS production in the order of EGCG > ECG > EGC = EC. A similar catechin-specific effect of green tea on adipocyte ROS levels was observed. In parallel to this, EGCG at 50 μM for 8 h was found to induce a 50% decrease in GSH levels from preadipocytes and adipocytes, whereas neither EC, ECG, nor EGC at the same concentrations and duration of treatment significantly decreased GSH levels. According to structural differences among them occurring in the number of hydroxyl groups and the presence of a galloyl group, our results supported the important roles of the galloyl group and the hydroxyl groups of the B ring of EGCG in regulating ROS and GSH levels of fat cells. This is consistent with previous results that green tea modulates cell functions of fat cells in catechin-specific ways [3, 11].

Distinct properties of both preadipocytes and adipocytes have been extensively described [27, 43 – 46]. Whether preadipocytes and adipocytes have different magnitudes of ROS levels was determined in this study. We found that control 3T3-L1 preadipocytes exhibited a three-fold lower magnitude of ROS levels (67 ± 8 absorbance/cell number *vs.* 20 ± 2 absorbance/cell number) than did control 3T3-L1 adipocytes. This is similar to results reported by Mouche *et al*. [46]. One of the possible explanations is attributable to changes in GSH levels since GSH levels were about two-fold higher in control preadipocytes (51 ± 5 nmol/mg protein) than in control adipocytes (24 ± 2 nmol/mg protein). Differences in GSH levels may be explained by the fact that γ-GCS activity in control preadipocytes (4.3 ± 0.55 mg phosphate formed/mg protein) was found to be significantly higher than that of control adipocytes (2.3 ± 0.55 mg phosphate formed/mg protein). We further demonstrated whether preadipocytes and adipocytes have different responses to EGCG at the level of oxidative stress. Although EGCG generally had similar effects in altering ROS production and GSH depletion between preadipocytes and adipocytes, a more significant increase in the magnitude of ROS production in preadipocytes than in adipocytes depended on the dosage range. This is supported by the findings that EGCG at 50 μM, but not at 5 – 20 μM, for 8 h increased ROS levels of preadipocytes and adipocytes by 300 – 500 and 180 – 300%, respectively, compared to the control. At those concentrations, preadipocytes (~4 h) had a more-acute response to EGCG in producing ROS than adipocytes (~8 h). The different degree of EGCG-induced ROS levels between preadipocytes and adipocytes might be partially explained by changes in GSH levels induced by EGCG since preadipocytes had a greater response to EGCG than adipocytes in terms of GSH depletion and since the rising ROS levels induced by EGCG parallel decreases in GSH levels caused by EGCG. Another explanation is that basal ROS and GSH levels as previously described differed between preadipocytes and adipocytes. This explanation is strengthened by our findings that the GSH depleter, BSO, alone more effectively caused GSH depletion and ROS production in preadipocytes than in adipocytes.

In this study, differential 67LR levels might also explain any difference in preadipocytes and adipocytes in response to EGCG in producing oxidative stress, since 67LR gene expression changed when 3T3-L1 cells differentiated (Fig. 6) and since 67LR antiserum treatment more effectively prevented EGCG-altered ROS and GSH levels in preadipocytes than in adipocytes (Fig. 7). These results, together with the findings that NRS did not prevent the increased ROS levels and decreased GSH levels by EGCG, suggest 67LR-dependent effects of EGCG on oxidative stress in fat cells. This was consistent with those reported in lung cancer cells [24]. Our further observations that 67LR antiserum, but not NRS, blocked the EGCG-stimulated translocation of this receptor from cytosol to membrane of 3T3-L1 preadipocytes and adipocytes indicate that EGCG transiently modifies the distribution of 67LR between cytosol and membrane of fat cells. This observation may explain for our findings that EGCG alone insignificantly increased 67LR mRNA and protein levels of preadipocytes and adipocytes by 5 – 15% and that the effects of 67LR antiserum on 67LR gene expression appeared insignificant. Other possible explanations are that the distinct signal transducers (*i.e.*, extracellularly regulated kinase (ERK)) of the 67LR in 3T3-L1 preadipocytes and adipocytes may be affected by EGCG. This assumption can be indirectly supported by the evidence that EGCG reduced ERK kinase activity in 3T3-

L1 preadipocytes and adipocytes [18, 21] and that EGCG stimulated expression of eukaryotic translation elongation factor 1A gene in tumor cells *via* the 67LR pathway [47].

GTCs have numerous biological effects *in vitro*, and generally effects are observed in the range of 10–100 μM [3]. *In vivo*, plasma concentrations of green tea EGCG or other catechins as generally reported in animals and humans are about 1 μM [3]. However, the absorption and distribution of administrated EGCG and other tea catechins are poor and dependent on catechin structure, purity, dosage, the route of administration, and the tissue involved. For example, after consumption of 1.5 g of decaffeinated green tea solids, the catechins in human plasma reached peak levels in 1.5–2.5 h [48]. At that time, the plasma levels (free and conjugated) of EGCG, EGC, and EC levels were 0.26, 0.48, and 0.19 μM, respectively, while ECG was not detected [48]. Consumption of a single high dose of green tea, equivalent to six cups of tea, can raise plasma levels of catechin to 2–4 μM in 60 min [49]. In a phase I pharmacokinetic study to determine the systemic availability of green tea EGCG after single oral administration of 800 mg EGCG (approximately eight cups of green tea at once) to healthy subjects, the average maximum plasma concentration and terminal elimination half-life are about 0.96 μM, 4 and 2 h, respectively [50]. A few minutes after two to three cups of green tea are consumed, the saliva levels of various catechins reach peaks of 39–144 μM EGCG, 11–48 μM EGCG, and 7–28 μM EC [51]. Sixty minutes after intragastric administration of EGCG at a dose of 500 mg/kg body weight to rats, the levels of EGCG were 10 μM in plasma, 48 μM in the liver, 0.5 μM in the brain, 565 μM in the small intestinal mucosa, and 68 μM in the colon mucosa [52]. When [^{3}H]EGCG is administered directly into the stomach of mice, radioactivity is found in the digestive tract, liver, lung, pancreas, mammary gland, skin, brain, kidney, uterus, ovary, and testis [53]. When EGCG was administrated at a dose of 100 mg/kg body weight to rats by intraperitoneal injection, the plasma concentrations of unmetabolized EGCG, determined by HPLC, were 24, 2, 1, and 1 μM at 0.5, 1, 2, and 24 h, respectively [52]. Unfortunately, what is not clear at this time is whether effective doses of catechins can be achieved in adipose tissues simply by consuming tea infusions. Accordingly, the doses (5–100 μM) of EGCG (the effective dose of this study is in the range of 10–100 μM) or other tea catechins generally used in this study compatible with the goal of helping regulating the ROS production of this study and the initiation and progression of obesity [3, 11] were a little bit higher, but might be acceptable for the physiological effect of EGCG in animals. This is indirectly supported by the evidence that green tea EGCG given to rats by intraperitoneal administration at a dosage of 70–92 mg *per* 1000 g body weight, which contained about 152–200 μM (if the density of rat body weight is assumed to be 1 g/cm^3), could within 8 days decrease body weight, body fat, adipose weight, and serum lipid levels in rats [54], and that EGCG given to mice by oral administration at a dosage of 0.5–1 g *per* 100 g diet, which contained about 10 900–21 800 μM of EGCG (if the density of diet is assumed to be 1 g/cm^3), could within 29 days reduce body weight, adipose fats, and serum triglycerides in mice [12].

In this study through the determination of LDH release into the culture medium, EGCG at 5–100 μM produced no cytotoxic effects. Because the time required for EGCG to cause increases in ROS production obtained from this study is earlier than that of decreases in the cell viability caused by EGCG [18], EGCG may act at 50 μM to inhibit cell proliferation [18] and apoptosis [19, 20] of 3T3-L1 cells *via* oxidative stress as reported for hapatoma [16]. Indeed, we observed in this study that NAC treatment reversed the effects of EGCG in reducing the number of 3T3-L1 preadipocytes and adipocytes (data not shown) with concurrent changes in ROS and GSH levels (Fig. 3) when the cells were examined by the trypan blue dye exclusion method. Firm conclusions as to whether any of these *in vitro* effects of EGCG can explain for its *in vivo* effects on body weight, body fat, and adipose weight in animals [52] and humans [11, 12] will require more thorough *in vivo* studies.

We concluded that the stimulatory effects of EGCG on ROS production of preadipocytes and adipocytes are mediated through a GSH-dependent pathway. While EGCG was demonstrated to increase ROS levels and decrease GSH levels, its signaling was altered by 67LR antibody treatment. This suggests a 67LR-mediated effect of EGCG. Increases in oxidative stress induced by EGCG in this study may have been related to the mechanism through which EGCG exerts its effects on fat cells [18–20]. In aspect of the antiobesity effect of EGCG [11, 12], the EGCG-induced ROS production in fat cells appears beneficial. While many benefits of GTCs have received great attention [3], it is also important to consider the adverse effects that may accompany heavy use of green tea or catechins. For example, increases in oxidative stress of fat cells induced by EGCG may be detrimental to health by interfering the adipocyte endocrine activity [12, 21] or causing normal cell apoptosis [3]. Accordingly, it would be worthwhile in a further *in vivo* study to explore whether the EGCG-induced ROS production is beneficial or detrimental to health.

This work was supported by the National Science Council, Taiwan, Grant NSC95-2311-B-008-008-MY3; the Armed Forces Tao-Yuan General Hospital, Taiwan; Veteran General Hospital and University System of Taiwan Joint Research Program, and Tsou's Foundation, Taiwan; and Cathay General Hospital and National Central University Joint Research Program, Taiwan to Y.–H. K.

The authors have declared no conflict of interest.

5 References

[1] Rusznyak, S., Szent-Gyorgyi, A., Vitamin P flavanols as vitamins, *Nature* 1936, *138*, 27.

[2] Roberts, E. A. H., The chemistry of tea fermentation, *J. Sci. Food Agric.* 1952, *3*, 193 – 198.

[3] Liao, S., Kao, Y. H., Hiipakka, R. A., Green tea: Biochemical and biological basis for health benefits, *Vitam. Horm.* 2001, *62*, 1 – 94.

[4] Lin, J. K., Liang, Y. C., Lin-Shiau, S. Y., Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade, *Biochem. Pharm.* 1999, *58*, 911 – 915.

[5] Yang, C. S., Wang, Z. Y., Tea and cancer, *J. Natl. Cancer Inst.* 1993, *85*, 1038 – 1049.

[6] Ahmad, N., Mukhtar, H., Green tea polyphenols and cancer: Biologic mechanisms and practical implications, *Nutr. Rev.* 1999, *57*, 78 – 83.

[7] Haqqi, T. M., Anthony, D. D., Gupta, S., Ahmad, N., *et al*., Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea, *Proc. Natl. Acad. Sci.* 1999, *96*, 4524 – 4529.

[8] Mendel, S., Youdim, M. B., Catechin polyphenols: Neurodegeneration and neuroprotection in neurodegenerative diseases, *Free Radic. Biol. Med.* 2004, *37*, 304 – 317.

[9] Mendel, S., Weinreb, O., Amit, T., Youdim, M. B., Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (–)-epigallocatechin-3-gallate: Implications for neurodegenerative diseases, *J. Neurochem.* 2004, *88*, 1555 – 1569.

[10] Song, E. K., Hur, H., Han, M. K., Epigallocatechin gallate prevents autoimmune diabetes induced by multiple low doses of streptozotocin in mice, *Arch. Pharm. Res.* 2003, *26*, 559 – 563.

[11] Kao, Y. H., Chang, H. H., Lee, M. J., Chen, C. L., Tea, obesity, and diabetes, *Mol. Nutr. Food Res.* 2006, *50*, 188 – 210.

[12] Wolfram, S., Wang, Y., Thielecke, F., Anti-obesity effects of green tea: From bedside to bench, *Mol. Nutr. Food Res.* 2006, *50*, 176 – 187.

[13] Lin, J. K., Lin-Shiau, S. Y., Mechanisms of hypolipidemic and anti-obesity effects of tea and tea polyphenols, *Mol. Nutr. Food Res.* 2006, *50*, 211 – 217.

[14] Dulloo, A. G., Duret, C., Rohrer, D., Girardier, L., *et al*., Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans, *Am. J. Clin. Nutr.* 1999, *70*, 1040 – 1045.

[15] Dulloo, A. G., Seydoux, J., Girardier, L., Chantre, P., *et al*., Green tea and thermogenesis: Interaction between catechin-polyphenols, caffeine and sympathetic activity, *Int. J. Obes. Relat. Metab. Disord.* 2000, *24*, 252 – 258.

[16] Waltner-Law, M. E., Wang, X. L., Law, B. K., Hall, R. K., *et al*., Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production, *J. Biol. Chem.* 2002, *277*, 34933 – 34940.

[17] Weinreb, O., Mandel, S., Youdim, M. B. H., cDNA gene expression profile homology of antioxidants and their antiapoptotic and proapoptotic activities in human neuroblastoma cells, *FASEB J.* 2003, *17*, 935 – 937.

[18] Hung, P. F., Wu, B. T., Chen, H. C., Chen, Y. H., *et al*., Antimitogenic effect of green tea (–)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways, *Am. J. Physiol. Cell Physiol.* 2005, *288*, C1094 – C1108.

[19] Wu, B. T., Hung, P. F., Chen, H. C., Huang, R. N., *et al*., The apoptotic effect of green tea (–)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the Cdk2 pathway, *J. Agric. Food Chem.* 2005, *53*, 5695 – 5701.

[20] Lin, J., Della-Fera, M. A., Baile, C. A., Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes, *Obes. Res.* 2005, *13*, 982 – 990.

[21] Liu, H. S., Chen, Y. H., Hung, P. F., Kao, Y. H., Inhibitory effect of green tea (–)-epigallocatechin gallate on resistin gene expression in 3T3-L1 adipocytes depends on the ERK pathway, *Am. J. Physiol. Endocrinol. Metab.* 2006, *290*, E273 – E281.

[22] Rudich, A., Tirosh, A., Potashnik, R., Khamaisi, M., Bashan, N., Lipoic acid protects against oxidative stress induced impairment in insulin stimulation of PKB and glucose transport in 3T3-L1 adipocytes, *Diabetologia* 1999, *42*, 949 – 957.

[23] Hwang, J. T., Park, I. J., Shin, J. I., Lee, Y. K., *et al*., Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase, *Biochem. Biophys. Res. Commun.* 2005, *338*, 694 – 699.

[24] Tachibana, H., Koga, K., Fujimura, Y., Yamada, K., A receptor for green tea polyphenol EGCG, *Nat. Struct. Mol. Biol.* 2004, *11*, 380 – 381.

[25] Mecham, R. P., Receptors for laminin on mammalian cells, *FASEB J.* 1991, *5*, 2538 – 2546.

[26] Niimi, T., Kumagai, C., Okano, M., Kitagawa, Y., Differentiation-dependent expression of laminin-8 (alpha 4 beta 1 gamma 1) mRNAs in mouse 3T3-L1 adipocytes, *Matrix Biol.* 1997, *16*, 223 – 230.

[27] Burton, G. R., Nagarajan, R., Peterson, C. A., McGehee, R. E. Jr., Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis, *Gene* 2004, *329*, 167 – 185.

[28] Araki, S., Dobashi, K., Kubo, K., Yamamoto, Y., *et al*., N-acetylcysteine attenuates TNF-α induced changes in secretion of interleukin-6, plasminogen activator inhibitor-1 and adiponectin from 3T3-L1 adipocytes, *Life Sci.* 2006, *79*, 2405 – 2412.

[29] Khamaisi, M., Kavel, O., Rosenstock, M., Porat, M., *et al*., Effect of inhibition of glutathione synthesis on insulin action: In vivo and in vitro studies using buthionine sulfoximine, *Biochem. J.* 2000, *349*, 579 – 586.

[30] Ferrer, A. S., Santema, J. S., Hilhorst, R., Visser, A. J. W. G., Fluorescence detection of enzymatically formed hydrogen peroxide in aqueous solution and in reversed micelles, *Anal. Biochem.* 1990, *187*, 129 – 132.

[31] Cohn, V. H., Lyle, J., A fluorometric assay for glutathione, *Anal. Biochem.* 1966, *14*, 434 – 440.

[32] Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 1976, *72*, 248 – 254.

[33] Sekura, R., Meister, A., γ-Glutamylcysteine synthetase: Further purification, "half of the sites" reactivity, subunits, and specificity, *J. Biol. Chem.* 1977, *252*, 2599 – 2605.

[34] Taussky, H. H., Shorr, E., A microcolorimetric method for the determination of inorganic phosphate, *J. Biol. Chem.* 1953, *202*, 675 – 685.

[35] Chen, Y. H., Lee, M. J., Chang, H. H., Hung, P. F., *et al*., 17β-Estradiol stimulates resistin gene expression in 3T3-L1 adipocytes via the estrogen receptor, extracellularly regulated kinase, and CCAAT/enhancer binding protein-α pathways, *Endocrinology* 2006, *147*, 4496 – 4504.

[36] Jackers, P., Minoletti, F., Belotti, D., Clausse, N., *et al*., Isolation from a multigene family of the active human gene of the metastasis-associated multifunctional protein 37LRP/p40 at chromosome 3p21.3, *Oncogene* 1996, *13*, 495 – 503.

[37] Makrides, S., Chitpatima, S. T., Bandyopadhyay, R., Brawerman, G., Nucleotide sequence for a major messenger RNA for a 40 kilodalton polypeptide that is under translational control in mouse tumor cells, *Nucleic Acids Res.* 1988, *16*, 2349.

[38] Harmon, A. W., Harp, J. B., Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis, *Am. J. Physiol. Cell Physiol.* 2001, *280*, C807 – C813.

[39] Yang, G. Y., Liao, J., Li, C., Chung, J., *et al*., Effect of black and green tea polyphenols on c-jun phosphorylation and H_2O_2 production in transformed and nontransformed human bronchial cell lines: Possible mechanisms of cell growth inhibition and apoptosis induction, *Carcinogenesis* 2000, *21*, 2035 – 2039.

[40] Hong, J., Lu, H., Meng, X., Ryu, J. H., *et al*., Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (–)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells, *Cancer Res.* 2002, *62*, 7241 – 7246.

[41] Nakagawa, H., Hasumi, K., Woo, J. T., Nagai, K., *et al*., Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (–)-epigallocatechin gallate, *Carcinogenesis* 2004, *25*, 1567 – 1574.

[42] Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., *et al*., Increased oxidative stress in obesity and its impact on metabolic syndrome, *J. Clin. Invest.* 2004, *114*, 1752 – 1761.

[43] Yamada, Y., Rosen, E. D., Spiegelman, B. M., Molecular regulation of adipogenesis, *Annu. Rev. Cell Dev. Biol.* 2000, *16*, 145 – 171.

[44] Gregoire, F. M., Smas, C. M., Sul, H. S., Understanding adipocyte differentiation, *Physiol. Rev.* 1998, *78*, 783 – 809.

[45] Zwirska-Korczala, K., Adamczyk-Sowa, M., Sowa, P., Pilc, K. *et al*., Role of leptin, ghrelin, angiotensin II and orexins in 3T3-L1 preadipocyte cells proliferation and oxidative metabolism, *J. Physiol. Pharmacol.* 2007, *58*, 53 – 64.

[46] Mouche, S., Mkaddem, S. B., Wang, W., Katic, M., *et al*., Reduced expression of the NADPH oxidase NOX4 is a hallmark of adipocyte differentiation, *Biochem. Biophy. Acta* 2007, *1773*, 1015 – 1027.

[47] Umeda, D., Yano, S., Yamada, K., Tachibana H., Green tea polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor, *J. Biol. Chem.* 2008, *283*, 3050 – 3058.

[48] Yang, C. S., Chen, L., Lee, M. J., Balentine, D., *et al*., Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers, *Cancer Epidemiol. Biomark. Prev.* 1998, *7*, 351 – 354.

[49] Unno, T., Kondo, K., Itakura, H., Takeo, T., Analysis of (–)-epigallocatechin gallate in human serum obtained after ingesting green tea, *Biosci. Biotechnol. Biochem.* 1996, *60*, 2066 – 2068.

[50] Chow, H. H. S., Cai, Y., Alberts, D. S., Hakim, I., *et al*., Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E., *Cancer Epidemiol. Biomark. Prev.* 2001, *10*, 53 – 58.

[51] Yang, C. S., Lee, M. J., Chen, L., Human salivary tea catechin levels and catechin esterase activities: Implication in human cancer prevention studies, *Cancer Epidemiol. Biomark. Prev.* 1999, *8*, 83 – 89.

[52] Nakagawa, K., Miyazawa, T., Chemiluminescence-high-performance liquid chromatographic determination of tea catechin (–)-epigallocatechin 3-gallate, at picomole levels in rat and human plasma, *Anal. Biochem.* 1997, *248*, 41 – 49.

[53] Suganuma, M., Okabe, S., Oniyama, M., Tada, Y., *et al*., Wide distribution of [3H](–)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue, *Carcinogenesis* 1998, *252*, 566 – 570.

[54] Kao, Y. H., Hiipakka, R. A., Liao, S., Modulation of endocrine systems and food intake by green tea epigallocatechin gallate, *Endocrinology* 2000, *141*, 980 – 987.

