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Sorghum (*Sorghum bicolor* (L.) Moench) Extract Enhances Thiazolidinedione-Induced 3T3-L1 Preadipocyte Differentiation but Inhibits Adipogenic Genes

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Sorghum bicolor **(L.) Moench is known as a healthful food. We examined whether a water-soluble sorghum extract (SE) from *S. bicolor* has an anti-diabetic effect through a mechanism that improves insulin sensitivity or anti-adipogenesis. Although the treatment of SE did not affect the adipogenesis of 3T3-L1 adipocytes induced by isobutyl methylxanthine/dexamethasone/insulin (MDI), it significantly enhanced MDI/thiazolidinedione (TZD)-induced adipogenesis in 3T3-L1 adipocyte differentiation. Real-time polymerase chain reaction analysis showed that treatment with SE reduced the expression of adiponectin, adipocyte protein 2 (aP2), and resistin in 3T3-L1 adipocyte cells. SE suppressed the expression of transcription factors, peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT enhancer-binding protein α in both MDI- and MDI/TZDs-treated 3T3-L1 adipocytes. SE treatment reduced tumor necrosis factor α protein in cell lysates from MDI-induced 3T3-L1 adipocytes but not those induced by MDI/TZD. Our results suggest that SE can serve as an effective food source that improves insulin sensitivity and an anti-obesity agent.**

Key words sorghum extract, 3T3-L1 cells, adipogenesis, insulin sensitivity

INTRODUCTION

Obesity is a global health problem characterized by an increase in adipose tissue mass. Adipogenesis is a key pathway for this increase, in which preadipocytes mature into adipocytes through cell differentiation.[1)] Several compounds from natural sources have been examined for the prevention of obesity. Among them, plants or foods that contain polyphenol appear to exhibit preventive effects against certain cancers, obesity, and diabetes.[2,3)]

Sorghum (*Sorghum bicolor* [L.] Moench) is an essential cereal crop worldwide with potential benefits for human health as it is rich in phytochemicals such as tannins, phenolic acids, anthocyanins, phytosterols, and policosanols.[4-6)] Some studies have reported that sorghum extract (SE) exhibits antidiabetic effects by improving insulin sensitivity and the activation of adenosine monophosphate-activated protein kinase (AMPK) in mice and rat models, respectively.[7-9)] These phytochemicals have gained increased research interest owing to their antioxidant activities, cholesterol-lowering properties, and anticarcinogenic and antidiabetic effects.[6,8)] However, no previous reports have investigated the effect of SE on antidiabetic effects in terms of the mechanism that improves insulin sensitivity in culture adipocytes. The present study was designed to examine whether the water-soluble fraction of sorghum powder can modulate adipocyte differentiation and function in an *in vitro* cell culture system. Mouse 3T3-L1 preadipocyte has been used for evaluation of natural compounds or food components parallel with that of mice model [2,10-13)]. In this study, we investigated the effect of water-soluble fraction of sorghum powder in a 3T3-L1 preadipocyte differentiation system.

MATERIALS AND METHODS

Materials 3T3-L1 mouse fibroblast cells was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma, St. Louis, MI, USA. Calf serum was obtained from Hyclone, penicillin, and streptomycin mixed solutions were obtained from Nakalai Tesque (Kyoto, Japan), and fetal bovine serum (FBS) was purchased from ICN biomedicals. Dimethyl sulfoxide (DMSO), troglitazone, and rosiglitazone were purchased from Wako Pure Chemicals. Adipocyte induction medium containing IBMX, insulin, and dexamethasone solution was supplied by Cayman Chemical (Ann Arbor, MI, USA). Bio-Rad Protein Assay kits were obtained from Bio-Rad Laboratories. Ready-SET-Go Mouse tumor necrosis factor alpha (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were supplied by Affymetrix eBioscience.

Preparations of SE Sorghum powder was supplied as a gift by Prof. Shin Sato of Aomori University of Health and Welfare.[9)] Dried sorghum was finely pulverized with a mill and then incubated with stirring in 80% ethanol. After centrifugation, the supernatant was concentrated under reduced pressure using an evaporator. The concentrated supernatant was lyophilized and dissolved in phosphate-buffered saline (PBS), and the solubilized fraction was filter-sterilized to produce SE in

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this study. The polyphenol concentration of the SE solution was 120 mg/g gallic acid monohydrate as measured using the Folin–Ciocalteu method. The polyphenol concentration of the SE solution was used as the SE concentration.

Cell Culture and Drug Treatment The 3T3-L1 cells were seeded in 96- or 24-well plates and grown to the confluence at 37°C in 5% CO_2 and 95% air in DMEM with 10% bovine calf serum (HyClone, South Logan, UT, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin. After confluence, cells were fed DMEM supplemented with 10% FBS and an adipocyte induction medium for 3 d. The medium was changed to DMEM with 10% FBS and an insulin solution for the other 6 d. From days 0 to 9, the cells were treated with various concentrations of SE in the presence of troglitazone, rosiglitazone, or DMSO. The culture medium was replaced every 2 d thereafter with DMEM containing 10% FBS and insulin (Cayman Chemical).

Viability Assay Cell proliferation was measured using an MTT assay kit (Roche diagnostics). Cells were seeded in 96-well plates at 1×10^3 cells per well in 100 µL of culture medium and allowed to adhere overnight. Compounds were added to the medium, and the cells were incubated for a further 24 to 48 h, with untreated cells used as controls. MTT reagent was added to each well. After 4 h of incubation, the solubilization solution was added to each plate, which was incubated overnight. Using a microplate reader, absorbance was measured at 550 nm.

Oil Red O Staining At day 9 after differentiation, the 3T3-L1 adipocytes seeded in 96-well cell culture plates were rinsed with PBS and fixed in formalin for 15 min at room temperature. The formalin was then removed, and the cells rinsed twice with a 60% isopropanol solution. An Oil Red O solution (Cayman Chemical) was added to the dried wells and incubated with the cells for 20 min. The cells were rinsed three times with water and twice with 60% isopropanol and then dried. Lipid droplet accumulation was observed under a microscope. The stained lipid droplets were dissolved in isopropanol, and the absorbance of the extracted solution was measured at 492 nm.

RNA Isolation and Quantitative PCR The cells were washed twice with PBS and dissolved in RNAiso Plus (Takara Bio, Shiga, Japan) and transcribed to cDNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio) according to the manufacturer's instructions. The cDNA was analyzed using a PowerUp SYBER Green PCR kit on an ABI StepOne real-time polymerase chain reaction (PCR) machine (Applied Biosystems, Foster City, CA, USA). The conditions for PCR amplification were 95°C for 2 min, followed by 40 cycles at 95°C for 30 s and 59°C for 30 s. Data were analyzed using the ΔΔCT method. All mRNA levels were normalized relative to β-actin expression levels and compared with untreated control cells. Primer sequences for real-time PCR were β-actin: 5′-CAGCCTTCCTTCTTGGGTATGG-3′, 5′-CTGTGTTGGCATAGAGGTCTTTACG-3′; C/EBPα: 5′-TTACAACAGGCCAGGTTTCC-3′, 5′-GGCTGGCGACATACAGTACA-3′; PPARγ: 5′-GGAGCCTAAGTTTGAGTTTGCTGTG-3′, 5′-TGCAGCAGGTTGTCTTGGATG-3′ Adiponectin: 5′-AGCCTGGAGAAGCCGCTTAT-3′, 5′-TTGCAGTAGAACTTGCCAGTGC-3′; aP2: 5′-CATGGCCAAGCCCAACAT-3′, 5′-CGCCCACTTTGAAGGAAATC-3′, resistin: 5′-TCAACTCCCTGTTTCCAAATGC-3′, 5′-TCTTCACGAATGTCCCACGA-3′.

Immunoblot Analysis For immunoblot analysis, 3T3-L1 cells were cultured in 24-well plates. The cells were rinsed twice with PBS, after which 200 µL of a cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, and 1% protease inhibitor cocktail) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the lysates were collected in Eppendorf tubes. Using a Quick Start Protein Assay kit (Bio-Rad, Hercules, CA, USA), the protein content of the cell lysate samples was measured. The samples were then mixed with a 4 × sample buffer (Sigma-Aldrich), and the samples (6-10 µg protein) were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE). The proteins were electro-transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA), which was blocked for 1 h at room temperature in 5% skim milk in PBS with 0.05% Tween-20 (PBS-T) and incubated with the primary antibody in PBS-T overnight at 4°C. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary donkey anti-rabbit immunoglobin G (IgG) (Cell Signaling Technology, Beverly, MA, USA) or HRP-conjugated secondary rabbit anti-mouse IgG (Cell Signaling Technology). Anti-PPARγ, anti-phospho-AMPKα (Thr172), anti-AMPK, and anti-β-actin antibodies were purchased from Cell Signaling Technology. An HRP substrate (Luminata Forte Western HRP Substrate; Millipore) was added to the membrane and chemiluminescence was detected using the LAS 3000 imaging system (Fujifilm, Tokyo, Japan). The blots were stripped and re-probed with other primary antibodies and subjected to chemiluminescence detection. Target protein band intensity was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

TNF-α Measured by ELISA For the quantitative determination of adipocytokines, production from preadipocytes to adipocytes into cells, TNF-α concentrations in the cell lysates were measured. Cells were lysed in an ice-cold lysis buffer (50 mM Tris, 0.15 M NaCl, 10 mM EDTA, and 0.1% NP-40 at pH 8.0 with HCl). The cell lysate was diluted with an ELISA buffer, and the contents were measured using a mouse TNF-α ELISA kit (Affymetrix). The values were normalized with the total protein content.

Statistical Analysis The results are presented as the means of at least triplicate determinations ± standard deviations. For cell culture, data were analyzed using a one-way analysis of variance followed by Dunnett's multiple comparison test. For PCR and western blot data, a Student's t test was conducted. In all statistical comparisons, p values of < 0.05 and < 0.01 were considered statistically significant.

RESULTS

Effect of SE on Cytotoxicity and Adipogenesis Effect in 3T3-L1 Cells To determine the effect of SE on cytotoxicity, 3T3-L1 cells were treated with 5-50 µg/mL SE, and cell viability was measured with an MTT assay. As shown in Fig. 1, SE was not associated with cytotoxicity at a concentration of 5 µg/mL for 48 h. We next measured the adipogenic effect of SE on adipocyte differentiation. Preadipocytes were incubated in a methylxanthine/dexamethasone/insulin (MDI) differentiation medium in the presence of SE for 3 d and then replaced with

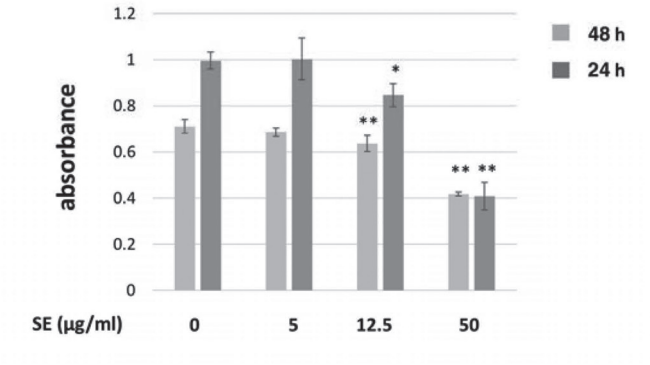

Fig. 1. Effects of SE on the Proliferation of 3T3-L1 Preadipocytes

Growing 3T3-L1 cells were treated with 5, 12.5, and 50 μg/mL SE for 24 and 48 h. Cell viability was determined using the MTT assay. Data of the three independent experiments are expressed as mean ± SD (n = 3-4). *p < 0.05, **p < 0.01.

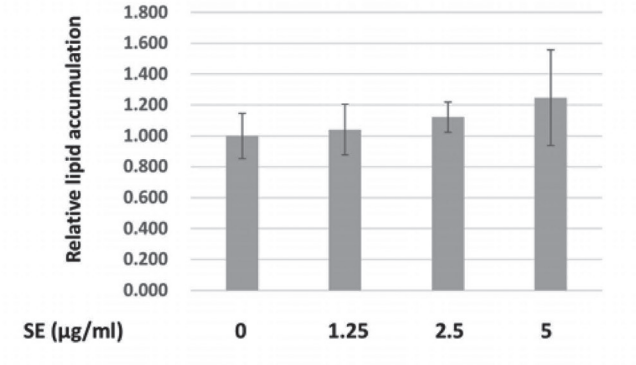

Fig. 2. Effect of SE on Lipid Accumulation in 3T3-L1 Cells

Post-confluent 3T3-L1 cells were induced to differentiate in the absence or presence of various concentrations of SE. On day 9, the cells were fixed and stained with Oil Red O to visualize lipid droplets. The triglyceride content was measured to quantify the intracellular lipid content at 492 nm using a microplate reader. Data are expressed as mean ± SD (n = 16).

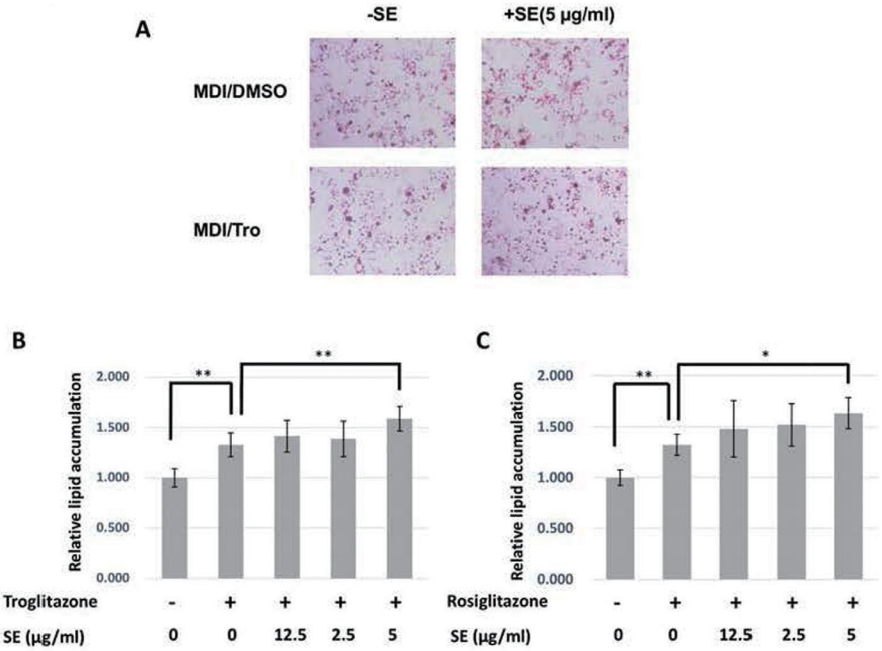

Fig. 3. Effect of SE on the Differentiation of 3T3-L1 Preadipocytes in the Presence of TZDs

Post-confluent mouse 3T3-L1 preadipocytes were cultured in DMEM containing MDI for 9 d. SE (5 μg/mL) and troglitazone (5 μM) (A, B) or rosiglitazone (10 μM) (C) were added to the cultures throughout the experiment. The cells were fixed and stained with Oil red O and the eluted fractions were analyzed at 492 nm (B, C). Oil red O-stained 3T3-L1 adipocytes were observed under a microscope (A). Data are expressed relative to the cells treated with DMSO/MDI control. Values are expressed as mean ± S.D (n = 7-8). *p < 0.05, **p < 0.01.

a fresh medium containing insulin in the presence of SE every 2 d until day 9. The adipocytes were fixed and then stained with Oil Red O as described in materials and methods. High lipid droplet accumulation was apparent in MDI-treated cells (Fig. 3A). In the MDI-treated cells, SE-treated cells exhibited no significant changes compared with those in untreated 3T3-L1 adipocytes (Figs. 2, 3A). We then treated preadipocytes with MDI and troglitazone as a PPARγ agonist to induce adipogenesis. Oil Red O staining showed that troglitazone treatment increased lipid droplet accumulation significantly when compared with MDI-treated 3T3-L1 cells. The addition of 5 μg/mL SE significantly enhanced troglitazone-induced lipid accumulation (Fig. 3A and B). Next, we treated preadipocytes with SE in the presence of rosiglitazone, which is also a PPARγ agonist. Rosiglitazone treatment increased lipid accumulation and 5 μg/mL SE enhanced rosiglitazone-induced lipid accumulation significantly in 3T3-L1 adipocytes (Fig. 3C). These indicate that SE can enhance MDI/thiazolidinedione (TZD)-induced lipid accumulation but not MDI-induced 3T3-L1 adipocytes.

SE Treatment Downregulates Adipogenesis-Related Genes in 3T3-L1 Adipocyte Differentiation The expression of adipogenesis-related genes in 3T3-L1 cells was analyzed to investigate the mechanism responsible for SE-induced adipogenesis in the presence of TZDs. Post-confluent cells were differentiated into adipocytes with MDI in the presence of troglitazone, and total RNA was isolated on day 6 from differentiated 3T3-L1 cells treated with SE. As shown in Fig. 4,

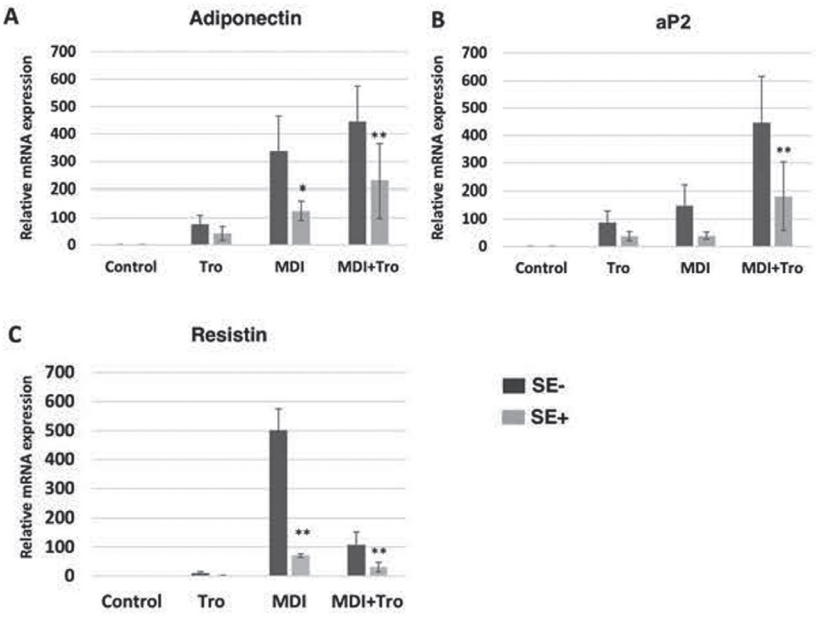

Fig. 4. Effect of SE on Expression of Adipocyte Specific Genes in 3T3-L1 Adipocytes

Post-confluent 3T3-L1 cells were induced to differentiate in the presence of SE and troglitazone (5 μM) or rosiglitazone (10 μM). Total RNA was extracted on day 6, and mRNA levels of adiponectin (A), aP2 (B), and resistin (C) were evaluated by real-time PCR. Each expression level was normalized to β-actin. Values are expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

mRNA of adiponectin, aP2, and resistin were significantly reduced in SE-treated cells compared with those in SE-untreated cells. Resistin expression was suppressed in MDI/TZDs-treated 3T3-L1 adipocytes compared with that in MDI-treated cells. SE treatment inhibited resistin expression in both MDI- and MDI/troglitazone-treated cells. The gene expression of PPARγ and C/EBPα, both of which are major transcription factors in the regulation of adipogenesis, was evaluated via real-time PCR to identify the inhibitory effects of SE on adipogenesis-related genes in 3T3-L1 cells (Fig. 5). SE treatment effectively suppressed the expression of PPARγ and C/EBPα mRNA levels in 3T3-L1 adipocytes independent of the presence or absence of troglitazone, indicating that SE treatment suppressed the expression of adipogenic genes by reducing the transcription of PPARγ and C/EBPα genes.

Recent *in vivo* data suggest that SE dietary treatment upregulates the phosphorylation of AMPK in diabetic rat livers.[9] Additionally, activated AMPK protects preadipocyte differentiation by inhibiting adipogenic gene expression. We investigated whether SE affects AMPK activity during 3T3-L1 adipocyte differentiation. MDI treatment induced AMPK phosphorylation compared to the nontreatment control, and SE treatment did not significantly affect AMPK activation in both MDI- and MDI/troglitazone-treated 3T3-L1 cells (Fig. 6A). We also found that PPARγ protein expression was downregulated in SE-treated 3T3-L1 adipocytes (Fig. 6B).

For quantitative determination of adipocytokine, production from preadipocytes to adipocytes into cells, TNF-α levels in cell lysate were measured using ELISA. Post-confluent cells were differentiated into adipocytes with MDI in the presence of troglitazone, and cell lysate was extracted on day 6 from differentiated 3T3-L1 cells treated with SE. TNF-α production increased in MDI and troglitazone/MDI-treated 3T3-L1 cells. As shown in Fig. 7, intracellular TNF-α production levels in MDI-treated 3T3-L1 cells decreased significantly with a 5 μg/mL dose of SE. TNF-α production was lower in MDI/troglitazone-induced cells than in MDI-induced 3T3-L1 cells, and compared with MDI-treatment cells, SE treatment did not change TNF-α content in troglitazone-treated cells.

DISCUSSION

Sorghum extract treatment significantly enhanced TZD-

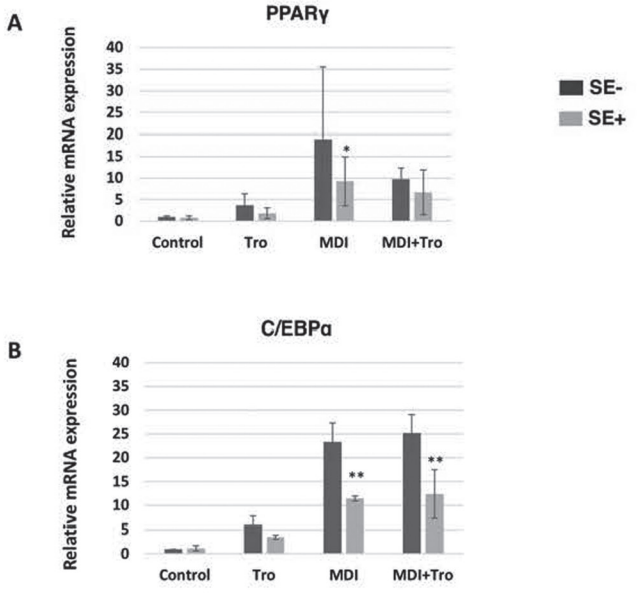

Fig. 5. Effect of SE Treatment on Adipocyte Transcription Factors in 3T3-L1 Adipocytes

Post-confluent 3T3-L1 cells were induced to differentiate in the presence of SE with 5 μM troglitazone. Total RNA was extracted on day 6, and mRNA levels of PPARγ (A) and C/EBPα (B) were evaluated by real-time PCR. Each expression level was normalized to β-actin. Values are expressed as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

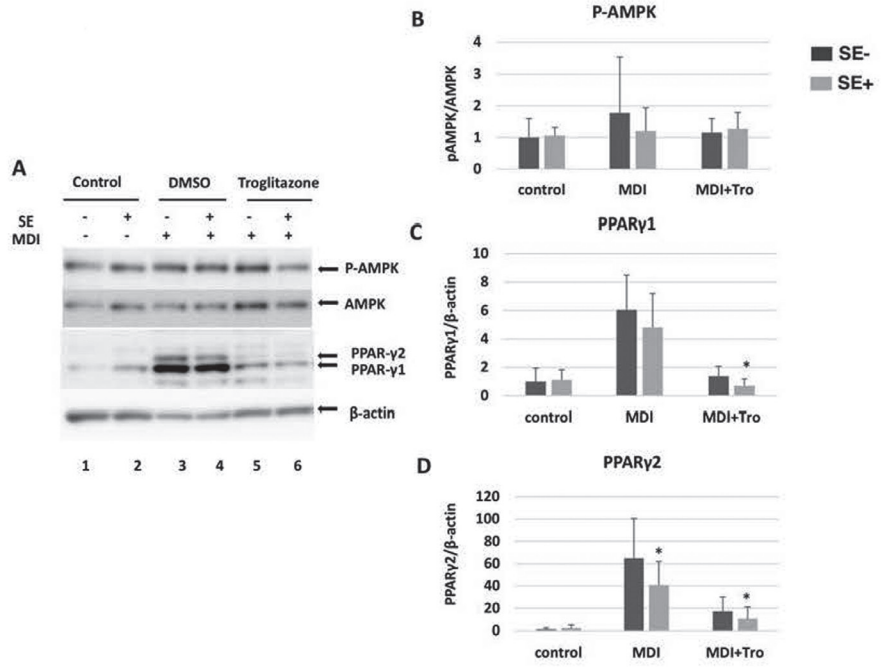

Fig. 6. Effect of SE on AMPK Activity in 3T3-L1 Adipocytes

Post-confluent 3T3-L1 cells were induced to differentiate in the presence of SE and 5 μM troglitazone. Cells were lysed on day 6 and subjected to a western blot analysis with p-AMPK, AMPK, PPARγ, and β-actin antibodies (A). Quantitation of p-AMPK (B), PPARγ1 (C), and PPARγ2 (D) protein expression levels using ImageJ were indicated. p-AMPK expression levels were normalized to that of AMPK. PPARγ expression levels were normalized to that of β-actin. Blots are representative of at least three independent experiments. Values are expressed as mean ± SD (n = 3). *$p < 0.05$.

increased lipid droplets in MDI-induced 3T3-L1 adipocytes, although it did not significantly enhance MDI-induced 3T3-L1 adipogenesis. A TZD were developed as a PPARγ agonist[14,15] and improves insulin sensitivity in the liver and skeletal muscles. TZDs such as troglitazone, rosiglitazone, and pioglitazone have been developed to treat type 2 diabetes. They activate the adipogenic transcription factor PPARγ and induce metabolic changes in adipose tissues.[16] However, TZDs have significant efficacy in increasing insulin sensitivity and are associated with adverse effects such as weight gain, edema, cardiac failure.[17] We recently reported that TZDs downregulate PPARγ genes and proteins in 3T3-L1 adipocytes.[18]

Treatment with SE reduced the expression of adipogenic genes in MDI/troglitazone-treated 3T3-L1 adipocytes, in which lipid accumulation increased. At this point, the difference between the mechanisms of the suppression of adipogenic genes in the presence and absence of troglitazone by SE treatment is unclear. Troglitazone increases lipid accumulation by increasing small adipocytes.[19] Further analysis is necessary to analyze the cooperative effect of SE and troglitazone in the 3T3-L1 adipocyte for size of adipocytes. SE enhances insulin sensitivity cooperating with TZDs and downregulates the expression of adipogenic-specific genes and anti-obesity agents. We suggest that SE has a beneficial effect on the treatment of both diabetes and obesity.

Sorghum powder contains high levels of polyphenol.[4-6] In the present study, we prepared SE (0.5 mg/mL polyphenol equivalent of gallic acid) as a water-soluble fraction from sorghum powder. DPPH analysis showed that SE exhibited effective antioxidant activity, which was slightly lower or equivalent to resveratrol (unpublished data). The water-soluble fraction of sorghum powder enhanced MDI/TZD-induced 3T3-L1 adipogenesis, although the effect of the water-insoluble fraction of sorghum powder is unknown.

Activated AMPK via phosphorylation acts as a cellular energy sensor by regulating fatty acid and glucose homeostasis.[20-22] Activated AMPK protects preadipocyte differentiation by inhibiting the expression of adipogenic genes. SE treatment did not induce AMPK activation in MDI- or MDI/TZD-treated 3T3-L1 cells. In our study, the inhibitory mechanism of adipogenic genes by SE does not appear to be due to AMPK activation.

Resistin is an adipocyte-derived adipokine linked to obesity, insulin resistance, and diabetes.[23] TNF-α is a pro-inflammatory cytokine produced by numerous cells and induced

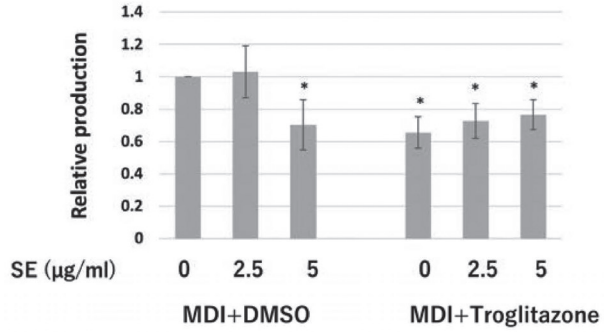

Fig. 7. Intracellular TNF-α is Inhibited by SE in 3T3-L1 Adipocytes

Intracellular adipokine TNF-α was measured on differentiated 3T3-L1 cells with SE. Cell lysate was harvested on day 7 and measured using an ELISA kit. The production level was normalized to total protein contents. Data are expressed relative to the cells treated with a DMSO/MDI control. TNF-α contents in DMSO/MDI controls are 120-250 ng/mg protein. Values are expressed as mean ± SD (n = 4). *$p < 0.05$, **$p < 0.01$.

insulin resistance. Increasing resistin expression and TNF-α activity were observed during 3T3-L1 adipocyte differentiation.[23,24] Troglitazone treatment with MDI reduced resistin mRNA compared with that of MDI-induced 3T3-L1 cells. SE treatment reduced MDI-induced resistin expression to levels comparable to those of troglitazone. Furthermore, at a concentration of 5 μg/mL, SE treatment inhibited TNF-α content in MDI-induced 3T3-L1 cells but not in MDI/troglitazone-treated 3T3-L1 cells, in which TNF-α production was decreased compared with that in MDI-treated cells.

In conclusion, our findings indicate that SE has an anti-obesity effect, reducing the expression of adipocytokines such as resistin and TNF-α and improving insulin sensitivity through cooperating PPAR-γ agonists, leading to suppression of adipogenic genes.

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Conflict of interest The authors declare no conflict of interest.

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