(-)-Epigallocatechin gallate suppresses adipocyte differentiation through the MEK/ERK and PI3K/AKT pathways

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Short Running Title: EGCG suppresses the clonal expansion of adipocytes

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Keywords: EGCG, 3T3-L1, ROS, FoxO1, MEK/ERK, PI3K/AKT

Abbreviations: EGCG, (-) epigallocatechin gallate; ERK, extracellular signal-regulated protein kinase**;** FoxO, Forkhead transcription factor class O**;** MEK, MAPK/ERK kinase**;** PI3K, phosphatidyl inositol 3 kinase

Abstract

Tea catechin, (-) epigallocatechin gallate (EGCG), is one of the compounds that have been reported to act against obesity and diabetes. In order to determine the effect of EGCG on adipocyte differentiation, we treated 3T3-L1 preadipocytes with different kinds of catechins. Oil Red O staining showed that catechins, especially EGCG, significantly reduced intracellular lipid accumulation. Cell cycle analysis revealed that EGCG inhibited cell proliferation by disturbing the cell cycle during the clonal expansion of 3T3-L1. Real-time polymerase chain reaction (PCR) revealed that EGCG noticeably reduced mRNA expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (C/EBPα), and forkhead box class O (FoxO1). EGCG also caused a significant decrease in the transcriptional activity of FoxO1, a forkhead transcription factor class O1 involved in adipocyte differentiation, via the phosphoinositide-3-kinase (PI3K)/AKT and mitogen-activated protein kinase kinase-extracellular-signal-regulated kinase (MEK/ERK) pathways. These results suggest that EGCG suppresses the clonal expansion of adipocytes by inactivating FoxO1 via insulin signaling and stress-dependent MAP kinase pathways.

Keywords: EGCG, 3T3-L1, ROS, FoxO1, MEK/ERK, PI3K/AKT

1. Introduction

Obesity is generally considered as a major risk factor for diabetes, hypertension, and heart disease (David et al., 2001; Lee et al., 2005; Balkau et al., 2007). Obesity is caused by both genetic and environmental factors (Famer and Auwerx, 2004; Lee et al., 2005). The development of obesity is characterized by an increased number of fat cells and their lipids as a result of the mitogenesis and differentiation processes (Famer and Auwerx, 2004; Liao et al., 2001). Further understanding of these mechanisms would aid in the prevention of obesity and associated diseases in humans.

Tea is used as a common beverage across the world. It is well known that tea prevents many diseases, and longevity is often associated with the habit of drinking tea (Ahmad and Mukhtar, 1999; Crespy and Williamson, 2004; Liao et al., 2001; Lin et al., 1999; Mitcher et al., 1997). Recently, it has been reported that (-) epigallocatechin gallate (EGCG) in green tea polyphenols prevents the incidence of cancer (Ahmad and Mukhtar, 1999; Lin et al., 1999; Mitcher et al., 1997), collagen-induced arthritis (Haqqi et al., 1999), oxidative stress-induced neurodegenerative diseases (Mendel and Youdim, 2004), and streptozotocin-induced diabetes (Song et al., 1999). In addition, EGCG can reduce body weight and body fat (Kao et al., 2000a). Moreover, injection of EGCG into rats reduces their food uptake; lipid absorption; and blood triglyceride, cholesterol, and leptin levels, as well as stimulates energy expenditure, fat oxidation, high-density lipoprotein levels, and fecal lipid excretion (Dulloo et al., 1999; Kao et al., 2000a; Kao et al., 2000b; Liao et al., 2001). EGCG was found to reduce cell number and triacylglycerol content during the differentiation of preadipocytes (Kao et al., 2000a; Kim et al., 2010) and decreased the growth of 3T3-L1 preadipocytes. These evidences suggest that EGCG regulates the mitogenic, endocrine, and metabolic functions of adipocytes (Kao et al., 2000a; Kim et al., 2010).

The clonal cell lines of preadipocytes, such as mouse 3T3-L1 and 3T3-F442A (Gregoire et al., 1998; Rosen et al., 2000), require sequential events, including growth arrest of proliferating preadipocytes, reentry into the cell cycle with limited clonal expansion, and growth arrest associated with terminal differentiation. These changes in cell cycle require the expression of several adipogenic transcription factors, including an early increase in the expression of CCAAT/enhancer-binding protein (C/EBP) β and C/EBPδ followed by the induction of peroxisome proliferator-activated receptor (PPARγ) and C/EBPα (Mandrup and Lane, 1997; Morrison and Farmer, 2000; Rosen et al., 2000; Srujana et al., 2007). In a recent study, Nakae et al. reported that the over-expression of activated FoxO1 in adipocyte progenitor cells induces the expression of the cell cycle arrest-associated factor p21 and C/EBP inhibitor CHOP10; these disturb the progression of clonal expansion, thereby inhibiting adipocyte differentiation (Nakae et al., 2003). In another study, Munekata et al. reported that knockdown of FoxO1 mRNA expression markedly suppressed adipocyte differentiation (Munekata and Sakamoto, 2009). These observations suggest that FoxO1 plays an essential role in adipocyte differentiation.

Insulin-like growth factor I (IGF-I) regulates mitogenesis, survival, and protein synthesis (Yin et al., 2009). IGF-I acts by binding to its cognate cell membrane receptor and then

activates several signaling intermediates, including members of the insulin receptor substrate (IRS) family and Shc (Src homolog and collagen homolog). Activation of IRS leads downstream to the activation of the PI3-kinase-Akt pathway, and the activation of Shc leads to the activation of the MEK/ERK pathway (Samani et al., 2007). Stimulation of the PI3-kinase-Akt pathway most often promotes cell growth, protein synthesis, and cell survival, whereas stimulation of the MEK/ERK pathway promotes cell proliferation and gene transcription (Yin et al., 2009).

In this study, we analyzed the suppressive effect of EGCG on adipocyte differentiation mediated by the cell growth arrest at clonal expansion. In particular, we focused on the transcription factor FoxO1 that participates in clonal expansion arrest under the control of MEK/ERK and PI3K/AKT pathways.

2. Materials and Methods

2.1. Materials

(-)-Epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-catechin gallate (CG) were provided by Mitsui Norin (Tokyo, Japan). The catechins used in this study were dissolved in methanol and stored at –20 °C. PI3-K inhibitor (LY294002) was purchased from Echelon Biosciences Inc. (UT, USA), and MEK inhibitor (PD98059) was purchased from Calbiochem (Dermtadt, Germany). These inhibitors were dissolved in methanol and stored at –20 °C.

2.2. Cell culture

The 3T3-L1 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM high-glucose) supplemented with 10% fetal bovine serum (FBS) (Sanko Junyaku, Tokyo, Japan). After the cells reached confluence, they were cultured for 2 days with 0.25 μM dexamethasone (Sigma-Aldrich, Missouri, U.S.A), 0.5 mM 3-iso-butyl-1-methylxanthine (Sigma-Aldrich), and 10 μg/ml insulin (Wako, Osaka, Japan) (DMI induction). Next, the cells were cultured in the media containing 5 μg/ml insulin for 2 days and in standard culture media for 4 days (Rubin et al., 1978).

2.3. Oil Red O staining

Cells were cultured in media containing DMI and catechin (100 μM of EGCG, ECG, EGC, or CG) for 2 days and in standard culture media for 6 days. Cells were fixed with 4% paraformaldehyde for 1 h and then dyed with 3 mg/ml Oil Red O (Sigma-Aldrich) (in 60% isopropanol) for 10 min. After washing, the dye was eluted for 10 min with 100% isopropanol. The concentration of the eluted dye was determined from absorbance measurements (optical density (O.D.) 420 nm).

2.4. Triglyceride assay

Cells were lysed in lysis buffer (20 mM HEPES [pH 7.6], 420 mM NaCl, 1% Triton X-100,

0.1% SDS), and total fat was extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). The cell extract (600 μl) was incubated in 2 ml methanol and 1 ml chloroform for 1 h, after which 1 ml chloroform and 1 ml of water were added, and samples were centrifuged briefly to collect the chloroform phase. This extract was dried overnight and dissolved in 10% triton-isopropanol solution. The quantity of triglyceride was measured according to the instructions for the triglyceride E-test Wako (Wako). The quantity of triglycerides (μg/μl) was normalized according to the each protein content.

2.5. Measurement of reactive oxygen species (ROS)

ROS were detected with the peroxide-sensitive fluorophore 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Wako). At the times indicated, DCF-DA dissolved in DMSO was added to cells at a final concentration of 5 μM. After incubation for 30 min at 37 °C, 3T3-L1 cells were washed with Hank's Balanced Salt (HBBS, Sigma-Aldrich) and suspended in HBBS. Cells were observed under a BZ-Analyzer fluorescence microscope (KEYENCE, Osaka, Japan).

2.6. Proliferation experiments

Cells were cultured in the presence and absence of DMI and either EGCG (100 μM) or *N*-acetylcystein (NAC, 10 mM) for 2 days. Cells were collected after 0 and 2 days culture and then counted.

2.7. FACS analysis

Cells were cultured in the presence and absence of DMI and either EGCG (100 μM) or NAC (10 mM). Cells were cultured for 1 or 2 days, fixed in 75% ethanol, and then stained with the Guava Cell Cycle Reagent (Guava Technologies, CA, USA). The cells were analyzed by using a flow cytometry system with Guava PCA (Guava Technologies).

2.8. Real-time PCR

Quantitative polymerase chain reaction (PCR) analysis was performed with Gene Amp 5700 Sequence Detection System (Applied Biosystems, CA, USA) and Thunder Birds SYBR qPCR mix reagent (TOYOBO, Osaka, Japan). PCR (95 °C for 15 s, 60 °C for 1 min, for 40 cycles) was performed using the specific primers (5′-AAA CTC TGG GAG ATT CTC CT-3′ and 5′-TGG CAT CTC TGT GTC AAC-3′) for PPARγ, (5′-GCC AAA CTG AGA CTC TTC-3′ and 5′-GGA AGC CTA AGT CTT AGC-3′) for C/EBPα, (5′-GAT CTA CGA GTG GAT GGT-3′ and 5′-CTT GCT GTG AAG GGA CAG-3′) for FoxO1, (5′-ACC AGG CTG AAG GAT CAC TGA GGA-3′ and 5′-GGC GTG GGA GTC TCA AAG GTG T-3′) for FoxO3a, and (5′- CTG TGC TGC TCA CCG AGG-3′ and 5′-AGC CTG GAT GGC TAC GTA-3′) for β-actin. β-actin was used as an internal standard for normalization of each sample.

2.9. Luciferase assay

COS7 cells were transfected with FoxO1-expressing plasmid (pcDNA3-FoxO1, 0.25 μg/4 cm

dish), luciferase reporter plasmid with consensus sequences of FoxO1 binding site (IRS-Luc, 0.5 μg/4 cm dish), and β-galactosidase-expressing plasmid (PCMV-β-gal, 0.25 μg/4 cm dish). After 45 h of incubation, the cells were treated with EGCG (0, 10, 20, 50, or 100 μM) for 3 h. The luciferase reporter plasmid with a FoxO binding site of the catalase promoter (Catalase-Luc) was constructed. The stable 3T3-L1 cells carrying Catalase-Luc were prepared (3T3-L1/Catalase-Luc). These cells were treated with catechin (100 μM each of EGCG, ECG, and EGC) and NAC (10 mM) for 2 days. After brief centrifugation (12,000 rpm; 2 min; MX-100; Tomy, Tokyo, Japan) of the cell lysate, supernatant was collected and mixed with Luciferase Assay Reagent (Promega, Wiscony, USA). Luciferase activity was measured with Luminometer Microlumat LB69p (Berthold Technology, Wildbad, Germany).

3. Results

3.1. Effect of catechin on fat accumulation

In our previous study, we have examined the effects of catechins (EGCG, ECG, EGC and CG) on 3T3-L1 cells at various doses (0, 5, 50, 100, 200 μM) and at various developmental periods (0-2, 2-4 and 0-8 days after DMI induction) (Kim et al., 2010). From these data, we identified that exposure of 100 μM EGCG at 0-2 days of differentiation reduced fat accumulation most efficiently, without toxicity. In this study, we have exposed 3T3-L1 cells with catechin (EGCG, ECG, EGC, or CG, 100 μM) and DMI for 2 days. After 6 days of culture, cells were stained with Oil Red O, and the absorbance of the extracted dye was measured (OD, 420 nm). As observed in Figure 1A, catechin suppressed Oil Red O-stained lipid level, especially ECG showed the strongest suppressive effect. Under the same conditions, we determined triglyceride accumulation (Fig. 1B) and found that catechin efficiently reduced intracellular level of triglyceride. Because catechins belong to antioxidant compounds, the fat level in adipocytes is possibly changed by their antioxidative activity. Accordingly, we treated cells with *N*-acetylcysteine (NAC), one of the commonly used antioxidants, which also resulted in reduced triglyceride accumulation (Fig. 1B). These results suggest that adipocyte differentiation was possibly prevented by antioxidant effect of catechin.

3.2. Effect of EGCG on intracellular ROS

3T3-L1 cells were treated with DMI and either EGCG (100 μM) or NAC (10 mM) for 2 days, after which cells were cultured in media containing insulin for 2 days. In the absence of EGCG or NAC, 3T3-L1 cells gradually produced intracellular reactive oxygen species (ROS) along with differentiation (Fig. 2). However, EGCG and NAC efficiently removed the intracellular ROS in differentiating 3T3-L1 cells. These results indicate that EGCG and NAC simultaneously reduced ROS and lipid accumulation in DMI-induced 3T3-L1 cells.

3.3. Effect of EGCG on proliferation

3T3-L1 cells were treated with EGCG (100 μM) or NAC (10 mM) in the presence and absence of DMI for 2 days. The proliferation of 3T3-L1 cells was not changed by EGCG and NAC in

the absence of DMI induction (DMI (-)) (Fig. 3). It is generally considered that, after the DMI induction, 3T3-L1 proliferates twice (so called clonal expansion) to lead to terminal differentiation (Nakae et al., 2003). Under the DMI induction, the cell number of 3T3-L1 cells increased drastically, and cell growth was significantly inhibited by EGCG treatment (DMI (+)). On the other hand, NAC exerted only weak suppressive effect on cell proliferation of DMI-induced 3T3-L1 preadipocytes. Accordingly, these results indicate that EGCG strongly suppressed the clonal expansion of differentiating 3T3-L1 cells.

3.4. Effect of catechin on cell cycle

Under the same conditions, the effect of EGCG on cell cycle status was analyzed using flow cytometry. As shown in Figure 4, EGCG and NAC did not affect the cell cycle rate of 3T3-L1 in the absence of DMI. On the other hand, after DMI-treatment, EGCG disturbed the cell cycle of 3T3-L1 cells (DMI (+)). In DMI-induced 3T3-L1, EGCG, and not NAC, reduced the cell population at the G0/G1 phase and increased the cell population at the S phase (DMI (+)). These results indicate that EGCG specifically inhibited the clonal expansion of differentiating 3T3-L1 cells.

3.5. Effect of catechin on mRNA expression of clonal expansion-related gene

To elucidate the signaling pathway through which EGCG acts on the clonal expansion of 3T3-L1, we performed real time-PCR and analyzed the endogenous mRNA level of clonal expansion-related genes, including PPARγ, C/EBPα, FoxO1, and FoxO3. As observed in Figure 5, EGCG significantly reduced the mRNA expression of PPARγ, C/EBPα, and FoxO1, but did not reduce the mRNA expression of FoxO3. In contrast, NAC did not change the mRNA level of these genes. These results suggest that EGCG was specifically involved in the clonal expansion by reducing the mRNA expression of PPARγ, C/EBPα, and FoxO1.

3.6. Effect of EGCG on the transcriptional activity of FoxO1

To further analyze the effect of EGCG on FoxO1, we analyzed the transcriptional activity of FoxO1. COS7 cells were transfected transiently with FoxO1-expressing plasmid (pcDNA3-FoxO1), luciferase reporter plasmid with consensus sequences of FoxO1 binding site (IRS-Luc), and β-galactosidase-expressing plasmid (PCMV-β-gal). These cells were treated with EGCG (0, 10, 20, 50, and 100 μM) for 3 h and analyzed for luciferase assay. At the concentration of 100 μM, EGCG reduced the transcriptional activity of FoxO1 in COS7 cells (Fig. 6A). We also prepared 3T3-L1 cells that stably expressed the luciferase reporter plasmids with a FoxO1 binding site of catalase promoter (3T3-L1/Catalase-Luc). As shown in Figure 6B, EGCG and NAC reduced the transcriptional activity of FoxO1. To elucidate the signaling mechanism underlying the effect of EGCG on FoxO1, we used the specific inhibitors against PI3K (LY294002) and MEK (PD98059). EGCG reduced the transcriptional activity of FoxO1 after the treatment with LY294002 or PD98059, whereas NAC failed to suppress FoxO1 activity in the presence of PD98059. In contrast to these results, EGCG lost its suppressive effect on FoxO1 after the co-treatment with LY294002 and PD98059. These

results suggest that EGCG suppresses the clonal expansion of differentiating adipocytes by reducing the transcriptional activity of FoxO1 via the PI3K/AKT and MEK/ERK pathways.

4. Discussion

Our results clearly indicate that catechin suppressed the differentiation of 3T3-L1 preadipocytes. NAC, which also has antioxidant activities, similarly suppressed the differentiation of 3T3-L1 cells (Fig. 1). These data suggest that antioxidant activities of EGCG suppress the differentiation of 3T3-L1 cells.

Mature adipocytes produce higher levels of ROS compared with other tissues, including liver, skeletal muscle, and aorta (Shimomura et al., 2007). Thus, it is possible that mature adipocytes could be major sources of ROS that accelerate the differentiation of preadipocytes. Recently, it was reported that ROS facilitate adipocyte differentiation by inducing mitotic clonal expansion (Lee et al., 2009). Our studies showed that intracellular ROS level increased along with differentiation of 3T3-L1 (Fig. 2), and EGCG and NAC clearly reduced the ROS generated from mitochondoria in developing adipocytes, suggesting that EGCG and NAC possibly suppress adipocyte differentiation by lowering intracellular ROS. However, these results did not clearly indicate whether EGCG and NAC reduce ROS level by inhibiting the differentiation or suppress differentiation by reducing the ROS level. Recently, it was reported that various phytochemicals like genistein, docosahexaenoic acid, epigallocatehin gallate, quercetin and resveratrol inhibit adipogenesis or induce apoptosis (Srujana et al., 2007). For instance, genistein suppressed mitotic clonal expansion of postconfluent 3T3-L1 preadipocytes (Harmon and Harp, 2001). To understand why EGCG suppresses adipocyte differentiation, we analyzed the effect of EGCG on cell proliferation and cell cycle (Figs. 3, 4). These analyses suggest that EGCG inhibited cell proliferation and disturbed the cell cycle, whereas NAC partially affected the cell cycle (Fig. 4), indicating that EGCG possibly reduces intracellular ROS by suppressing clonal expansion of the DMI-treated 3T3-L1 cells.

Furthermore, we examined the effect of EGCG on the endogenous mRNA expression of clonal expansion-related genes, including PPARγ, C/EBPα, and FoxO1. EGCG substantially reduced mRNA expression of PPARγ, C/EBPα, and FoxO1 (Fig. 5), whereas NAC did not. These data suggest that EGCG may arrest clonal expansion by reducing the expression of clonal expansion-related genes and thereby suppress differentiation. Next, we focused on FoxO transcription factors. It has been reported that the FoxO family inhibits the clonal expansion stage (Nakae et al., 2003). Nakae et al. reported that FoxO1 suppresses adipocyte differentiation, whereas we clarified the fact that FoxO1 is essential to facilitate the differentiation of 3T3-L1 (Munekata and Sakamoto, 2009). Because EGCG reduced FoxO1 transcriptional activity in COS7 cells (Fig. 6A) and 3T3-L1 cells (Fig. 6B), it was suggested that suppressive effect of EGCG on adipocyte differentiation is at least partially caused by the inactivation of FoxO. Our data suggested that EGCG possibly reduces intracellular ROS level by inhibiting clonal expansion of 3T3-L1. In our previous study, we found that HepG2 cells externally exposed to ROS showed increased lipid accumulation (Sekiya et al., 2008). Because

NAC shows strong antioxidant activity, it possibly reduces lipogenic activity by lowering intracellular ROS level in 3T3-L1 cells.

To clarify the mechanism underlying the inactivation of FoxO1, we analyzed the involvement of PI3K/AKT and MEK/ERK pathways in EGCG-treated 3T3-L1 cells (Fig. 6B). The suppressive effect of EGCG on FoxO1 remained unchanged by the treatment with PI3K inhibitor (LY294002) or MEK inhibitor (PD98059). However, EGCG lost its effect on FoxO1 in the presence of both the inhibitors. We speculated that insulin activates the IRS family and Shc: activated IRS leads to the activation of the PIK/AKT pathway, and activated Shc leads to the activation of the MEK/ERK pathway. These results indicate that EGCG activates the PI3K/AKT pathway and inactivates the MEK/ERK pathway, thereby reducing the transcriptional activity of FoxO1. Because catechin did not affect the expression of Gadd45 (data not shown), we ignored the participation of WNT signaling pathway.

These findings indicate that EGCG specifically suppresses differentiation by lowering the endogenous mRNA expression of clonal expansion-related genes, and particularly, that EGCG reduces the transcription activity of FoxO1 via the MEK/ERK and PI3K/AKT pathways. Future studies on the association between EGCG and FoxO1 may aid in the prevention of diabetes and obesity.

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Figure Legends

Fig. 1. Effect of catechin on adipocyte differentiation
(A) Adipose accumulation was observed in the cells treated with catechin. Cells were treated with catechin (100 μM of EGCG, ECG, EGC, or CG) and DMI for 2 days. Then, cells were cultured for 2 days with insulin (5 μg/ml) and for 4 days in standard medium, after which cells were fixed for Oil Red O staining. The cells stained with Oil Red O were treated with isopropanol to extract the dye, and the concentration of eluted dye was determined by measuring the absorbance at an optical density of 420 nm. An error bar shows the standard deviation of the mean, $n = 3$; $^{**}P < 0.05$. (B) Cells were treated by EGCG (100 μM), ECG (100 μM), or NAC (10 mM) with DMI for 2 days. Then, cells were cultured for 2 days with insulin (5 μg/ml) and for 4 days in standard medium. Lipid was then extracted using the Bligh and Dyer methods (Bligh and Dyer, 1959). Triglyceride level was corrected by total protein level. An error bar shows the standard deviation of the mean, $n = 3$; $^{**}P < 0.05$.

Fig. 2. Effect of EGCG on the level of ROS
Cells were treated with either EGCG (100 μM) or NAC (10 mM) with DMI for 2 days. Then, cells were cultured for 2 days with insulin (5 μg/ml) in the standard medium. After 0, 2, or 4 days culture, cells were treated with DCF-DA (5 μM) for 30 min and observed under a fluorescence microscope.

Fig. 3. Effect of EGCG on cell proliferation
Cells were treated with EGCG (100 μM) or NAC (10 mM) in the absence and presence of DMI. After 0 or 2 days culture, cells were detached and counted. Error bars show standard deviation of the mean, $n = 3$; $^{**}P < 0.05$.

Fig. 4. Effect of EGCG on cell cycle
Cells were treated with EGCG (100 μM) or NAC (10 mM) in the absence and presence of DMI. After 1 or 2 days culture, cells were detached and fixed. Changes in DNA content were analyzed by a flow cytometry system with Guava PCA.

Fig. 5. Effect of catechin on mRNA expression
The mRNA level of each gene was analyzed by real-time PCR. Cells were treated with EGCG (100 μM) or NAC (10 mM) with DMI for 2 days and then cultured for 6 days. RNA was prepared and subjected to cDNA synthesis for real-time PCR assay.

Fig. 6. Effect of EGCG on transcriptional activity of FoxO1

(A) Luciferase activity of the ECGC-treated cells is shown. Cells were transfected with FoxO1-expressing plasmid (pcDNA3-FoxO1, 0.25 μg/4-cm dish), luciferase reporter plasmid with a consensus sequence of FoxO1 binding site (IRS-Luc, 0.5 μg/dish), and β-galactosidase expressing plasmid (PCMV-β-gal, 0.25 μg/dish). After 45 hours of incubation, cells were treated with EGCG (0, 10, 20, 50, or 100 μM) for 3 h and analyzed for luciferase activity. Luciferase activity was corrected by β-galactosidase activity. An error bar shows the standard deviation of the mean, $n = 6$; $^{**}P < 0.05$. (B) The luciferase activity of the EGCG-treated cells is shown. 3T3-L1 cells carrying luciferase reporter plasmid with a FoxO1 binding site (3T3-L1/Catalase-Luc) were pretreated with PI3-K inhibitor (20 μM; LY294002) and MEK inhibitor (50 μM; PD98059) for 2 h, and then treated with EGCG (100 μM) and NAC (10 mM) for 2 days. An error bar shows the standard deviation of the mean, $n = 3$; $^{**}P < 0.05$.

Fig. 1

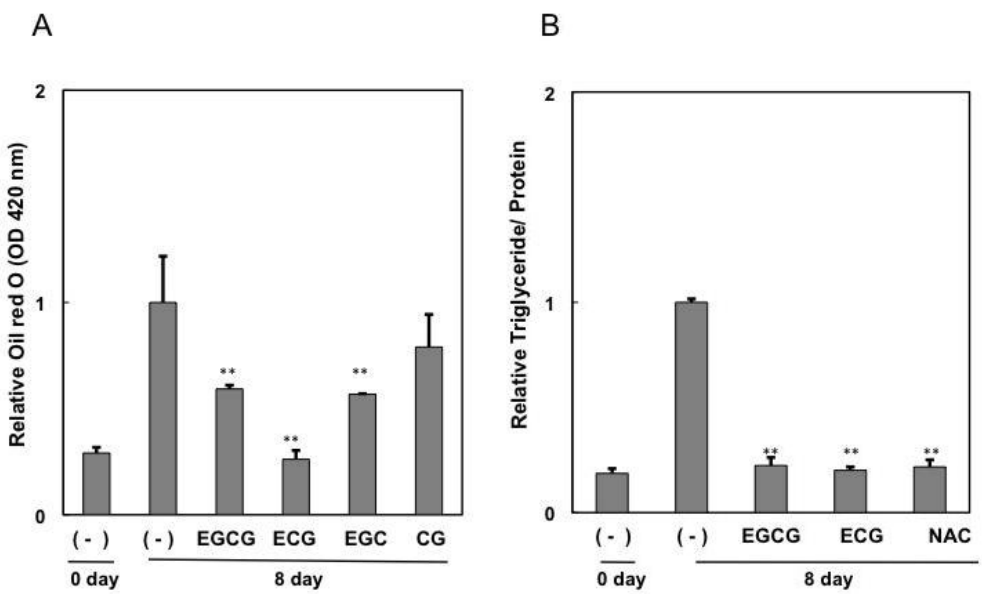

A
B
Relative Oil red O (OD 420 nm)
Relative Triglyceride/ Protein
2
1
0
**
(-)
(-)
EGCG
ECG
EGC
CG
NAC
0 day
8 day

Fig. 2

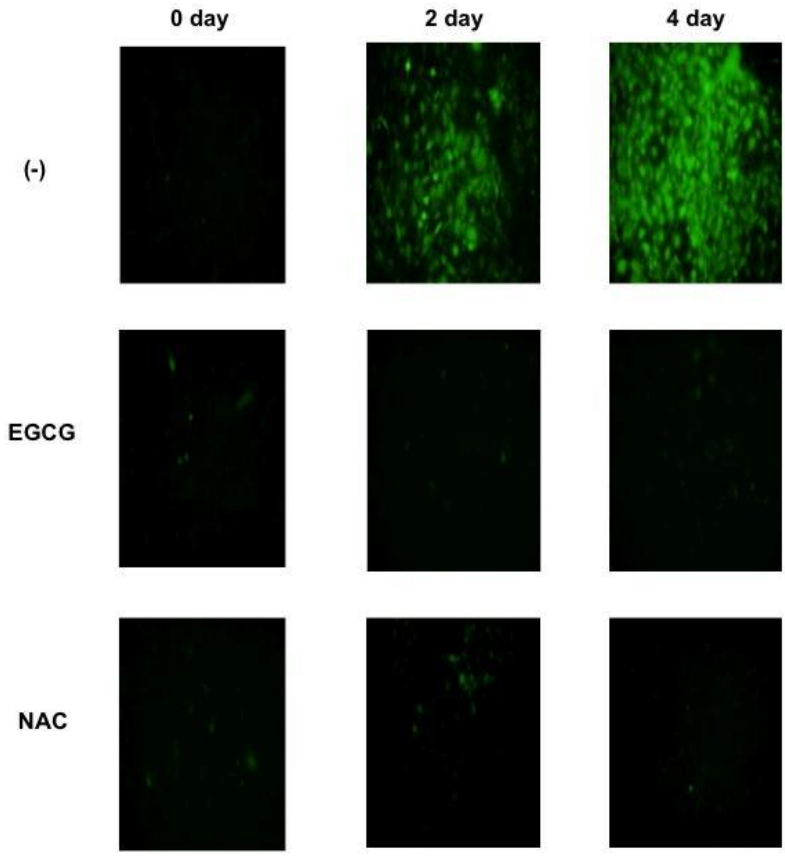


Fig. 3

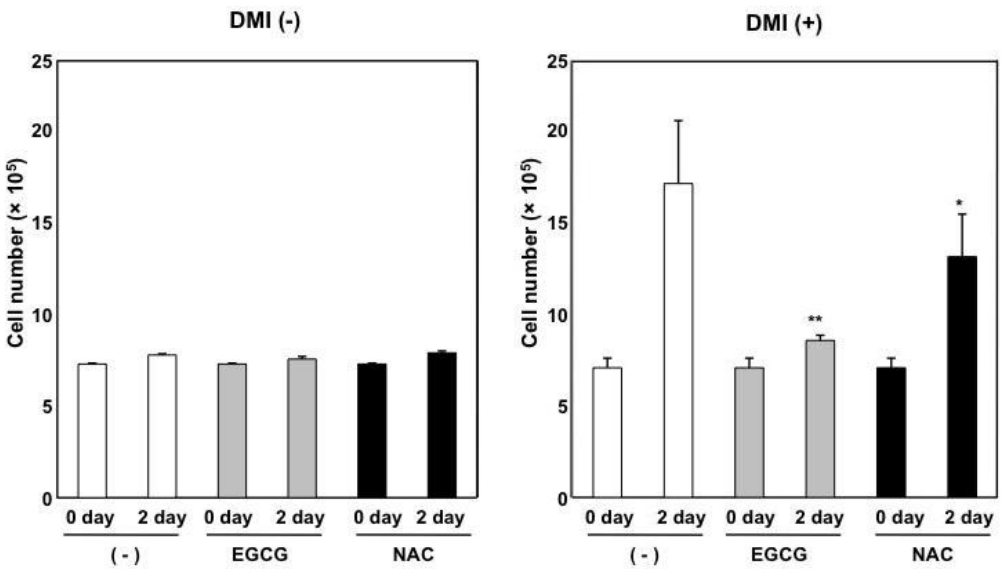


Fig. 4

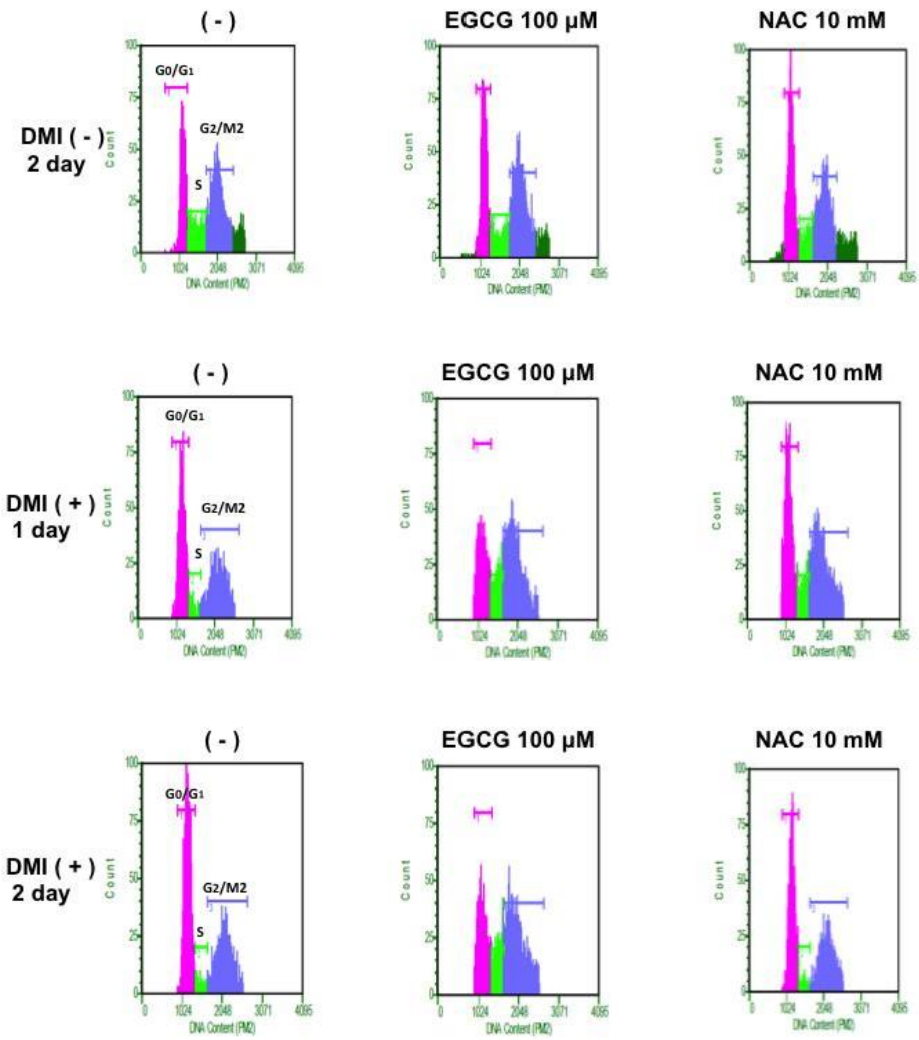

(-)
EGCG 100 μM
NAC 10 mM
DMI (-)
2 day
DMI (+)
1 day
DMI (+)
2 day
G0/G1
G2/M2
S
Count
DNA Content (FM2)

Fig. 5

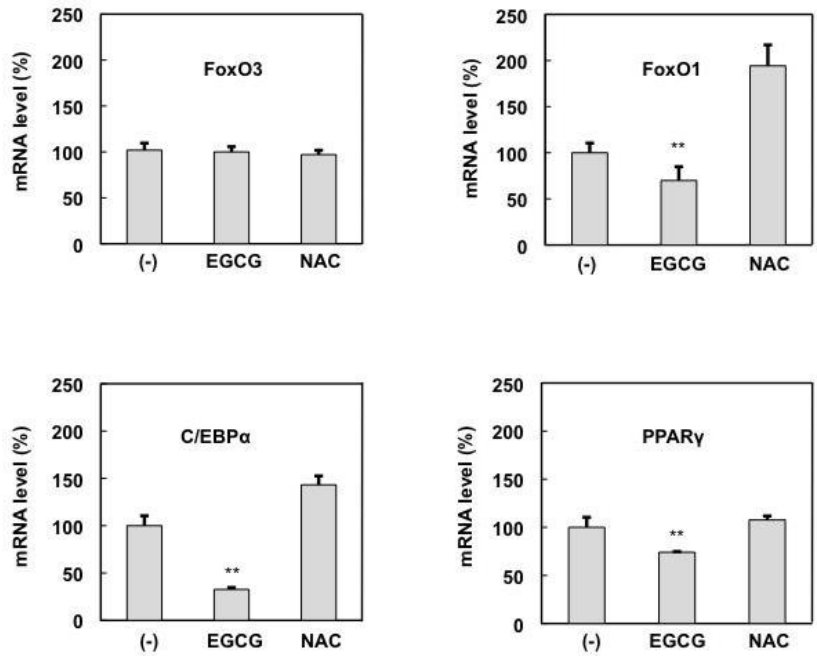
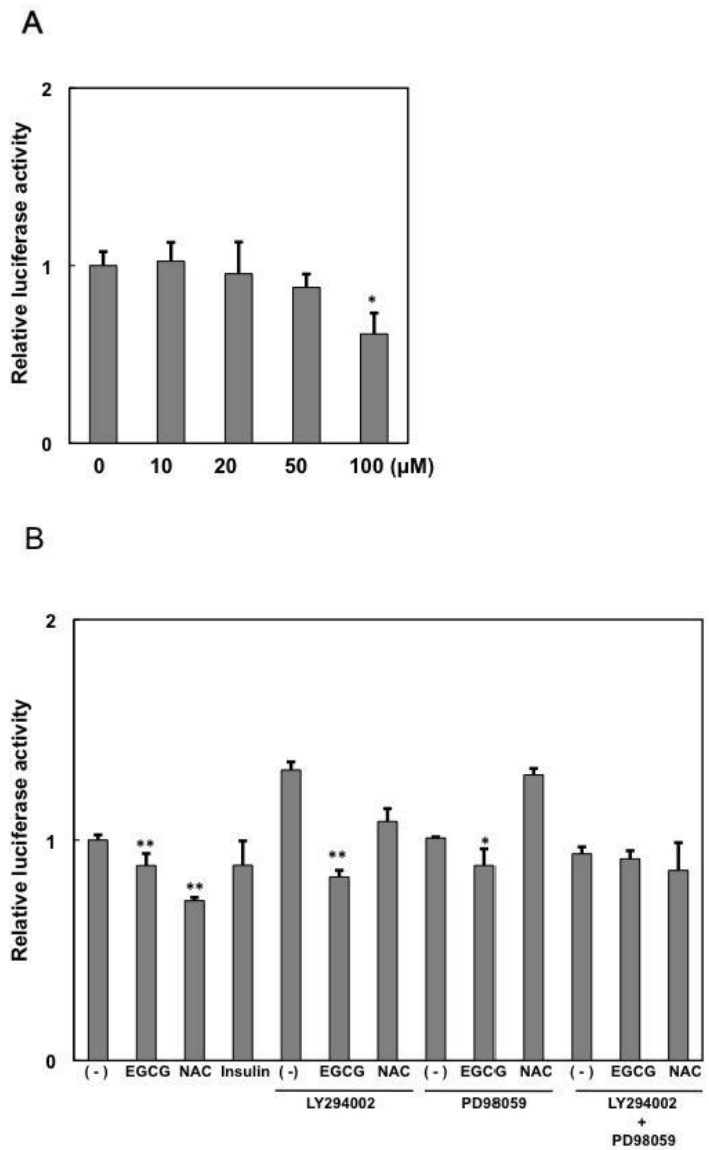

FoxO3
FoxO1
C/EBPα
PPARγ
mRNA level (%)
250
200
150
100
50
0
**
(-)
EGCG
NAC

Fig. 6


A
Relative luciferase activity
2
1
0
0
10
20
50
100 (μM)
*
B
Relative luciferase activity
2
1
0
**
**
**
*
(-)
EGCG
NAC
Insulin
(-)
EGCG
NAC
(-)
EGCG
NAC
(-)
EGCG
NAC
LY294002
PD98059
LY294002
+
PD98059