A Doctoral Dissertation

Role of Active Compounds of *Citrus sunki* Peel in Hepatic Lipid Metabolism

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간조직 지질대사에서 진귤과피 활성성분의 기능

지도교수 박 덕 배

최 지 강

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Role of Active Compounds of *Citrus sunki* Peel in Hepatic Lipid Metabolism

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(Supervised by Professor Deok-Bae Park)

A thesis submitted in partial fullfillment of the requirement

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ABSTRACT

Plants of the genus citrus are primarily valued for their edible fruit, but they also have traditional medicinal value. Although a number of previous studies have investigated their pharmacological activities of extracts isolated from the peel of citrus fruits, little is known about pharmacologically active compounds and related action mechanism underlying each pathological condition. The present study aimed to investigate the effect of extract isolated from the peel of *citrus sunki* hort.ex Tanaka, one of Jeju-native citrus fruits, on the hepatic lipid metabolism. Additionally, we performed compositional analysis to find out any leading compounds that is active in regulating lipid metabolism in liver cells. Finally, action mechanism of each compound to suppress hepatic lipid accumulation was also investigated.

Treatment of $HepG_2$ liver cells with the extract of fermented *citrus sunki* peel (FSE) suppressed free fatty acid (FFA)-induced lipid accumulation. *In vivo* studies using high fat diet rats also found that FSE can suppress the increased levels of a number of pathological indicators including AST, ALT, γ-GT, triglyceride within plasma, weight of liver and spleen, and the degree of hepatic fat accumulation that were induced by high fat diet. From the compositional analysis, five rich compounds were characterized; synephrine (alkaloid), tangeritin and nobiletin (polymethoxyflavones), rutin and hesperidin (flavonone glycoside) except other

trace elements.

All five compounds suppressed FFA-induced accumulation of triglyceride (TG) in the cytoplasm of $HepG_2$ cells. The hypolipidemic activity of four compounds except tangeritin was sensitive to the inhibition of AMPK. They also decreased protein levels as well as mRNA levels of SREBP1c (a mediator of *de novo* lipogenesis) and stimulated the phosphorylation of AMPK, however, decreased the mRNA levels of FAS (fatty acid synthase), one of SREBP1c-regulated target gene product. Hypolipidemic activity of tangeritin was sensitive to the inhibition of PI3-kinase in $HepG_2$ cells. Although tangeritin marginally stimulated the phosphorylation of AMPK, the inhibition of AMPK failed to suppress FFA-induced TG accumulation. Tangeritin also decreased protein levels as well as mRNA levels of SREBP1c that were increased by FFA.

Taken together, 5 major compounds were totally active to suppress FFA-induced TG accumulation in the cytoplasm of $HepG_2$ liver cells. However, action mechanism of each compound is different in part, although it shares a few signaling protein and transcription factor(s) like as AMPK and SREBP1c, respectively. The peel of *citrus sunki per se.* and leading compounds within the peel have a good pharmacological value to prevent or treat hepatic steatosis and more progressed liver disease like as nonalcoholic steatohepatitis (NASH).

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the most common liver disease, is defined by a hepatic triglyceride content exceeding 5% of liver weight in the absence of excess alcohol intake (Kleiner *et al.*, 2005) and is strongly associated with obesity, type2 diabetes and insulin resistance (Lazo and Clark, 2008). NAFLD is now considered as the hepatic manifestation of the metabolic syndrome. NAFLD encompasses a wide spectrum ranging from simple hepatic steatosis to hepatic steatosis with nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and in severe cases, hepatocarcinoma (Neuschwander-Tetri and Caldwell, 2003).

NAFLD prevalence is 10-24%, increasing to 25-75% in obesity and type2 diabetes mellitus (Angulo, 2002; Moscatiello *et al*., 2007). Fatty liver disease is a major contributor to cardiovascular and overall obesity-related morbidity and mortality (Angulo, 2007; Loria *et al*., 2007). Most of patients with NAFLD are asymptomatic and the symptoms are usually non-specific when they are occurring. So NAFLD accounts for about 90% of cases of asymptomatic elevation of transaminases when other cases of liver disease are excluded (Moscatiello *et al*., 2007). Nonetheless, transaminases are normal in >80% of subjects with NAFLD (Browning, 2006). Although a “benign” condition, hepatic steatosis is a risk factor for more serious liver alterations (necroinflammation, hepatocyte ballooning, Mallory bodies formation, enlargement and dysfunction of mitochondria), eventually leading to fibrosis and to NASH. NASH in turn affects about 3% of the general lean population, about 20% of obese subjects and

about 50% of morbidly obese subjects (Moscatiello *et al*., 2007). About 10% of NAFLD patients eventually progress to NASH. NASH may progress to cirrhosis and to liver-related death (mostly hepatocellular carcinoma) in about 25% and about 10% of cases, respectively (Angulo, 2002).

In 1998, Day *et al.* launched the "two-hit theory" stating that two succeeding wallops have to be delivered to the liver to cause NASH. The "first hit" consists in the accumulation of excessive hepatic fat owing to insulin resistance. This step is often present in patients with the metabolic syndrome, and although it is not sufficient to cause NASH, it is enough to predispose the liver to chronic inflammation. The oxidative stress owing to reactive oxygen species (ROS), gut-derived lipolysaccharide, and soluble mediators synthesized both from cells of the immune system and from cells of the adipose tissue (Lalor *et al.,* 2007) have been indicated as factors of risk responsible for the "second hit". Although the model of the "two-hit theory" quickly spread through the scientific world, it seems obvious that different factors are necessarily interacting.

Several lines of evidences show that the status of insulin resistance is the key factor in the pathogenesis and potential evolution of hepatic steatosis. The combination of obesity, increased visceral white adipose tissue and peripheral insulin resistance are associated with increased gluconeogenesis, enhanced peripheral lipolysis with release of free fatty acid (FFA) from visceral fat, increased hepatic uptake of FFA and hepatocellular triglyceride (TG) synthesis and accumulation (Shoelson*, et al,* 2007) (Gastaldelli *et al.,* 2007; Jensen *et al.,* 1989). It is worth noting that insulin resistance also seems able to promote the progression of simple steatosis to NASH and fibrosis. Clinical studies have shown that in NAFLD patients insulin resistance is significantly associated with the severity of fibrosis (Bugianesi *et al.,* 2006). Experimental data have

highlighted that high glucose levels and hyperinsulinemia cause up-regulation of connective growth factor (Paradis *et al.,* 2001), and that hyperinsulinemia can both directly induce oxidative stress (Özcan *et al.,* 2004) and stimulate hepatic stellate cells to proliferate and secrete extracellular matrix (Svegliati-Baroni *et al.,* 1999). Insulin resistance is the disruption of signaling pathways in cells, leading to diminished ability to execute normal cellular responses to insulin. Details of insulin signaling pathway are that the insulin binding to its receptor activates the phosphorylation of insulin receptor, which in turn causes tyrosine phosphorylation of the insulin substrate (IRS) proteins. There are two important IRS: IRS1 and IRS2. IRS1 is the initiator in the pathway of glucose metabolism. Upon phosphorylation, IRS1 induces stimulation of the PI3K-AKT/ protein kinase B pathway, resulting in recruitment of glucose transporters (GLUT). IRS2 cranks up lipid metabolism in cells and is a main regulator in *de novo* lipogenesis (DNL) via sterol regulatory element binding protein 1c (SREBP1c). (Herman and Kahn, 2006; Taniguchi *et al.,* 2006).

The occurrence of insulin resistance in the liver, characterized by reduced insulin-suppressing effect in hepatic glucose production (Bugianesi *et al.*, 2005). Strong evidence exists demonstrating that in NAFLD patients, insulin does not suppress adipose tissue lipolysis to the same extent that it does in healthy individuals (Lewis *et al.,* 2002). Because insulin has a potent suppressive effect on HSL (hormone-sensitive lipase), which hypolyzes TG and liberates FFA in adipose tissue. In insulin resistance state there is an increase in lipolysis in adipose depots and increasing FFA delivery to the liver. Consequently, insulin resistance-induced-hyperinsulinemia determines an

increased hepatic synthesis of FFAs and a decreased synthesis of apolipoprotein B100, leading to TG accumulation in the liver (Charlton *et al.,* 2002). The mechanism whereby FFAs induce hepatic insulin resistance is mediated by translocation of the protein kinase C (PKC) isoforms from the cytosol to the membrane compartment, resulting in impairment of hepatic IRS-associated PI3K activity (Shulman, 2000). In NAFLD patients, primary defect in insulin receptors causes the downregulation of IRS1 signaling by excess FFAs, which activate the stress/inflammatory kinases JNK, IKKβ, and suppressors of cytokine signaling-3 (SOCS-3) contributing to insulin resistance (Ghanim *et al.,* 2007; Nguyen *et al.,* 2005).

Development of hepatic steatosis, the first hit, is the accumulation of TG consisting of FFA and a glycerol backbone in the hepatocytes (Schreuder *et al.,* 2008). From a kinetic standpoint, hepatic steatosis develops when there is an imbalance in which fatty acid uptake and *de novo* synthesis exceed oxidation and resecretion. The source of fatty acids potentially contribute to fatty liver including: (1) peripheral fat stored in adipose tissue that is delivered to the liver by way of the plasma non-esterified fatty acid (NEFA) pool. (2) Fatty acid newly made within the liver through the DNL. (3) Dietary fatty acids, which can enter the liver through spillover into the plasma NEFA pool and through the uptake of intestinally-derived chylomicron remnants. Donnelly *et al.* (2005) combining the multiple-stable-isotope approach with liver biopsy, stimultaneously assessed the contribution of different sources of fatty acids to liver TG accumulation in patients with NAFLD. They found that of the TG labeled in liver, 59% of TG arose from circulating NEFA pool, 26% from DNL and 15% from the diet. Notably, in healthy individuals, DNL is a minor supplier of fatty acids to hepatocytes in the fasting state, less than 5% of the total supply of fatty acids originates from DNL. In the

postprandial state, insulin stimulates DNL which then accounts for over 26% of the fatty acids supplies. This more or less diurnal rhythm is not seen in NAFLD patients where the contribution of DNL is continuously 26% (Donnelly *et al.,* 2005). DNL is the metabolic pathway leading to the conversion of an excess of carbohydrates into fatty acid, which are ultimately esterified with glycerol-3-phosphate to form TG. The activity of the lipogenic pathway is strongly dependent upon the nutritional conditions. A diet rich in carbohydrates stimulates the lipogenic pathway, whereas, starvation decreases its activity. Lipogenic enzyme activities are controlled by post-translational mechanisms but the main control is at the transcriptional level (Ferré and Foufelle. 2010). It is now clearly established that lipogenic enzyme transcription requires both insulin and glucose to be fully induced (Foufelle and Ferré, 2002). SREBP1c have been identified as mediator of the transcriptional effect of lipogenic gene expression (Ferre and Foufelle, 2007). SREBP1c is the isoform that plays a role in synthesis of fatty acids and TG in the liver, by stimulating the formation of enzymes, most important acetyl CoA carboxylase (ACC), and fatty acid synthase (FAS),etc (Dentin *et al.,* 2004). SREBP1c belongs to the basic-loop-helix-leucine zipper (bHLH-LZ) family of transcription factors. Two other isoforms, SREBP2 and SREBP1a, are involved in the regulation of genes involved in cholesterol synthesis (Brown and Goldstein, 2009). SREBPs are synthesized as inactive precursors bound to the membranes of the endoplasmic reticulum (ER) and thus must undergo proteolytic cleavage to liberate their N-terminal domain, which constitutes the mature transcription factor. Once the mature, active nuclear form of SREBP is translocated into the nucleus, it binds to sterol regulatory element (SRE), present in the promoters of its own and target genes, thereby promoting the lipogenic process in the liver. SREBP1c expression is transcriptionally controlled by various nutritional and

hormonal factors, insulin being one of the most potent activator. Nevertheless, recent studies suggest that SREBP1c could be activated independently of insulin due to the presence of increased stress of the ER, as observed in steatosis liver in response to fat overload and, in particular, with saturated fatty acids, leading to increasing lipogenesis (Ferre and Foufelle, 2007; Ji, 2008). Recently, it has been reported that AMPK (AMP-activated protein kinase) inactivates SREBP1 and inhibits hepatic steatosis in high-fat diet-induced animal models (Kim *et al.,* 2010; Lee *et al.,* 2010). AMPK is a major cellular energy sensor and a master regulator of metabolic homeostasis. AMPK is a heterotrimer comprising a α-catalytic subunit with βγ-regulatory subunits. AMPK are activated by two distinct signals: a Ca^{2+}-dependent pathway mediated by CAMKKβ and an AMP-dependent pathway mediated by LKB1 (Sanders *et al.,* 2007). These and other upstream kinases, including TGF-β-activated kinase-1(TAK-1), phosphorylate Thr172 on the a subunit of AMPK. Binding of AMP to the g subunit leads to allosteric activation of AMPK as well as protection of Thr172 from dephosphorylation, thereby maintaining the enzyme in the activated state. AMPK thus serves as a unique metabolic control node as it senses cellular energy status through modulation of its activities via phosphorylation and allosteric activation by AMP. (Zhang *et al.,* 2009) in response to a reduction in the energy charge, AMPK switches off anabolic pathways such as fatty acid, TG and cholesterol synthesis as well as protein synthesis and transcription that consume ATP, and switch on catabolic pathways that generate ATP, such as fatty acid oxidation and glycolysis. The effects of AMPK activation are pleiotropic in key metabolically relevant tissues, such as liver, skeletal muscle, adipose, and hypothalamus. Liver AMPK decreases hepatic lipogenesis. AMPK phosphorylates and inactivates acetyl-CoA carboxylase1 (ACC1) and 3-hydroxy-3-methylglutaryl-CoA reductase,

leading to inhibition of *de novo* fatty acid and cholesterol synthesis. Phosphorylation of ACC2 by AMPK, on the other hand, causes increases of fatty acid oxidation. (Viollet *et al.,* 2009) ACC is a rate-determing enzyme for the synthesis of malonyl-CoA, both a critical substrate for fatty acid biosynthesis and a potent inhibitor of fatty acid oxidation. AMPK can also increase the activity of malonyl-CoA decarboxylase (MCD) to further decrease hepatic malonyl-CoA levels (Assifi *et al.,* 2005). Hypertriglyceridemia has been documented in both whole-body AMPKα2-/- and liver specific AMPKα2-/- mice (Andreelli *et al.,* 2006). In addition, AICAR (AMPK activator) infusion has been shown to decrease plasma TG (Bergeron *et al.,* 2001). AMPK also mediates suppression of lipogenic gene expression (FAS and ACC) via decreasing the actions of transcriptional factors SREBP1c (Foretz *et al.,* 2005; Zhou *et al.,* 2001).

Plants of the genus citrus are primarily valued for their edible fruit, but they also have traditional medicinal value. The peel of citrus fruits has been used in traditional asian medicine for centuries. In modern European herbal medicine, the fruit peel of *C.aurantium* is used to treat dyspepsia and related conditions (Blumenthal. 1998). In our previous study, we showed that fermented peel of *citrus sunki* Hort.ex Tanaka promotes ethanol metabolism and suppresses body fat accumulation (Cui *et al*. 2007). The peel of citrus sunki has many active compounds. (Kurowska and Manthey, 1999). Especially, the peel of citrus sunki is a rich source of flavanones, as well as many polymethoxylated flavones, which are very rare in other plants. (Nogata *et al*. 2006) The most prevalent flavanones are hesperidin, rutin and naringenin. Tangeritin and nobiletin are two polymethoxylated flavones that are commonly found in citrus fruit peel. These compounds not only play important physiological and ecological roles but are also of commercial interest because they have a multitude of applications in the food and

pharmaceutical industries. For example, naringenin and hesperidin may act as antioxidants and anti-inflammatory agents. (Chen *et al*. 1990; Emin *et al.*,1994; Kobayashi and Tanabe, 2006). Polymethoxylated flavones are also of interest for their various pharmacological potentials, the most important of which are antitumor, anti-inflammatory, antimutagenic, and antiallergic properties. (Kandaswami *et al.,* 1991; Kandaswami *et al*., 1991; Kawaii *et al*., 1999; Murakami *et al*., 2000a; Murakami *et al*., 2000b). Rutin, a flavonol glycoside commonly found in citrus fruit peels, has been shown to have significant anti-inflammatory properties. (Guardia *et al*., 2001) other compounds of the extract of citrus peel are alkaloids. The primary alkaloid is synephrine in the extract of citrus peel extract. Studies have suggested that synephrine has thermogenic and lipolytic activities (Arch. 2002; Gougeon *et al*., 2005; Sale *et al*., 2006), increasing energy metabolism (Haaz *et al*., 2006; Stohs and Shara, 2007) as well as athletic performance (Haller *et al*., 2005).

The purpose of the study was to investigate the effect of compounds in the peel of *citrus* sunki, one of citrus fruits grown uniquely in Jeju island, in Korea, on hepatic lipid metabolism. Previous studies showed that the compounds in the citrus fruits could ameliorate insulin resistance and lower hepatic TG contents (Jung *et al.,* 2006; Jung *et al.,* 2003; Kurowska and Manthey, 2004). However, the exact mechanism of the active compounds in the citrus fruits in lowering TG levels has been not answered yet.

The present study investigated the effect of extract of the fermented peel of *citrus sunki* on FFA-induced lipid accumulation in $HepG_2$ liver cells. Additionally, the effect of extract on plasma lipid levels and hepatic steatosis in high fat diet (HFD) animals was also evaluated.

Based on results found in experiments using extracts, we performed compositional

analysis to determine the major active compounds in the peel of *citrus sunki* hort. ex Tanaka, one of Jeju-native citrus fruits. We identified five major compounds; one of alkaloids (synephrine), two of polymethoxyflavones (PMFs, tangeritin and nobiletin) and two of flavonoids (rutin and hesperidin). We investigated the effect of the five compounds on decreasing TG accumulation and explored the mechanism of decreasing TG accumulation in HepG2 cells.

MATERIALS AND METHODS

1. Reagents

$HepG_2$ cells were obtained from the Korean cell line bank (KCLB, Seoul, Korea). Dulbecco's Minimal Essential Medium (DMEM), palmitic acid, sodium oleate, Thiazolyl Blue Tetrazolium Bromide (MTT), compound C, synephrine, tangeritin, nobiletin, rutin and hespridin were from Sigma (St. Louis, USA). Fetal Bovine Serum (FBS) was from PAA (Etobicoke, Ontario, Canada) and penicillin streptomycin was from GIBCO (N.Y., USA). Lactate dehydrogenenase (LDH) cytotoxicity kit was from TAKARA (Otsu, Shiga Japan) and AICAR was from (Cayman, Ann Arbor, MI, USA).

2. Cell culture

$HepG_2$ cells were maintained in DMEM containing glucose (4.5 g/L), FBS (10%, v/v), and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37℃ atmosphere and 5% CO_2. Confluent cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS) and further incubated or treated with various compounds for purposes.

3. Animals

6-week-old male Sprague-Dawley rats were fed with high-cholesterol diets (Table 1) for 8 weeks to induce steatosis, and further fed with FSE (oral, 2g/kg,1time/day) and high-fat (20% cholesterol) diet (HFD) for an additional 8 weeks and control group fed

with normal diet. All animals were kept in a temperature and humidity-controlled environment in a 12 h light-dark cycle. At all times, they were allowed free access to water and diet.

Table 1. Composition of high-fat diet

Ingredient	g/kg
Casein	200
DL-Methionine	3
Sucrose	475
Cornstarch	150
Corn Oil	50
Cellulose	50
Mineral Mix #200000	35
Vitamin Mix #300050	10
Cholesterol	**20**
Cholic Acid	**5**
Choline Bitartrate	2
계	1000.00

4. Lactate dehydrogenase assy (LDH assay)

Lactate dehydrogenase leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate. (FERNÁNDEZ-LÓPEZ *et al.,* 2006) The cell-free culture medium was collected and then incubated with the same volume of reaction mixture of LDH kit in a 96 well microwell plate for 10 min at room temperature. The optical density at 492 nm was measured by ELISA plate reader (Sunrise, Tecan, Austria).

5. MTT assay

MTT assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membrane, thus resulting in its accumulation within healthy cells. Briefly, the same volume of MTT solution (1 mg/ml) equivalent to cell-free medium of each well was added and incubated for 30 min at 37℃. The supernatant was removed and 2-propanol was added to each well. The optical density at 570 nm was measured by ELISA plate reader (Sunrise, Tecan, Austria).

6. TG assay

Methods for triglyceride determination generally involve enzymatic or alkaline hydrolysis of triglycerides to glycerol and free fatty acids followed by either chemical or enzymatic measurement of the glycerol released. Treated $HepG_2$ cells were scrapped from culture dishes and displaced to 1.5 ml microcentifuge tube. The tubes were centrifuged at 3,000 rpm for 5 min. The supernatant was discarded and the pellet was with PBS. TG concentration was measured with TG assay reagents (Sigma, Louis USA) by a manufacturer's protocol. The optical density at 540 nm was measured by ELISA plate reader. (Sunrise, Tecan, Austria).

7. Nile red staining

Nile red is an ideal probe for the detection of lipids as it exhibits high affinity, specificity and sensitivity to the degree of hydrophobicity of lipids, the latter feature results in a shift of the emission spectrum from red to yellow, in the presence of polar and non-polar lipids, respectively (Greenspan *et al*., 1985). After treatment with compounds, $HepG_2$ cells were washed twice with PBS. Cells were stained with in PBS (200 ng/ml nile red, 2% acetone) for 5 min. Cells were washed with PBS to remove excess stain. The emitted fluorescence from cells were observed under the inverted microscope (IX70, Olympus, Tokyo, Japan) equipped with fluorescence filters and digital camera.

8. Western blot

$HepG_2$ cells were preincubated in serum-free medium overnight and then treated with various compounds for purposes. Cells were collected by scrapping and washed with D-PBS. Collected cell pellets were lysed in a lysis buffer (RIPA, Millipore, Billerica, USA) supplemented with inhibitors for various proteases and phosphatases (Sigma, Louis, USA) and kept on ice for 15 min. Lysates were centrifuged at 15,000 rpm at 4℃ for 15 min and the supernatant was stored at -20℃ until use. Protein concentration was determined with BCA protein assay reagent (Pierce, USA). Aliquots of the lysates (15 μg protein) were separated on a 4-12% Tris-Bis gel (Invitrogen, Carlsland, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) with transfer buffer (Invitrogen, Carlsland, USA) . After blocking the nonspecific

site with 5% non-fat dry milk (Santa cruz, CA, USA) in TBS-T buffer. The membrane was incubated with specific primary antibodies at 4℃ for overnight. Primary antibodies against p-AMPK, AMPK, p-ACC were from Millipore (Bedford, USA), and antibodies against SREBP1c, PPARg, GAPDH were from Santa cruz (CA, USA). The membranes were further incubated with secondary antibodies. The bands of immunoactive protein was visualized with western lightning Plus-ECL reagents (Perkin Elmer, MA, USA) and exposed onto a x-ray film.

9. Reverse transcription - Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using a TRI reagent according to the manufacturer's instruction. First-strand cDNA synthesis was performed in a reaction mixture (20 μl) containing 4 μg total RNA, oligo(15)-dT primers, and reverse transcriptase (Promega, Madison USA) . PCR reactions were performed in a reaction mixture (20 μl) containing 2 ul cDNA product, 10 pmol of each primer, and 0.5 unit Taq polymerase (iNtRON, Korea). Oligonucleotide primer sequences (Bioneer, Korea) used in PCR amplification are as follow:

GAPDH; (forward) 5'-TCCACCACCCTGTTGCTGTA-3',

(reverse) 5'-ACCACAGTCCATGCCATCAC-3'

SREBP1c; (forward) 5'-GTGGCGGCTGCATTGAGAGTGAAG-3',

(reverse) 5'-AGGTACCCGAGGGCATCCGAGAAT-3';

PPARγ; (forward) 5'-TCCGACTCCGTCTTCTTGAT-3',

(reverse) 5'-GCCTAAGGAAACCGTTCTGTG-3';

FAS; (forward) 5'-CAAGAACTGCACGGAGGTGT-3',

(reverse) 5'-AGCTGCCAGAGTCGGAGAAC-3'.

PCR was performed at 94℃ for 20 s (denaturation), followed by 60℃ for 20s (annealing), and 72℃ for 50s (polymerization). The last cycle was followed by a final extension step at 72℃ for 10 min. The RT-PCR products were separated with an electrophoresis in a 1% agarose gel.

10. Statistical analysis

All data were represented as a mean ± standard errors (S.E.) of at least three independent experiments. The student's t-test was used to determine the differences between groups. $P<0.05$ was statistically significant.

RESULTS

I. Effect of extract of Citrus sunki peel on lipid metabolism in $HepG_2$ cells

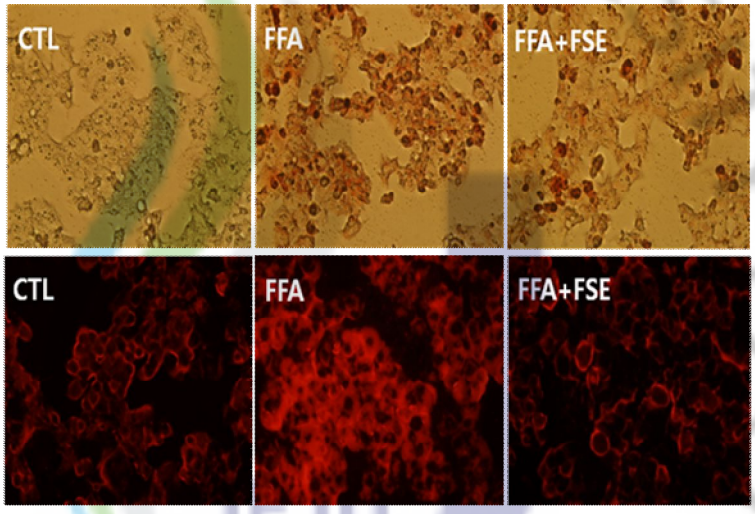
1. Effect of FSE on decreasing the TG accumulation in $HepG_2$ cells

Although citrus fruits have been reported to have antioxidant, antitumor, and anti-inflammation activities, little is known about their involvement in the regulation of hepatic lipid metabolism. At first, the effect of extract of *citrus sunki* peel on intracellular lipid accumulation in $HepG_2$ liver cells. To induce lipid accumulation, mixture of palmitate and oleate (1:2) (FFA) was added to the culture medium. Two different extracts were used (SE, extract of *citrus sunki* peel; FSE, extract of fermented *citrus sunki* peel). *Citrus sunki* peel has strong bitter taste, and the fermentation process can remove the bitterness. Because the extract will be applied to develop a diet supplement or the pilot compounds for the pharmacological purposes, such application is necessary. SE or FSE was pretreated before FFA addition. FFA treatment increased the amount of lipid within cytoplasm of $HepG_2$ cells and this FFA-induced lipid accumulation was clearly suppressed by SE (data not shown) and FSE (Fig. 1A).

2. Effect of FSE on AMPK signaling pathway in $HepG_2$ cells

Because AMPK- ACC signaling step plays an important role in synthesizing neutral fat (triglyceride, TG) using FFA as a substrate, it is tested whether SE or FSE can affect AMPK-ACC signaling pathway. FFA treatment decreased the basal levels of phosphorylated AMPK and ACC, suggesting the stimulation of ACC activity and biosynthesis of TG. SE, FSE, and extract of *citrus unshiu* peel (UE) were all restored FFA-induced decrease of phosphorylation of AMPK and ACC over the FFA-free control (Fig. 1B).

A.



B.

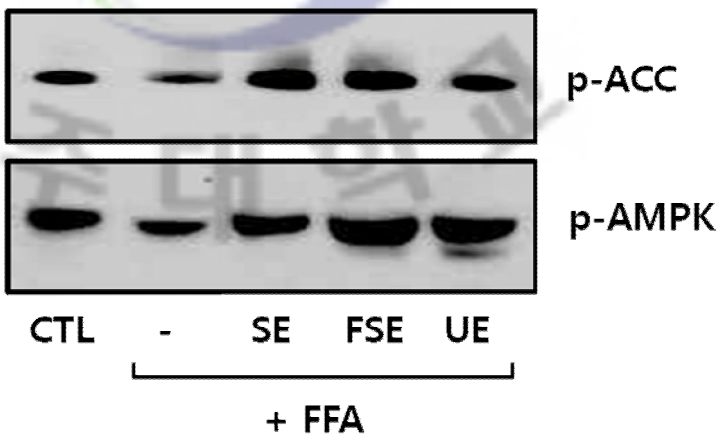


Fig. 1. Effect of FSE on lipid accumulation and phosphorylation of AMPK/ACC in $HepG_2$ cells. (A) $HepG_2$ cells were serum-starved for 4 h and pretreated with FSE (100 μg/ml) for 30 min before FFA (1 mM) treatment (24h) After treatment, cells were stained with oil red or nile red to visualize intracellular lipid as described in "Materials and Methods". (B) $HepG_2$ cells were serum-starved for 4 h and pretreated with FSE (200 μg/ml), SE (200 μg/ml), and UE (200 μg/ml) for 30 min before FFA (0.5 mM) treatment for 4 h. SE, extract of *citrus sunki* peel; FSE, extract of *citrus sunki* peel (fermented); UE, extract of *citrus unshiu* peel; FFA, free fatty acid

(palmitate:oleate=1:2)

II. Effect of FSE on high-cholesterol diet-induced steatosis in rats

1. Effect of FSE on the weight of liver and spleen

HFD increased the weight of organs (liver and spleen). Metformin, the drug for the treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function.(American Diabetes Association. 2009, American Diabetes Association. 2009) was used as a positive control in experiments. Diet with FSE decreased the weight of liver and spleen that was increased by HFD (Fig. 2).

2. Effect of FSE on the plasma pathological profiles

Hepatic steatosis can manifest with mild chronic elevation of liver aminotransferases detected on routine laboratory testing (Aubé *et al.*, 1999). NAFLD accounts for about 90% of cases of asymptomatic elevation of transaminases when other causes of liver disease are excluded (Moscatiello *et al*. 2007). HFD increased plasma levels of AST, ALT and γ-GT and FSE significantly suppressed such increases (Fig. 3). Plasma TG levels were also elevated by HFD and FSE again significantly suppressed HFD-induced elevation of TG (Fig. 4).

3. Effect of FSE on intrahepatic lipid accumulation

Lipid accumulation in hepatocytes and blood plasma is a critical event in hepatic steatosis. We observed the degree of TG accumulation in the liver by Hematoxylin-eosin staining. HFD lead to a lipid accumulation in the liver and FSE suppressed the lipid accumulation (Fig. 5). These hypolipidemic activities of FSE was observed clearly in the liver of HFD rats as well as $HepG_2$ liver cells, suggesting the strong potential of the peel of *citrus sunki* as a new supplements for health purposes especially in regulating hepatic lipid metabolism.

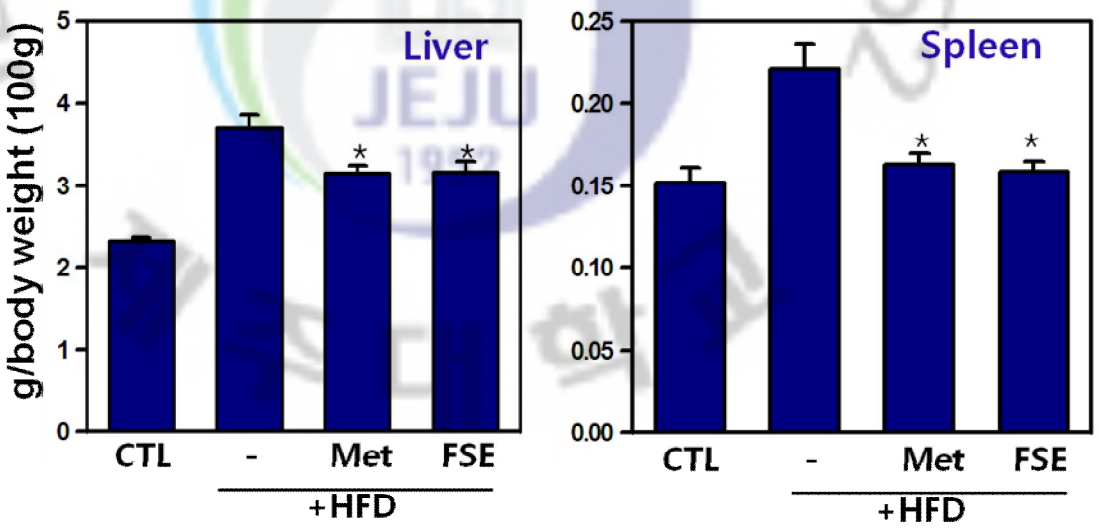


Fig. 2. Effect of FSE on the weight of liver and spleen. SD rat were fed with HFD for 8 weeks before feeding with metformin or FSE for an additional 8 weeks. Results are expressed as the mean±SE. *Significantly different from HFD ($P<0.05$). CTL, control; HFD, high-fat diet; Met, metformin; FSE, hot-water extract of fermented *Citrus sunki* peel

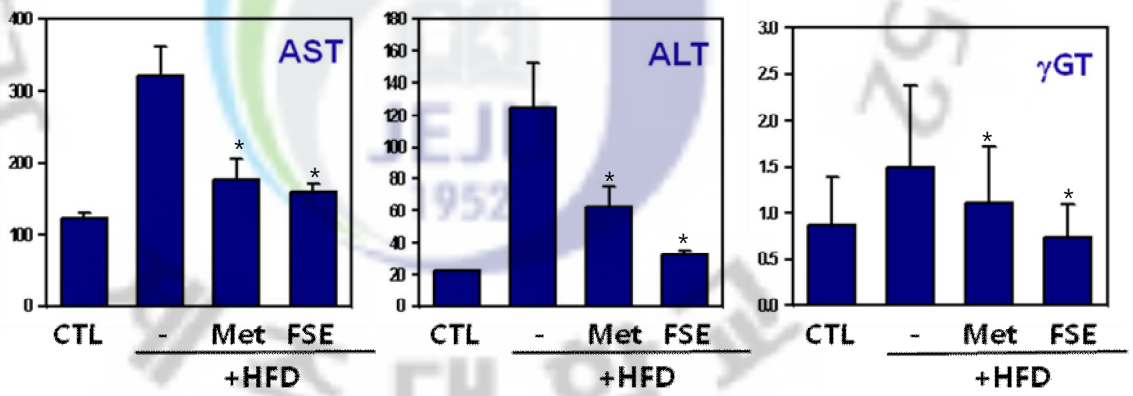


Fig. 3. Effect of FSE on the plasma pathological profiles. SD rat were fed with HFD for 8 weeks before feeding with metformin or FSE for an additional 8 weeks. Blood plasma concentrations of AST, ALT and γ–GT were measured. Results are expressed as the mean±SE. *Significantly different from HFD ($P<0.05$). CTL, control; HFD, high-fat diet; Met, metformin; FSE, hot-water extract of fermented *Citrus sunki* peel

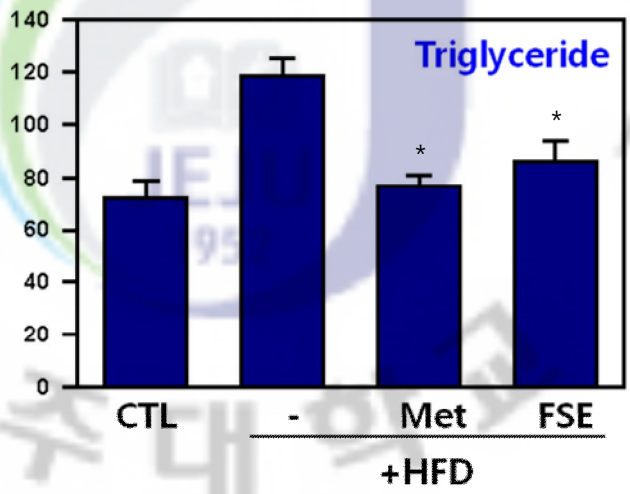


Fig.4. Effect of FSE on blood plasma triglyceride content. SD rat were fed with HFD for 8 weeks before feeding with metformin or FSE for an additional 8 weeks. Blood plasma TG levels were measured. Results are expressed as the mean±SE. *Significantly different from HFD ($P<0.05$). CTL, control; HFD, high-fat diet; Met, metformin; FSE, hot-water extract of fermented *Citrus sunki* peel

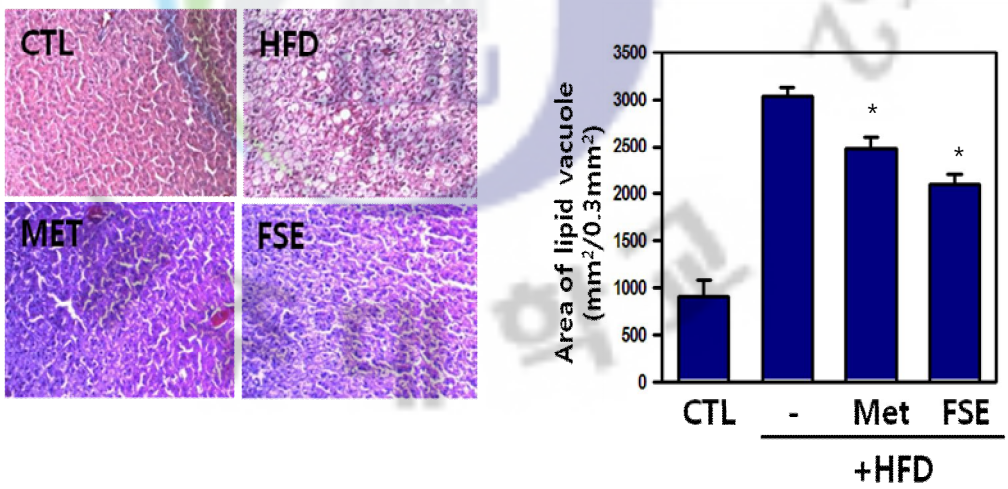


Fig. 5. Effect of FSE on intrahepatic lipid accumulation. SD rat were fed with HFD for 8 weeks before feeding with metformin or FSE for an additional 8 weeks. Liver tissues were stained with hematoxylin and eosin as described in "Materials and Methods". The areas of lipid droplets within same rectangles were calculated with the aid of image analysis. Results are expressed as the mean±SE. *Significantly different from HFD ($P<0.05$). CTL, control; HFD, high-fat diet; Met, metformin; FSE, hot-water extract of fermented *Citrus sunki* peel.

III. Active compounds in extract of *Citrus sunki* peel and their roles in the lipid metabolism in liver cells

1. Composition analysis

Extracts were further fractionated for the composition analysis. First, analysis for alkaloids was carried out (Table 2). Among tested four major compounds, only one alkaloid compound (synephrine) was present in extracts of peels of various citrus fruits (Table 2). Synephrine is also abundant in the peel of *citrus aurantum* and known to be helpful for weight loss (Fugh-Berman and Myers, 2004). However, its adverse effect (arrhythmias etc) limits its application for the health purposes (Marchei *et al*., 2006). In addition, the action mechanism at cellular levels has been not fully understood yet. Two polymethoxyflavones (PMFs, tangeritin and nobiletin) and two flavonoids (rutin and hesperidin) were also present in the extracts of peels of various citrus fruits (Table 3). Another PMFs or flavonoids were absent or just little.

From these results, the following experiments were designed as to investigate the role of the five major compounds in the hepatic lipid metabolism and their action mechanism.

Table 2. Alkaloids in the extract of peel of various citrus fruits

Extraction		Synephrine	Octopamine	Tyramine	Norepinephrine
Immature Unchiu	80% EtOH	25.34±0.35	ND	ND	ND
	Hot water	15.26±0.17	ND	ND	ND
Mature Unshiu	80% EtOH	6.62±0.12	ND	ND	ND
	Hot water	4.53±0.04	ND	ND	ND
Immature sunki	80% EtOH	27.92±0.27	ND	ND	ND
	Hot water	15.28±0.35	ND	ND	ND
Mature sunki	80% EtOH	10.34±0.04	ND	ND	ND
	Hot water	6.75±0.83	ND	ND	ND
Immature sunki (fermented)	80% EtOH	29.79±0.12	ND	ND	ND
	Hot water	15.53±1.64	ND	ND	ND
Mature sunki (fermented)	80% EtOH	11.73±0.05	ND	ND	ND
	Hot water	7.44±0.10	ND	ND	ND

*Structure of synephrine

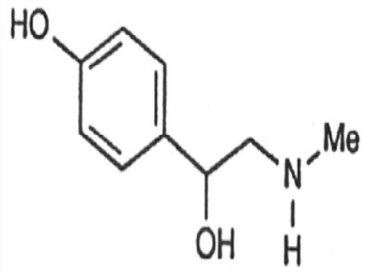
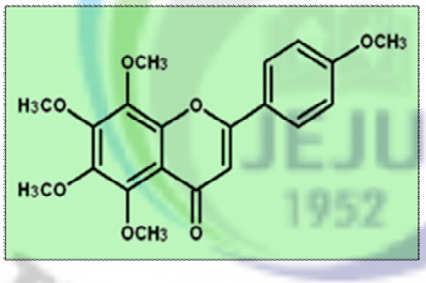


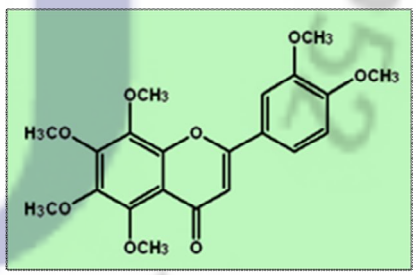
Table 3. Polymethoxyflavones and flavonoids in the extract of peel of various citrus fruits

	Immature Unchiu		Mature Unshiu		Immature sunki		Mature sunki		Immature sunki (fermented)		Mature sunki (fermented)	
	80% EtOH	Hot Water	80% EtOH	Hot Water	80% EtOH	Hot Water	80% EtOH	Hot Water	80% EtOH	Hot Water	80% EtOH	Hot Water
Rutin	4.70±0.05	2.69±0.22	2.58±0.15	1.94±0.13	17.02±0.09	8.38±0.11	5.32±0.15	6.15±0.54	14.67±0.09	7.60±0.16	6.10±0.14	6.20±1.08
Naringin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hesperedin	12.55±0.50	13.58±1.06	13.64±0.72	6.45±0.07	17.11±0.08	15.45±023	14.15±0.24	12.38±0.77	16.35±0.46	14.76±0.40	14.73±0.13	14.73±0.13
Quercetin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Naringinin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hesperetin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sinencetin	0.14±0.00	0.08±0.00	0.06±0.00	0.04±0.00	4.23±0.09	1.05±0.02	0.91±0.03	0.37±0.01	4.37±0.16	1.00±0.02	1.00±0.03	0.42±0.01
Nobiletin	1.76±0.03	0.73±0.05	0.58±0.03	0.35±0.00	38.83±0.89	7.32±0.15	8.67±0.28	2.89±0.07	40.90±1.74	6.99±0.17	9.43±0.29	3.24±0.08
Tangeretine	0.90±0.02	0.26±0.02	0.27±0.01	0.10±0.00	55.13±1.30	6.11±0.08	14.52±0.57	2.97±0.10	59.32±2.79	5.64±0.16	15.48±0.50	3.43±0.08

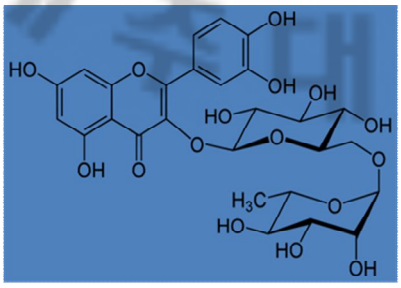
*Structure of tangeritin, nobiletin, rutin and hesperidin



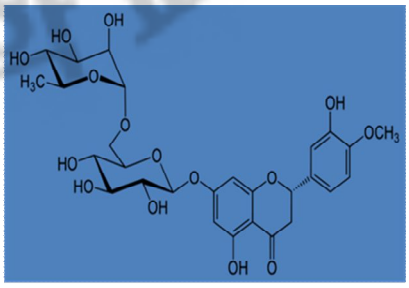
tangeretin



nobiletin



rutin



hesperidin

2. Hypolipidemic activity and action mechanism of synephrine in $HepG_2$ cells

2-1. Effect of synephrine on cell viability

The cytotoxicity of synephrine was evaluated with LDH assay and MTT assay. Synephrine was not cytotoxic at doses tested (0-100 μM) at least with 36 h (Fig. 6)

2-2. Effect of synephrine on FFA-induced TG (triglyceride) accumulation

Synephrine is a drug commonly used for weight loss. But the effectiveness is widely debated. In the present study, cytoplasmaic TG accumulation was induced by FFA treatment in HepG2 cells after pretreatment for 30min. Cytoplasmic TG was visualized by a calorimetric (oil red) and fluorogenic (nile red) staining and with the aid of digitized fluorescence microscopy. TG content was also measured by assay reagents. Synephrine (50 μM) suppressed FFA (0.1 mM)-induced TG accumulation (Fig.7). From the TG assay, FFA-induced elevation of TG content was also reduced by treatment with synephrine. AICAR, an AMPK activator, also reduced TG content (positive control). When the AMPK inhibitor compound C was treated together with synephrine, hypolipidemic activity of synephrine was blocked, suggesting the involvement of AMPK in this process by synephrine (Fig. 8).

2-3. Role of AMPK-SREBP1c in the hypolipidemic activity of synephrine

SREBP1c (sterol regulatory element-binding protein-1c), which is transcription factor, controls the DNL. When SREBP1c was activated, it will in turn stimulate several lipogenic enzymes, such as liver Pyruvate kinase (L-PK), ACC, FAS, etc.

The present study investigated whether SREBP1c is involved in the hypolipidemic function of synephrine. From the immunoblot analysis, treatment of cells with FFA reduced the levels of AMPK phosphorylation but increased SREBP1c protein levels (Fig. 9). However, pretreatment with synephrine again increased the phosphorylation of AMPK and decreased SREBP1c protein levels. The reduction of SREBP1c protein levels by synephrine was blocked by the inhibitor of AMPK (compound c). These results imply that the steady-state levels of SREBP1c protein expression was under the control of AMPK. From the RT-PCR analysis, mRNA levels of SREBP1c and FAS were increased by FFA, however, FFA-induced increase of SREBP1c mRNA levels was reduced by synephrine (Fig. 10). AICAR also reduced SREBP1c mRNA levels like as synephrine.

In summary, synephrine can suppress FFA-induced TG accumulation through the stimulation of AMPK, suppression of SREBP1c production at the transcriptional level and the resultant reduction of FAS expression.

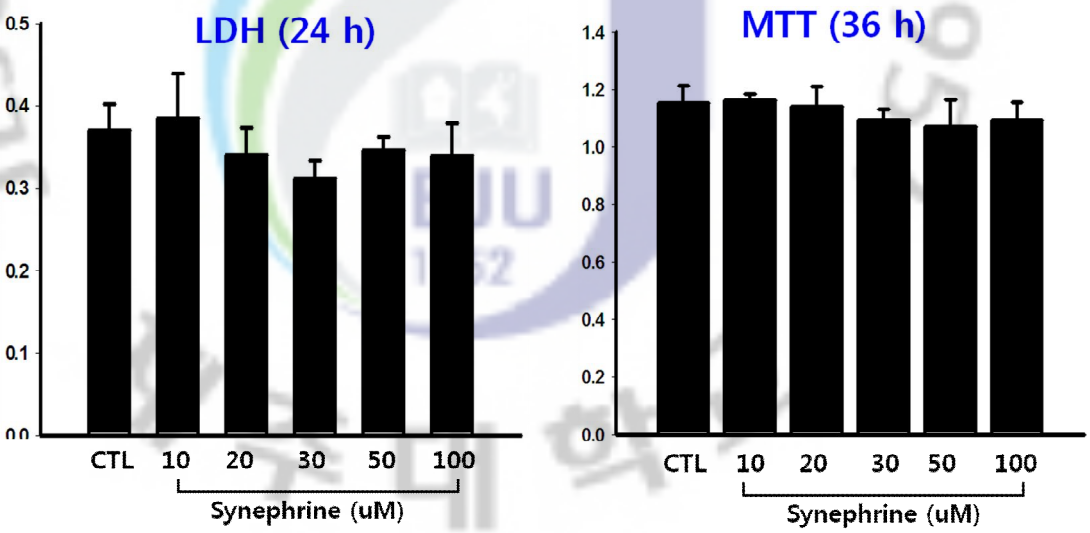


Fig. 6. Effect of synephrine on cell viability in $HepG_2$ cells. Cells were serum-starved for overnight and treated with synephrine (10- 100 μM) for 24 h. The cytotoxicity was determined with LDH release and MTT assay as described in "Materials and Methods. CTL, control.

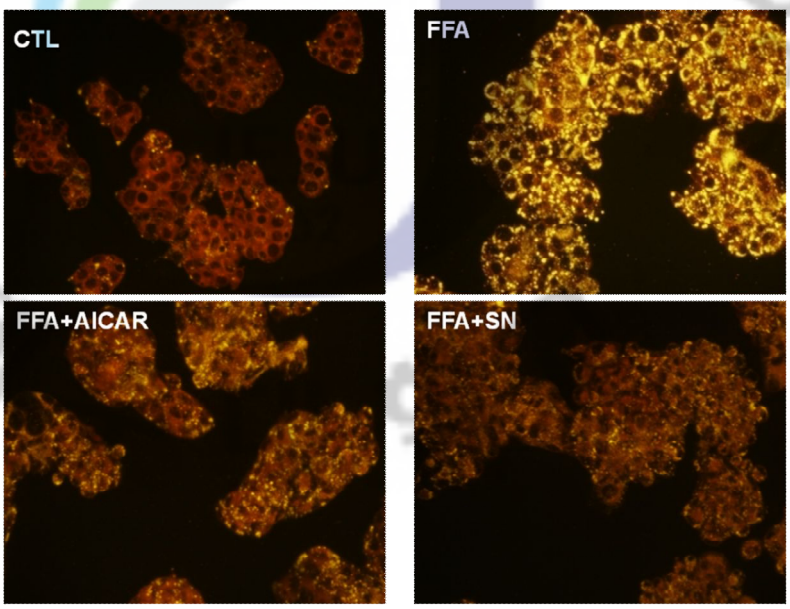


Fig. 7. Effect of synephrine on FFA-induced lipid accumulation in $HepG_2$ cells. Cells were serum-starved for 12 h and pretreated with synephrine (50 μM) or AICAR (1 mM) before the addition of FFA (0.1 mM). After 12 h treatment, lipid accumulation was observed under a fluorescent microscope after staining with nile red. FFA, free fatty acid; AICAR, AMPK activator; SN, synephrine.

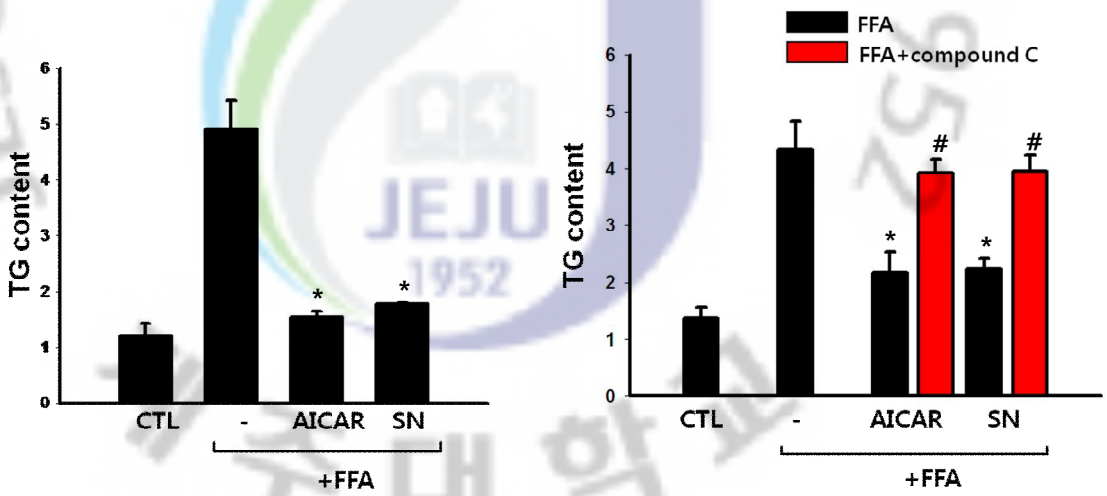


Fig. 8. Effect of synephrine on FFA-induced elevation of TG content in HepG2 cells

Cells were serum-starved for 12 h and pretreated with compound C (10 μM) for 30 min, then further treated with AICAR (1 mM) or synephrine (50 μM) for 30 min before FFA (1 mM) treatment for 12 h. TG content was determined as described in "Materials and methods". *Significantly different from FFA alone ($p<0.05$), # compared with AICAR or SN treatment without treatment compound C ($P<0.05$).

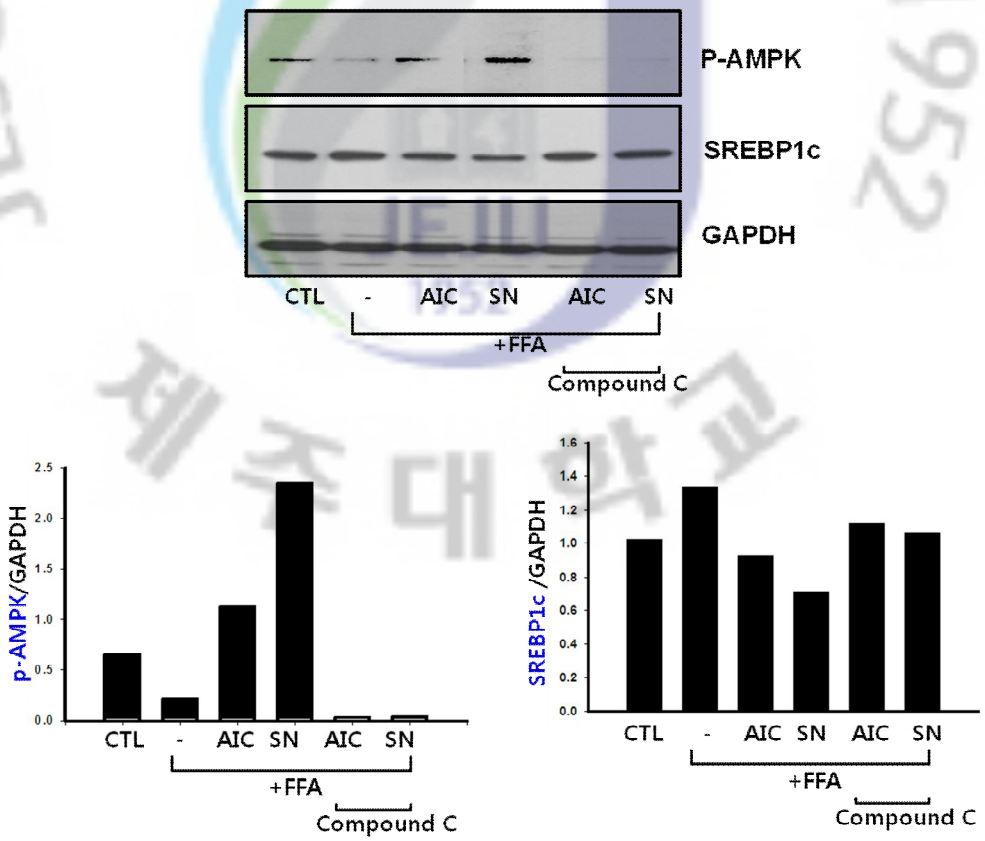


Fig. 9. Effect of synephrine on AMPK-SREBP1c protein levels in HepG2 cells. Cells were serum-starved for 12 h and pretreated with compound C (10 μM) for 30 min and further treated with AICAR (1 mM) or synephrine (50 μM) 30 min before FFA (0.2 mM) treatment for 15 h. The levels of phospho-AMPK and SREBP1c proteins were detected by western blot. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; SN, synephrine; compound C, AMPK inhibitor.

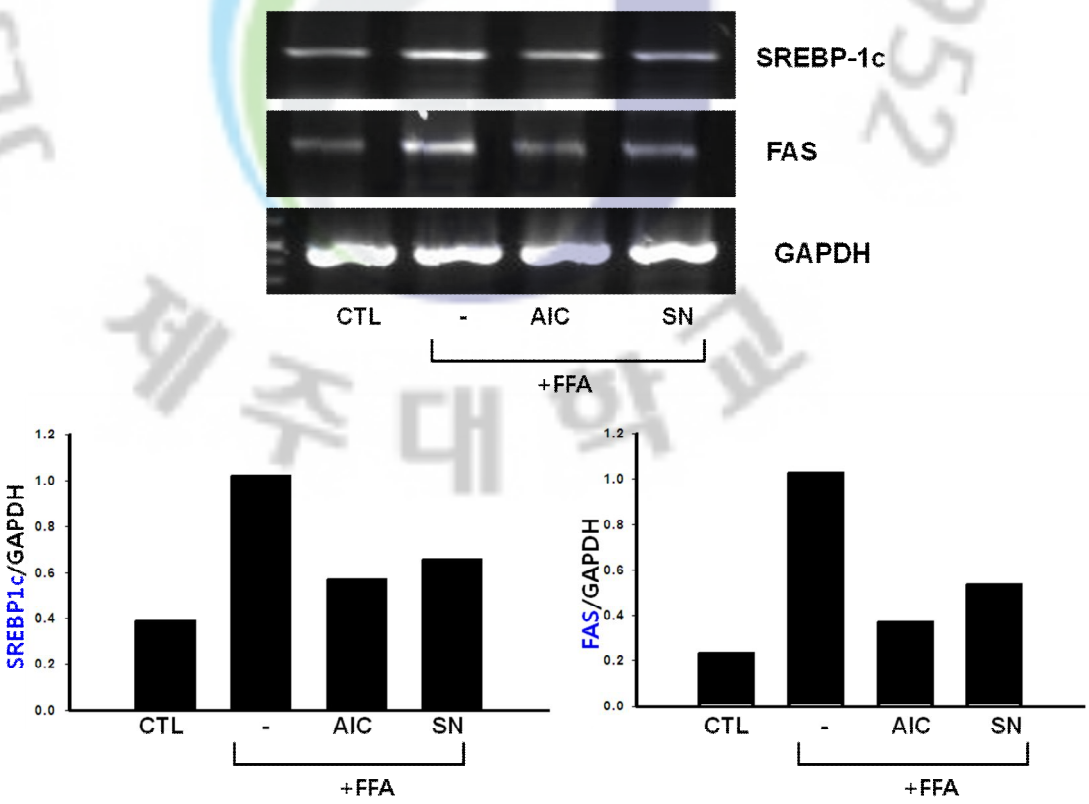


Fig. 10. Effect of synephrine on AMPK-SREBP1c mRNA levels in HepG2 cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or synephrine (50 μM) 30 min before FFA (0.2 mM) treatment for 15 h. The levels of SREBP1c mRNA were detected by RT-PCR. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; SN, synephrine.

3. Hypolipidemic activity and action mechanism of tangeritin in $HepG_2$ cells

3-1. Effect of tangeritin on cell viability

Effect of tangeritin on the viability of $HepG_2$ cells was examined. Any significant differences were not observed between the control group and the tangeritin-treated group, regardless the presence of FFA in the culture medium (Fig. 11).

3-2. Effect of tangeritin on FFA-induced TG accumulation in $HepG_2$ cell

As synephrine, treatment of cells with tangeritin (50 μM) clearly suppressed FFA-induced lipid accumulation within cells stained with nile red (Fig. 12). FFA-induced increase of TG content was completely blocked by tangeritin (50 μM) as well as by AICAR, an AMPK stimulator (Fig. 13A). From these results, we tested whether such effect of tangeritin is mediated by AMPK. To do this, compound c, an inhibitor of AMPK, was treated together with tangeritin. Although AICAR induced-reduction of TG was completely blocked by compound c, the TG-lowering activity of tangeritin was not changed by compound c, suggesting that TG-lowering activity of tangeritin is independent of AMPK activity (Fig. 13B). One previous study (Azzout-Marniche *et. al.,* 2000) reported that the effect of insulin on SREBP1c expression is mediated by PI3K-dependent pathway. From this, it was tested whether TG-lowering activity of tangeritin is mediated by PI3K-dependent pathway. Treatment with wortmannin, a PI3K inhibitor, significantly suppressed the TG-lowering activity of tangeritin (Fig. 13C). These results

provide an evidence that tangeritin can suppress FFA-induced lipid accumulation through PI3K activity, independent of AMPK activity.

3-3. Role of AMPK-SREBP1c in the hypolipidemic activity of tangeritin

The present study investigated whether SREBP1c is involved in the hypolipidemic function of tangeritin. From the immunoblot analysis, treatment of cells with FFA reduced the levels of AMPK phosphorylation but increased SREBP1c protein levels (Fig. 14). Although pretreatment with tangeritin failed to increase the phosphorylation of AMPK, it decreased SREBP1c protein levels. From the RT-PCR, SERBP1c mRNA levels were increased by FFA and this increase was totally reduced to the basal level by tangeritin (Fig. 15). These results suggest that tangeritin can suppress FFA-induced TG accumulation through the suppression of SREBP1c production at the transcriptional level and AMPK is not involved in this process.

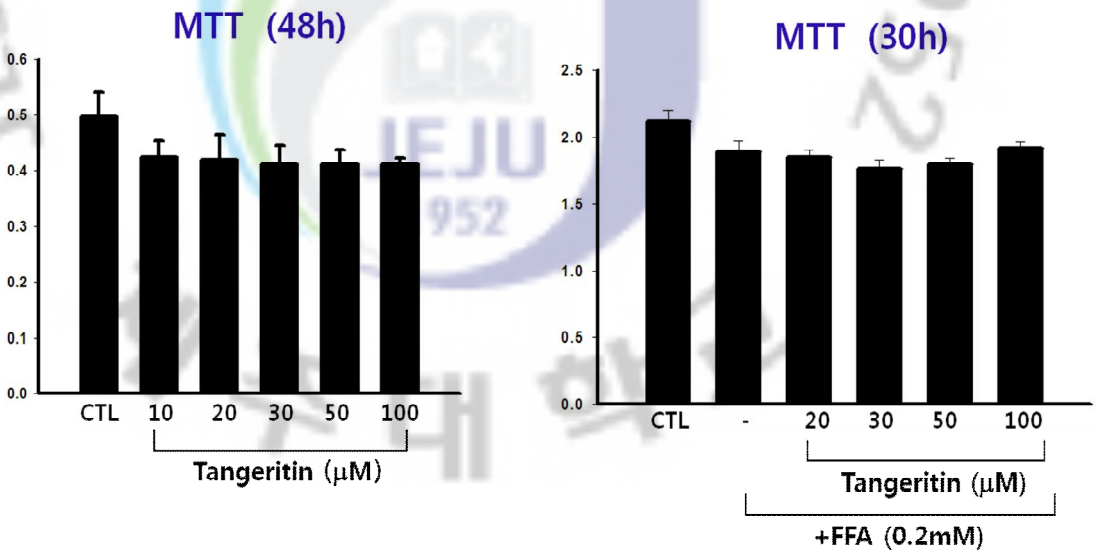


Fig. 11. Effect of tangeritin on cell viability in HepG$_2$ cells. Cells were serum-starved for 12 h and pretreated with tangeritin (20μM – 100 μM) for 30 min and further treated with or without FFA (0.2 mM) for 30 h. FFA, free fatty acid; TN, tangeritin.

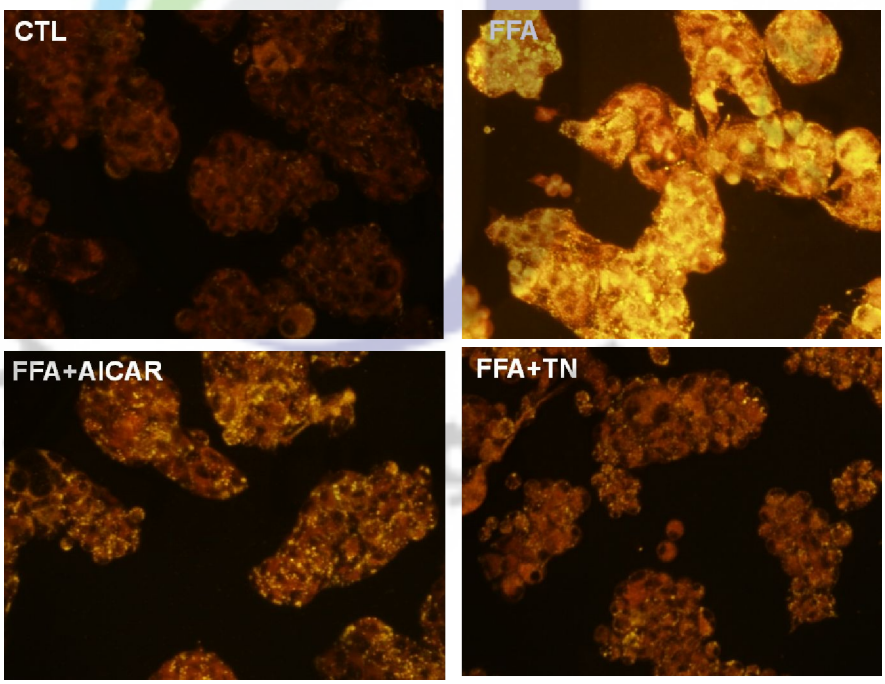


Fig. 12. Effect of tangeritin on FFA-induced lipid accumulation in HepG2 cells. Cells were serum-starved for 12 h and pretreated with tangeritin (50 μM) or AICAR (1 mM) before FFA (0.1 mM) treatment for an additional 12 h. After treatment, cells were stained with nile red and observed under a fluorescent microscope. FFA, free fatty acid; AICAR, AMPK activator; TN, tangeritin.

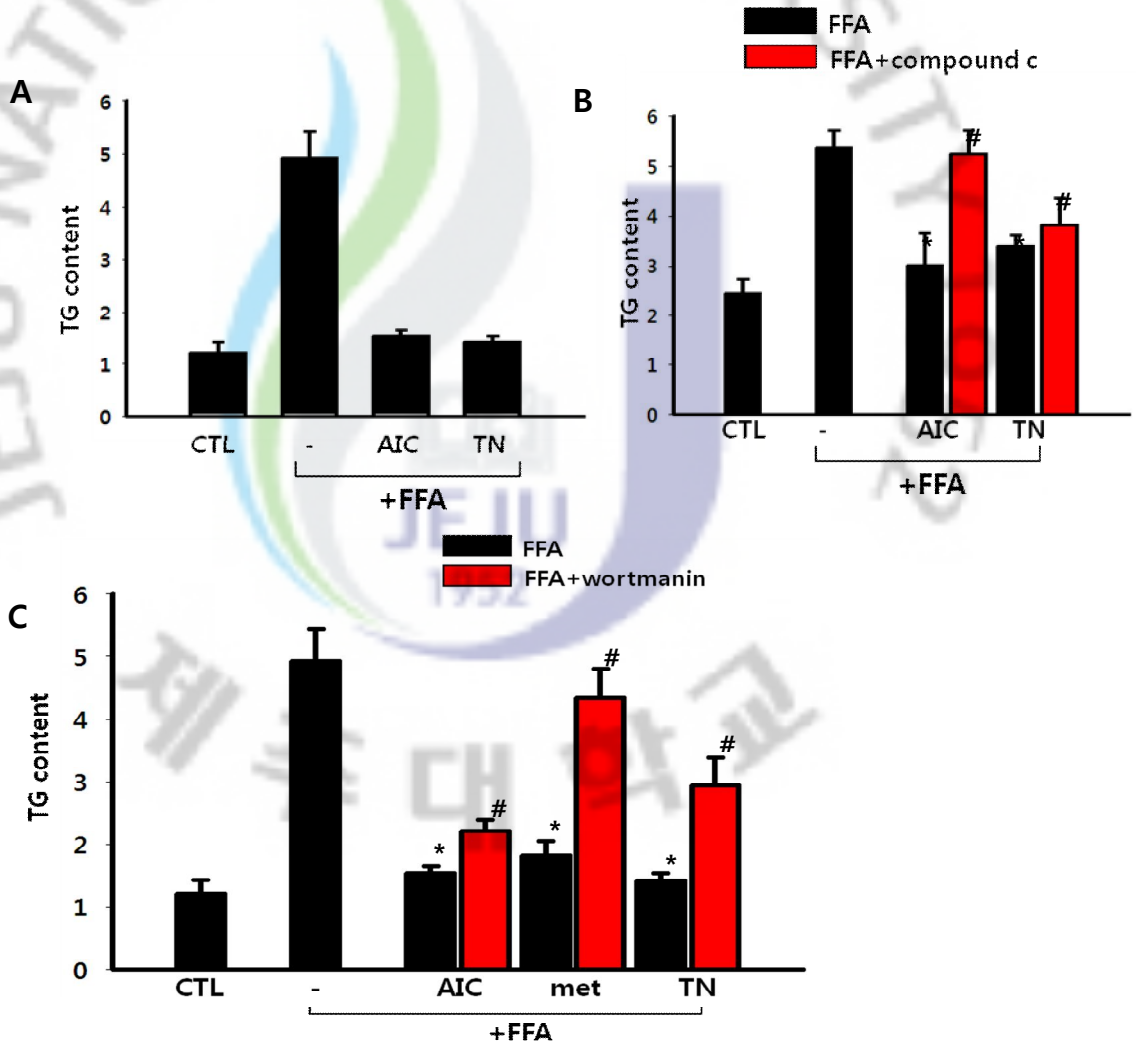


Fig. 13. Effect of tangeritin on TG content and its action mechanism Cells were serum-starved for 12 h and pretreated with compound c (10 μM) or wortmanin (100 uM) for 30 min and further treated with AICAR (1 mM), metformin or tangeritin (50 uM) for 30 min before FFA (1mM) treatment for an additional 12 h. TG content was measured by TG assay reagent as described in "Materials and methods". *Significantly different from FFA alone ($p<0.05$), # compared with AICAR + FFA, metformin + FFA, and TN + FFA, respectively within each pair ($P<0.05$). CTL, control; AIC, AICAR, AMPK activator; met, metformin; TN, tangeritin; FFA, free fatty acid; wortmanin, PI3-kinase inhibitor.

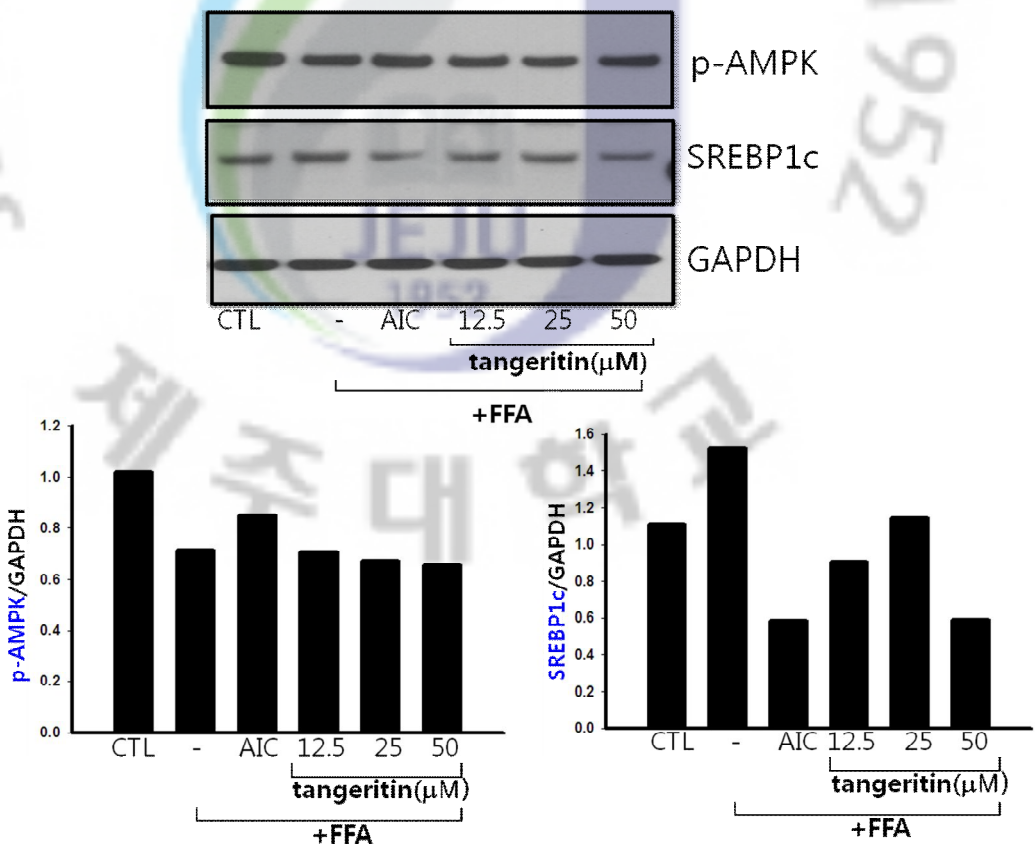


Fig. 14. Effect of tangeritin on SREBP1c in HepG2 cells. Cells were serum-starved for 12 h and pretreated AICAR (1 mM) or tangeritin for 30 min before FFA (0.1 mM) treatment for 15 h. Phospho-AMPK and SREBP1c proteins were detected by western blot. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; TN, tangeritin.

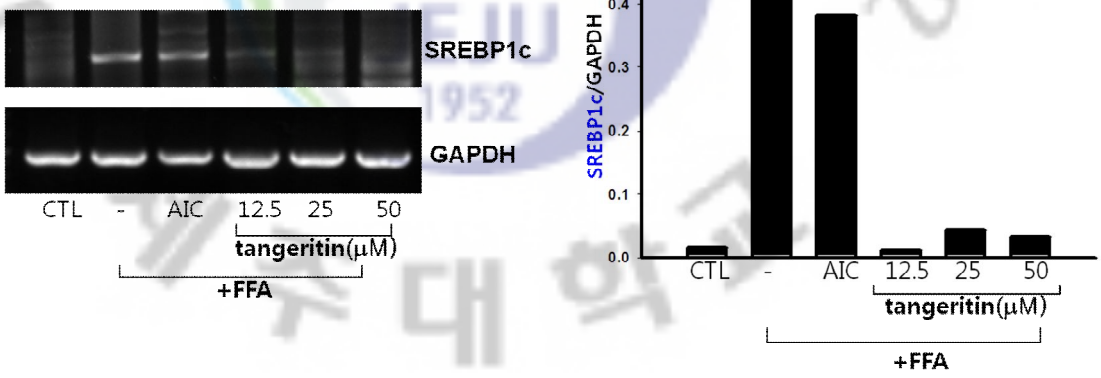


Fig. 15. Effect of tangeritin on SREBP1c mRNA levels in HepG2 cells. Cells were serum-starved for 12 h and treated AICAR (1 mM) or tangeritin for 30 min before FFA (0.1 mM) treatment for 15 h. The mRNA levels of SREBP1c and GAPDH were detected by RT-PCR. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; TN, tangeritin.

4. Hypolipidemic activity and action mechanism of nobiletin in $HepG_2$ cells

4-1. Effect of nobiletin on cell viability

Effect of nobiletin on the viability of $HepG_2$ cells was examined. Any significant differences were not observed between the control group and the nobiletin-treated group, regardless the presence of FFA in the culture medium (Fig. 16).

4-2. Effect of nobiletin on the FFA-induced TG accumulation and its action mechanism

Like as previous experiments using synephrine and tangeritin, effect of nobiletin on the FFA-induced TG accumulation was evaluated. First, FFA-induced cellular lipid accumulation was suppressed by nobiletin treatment (nile red staining) (Fig. 17). FFA-induced elevation of TG content was also significantly decreased by nobiletin treatment and the nobiletin's TG-lowering activity was sensitive to the inhibition of AMPK with compound C (Fig. 18). Levels of phospho-AMPK and SREBP1c proteins were decreased and increased, respectively, by FFA. Nobiletin restored FFA-induced decrease of phospho-AMPK and blocked FFA-induced increase of SREBP1c protein levels (Fig. 19). mRNA levels of SREBP1c were also increased by FFA but suppressed by co-treatment of nobiletin (Fig. 20). Although the chemical structure of nobiletin is highly similar to that of tangeritine, action mechanism of each PMF compounds seems to be quite different in terms of the usage of AMPK to suppress TG accumulation.

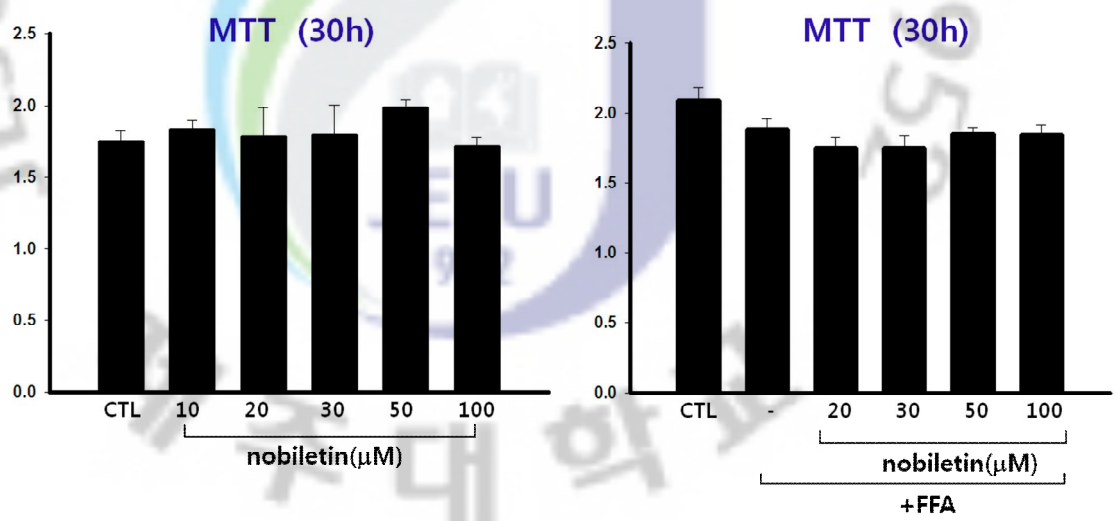


Fig. 16. Effect of nobiletin on cell viability in HepG$_2$ cells. Cells were serum-starved for 12 h and pretreated with nobiletin (20-100 μM) for 30 min and further treated with or without FFA (0.2 mM) for 30 h. FFA, free fatty acid; Nb, nobiletin.

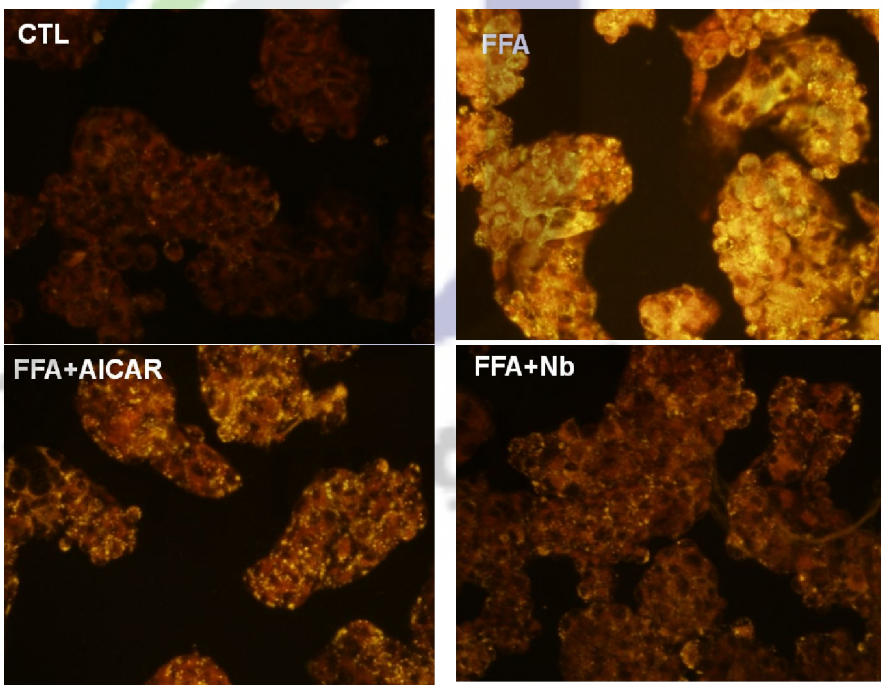


Fig. 17. Effect of nobiletin on FFA-induced lipid accumulation in HepG2 cells. Cells were serum-starved for 12 h and pretreated with nobiletin (50 μM) or AICAR (1 mM) before FFA (0.1 mM) treatment for 12 h. After treatment, cells were stained with nile red and observed under a fluorescent microscope. FFA, free fatty acid; AICAR, AMPK activator; Nb, nobiletin.

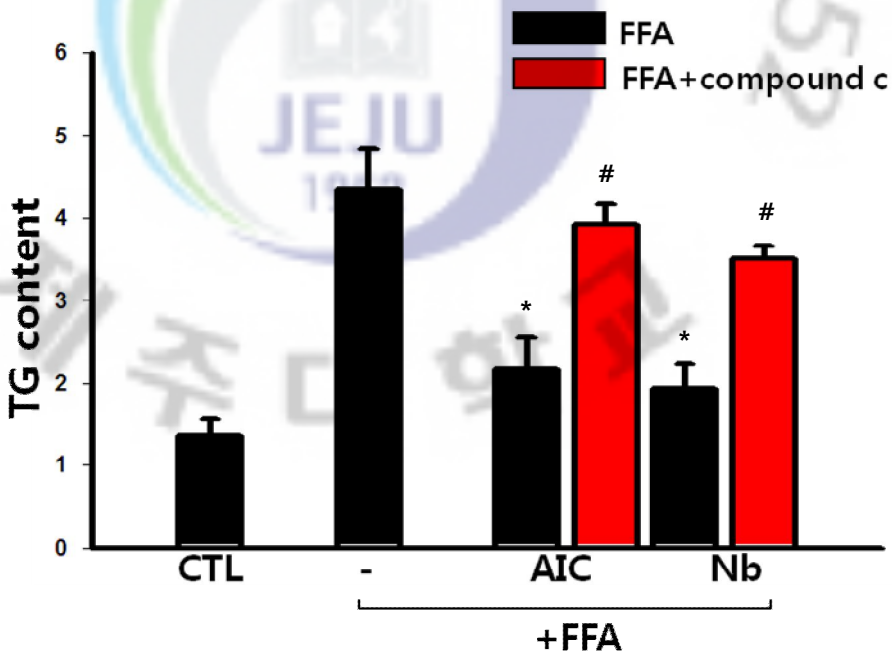


Fig. 18. Effect of nobiletin on FFA-induced elevation of TG content in HepG2 cells.

Cells were serum-starved for 12 h and pretreated compound C (10 μM) for 30min and further treated with AICAR (1 mM) or nobiletin (50 μM) for 30 min before FFA (1mM) treatment for an additional 12 h. TG content was measured by TG assay reagent as described in "Materials and methods". . *Significantly different from FFA alone ($p<0.05$), # compared with AICAR + FFA, and Nb + FFA, respectively within each pair ($P<0.05$). CTL, control; AIC, AICAR, AMPK activator; FFA, free fatty acid; Nb, nobiletin.

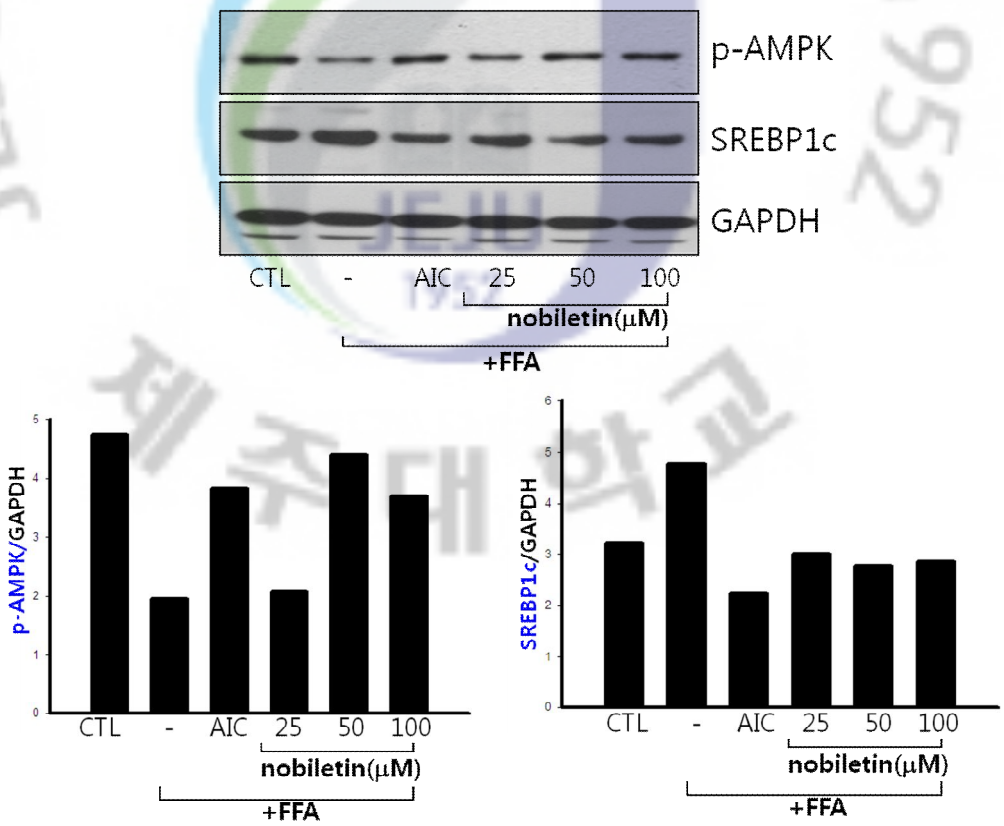


Fig. 19. Effect of nobiletin on AMPK-SREBP1c in HepG2 cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or nobiletin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. Phospho-AMPK and SREBP1c proteins were detected by western blot. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; Nb, nobiletin.

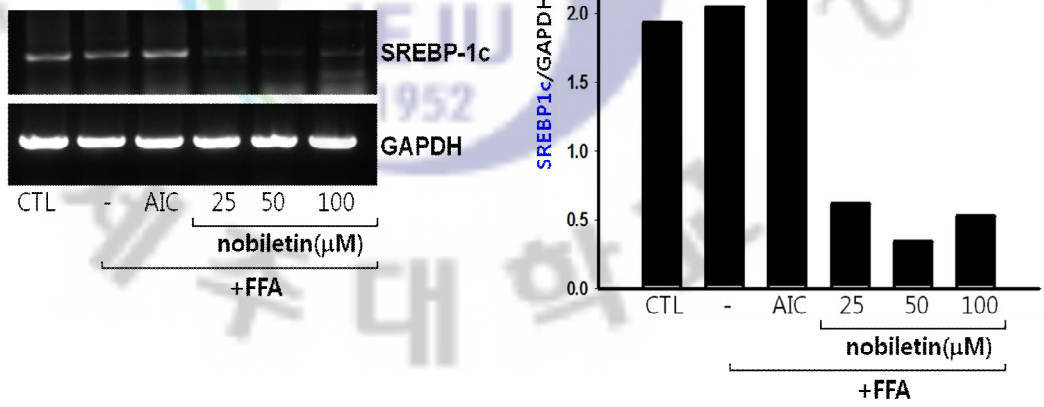


Fig. 20. Effect of nobiletin on AMPK- SREBP1c mRNA levels in HepG2 cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or nobiletin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. The mRNA levels of SREBP1c and GAPDH were detected by RT-PCR. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator; Nb, nobiletin.

5. Hypolipidemic activity and action mechanism of rutin in $HepG_2$ cells

5-1. Effect of rutin on cell viability

Effect of rutin on the viability of $HepG_2$ cells was examined. Any significant differences were not observed between the control group and rutin-treated group, regardless the presence of FFA in the culture medium (Fig. 21).

5-2. Effect of rutin on FFA-induced TG accumulation in HepG2 cells

FFA-induced increase in TG content (from TG assay) as well as lipid accumulation within cells (from nile red staining) were all suppressed by rutin treatment (Fig. 22, 23). Rutin's TG lowering activity was also sensitive to the inhibition of AMPK (Fig. 23).

5-3. Role of AMPK-SREBP1c in the hypolipidemic activity of rutin

We examined the effect of rutin on the phosphorylation of AMPK, matured SREBP1c and PPARγ protein levels and on the mRNA levels of SREBP1c, FAS and PPARγ. PPARγ is a member of the nuclear hormone receptor superfamily that is required for normal adipocytes differentiation.(Tontonoz, *et al*. 1994) Normally, PPARγ is expressed at very low levels in the liver; however, in animal models with insulin resistance and fatty livers, the expression of PPARγ is markedly increased.(Chao et al. 2000, Edvardsson *et al*. 1999)

Levels of phospho-AMPK and SREBP1c proteins were decreased and increased, respectively, by FFA. Rutin restored FFA-induced decrease of phospho-AMPK and blocked FFA-induced increase of SREBP1c protein levels (Fig. 24). Notably, at higher concentration (100 μM), rutin decreased PPARγ protein and mRNA levels that was increased by FFA induction (Fig. 24, 25). mRNA levels of FAS (downstream gene of SREBP1c) that were also increased by FFA induction and decreased by rutin (Fig. 25).

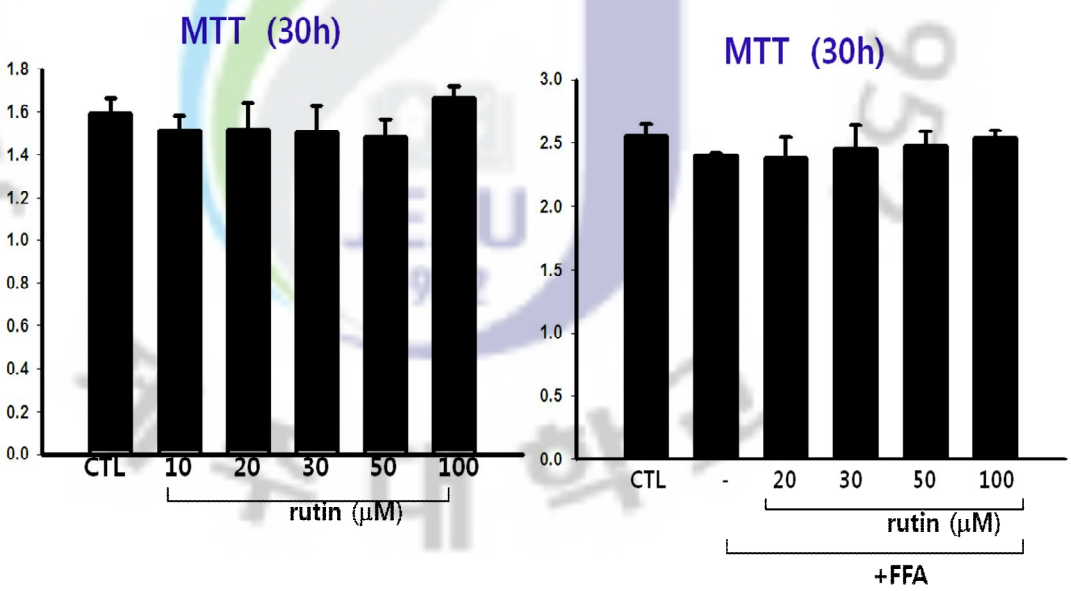


Fig. 21. Effect of rutin on cell viability in HepG2 cells. Cells were serum-starved for 12 h and pretreated with rutin (10-100 μM) for 30 min and further treated with or without FFA (0.2 mM) for 30 h. FFA, free fatty acid.

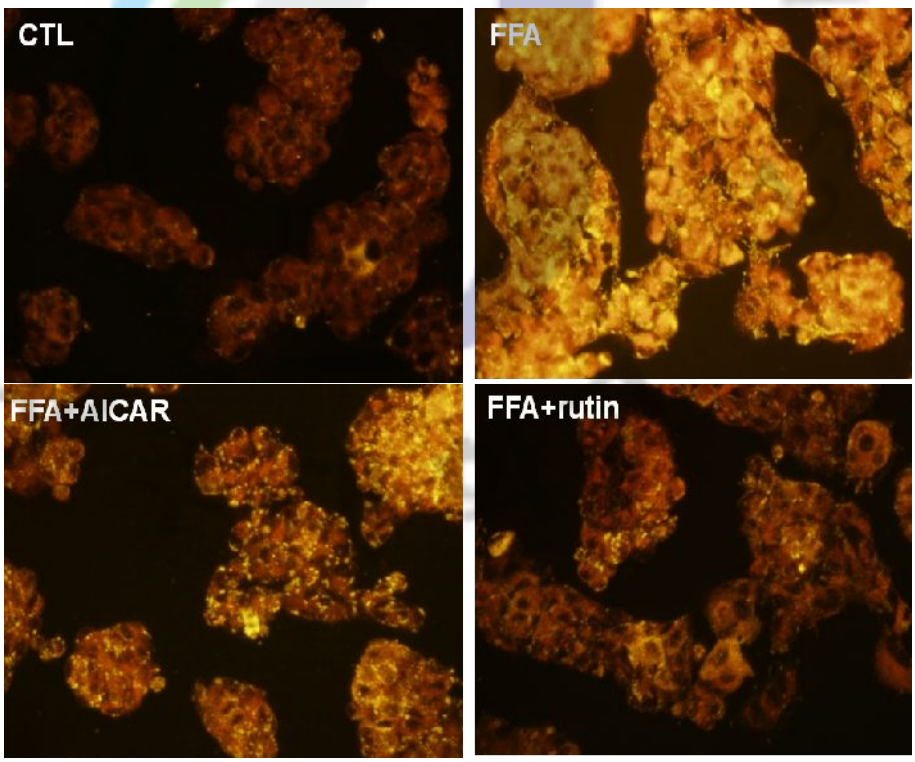


Fig. 22. Effect of rutin on FFA-induced lipid accumulation in HepG2 cells. Cells were serum-starved for 12 h and pretreated with rutin (50 μM) or AICAR (1 mM) before FFA (0.1 mM) treatment for 12 h. After treatment, cells were stained with nile red and observed under a fluorescent microscope. FFA, free fatty acid; AICAR, AMPK activator.

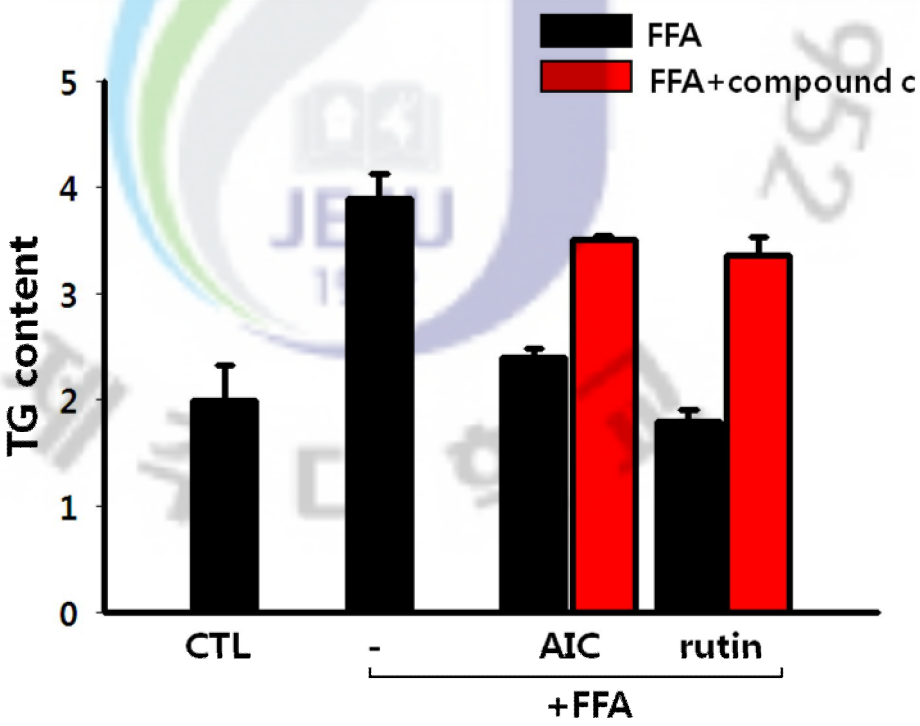


Fig23. Effect of rutin on FFA-induced elevation of TG content in HepG2 cells. Cells were serum-starved for 12 h and pretreated compound C (10 μM) for 30 min and further treated with AICAR (1 mM) or rutin (50 μM) for 30 min before FFA treatment for an additional 12 h. TG content was measured by TG assay reagent as described in "Materials and methods". . *Significantly different from FFA alone ($p<0.05$), # compared with AICAR + FFA, and rutin + FFA, respectively within each pair ($P<0.05$).

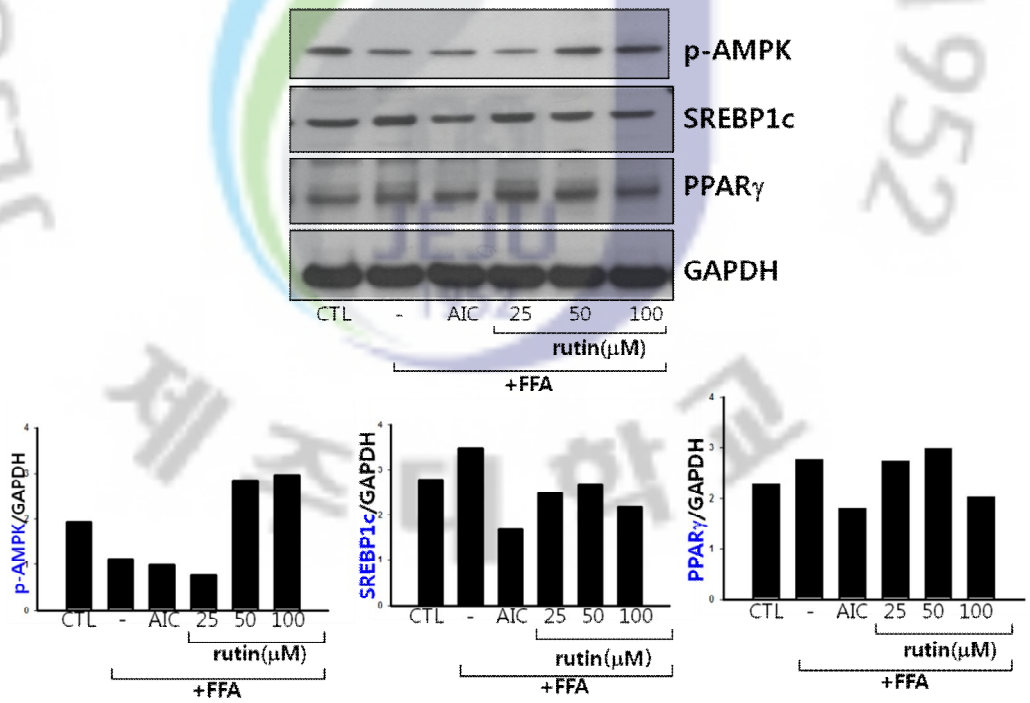


Fig. 24. Effect of rutin on AMPK-SREBP1c in HepG$_2$ cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or rutin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. Phospho-AMPK, PPARγ and SREBP1c proteins were detected by western blot. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator.

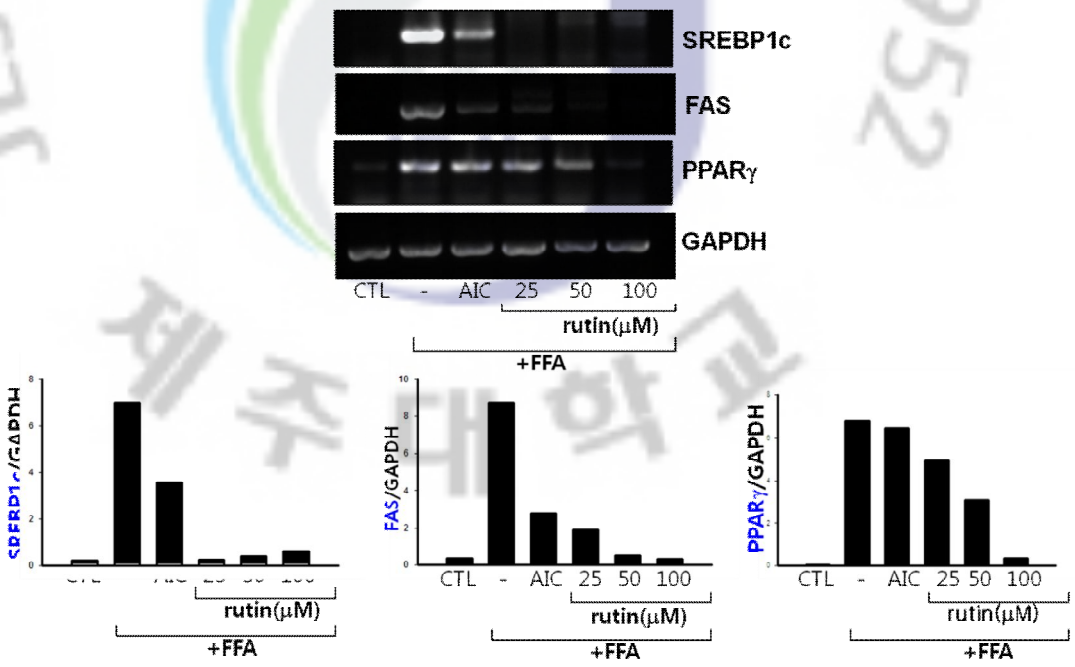


Fig. 25. Effect of rutin on AMPK-SREBP1c mRNA levels in HepG$_2$ cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or rutin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. The mRNA levels of SREBP1c, FAS and GAPDH were detected by RT-PCR. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator.

6. Hypolipidemic activity and action mechanism of hesperidin in $HepG_2$ cells

6-1. Effect of hesperidin on cell viability

Effect of hesperidin on the viability of $HepG_2$ cells was examined. Any significant differences were not observed between the control group and hesperidin-treated group, regardless the presence of FFA in the culture medium (Fig. 26).

6-2. . Effect of hesperidin on FFA-induced TG accumulation in HepG2 cells

FFA-induced lipid accumulation was decreased by hesperidin (Fig. 27). FFA-induced elevation of TG content was reduced by hesperidin (Fig. 28). Hesperidin's TG-lowering activity was also sensitive to the inhibition of AMPK (Fig. 28).

6-3. Role of AMPK-SREBP1c in the hypolipidemic activity of hesperidin in $HepG_2$ cells

Levels of phospho-AMPK and SREBP1c protein were decreased and increased, respectively, by FFA. Hesperidin restored FFA-induced decrease of phospho-AMPK and blocked FFA-induced increase of SREBP1c protein levels (Fig. 29). mRNA levels of SREBP1c and FAS were also increased by FFA but suppressed by co-treatment with hesperidin (Fig. 30). From results, action mechanism of rutin and hesperidin is definitely common in terms of usage of AMPK, SREBP1c, and FAS in $HepG_2$ liver cells.

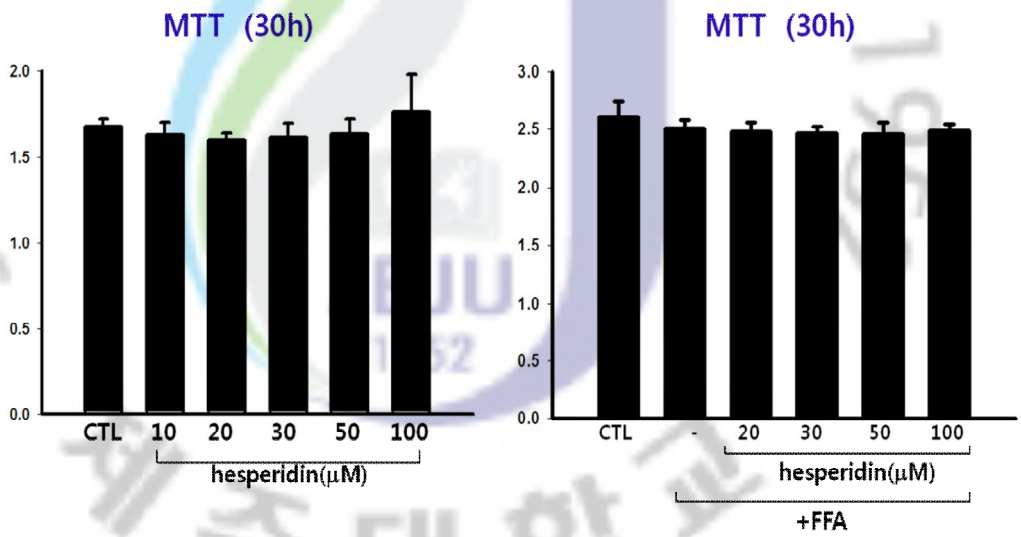


Fig. 26. Effect of hesperidin on cell viability in $HepG_2$ cells. Cells were serum-starved for 12 h and pretreated with hesperidin (10-100 μM) for 30 min and further treated with or without FFA (0.2 mM) for 30 h. FFA, free fatty acid; hesp, hesperidin.

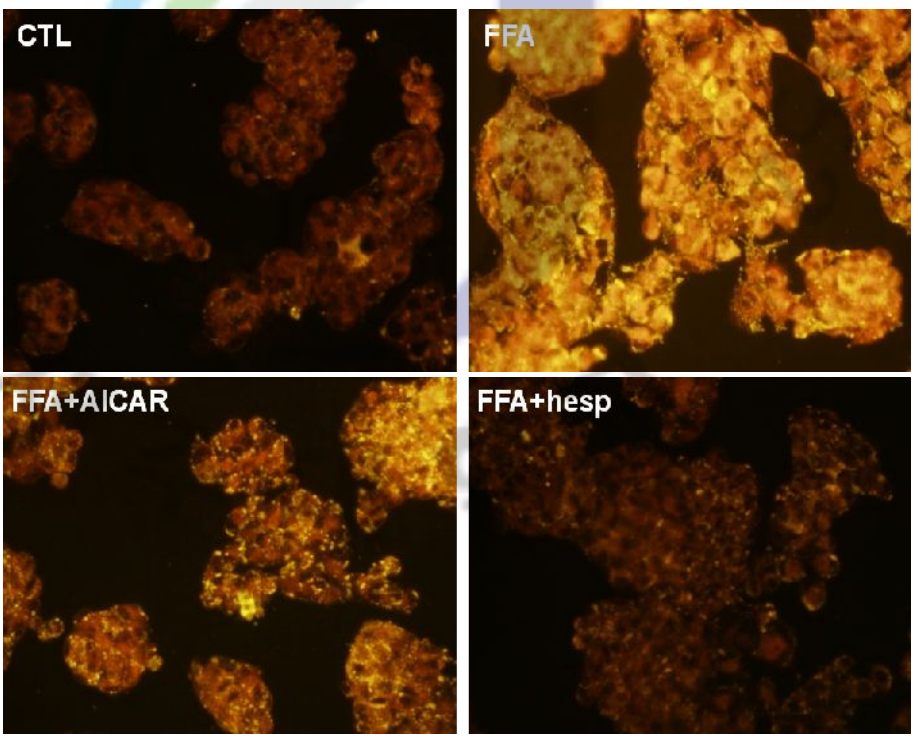


Fig. 27. Effect of hesperidin on FFA-induced lipid accumulation in HepG2 cells. Cells were serum-starved for 12 h and pretreated with hesperidin (50 μM) or AICAR (1 mM) before FFA (0.1 mM) treatment for 12 h. After treatment, cells were stained with nile red and observed under a fluorescent microscope. FFA, free fatty acid; AICAR, AMPK activator; hesp: hesperidin.

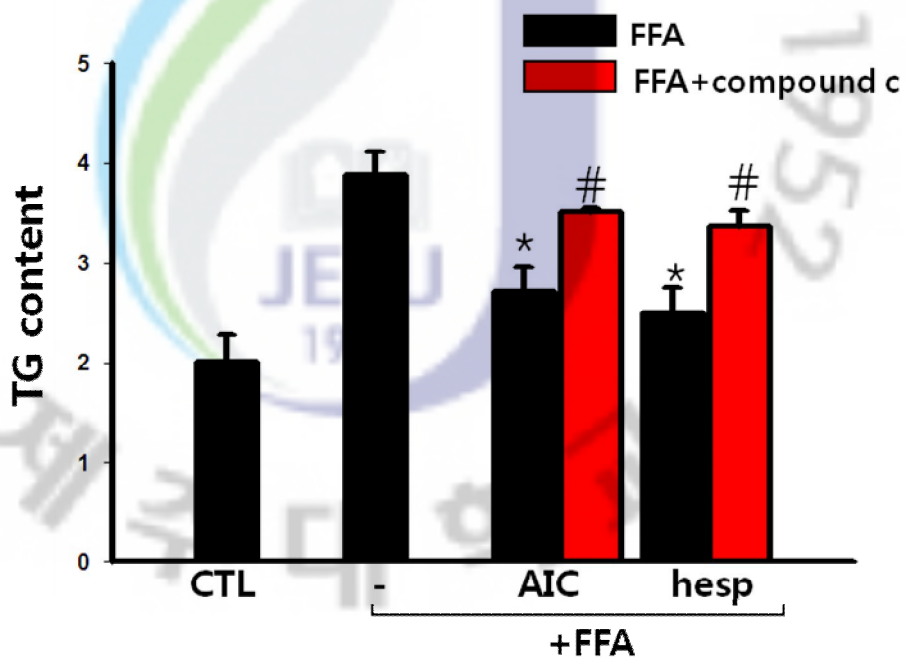


Fig. 28. Effect of hesperidin on FFA-induced elevation of TG content in HepG2 cells. Cells were serum-starved for 12 h and pretreated compound C (10 μM) for 30 min and further treated with AICAR (1 mM) or hesperidin (50 μM) for 30 min before FFA treatment for an additional 12 h. TG content was measured by TG assay reagent as described in "Materials and methods". . *Significantly different from FFA alone ($p<0.05$), # compared with AICAR + FFA, and hesperidin + FFA, respectively within each pair ($P<0.05$). CTL, control; AIC, AICAR, AMPK activator; met, metformin; hesp, hesperidin; FFA, free fatty acid.

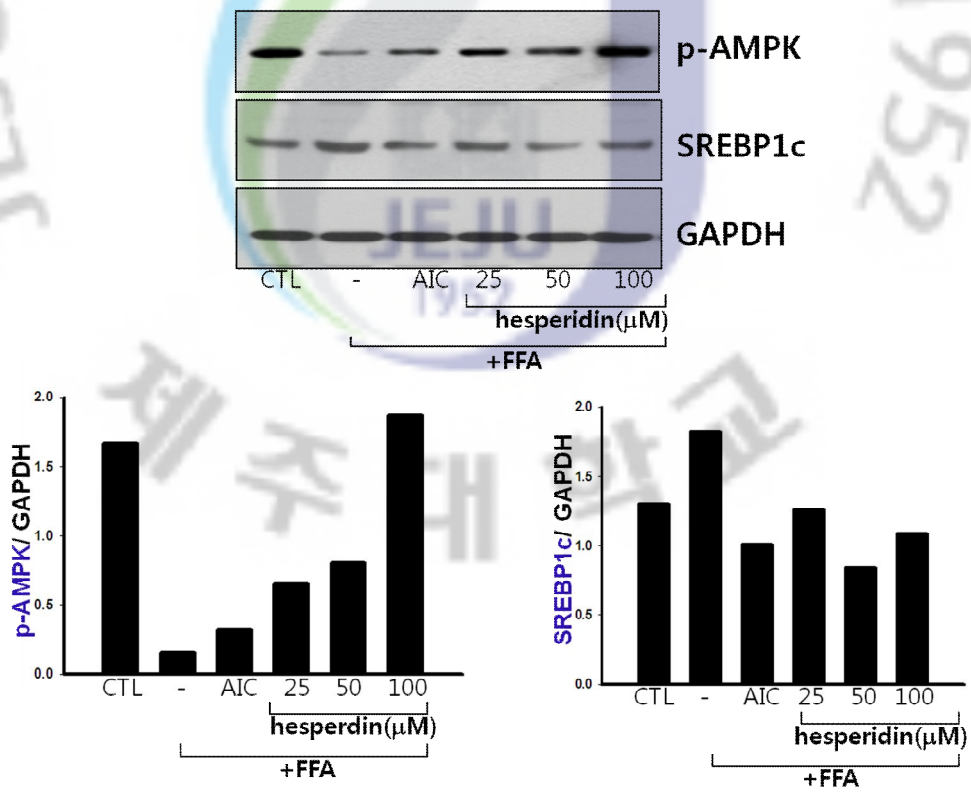


Fig. 29. Effect of hesperidin on AMPK-SREBP1c in HepG$_2$ cells. Cells were serum-starved for 12 h and treated with AICAR (1 mM) or rutin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. Phospho-AMPK and SREBP1c proteins were detected by western blot. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator.

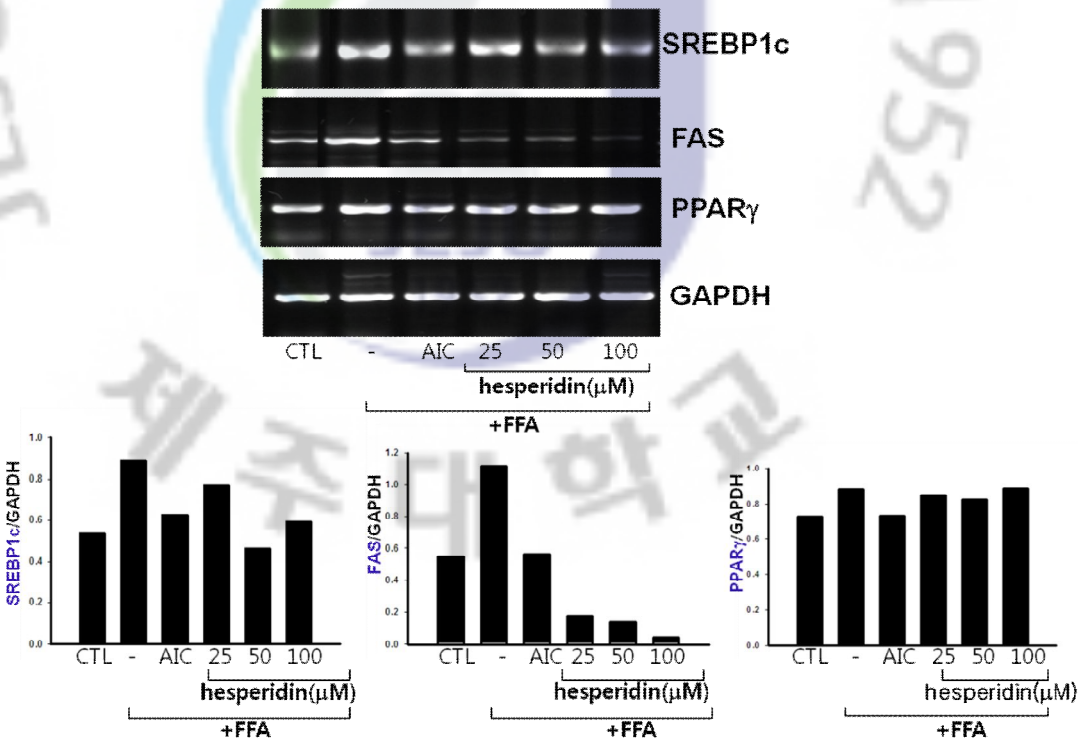


Fig. 30. Effect of hesperidin on AMPK-SREBP1c mRNA levels in HepG$_2$ cells Cells were serum-starved for 12 h and treated with AICAR (1 mM) or hesperidin (25-100 μM) for 30 min before FFA (0.1 mM) treatment for an additional 15 h. The mRNA levels of SREBP1c, FAS and GAPDH were detected by RT-PCR. CTL, control; FFA, free fatty acid; AIC, AICAR, AMPK activator.

DISCUSSION

In our study, the active compounds of *citrus sunki* extract ameliorated the TG accumulation by DNL in $HepG_2$ liver cells. DNL with fatty acids in liver is regulated independently by insulin and glucose (Koo *et al*., 2001; Stoeckman and Towle, 2002). In the nucleus, SREBP1c transcriptionally activates all genes required for lipogenesis (Horton *et al*.,. 2002; Horton *et al*., 2003). The role of SREBP1c in the pathogenesis of fatty liver has been explored in different animals' models. SREBP1c levels are elevated in the fatty livers of obese, insulin resistant and hyperinsulinaemic ob/ob mice (Shimomura *et al*., 1999; Shimomura *et al*. 2000). The overexpression of SREBP1c in transgenic mouse livers leads to the development of a classic fatty liver due to increased lipogenesis. (Shimano *et al*., 1997) SREBP1c expression is depressed during fasting but increases markedly when animals are refed a high carbohydrate diet. Data from adipocytes hepatocytes and from human subjects showed that the transcription of SREBP1c is induced by insulin through PI3K dependent pathway (Azzout-Marniche *et al*., 2000; Ducluzeau *et al*., 2001; Fleischmann and Iynedjian, 2000; Sewter *et al*., 2002; Sewter *et al*., 2002). The effect of insulin on SREBP1c transcription is opposed in the liver by glucagon via cAMP. AMPK-activated protein kinase has also been shown to inhibit SREBP1c transcription (Foretz *et al*., 1999; Tomita *et al*., 2005). In our study,

tangeritin, one of PMFs, decreased the FFA-induced TG accumulation in $HepG_2$ cells. Although tangeritin slightly increased the phosphorylation of AMPK, such effect of tangeritin was not blocked by the inhibition of AMPK with compound C, but were blocked by the inhibition of PI3K with wortmannin. Although the blockade by wortmannin is not complete, we believe that the TG-lowering activity of tangeritin is significantly dependent on the PI3K pathway in $HepG_2$ cells. Nobiletin is very similar with tangeritin in their chemical structure. In $HepG_2$ cells, nobiletin decreased the FFA-induced TG accumulation through activation of AMPK, thereby down-regulating SREBP1c, at both of protein and mRNA levels. Thus, nobiletin should be dependent on AMPK activity to down-regulate SREBP1c and the subsequent blockade of TG accumulation. In addition, the TG-lowering activity of other active compounds synephrine, rutin and hesperidin share common properties with that of nobiletin, in terms of the dependence on AMPK and SREBP1c in $HepG_2$ cells.

Notably, high concentration (100 μM) rutin decreased PPARγ protein levels and the mRNA levels that were increased by FFA induction. PPARγ plays a role in increasing insulin sensitivity as well as in promoting fatty acid uptake into adipocytes and adipocytes differentiation. However, in animal models with insulin resistance and fatty livers, the expression of PPARγ is markedly increased (Chao *et al*., 2000; Edvardsson *et al*., 1999). The importance of PPARγ expression in the development of liver-specific gene deletions of PPARγ in two different insulin-resistant mouse models,

the ob/ob mouse and the lipodystrophic transgenic mouse, named AZIP/F-1, AZIP/F-1 mice are insulin resistant due to a near absence of white adipose tissue and leptin deficiency (Chao *et al*., 2000). The genetic deletion of hepatic PPARγ in livers of either ob/ob (Matsusue *et al*., 2003) or AZIP/F-1 (Gavrilova *et al*., 2003) mice markedly attenuates the development of hepatic steatosis, independently of the presence of hyperinsulinemia or hyperglycemia. Previous studies have demonstrated that SREBP1c can transcriptionally activate PPARγ and it has been suggested that SREBP1c may activate PPARγ by stimulating production of an activating ligand for the nuclear receptor (Kim *et al.*, 1998). Although all compounds except of rutin decreased the proteolytic cleavage of matured SREBP1c protein and mRNA levels of SREBP1c, they didn't reduce the expression of PPARγ protein and mRNA levels. Recently, one study showed that with high-fat diet, PPARγ2 knockdown C57BL/6J mice reduced mRNAs of lipogenic genes (fatty acid synthase, steraroyl-CoA desaturase1, acyetyl-CoA carboxylase1) without alteration of SREBP-1c mRNA (Yamazaki *et al*. 2010). Thus, we suggest that rutin does not transcriptionally activate PPARγ by SREBP1c. Rutin might activate PPARγ by other unknown mechanisms to modulate the lipogenic genes such as FAS.

Previous studies have shown an inverse correlation between AMPK and SREBP1c activities in hepatocytes and in livers of refed mice and ethanol-fed mice (Foretz *et al*., 2005; You *et al*., 2004; Young, 2009; Zhou *et al*., 2001). However, it is largely

unknown how AMPK regulates SREBP activity to control lipid homeostasis. Recently, a study showed that attenuation of ER stress by molecular chaperones in livers of ob/ob mice slowed down lipogenesis by inhibiting SREBP1c proteolytic cleavage and thus improved steatosis and insulin sensitivity of these animals (Kammoun *et al*., 2009a). Moreover, activation of AMPK by AICAR inhibits palmitate-induced endoplasmic reticulum (ER) stress (Cacicedo *et al*., 2008). Thus, AMPK might inhibit the proteolytic cleavage of SREBP1c by attenuation of ER stress. Other study showed that AMPK inhibits cleavage and transcriptional activation of SREBP via direct phosphorylation. SREBP1c and SREBP2, but not SREBP1a, are characterized as conserved substrates of AMPK. AMPK is sufficient and necessary for the suppression of SREBP1c proteolytic processing, nuclear translocation, and gene expression of target lipogenic enzymes in response to AMPK activators, such as metformin, in primary hepatocytes or in $HepG_2$ cells under conditions mimicking *in vivo* hyperglycemia and hyperinsulinemia. The AMPKα subunits strongly associate with and highly phosphorylates the precursor and nuclear forms of SREBP1c or SREBP2. SREBP1c Ser372 phosphorylation is required for AMPK activators to inhibit SREBP1c cleavage and prevent SREBP1c gene autoregulation in a SRE-dependent manner (Li *et al*., 2011).

Recent evidence indicates that, in the face of increased fatty acid delivery to hepatocytes, hepatic DNL is increased in NAFLD as the result of relatively preserved sensitivity of the DNL pathways to insulin and overexpression of SREBP1c as a result

of hyperinsulinemia (Chen *et al*., 2004). ER stress is an additional potential actor explaining a high lipogenic rate in obese insulin-resistance rodents (Ferré and Foufelle, 2010). Saturated fatty acids such as palmitate are excellent activators of an ER stress in several cellular models including hepatocytes (Wei *et al*., 2006). Using cultured primary rodent hepatocytes, some data showed that ER stress rapidly induces cleavage of the precursor of SREBP1c and expression of SREBP1c target genes independent of insulin (Kammoun *et al*., 2009b).

NAFLD is deeply associated with insulin resistance or other pathological disorders like as metabolic syndrome. However, the strategy to prevent or to treat NAFLD is limited. Because the liver is multifunctional, detoxifying organ, the health of liver is essential for the maintenance of body homeostasis. Any drugs should not be harmful to liver cells. In this context, some natural products are good pharmacological candidates in the liver because of the safety and minimal toxicity

Plants of the genus *citrus* have traditional medicinal value. Although a number of previous studies have investigated their pharmacological activities of extracts isolated from the peel of citrus fruits, little is known about pharmacologically active compounds and related action mechanism of each pathological condition. The present study aimed to investigate the effect of extract isolated from the peel of *citrus sunki* hort.ex Tanaka, one of Jeju-native citrus fruits, on the hepatic lipid metabolim. *In vivo* studies using high fat diet rats also found that the extract of *citrus sunki* peel can suppress the

increased levels of a number of pathological indicators including AST, ALT, γ-GT, triglyceride within plasma and the degree of hepatic fat accumulation that were induced by high fat diet. We characterized five major compounds rich in the extract. All five compounds suppressed FFA-induced accumulation of TG in the cytoplasm of $HepG_2$ cells. The hypolipidemic activity of four compounds except tangeritin was sensitive to the inhibition of AMPK. They also decreased protein levels as well as mRNA levels of SREBP1c (a mediator of DNL) and stimulated the phosphorylation of AMPK, however, decreased the mRNA levels of FAS, one of SREBP1c-regulated target gene product. Hypolipidemic activity of tangeritin was sensitive to the inhibition of PI3-kinase in $HepG_2$ cells. Although tangeritin marginally stimulated the phosphorylation of AMPK, the inhibition of AMPK failed to suppress FFA-induced TG accumulation. Tangeritin also decreased protein levels as well as mRNA levels of SREBP1c that were increased by FFA.

Taken together, 5 major compounds were totally active to suppress FFA-induced TG accumulation in the cytoplasm of $HepG_2$ liver cells. However, action mechanism of each compound is different in part, although it shares a few signaling protein and transcription factor(s) like as AMPK and SREBP1c, respectively. The peel of *citrus sunki per se.* and leading compounds within the peel have a good pharmacological value to prevent or treat hepatic steatosis and more progressed liver disease like as NASH.

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