Fermented Soymilk Alleviates Lipid Accumulation by Inhibition of SREBP-1 and Activation of NRF-2 in the Hepatocellular Steatosis Model

Sang Bong Ahn¹, Wen Hao Wu², Jong Hun Lee²³, Dae Won Jun⁴, Jihyun Kim⁵, Riji Kim⁵, Tae-bok Lee⁵⁶, and Jin Hyun Jun²⁵⁷*

¹Department of Internal Medicine, Eulji University School of Medicine, Daejeon 34824, Republic of Korea
²Eulji Medi-Bio Research Institute (EMBRI), Eulji University, Daejeon 34824, Republic of Korea
³Department of Plastic and Reconstructive Surgery, Eulji University School of Medicine, Daejeon 34824, Republic of Korea
⁴Department of Internal Medicine, Hanyang University College of Medicine, Seoul 04763, Republic of Korea
⁵Department of Senior Healthcare, BK21 Plus Program, Graduate School of Eulji University, Daejeon 34824, Republic of Korea
⁶Biomedical Research Institute, Seoul National University Hospital, Seoul 03080, Republic of Korea
⁷Department of Biomedical Laboratory Science, Eulji University, Seongnam 13135, Republic of Korea

Introduction

In recent decades, metabolic diseases have emerged as a serious health problem in developed countries regardless of age, sex, and region, due in part to high calorie consumption among the current generation. Continuous abnormal lipid accumulation in the liver causes hepatic steatosis, in which total fat comprises more than 5–10% of the organ’s weight. Several studies have focused on the two major types of fatty liver diseases, including alcoholic liver disease and non-alcoholic fatty liver disease (NAFLD), which is the most common liver disease [1, 2]. NAFLD has an estimated prevalence of 34% among adults in the United States, and is recognized as the primary cause of liver dysfunction in children [3, 4]. The hepatic features of patients with NAFLD range from simple fat deposition to non-alcoholic steatohepatitis (NASH), fibrosis, severe cirrhosis, and hepatocellular carcinoma. About 10% of cases with benign steatosis are at risk to develop more severe NASH, which indicates the characteristic increase of inflammatory and apoptotic cells in the liver, and may result in cirrhosis in up to 25% of cases [5, 6]. Pharmacologic therapies for NAFLD patients, such as thiazolidinediones, pioglitazone, rosiglitazone, and metformin have not been...

Ingredients of soy and fermented soy products have been widely utilized as food supplements for health-enhancing properties. The aim of this study was to evaluate the effects of fermented soymilk (FSM) and soymilk (SM) on free fatty acid-induced lipogenesis in the hepatocellular steatosis model. HepG2 cells were incubated with palmitic acid (PA) for 24 h to induce lipogenesis and accumulation of intracellular lipid contents. The PA-treated cells were co-incubated with FSM, SM, genistein, and estrogen, respectively. Lipid accumulation in the PA-treated HepG2 cells was significantly decreased by co-incubation with FSM. Treatment of HepG2 cells with PA combined with genistein or estrogen significantly increased the expression of SREBP-1. However, FSM co-incubation significantly attenuated SREBP-1 expression in the PA-treated HepG2 cells; in addition, expression of NRF-2 and phosphorylation of ERK were significantly increased in the PA and FSM co-incubated cells. PA-induced ROS production was significantly reduced by FSM and SM. Our results suggested that the bioactive components of FSM could protect hepatocytes against the lipid accumulation and ROS production induced by free fatty acids. These effects may be mediated by the inhibition of SREBP-1 and the activation of NRF-2 via the ERK pathway in HepG2 cells.

Keywords: Fermented soymilk, steatosis model, HepG2, SREBP-1, NRF-2
Recently, the antihyperlipidemic effects of LDL cholesterol levels in high-fat diet-induced SD rats [11]. To cell differentiation in 3T3-L1 cells, and decreased serum contents and the expression of transcription factors related soymilk (FSM) significantly inhibited cellular triglyceride which cause flatulence [19,20]. Treatment with fermented of its peculiar flavor and high levels of oligosaccharides, lactic acid bacteria, since SM itself has limitations in terms several studies have been conducted on SM fermented with vegetarians, and patients with milk allergies; however, dietary supplement for lactose-intolerant consumers, thus contributes to the mitigation of hepatocellular steatosis. These effects may be mediated by the regulation of SREBP-1 and NRF-2 expression with the antioxidant capacity of FSM in PA-treated HepG2 cells.

Materials and Methods

Cell Culture Model of Steatosis and MTT Assay
The human hepatoma cell line HepG2 was cultured under 5% CO₂ and 95% air at 37°C in RPMI 1640 medium (Gibco, USA), supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. Lipid accumulation was induced by incubation with 0–1,000 μM palmitic acid (PA; Sigma-Aldrich, USA) for 24 h (PA group). HepG2 cells (2.5 × 10⁴ cells/ml) were seeded in microplate wells and incubated with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) for 4 h at 37°C under 5% CO₂ and 95% air. Then, 250 μl dimethyl sulfoxide was added to extract the MTT formazan and the absorbance of each well was read at 540 nm using an automatic microplate reader.

Preparation of Lyophilized Samples
The liquid FSM of Soypro and the liquid SM were obtained from Duduwon Co. (Korea). These were lyophilized by following the lyophilization protocol for this study. Both FSM and SM were prepared by lyophilization of the supernatants after centrifugation (141,000 ×g, 1 h) of the crude liquid materials. The yield of FSM and SM from the initial crude liquid materials was about 2.9% (16.6 g from 580 ml of liquid Soypro) and 3.1% (153 g from 500 ml of liquid SM), respectively. After lyophilization, FSM and SM were stored at −20°C.

Oil Red O Staining for Intracellular Lipid Accumulation and Lipid Quantification
After co-incubation with 200 μM PA and 0.04%–1.0% FSM, the HepG2 cells were washed three times with PBS, and then fixed with 10% formalin for 1 h. The cells were washed with 60%
isopropanol briefly, and incubated with 60% filtered Oil Red O solution (0.7 g per 200 ml of isopropanol, purchased from Sigma Chemical, USA) for 30 min at room temperature after washing three times with distilled water. Subsequently, the stained cells were observed under a microscope (Nikon, Japan). For the quantitative assay, neutral lipid accumulation in HepG2 cells was measured using a steatosis colorimetric assay kit (Cayman Chemical, USA) according to the manufacturer’s instructions, as previously reported [25].

**Treatment of Various Reagents Co-Incubated with PA**

The steatosis model of PA (200 μM)-treated HepG2 cells was co-incubated with FSM, SM, genistein, and estrogen, respectively. The treatment concentration of FSM was determined as 1.0% on the basis of the results of Oil Red O staining; this concentration was also applied for the SM treatment. Concentrations of genistein (50 μM) and estrogen (50 nM) were applied as previously reported [26, 27].

**Western Blot Analysis to Evaluate the Role of Lipogenesis-Related Molecular Pathways**

Cellular proteins were extracted using an extraction buffer of the following composition: 50 mM Tris-HCl, 0.5% sodium deoxycholate, pH 8.0, 1% nonidet P-40, 5 mM EDTA, 0.1% SDS, 150 mM NaCl, 1 mM PMSF, 1 mM NaVO₄, 1 mM NaF, and protease inhibitor cocktail (Roche, Germany). The protein extracts were separated on 10% polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Gelman Laboratory, USA). After blocking, the membranes were incubated with a primary antibody, and then with horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, USA). The blots were developed using an ECL Detection Kit (Amersham Pharmacia, UK). Primary antibodies to SREBP-1, NRF-2, and extracellular signal-regulated kinase (ERK) were purchased from Santa Cruz Biotechnology, and to β-actin from Sigma. To evaluate the signaling pathway of FSM, the ERK inhibitor U0126 (10 mM) was treated before PA and FSM treatment [28]. It was confirmed by western blot analysis. Positive protein bands were quantified by densitometry with the accompanying software (Image J). The western blotting results were normalized with those obtained for β-actin.

**Measurement of the Production of Cellular Reactive Oxygen Species (ROS)**

The production of cellular ROS levels was measured at 5 h post-treatment with PA or the combination of various reagents, using a commercial DCFDA assay kit (Invitrogen, USA) according to the manufacturer’s recommendations. After removing the medium, 10 mM CM-H₂DCFDA dissolved in 20 ml of phenol-red-free medium was added to the wells; the cells were then incubated at 37°C and washed three times with 50 ml of PBS per well. Fluorescence was measured at the excitation/emission wavelengths of 485/530 nm using the Accuri C6 device (BD Biosciences, USA).

---

**Fig. 1.** Growth of HepG2 cells cultured with palmitic acid (PA) (0 to 1,000 μM).

(A) Cell proliferation was determined by MTT assay, and no cytotoxicity was found at up to 200 μM of PA. Intracellular lipid droplets were stained with the lipophilic dye Oil Red O in (B) the untreated control and (C) the 200 μM PA treatment group. *p < 0.05, **p < 0.01.
Statistical Analysis
Data were analyzed using a two-tailed Student’s t-test to assess statistical significance. A probability value of $p < 0.05$ was considered statistically significant.

Results

Effects of Palmitic Acid on the Proliferation of and Lipogenesis in HepG2 Cells
To evaluate the cytotoxicity of PA, HepG2 cells were treated with increasing concentrations of PA from 0 to 1,000 μM. Cell proliferation was determined by MTT assay and no cytotoxicity was found at up to 200 μM of PA (Fig. 1A), whereas concentrations of more than 400 μM showed cytotoxic effects. Lipid accumulation in HepG2 cells was qualitatively evaluated with the lipophilic dye, Oil Red O staining method. As shown in Fig. 1C, intracellular lipid accumulation was substantially increased in the 200 μM PA treatment group, as compared with the untreated group of Fig. 1B. We found that the optimal concentration of PA treatment for the steatosis model was 200 μM in this study.

Effect of Fermented Soymilk on Palmitic Acid Induced Lipogenesis
HepG2 cells were incubated with 200 μM PA for 24 h, which could simultaneously lead to steatogenesis and lipogenesis in hepatocytes. Then, the cells were stained with Oil Red O solution for 30 min. The intracellular lipid contents were stained pink, as visualized under microscopy (Fig. 2A). The results indicated that treatment with 1.0% of FSM significantly decreased the lipid droplets and neutral lipid contents in HepG2 cells cultured with PA (Figs. 2D and 2E).

Effects of Genistein and Estrogen on Palmitic Acid-Induced Lipogenesis
Genistein is enriched in soy products, and is an estrogenic isoflavone and an effective reagent for various metabolic diseases. Genistein binds to the estrogen receptors and activates the estrogenic pathway. We determined the effects of genistein and estrogen on lipid accumulation in HepG2 cells cultured with PA for 24 h (Fig. 3). The genistein and estrogen treatment groups showed no significant differences as compared with the untreated control group (Figs. 3D and 3E); moreover, treatment with SM showed no significant difference in PA-induced lipogenesis (Fig. 3F). However, an anti-lipogenesis effect was observed in the 1.0% FSM treatment group alone (Figs. 3C and 3G). This effect was not related to the estrogenic pathway, but was potentially mediated by the bioactive components after fermentation of SM with lactic acid bacteria from kimchi.

Effect of FSM on the Expression of SREBP-1, NRF-2, and Phosphorylated ERK
We investigated the effect of FSM treatment on the expression of SREBP-1, NRF2, and ERK in PA-treated HepG2 cells by western blot analysis (Fig. 4). SREBP-1 is a transcription factor that belongs to the basic helix-loop-helix-leucine zipper family. SREBP-1 target genes are mostly related to the synthesis and regulation of cholesterol, triglyceride, and fatty acid. Our results indicated that the
expression level of SREBP-1 was significantly ($p < 0.05$) decreased in the FSM co-treated HepG2 cells, as compared with the PA-induced lipogenic cells (Fig. 4A). We next evaluated the expression of NRF-2, which has emerged as a key modulator of the cell’s primary defense mechanism to many harmful environmental toxicants and carcinogens [23]. NRF-2 has a role in preventing the activation of carcinogens to toxic metabolites, especially by induction of the phase II detoxification enzyme, NAD(P)H: quinone reductase (NQO1) [24]. Fig. 4B shows that in contrast to SREBP-1, the protein levels of NRF-2 were increased in the FSM-treated HepG2 cells, as compared with the PA-induced lipogenic cells.

Accumulating evidence indicates that the activation of ERK is associated with cell cycle arrest and apoptosis induction. In this study, we found ERK phosphorylation was increased at 1 h (Fig. 4C), and the phosphorylation was maintained for 24 h (Fig. 4D) by FSM treatment in PA-treated HepG2 cells. We found that the ERK inhibitor directly blocked the phosphorylation of ERK by FSM treatment. HepG2 cells with PA and FSM had significantly decreased p-ERK levels both at 1 and 24 h after U0126 treatment (Figs. 4E and 4F).

**Production of Reactive Oxygen Species in HepG2 Cells**

Cellular production of ROS was measured by DCFDA staining and flow cytometry. In Fig. 5, ROS production was non-detectable in the untreated control cells, whereas PA treatment significantly increased ROS production, as compared with the untreated control group ($p < 0.01$). Both FSM and SM co-treatment with PA significantly counteracted this effect in HepG2 cells ($p < 0.01$) (Fig. 5). Genistein and
Fig. 4. Expression of SREBP-1, NRF-2, and phosphorylated ERK (p-ERK) in palmitic acid (PA)-induced lipogenesis and fermented soymilk (FSM) treatment.

(A) PA significantly increased the expression of SREBP-1, whereas FSM significantly decreased the expression of SREBP-1 after 24 h of treatments in HepG2 cells. (B) Expression of NRF-2 and (C and D) phosphorylation of ERK at 1 and 24 h were significantly increased in FSM and PA co-treated cells, as compared with PA-treated HepG2 cells. Phosphorylation of ERK with FSM was blocked by ERK inhibitor U0126 treatment (E and F).
Discussion

Anti-inflammatory properties of FSM have been previously studied [29, 30]. However, the anti-obesity activity of FSM products remains unclear; moreover, its regenerative health effects on impaired hepatocytes caused by lipid overloading have not yet been fully described [11]. To investigate the effect of FSM in the prevention of lipid accumulation in liver cells, HepG2 cells that were cultured with PA for 24 h were treated with FSM. In contrast to SM, genistein, and estradiol, the bioactive components in FSM alone could protect hepatocytes against lipid accumulation, and FFA-induced ROS production in HepG2 cells. These protective mechanisms are possibly mediated by the inhibition of SREBP-1 and the activation of NRF-2 via the phosphorylated ERK pathway in hepatocytes.

Generally, SM is produced by soaking dried soybeans and grinding them in water. A traditional staple of East Asian cuisine, SM is a stable emulsion of oil, isoflavones, water, and protein. It contains appreciable levels of proteins, peptides, amino acids, isoflavones, and vitamins in the range of 10–45% of the Daily Value, with significant content of calcium and magnesium. Genistein is an abundant isoflavone in SM, and alternately called phytoestrogen owing to its similar molecular structure to human estrogen, and also binds to the estrogen receptors. Many reports suggest that consumption of genistein prevents the development of NASH in the animal model; in addition, reported data suggest the alleviation of cytotoxic stresses such as oxidative stress and ER stress [31–33].

In this study, we comparatively evaluated the anti-lipogenesis effects of genistein, estrogen, SM, and FSM on hepatic cells. The HepG2 cells cultured with PA for 24 h were subjected to treatment with genistein or estrogen, respectively. The genistein and estrogen treatment groups showed no significant differences, as compared with the untreated control group; however, an anti-lipogenesis effect was clearly observed in the FSM treatment group alone. Thus, the anti-lipogenesis effect might not be related to the isoflavonic and estrogenic pathway. SM, which was not fermented, also showed no significant effects on anti-lipogenesis. FSM contains certain bioactive components by fermentation with kimchi lactic acid bacteria that could inhibit SREBP-1 and activate the NRF-2 pathway. Further studies for the identification of specific bioactive components and confirmation with in vivo experiments are required.

SREBP-1 is a transcription factor that belongs to the basic helix-loop-helix-leucine zipper family. It regulates genes required for glucose metabolism, and fatty acid and lipid production. SREBP-1 expression is regulated by insulin [36]. SREBP-1a regulates genes related to lipid and cholesterol production and its activity is regulated by sterol levels in the cells [35]. In the inactivated state, SREBP-1 is found on the ER membrane in the cytoplasm. When cells are exposed to lack of sterol or high-dose lipid treatment, the N-terminal domain of SREBP-1 is cleaved and moves to the nucleus where it functions as a transcription factor to upregulate the expression level of genes that are implicated in sterol synthesis. Subsequently, sterol is over-accumulated in the cytoplasm, which leads to the inactivation of SREBP-1 through a negative feedback loop. In our study, the SREBP-1 protein level was decreased in the FSM and PA co-treatment groups, as compared with the PA-treated control group and the SM, genistein, and estrogen co-treatment groups. However, there was no difference observed between the FSM with PA co-treatment group and FSM-only treated group. Therefore, FSM showed sufficient capacity to alleviate obesity via SREBP-1 regulation, in the natural state without exposure to PA; however, SM could not reduce the SREBP-1 expression level, as compared with the untreated group.

NRF-2 is a basic leucine zipper protein that regulates the
expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation [36]. Several bioactive molecules that stimulate the NRF-2 pathway are currently being studied for the treatment of oxidative stress-related diseases [37]. Therefore, there is increasing interest in the potential effects of exogenous antioxidants on the prevention of oxidative gastrointestinal disorders. Recently, upregulation of endogenous antioxidant and phase II antioxidant enzymes by NRF-2 has emerged as a novel target for the prevention of colorectal cancer, since chronic inflammation is a known contributing factor in 15–20% malignancies, including colorectal cancer, and the inflammation is mediated by several factors including oxidative stress, ROS, and reactive nitrogen species [38, 39]. Increase of NRF-2 nuclear translocation due to LPS exposure may be due to the endogenous defensive mechanism in cells [24]. Phytoncides are naturally occurring anti-inflammatory compounds derived from plants and trees. They could attenuate ROS production that was associated with increased NRF-2 expression [24]. Similarly, our data demonstrated that reduced production of ROS with FSM was associated with the upregulation of NRF-2 expression.

The elucidation of the mechanism by which NRF-2 acts as a cytoplasmic “switch” to activate a battery of cytoprotective genes is a new paradigm in nutrition science. Identification of NRF-2 provided the first clue that bioactive diet-derived compounds like sulforaphane have the potential to coordinately influence large sets of function-specific genes [40]. NRF-2 has been variously described as an activator of cellular defense mechanisms, the master redox switch, and a guardian of health span and gatekeeper of species longevity [41–43]. In this study, NRF-2 was significantly increased by FSM in both PA untreated and treated HepG2 cells. Except for genistein, certain modified isoflavones by fermentation of SM showed similar action to that of sulforaphane and luteolin. Soy isoflavones such as genistein, daidzein, and equol show potential antioxidant effects, and daidzein and S-equol act via strong binding of both ER-β and NRF-2 to the quinone reductase antioxidant response element [44]. Daidzein can be converted to its end metabolite S-equol in some individuals, based on the presence of certain intestinal bacteria [45]. Several decades of research suggest that S-equol has potential for significant health benefits. A study showed that pre-incubation of human umbilical vein endothelial cells and EA.hy926 cells followed by co-treatment with S-equol significantly improved cell survival in response to H₂O₂ and reduced apoptotic cells by activation of the NRF-2 pathway [46]. In our study, it is likely that FSM contained daidzein and S-equol, which may occur at increased levels due to fermentation with lactic acid bacteria.

ERK is a well-studied protein kinase, which is widely expressed in most eukaryotic cells. It is involved in many intracellular signaling pathways as a mediator of cell signaling from the cell membrane via adding phosphate groups to the target protein [47]. Recently, studies have shown that ERK 1/2 is also involved in cellular fatty acid uptake and stearoyl-CoA desaturase 1 protein expression, a key enzyme for lipid metabolism in liver [48]. In our experiments, active ERK (p-ERK) was significantly increased at both 1 and 24 h after FSM co-treatment with PA in HepG2 cells. However, increasing p-ERK levels by FSM were directly suppressed after treatment with an ERK inhibitor (U0126). Collectively, the results indicated that FSM treatment in HepG2 cells contributed to maintaining long-term ERK activation for 24 h, followed by phosphorylation of SREBP-1. The N-terminal domain of SREBP-1a and SREBP-2 is a substrate for phosphorylation by ERK [49]. Activation of SREBP-1a by phosphorylation of the N-terminal domain is a necessary step for sterol accumulation in cells. Moreover, lipid overloading in the cytoplasm triggers negative feedback, by which SREBP-1 undergoes inactivation with reduced protein expression level.

Taken together, our results suggest that bioactive components in FSM (but not SM, genistein, and estradiol) could protect hepatocytes against lipid accumulation and ROS production induced by FFAs. These effects may be mediated by the inhibition of SREBP-1 expression and the activation of NRF-2 via the ERK pathway in PA-treated HepG2 cells. We suggest that FSM from SM with lactic acid bacteria isolated from kimchi might have bioactive ingredients for the prevention of NASH and lipogenesis in liver cells.

Acknowledgments

This research was supported by EMBRI Grants 2014 EMBRI-SN0002 from Eulji University.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

challenges and new opportunities. World J. Gastroenterol. 20: 5320-5330.


