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RESEARCH ARTICLE

Green tea (–)-epigallocatechin gallate inhibits IGF-I and IGF-II stimulation of 3T3-L1 preadipocyte mitogenesis via the 67-kDa laminin receptor, but not AMP-activated protein kinase pathway*

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Scope: This study investigated the pathways involved in epigallocatechin gallate (EGCG) modulation of insulin-like growth factor (IGF)-I-stimulated and IGF-II-stimulated mitogenesis in 3T3-L1 preadipocytes.

Methods and results: We found that this process was dose and time dependent, and caused by suppression of IGF-I-stimulated and IGF-II-stimulated phosphorylation of p66Shc and mitogen-activated protein kinase (MAPK) pathway proteins, including MEK1 kinase (RAF1), extracellular signal-regulated protein kinase (ERK) kinase (MEK1), and ERK 1 and ERK 2 (ERK1/2), but not phospho-Jun-N-terminal kinase, protein kinase B, p52Shc, or p46Shc. Furthermore, EGCG inhibited the IGF-I-stimulated phosphorylation of the IGF-I receptor-beta (IGF-IR β), the association of IGF-IR with the p66Shc protein, and the IGF-II-stimulated associations of the IGF-II receptor with $G_{\alpha i\text{-}2}$ and p66Shc proteins, suggesting that EGCG selectively affects particular types of Shc and MAPK family members. Pretreatment with antiserum against the EGCG receptor (also known as the 67-kDa laminin receptor; 67LR), but not with an adenosine monophosphate (AMP)-activated protein kinase (AMPK) inhibitor, prevented the inhibitory actions of EGCG on IGF-I- and IGF-II-stimulated ERK1/2 phosphorylation and subsequent preadipocyte proliferation.

Conclusion: The results of this study suggest that EGCG mediates anti-IGF-I and anti-IGF-II signals in preadipocyte mitogenesis via the 67LR but not the AMPK pathway.

Keywords:
Epigallocatechin gallate / Green tea / Insulin-like growth factor / Laminin receptor / Preadipocyte

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Abbreviations: **AKT**, protein kinase B; **AMP**, adenosine monophosphate; **AMPK**, AMP-activated protein kinase; **BrdU**, bromodeoxyuridine; **CS**, calf serum; **DMEM**, Dulbecco's modified Eagle's medium; **DMSO**, dimethyl sulfoxide; **EC**, epicatechin; **ECG**, epicatechin gallate; **EGC**, epigallocatechin; **EGCG**, epigallocatechin gallate; **ERK1/2**, extracellular signal-regulated protein kinase 1 and 2; **FOXO1**, forkhead box O 1; **IGFBP-3**, IGF-binding protein 3; **IGF-I/-II**, insulin-like growth factor I and II; **IGF-IIR**, IGF-II receptor; **IGF-IR**, IGF-I receptor; **IOD**, integrated optical density; **MAPK**, mitogen-activated protein kinase; **MEK1/2**, MAPK or ERK kinase; **PI3K**, phosphatidylinositol 3-kinase; **pJNK**, phospho-Jun-N-terminal kinase; **pRAF1**, phospho-RAF1; **RAF1**, MEK1 kinase; **Shc protein**, Src homologous and collagen (Shc) protein; **67LR**, 67-kDa laminin receptor.

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1 Introduction

Green tea catechins have recently been subject of increasing attention due to their chemopreventive roles in regulating obesity and fat cell functions. In particular, (-)-epigallocatechin gallate (EGCG) was demonstrated to reduce body weight, body fat, and blood lipid levels in vivo [1]. Other in vivo findings indicated that EGCG reduced food uptake and lipid absorption and stimulated fat oxidation and fecal lipid excretion [see details in reviews by Kao et al., 2006; Lin and Lin-Shiau, 2006; Wolfram et al., 2006; 2–4]. These findings were supported by in vitro data showing that EGCG is responsible for the following: (1) increasing the rate of oxygen consumption in brown adipose tissue, synergistically with caffeine and norepinephrine [5]; (2) regulating the activities and expression of various enzymes related to lipid anabolism and catabolism [2], including acetyl-CoA carboxylase [6], fatty acid synthase [7], glycerol-3-phosphate dehydrogenase [8], pancreatic lipase [9], lipoxygenase [10], hormone-sensitive lipase [11], and adenosine monophosphate (AMP)-activated protein kinase (AMPK) [12]; (3) inhibiting the adipogenic differentiation of preadipocytes into adipocytes [2–4]; (4) regulating adipokine secretion [13] and glucose uptake [14] of adipocytes; and (5) reducing serum- and insulin-induced increases in cell numbers [15, 16]. The antiobesity effects of EGCG may be also explained by its ability to induce functional changes in other target tissues (e.g., the digestive organs, liver, pancreas, brain, heart) and proteins (e.g., catechol-*O*-methyltransferase, caspase-3, cyclin-dependent kinases, fatty acid transporter, p53, sodium-dependent glucose transporters, uncoupling proteins, vimentin) [2–4, 17–23]. These changes are likely related to specific pathways, possibly through modulations of the endocrine system (e.g., insulin and IGF-I levels), the nervous system, the circulatory system, thermogenesis, carbohydrate metabolism, the redox status, and activities (e.g., apoptosis and cell proliferation) of different types of cells (e.g., liver, muscle, endothelial, and β-pancreatic cells) [2–4, 17–22].

EGCG influences serum- and insulin-regulated mitogenesis in preadipocytes [15, 16] and insulin-like growth factors (IGFs) are important factors to stimulate preadipocyte mitogenesis [23]; however, little is known about the mechanisms of EGCG's action in regulating IGF stimulation of preadipocyte growth. Some studies have shown that EGCG increased the tyrosine phosphorylation of IGF-I receptor (IGF-IR) in H4IIEC cells [24] and activated FOXO1a phosphorylation in HEK-293 cells through an IGF-I-like mechanism [25]. Others found that IGF expression and signaling in cancer cells can be inhibited by EGCG [26–28]. Thus, the involvement of EGCG in IGF-I signaling is still controversial and varies with cell types. Accordingly, a careful examination of IGF-I and IGF-II signaling molecules involved in the anti-IGF effects of EGCG on preadipocytes might elucidate the mechanisms that underlie the antimitogenic effect of EGCG on fat cells.

IGFs regulate fat cell activity through at least two pathways, the MAPK kinase (MEK1) and extracellular signal-regulated protein kinase (ERK)-regulated pathway and the phosphatidylinositol (PI3K) and protein kinase B (AKT)-mediated pathway [23]. The MEK1/ERK pathway plays a central role in controlling growth; the PI3K/AKT-mediated pathway appears to regulate metabolic enzymes. In both pathways, IGF-I acts by phosphorylating its own receptor and several downstream Shc proteins [23, 29], while IGF-II acts by associating its own receptor with the downstream $G_{\alpha i\text{-}2}$ protein and phosphorylating downstream mitogen-activated protein kinases (MAPKs) [30, 31]. Green tea EGCG was found to regulate the phosphorylation of MAPK in fat cells [15, 16], and MAPK is one of the downstream targets of IGF-IR, IGF-II receptor (IGF-IIR), G protein, and Shc proteins [20, 26–28]. The hypothesis thus arose that EGCG may regulate the signaling and phosphorylation of IGF-IR and all three of the Shc proteins in fat cells as well as the association of IGF-IIR with $G_{\alpha i\text{-}2}$ protein.

A putative EGCG receptor known as the 67-kDa laminin receptor (67LR) was discovered in cancer cells [32]. Recently, the 885-bp nucleotide sequence of the 67LR was identified in preadipocytes and adipocytes, and its expression was sensitive to the growth phase [16]. EGCG was found to increase insulin-stimulated association between 67LR and insulin receptor in preadipocytes, and pretreatment with the 67LR antiserum prevented the effect of EGCG on insulin signaling [16]. In adipocytes, EGCG was reported to suppress insulin-increased levels of glucose uptake via the 67LR and AMPK pathways [14]. Despite the demonstration that 67LR mediated antiinsulin actions of EGCG on preadipocytes and adipocytes, it is still unknown whether 67LR is responsible for EGCG-mediated effects on IGF-I and IGF-II signaling in preadipocyte mitogenesis.

The present study was designed to examine the mechanism underlying EGCG inhibition of IGF-I- and IGF-II-stimulated mitogenesis in 3T3-L1 preadipocytes. We confirmed that EGCG significantly suppressed IGF-I and IGF-II signaling and that this effect was prevented by pretreatment with the 67LR antiserum. We also found that EGCG retained the IGF-I-stimulated association between 67LR and IGF-IR and the IGF-II-stimulated association between 67LR and IGF-IIR. We discovered that treatment with the AMPK inhibitor, Compound C, did not block the anti-IGF-I and anti-IGF-II effects of EGCG. These results indicated that the 67LR, but not AMPK, mediated the effects of EGCG on IGF-I and IGF-II signaling in preadipocytes.

2 Materials and methods

2.1 Chemical reagents

All reagents (e.g., IGF-I, IGF-II, and PD98059) were purchased from Sigma Chemical (St. Louis, MO), unless noted

otherwise. EGCG and other catechins (> 98% pure), which were isolated from green tea (*Camellia sinensis*) as previously described [16], were dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in sterile media such that the final concentration of DMSO used was 0.1%. Dulbecco's modified Eagle's medium (DMEM), calf serum (CS), trypsin, agarose, and penicillin-streptomycin were purchased from GibcoBRL of Life Technologies (New York, NY). The BenchMark™ prestained protein molecular weight marker was purchased from Invitrogen Life Science Technologies (Carlsbad, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise stated.

2.2 Cell culture

As previously described by Ku et al. [16], 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA; ATCC-CL-173) and rat H4IIEC3 hepatoma cells (ATCC-CRL-1600) were grown in DMEM (pH 7.4) containing 10% CS, 100 μg/mL streptomycin and 100 units/mL of penicillin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was replaced every 2 days. Preadipocytes were subcultured before reaching confluence because serum components contain factors that facilitate adipogenesis of confluent 3T3-L1 preadipocytes to adipocytes.

2.3 Growth inhibition experiments

According to a previously published method [16], 3T3-L1 cells (15 000∼20 000/cm^2) were plated in triplicate 12-well plates. To study dose- and time-dependent effects of EGCG on IGF-I- or IGF-II-stimulated growth of 3T3-L1 preadipocytes, the log-phase cells (2∼3 days after inoculation) were treated with EGCG (0∼50 μM) in the presence or absence of either IGF-I or IGF-II (0∼10 nM) for the indicated time periods. The number of cells was counted with a hemocytometer using the 0.4% trypan blue exclusion method. Cell viability remained at 90∼100% during the 48-h treatment with 10∼50 μM EGCG. Cellular proliferation was measured with a commercially available BrdU enzyme-linked immunosorbent assay kit (Roche Applied Science, Mannheim, Germany). We should note that 3T3-L1 cells (∼2 000 cells/well) were preincubated with EGCG at the indicated concentrations for 2 h at 37°C, exposed to IGF-I or IGF-II for 12 h, and then incubated with 10 μM BrdU for 16 h.

2.4 Experimental treatments

We determined the effect of EGCG at different time points for each experimental condition using the method described by Ku et al. [16]. Serum-starved preadipocytes were pretreated with EGCG (0 and 20 μM) for 2 h and then either IGF-I or IGF-II (0 and 10 nM) was added, unless otherwise stated. In order to compare the effects of different catechins on IGF-I- and IGF-II-induced changes in IGF signaling proteins, serum-starved cells were pretreated for 2 h with 20 μM of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), or EGCG, and then 10-nM IGF-I or IGF-II was added. After 60 min of IGF-I and IGF-II incubation, levels of ERK1/2, phosphorylated ERK1/2 (pERK1/2), phosphorylated JNK (pJNK), JNK, and actin proteins were measured by a Western blot analysis. After 12 and 48 h of IGF-I and IGF-II treatments, BrdU incorporation and cell numbers were determined, respectively.

To study the 67LR-dependent effect of EGCG on IGF-I- and IGF-II-stimulated preadipocyte mitogenesis, 3T3-L1 preadipocytes were pretreated with 5 μg/mL of either preimmunized normal rabbit serum (NRS) or 67LR antiserum for 1 h, exposed to 20 μM EGCG for 2 h, and then incubated with 10 nM IGF-I or 10 nM IGF-II. After 1, 12, and 48 h of IGF-I and IGF-II treatments, protein kinase levels, BrdU incorporation, and cell numbers were determined, respectively.

In order to study the AMPK-dependent effects of EGCG on IGF-I- and IGF-II-stimulated growth of 3T3-L1 preadipocytes, serum-starved cells were pretreated for 1 h with the AMPK inhibitor Compound C (10 μM) [14], exposed to 20 μM EGCG for 2 h, and then incubated with 10 nM IGF-I or 10 nM IGF-II. Compound C was dissolved in 100% DMSO and then diluted in culture media such that the final concentration of DMSO used was 0.1%. After 1 and 48 h of 10 nM IGF-I and IGF-II incubation, we measured protein kinase levels and the number of cells, respectively.

2.5 Immunoprecipitation

IGF-IR, IGF-II receptor, and 67LR proteins were immunoprecipitated according to the methods described by Chen et al. [33] and Ku et al. [16]. Briefly, we obtained whole-protein extracts from predipocytes using lysis buffer, which contained 50 mM Tris-Cl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM 2-mercaptoethanol, 0.25% NP-40, 250 mM NaCl, 5% glycerol, and protein inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). An aliquot of the supernatant (1 mg of protein) was preincubated for 1 h at 4°C with antibodies specific for IGF-IR, IGF-IIR, and 67LR. A control supernatant aliquot was incubated with preimmunized NRS. Samples were then incubated with 20 μL of protein A-agarose overnight at 4°C. Total amounts of IGF-IR, IGF-IIR, phosphorylated IGF-IR (pIGF-IR), pShc, $G_{\alpha i-2}$, and 67LR in the immunoprecipitates were measured by a Western blot analysis with isoform-specific or phosphotyrosine antibodies. After normalization to the total IGF-IR or IGF-IIR protein, amounts of pShc, $G_{\alpha i-2}$, and 67LR were expressed as a percent of the controls to indicate changes in binding to IGF-IR and IGF-IIR. After normalization to the

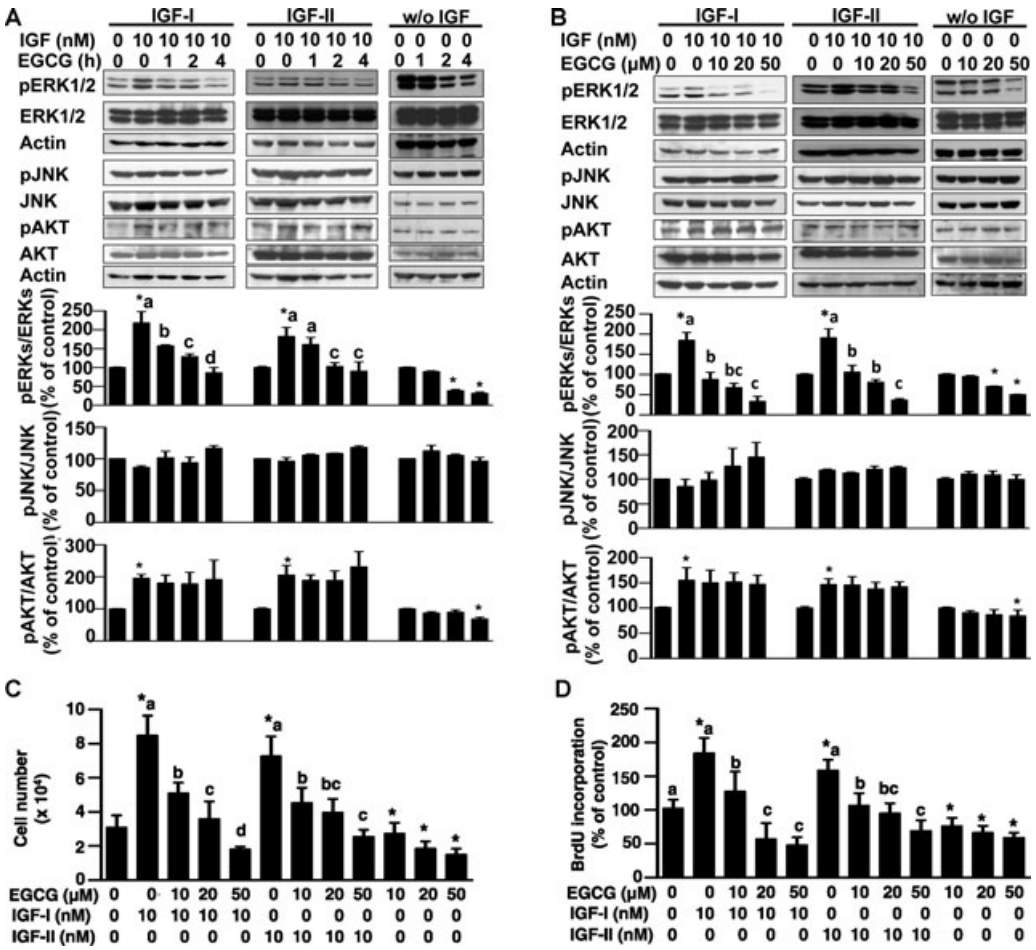

Figure 1. Effects of EGCG on IGF-I- and IGF-II-induced changes in the phosphorylation status of signaling proteins (A and B), cell number (C), and BrdU incorporation (D). 3T3-L1 cells were preincubated with 20 μM (A) or the indicated concentration of EGCG (B–D) for the indicated times or for 2 h (B) and then cells were treated with IGF-I or IGF-II as described in the section "Materials and methods." Cell extracts were prepared as described in the section "Materials and methods" and the levels of various proteins were determined by Western blot using specific antibodies. In (A), the control was evaluated at time 0, when EGCG was first added; while, in (B–D), control experiments were conducted without IGF-I or IGF-II treatment in the absence of EGCG. All graphical data are expressed as the mean ± SEM from triplicate determinations. In some data, standard error bars are too small to be seen. Groups with different letters significantly differ ($p < 0.05$) from each other. *$p < 0.05$ versus the control.

total 67LR protein, the amounts of IGF-IR and IGF-IIR were expressed as a percent of the controls to indicate changes in binding to 67LR. Data obtained from NRS were not presented due to insignificant changes.

2.6 Western blot analysis

Immunoblot analysis was performed as described by Chen et al. [33] and Ku et al. [16]. Briefly, 50∼75 μg of protein was separated by 12% SDS polyacrylamide gel electrophoresis with 2× gel-loading buffer (100 mM Tris-HCl (pH 6.8), 0.2% bromophenol blue, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol). Electrophoresis-separated proteins were blotted onto Immobilon-NC transfer membranes (Millipore, Bedford, MA), which were then incubated with primary antibody at a dilution of 1:1000 (∼0.2 μg/mL), followed by secondary antibody (e.g., donkey antimouse IgG, donkey antigoat IgG, or donkey antirabbit IgG conjugated with horseradish peroxidase) at a dilution of 1:2000 (∼0.2 μg/mL). The immunoblots were visualized by adding Western Lightning™ chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA) for 3 min, followed by exposure to Fuji film for 2∼3 min and scanning with a Microtek ScanMaker i800 (Microtek International Inc., Hsinchu, Taiwan). With a Gel-Pro Analyzer (Media Cybernetics Inc., Bethesda, MD), each scanned band was quantified, the integrated optical density (IOD) was calculated, and data were normalized to β-actin according to the IOD values. Protein levels are expressed as a percent of the controls, unless otherwise noted.

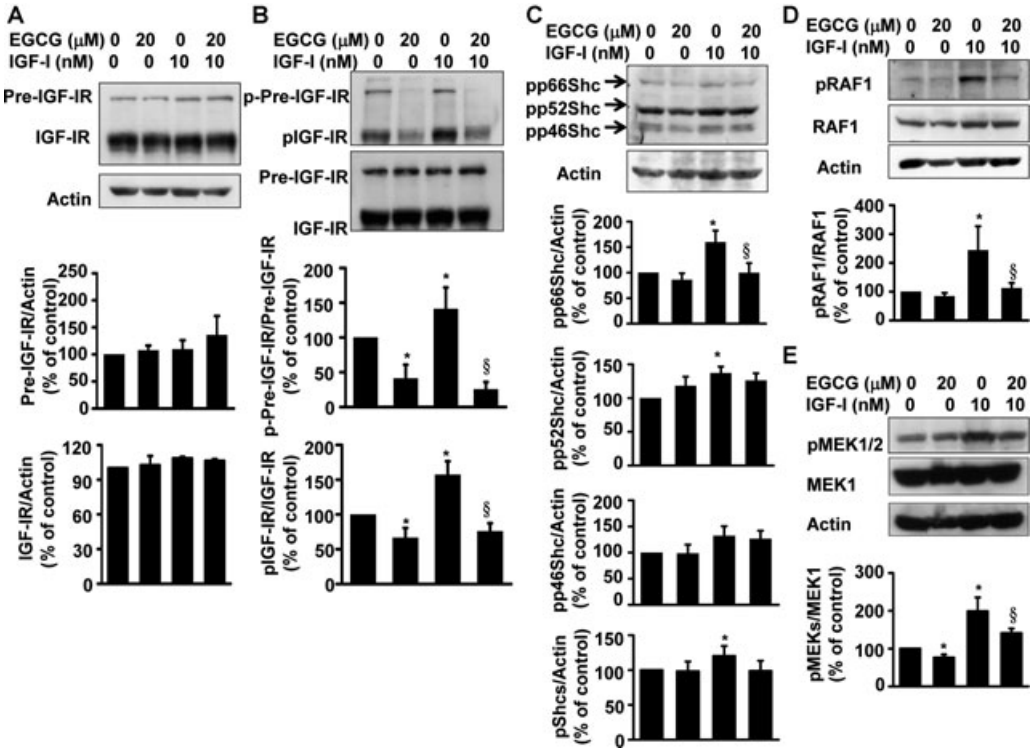

Figure 2. EGCG altered IGF-I-stimulated phosphorylation of IGF-I signaling molecules in 3T3-L1 preadipocytes. All experiments included 2 h of pretreatment with EGCG, followed by the addition of IGF-I, and incubation for 60 min. Total levels of the pre-IGF-I receptor (Pre-IGF-IR) and IGF-IR proteins (A) and protein phosphorylation levels of p66Shc, p52Shc, p46Shc, RAF1, and MEK1 proteins (C–E) were determined by a Western blot analysis as described in the section "Materials and methods." But, levels of phosphorylated Pre-IGF-IR (p-Pre-IGF-IR) and phosphorylated IGF-IR (pIGF-IR) proteins (B) were determined when cytosolic protein lysates were subjected to immunoprecipitation with IGF-IR antiserum and then probed with either phosphotyrosine or IGF-IRβ antisera. Control experiments were conducted without IGF-I or EGCG treatments. Data are expressed as the mean ± SEM of triplicate determinations. In some data, standard error bars are too small to be seen. $^{*}p < 0.05$ versus the control. $^{\S}p < 0.05$ IGF-I versus EGCG + IGF-I.

2.7 Statistical analysis

Data are expressed as the mean ± SEM. A statistical analysis was performed as described by Ku et al. [16].

3 Results

3.1 Green tea EGCG inhibited IGF-stimulated mitogenesis of 3T3-L1 preadipocytes

We optimized conditions for IGF-I- or IGF-II-stimulated mitogenesis of 3T3-L1 preadipocytes via the ERK and PI3K pathways (Supporting Information Figs. 1 and 2), as previously described [16]. IGF-I and IGF-II dose dependently increased the proliferation of 3T3-L1 cells (Supporting Information Fig. 1). Using specific inhibitors of MEK1/2, PI3K, and p38 kinases, we found that the mitogenic effects of IGF-I and IGF-II on 3T3-L1 cells were inhibited by inhibitors of MEK1/2 and PI3K but not p38 (Supporting Information Fig. 2). Treatment with 20 μM EGCG significantly inhibited the IGF-I-induced increase in MEK1/2 activity between 1∼4 h, as indicated by the decreased amounts of pERK1 and pERK2 in samples treated with EGCG (Fig. 1); this inhibition was time (Fig. 1A) and dose dependent (Fig. 1B). The effect of EGCG on the IGF-I-induced increase in phosphorylated AKT (pAKT) was not statistically significant when we compared data from IGF-I treatment alone with the combination of EGCG and IGF-I. Total protein levels of ERK1/2 and AKT were not significantly changed by 20 μM EGCG in the presence of IGF-I.

Treatment with 20 μM EGCG for 1, 2, or 4 h did not significantly alter the amounts of pJNK in the presence or absence of IGF-I, indicating that the effects were selective for MEK1/2 (Fig. 2). We also found that EGCG dose dependently prevented IGF-I-stimulated increases in both cell number (Fig. 1C) and BrdU incorporation (Fig. 1D). Similar results were obtained when 3T3-L1 preadipocytes were pretreated with EGCG and then incubated with IGF-II. EGCG inhibited IGF-II-induced increases in MEK1/2 activity (Fig. 1A and B), cell number (Fig. 1C), and BrdU incorporation (Fig. 1D), but had no effects on total protein levels of ERK1/2, pAKT, AKT, JNK, and pJNK.

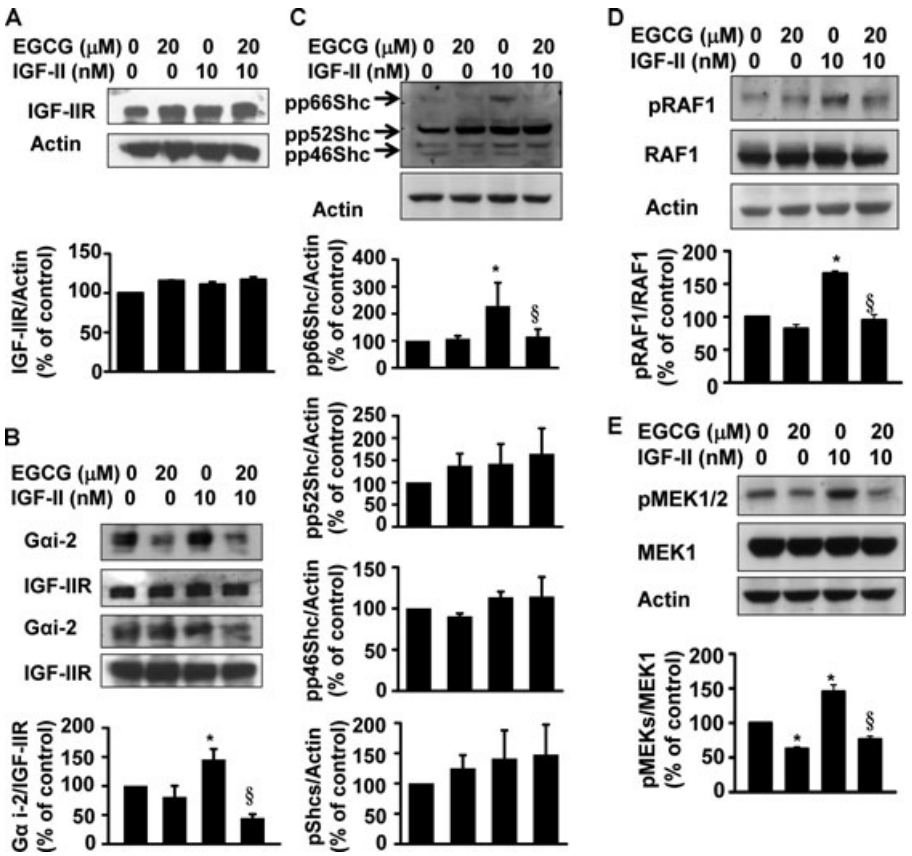

Figure 3. EGCG altered IGF-II-stimulated phosphorylation of IGF-II signaling molecules in 3T3-L1 preadipocytes. All experiments included 2 h of pretreatment with EGCG, followed by the addition of IGF-II, and incubation for 60 min. Total levels of the IGF-II receptor (IGF-IIR) (A) and protein phosphorylation levels of p66Shc, p52Shc, p46Shc, RAF1, and MEK1 proteins (C–E) were determined by a Western blot analysis as described in the section "Materials and methods." But, EGCG reduced the IGF-II-stimulated association between the IGF-IIR and $G_{\alpha i-2}$ proteins (B) when cytosolic protein lysates were subjected to immunoprecipitation with IGF-IIR antiserum and then probed with either IGF-IIR or $G_{\alpha i-2}$ antisera. Duplicate panels presented in Fig. 3B were obtained from two separate experiments. Control experiments were conducted without IGF-II or EGCG treatment. Data are expressed as the mean ± SEM of triplicate determinations. In some data, standard error bars are too small to be seen. $^{*}p < 0.05$ versus the control. $^{\S}p < 0.05$ IGF-II versus EGCG + IGF-II.

3.2 EGCG inhibited the phosphorylation of IGF-I signaling molecules

In the presence and absence of IGF-I, EGCG did not alter total protein levels of IGF-IR or Pre-IGF-IR, the long-chained precursor of IGF-IR (Fig. 2A). However, EGCG, both alone and in the presence of IGF-I, reduced basal levels of phosphorylated Pre-IGF-IR (p-Pre-IGF-IR) and pIGF-IR (Fig. 2B). Incubation with 20 μM EGCG significantly reduced IGF-I-stimulated phosphorylation of p66Shc, but not p52Shc or p46Shc (Fig. 2C). Additionally, EGCG significantly reduced IGF-I-increased levels of pRAF1 and pMEK1/2 proteins, but did not alter the total amounts of RAF1 or MEK1 (Fig. 2D and E).

3.3 EGCG inhibited the phosphorylation of IGF-II signaling molecules

In the presence and absence of IGF-II, EGCG did not alter total protein levels of IGF-IIR (Fig. 3A). However, EGCG pretreatment did inhibit the IGF-II-stimulated association of the IGF-IIR with $G_{\alpha i-2}$ protein (Fig. 3B). Furthermore, EGCG suppressed IGF-II-stimulated phosphorylation of p66Shc, RAF1, MEK1/2, but not p52Shc or p46Shc proteins (Fig. 3C, D, E). EGCG treatment did not alter total protein levels of RAF1 or MEK1 in either the presence or absence of IGF-II.

3.4 EGCG altered IGF-stimulated associations of IGF-IR and IGF-IIR proteins with Shc proteins

EGCG pretreatment inhibited the IGF-I-stimulated association of the IGF-IR with p66Shc (Fig. 4). Although EGCG in the presence of IGF-I decreased the IGF-I-stimulated association of IGF-IR with p52Shc and p46Shc, we found no statistically significant differences between IGF-I and the combination of IGF-I and EGCG on the binding of IGF-I with either Shc protein (Fig. 4A). We observed similar effects of EGCG on the IGF-II-stimulated association of the IGF-IIR with p66Shc, p52Shc, and p46Shc proteins (Fig. 4B).

3.5 EGCG effects on IGF signaling compared to other green tea catechins

After 48 h of IGF-I treatment, EGCG was generally the most effective tea catechin in reducing IGF-stimulated increases in the number of preadipocytes (Fig. 5A), BrdU incorporation (Fig. 5B), and phosphorylation of ERK1/2 proteins (Fig. 5C). Total amounts of ERK1/2, pJNK, and JNK proteins did not

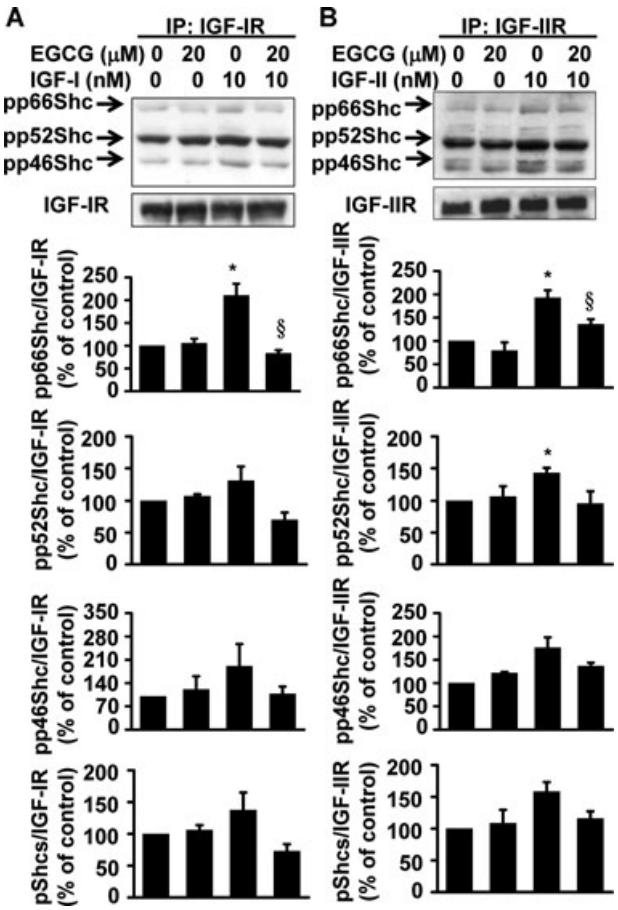

Figure 4. EGCG differentially altered IGF-I-stimulated associations between the IGF-I receptor (IGF-IR) and various Shc molecules in 3T3-L1 preadipocytes, as well as the IGF-II-stimulated associations between the IGF-II receptor (IGF-IIR) and Shc molecules. Cytosolic protein lysates were subjected to immunoprecipitation with antisera against (A) IGF-IR and (B) IGF-IIR proteins and then probed with the indicated antibodies on Western blots. Levels of pShc proteins are expressed as a percentage of the controls after normalization to immunoprecipitated proteins. Control experiments were conducted without IGF-I, IGF-II, or EGCG treatments. Data are expressed as the mean ± SEM of triplicate determinations. $^{*}p < 0.05$ versus the control. $^{\S}p < 0.05$ IGF-I versus EGCG + IGF-I or IGF-II versus EGCG + IGF-II.

change with IGF-I or IGF-II in the absence or presence of each catechin.

3.6 The anti-IGF activity of EGCG depended on the 67LR pathway

Pretreatment with a 67LR antibody prevented the inhibitory effects of EGCG on IGF signaling, as indicated by uninhibited IGF-mediated increases in the phosphorylation of ERK1/2 proteins (Fig. 6A), the number of preadipocytes (Fig. 6B), and the magnitude of BrdU incorporation (Fig. 6C).

By immunoprecipitation, we determined that IGF-IR and IGF-IIR proteins individually associated with the 67LR protein in the absence of IGF-I, IGF-II, and EGCG. IGF-I and IGF-II significantly increased the associations of IGF-IR (Fig. 7A and C) and IGF-IIR (Fig. 7B and D) with 67LR, respectively. EGCG alone tended to stimulate these associations. Although EGCG caused a 19∼26% increase in the IGF-I-stimulated association of IGF-IR with the 67LR, we found no statistically significant differences between IGF-I and the combination of IGF-I and EGCG. In the presence of IGF-II, EGCG did not alter the IGF-II-stimulated association of IGF-IIR with 67LR.

3.7 The anti-IGF activity of EGCG was independent of the AMPK pathway

Treatment with EGCG alone stimulated phosphorylation of AMPK, while treatment with the AMPK inhibitor Compound C alone inhibited phosphorylation of AMPK (Fig. 8A and B); however, pretreatment with Compound C did not prevent the inhibitory effect of EGCG on IGF signaling. The EGCG-mediated decreases in the IGF-stimulated phosphorylation of ERK1/2 and MEK1/2 proteins tended to be enhanced by Compound C treatment (Fig. 8A and B). In parallel, Compound C did not block the inhibitory effects of EGCG on IGF-stimulated preadipocyte proliferation (Fig. 8C).

3.8 EGCG effects are cell type-specific

We additionally compared the results for 3T3-L1 preadipocytes (Fig. 1) with results from the same assays performed in rat H4IIEC3 hepatoma cells (Fig. 9). In H4IIEC3 hepatocytes, 20 μM EGCG alone for 2 h did not significantly alter levels of pJNK, JNK, pAKT, AKT, or ERK1/2, but tended to increase the phosphorylation of ERK1/2 proteins (Fig. 9A). EGCG pretreatment of H4IIEC3 cells did not reduce the levels of pERK1/2 following a 60-min incubation with 10 nM of either IGF-I (Fig. 9A) or 10 nM IGF-II (Fig. 9B).

4 Discussion

In this study, we presented an in-depth analysis of the mechanism underlying the effects of EGCG on IGF regulation of preadipocyte mitogenesis. Our observations suggest that the anti-IGF-I and anti-IGF-II effects of EGCG on preadipocyte mitogenesis are mediated through the Shc/RAF1/MEK1/ERK pathway. Plant polyphenols, such as resveratrol, quercetin, curcumin, and EGCG, reportedly affect cancer cell proliferation [17, 18, 34–38]. High doses of resveratrol (100∼150 μM) and quercetin (80 μM) inhibited growth of human colon cancer cells through the IGF-I/AKT/Wnt/cyclin D1 signaling pathways [35, 36]. Data have also indicated that resveratrol decreases IGF-I secretion [35], activates the FOXO1 and p53 pathways [35], inhibits

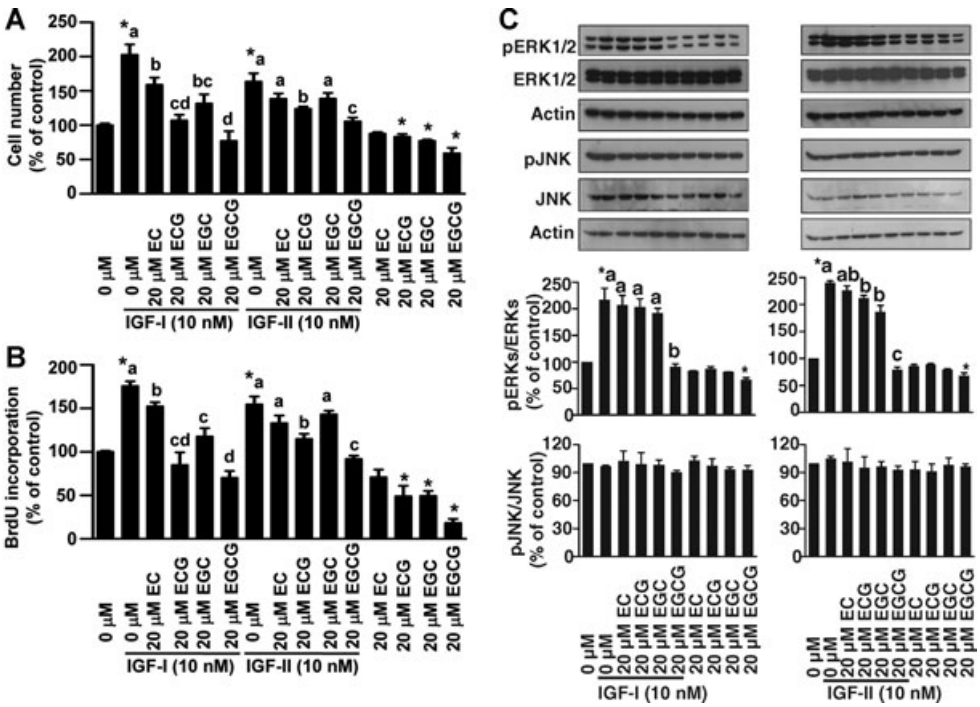

Figure 5. Green tea catechins displayed effects on (A) cell number, (B) cell proliferation, and (C) amounts of phosphorylated (ERK)1/2 (pERK1/2) proteins but not the amounts of total ERK1/2, phosphorylated JNK (pJNK), or JNK proteins. Total amounts of pERK1/2 and pJNK proteins, as determined by a Western blot analysis, are expressed as a percentage of the control after normalization to individual total protein levels. Control experiments were conducted without IGF-I, IGF-II, or EGCG treatments. In all IGF-I and IGF-II treatments, groups with different letters significantly differ ($p < 0.05$) from each other. *$p < 0.05$ versus the control. Data are expressed as the means ± SEM of triplicate determinations. EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; and EGCG, epigallocatechin gallate.

IGF-I-stimulated ERK1/2 phosphorylation [34], and suppresses insulin signaling in a Sirt-1-independent manner [34]. Additionally, resveratrol and quercetin inhibit 3T3-L1 preadipocyte growth through their antioxidant activity and a caspase-3-related pathway [39, 40]. In our study, 2 h of treatment with 10∼50 μM of EGCG in the presence of either 10 nM IGF-I or 10 nM IGF-II did not cause significant reductions in IGF-I- or IGF-II-increased pAKT levels, thus excluding a possible role of the PI3K/AKT pathway in the anti-IGF effects of EGCG on preadipocyte mitogenesis. This difference between EGCG and other plant polyphenols could be because a different IGF signal transducer, besides MAPK and PI3K, is responsible for the distinct anti-IGF effects of resveratrol and EGCG, or because the levels and responses of IGF signaling proteins required for the actions of resveratrol and EGCG vary with the type of cells. We should note that EGCG reportedly reduces FOXO1 activity in HEK-293 cells [25] and in differentiating 3T3-L1 adipocytes [41] and increases IGF-binding protein 3 (an endogenous antagonist of IGF) levels in human colon cancer cells and hepatocellular carcinoma cells [26–28]. Further studies are required to determine whether any changes in the activity and expression of FOXO1, Sirt-1, cyclin D1, IGFBP-3, Wnt signaling proteins, and other target proteins (e.g., vimentin and cyclin-dependent kinases) [17, 18, 26–28, 34–41] are necessary for the anti-IGF effects of EGCG on 3T3-L1 preadipocyte mitogenesis.

Our findings were partially consistent with previous observations of anti-IGF-I signaling of EGCG [27, 28, 38]. We found that EGCG inhibited IGF signaling in 3T3-L1 preadipocytes by reducing the activities of the IGF receptors and their downstream proteins Shc, RAS, RAF1, and MEK1/2, as indicated by reduced levels of phosphorylated IGF-IRβ, p66Shc, RAF1, MEK1/2, and ERK1/2 proteins and by reduced association of IGF-IIR with the $G_{\alpha i-2}$ protein. The anti-IGF effects of EGCG were not due to changes in the total amount of protein; they could be attributable to EGCG inhibition of associations between IGF receptors and Shc. EGCG significantly suppressed IGF stimulation of the phosphorylation of p66Shc, but not p52Shc or p46Shc proteins. Significant alterations in p-Pre-IGF-IR protein levels induced by EGCG also explained the decreased amount of pIGF-IR protein observed following EGCG treatment.

Many mechanistic studies of green tea have reported that EGCG has cell type-dependent effects [17, 18]. For example, the strength of EGCG in reducing the serum-stimulated growth of 3T3-L1 preadipocytes differed from that of 3T3 fibroblasts, presumably due to inactivation of different types of cyclin-dependent kinases [15]. In our study, using the same experimental assay methods, we observed different effects of

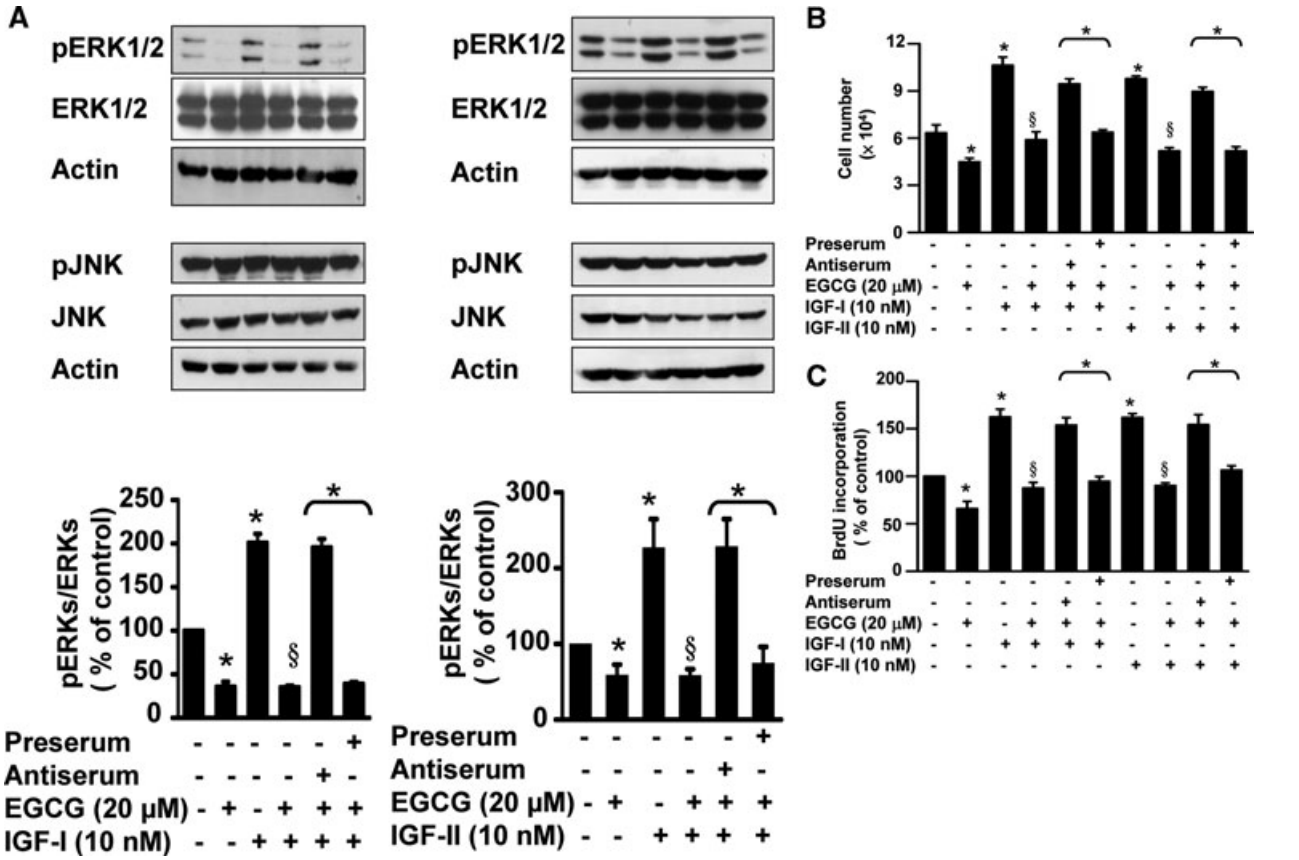

Figure 6. Antiserum against 67-kDa laminin receptor (67LR) antagonized the effects of EGCG on IGF-I-stimulated and IGF-II-stimulated changes in (A) phosphorylated ERK1/2 (pERK1/2), (B) number of cells, and (C) BrdU incorporation in 3T3-L1 preadipocytes. Data are expressed as the means ± SEM from triplicate determinations. $^{*}p < 0.05$ versus the control; brackets indicate comparisons of EGCG + IGF-I + NRS versus EGCG + IGF-I + antiserum, or EGCG + IGF-II + NRS versus EGCG + IGF-II + antiserum. $^{\S}p < 0.05$ IGF-I versus EGCG + IGF-I, or IGF-II versus EGCG + IGF-II.

EGCG on downstream IGF-I and IGF-II signaling molecules between 3T3-L1 preadipocytes and H4IIEC3 cells. Normal and transformed cell lines may have different sensitivities to EGCG; a notion that may be supported by the findings that 67LR expression depended on tissue type and that inhibition of insulin-induced phosphorylation of ERK1/2 proteins by EGCG treatment was much less in 3T3-L1 cells than that observed in H4IIEC3 cells and KB oral cancer cells [16]. It would be worthwhile to explore whether the differences in EGCG effects among cell lines are due to disparate actions of IGF-I and IGF-II on other kinases besides MAPK (e.g., cyclin-dependent kinases) and whether the activity and expression of 67LR are different among 3T3-L1, H4IIEC3, and KB cells following the EGCG treatment.

The biological activities of green tea catechins that provide various health benefits vary according to the type of catechin [2–4, 17, 18]. EGCG works differently from EC, EGC, and ECG in reducing the body weight and plasma levels of insulin and IGF-I in rats [1], and in suppressing insulin stimulation of 3T3-L1 preadipocyte mitogenesis [16]. In parallel, we found that EGCG was generally more effective than EC, ECG, and EGC at changing IGF-stimulated increases in the number of cells, the amount of incorporated BrdU, and levels of pERK1/2, suggesting that EGCG might act through a different mechanism. Among tea catechins, EGCG contains the largest number of hydroxyl groups on its three aromatic rings, which confer hydrogen bonding [42]. Also, EGCG has both gallyl and galloyl groups that are important for conformational flexibility, while neither EC nor EGC have galloyl group [43–45]. The *ortho*-trihydroxy group of EGCG is reportedly important for its apoptotic action, antioxidant activity, radical scavenging, and metal-chelating activity [17, 18, 43–45]. Although the chemical basis for the anti-IGF effects of EGCG was not determined in this study, the *ortho*-trihydroxy structures on the gallyl and galloyl groups of EGCG may be capable of intramolecular and intermolecular interactions, forming relatively stable structures [17, 43–45] for mediating IGF signaling; however, this requires further investigation.

The 67LR could mediate the effect of EGCG on mitogenesis in cancer cells and nonadipocyte cells [32, 46] and 67LR signaling may be related to certain pathways, such as through the modulations of the cytoskeleton system,

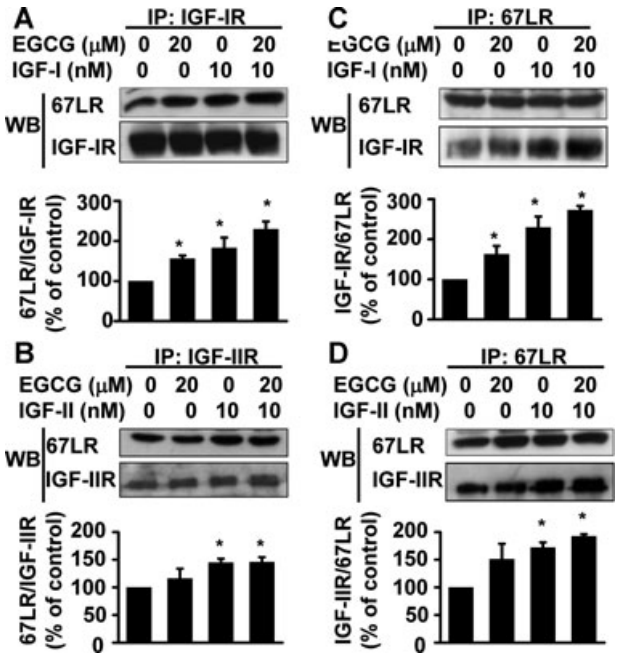

Figure 7. EGCG affected the IGF-I-stimulated association between the IGF-I receptor (IGF-IR) protein and the 67-kDa laminin receptor (67LR) in 3T3-L1 preadipocytes, as well as the IGF-IIR-stimulated association between the IGF-IIR and 67LR. Serum-starved preadipocytes were pretreated with EGCG for 2 h and then incubated with either IGF-I or IGF-II for 60 min. Cytosolic protein lysates were subjected to immunoprecipitation with antisera against the (A) IGF-IR protein, (B) IGF-IIR protein, or (C and D) 67LR protein. Immunoprecipitated proteins (IPs) were transferred to membranes and then probed with the indicated antibodies on Western blots (WB). Levels of 67LR, IGF-IR, and IGF-II proteins are expressed as a percentage of the control after normalization to immunoprecipitated proteins. Control experiments were conducted without IGF-I, IGF-II, or EGCG treatment. Data are expressed as the means ± SEM from triplicate determinations. $^{*}p < 0.05$ versus the control.

membrane lipid raft, and activity of eukaryotic translation elongation factor-1A [2, 47]. In 3T3-L1 preadipocytes and adipocytes, the 67LR mediates the effect of EGCG on reactive oxygen species (ROS) levels through the glutathione-dependent pathway [48], and can also mediate antiinsulin signaling of EGCG in preadipocyte mitogenesis and adipocyte glucose uptake [14, 16]. In this study, using the 67LR antiserum or a purified 67LR antibody (Supporting Information Fig. 5), we demonstrated that EGCG's effects on IGF stimulation of preadipocyte mitogenesis were dependent on 67LR protein. Blocking 67LR with the antibody also blocked the inhibitory effects of EGCG on IGF-increased levels of pERK1/2, number of cells, and BrdU incorporation. Interestingly, EGCG tended to increase the IGF-stimulated hydrogen peroxide levels in the culture medium when incubated with 3T3-L1 preadipocytes (Supporting Information Fig. 3) and pretreatment with *N*-acetylcysteine (NAC; see Supporting Information) did not

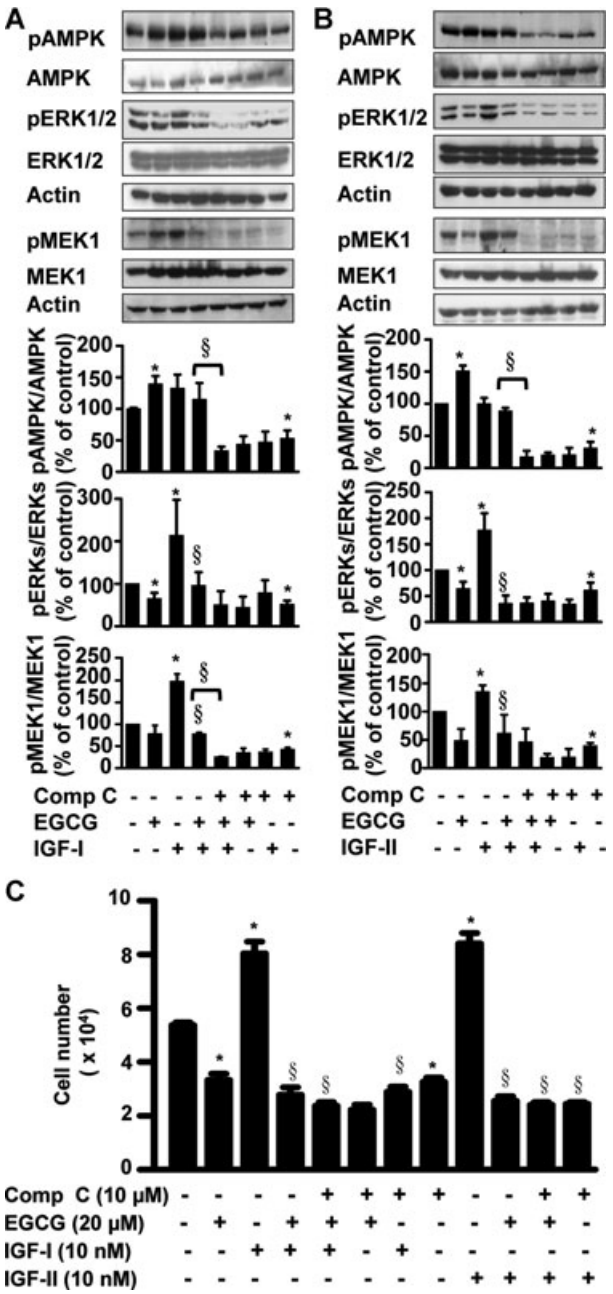

Figure 8. The AMPK inhibitor, Compound C (Comp C), did not prevent the inhibitory effects of EGCG on IGF-I- (A) or IGF-II-increased (B) levels of pERK1/2 and pMEK1 proteins, or the number of preadipocytes (C). Amounts of total phosphorylated AMPK (pAMPK), pERK1/2, and pMEK1, as determined by a Western blot analysis, are expressed as a percentage of controls after normalization to the individual total protein levels. Control experiments were conducted without IGF-I, IGF-II, EGCG, or Comp C treatment. Data are expressed as the means ± SEM from triplicate determinations. $^{*}p < 0.05$ versus the control; $^{\S}p < 0.05$ versus IGF-I, or versus IGF-II.

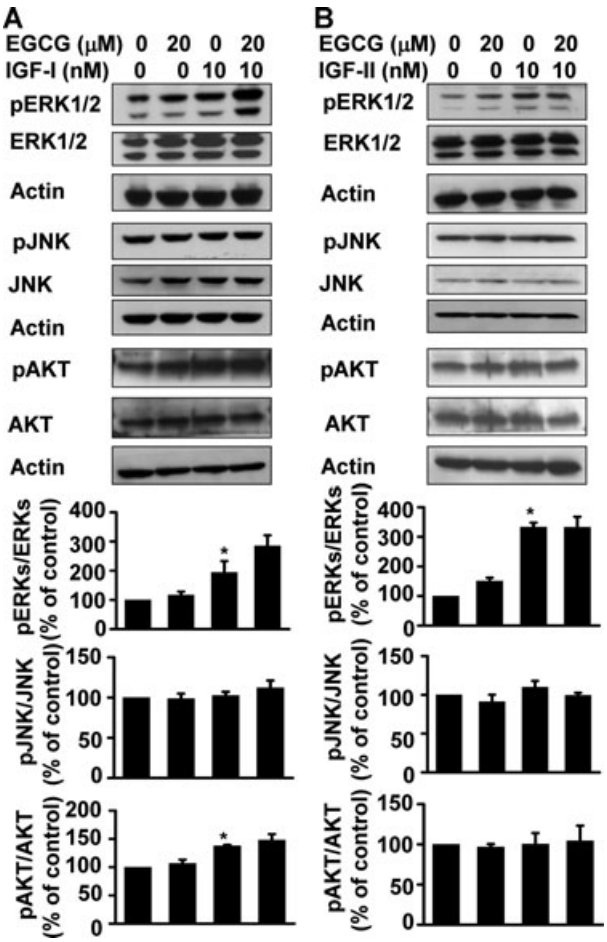

Figure 9. EGCG altered IGF-I- (A) and IGF-II-stimulated (B) protein expression and phosphorylation of ERK, JNK, and AKT of rat H4IIEC3 hepatoma cells when serum-starved cells were treated for 2 h with EGCG in the presence and absence of an additional 60 min of IGF-I or IGF-II treatment. Control experiments were without IGF-I, IGF-II, or EGCG treatment. Data are expressed as the mean ± SEM of triplicate determinations. Amounts of total phosphorylated ERK1/2 (pERK1/2), phosphorylated JNK (pJNK), and phosphorylated AKT (pAKT), as determined by a Western blot analysis, are expressed as a percentage of controls after normalization to the individual total protein levels. In some data, standard error bars are too small to be seen. $*p < 0.05$ versus the control.

block the anti-IGF effects of EGCG (Supporting Information Fig. 4). In addition, NAC in the presence of IGF blocked the mitogenic effect of IGF on 3T3-L1 preadipocytes. A recent report using 3T3-L1 adipocytes indicated that IGF-I stimulated ROS production and inhibited insulin-dependent glucose uptake via the ROS pathway [49]. Since NAC and EGCG can act as antioxidants and since they have the same inhibitory effect on IGF signaling in 3T3-L1 cells, the results of our study could not exclude the possibility that alterations in the levels of ROS and glutathione caused by EGCG may help explain the anti-IGF signaling mechanism of EGCG. Further studies are needed to determine whether other 67LR signaling pathways [2, 47] are necessary for the anti-IGF effects of EGCG.

The AMPK pathway is responsible for EGCG inhibition of insulin-stimulated glucose uptake in HepG2 liver cells [12] and 3T3-L1 adipocytes [14]. However, using Compound C, we demonstrated that EGCG's effect on IGF stimulation of preadipocyte mitogenesis was independent of the functional AMPK. The requirement of AMPK for EGCG's actions on fat cells may vary with the growth phase of cells, the presence of hormone type, or the type of cells.

Green tea EGCG has low bioavailability, possibly dependent on the duration and dosage of treatment, the route of administration; the tissues; and the assay models employed [1, 2, 17, 19–21]. Maybe for these reasons, the average maximal plasma levels and tissue levels of EGCG reported in humans and animals are in the wide range of 0.04∼515 μM and 0.002 to 565 μM, respectively [1, 2, 17, 19–21]. The dose of EGCG that is effective in altering activity of cultured cells in vitro is generally in the range of 10∼100 μM [17]. However, what is not clear at this time is whether effective doses of EGCG can be achieved in fat cells simply by consuming green tea infusions or EGCG capsule. Accordingly, further studies are needed to demonstrate whether the 10∼50 μM doses of EGCG used in this study are compatible with the goal of helping to regulate the initiation and progression of obesity. In our collaborative study, daily intraperitoneal administration of EGCG to rats at a dosage of 70∼92 mg/1 000 g body weight resulted in decreased body weight and adipose weight within 8 days [1]. Additionally, a recent review of epidemiological and intervention studies of green tea summarized that daily consumption of 5∼6 or more cups of green tea, containing a total of 200∼300 mg EGCG, may confer the beneficial effects of maintaining cardiovascular and metabolic health [50].

Previous research has shown that the development of obesity and obesity-related diseases can be regulated by IGFs and EGCG [2, 22, 23, 29, 50–52]. The IGF-I system can increase the number of fat cells; it remains hyperactive in the obese condition, and plays important roles in regulating obesity, diabetes, cancer, metabolic syndrome, and lipid metabolism [23, 51]. Green tea EGCG has been reported to reduce body weight, body fats, adipose tissue weight, and blood levels of glucose, triglyceride, and IGF-I in obese rats [1–4, 17, 22, 50, 52], implying possibility of treating obesity and obesity-associated diseases through alterations of the circulating IGF-I levels, inactivation of the IGF-I signaling pathway, and activation of the EGCG signaling pathway. Here, we have discovered that EGCG inhibition of IGF-I/II's actions may be due to its suppressive effects on the activities of the IGF-IR, RAF1, and MEK1/2, and to the associations of IGF-IR/IIR with particular members of the Shc family. We further showed that EGCG-induced changes in IGF-I and IGF-II signaling in preadipocytes were mediated through the 67LR, but not AMPK, pathway. This information may help clarify the mechanism by which EGCG operates to modulate obesity, diabetes, and metabolic syndrome [1–4, 17, 22, 50, 52].

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