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Note

Bi-Phasic Effect of Equol on Adipocyte Differentiation of MC3T3-L1 Cells

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We investigated the effects of equol on adipogenesis by measuring lipid accumulation and analyzing the change in adipocyte-related gene expression in MC3T3-L1 cells. Treatment with 10 μM equol tended to increase adipocyte-related gene expression, whereas 100 μM equol reduced lipid accumulation and suppressed the expression of these genes and proteins. Our results suggest that equol regulated adipogenesis in a bi-phasic fashion.

Key words: isoflavone; equol; MC3T3-L1; adipogenesis

Isoflavones are structurally similar to estrogens and bind to estrogen receptors (ER), suggesting they exhibit estrogenic action in various tissues. Isoflavones can therefore be expected to prevent menopausal symptoms, osteoporosis, cardiovascular diseases, and cancers.[1)] Furthermore, a diet rich in isoflavones exerts beneficial effects on lipid metabolism and obesity.[2,3)] Daidzein, a major soybean isoflavone, is metabolized to equol in the gastrointestinal tract by gut microflora.[4)] Equol binds to ER with greater affinity than its precursor.[5)] The preventive effects of isoflavones on fat accumulation in early postmenopausal women depend on an individual's equol-producing capacity.[6)] In addition, dietary equol supplementation reduces whole body fat mass and plasma lipids in ovariectomized (OVX) mice.[2)] However, the effects of equol on adipogenesis are not fully understood. We used in this study MC3T3-L1 cells to examine the effects of equol on lipid accumulation and the expression of several genes and proteins associated with adipogenesis.

(*R*,*S*)-Equol was purchased from LC Laboratories (MA, USA). Anti-peroxisome proliferator-activated receptor gamma (PPARγ) and anti-TFIIB antibodies were obtained from Santa Cruz Biotechnology (CA, USA), and anti-fatty acid synthase (FAS) antibodies were from Cell Signaling Technology (MA, USA). MC3T3-L1 cells (Japanese Collection of Research Bioresources, Osaka, Japan) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Tokyo, Japan) with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Two days after confluence (designated day 0), cell differentiation was induced with an induction medium containing 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 μM dexamethasone (DEX), and 10 μg/mL of insulin (Sigma Aldrich, Tokyo, Japan) in DMEM with 10% FBS. The induction medium was removed after 48 h (day 2) and replaced with DMEM containing 10% FBS supplemented with 5 μg/mL of insulin. This medium was changed every 2 d. Samples were added to the adipocyte culture medium on day 0. Cell proliferation was determined by an MTS assay (Promega, Tokyo, Japan). Preadipocytes were induced to differentiation by the induction medium with or without equol for 3 d. The absorbance was measured at 450 nm by a microplate reader. Cellular lipid accumulation was assessed by the optical density (OD) value after Oil Red O staining (Sigma-Aldrich). The cells were fixed in formalin on day 6 and stained with an Oil Red O working solution. Lipids and Oil Red O were extracted with isopropanol, and the absorbance was measured at 520 nm by the microplate reader. Total RNA was isolated from the cells by using Isogen II (Nippon Gene, Tokyo, Japan) on day 4. Complementary DNA (cDNA) was synthesized from total RNA by using a Prime Script RT master mix (Takara, Shiga, Japan), and cDNA was quantified by real-time PCR, using SYBR Premix Ex Taq II (Takara). The cycling conditions were 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The primer sequences were as follow: PPARγ, 5′-TTTTCAAGGGTGCCAGTTTC-3′ and 5′-AATCCTTGGCCCTCTGAGAT-3′; CCAAT/enhancer-binding protein alpha (C/EBPα), 5′-TTGAAGCACAATCGATCCATCC-3′ and 5′-GCACACTGCCATTGCACAAG-3′; FAS, 5′-TTGCTGGCACTACAGAATGC-3′ and 5′-AACAGCCTCAGAGCGACAAT-3′; CD36, 5′-TCCATTTTGCCTCTGCCTTGA-3′ and 5′-AACATGTGTTTGTGGGGCTCCT-3′; β-actin, 5′-CCACAGCTGAGAGGGAAATC-3′ and 5′-AAGGAAGGCTGGAAAAGAGC-3′. The results are expressed as the number-fold increase relative to the controls after normalizing to β-actin. A Western blot analysis was conducted by lysing the cells in an RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich) on day 4. The extracted proteins were separated by SDS–PAGE on 4–12% gradient gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. After the membrane had been blocked for 1 h with Block Ace (DS Pharma Biomedical, Tokyo, Japan), the membrane

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Abbreviations: ER, estrogen receptor; OVX, ovariectomized; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer-binding protein alpha; FAS, fatty acid synthase; IBMX, 1-methyl-3-isobutylxanthine; DEX, dexamethasone; OD, optical density; cDNA, complementary DNA

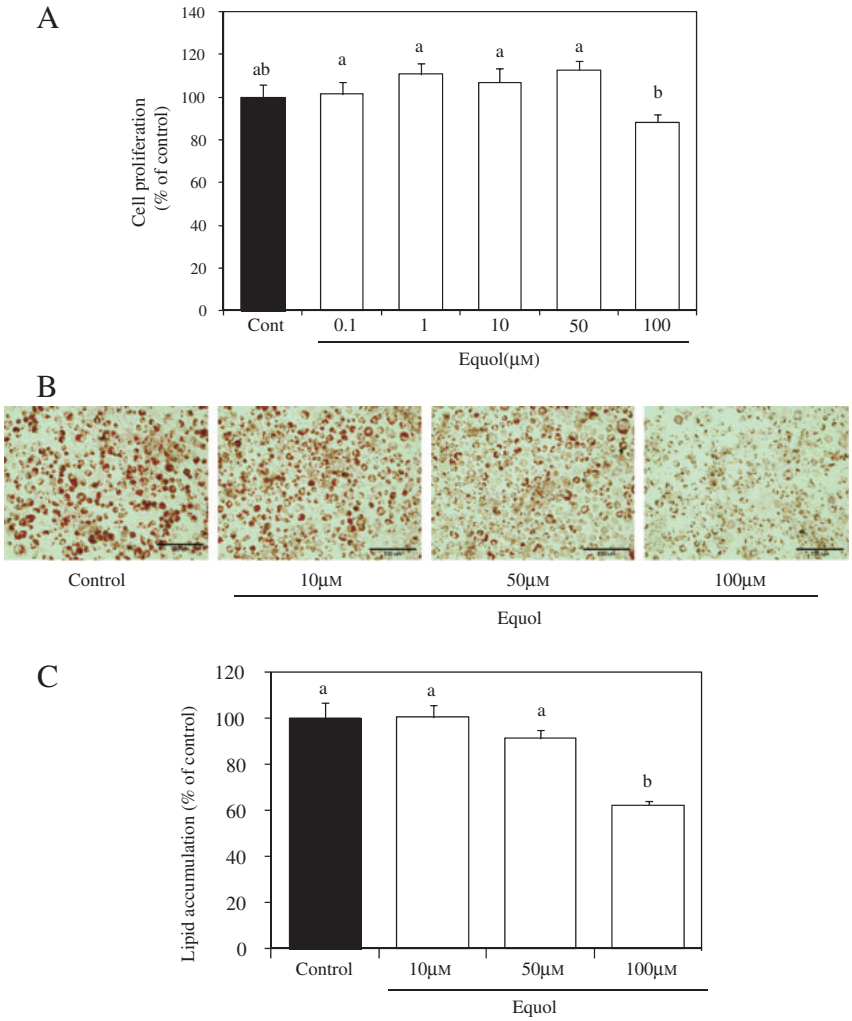

Fig. 1. Effect of Equol on Cell Proliferation and Lipid Accumulation in Adipocytes.

A, Cell proliferation of differentiated MC3T3-L1 adipocytes. Equol was added with the differentiation medium on day 0. Cell proliferation was measured by the MTS assay after 3 d of treatment. B, Effect of different doses of equol on lipid accumulation in MC3T3-L1 adipocytes. MC3T3-L1 adipocytes were incubated with equol during days 0–6, and lipid droplets were stained with Oil Red O. The stained adipocytes were photographed. Oil Red O was eluted and quantified at 450 nm. The data are representative of three independent experiments and are expressed as the mean ± SD. Means with different letters are significantly different ($p < 0.05$).

was incubated overnight with the antibody against PPARγ, FAS, or TFIIB, and then for 1 h with the anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology). Immunoactive bands were detected with the LumiGLO reagent and peroxide (Cell Signaling Technology) and documented with a Light Capture system II (Atto, Tokyo, Japan). Data are expressed as the mean ± SD. A statistical analysis was performed by using a one-way analysis of variance with Tukey's *post-hoc* test. p values < 0.05 were considered significant.

The MTS assay showed that treating with 100 μM equol significantly suppressed proliferation, but that 0.1–50 μM equol did not influence the growth of MC3T3-L1 cells (Fig. 1A). This result indicates that high-dose equol suppressed cell proliferation in differentiating MC3T3-L1 cells. To examine the effect of equol on the lipid accumulation in adipocytes, MC3T3-L1 cells were treated with equol at a higher concentration than had previously been examined.[7] The accumulated lipid droplets were detected by staining with Oil Red O after 6 d of culture. Microscopic observation of the Oil-red O staining revealed a reduction in the amount of oil droplets with increasing concentration of equol in a dose-dependent manner (Fig. 1B). The OD value for the Oil Red O-eluted solution of cells treated with 50 μM equol reduced and was decreased to 60% of the control level by 100 μM equol, suggesting that higher concentrations of equol inhibited adipogenesis during adipocyte differentiation (Fig. 1C).

To determine whether equol affected the expression of genes associated with adipogenesis, we used real-time PCR to examine the mRNA expression of PPARγ and C/EBPα which stimulate the transcription of many adipocyte-related genes. The expression of these regulators increased in MC3T3-L1 cells that had been treated with 10 μM equol; however, the effect of 50 μM equol was no different from that of the control, and 100 μM equol had significantly decreased the expression of these mRNA on day 4 (Fig. 2A and B). In addition, the expression of FAS, an identified downstream target gene of PPARγ, showed the same trends as PPARγ and C/EBPα (Fig. 2C). Transcripts of CD36 were not changed by treating with 10 μM equol, but were reduced by treating with 50 μM and 100 μM equol when compared

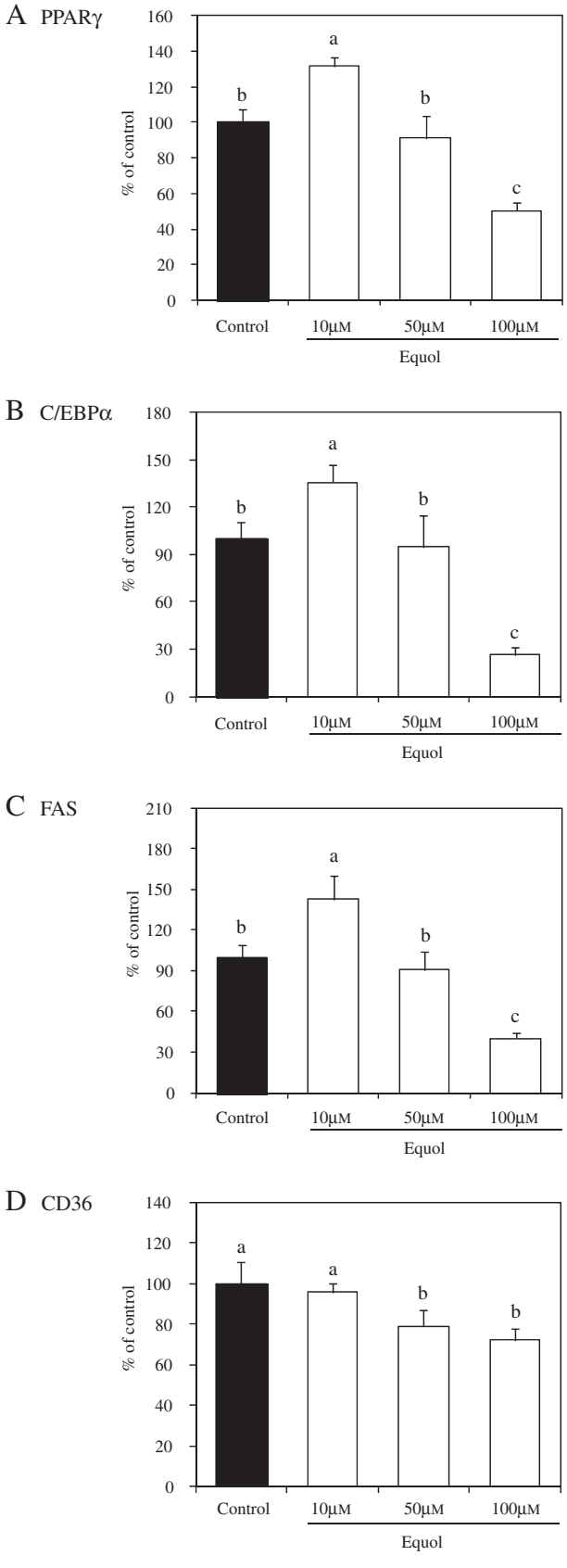

Fig. 2. Effect of Equol on Adipogenesis Genes.

MC3T3-L1 cells were cultured in a medium containing different concentrations of equol from day 0 to day 4. Total RNA was isolated on day 4 to quantify the PPARγ (A), C/EBPα (B), FAS (C), and CD36 (D) expression in MC3T3-L1 cells. All genes were normalized to β-actin. The data are representative of three independent experiments and are expressed as the mean $\pm$ SD. Means with different letters are significantly different ($p < 0.05$).

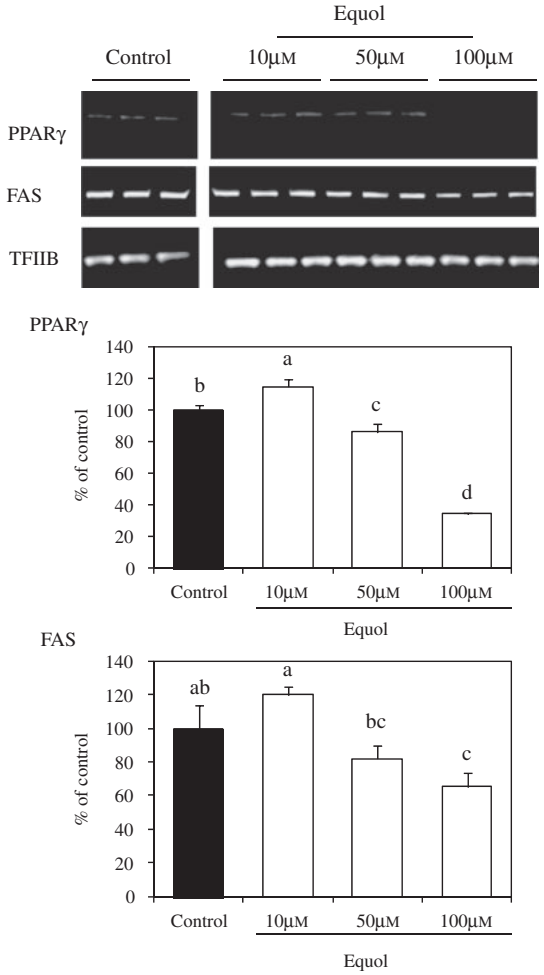

Fig. 3. Effects of Equol on PPARγ and FAS Protein Synthesis in MC3T3-L1 Cells.

After MC3T3-L1 cells had been differentiated with or without equol until day 4, PPARγ and FAS protein synthesis were analyzed by Western blotting. TFIIB was used as a loading control. The data are representative of three independent experiments and are expressed as the mean $\pm$ SD. Means with different letters are significantly different ($p < 0.05$).

with the control (Fig. 2D). Whole cell lysates were extracted on day 4 and subjected to Western blotting to determine whether equol affected the synthesis of PPARγ and FAS protein in MC3T3-L1 cells. PPARγ and FAS protein synthesis tended to increase with 10 μM equol compared with the control, and decreased with 50 μM and 100 μM equol in a dose-dependent manner (Fig. 3). The results of a protein synthesis in the treatment with 10 μM or 100 μM equol were consistent with those of mRNA expression (Fig. 2A and C). The inhibition of mRNA expression and protein synthesis by 100 μM equol seemed not to be toxic, because the result of the MTS assay showed no difference between the control and 100 μM equol treatment (Fig. 1A).

Cho *et al.*[7] have reported that equol enhanced adipocyte differentiation of mesenchymal stem cell 10T1/2 and increased PPARγ expression up to 20 μM. Consistent with these findings, our results show that 10 μM equol tended to enhance adipocyte differentiation by transactivating the expression of adipocyte-related genes in MC3T3-L1. Daidzein has been reported to enhance adipocyte differentiation up to 100 μM in a dose-dependent manner through the PPAR pathway *in*

vitro.[7] On the other hand, 50 μM genistein, an isoflavone, has inhibited MC3T3-L1 adipocyte differentiation *via* suppressing FAS and C/EBPα expression,[8] suggesting that a higher concentration of equol may inhibit adipogenesis by regulating adipocyte-related genes in a manner similar to genistein. In fact, high-dose isoflavones have prevented obesity by modulating the expression of genes related to lipid metabolism in OVX rats.[9] Equol showed a bi-phasic effect that enhanced adipocyte differentiation at 10 μM and inhibited it at 100 μM in this study. Each isoflavonoid has different binding affinity for both ERα and ERβ.[10,11] The various effects of isoflavones on adipogenesis may therefore have been due to the different binding affinity for ERs. Although further studies are needed to investigate how equol regulated adipogenesis gene expression, our results suggest that equol had a unique action on adipocyte differentiation. These results provide insight into the molecular mechanisms for equol in adipogenesis and lipid metabolism in adipocytes.

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