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Epigallocatechin-3-gallate가 지방세포 분화와 포도당 수송 및 지질대사 유전자에 미치는 영향

최 혁[1] · 명규호*

[1]국립축산과학원 영양생리팀, *전남대학교 동물자원학부

The Effects of Epigallocatechin-3-gallate on Adipocyte Glucose Transport and Differentiation, and Aberrant Lipid Metabolism-related Genes Expression

Hyuck Choi[1] and Kyuho Myung[2,*]

[1]*Animal Nutrition & Physiology Team, National Institute of Animal Science, Rural Development Administration, Wanju-gun 565-851, Korea*
[2]*Department of Animal Science, Chonnam National University, Gwangju 500-757, Korea*
**Corresponding author: khmyung@jnu.ac.kr*

ABSTRACT

Background: Green tea catechins have been shown to promote loss of body fat and to inhibit growth of many cancer cell types by inducing apoptosis. **Objective:** The objective was to determine if epigallocatechin-3-gallate (EGCG) has ability to reduce differentiation in 3T3-L1 adipocytes and alter expression of aberrant lipid metabolism-related genes in HepG2 hepatocytes. **Results:** EGCG attenuated differentiation related gene expressions of adipocytes likely due to retarding glucose uptake in 3T3-L1 cells. EGCG decreased the concentration of cholesterol in HepG2 cell, resulting in the similar degree of suppression in apoB 100, and of enhancement in LDL receptor and HMG-CoA reductase mRNA levels. **Conclusion:** These results demonstrated that EGCG can act directly to inhibit differentiation of 3T3-L1 adipocytes and reduce abnormality in lipid metabolism-related genes in HepG2 hepatocytes, thus, could be a potent adjuvant in the treatment of obesity and metabolism disease.

Additional key words: Epigallocatechin-3-gallate, Adipocyte differentiation, ApoB 100, LDL receptor, HepG2

Introduction

An excessive amount of body fat is still considered to be one of the major health problems such as obesity, diabetes mellitus, hyperlipidemia and cardiovascular disease associated with an abnormality in lipid metabolism (Laakso, 1999; Yamagishi, 2011). Therefore, over the past decades the prevention of the fat accumulation in adipose tissues has been intensively studied. Among them, herbal medicines have been widely investigated for their clinical potential to treat inhibit adipogenesis (Takahashi *et al*., 2002).

Green tea catechins have been shown to promote loss of body fat and to inhibit growth of many cancer cell types by inducing apoptosis (Lin *et al*., 2005). Particularly, epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea (*Camellia sinesis,* Theaceae, etc.) and strongest bioactive chemical (Hara, 2001). Some studies revealed that EGCG also has preventive activities in fatty liver disease and cardiovascular disease (Nakachi *et al*., 1995; Santamarina *et al*., 2015). Furthermore, numerous potential mechanisms have been proposed to account for these activities including: decreasing plasma cholesterol and triglyceride concentrations, and the ratio of low and very low density lipoprotein cholesterol concentrations to high density lipoprotein cholesterol concentrations in animal studies (Yoshizawa *et al*., 1987; Castelli *et al*., 1990). However, their studies only demonstrated one aspect role of the EGCG. Furthermore, no clear exact mechanism for protective effect of EGCG against abnormality in lipid metabolism in humans has been reported.

Thus, this study was conducted to evaluate the ability of EGCG to retard glucose transport and differentiation-related gene expression in 3T3-L1 adipocytes, and alter expression of aberrant lipid metabolism-related genes in HepG2 hepatocytes.

Materials and methods

1. Cell culture and treatment

3T3-L1 murine pre-adiocytes (from ATCC) were cultured in 25 cm^3 flasks and induced to differentiate as described by Chen *et al* (1997). Briefly, the cells were placed on culture and grown to 80% confluence before induction. The cells were then induced to differentiate into adipocytes in the presence of DMEM-F12 (Invitrogen, Carlsbad, CA, USA), 10% heat-inactivated fetal calf serum (FCS) and 5 g/ml insulin with 390 ng/ml dexamethasone and 115 g/ml 3-iosbutyl-1-methylxanthine. The cells were then differentiated with treatment media of 0, 20 or 100 μM EGCG (Sigma-Aldrich, St Louis, MO, USA) for 24 h and terminal differentiation occurred by d 10. The HepG2 cells were grown in 75 cm^3 flasks containing MEM (Invitrogen), supplemented with 2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L sodium bicarbonate, 110 mg/L sodium pyruvate and 10 % FCS. They were then subcultured from 75 cm^3 flasks into 25 cm^3 flasks and allowed to grow without disruption for 24 h in a medium of MEM with FCS. The medium was replaced with fresh, pre-warmed serum-free medium and cells were incubated for another 24 h. Serum-free MEM was made by supplementing MEM with 80 g/L BSA (fatty acid free) complexed to 5 uM/L oleic acid (sodium salt), 22.2 M/L glucose, 45.5 M/L Na_2 CO_3 and 1 M/L sodium pyruvate as in Pal *et al*. (2003). The cells were then incubated with treatment media for 24 h.

HepG2 human hepatocarcinoma cell line (from ATCC), was cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, USA) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37oC in a humidified atmosphere of 5% CO_2. At 80% confluent and the cells were then incubated with treatment media for 24 h.

2. Glucose uptake assay and confocal microscopy

A modification of a method was used for the measurements of 2-Deoxy[^{3}H]glucose (Deoxy-D-glucose,2-[^{3}H(glucose)]; 6.0 Ci/mmol; NEN Life Science Products, Inc.,) uptake (Bernier *et al*., 1988). Briefly, 3T3-L1 cells were grown and differentiated in 24-well culture plates. After 24 h treatment in starved (DMEM without glucose; Invitrogen) media, cells were washed twice with phosphate buffered saline solution (PBS). This was followed by the addition of 0.5 ml pre-warmed starvation media containing 0.5 Ci 2-Deoxy[^{3}H] glucose for 30 min at 37^{o}C. Uptake was terminated by rapid removal of the 2-Deoxy[^{3}H]glucose, followed by three washes in ice-cold PBS. Cells were lysed with 300 μl of 5% trichloroacetic acid; the lysates were added 3 ml of scintillant and counted in a LKB 1218 Rackbeta Liquid Scintillation counter (Amersharm bioscience Inc., Piscataway, NJ, USA). The confocal microscopy was performed as described by Liu *et al*. (2005) with minor modifications. The cells were incubated in each treatments media at 37^{o}C for 15 min for induction of GLUT 4 translocation, and photographed with an Olympus Fv 500, Confocal Laser Scanning Microscope (Olympus Co, Ltd, Tokyo, Japan).

3. RNA isolation and real-time reverse transcriptase-PCR (real time RT-PCR)

Total RNA was isolated from the cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was used to measure the quantities of LPL (Accession No. BC 003305), C/EBP-α (Accession No. NM 007678), mouse PPAR-γ2 (Accession No. Y 12882), and mRNA relative to the quantity of 18S (Accession No. S 56974) mRNA of 3T3-L1 cells, and the quantities of LDL receptor (Accession No. NM 000527) and HMG-CoA reductase (Accession No. M 62633) relative to the quantity of Cyclophilin (Accession No. Y 00052) mRNA for HepG2 cells. Measurement of the relative quantities of cDNA was conducted using a SYBER Green real-time RT-PCR Master Mix (Qiagen, Valencia, CA, USA), appropriate forward and reverse primers (0.5 μM) (Table 1, 2), and 0.2 μg RNA. Assays were performed in the Rotor-Gene 2000 Real-Time Cycler using appropriate analysis software (Corbett Research, Sydney, Australia) and the thermal

Table 1. Forward and reverse primers for real-time PCR for mouse LPL, C/EBP-α and PPAR-γ2

Item		Primer
LPL		
	Forward	5'-ACAAGGTCAGAGCCAAGAGAAGCAG-3'
	Reverse	5'-GTTGCTTGCCATTCTCAGTCCCAG-3'
C/EBP-α		
	Forward	5'-GGTGCGCAAGAGCCGAGATAAAG-3'
	Reverse	5'-AGTTCACGGCTCAGCTGTTCCAC-3'
PPAR-γ2		
	Forward	5'-TGAACGTGAAGCCCATCGAGGAC-3'
	Reverse	5'-TCTGTCATCTTCTGGAGCACCTTGG-3'
18S		
	Forward	5' GATCCATTGGAGGGCAAGTCTGG 3'
	Reverse	5' TACCCACTGAGCCATCTCACCAGC 3'

Table 2. Forward and reverse primers for real-time PCR for Human LDL receptor and HMG-CoA reductase

Item	Primer
LDL receptor	
Forward	5'-CACAGCCGTAAGGACACAGCACAC-3'
Reverse	5'-GCCCAGAGCTTGGTGAGACATTC-3'
HMG-CoA reductase	
Forward	5'-GCCTGGGCCAGAGAAGATAATGTTC-3'
Reverse	5'-GCACAGTTCTAGGGCCATTCACG-3'
Cyclophilin	
Forward	5'-CAGGGTTTATGTGTCAGGGTGGTG-3'
Reverse	5'-AGATGCCAGGACCCGTATGCTTTAG-3'

cycling parameters recommended by the manufactures (40 cycles of 15 s at 94°C and 30 s at 55°C). Titrations of all the genes (0.5 μM) forward and reverse primers against increasing amounts of cDNA gave linear responses with slopes of -0.24.

4. Western blot analysis

After completion of treatments in HepG2 cells washed twice with PBS and cells were harvested in a lysis buffer (Cell Signaling, Beverly, MA, USA) and the protein concentration was determined by the BCA assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved on 10% SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), the membrane was incubated overnight at 4°C with specific primary antibodies for polyclonal anti-human LDL (Chemicon International, Inc, Temecula, CA, USA) (1:2,000 dilution in the incubating buffer) or ApoB 100 (Biodesign International, Saco, ME, USA) (1:2,000 dilution in the incubating buffer). After a series of washes, the membranes were incubated in the incubating buffer containing goat anti-human antibodies (1:2,000 dilution in the incubating buffer) conjugated with horseradish peroxide (Sigma-Aldrich, Inc, Saint Louis, MO, USA), Signals were detected by an enhanced chemiluminescence reagent (Santa Cruz Biotech, CA, USA), and a LAS-4000 lumino-image analyzer system (Fujifilm, Tokyo, Japan). The data were normalized by blotting the same membranes with mouse anti-β-actin (Sigma-Aldrich, Inc, Saint Louis, MO, USA) (1:10,000 dilution in the incubating buffer) as a house keeping gene using image reading and analyzing programs (Fujifilm).

5. Total cholesterol analysis

To measure total cholesterol in HepG2 cells, 1 mL of 1 M/L KOH in methanol was added to cells to saponify cholesterol esters. The tubes were then flushed with N_2 and heated at 45°C for 1 h. After hydrolysis was completed, the solution was diluted with 2 ml of water and the lipids were extracted twice with hexane (1 ml). Total cholesterol was determined with an enzymatic cholesterol assay kit (BC 180-E, YD-Diagnostic, Inc, Seoul, Korea). The absorbance was read using a spectrophotometer (Smartspec 3000, Bio-Rad Laboratories, Hercules, CA, USA) at 500 nm.

6. Statistical analysis

All experiments were repeated at least three times and statistical analysis was performed using

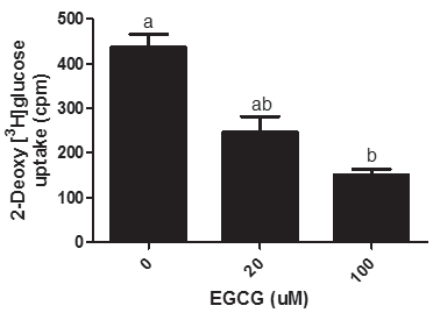
one-way analysis of variance and Duncan's multiple comparisons using the Graph Pad Prism 4 for Windows statistical software package (Graph Pad Software Inc., La Jolla, CA, USA). All the data presented is expressed as the mean SEM from three independent measurements. A P-value of < 0.01 or < 0.05 was considered to be statistically significant.

Results and discussion

1. Glucose uptake and GLUT 4 translocation

Results showed that 100 μM EGCG treatment significantly ($p < 0.01$) increased 2-Deoxy[^{3}H]glucose uptake compared to 0 μM EGCG treatment (Fig. 1). There has been no report on the effect of EGCG on glucose transport in adipocytes so far. However, it was assumed that EGCG could stimulate glucose transport in adipocyte cell due to its ability of enhancing cell viability by antioxidant capabilities (Lee *et al*., 2005). To further substantiate for its glucose-stimulatory activity, pre-adipocyte were incubated with certain amounts of EGCG. The treated cells were then fixed and immunostained with an antibody specific against GLUT 4, and photographed with a confocal fluorescence microscope. The immunostained fluorescence signal were more intensified and translocated to cytosol outer membrane in lower EGCG treatments, that is very accordingly to 2-Deoxy[^{3}H]glucose uptake

A.



B.

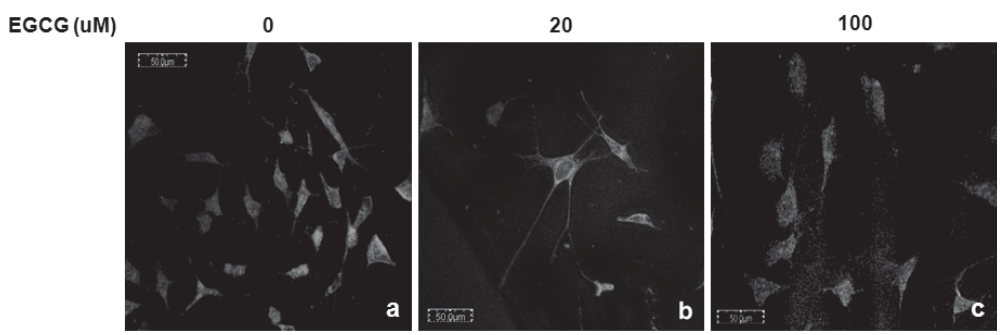


Fig. 1. The effects of EGCG treatment on glucose transport in 3T3-L1 cells. (A) Cells were treated for 24 h with different levels of EGCG. The bar graph indicates the mean ± SEM of three independent analyses. [a,b,]Means above the columns followed by different letters differ significantly ($p < 0.01$). (B) The treated cells were fixed and immunostained with an antibody specific against GLUT 4, and photographed with a confocal fluorescence microscope. The signal are more intensified and translocated to cytosol outer membrane with lower EGCG level.

measurement (Fig. 1).

2. Adipocyte differentiation-related gene expression

3T3-L1 cells were cultured in adipogenic medium for 10 days and then adipocyte differentiation maker gene was analyzed by real-time RT- PCR. Results showed that 20 and 100 μM EGCG significantly reduced expression of LPL (50 and 69%, $p < 0.05$) compared with 0 μM EGCG treatment (Table 3). Furthermore, 100 μM EGCG treatments also reduced (43%, $p < 0.05$) expression of C/EBP-α (Table 3). In addition, both 20 and 100 μM EGCG reduced expression of PPAR-γ2 (78 and 73%, $p < 0.01$) compared with 0 μM EGCG treatment (Table 3). Inhibition of PPAR-γ2 by both higher levels of EGCG, rather than over LPL is more consistent with the role of this gene. PPAR-γ2 is a major adipogenic transcription factors expressed during whole differentiation period, while LPL is one of adipocyte expressed genes at terminal differentiation duration (Cowherd *et al*., 1999). It is unclear why both higher levels of EGCG did not inhibit C/EBP-α expression given that it is also a major adipogenic transcription factors expressed during whole differentiation period.

3. Aberrant lipid metabolism-related gene expression and cholesterol analysis

Twenty and 100 μM EGCG significantly ($p < 0.05$) decreased the level of HMG-CoA reductase mRNA compared with 0 μM EGCG cells (Table. 4). However, neither 20 μM EGCG altered mRNA expression nor protein abundance of LDL receptors (Table 4). One of aims of the present study was to determine whether EGCG would reduce expression of apoB 100 in HepG2 cells. When cells were incubated with 20 and 100 μM EGCG, apoB 100 production were suppressed by 5 and 10%, respectively compared with 0 μM EGCG cells (Table 4). Both 20 and 100 μM EGCG significantly decreased ($p < 0.05$) cholesterol availability, which regulates VLDL synthesis and secretion (Mohammadi *et al*., 1998), in the cells (Table 4). Generally, a decrease in intra cellular cholesterol triggers the cell to up-regulate the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, and also LDL receptor gene expression, to increase cholesterol concentrations on the cells (Brown and Goldstein, 1986). Collectively, our data suggest that 20 μM EGCG was similar to 100 μM EGCG in decreasing availability of cholesterol to these cells, which resulted in a suppression of apoB 100, and has

Table 3. Concentrations of mRNA for LPL, C/EBP-α, and PPAR-γ2 in 3T3-L1 cells grown with 0, 20 and 100 uM EGCG.

	EGCG (μM)				
Item	**0**	**20**	**100**	**SEM**	***p*-value**
mRNA[a]					
LPL	100[b]	50[c]	31[c]	4.7	0.01
C/EBP-α	100[b]	100[b]	57[c]	1.6	0.04
PPAR-γ2	100[b]	22[c]	27[c]	1.3	0.01

[a] The data are expressed as a percentage of the value observed in the 0 treatment. Each datum was normalized by the quantity of cyclophilin mRNA detected in the same cell by real-time reverse transcriptase PCR.

[b,c,d] Within a row, means not bearing a common superscript differ ($p < 0.05$).

Table 4. Concentrations of mRNA for HMG CoA and LDL receptor and protein for LDL receptor and ApoB 100, and total cholesterol in HepG2 cells grown with 0, 20 and 100 μM EGCG

	EGCG (μM)				
Item	**0**	**20**	**100**	**SEM**	***p*-value**
mRNA[a]					
HMG CoA	100[c]	7.5[d]	7.4[d]	0.9	0.01
LDL receptor	100[c]	105[c]	50[d]	2.4	0.02
Protein[b]					
LDL receptor	100	113	115	8.3	0.25
ApoB 100	100[c]	95[cd]	90[d]	2.5	0.04
Total cholesterol[e]	100[c]	52[d]	52[d]	2.6	0.01

[a] The data are expressed as a percentage of the value observed in the 0 treatment. Each datum was normalized by the quantity of cyclophilin mRNA detected in the same cell by real-time reverse transcriptase PCR.

[b] The data are expressed as a percentage of the value observed in the 0 treatment. Each datum was normalized by the quantity of ß-actin detected in the same tissue by Western blot analysis.

[c,d] Within a row, means not bearing a common superscript differ ($p < 0.05$).

[e] The data are expressed as a percentage of the value observed in the 0 treatment.

either a tendency towards, or significantly increases in expression of LDL receptors and HMG-CoA reductase. However, we are not able to explain why there is an inverse relationship between expression of LDL receptor mRNA and protein abundance in HepG2 cells after the 100 μM EGCG treatment.

Conclusion

Our present findings suggest that both 20 and 100 μM EGCG could down regulate major adipogenic transcription factors and adipocyte expressed genes such as C/EBP-α, PPAR-γ2 and LPL through retarding glucose uptake action in 3T3-L1 cells. Furthermore, those levels of EGCG likely have similar effect on decreasing cholesterol availability in HepG2 cells, which resulted in suppression of apoB 100 and either tendency towards a significant decrease in LDL receptor or of HMG-CoA reductase. Thus, EGCG may be of great benefit in anti-obesity drug and treatment of abnormality in lipid metabolism.

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