(--)-Catechin suppresses expression of Kruppel-like factor 7 and increases expression and secretion of adiponectin protein in 3T3-L1 cells

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Am J Physiol Endocrinol Metab 292: E1166–E1172, 2007. First published December 12, 2006; doi:10.1152/ajpendo.00436.2006.—Adiponectin is an adipocyte-derived hormone that can increase insulin sensitivity and promote adipocyte differentiation. Administration of adiponectin to obese or diabetic mice reduces plasma glucose and free fatty acids levels. Green tea polyphenols possess many pharmacological activities such as antioxidant, anti-inflammatory, antiobesity, and antidiabetic activities. To investigate whether green tea polyphenols have an effect on the regulation of adiponectin, we measured expression and secretion levels of adiponectin protein after treatment of each green tea polyphenol in 3T3-L1 adipocytes. We found that (--)-catechin enhanced the expression and secretion of adiponectin protein in a dose- and time-dependent manner. Furthermore, treatment of (--)-catechin increased insulin-dependent glucose uptake in differentiated adipocytes and augmented the expression of adipogenic marker genes, including PPARγ, C/EBPα, FAS, and SCD-1, when (--)-catechin was treated during adipocyte differentiation. In search of the molecular mechanism responsible for inducible effect of (--)-catechin on adiponectin expression, we found that (--)-catechin markedly suppresses the expression of Kruppel-like factor 7 (KLF7) protein, which has recently been reported to inhibit the expression of adiponectin and other adipogenesis related genes, including leptin, PPARγ, C/EBPα, and ap2 in adipocytes. KLF7 is a transcription factor in adipocyte and plays an important role in the pathogenesis of type 2 diabetes. Taken together, these data suggest that the upregulation of adiponectin protein by (--)-catechin may involve, at least in part, suppression of KLF7 in 3T3-L1 cells.

Adiponectin (also known as Acrp30, AdipoQ, apM1, and GBP28) is a member of the adipocyte-derived hormones (11, 17, 19, 22, 23). The mouse adiponectin gene encodes 247 amino acids that form oligomers and is believed to circulate in the plasma as a homotrimer or as larger complexes of 12–15 subunits (11). The expression of adiponectin is limited to adipose tissue but is reduced in obesity. Plasma adiponectin concentration and mRNA expression are decreased in obese humans despite increased adiposity and in patients with type 2 diabetes with insulin resistance (1, 10, 25). Furthermore, administration of adiponectin to obese or diabetic mice causes weight loss and reduces the circulating free fatty acid level by enhanced skeletal muscle fat oxidation (7) and also enhances insulin sensitivity and reduces the plasma glucose level by suppressing hepatic glucose production (2, 6, 28). In addition, adiponectin has antiatherogenic and anti-inflammatory properties (11, 21).

Adiponectin gene expression is regulated by several transcription factors, including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT enhancer-binding protein (C/EBP)α, liver receptor homolog-1, Kruppel-like factor 7 (KLF7), and sterol regulatory element-binding protein-1c (SREBP-1c) (14, 17, 20, 24). Although there has been rapid progress in the understanding of the physiological functions of adiponectin and the signaling pathways mediating adiponectin action, relatively little is known about the mechanisms of its transcriptional regulation, and only a few agents are known to regulate the production of adiponectin.

Green tea, a beverage commonly consumed in Asian countries, is a significant source of a type of polyphenolic flavonoids known as catechins. In a recent report, an injection of catechins into lean and obese Zucker rats significantly lowered blood glucose and insulin levels (13), and green tea extract has been shown to increase glucose metabolism in adipocytes (5). Whether green tea catechins have any effect on the production of adiponectin or the modulation of the action of adiponectin is unknown.

In this study, we used 3T3-L1 adipocytes to examine the effects of green tea components on adiponectin protein expression. We show that (--)-catechin markedly enhances the expression of adiponectin and increases glucose uptake into 3T3-L1 adipocytes. The effects are accompanied by the down-regulation of KLF7, which is a recently identified transcription factor involved in the pathogenesis of type 2 diabetes.

MATERIALS AND METHODS

Chemical reagents. (--)-Epigallocatechin 3-gallate, (--)-epigallocatechin, (--)-epicatechin 3-gallate, (--)-epicatechin, (--)-epicatechin, (--)-gallocatechin 3-gallate, (--)-gallocatechin, (--)-catechin 3-gallate, (--)-catechin, (+)-catechin, quercetin, gallic acid, theobromine, theophylline, theanine, and kaempferol were all purchased from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO). Penicillin-streptomycin, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and calf serum were purchased from GIBCO-BRL of Life Technologies (New York, NY). Fatty acid-free bovine serum albumin, isobutylmethylxanthine, insulin, dexamethasone, and monoclonal anti-β-actin antibody were also purchased from Sigma. The antibody to PPARγ was purchased from Upstate (Lake Placid, NY), that to C/EBPα from Affinity BioReagents (Golden, CO), that to adiponectin from Chemicon International (Temecula, CA), that to SREBP-1c from BD Biosciences Pharmingen (San Diego, CA), and that to KLF7 from Abanova (Neihu District, Taipei, Taiwan).

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Table 1. Effect of purified compound from green tea on adiponectin secretion in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Purified Compounds From Green Tea, 50 μM</th>
<th>Adiponectin Concentration ± SE (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 0.374</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>256.6 ± 10.55</td>
</tr>
<tr>
<td>(-)-Epigallocatechin 3-gallate</td>
<td>101.6 ± 6.89</td>
</tr>
<tr>
<td>(-)-Epigallocatechin</td>
<td>101.3 ± 2.57</td>
</tr>
<tr>
<td>(-)-Epicatechin 3 gallate</td>
<td>79.2 ± 4.77**</td>
</tr>
<tr>
<td>(-)-Epicatechin</td>
<td>95.1 ± 11.25</td>
</tr>
<tr>
<td>(+)-Epicatechin</td>
<td>103.2 ± 3.40</td>
</tr>
<tr>
<td>(+)-Gallocatechin 3-gallate</td>
<td>100.8 ± 3.25</td>
</tr>
<tr>
<td>(-)-Gallocatechin</td>
<td>95.2 ± 5.67</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>99.7 ± 3.47</td>
</tr>
<tr>
<td>(-)-Catechin</td>
<td>213.4 ± 8.56**</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>101.9 ± 3.56</td>
</tr>
<tr>
<td>Quercetin</td>
<td>54.1 ± 5.63**</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>69.9 ± 6.52**</td>
</tr>
<tr>
<td>Theobromine</td>
<td>74.9 ± 7.35**</td>
</tr>
<tr>
<td>Theophylline</td>
<td>78.2 ± 6.99</td>
</tr>
<tr>
<td>Theanine</td>
<td>142.2 ± 11.2**</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>42.1 ± 2.92**</td>
</tr>
</tbody>
</table>

Results are represented as means ± SE of 7 separate experiments and are expressed as %control. Fully differentiated adipocytes were treated with 50 μM green tea components for 24 h, and adiponectin concentrations in cell culture supernatants were determined with a Quantiline adiponectin immunoassay kit. Control experiments were those not treated with (-)-catechin. Pioglitazone was used as positive control. Statistical significance of the difference was established by Student’s t-test. Significantly different from control treated with DMSO: *P < 0.05, **P < 0.01.

Cell culture and treatment. Mouse 3T3-L1 (ATCC CL173) preadipocyte cells were maintained in DMEM containing 10% calf serum. For the differentiation, postconfluent 3T3-L1 preadipocytes (referred to as day 0) were treated with DMEM containing 10% FBS, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone for 2 days and were then treated for 2 days with DMEM containing 10 μg/ml insulin and 10% FBS. Thereafter, cells were maintained in and refed every 2 days with DMEM containing 10% FBS. With this protocol, >80% adipocyte differentiation was achieved.

Day 7 adipocytes were incubated in low-glucose DMEM (GIBCO-BRL) containing 2% (wt/vol) fatty acid-free BSA for 24 h. After 24 h, the cells were treated with (-)-catechin at the indicated concentrations for 24 h. Depending on the purpose of the experiment, 10 μM pioglitazone was used as a positive control.

To examine the effect of (-)-catechin on adiponectin differentiation, postconfluent 3T3-L1 preadipocytes were treated with 50 μM (-)-catechin every 2 days during adipocyte differentiation.

Mouse adiponectin immunoassay. For a quantitative determination of adiponectin concentrations in cell culture supernatants, Quantikine immunoassay kit was used (R&D Systems, Minneapolis, MN). The media of the cells treated with green tea components were centrifuged for 5 min at 1,000 g, and the supernatants were diluted 2,000-fold. The adiponectin levels in cell culture supernatants were determined according to the method recommended by the manufacturer. A standard curve was obtained in the range of adiponectin from 0.16 to 10 ng/ml. The adiponectin levels in cell culture supernatants were determined according to the method recommended by the manufacturer. A standard curve was obtained in the range of adiponectin from 0.16 to 10 ng/ml. The adiponectin concentration was calculated from the standard curve and was normalized by determining each total protein concentration.

2-Deoxyglucose uptake. Uptake of 2-deoxyglucose by the 3T3-L1 adipocytes was measured as previously described (4). Briefly, cells were incubated in low-glucose DMEM containing 0.1% BSA for 16 h at 37°C. Cells were treated with or without 50 μM (-)-catechin for 24 h at 37°C and then stimulated with or without 100 nM insulin for 1 h at 37°C. Glucose uptake was initiated by the addition of 2-deoxy-d-[14C]glucose at a final concentration of 3 μmol/l for 10 min in HEPES buffer-saline (140 mM NaCl, 5 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, 20 mM HEPES, pH 7.4). The reaction was terminated by separating cells from the HEPES buffer saline and 2-deoxy-d-[14C]glucose. After three washes in ice-cold PBS, the cells were extracted with 0.1% SDS and subjected to scintillation counting for 14C radioactivity. The protein concentration was determined with a BCA assay kit (Pierce, Rockford, IL), and the radioactivities were normalized by determining each total protein concentration.

Western blot analysis. 3T3-L1 cells were lysed in RIPA buffer (PBS, pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail). Forty micrograms of proteins were resolved on 10% NuPAGE gels run in an MES buffer system (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes according to the manufacturer’s protocol. Immunoreactive proteins were revealed by enhanced chemiluminescence with ECL+ (Amersham Biosciences, Piscataway, NJ). Anti-β-actin antibody was used to assess equal loading of the protein. Blots were quantified using an ImageMaster (Amersham Biosciences).

Sudan II staining. Intracellular lipid accumulation was also determined by Sudan II staining. The cells were washed twice with ice-cold PBS, fixed with 4% formaldehyde in PBS for 20 min on ice, and stained with 0.5% (wt/vol) Sudan II (Wako Japan) in 60% (vol/vol) isopropanol for 1 h at room temperature. After the staining, the cells...
were washed with 70% ethanol to remove excess stain. Stained oil droplets in the cells were dissolved in isopropanol containing 4% (vol/vol) Nonidet-P40 and spectrophotometrically measured at an absorbance of 490 nm.

**RESULTS**

Inducible effects of (-)-catechin on expression and secretion of adiponectin in 3T3-L1 adipocytes. Fully differentiated adipocytes (at 7 days after differentiation induction) were treated with the purified compound from green tea at 50 μM for 24 h, and the adiponectin concentrations in cell culture supernatants were determined with a quantitative sandwich enzyme immunoassay kit. It is well known that pioglitazone, a synthetic ligand of PPARγ, increases adiponectin expression. Thus, pioglitazone was used as a positive control (15). (-)-Epicatechin 3-gallate, quercetin, gallic acid, theobromine, theophylline, and kaempferol at 50 μM suppressed the adiponectin protein release to 79, 54, 69, 74, 78, and 42% respectively, compared with DMSO-treated control cells, whereas (-)-catechin and theanine significantly increased the adiponectin protein secretion to 213 and 142%, respectively (Table 1). In particular, (-)-catechin increased the release of adiponectin protein in a concentration- and time-dependent manner (Fig. 1). To determine whether the (-)-catechin-induced mRNA was calculated by the comparative critical threshold (C_T)

method.

**Adenovirus infection.** KLF7 adenovirus was kindly provided by Yoon BJ (Korea University). Briefly, human KLF cDNA was generated by amplification form Human Adipocyte Marathon-Ready cDNA (Clontech) using the following primers: sense, 5’-TGA GCC AGA CGA ACT GAC AA-3’; anti-sense, 5’-CCT TTA GAC ACT AGC CGA TG-3’. KLF7 cDNA was in-frame fused with HA epitope tag in its NH2 terminus, and recombinant adenovirus was constructed with Adeno-X® System 1 (Clontech). For adenovirus infection, adipocytes (at day 6 after differentiation) were infected with adenovirus at 20 PFU/cell for 16 h at 37°C. Then, the culture medium was replaced with fresh medium. At 72 h after viral infection, adenovirus-infected 3T3-L1 adipocytes were incubated in low-glucose DMEM containing 2% (wt/vol) fatty acid-free BSA for 16 h. The cells were treated with 50 μM (-)-catechin for 24 h.

**Statistical analysis.** Data are presented as means ± SE. Two-tailed Student’s t-tests were used to calculate statistical significance.
The increase in glucose uptake in untreated control cells. However, without insulin stimulation, not statistically significant. Fig. 3, there was a 1.8-fold increase in insulin-stimulated glucose uptake in (-)-catechin-treated cells compared with untreated cells.

Intracellular adiponectin mRNA and protein significantly increased in the presence of 50 μM (-)-catechin 24 h after treatment.

Elevation of insulin-stimulated glucose uptake by (-)-catechin in 3T3-L1 adipocytes. Adiponectin is an insulin-sensitizing adipokine, and its overexpression increases the insulin-stimulated glucose transport (8, 12). As treatment with (-)-catechin increased the adiponectin protein expression and secretion, it was hypothesized that (-)-catechin would have an insulin-sensitizing action. To test this hypothesis, adipocytes were pretreated with or without (-)-catechin for 24 h, and cells were then treated in the presence or absence of 100 nM insulin. As shown in Fig. 3, there was a 1.8-fold increase in insulin-stimulated glucose uptake in (-)-catechin-treated cells compared with untreated control cells. However, without insulin stimulation, the increase in glucose uptake in (-)-catechin treated cells was not statistically significant.

Inhibitory effects of (-)-catechin on KLF7 expression. Despite the importance of adiponectin, there is little information regarding the mechanisms involved in adiponectin gene expression in adipocytes. To understand the molecular mechanism for the (-)-catechin-induced upregulation of adiponectin expression, we examined the expression patterns of various transcription factors that had been reported to regulate transcription of adiponectin gene. SREBP-1c controls adiponectin gene expression in differentiated adipocytes (24). PPARγ and liver receptor homolog-1 also increase the expression of adiponectin (17). Adiponectin mRNA expression is also regulated via C/EBPα and KLF7 (14, 20).

Pioglitazone increases the expression of adiponectin gene through activating PPARγ. Fully differentiated adipocytes were treated with either (-)-catechin or pioglitazone for 24 h, and the protein extracts were then subjected to Western blot with antibodies raised to SREBP-1c, PPARγ, C/EBPα, and KLF7. The expression of SREBP-1c, PPARγ, and C/EBPα was significantly increased in the pioglitazone-treated cells, whereas that of KLF7 was not affected by treatment with pioglitazone. However, the treatment of (-)-catechin remarkably reduced the KLF7 protein expression but did not alter the expression levels of the SREBP-1c, PPARγ, and C/EBPα proteins in differentiated adipocytes (Fig. 4).

Effect of (-)-catechin on adipogenesis. It has been reported that KLF7 contributes to the pathogenesis of type 2 diabetes by inhibiting adipogenesis and adipokine secretion in adipocytes (14). The expression of KLF7 markedly decreased at 6 h after inducing differentiation, and its overexpression resulted in the reduction of adipogenic factors (PPARγ and C/EBPα) and adipokines (adiponectin and leptin). As (-)-catechin led to a decrease in KLF7 expression, the effect of (-)-catechin on adipocyte differentiation was examined in 3T3-L1 preadipocytes. During differentiation, postconfluent 3T3-L1 preadipocytes were treated with 50 μM (-)-catechin every 2 days for 6 days.

Given the importance of KLF7, the level of KLF7 protein in (-)-catechin-treated or untreated cells was determined at different stages of the adipogenesis process. The KLF7 protein was abundant in the 3T3-L1 preadipocytes (day 0); however, when the cells were induced to differentiate, KLF7 protein levels were determined by Western blot with an anti-KLF7 antibody. Data are expressed as means ± SE from 3 independent experiments. **P < 0.01 with untreated cells.
levels drastically decreased at 24 h and gradually increased afterward along the progression of the differentiation process until the expression levels of KLF were restored to that of KLF in preadipocytes at day 7 (Fig. 5). On the other hand, the treatment of (-)-catechin maintained the decreased expression level of KLF7 during the adipocyte differentiation. Cellular triglycerides were measured to confirm the lipid accumulation. As shown in Fig. 6A, (-)-catechin increased cellular triglyceride to ~17.8% compared with the untreated cells. The expressions of gene transcripts known to be associated with adipocyte differentiation, including PPARγ, CEBPα, FAS, adiponectin, and SCD-1, were also increased by (-)-catechin treatment (Fig. 6B). Interestingly, as a result of the (-)-catechin treatment, the expressions of the adipocyte differentiation markers, including PPARγ, CEBPα, FAS, and SCD-1 and the cellular triglycerides were markedly increased at 24 h after differentiation was induced, and the increased levels were sustained until day 7 of differentiation.

Effect of (-)-catechin on adiponectin expression in KLF7-overexpressing cells. To further examine whether KLF7 is involved in (-)-catechin-stimulated adiponectin expression, differentiated adipocytes were infected with KLF7 adenovirus (AdKLF7) or mock and then treated with or without 50 μM (-)-catechin for 24 h (Fig. 7). The expression level of adiponectin in KLF7 overexpressing cells was significantly reduced compared with that in control cells. Although (-)-catechin significantly increased adiponectin protein levels in mock adenovirus-infected adipocytes, its effect was prevented by the overexpression of KLF7.

DISCUSSION

(-)-Catechin is a flavanol antioxidant extracted from green tea. The present study demonstrates the novel effect of (-)-catechin of enhancing the expression and secretion of adiponectin in adipocytes. A recent study showed that overexpression of adiponectin accelerated adipocyte differentiation and augmented the insulin-responsive glucose transport (8). Thus, we attempted to examine whether the (-)-catechin-induced increase in adiponectin expression displays an increase in the insulin-stimulated glucose transport. Treatment with (-)-catechin increased insulin-stimulated glucose uptake into 3T3-L1 adipocytes, which suggests that (-)-catechin serves as an insulin-sensitizing material.

![Graph showing effects of (-)-catechin on adipogenesis.](image1)

![Graph showing effects of (-)-catechin on adiponectin expression.](image2)

![Western blot showing expression levels of adiponectin and β-actin](image3)
Adiponectin gene expression is regulated by several extra-cellular signaling factors, including insulin, TNF-α, and β-adrenergic agonists, although the link between these signals and adiponectin gene expression, in most cases, has yet to be elucidated. PPARγ has been shown to induce adiponectin promoter activity through an unidentified element responsive to PPARγ (18), and C/EBPα has been reported to regulate adiponectin gene transcription through an intronic enhancer (20). SREBP-1c binds to the adiponectin promoter and mediates the insulin-dependent adiponectin expression (24). KLF7 has recently been reported to inhibit adiponectin gene expression in adipocytes (14). We tested whether the (−)-catechin-induced increase in adiponectin expression is mediated by these transcription factors. Unlike pioglitazone, the (−)-catechin treatment significantly decreased the expression of KLF7 but did not induce a significant change in the expressions of PPARγ, C/EBPα, or SREBP-1c in fully differentiated adipocytes. This observation suggests that the mechanism responsible for the increase of adiponectin expression resulting from (−)-catechin treatment may differ from that of pioglitazone treatment. Unlike pioglitazone, which enhances adiponectin expression by directly activating PPARγ, the ability of (−)-catechin to enhance adiponectin expression may be attributable to the suppression of KLF7 expression.

KLF7, a member of the KLF family, is reported to be a novel candidate for conferring susceptibility to type 2 diabetes. The KLF7 gene is expressed in adipocytes and various human tissues, including the pancreas, liver, and skeletal muscle. In human adipocytes overexpressing KLF7, the expressions of adiponectin, leptin, PPARγ, C/EBPα, and aP2 were significantly reduced. In the insulin-secreting cell line, the expression and glucose-induced secretion of insulin were suppressed in KLF7-overexpressed cells. Therefore, it is reported that the inhibition of KLF7 expression and/or activity may be a target for a new therapeutic and/or preventative approach for treating type 2 diabetes. Herein, we have shown that (−)-catechin inhibits the KLF7 expression and maintains the low levels of KLF7 expression during adipocyte differentiation. Furthermore, when (−)-catechin was treated during adipocyte differentiation, it augmented lipid accumulation and increased the expression of the adipocyte differentiation markers, including FAS, SCD-1, PPARγ, and C/EBP. Moreover, overexpression of KLF7 prevented (−)-catechin-stimulated adiponectin expression. The evidence obtained in the present study suggests that (−)-catechin promotes adipogenesis and augments insulin sensitivity by suppressing the KLF7 expression. Additional studies are needed to investigate the exact molecular mechanism underlying the increase of adiponectin expression via the suppression of the KLF7 gene by (−)-catechin.

Green tea extracts have antidiabetic and antiobesity activities among their many known pharmacological activities. Although (−)-catechin displays different effects on the regulation of adiponectin expression from the major green tea polyphenols, it would be worthwhile in a further in vivo study to explore whether the effect of (−)-catechin on adiponectin gene expression in our in vitro study is related to the mechanism by which green tea extracts exert their antidiabetic (26) and antiobesity (16) actions in animals.

In summary, we have shown for the first time that (−)-catechin stimulates adiponectin protein expression and secretion in adipocytes. We have also provided evidence that (−)-catechin suppresses KLF7 gene expression, which may contribute to promoting adipocyte differentiation and induce the production of adiponectin. Thus, these results provide a rationale for the use of (−)-catechin in the development of new therapeutic agents in the treatment of type 2 diabetes.

REFERENCES


