

Inhibitory effect of green tea (–)-epigallocatechin gallate on resistin gene expression in 3T3-L1 adipocytes depends on the ERK pathway

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Liu, Hang-Seng, Yen-Hang Chen, Pei-Fang Hung, and Yung-Hsi Kao. 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* 290: E273–E281, 2006. First published September 13, 2005; doi:10.1152/ajpendo.00325.2005.—Resistin (Rstn) is known as an adipocyte-specific secretory hormone that can cause insulin resistance and decrease adipocyte differentiation. By contrast, green tea catechins, especially (–)-epigallocatechin gallate (EGCG), have been reported as body weight and diabetes chemopreventatives. Whether EGCG regulates production of Rstn is unknown. Using 3T3-L1 adipocytes, we found that EGCG at 20 and 100 μ M suppressed Rstn mRNA levels by \sim 35 and 50%, respectively, after 3 h. The basal half-life of Rstn mRNA induced by actinomycin D was >12 h but shifted to 3 h in the presence of EGCG. This suggests that EGCG regulates the stability of Rstn mRNA. Treatment with cycloheximide did not prevent EGCG-suppressed Rstn mRNA levels, which suggests that the effect of EGCG does not require new protein synthesis. Intracellular Rstn protein significantly decreased in the presence of 100 μ M EGCG 3 h after treatment, whereas the release of the Rstn protein did not significantly change. This suggests that EGCG may modulate the distribution of Rstn protein between the intracellular and extracellular compartments. EGCG did not affect the amounts of extracellular signal-related kinase-1/2 (ERK1/2), phospho-JNK, phospho-p38, and phospho-Akt proteins but reduced the amounts of phospho-ERK1/2 proteins. Overexpression with MEK1 blocked EGCG-inhibited Rstn mRNA expression. These data suggest that EGCG downregulates Rstn expression via a pathway that is dependent on the ERK pathway.

genistein; mitogen-activated protein kinase; extracellular signal-related kinase

RESISTIN (RSTN) IS A CYSTEINE-RICH POLYPEPTIDE HORMONE (20, 38) that, depending on the species, contains 4–5 exons, 3–4 introns, 575–1217 bp of mRNA, and 108–114 amino acids of protein (10–12.5 kDa) (16, 23, 44, 45). Rstn is first isolated from adipose tissues and found to link obesity to type 2 diabetes (44). In particular, Rstn mRNA expression in adipose tissues of obese humans is higher than that in normal subjects (12). In addition, a single nucleotide polymorphism in the Rstn gene promoter is associated with obesity (14) and diabetes (35), and the plasma Rstn levels are elevated in patients with obesity (12) and type 2 diabetes (59). Moreover, the dominant negative form of Rstn enhances adipogenesis and improves insulin sensitivity (24). Ever since its discovery, Rstn also has been found to possess numerous other actions. For example, Rstn regulates fasted blood glucose (6), causes dyslipidemia (39), suppresses insulin-stimulated glucose uptake in adipo-

cytes (44) and muscle cells (30), inhibits dopamine and nor-epinephrine release in the hypothalamus (8), and promotes endothelial cell activation (50) and smooth muscle proliferation (9). To our knowledge, the expression of Rstn gene can be regulated by nutritional, endocrine, genetic, pharmacological, and developmental factors (5), and the mechanisms of action of Rstn are emerging. For example, Rstn promotes smooth muscle cell proliferation through the activation of extracellular signal-regulated kinase (ERK1/2) and phosphatidylinositol 3-kinase (PI3K) (9) and inhibits insulin signaling through induction of the gene expression of suppressor of cytokine signaling 3 (46).

Despite the importance of Rstn, relatively little is known about the control of production of Rstn and the modulation of its actions (5) by vitamin nutrients. Although a recent study (15) showed that vitamin A reduced Rstn mRNA levels in white and brown adipocytes, the results did not demonstrate whether any of the other vitamins is responsible for the regulation of Rstn gene expression. Green tea catechins (GTCs) are polyphenolic flavonoids that were once called vitamin P (38). Whether GTCs are able to regulate Rstn gene expression is unknown.

GTCs possess multiple functions, including lowering the incidence of certain cancers and diseases in animal models (27, 56). In particular, green tea (–)-epigallocatechin gallate (EGCG; Fig. 1), the majority of GTC, lowers the incidence of streptozotocin-induced diabetes (42) and reduces body weight, body fat, and blood levels of glucose and lipid in leptin receptor-defective obese rats (20, 21). In addition, EGCG protects pancreatic cells (42), enhances insulin activity (2), represses hepatic glucose production (51), reduces food uptake and absorption (20, 27), stimulates thermogenesis (13) and lipid excretion (27), and modulates insulin-leptin endocrine systems (20). Moreover, EGCG inhibits the sodium-dependent glucose transporter (25) and represses various enzymes related to lipid metabolism, such as acetyl-CoA carboxylase, fatty acid synthase, pancreatic lipase, gastric lipase, and lipooxygenase (27, 52). It is also a potent antioxidant (27–29, 56) and reduces serum- or insulin-induced increases in the cell number and the triacylglycerol content of 3T3-L1 adipocytes during a 9-day period of differentiation (21). Whether EGCG has an effect on the production of Rstn or modulation of action of Rstn is unknown. In addition, EGCG has been reported to mimic the effect of insulin on hepatic glucose production through the reduction of phosphoenolpyruvate carboxykinase gene expression (51). These findings, together with observations of insulin (22, 41) to alter gene and protein expression of adipocyte Rstn,

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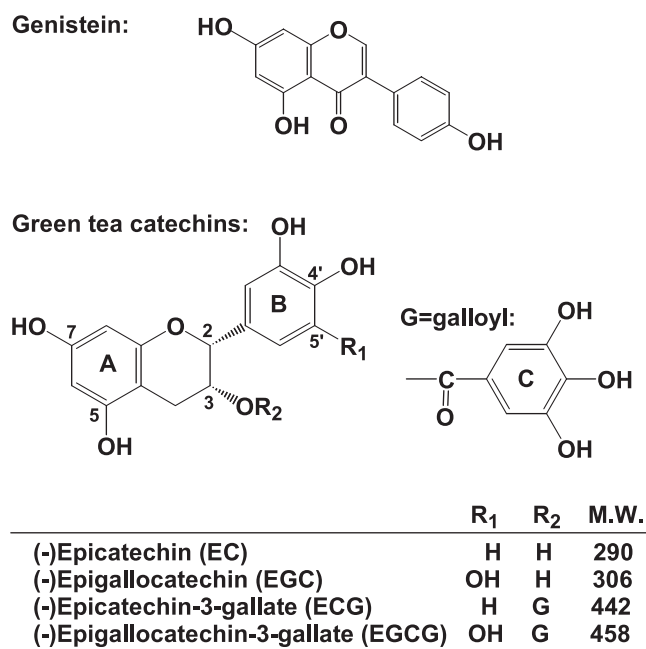


Fig. 1. Structures of genistein and 4 major green tea catechins. Differences among these flavonoids occur in the number of hydroxyl groups, aromatic rings (A, B, C), molecular weight (MW), and presence of a galloyl group.

suggest that EGCG may regulate Rstn gene expression, but this requires further demonstration. Finally, the fact that the signal element(s) responsible for transducing the action of insulin on Rstn gene expression (22) has not been identified has caused much controversy. Accordingly, an examination of the signal element through which EGCG carries out its modulation of Rstn expression should help clarify these observations.

In this study, we used 3T3-L1 adipocytes to examine the effects of green tea EGCG on Rstn gene and protein expression. We hypothesized that EGCG would downregulate Rstn gene and protein expression and that its effects would be dependent on mitogen-activated protein kinase (MAPK) pathways. MAPKs were chosen because they had been reported (1, 11, 27–29) to be essential signal transducers of EGCG in the regulation of other genes.

MATERIALS AND METHODS

Chemical reagents. Green tea EGCG and other catechins (>98% pure) were isolated from green tea (*Camellia sinensis*) in our laboratory, as described previously (20). Catechins were dissolved in sterile medium for cell treatment. Other materials, such as dexamethasone (Dex) and genistein (Fig. 1), were purchased from Sigma (St. Louis, MO) unless otherwise mentioned. Penicillin-streptomycin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, agarose, and 1 Kb Plus DNA ladder marker were purchased from GIBCO-BRL of Life Technologies (New York, NY). Except for the Rstn antibody, which was obtained from Linco Research (St. Charles, MO), all antibodies [ERK1, ERK2, phospho (p)-ERKs, etc.] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The 3'-rapid amplification of cDNA ends system, TRIzol, *Taq* polymerase, and BenchMark prestained protein marker were purchased from Invitrogen Life Science Technologies (Carlsbad, CA).

Cell culture. 3T3-L1 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM (pH 7.4) containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified

atmosphere of 95% air-5% CO₂ at 37°C. 3T3-L1 cells were plated at a density of 12,000–15,000/cm² on a 10-cm plate (18). Every 2 days, 10 ml of medium were replaced before these cells became confluent. To obtain 3T3-L1 adipocytes, 2-day-postconfluent 3T3-L1 preadipocytes (3 × 10⁶ cells/10-cm plate, designated *day 0*) were treated with DMEM containing a final concentration of 10 µM Dex, 0.5 mM 1-methyl-3-isobutylxanthine, and 10% FBS for 48 h (10). The medium was then changed to DMEM containing 10% FBS for an additional 6–10 days (replaced every other day). With this protocol, >90% adipocyte differentiation was achieved, as indicated by phenotypic appearance and triglyceride accumulation (10). Differentiated adipocytes expressed more Rstn mRNA than did preadipocytes or differentiating preadipocytes (10, 44).

For all experiments, adipocytes were incubated with or without green tea flavonoids, Dex, troglitazone, cycloheximide (CHX), actinomycin D (Acti-D), and kinase inhibitors, which were dissolved in 0.1% dimethyl sulfoxide (DMSO) and tested in DMEM with or without 10% FBS. Acti-D (5 µg/ml) and CHX (5 µg/ml) were used to block transcriptional and translational activities, respectively (10, 22). In experiments, 3T3-L1 adipocytes were pretreated with and without either Acti-D for 30 min or CHX for 90 min. Adipocytes were then stimulated with or without EGCG (100 µM) for the indicated time period. After treatment, Rstn mRNA and protein levels were measured. Despite the high dose of some inhibitors used in the experiment, no adverse effects on cell viability of adipocytes for 12–24 h were noted, as examined by the trypan blue exclusion method (data not shown). Concentrations of EGCG at 20 and 100 µM used in this study were determined as reported by Hung et al. (18).

Cell transfection. We followed the methods reported by Hung et al. (18) and transfected transiently for 24 h into 3T3-L1 adipocytes (3 × 10⁶ cells in a 6-cm plate), with 10 µg of pBabe puro vector containing the wild type of MEK1 or a constitutively active mutant MEK1S217E/S221E (designated MEK1EE) cDNA. The empty pBabe vector was considered to be the control. The TransFast transfection reagent (Promega) was in a volume of 45 µl and comprised the synthetic cationic lipid, (+)-*N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[2,3-di(tetradecanoyloxy)propyl] ammonium iodide and the neutral lipid *L*-dioleoyl-phosphatidylethanolamine. The adipocytes were co-transfected with pSV-β-gal for determining the efficiency of the transfection. The protein amounts of MEK1 and p-ERKs were measured by Western blot analysis.

Digoxigenin-PCR ELISA for Rstn mRNA. Rstn mRNA levels were measured using a commercial PCR ELISA kit with digoxigenin (DIG) label and detection (Roche Applied Science, Mannheim, Germany) (10). Total RNA was isolated with the TRIzol kit, and cDNA was then synthesized from equal amounts (5 µg) of RNA by using MMLV reverse transcriptase (Invitrogen). PCR was performed under the following conditions: an initial denaturing cycle at 94°C for 5 min, followed by 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing for Rstn at 60°C and for β-actin at 53°C for 90 s, and extension at 72°C for 90 s. A final extension cycle at 72°C for 10 min was added after the last cycle. The PCR product was run on 1.5% (wt/vol) agarose gel electrophoresis using 40 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA and visualized by 0.5 µg/ml ethidium bromide. The forward and reverse primers were 5'-GTACCCACGGGATGAAGAACC-3' and 5'-GCAGAGCCA-CAGGAGCAG-3' for mouse Rstn (acc. no. AF323080) and 5'-CCAGGGTGTGATGGTGGGAATG-3' and 5'-CGCAGGATTC-CCTCTCAGCTG-3' for actin (acc. no. X03672), respectively. The Rstn and actin PCR products, projected to be 252 and 500 bp, respectively, were cloned into pTargetT vector for subsequent nucleotide sequencing performed by Academia Sinica (Taipei, Taiwan). For the quantification of Rstn and β-actin (the control), they were identified by DIG-labeling RT-PCR and then hybridized with a biotin-labeled Rstn or β-actin cDNA probe. After immobilization on a streptavidin-coated well, they were visualized with an anti-DIG

peroxidase conjugate and the colorimetric substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) at 405 nm. Sample Rstn mRNA levels were determined by relationship to a standard curve of Rstn cDNAs, ranging from 3 to 200 ng/well [OD 405 nm = (0.1141 + 0.0031) × ng DNA/well; $R^2 = 0.998$]. An almost linear range in the number of PCR amplifications for Rstn was observed at between 20 and 40 cycles compared with the β -actin standard. Thus 30 cycles of PCR amplification were later used for all experiments. After normalization to β -actin mRNA, Rstn mRNA levels were expressed as percentage of control.

Western blot analysis. Immunoblot analysis was as described by Chen et al. (10) to obtain whole cell protein extracts. After adipocytes were washed twice with cold 10 mM phosphate-buffered saline (PBS) and lysed in the 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 phenylmethylsulfonyl fluoride. Protein concentration of the lysate supernatant was determined by the Bradford method at 595 nm (7). Fifty micrograms of supernatant proteins were loaded on 12% SDS-PAGE. Gel protein was then blotted onto Immobilon-NC transfer membrane (Millipore, Bedford, MA). The immunoblots were blocked for 1 h at room temperature with 10 mM PBS containing 0.1% Tween 20 (PBST) and 5% defatted milk. After the wash with PBST, immunoblot analyses with primary antibody were performed. All primary antibodies (ERK1, ERK2, p-ERKs, p-p38, p-JNK, p-Akt, β -actin, and Rstn antisera) were used at a dilution of 1:1,000 (~0.2 $\mu\text{g}/\text{ml}$). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-goat IgG, or goat anti-guinea pig were used as the secondary antibodies at a dilution of 1:2,000 (~0.2 $\mu\text{g}/\text{ml}$). The immunoblots were visualized using Western Lighting Chemiluminescence Reagent Plus kit (PerkinElmer Life Science, Boston, MA) for 3 min followed by exposure to Fuji film for 2–3 min. Blots were quantified using a Molecular Imager (Bio-Rad Laboratories, Hercules, CA). After normalization to β -actin protein, levels of the intracellular Rstn protein and kinases were expressed as a percentage of the control unless noted otherwise.

Rstn protein ELISA. A homologous ELISA procedure was adapted from Chen et al. (10) to analyze secreted Rstn protein. The primary antiserum (guinea pig anti-mouse Rstn serum) was used at a dilution of 1:1,000, and the second antiserum (donkey anti-guinea pig IgG serum) was used at a dilution of 1:3,000. The lowest detectable level of Rstn was 5 ng/well. The interassay and intra-assay coefficients of variation in ELISA were 7–9 and 3–4%, respectively. The reproducible results were obtained in the range of Rstn from 5 to 80 ng/well. The resulting dilution curve from the culture medium for testing the specificity of Rstn antiserum was parallel to the Rstn standard. Sample Rstn protein was calculated from the standard curve. Ten percent FBS and Rstn hormone (expressed in *Escherichia coli* and purified with a Ni column) (10) were used as the blank control and the standard, respectively.

Statistical analysis. Data are expressed as means \pm SE. An 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. $P < 0.05$ indicated significance. All statistics were performed using SigmaStat (Jandel Scientific, Palo Alto, CA).

RESULTS

Inhibitory effects of EGCG on Rstn mRNA expression and stability. EGCG inhibited the steady-state levels of Rstn mRNA in a concentration- (Fig. 2A) and time-dependent (Fig. 2B) manner. EGCG at 20 and 100 μM suppressed Rstn mRNA levels by ~35 and 50%, respectively, 3 h after treatment. In addition, treatment of adipocytes with EGCG in the presence or absence of 10% FBS caused similar inhibition of Rstn mRNA expression (Fig. 2A). The possibility that the EGCG-

induced reduction in Rstn mRNA expression resulted from increased Rstn mRNA degradation was also examined (Fig. 2C). 3T3-L1 adipocytes were pretreated with the transcriptional inhibitor Acti-D and then treated with or without 100 μM EGCG. The basal half-life of Rstn mRNA was >12 h, but it rapidly shifted to 3 h in the presence of EGCG (Fig. 2).

Effects of EGCG on Rstn protein expression, secretion, and stability. To determine whether EGCG-suppressed Rstn gene expression also occurred at the level of translation, changes in the intracellular Rstn protein content, as well as release of Rstn into culture medium, were measured (Fig. 3). Intracellular Rstn protein significantly decreased in the presence of 100 μM EGCG 3 h after treatment (Fig. 3A). EGCG had no significant effect on Rstn protein release (Fig. 3B). The possibility that EGCG-induced reduction in Rstn protein expression was related to changes in Rstn protein stability also was examined (Fig. 3, C and D). 3T3-L1 adipocytes were pretreated with the translational inhibitor CHX and then treated with or without 100 μM EGCG (Fig. 3, C and D). The basal half-life of the intracellular Rstn protein was more than 25 h, and EGCG had no further effect on protein levels (Fig. 3C). Similarly, EGCG did not affect the half-life of Rstn protein released into the extracellular medium (Fig. 3D). CHX tended to decrease Rstn protein secretion from 150 to 117 ng/ml after 3 h ($P = 0.09$), from 280 to 140 ng/ml after 12 h ($P < 0.05$), and from 405 to 175 ng/ml after 24 h ($P < 0.05$, data not shown).

Effect of CHX on EGCG-suppressed Rstn mRNA expression. To further examine whether EGCG-suppressed expression of the adipocyte Rstn gene is mediated via other proteins, adipocytes were pretreated with or without CHX for 90 min, and cells were then treated with or without 100 μM EGCG for 3 h (Fig. 4). Treatment with CHX alone did not alter Rstn mRNA expression of adipocytes compared with the control. In addition, pretreatment with CHX did not prevent EGCG-suppressed Rstn mRNA levels.

Changes in ERK MAPKK, p38 MAPKK, JNK MAPKK, and PI3K activities. Whether EGCG-induced downregulation of Rstn mRNA expression depends on the MAPK and PI3K pathways was assessed by changes in the activities of MEK1, p38 MAPKK, JNK MAPKK, and PI3K (Fig. 5). The activities of MEK1, p38 MAPKK, JNK MAPKK, and PI3K were assessed by changes in the amount of the phosphorylated form of their own protein substrates of ERK1 and ERK2, p38, JNK, and Akt, respectively. EGCG significantly reduced the amounts of p-ERK1 and p-ERK2 but did not alter the amounts of total proteins of ERK1, ERK2, p-p38, p-JNK, and p-Akt (Fig. 5A).

Overexpression of MEK1 prevented EGCG-suppressed Rstn expression. To further demonstrate whether EGCG-induced downregulation of Rstn mRNA expression was prevented by overexpression of MEK1, adipocytes were transiently overexpressed with MEK1 or a constitutively active mutant of MEK1S217E/S221E (designated MEK1EE) and then stimulated with or without 100 μM EGCG for 3 h (Fig. 5B). Although EGCG significantly reduced Rstn mRNA levels in empty vector-transfected adipocytes, its effect was prevented by the overexpression of either MEK1 or MEK1EE (Fig. 5B). In the presence or absence of EGCG, changes in β -galactosidase (β -Gal) production (from cotransfection with pSV- β -Gal) in any of the transfected adipocytes did not significantly differ. The amounts of MEK1 and p-ERK proteins in MEK1- or

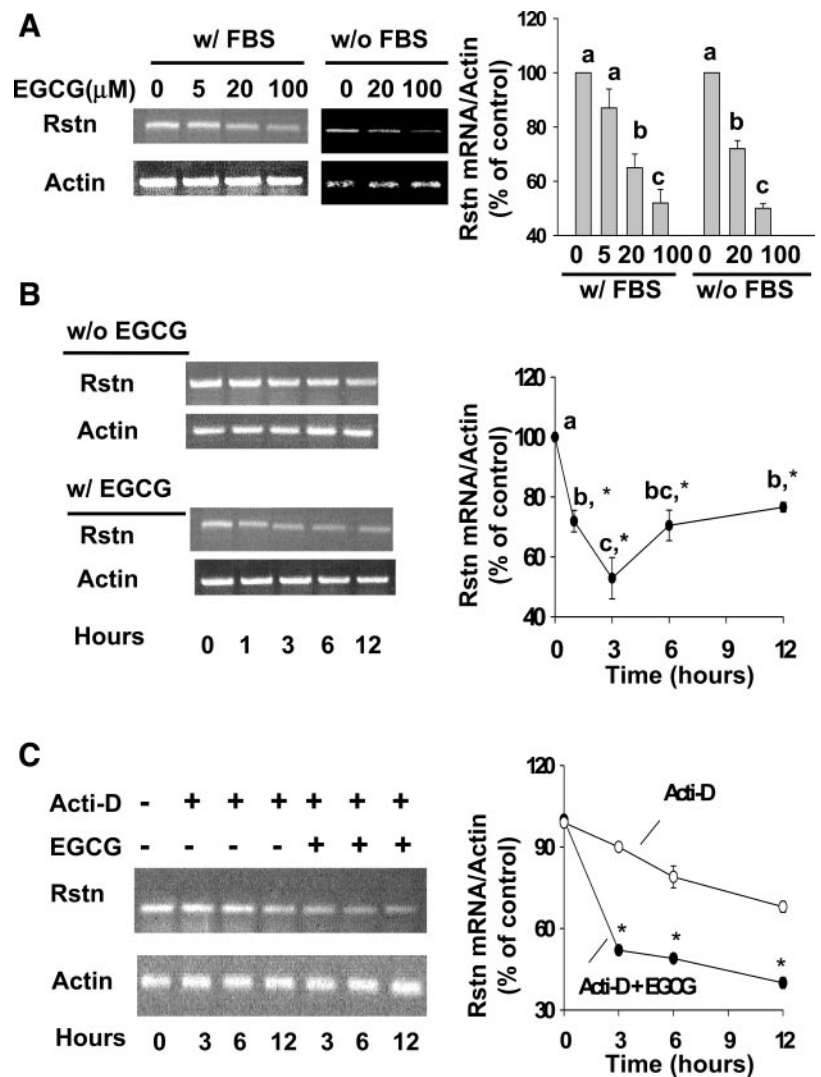


Fig. 2. Inhibitory effect of (–)-epigallocatechin gallate (EGCG) and actinomycin D (Acti-D, 5 μg/ml) in the presence and absence of FBS on resistin (Rstn) mRNA expression in 3T3-L1 adipocytes. **A**: dose-dependent effect of EGCG in the presence and absence of 10% FBS was observed 3 h after treatment. **B**: time-dependent effect of EGCG (100 μM) was observed. **C**: EGCG (100 μM) decreased Rstn mRNA stability. Data are expressed as means ± SE from duplicates of 3 independent experiments. SE bars are too small to be seen in **B** and **C**. In **A**, control experiment was that not treated with EGCG, whereas in **B** and **C**, controls were set at time 0 when EGCG was added in the beginning; a, b, and c: groups with different letters are significantly different ($P < 0.05$) from each other. In **B**, * $P < 0.05$ with EGCG vs. without EGCG at a given time. In **C**, * $P < 0.05$ Acti-D vs. Acti-D + EGCG. *Right*: digoxigenin (DIG)-PCR ELISA; *left*: RT-PCR.

MEK1EE-transfected adipocytes were respectively confirmed, as indicated in Fig. 5C.

Differential regulation of Rstn gene expression by green tea flavonoids, troglitazone, and Dex. We also investigated whether EGCG differed from the two Rstn regulators, Dex (10 μM) and troglitazone (a PPARγ ligand; 0.1 μM), in suppressing Rstn mRNA expression (Fig. 6, A and B). Treatment with either EGCG or troglitazone decreased the level of Rstn mRNA by ~50%, whereas Dex significantly increased the Rstn mRNA level by 125%. Treatment with EGCG and Dex reduced Rstn mRNA expression by ~15% compared with Dex alone, but it was still an 80% increase over control. The flavonoid-specific effect of green tea on the regulation of Rstn mRNA expression was also assessed (Fig. 6, A and C). Genistein at 100 μM stimulated Rstn mRNA expression (Fig. 6A). Actually, genistein at 1 nM effectively induced increases of Rstn mRNA levels (data not shown). By contrast, (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (–)-epicatechin-3-gallate (ECG), and EGCG individually at 100 μM reduced the Rstn mRNA level (Fig. 6C). The latter three catechins seemed to be more effective than EGC in downregulating Rstn mRNA expression.

DISCUSSION

The present study demonstrates a novel effect of green tea EGCG in inhibiting Rstn gene expression in adipocytes. The effects of EGCG were dose and time dependent. It is likely that EGCG regulates Rstn mRNA levels by destabilizing Rstn mRNA. This conclusion is supported by our findings that EGCG shortens the basal half-life of Rstn mRNA induced by Acti-D alone. In higher eukaryotes, the control of mRNA stability is the result of a complex set of events and can be regulated by exogenous factors (37). Unfortunately, the mechanism of the altered stability of Rstn mRNA by EGCG (Fig. 3) is still not clear in this study. However, it was evident that the mechanism of action of different concentrations of EGCG for regulating the expression of a particular gene has consistently been reported to depend on the types of cells (18) and the antioxidant and prooxidant activities of EGCG (26–29). This contention is also supported by the reported dose-dependent effect of EGCG on human neuroblastoma cells, in which 50 μM EGCG induced oxidative stress and radical formation and upregulated proapoptotic genes, whereas at <20 μM it upregulated antiapoptotic genes (26, 29). In support of this observa-

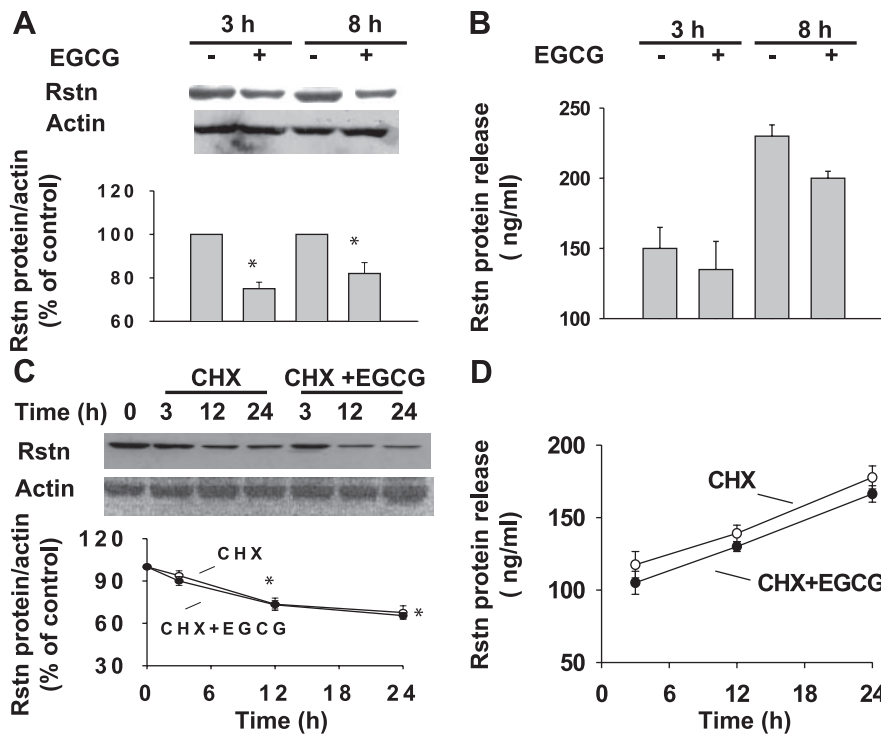


Fig. 3. Effects of EGCG (A and B) and cycloheximide (CHX, C and D) on intracellular (A and C) and extracellular (B and D) Rstn protein levels in 3T3-L1 adipocytes. A and B: effects of EGCG (100 μM) were observed 3–8 h after treatment. C and D: EGCG did not affect Rstn protein stability during the 24-h treatment. Data are expressed as means \pm SE from duplicates of 3 independent experiments. In some cases, SE bars are too small to be seen. In A and B, control experiments were those without EGCG treatment; in C and D, control was set at time 0 when EGCG was added in the beginning. * $P < 0.05$ vs. control at given time.

tion, EGCG at 50 μM was found to exert an effect on phosphoenolpyruvate carboxykinase gene expression in rat hepatoma cells via modifying the redox state of the cell (51). Accordingly, the possibility still remains that EGCG acts at 100 μM to change the stability of Rstn mRNA via oxidative stress or prooxidant activity, as was reported for hepatoma (51) and neuroblastoma cells (26, 29). But this needs further study.

We also attempted to examine whether EGCG-suppressed expression of the adipocyte Rstn gene is indirectly mediated via other proteins. Treatment with CHX did not prevent EGCG-induced suppression of Rstn mRNA expression, which suggests that new protein synthesis is not required for the effect

of EGCG. The observation that acute (3 h) exposure to EGCG induced a 50% decrease in Rstn mRNA also supports this suggestion. In a parallel line, the absence of an effect of Acti-D on EGCG-induced degradation of Rstn mRNA demonstrates that new mRNA synthesis is not required for the effect of EGCG. Taken together, EGCG may directly stimulate the instability of Rstn mRNA, and/or there is an argument for the presence of preexisting mRNA and protein. The latter contention is supported by recent discoveries that an EGCG receptor, the so-called laminin receptor, is located on the membrane of human cancer cells (48), that the specific isoform of laminin called laminin-8 ($\alpha 4$ - $\beta 1$ - $\gamma 1$) is present in 3T3-L1 adipocytes (33), and that EGCG can regulate the expression and activity of membrane transporters, kinases, and proteasomes (1, 18, 26–29, 32, 51).

Although many actions of green tea EGCG are mediated by phosphorylation/dephosphorylation mechanisms that are dependent on kinase pathways (26–29), there has been no report that EGCG signaling is directly related to the activity of MAPKs known to be involved with Rstn biosynthesis and secretion. To support our hypothesis that EGCG would downregulate Rstn gene and protein expression and that its effects would be dependent on MAPK pathways, we herein demonstrated that EGCG selectively decreases the amounts of p-ERKs, but not p-p38, p-JNK, or p-Akt, in adipocytes. Furthermore, overexpression of either MEK1 or its active mutant completely counteracted EGCG-inhibited Rstn mRNA levels but did not affect Rstn mRNA expression in the absence of EGCG. Together, these observations suggest that EGCG downregulates gene expression of adipocyte Rstn via a pathway that is dependent on the ERK pathway. To our knowledge, the inhibition of EGCG on MAPK activity in cell-free systems is competitive with the myelin basic protein substrate and is noncompetitive with ATP (57). In cultured preadipocytes, we

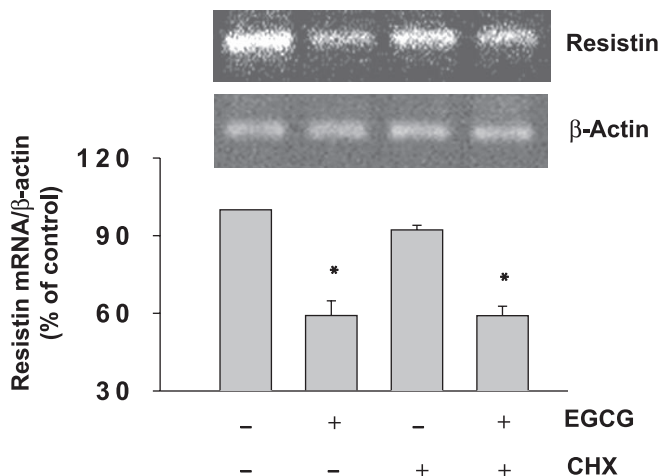


Fig. 4. CHX did not prevent an EGCG-induced decrease in Rstn mRNA expression in 3T3-L1 adipocytes. Adipocytes were pretreated with or without CHX (5 μg/ml) for 90 min and then stimulated with or without 100 μM EGCG for 3 h. Top: RT-PCR. Bottom: DIG-PCR ELISA. Control experiments were those treated with neither EGCG nor CHX. Data are expressed as means \pm SE from duplicates of 3 independent experiments. * $P < 0.05$ vs. control.

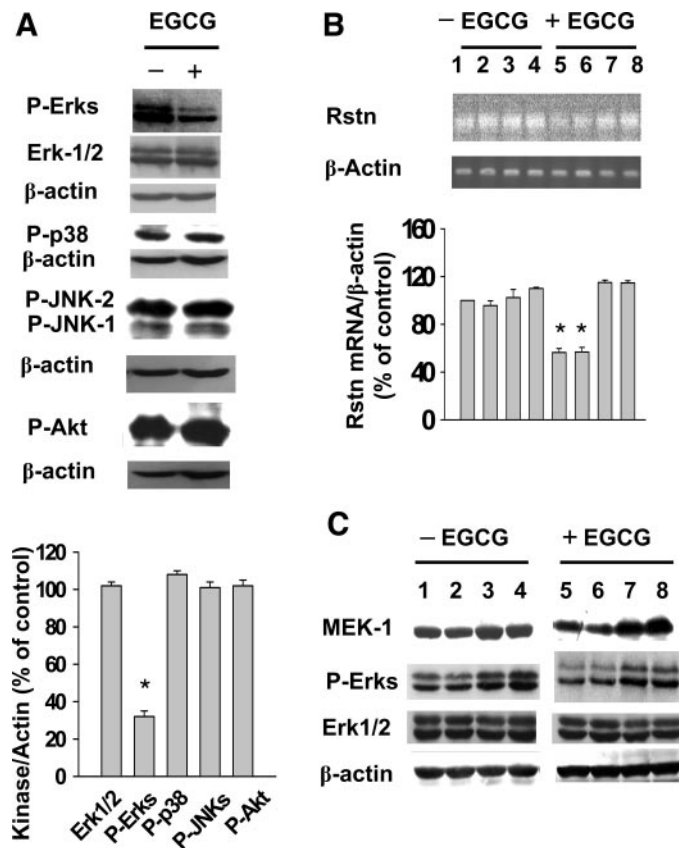


Fig. 5. EGCG-induced decreases in Rstn mRNA of 3T3-L1 adipocytes related to extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathways. **A:** green tea EGCG reduced the amount of phospho (p)-ERK protein, but did not alter the amounts of ERK1, ERK2, p-p38, p-JNKs, or PI3K proteins. After 3 h of 100 μ M EGCG treatment, total cell lysates were measured by Western blot analysis. Kinase protein levels are expressed relative to β -actin protein levels. **B:** transient overexpression with 10- μ g pBabe puro vector containing wild-type MEK1 or constitutively active mutant of constitutively active mutant MEK1S217E/S221E (designated MEK1EE) cDNA did not affect Rstn mRNA levels and counteracted EGCG-suppressed Rstn mRNA expression. Rstn mRNA was examined by RT-PCR (bands) and by DIG-PCR ELISA (bars), respectively, after 30 cycles of PCR amplification. **C:** effects of transient overexpression with wild-type MEK1 or its active mutant on the amounts of MEK1, p-ERKs, and ERK1/2 were confirmed. Data are expressed as means \pm SE (A and B) from duplicates of 3 independent experiments. * $P < 0.05$, with EGCG vs. without EGCG (A and B, control), vector vs. MEK1 or MEK1EE transfected (C). In C, control group is expressed as normal cells without transfection. Lanes 1 and 5, no vector; lanes 2 and 6, empty vector transfected; lanes 3 and 7, MEK1 transfected; lanes 4 and 8, MEK1EE transfected.

found that EGCG did significantly prevent the increase in phosphorylated ERK1/2 from 3T3-L1 preadipocytes by FBS, IGF-I, and IGF-II (18) and that EGCG concomitantly reduced IGF-I receptor activity, as indicated by a decrease in the phosphotyrosine-IGF-I receptor and an association of the IGF-II receptor with $G\alpha_{i-2}$ protein (unpublished observations). In contrast with kinases, the activities of certain protein phosphatases are stimulated 15% by 10–50 μ M EGCG but not altered by 100 μ M EGCG (58). Accordingly, confirming whether EGCG induces a decrease in phosphorylated ERK1/2 from 3T3-L1 adipocytes, via reducing the activity of growth factor receptor as reported for preadipocytes (18) or stimulat-

ing the activity of protein phosphatase as reported for oocytes (58), requires more thorough studies.

Although 50% increases in the rate of Rstn mRNA degradation by EGCG were consistent with 50% decreases of total Rstn mRNA levels (Fig. 2), this study could not exclude the possibility that EGCG-induced decreases in the steady-state levels of Rstn mRNA result from decreases in rates of transcription. To our knowledge, other signaling mechanisms such as hormones, receptor kinases, transcriptional factors, androgen 5 α -reductase, and CCAAT enhancer-binding protein (C/EBP), with the effects of EGCG on cancer growth inhibition and keratinocyte differentiation, have been reported (4, 11, 26–29). In connection with this observation, fat cell adipogenesis (19, 36) and the promoter activity and expression of the Rstn gene (5, 15–17, 40–41, 43–45) are regulated by a variety of hormones, cytoplasmic receptors, nuclear receptors, and coactivator systems. Here, EGCG alone at 100 μ M for 3 h reduced Rstn mRNA levels by 50% compared with the control and, in combination with Dex, decreased Rstn mRNA by

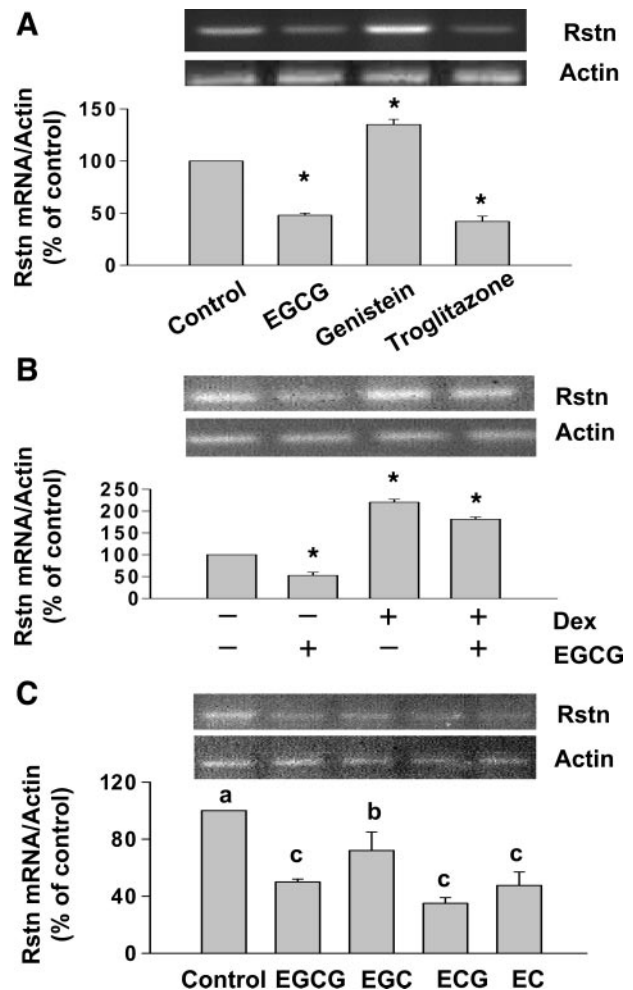


Fig. 6. Assessment of differences among green tea catechins (100 μ M), dexamethasone (Dex, 10 μ M), genistein (100 μ M), and troglitazone (0.1 μ M) in reducing Rstn mRNA expression 3 h after treatment. Bands: RT-PCR. Bars: DIG-PCR ELISA. Data are expressed as means \pm SE from duplicates of 3 independent experiments. *Significant difference ($P < 0.05$) from control; a, b, and c: groups with different letters are significantly different ($P < 0.05$) from each other.

~15% compared with Dex treatment alone, and still an 80% increase over control. This suggests that the effect of EGCG may be independent of a Dex-associated pathway. We observed a much lower extent of the effect of EGCG compared with troglitazone (a PPAR γ ligand) in decreasing Rstn mRNA expression, which implies that adipocytes have different sensitivities to these two compounds. It should be noted that EGCG can inhibit androgen 5 α -reductase (27) and that androgen stimulates Rstn mRNA expression (5). Determining whether the androgen receptor (which also serves as a transcriptional factor) and other component receptors or signal transducers (i.e., C/EBP) might be responsible for the effect of EGCG on Rstn gene expression, including the de novo transcription of the Rstn gene, requires further study via using Rstn promoter reporter assays.

Expression and secretion of Rstn protein are regulated differently by certain endocrine and nutritional factors, such as insulin, IGF-I, growth hormone, Dex, and vitamin A (4, 15, 22, 41). We report herein that EGCG at 100 μ M significantly altered the amount of intracellular Rstn protein within 12 h (Fig. 3A), although it had no significant effect on Rstn protein release within 8 h (Fig. 3B). This observation suggests that EGCG may transiently modify the distribution of Rstn protein between the intracellular and extracellular compartments. The effect of EGCG on decreasing intracellular Rstn protein levels may be partially explained by decreased Rstn mRNA stability and levels because the falling time (3 h) of Rstn protein initiated by EGCG parallels the decreases in Rstn mRNA levels caused by EGCG. An explanation for the lack of observed changes in the Rstn protein release by EGCG is that Rstn might feed back to inhibit its secretion in the experimental culture system. This assumption can be indirectly supported by the evidence that conditioned medium from COS cells transfected with hemagglutinin-tagged murine Rstn expression vector inhibits 3T3-L1 adipocyte differentiation (23) and that the inhibitory effect of Rstn on adipocyte differentiation is prevented by the medium containing a dominant negative form of Rstn (24). This suggests the importance of the dimerization of Rstn in the regulation of adipogenesis. Recent evidence showed that secretable and nonsecretable Rstn isoforms were reported in adipose tissues of rats, mice, and humans (3, 34). Thus it would be worthwhile in a future study to explore whether EGCG modulates the respective splicing and processing events of Rstn mRNA and protein and thereby leads to translocation of the Rstn protein.

Flavonoid-specific effects (25–29) of green tea on cell functions, including adipocytes (18, 20), have been reported. We report herein that four major GTCs inhibited Rstn mRNA expression in the order ECG > EGCG = EC > EGC. This suggests that the structural differences of catechins observed in the number of hydroxyl groups and the presence of a galloyl group (Fig. 1) might be important for their regulation of adipocyte Rstn gene expression. An additional finding that all major GTCs (inhibition) differed from genistein (stimulation) in regulating expression of the adipocyte Rstn gene suggests that the nature of the chemical structures of the aromatic rings (Fig. 1) is responsible for their differential effects. Distinct structures may contribute to specific binding with their own receptors, such as the laminin receptor (48) and estrogen

receptor (47), thereby resulting in the signaling that alters Rstn mRNA levels.

GTCs have numerous biological effects *in vitro*, and the effects are generally observed in the range of 10–100 μ M (27). *In vivo*, plasma concentrations of green tea EGCG or other catechins, as generally reported in animals and humans, are about 1 μ M (27). However, the absorption and distribution of administrated EGCG and other tea catechins are poor and dependent on catechin structure, purity, dosage, 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 (54). At that time, the plasma levels (free and conjugated) of EGCG, EGC, and EC levels were 0.26, 0.48, and 0.19 μ M, respectively, whereas ECG was not detected (54). Consumption of a single high dose of green tea, which is equivalent to six cups of tea, can raise plasma levels of catechin to 2–4 μ M in 60 min (49). A few minutes after two to three cups of green tea are consumed, the saliva levels of various catechins reach peaks of 39–144 μ M EGCG, 11–48 μ M EGC, and 7–28 μ M EC (55). Sixty minutes after intragastric administration of EGCG at a dose of 500 mg/kg body wt to rats, the levels of EGCG were 10 μ M in plasma, 48 μ M in the liver, 0.5 μ M in the brain, 565 μ M in the small intestinal mucosa, and 68 μ M in the colon mucosa (31). When EGCG was administrated at a dose of 100 mg/kg body wt to rats by intraperitoneal injection, the plasma concentrations of unmetabolized EGCG, determined by HPLC, were 24, 2, 1, and 1 μ M at 0.5, 1, 2, and 24 h, respectively (20). 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 μ M) of EGCG (the effective dose of this study is in the range of 20–100 μ M) or other tea catechins generally used in this study that are compatible with the goal of helping to regulate the initiation and progression of obesity and diabetes (27) 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 extract given to rats by oral administration at a dosage of 0.5 g/100 ml water, which contained ~2,183 μ M EGCG, could within 12 wk improve insulin sensitivity in rats and that green tea extract containing 327 μ M EGCG enhanced glucose uptake in rat adipocytes *in vitro* (53).

We conclude that EGCG suppresses Rstn gene expression in 3T3-L1 adipocytes. The signal element through which EGCG carries out its modulation of Rstn expression is dependent on the ERK pathway. Further studies on the discovery of the EGCG receptor (48) in adipocytes are required to elucidate similar and different mechanisms of actions by EGCG, vitamin A (15), genistein, and insulin (22, 41) in Rstn gene expression. Rstn has been reported to regulate glucose production (6) and transport (44), insulin resistance (44), and fat cell adipogenesis (23, 24). Accordingly, it would be worthwhile in a further *in vivo* study to explore whether significant decreases in Rstn mRNA and protein expression and transient alterations in the distribution of Rstn protein between the intracellular and extracellular compartments induced by EGCG in our *in vitro* study are related to the mechanism by which EGCG and EGCG-containing tea extracts exert their antidiabetic (53) and antiobesity (27) actions in animals. It would also be of interest

whether an effect of EGCG on Rstn gene expression could be demonstrated in humans in a future study.

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