Green tea (−)-epigallocatechin gallate inhibits insulin stimulation of 3T3-L1 preadipocyte mitogenesis via the 67-kDa laminin receptor pathway

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Ku HC, Chang HH, Liu HC, Hsiao CH, Lee MJ, Hu YJ, Hung PF, Liu CW, Kao YH. Green tea (−)-epigallocatechin gallate inhibits insulin stimulation of 3T3-L1 preadipocyte mitogenesis via the 67-kDa laminin receptor pathway. Am J Physiol Cell Physiol 2009; doi:10.1152/ajpcell.00272.2008.—Insulin and (−)-epigallocatechin gallate (EGCG) have been reported to regulate fat cell mitogenesis and adipogenesis, respectively. This study investigated the pathways involved in EGCG modulation of insulin-stimulated mitogenesis in 3T3-L1 preadipocytes. EGCG inhibited insulin stimulation of preadipocyte proliferation in a dose- and time-dependent manner. EGCG also suppressed insulin-stimulated phosphorylation of the insulin receptor-β, insulin receptor (IR) substrates 1 and 2 (IRS1 and IRS2), and mitogen-activated protein kinase pathway proteins, RAF1, MEK1/2, and ERK1/2, but not JNK. Furthermore, EGCG inhibited the association of IR with the IRS1 and IRS2 proteins, but not with the IRS4 protein. These data suggest that EGCG selectively affects particular types of IRS and MAPK family members. Generally, EGCG was more effective than epicatechin, epicatechin gallate, and epigallocatechin in modulating insulin-stimulated mitogenic signaling. We identified the EGCG receptor [also known as the 67-kDa laminin receptor (67LR)] in fat cells and found that its expression was sensitive to growth phase, tissue type, and differentiation state. Pretreatment of preadipocytes with 67LR antiserum prevented the effects of EGCG on insulin-stimulated phosphorylation of IRS2, RAF1, and ERK1/2 and insulin-stimulated preadipocyte proliferation (cell number and bromodeoxyuridine incorporation). Moreover, EGCG tended to increase insulin-stimulated associations between the 67LR and IR, IRS1, IRS2, and IRS4 proteins. These data suggest that EGCG mediates anti-insulin signaling in preadipocyte mitogenesis via the 67LR pathway.

insulin receptor; insulin receptor substrate; mitogen-activated protein kinase

The development of obesity is characterized by an increased number of fat cells and lipid accumulation due to mitogenesis and differentiation. In turn, these processes are regulated by endocrine, genetic, metabolic, neurological, pharmacological, environmental, and nutritional factors (27). Insulin is a key factor in stimulating fat cell mitogenesis and adipogenesis (18). Recently, green tea catechins, polyphenolic flavonoids (37), also known as vitamin P (38), have been proposed as chemopreventative agents for obesity and modulators of fat cell growth (22, 24–26, 29, 31, 32, 49, 50). In particular, (−)-epigallocatechin gallate (EGCG; Fig. 1) has been shown to reduce body weight and body fat in vivo (24–26, 29–31, 49). Other in vivo data (14, 24, 29, 31, 49) have shown that EGCG, or an EGCG-containing green tea extract, reduced food uptake, lipid absorption, blood triglyceride levels, and cholesterol levels; furthermore, EGCG has been shown to stimulate energy expenditure, fat oxidation, high-density lipoprotein levels, and fecal lipid excretion. These in vivo observations may be explained by the following in vitro findings: 1) EGCG, caffeine, and norepinephrine synergistically stimulated thermogenesis in brown adipose tissue (15); 2) EGCG regulated various enzymes related to lipid anabolism and catabolism (24), including acetyl-CoA carboxylase (45, 49), fatty acid synthase (52), pancreatic lipase (40), and lipoxygenase (21); 3) EGCG inhibited the differentiation of preadipocytes to adipocytes (24); and 4) EGCG reduced serum- or insulin-induced increases in cell number and triacylglycerol content during a 9-day period of differentiation (24). Despite the demonstration that EGCG had an influence on the insulin-regulated number of preadipocytes, no studies have demonstrated whether EGCG acted on the insulin signaling pathway for stimulating mitogenesis in preadipocytes. Accordingly, a careful examination of the insulin signaling molecules involved in the anti-insulin effects of EGCG might elucidate the mechanisms that underlie the anti-mitogenic effect of EGCG on fat cells.

Insulin regulates fat cell activity through at least two pathways, the phosphatidylinositol 3-kinase (PI3K)/AKT-mediated pathway and the MEK1/ERK-regulated pathway (48). The PI3K/AKT-mediated pathway plays a central role in regulating metabolic enzymes; the MEK1/ERK pathway appears to control growth (48). In both these pathways, insulin acts by phosphorylating its own receptor and several downstream insulin receptor (IR) substrate (IRS) proteins. Green tea EGCG was found to regulate the phosphorylation of MAP kinase in fat cells (22), and MAP kinase is one of the downstream targets of IR and IRS proteins (48); thus the hypothesis arose that EGCG may regulate the phosphorylation of IR and all four of the IRS proteins.

EGCG stimulation of mitogenesis has been extensively described previously in cancer cells and other cells (1, 3, 11–12, 24, 28–31, 41, 44, 46, 49) but not in fat cells. A putative EGCG receptor known as the 67-kDa laminin receptor (67LR) was discovered in cancer cells (41) and healthy muscle cells, hepatocytes, and nerve cells (34). However, no studies have identified a specific EGCG receptor in fat cells (41). The 67LR was involved in the anti-mitogenic effect of EGCG on cancer cell growth (39), but it is unknown whether the 67LR mediates EGCG effects on healthy cells. It is evident that some types of laminin receptors and laminins are present in preadipocytes and adipocytes and are involved in fat cell differentiation (7, 34–35). However, the nucleotide sequence of a 67LR has not been identified in adipocytes. Thus, it is unknown whether the 67LR plays a role in mediating the actions of...
EGCG on preadipocytes and adipocytes. Further studies are required to determine whether the 67LR is responsible for EGCG-mediated effects on insulin signaling in preadipocyte mitogenesis.

The present study was designed to understand the mechanism underlying EGCG inhibition of insulin-stimulated mitogenesis in 3T3-L1 preadipocytes. We confirmed that EGCG significantly suppressed insulin signaling, and we identified the nucleotide sequence of the 67LR in adipocytes. We showed that pretreatment with the 67LR antiserum prevented the effect of EGCG on insulin signaling. We also found that EGCG increased the insulin-stimulated associations between 67LR and IR, IRS1, IRS2, and IRS4. Our results indicated that the 67LR mediated the effects of EGCG on insulin signaling in preadipocytes.

MATERIALS AND METHODS

Chemical reagents. All active reagents (e.g., insulin and PD-98059) were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. Green tea EGCG and other catechins (>98% pure) were isolated from green tea (*Camellia sinensis*) in our laboratory as described previously (22). Catechins were dissolved in 0.1% DMSO and sterile medium for cell treatment. Penicillin-streptomycin, DMEM, calf serum (CS), trypsin, agarose, and the DNA molecular weight marker were purchased from GibcoBRL of Life Technologies (New York, NY). The 3′-RACE system, TRIzol, Taq polymerase, and the BenchMark pretained protein molecular weight marker were purchased from Invitrogen Life Science Technologies (Carlsbad, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture. According to a previously published method (22), 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA; ATCC-CL-173), rat H4IEC3 hepatoma cells (ATCC-CRL-1600), and human KB oral cancer cells (a gift from Dr. Rong-Nan Huang, a professor of the National Taiwan University, Taiwan) were grown in DMEM (pH 7.4) containing 10% CS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GibcoBRL) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Medium was replaced every 2 days. Because serum components contain factors that facilitate 3T3-L1 differentiation from preadipocytes to adipocytes when cells are confluent, these cells were subcultured before reaching confluency.

Growth inhibition experiments. 3T3-L1 cells (15,000–20,000/cm²) were plated in triplicate wells of 12-well plates. To determine dose- and time-dependent effects of EGCG on insulin-stimulated growth of 3T3-L1 preadipocytes, cells in the log phase of growth (2–3 days after inoculation) were treated with EGCG (0–50 μM) in the presence and absence of insulin (0–1,000 nM) for the indicated time periods. After incubation, cells were trypsinized and counted with a hemocytometer using the 0.4% Trypan blue exclusion method. Cell viability of preadipocytes remained at 90–100% during the 48-h treatment of 10–50 μM EGCG.

Cellular proliferation was measured according to the method described by Hung et al. (22) with a commercially available bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay kit (Roche Applied Science, Mannheim, Germany). Briefly, 3T3-L1 cells (2,000 cells/well) were plated with DMEM supplemented with 10% fetal bovine serum. After allowing 24 h for attachment, cells were starved with serum-free DMEM for 36 h; the medium was then replaced with fresh DMEM containing EGCG (at the indicated concentrations) for 2 h at 37°C. After 12 h of insulin treatment, BrdU (10 μM) was added for another 12 h. This allowed the BrdU to be incorporated newly synthesized DNA of dividing cells during the S-phase of the cell cycle. After incubation, cells were washed with 10 mM PBS and then collected by centrifugation at 1,500 rpm for 5 min. Cell pellets were dried at 60°C for 1 h, fixed with FixDenat solution at 25°C for 30 min, probed with mouse-anti-BrdU-POD (1 h), and visualized with the addition of 3,3,5,5-tetramethylbenzidine substrate for 5 min. An aliquot of 100 μl of 1 N H₂SO₄ was added to stop the reaction with a 1-min incubation on a 300-rpm shaker. The absorbance was read at 450 nm using an MRX microtiter plate reader (Dynatech Laboratories, Chantilly, VA). Culture medium alone and cells incubated with anti-BrdU-POD in the absence of BrdU were used as blank controls for nonspecific binding.

As previously described by Harmon and Harp (20), cytotoxicity was measured by measuring lactate dehydrogenase (LDH) release into the culture medium. Briefly, 3T3-L1 preadipocytes (15,000–20,000 cells/cm²) were plated in triplicate wells of 12-well plates. Vehicle or 20–50 μM EGCG was added to phenol red-free culture medium with or without cells that had been plated with or without 100 nM insulin. At 24 h after plating, all media were collected from the wells and stored for later analysis of the LDH activity. The protein concentrations in the media and lysates were determined in duplicate with a dye-binding method (5) using BSA (Sigma) as the standard. The LDH release assays were performed at 25°C with 50 μl of protein lysate (~5 μg protein) and 200 μl of substrate solution in a final volume of 250 μl per well. The substrate mixture was added to final concentrations of 0.3 mM sodium pyruvate, 0.25 mM β-NADH, and 20 mM Tris·HCl buffer (pH 7.4). The kinetic reaction was measured by absorbance at 340 nm within 5 min from the addition of substrate. One unit of LDH activity was defined as that which caused an initial rate of oxidation of one micromole of NADH per minute under the conditions specified at 25°C. Reproducible release rates were obtained in the range observed with a rabbit muscle LDH (Sigma) standard at 0.00098 to 0.098 micrograms per well [optical density at 340 nm = 0.0098 + 0.003121 × log (μg protein); r² = 0.97]. The LDH protein amounts in the media and lysates were determined in duplicate using rabbit muscle LDH (Sigma) protein as the standard. The percent LDH release was calculated as follows: %LDH release = (LDH in culture medium/LDH in cell lysate) × 100 (20).

Experimental treatments. As previously described by Hung et al. (22), we determined the effect of EGCG at different time points for each experimental condition. This allowed us to determine optimal conditions for EGCG inhibition of the insulin signaling pathway in 3T3-L1 preadipocytes. Serum-starved preadipocytes were pretreated with EGCG (0 and 20 μM) for 1.5 h, and then insulin (0 and 100 nM) was added, unless otherwise stated. To determine the effect of EGCG on the insulin-stimulated association of IRS proteins with the IR and downstream proteins, we used a 5-min insulin incubation. After the
EGCG followed by the addition of insulin, and incubations for the indicated times. To compare the effects of different catechins on the insulin-induced changes in insulin signaling proteins, serum-starved cells were pretreated for 1.5 h with 20 μM of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (ECG), or EGCG, and then 100 nM of insulin was added. After 10 or 60 min of insulin incubation, the levels of phosphorylated IR (pIR), pIRS2, ERK1/2, pERK1/2, pMEK1/2, MEK1, and actin proteins were measured by Western blot analysis. After 12 and 24 h of insulin treatment, BrdU incorporation and the cell number were determined, respectively.

To study the effect of kinase inhibitors on insulin-stimulated growth of 3T3-L1 preadipocytes, serum-starved cells were pretreated for 2 h with PD-98059 (50 μM) (2), U-0126 (10 μM) (17), SB-203580 (10 μM) (16), LY-294002 (50 μM) (6), or wortmannin (100 nM) (17). These compounds were dissolved in 100% DMSO (at a final concentration of 0.1%). After 1, 12, and 48 h of 100-nM insulin incubation, we measured protein kinases levels, BrdU incorporation, and the number of cells, respectively.

To study the 67LR-dependent effect of EGCG on insulin-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 1.5 h, and then incubated with 100 nM insulin. After 5 min, 12 h, and 24 h of insulin treatment, insulin signaling protein levels, BrdU incorporation, and cell numbers were determined, respectively.

Immunoprecipitation. Insulin receptor and IRS proteins were immunoprecipitated according to the method described by Hung et al. (22) and Chen et al. (9, 10), with a minor modification; to obtain whole-protein extracts from preadipocytes, we used Wito’s lysis buffer, which contained 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 Na3VO4, 1 mM PMSF, 15 mM 2-mercapto-ethanol, 0.25% NP-40, and protein inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). The lysate was agitated for 15 min at 4°C and then centrifuged at 14,000 rpm for 10 min. An aliquot of the supernatant (1 mg of protein) was preincubated for 1 h at 4°C with antibodies specific for the IR, IRS, or phosphotyrosine. A control supernatant aliquot was incubated with preimmunized NRS for 1 h at room temperature. Then, the samples were incubated with 20 μl protein A-agarose overnight at 4°C. Total amounts of IR, pIR, IRS, pIRS, IRS, pRAF1, pMEK1/2, and 67LR in the immunoprecipitates were measured by Western blot analysis with isofrom-specific antibodies. After normalization to the total IR or IRS protein, the amounts of insulin signaling proteins and 67LR were expressed as the percentage of control to indicate changes in the binding to IR or IRS. Data obtained from NRS were not shown due to insignificant changes.

Western blot analysis. Immunoblot analysis was performed as described by Hung et al. (22). An aliquot of 50 μg protein was separated by 12% SDS-PAGE with 2x gel-loading buffer [100 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 10% β-mercaptoethanol] and then blotted onto Immobilon-NC transfer membranes (Millipore, Bedford, MA). The membranes were

Fig. 2. Epigallocatechin gallate (EGCG) prevented the mitogenic effect of insulin in 3T3-L1 preadipocytes. All experiments included 1.5 h pretreatment with EGCG, followed by the addition of insulin, and incubations for the indicated times. A: EGCG (20 μM) time dependently blocked insulin (100 nM)-induced increases in MEK1 activity in preadipocytes, as indicated by changes in phosphorylated (p)ERK1/2. The levels of unphosphorylated proteins (ERK1/2) remained unaltered. The control was evaluated at time 0, when EGCG was first added in the absence of insulin. B: EGCG dose dependently blocked insulin-induced increases in MEK1 activity. After 10, 60, and 240 min incubations with 10 or 100 nM insulin, all concentrations of EGCG reduced the insulin effect on pERK. In contrast, EGCG-mediated changes in pAKT levels required longer insulin incubations and higher concentrations of EGCG. C: EGCG dose dependently blocked insulin-induced increases in preadipocyte bromodeoxyuridine (BrdU) incorporation after 12 h of insulin. D: EGCG dose dependently blocked insulin-induced increases in preadipocyte bromodeoxyuridine (BrdU) incorporation after 12 h of insulin. In B–D, control experiments were conducted without insulin treatment in the absence of EGCG. All graphical data are expressed as means ± SE from triplicate determinations. In some data, standard error bars are too small to be seen. In C and D, groups with different letters differ significantly (P < 0.05) from each other. *P < 0.05 vs. control. §P < 0.05 insulin vs. EGCG + insulin.
blocked for 1 h at room temperature with 10 mM PBS containing 0.1% Tween 20 (PBST) and 5% fat-free milk. After washing with PBST, membranes were incubated with specific antibodies. All primary antibodies (IR, pIR, IRS, pIRS, RAS, RAF1, pRAF1, ERK-1, ERK-2, pERKs, MEK1, pMEK1/2, pp38, JNK, pJNK, pAKT, p/67LR, and β-actin antisera) were used at a dilution of 1:1,000 (~0.2 μg/ml). The secondary antibody (donkey anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-goat IgG, or goat anti-guinea pig IgG conjugated with horseradish peroxidase) were used at a dilution of 1:2,000 (~0.2 μg/ml). The immunoblots were visualized by adding a Western Lightning chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA) for 3 min and exposing to Fuji film for 2–3 min. They were then quantified with a Molecular ImagerFX Pro Plus (Bio-Rad Laboratories, Hercules, CA). After normalization to β-actin protein, the levels of intracellular proteins were expressed as a percentage of the control, unless noted otherwise.

Statistical analysis. Data are expressed as means ± SE, unless noted otherwise. The 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. Statistical analyses were performed using SigmaStat (Jandel Scientific, Palo Alto, CA) and data that were logarithmically transformed.

RESULTS

Green tea EGCG inhibited insulin-stimulated mitogenesis of 3T3-L1 preadipocytes. We optimized the conditions for insulin-stimulated mitogenesis of 3T3-L1 preadipocytes via the ERK pathway (supplemental Figs. 1 and 2; supplemental material for this article is available online at the American Journal of Physiology: Cell Physiology website), as previously described (8, 47). In a previous report, we showed that green tea EGCG inhibited MEK1/2 activity in 3T3-L1 preadipocytes (22) and reduced the number of cells during insulin-stimulated differentiation (26). Here, we examined the concentration- and time-dependence of these EGCG effects (Fig. 2). First, we observed that 20 μM EGCG significantly inhibited the insulin-induced increase in MEK1/2 activity within 1 h (indicated by increased amounts of pERK1 and pERK2 proteins with 100 nM insulin). This EGCG inhibition was time dependent (Fig. 2, A and B) and dose dependent (Fig. 2B). Independent of insulin dosage, about 20 μM of EGCG was able to reduce insulin-stimulated MEK1/2 activity by 50% within 10 min of insulin treatment. The EGCG effects on insulin stimulation of pERKs (~10 min) were significantly more acute than the EGCG effects on insulin stimulation of pAKT (~4 h). The total

![Fig. 3. EGCG altered insulin-stimulated phosphorylation of insulin signaling molecules in 3T3-L1 preadipocytes. All experiments included 1.5 h pretreatment with EGCG, followed by the addition of insulin, and incubations for the indicated times. A: EGCG (20 μM) time dependently reduced insulin-stimulated levels of phosphorylated insulin receptor (pIR) substrate 1 (pIRS1), pIRS2, and pIR proteins. The control was evaluated at time 0, when EGCG was first added. B: EGCG dose dependently reduced insulin-stimulated levels of pIRS1, pIRS2, and pIR proteins in the presence or absence of 5 min of insulin. C and D: EGCG reduced the insulin-stimulated levels of pRAF and pMEK1/2 after 5 min of insulin, but not total RAF1 or MEK1 proteins. E: EGCG did not significantly reduce the insulin-stimulated levels of pJNK or JNK. However, 5 min of insulin did not significantly stimulate these proteins. In B–E, control experiments were conducted without insulin or EGCG treatments. Data are expressed as means ± SE of triplicate determinations. Total protein levels were determined with cytosolic protein lysates. Protein phosphorylation levels of the IR, IRS1, IRS2, RAF1, MEK1, and JNK were determined by Western blot analysis and are expressed as a percentage of the control after normalization to the individual total protein levels. In some data, standard error bars are too small to be seen. *P < 0.05 vs. control. §P < 0.05 insulin vs. EGCG insulin.]
protein levels of ERK1/2 and MEK1 were not significantly changed by 20 μM EGCG in the presence of insulin over the 4-h experiment. To further investigate the selectivity of EGCG inhibition, we assessed changes in the activities of MKK4/7 by examining changes in the phosphorylation of their protein substrate, JNK MAPK (data are shown in Fig. 3). We found that 20 μM EGCG for 1 h did not significantly alter the amounts of pJNK. Thus, the EGCG effects appeared to be selective for MEK1/2. When we examined the effects of EGCG on preadipocyte proliferation, we found that EGCG dose dependently prevented insulin-stimulated increases in both cell number (Fig. 2C) and BrdU incorporation (Fig. 2D).

**EGCG inhibited the phosphorylation of insulin signaling molecules.** To further characterize the effects of EGCG on insulin-stimulated preadipocyte proliferation, we examined changes in the activities of the IR and downstream signaling proteins, including RAF1 (an upstream kinase for MEK1), MEK1/2, and phosphorylated IRS proteins (8, 19, 36, 47). To investigate EGCG effects on IR activation, we pretreated preadipocytes in the presence and absence of 20 μM EGCG for 0.5–1.5 h, then added 100 nM insulin for 5-min (supplemental Figs. 2 and 3). We found that EGCG time dependently reduced insulin-increased levels of pIRβ, pIRS1, and pIRS2 proteins (Fig. 3A). At 20 μM EGCG, insulin-stimulated phosphorylation of IRβ, IRS1, and IRS2 was significantly reduced with 0.5, 0.5, and 1 h of insulin treatment, respectively. At a given duration of insulin treatment, EGCG also dose dependently prevented insulin-induced increases in the levels of these phosphorylated proteins (Fig. 3B). EGCG treatment did not alter total protein levels of IR, IRS1, or IRS2.

To examine whether EGCG altered the insulin-stimulated activities of proteins downstream of IRS, we measured changes in the phosphorylated forms of RAF1 and its substrates, the MEK1/2 proteins (Fig. 3, C and D). The RAS signaling pathway is required for the phosphorylation of RAF1 (42); thus the activity of RAS was also assessed by changes in levels of phosphorylated RAF1. We observed that EGCG significantly reduced the insulin-increased levels of pRAF1 (Fig. 3C) and pMEK1/2 (Fig. 3D) proteins. In the presence and absence of insulin, EGCG did not alter the total amounts of RAS (data not shown), RAF1 (Fig. 3C), or MEK1 (Fig. 3D). Treatment with EGCG alone did not significantly alter the phosphorylation of RAF1 or MEK1/2. When we measured changes in the amounts of RAF1 and MEK1/2 proteins (Fig. 3, C and D), we observed that EGCG at 20 μM for 1 h did not significantly alter the insulin-stimulated amounts of pRAF1.

Next, we examined the effects of EGCG on insulin regulation of the IRS4 protein (Figs. 3 and 4). Preadipocytes were pretreated with 20 μM EGCG for 90 min, and then 100 nM insulin was added for 5 min. We measured changes in the amounts of IRS4 and pIRS4 proteins. In contrast to the insulin-stimulated stimulation of IRS1 (Fig. 3) and IRS2 (Fig. 4), insulin reduced the phosphorylation of IRS4 (Fig. 4). The insulin reduction of IRS4 phosphorylation was further inhibited by EGCG. Similar to the results for IRS1 (Fig. 3B) and IRS2 (Figs. 3B and 4A), the total protein amounts of IRS4 were not significantly altered by treatments with EGCG, insulin, or both (Fig. 4B). Treatment with EGCG alone did not significantly alter the phosphorylation of IRS2, but reduced the levels of pIRS4.

**EGCG altered insulin-stimulated associations of IRS proteins with the IR and downstream proteins.** Changes in the phosphorylation of IRS1 and IRS2 can be regulated by an association with the IR (4, 8, 19, 36). Accordingly, we examined the possibility that EGCG effects on insulin-stimulated phosphorylation of IRS1 and IRS2 proteins were mediated through associations with the IR (Fig. 5). Cytosolic protein lysates were subjected to immunoprecipitation with IR antisera, and the associated proteins were probed with antibodies specific for pIRS1 and pIRS2 on a Western blot. We found that
EGCG pretreatment inhibited the insulin-stimulated association of the IR with either pIRS1 or pIRS2 (Fig. 5, A and E). Similar results were obtained when cytosolic protein lysates were subjected to immunoprecipitation (IP) with antisera against the IR protein (A); the IRS1 protein (B); the IRS2 protein (C); or the IRS4 protein (D) and then probed with the indicated antibodies on Western blots (WB). E: the levels of pIR, pIRS1, pIRS2, pIRS4, RAS, pRAF1, and pMEK1/2 proteins are expressed as a percentage of the controls after normalization to the immunoprecipitated proteins (shown in the top rows in A–D). Control experiments were conducted without insulin or EGCG treatments. Data are expressed as means ± SE of triplicate determinations. *P < 0.05 vs. control. §P < 0.05 insulin vs. EGCG + insulin. ND, not determined.

EGCG pretreatment inhibited the insulin-stimulated association of the IR with either pIRS1 or pIRS2 (Fig. 5, A and E). Similar results were obtained when cytosolic protein lysates were subjected to immunoprecipitation with antibodies to either IRS1 or IRS2 and then probed with antibodies to IR (data not shown), pIRβ, pIRS1, or pIRS2 on a Western blot (Fig. 5, B, C, and E). Finally, EGCG treatment also partially inhibited the insulin suppression of the IRS4 association with pIRβ (Fig. 5, D and E). Although EGCG increased by 27% the insulin-stimulated association between IRS4 and RAS protein, the effect was not statistically significant.

EGCG effects on insulin signaling compared with other green tea catechins. Next, we investigated the specific effects of green tea catechins on insulin-stimulated mitogenesis of preadipocytes. Serum-starved 3T3-L1 preadipocytes were pretreated with 20 μM of one of the four green tea catechins: EC, EGC, ECG, or EGCG (Fig. 1) for 1.5 h, followed by the addition 100 nM of insulin (Fig. 6). After 48 h of insulin treatment, we observed that EGCG was generally the most effective in reducing insulin-stimulated increases in the number of preadipocytes (Fig. 6A). Similarly, EGCG caused the greatest reduction of BrdU incorporation compared with the other tea catechins (Fig. 6B). We also examined the effects of green tea catechins on insulin signaling molecules in insulin-stimulated preadipocyte mitogenesis by measuring changes in the levels of pERK1/2, pMEK1/2, pIRS2, and pIRβ proteins (Fig. 6C). In general, we found that EGCG was more effective than the other tea catechins at reducing insulin-stimulated phosphorylation of these proteins. The total amounts of ERK1/2, MEK1, and pJNK (data not shown) proteins did not
change with insulin in the absence or presence of each cate-
chin.

The anti-insulin activity of EGCG depended on the 67LR pathway. An EGCG receptor was previously discovered in lung cancer cells; it is a nonintegrin receptor with an 885-bp coding region, and is also known as the 67-kDa laminin receptor (67LR) (41). We identified the 67LR in fat cells and found that its gene expression was sensitive to growth phase, tissue type, and differentiation state (supplemental Figs. 4 and 5). We investigated whether the anti-insulin effect of EGCG on preadipocyte mitogenesis was mediated through this EGCG receptor pathway (41). 3T3-L1 preadipocytes were pretreated with 67LR antiserum for 1 h, incubated with or without EGCG for 1.5 h, and then stimulated with 100 nM insulin for 5 min (Fig. 7). We found that pretreatment with a 67LR antibody prevented the inhibitory effect of EGCG on insulin signaling, as indicated by unaltered insulin-mediated increases in the phosphorylation of IRS2, RAF1, and ERK1/2 proteins (Fig. 7, A–C). In parallel, we found that the 67LR antiserum blocked the inhibitory effects of EGCG on insulin-stimulated preadi-

Fig. 6. Catechin-specific effects of green tea on the insulin-induced increases in cell viability (A), cell proliferation (B), and amounts of pERK1/2, pMEK1/2, pIR, and pIRS2 (C), but not the total proteins of ERK1/2 or MEK1, in 3T3-L1 preadipocytes. A: cell viability was examined by the Trypan blue exclusion method after 48 h of 100 nM insulin treatment in the presence and absence of 20 μM of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), or EGCG. B: cell proliferation was examined by the BrdU incorporation method after 12 h of 100 nM insulin treatment. C: cells were pretreated with or without 20 μM of EC, ECG, EGC, or EGCG, and then stimulated with 100 nM insulin for 10 or 60 min. Amounts of total proteins of ERK1/2, pERK1/2, MEK1, pMEK1, pIRS2, and pIR were determined by Western blot analysis and then expressed as a percentage of the control after normalization to actin protein. Control experiments were those without insulin or EGCG treatment. In all insulin-treated groups, groups with different letters are significantly different (P < 0.05) from each other. *P < 0.05 vs. control. Data at 10 min are expressed as means ± SE from triplicate determinations, while some of those at 60 min are representative of one determination pooled from four 10-cm culture plates. For pERK data at 10-min condition, the summary histogram was calculated from the five determinations.
No cytotoxic effect of EGCG. To determine whether EGCG induced alterations in insulin signaling through cytotoxic effects, we measured LDH release into the culture medium in response to a 24-h EGCG treatment (Fig. 9). Although 3T3-L1 preadipocytes released roughly 11–22% LDH over 24 h of incubation with 20 \( \mu \)M EGCG alone or 100 nM of insulin alone, the difference in %LDH release between the two treatments was not statistically significant (Fig. 9C). However, EGCG alone at 50 \( \mu \)M reduced LDH release by 50%. In insulin-treated preadipocytes, 20 or 50 \( \mu \)M EGCG treatments tended to reduce the release of LDH; however, the reductions were not statistically significant (Fig. 9C).

**EGCG effects are cell type specific.** We examined whether the effects of EGCG on downstream insulin signaling was cell type specific. We compared the results in 3T3-L1 preadipocytes (Fig. 3) with results from the same assays performed in rat H4IIEC3 hepatoma cells (Fig. 10A) and human KB oral cancer cells (Fig. 10, E–H). In contrast to the EGCG effects observed in 3T3-L1 cells (Figs. 2 and 3), we found in H4IIEC3 hepatocytes that 20 \( \mu \)M EGCG alone for 1.5 h tended to reduce the levels of pMEK1/2 (Fig. 10B), pAKT (Fig. 10C), and pJNK (Fig. 10D), but not pERK1/2 (Fig. 10A) proteins. Interestingly, in KB cells, EGCG alone induced significant increases in the phosphorylation of ERK1/2 (Fig. 10E) and MEK1/2 (Fig. 10F), but not AKT (Fig. 10G) or JNK (Fig. 10H) proteins. In H4IIEC3 cells, with the same EGCG treatment followed by a 5-min incubation of 100 nM insulin, the insulin-stimulated phosphorylation of JNK was significantly suppressed (Fig. 10D), but not that of ERK (Fig. 10A), MEK1/2 (Fig. 10B), or AKT (Fig. 10C) proteins. In KB cells, EGCG tended to increase insulin-stimulated levels of pERK (Fig. 10E), pMEK1/2 (Fig. 10F), pAKT (Fig. 10G), and pJNK (Fig. 10H) proteins, but we found no statistically significant differences between insulin alone and the combined insulin and EGCG effects on these phosphoprotein levels.

**DISCUSSION**

Various laboratory studies have contributed to the proposal that green tea EGCG is a chemopreventative for obesity and a modulator of fat cells (22, 24–26, 29, 31, 32, 49, 50). To extend the findings of a preliminary report (26), this study provided an in-depth understanding of the mechanism underlying the effects of EGCG on insulin regulation of preadipo-
cyte mitogenesis. The insulin-induced increase in the number of preadipocytes was prevented by EGCG, and this preventative effect was attributable to its inhibition of BrdU incorporation, a measure of DNA replication. We found that the anti-insulin effect of EGCG on the growth of 3T3-L1 preadipocytes depended on the ERK pathway. This contention was supported by the findings that exposure to EGCG inhibited the insulin-induced increases in phosphorylated ERK1/2 proteins in 3T3-L1 preadipocytes; furthermore, EGCG did not alter the total amounts of MEK1, ERK1, ERK2, or pJNK proteins. This suggested that EGCG acted on a specific type of MAPK that was stimulated by insulin. In support of this notion, EGCG treatment significantly blocked the insulin-stimulated phosphorylation of the upstream signaling molecules, pMEK1/2, pRAF1, and the IRS proteins. Moreover, EGCG treatment significantly blocked the insulin-stimulated associations of IRS1 and IRS2 proteins with RAF1. Taken together, these observations suggest that the anti-insulin effect of EGCG on preadipocyte mitogenesis is mediated through the IRS/RAS/MEK1/ERK pathway. We also found that 20–50 μM EGCG in the presence of 100 nM insulin for 4 h, but not for 10 min, caused reductions in insulin-increased pAKT levels. Thus, we could not completely exclude a possible role of the PI3K/AKT pathway in the anti-insulin effect of EGCG on preadipocyte mitogenesis. We did not determine whether the PI3K/AKT pathway was affected by EGCG cross-talk or by changes in the IRS/RAS/MEK1/ERK pathway.

We showed that EGCG significantly inhibited insulin signaling by reducing the activities of the IR and its downstream proteins, RAS, RAF1, and MEK1/2, as indicated by the reduced levels of phosphorylated IRβ, IRS1, IRS2, IRS4, RAF1, MEK1/2, and ERK1/2 proteins. This effect of EGCG was not due to changes in the total amount of protein because the total levels of unphosphorylated IR, IRS1, IRS2, IRS4, RAS, RAF1, MEK1, or ERK1/2 proteins were unaltered. A possible explanation for the inhibitory effects of EGCG was the observation that EGCG inhibited the association between the IR and IRS1 and IRS2. Interestingly, EGCG reversed insulin inhibition of the association between IRS4 and pIRβ. These data suggest that EGCG may use different mechanisms for regulating the insulin-stimulated associations of IRS1, IRS2, and IRS4 with the IR. In support of this contention, we observed that EGCG treatment had different effects on the insulin-stimulated associations of IRS1, IRS2, and IRS4 with the IR. We also found that 20 –50 μM EGCG in the presence of 100 nM insulin for 4 h, but not for 10 min, caused reductions in insulin-increased pAKT levels. Thus, we could not completely exclude a possible role of the PI3K/AKT pathway in the anti-insulin effect of EGCG on preadipocyte mitogenesis. We did not determine whether the PI3K/AKT pathway was affected by EGCG cross-talk or by changes in the IRS/RAS/MEK1/ERK pathway.

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Fig. 8. EGCG affected the insulin-stimulated associations between insulin signaling proteins and the 67LR in 3T3-L1 preadipocytes. Serum-starved preadipocytes were pretreated with 5 μg/ml 67LR antiserum for 1 h, exposed to 20 μM EGCG for 1.5 h, and then incubated with 100 nM insulin for 5 min. Cytosolic protein lysates were subjected to immunoprecipitation with antisera against the IR protein (A); the IRS1 protein (B); the IRS2 protein (C); or the IRS4 protein (D). Left: immunoprecipitated proteins were transferred to membranes and then probed with the indicated antibodies on Western blots. Right: levels of the 67LR protein are expressed as a percentage of the control after normalization to the immunoprecipitated proteins (shown in the bottom left rows in A–D). Control experiments were conducted without insulin or EGCG treatments. Data in A are representative of one determination pooled from four 10-cm culture plates; data in B–D are expressed as means ± SE from triplicate determinations. *P < 0.05 vs. control, or insulin vs. EGCG + insulin. 

Fig. 9. No cytotoxicity of (−)-epigallocatechin-3-gallate (EGCG) in 3T3-L1 preadipocytes. Serum-starved cells were pretreated with 20–50 μM EGCG or vehicle for 1.5 h, and then 100 nM insulin was added for 24 h. Lactate dehydrogenase (LDH) was measured in the culture medium (A) and in cell lysates (B). C: cytotoxicity was expressed as %LDH release. Data are expressed as means ± SE from triplicate determinations. *P < 0.05 vs. control, or insulin vs. insulin + EGCG (bracket).
ciations of individual IRS proteins with RAS, pRAF1, and pMEK1/2. Indeed, the fact that IRS4 has been shown to negatively regulate the action of IRS1 and IRS2 (42) may also support our findings.

Mechanistic studies of green tea indicated that EGCG has cell type-dependent effects (29, 30); however, these variations may have been due to differences in cell culture techniques or assay methods (29). In this study, we looked at whether EGCG effects on downstream insulin signaling were cell type specific. We used the same experimental assay methods to compare our results in 3T3-L1 preadipocytes to those in rat H4IIEC3 hepatoma cells and human KB oral cancer cells. Despite our findings that EGCG alone did not affect phosphorylation of insulin signaling molecules in 3T3-L1 preadipocytes, we found that EGCG alone reduced the levels of pMEK1/2, pAKT, and pJNK proteins in H4IIEC3 hepatocytes. In contrast, EGCG alone induced significant increases in pERK1/2 and pMEK1/2 in KB cells. In 3T3-L1 preadipocytes, EGCG suppressed insulin-stimulated phosphorylation of ERK1/2, MEK1/2, but not AKT or JNK, proteins. In H4IIEC3 cells, EGCG significantly suppressed insulin-stimulated phosphorylation of JNK, but not ERK, MEK1/2, or AKT proteins. In KB cells, we found no statistically significant effects of EGCG on insulin-stimulated phosphoprotein levels. Taken together, these findings demonstrate that the effects of green tea EGCG on downstream insulin signaling are cell type dependent. One possible explanation for the disparate findings among the three cell lines studied here is that normal, transformed, and cancer cell lines may have different sensitivities to green tea EGCG. To support these observations, our previous report showed that the IC50 value of EGCG for reducing the cell number was lower in 3T3-L1 cells than in 3T3 fibroblast and KB cells (22). Furthermore, the inhibition of insulin-induced phosphorylation of ERK1/2 proteins after 48-h EGCG treatment was much less in...
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3T3-L1 cells than that observed in KB cells (22). An alternative explanation is that the differences in EGCG effects among cell lines may be due to disparate actions of insulin on kinases in different cells. This statement is supported by our findings that 100 nM insulin alone for 5 min induced a significant increase in the phosphorylated JNK of H4IIEC3 cells (but not KB or 3T3-L1 cells), and significant increases in the phosphorylated ERK1/2 and MEK1/2 of KB and 3T3-L1 cells (but not H4IIEC3 cells).

Green tea catechins have numerous biological activities that provide various health benefits (22, 24–26, 29, 31, 39, 49–52). In most cases, but not all, gallated catechins, especially EGCG, are more active than other catechins. This contention is supported by our findings in 3T3-L1 preadipocytes that at the same dose and duration of treatment, EGCG was generally more effective than EC, ECG, and EGC at changing insulin-stimulated increases in the number of cells, the amount of incorporated BrdU, and the levels of pIR, pIRS2, pMEK1/2, and pERK1/2. The observed catechin-specific effects suggested that EGCG may act through a different mechanism than EC, EGC, and EGC in regulating the insulin signaling molecules involved in preadipocyte growth. The catechins tested have a common structure of three aromatic rings with different numbers of hydroxy groups that may be important for hydrogen bonding (Fig. 1) (22, 24, 29, 31, 37, 49). EGCG is unique among the tea catechins because it contains the largest number of hydroxy groups (37) and has both gallyl and galloyl groups (37), which confer conformational flexibility. Thus, the different structural aspects of the catechins may be important for interactions with other molecules.

We identified an 885-bp sequence in murine primary and secondary adipocytes that encoded the adipocyte 67LR with 99–99.7% amino acid identity and 88.6–95.8% nucleotide identity with the 67LR of the rat, human, and pig (13, 23, 33). Interestingly, our results showed that 67LR expression varied with the location of adipose tissue and with the growth and differentiation of fat cells. We found different levels of 67LR mRNA and protein in preadipocytes and adipocytes, suggesting that this may underlie their different responses to EGCG. In support of this notion, we observed that 20 μM EGCG significantly reduced the insulin-stimulated phosphorylation of ERK1/2, IRβ, and IRS1 proteins in 3T3-L1 preadipocytes; however, in 3T3-L1 adipocytes, 20 μM EGCG only significantly reduced insulin stimulation of ERK1/2 phosphorylation, while 50 μM EGCG was required to suppress insulin-stimulated increases of IRβ and IRS1 phosphorylation (data not shown).

The 67LR-mediated effect of EGCG on mitogenesis in cancer cells and nonadipocyte cells has received increasing attention (41, 43). To our knowledge, no information is available concerning the 67LR-mediated effect of EGCG on insulin signaling. In this study, we identified the existence of the 67LR in preadipocytes and adipocytes. With the 67LR antibody, we demonstrated that the EGCG effect on insulin stimulation of preadipocyte mitogenesis was dependent on the functional 67LR. When the 67LR was blocked with the antibody, the inhibitory effect of EGCG on insulin-increased levels of pIRS2, pRAF1, and pERK1/2 was blocked. This was most likely due to the fact that EGCG tended to enhance the insulin-stimulated association of IR, IRS1, IRS2, or IRS4 with the 67LR protein. These results suggest that the 67LR may act on insulin signaling, possibly through the association of the 67LR protein with the IR or with a particular type of IRS protein.

We conclude that the stimulatory effect of insulin on mitogenesis of 3T3-L1 preadipocytes is dependent on the ERK MAPK pathway and is likely mediated through increases in MEK1 activity. While it has previously been shown to be mainly mediated by the ERK pathway, we showed that insulin signaling was independent of the p38 MAPK pathway. We demonstrated that the mitogenic effect of insulin on preadipocytes is inhibited by green tea EGCG in a dose- and time-dependent manner. EGCG inhibition of insulin actions may be due to its suppressive effects on the activities of the insulin receptor, RAS, RAF1, and MEK1/2 proteins, and on the association of these proteins with particular members of the IRS family. Among the four major green tea catechins, EGCG is more effective than EC, ECG, and EGC at regulating insulin-mediated mitogenic signals. Finally, we showed that EGCG-induced changes in insulin signaling in preadipocytes can be mediated through the 67LR.

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