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Effects of Piperine on Insulin Resistance and Lipid Accumulation in Palmitate-treated HepG2 Cells

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Hepatic lipid accumulation and insulin resistance increases in patients with non-alcoholic fatty liver disease. Piperine is a major compound found in black pepper (*Piper nigrum*) and long pepper (*P. longum*). Piperine has been used in fine chemical for its anti-cancer, anti-obesity, anti-diabetic, anti-inflammatory and anti-oxidant properties. However, the signaling-based mechanism of piperine and its role as an inhibitor of lipogenesis and insulin resistance in human hepatocyte cells remains ill-defined. In the present study, we explored the effects of piperine on lipid accumulation and insulin resistance, and explored the potential underlying molecular mechanisms in palmitate-treated HepG2 cells. Piperine treatment resulted in a significant reduction of triglyceride content. Furthermore, piperine treatment decreased palmitate-treated intracellular lipid deposition by inhibiting the lipogenic target genes, sterol-regulatory-element-binding protein 1c (SREBP-1c) and fatty acid synthase (FAS); whereas the expression of carnitine palmitoyl transferase (CPT-1) and phosphorylation of acetyl coenzyme A carboxylase (ACC) gene involved in fatty acid oxidation was increased. Moreover, piperine also inhibited the phosphorylation of insulin receptor substrate (IRS)-1 (Ser307). Piperine treatment modulated palmitate-treated lipid accumulation and insulin resistance in HepG2 cells with concomitant reduction of lipogenic target genes, such as SREBP-1 and FAS, and induction of CPT-1-ACC and phosphorylation of IRS-1 (Tyr632)-Akt pathways. Therefore, piperine represents a promising treatment for the prevention of lipid accumulation and insulin resistance.

Key words : Insulin resistance, lipid accumulation, palmitate, piperine

Introduction

Obesity and associated conditions, such as type 2 diabetes, coronary heart disease, and nonalcoholic fatty liver disease (NAFLD) are presently a global health problem [6, 28]. Hepatic steatosis is a metabolic disorder that is an early form of NAFLD, and is defined by an excessive amount of hepatic lipid accumulation. Palmitate (C16:0), are the most plentiful free fatty acids in liver triglycerides of patients with NAFLD, that express excess accumulation of cytosolic lipid and activate lipogenic genes in the liver tissue [17]. Palmitate also can affect insulin resistance in insulin target tissues, both *in vitro* and *in vivo* [15, 22, 34, 44]. Treatment with palmitate and lipogenic transcription factors; such as sterol-regulatory-element-binding protein 1c (SREBP-1c), plays a role in lipid accumulation by upregulating many lipogenic genes. This lipogenic gene expression is related to fatty acid synthase (FAS) activity and leads to triglyceride synthesis [22]. In addition, acetyl coenzyme A carboxylase (ACC) inactivation suppresses malonyl-CoA synthesis and, depresses carnitine palmitoyl transferase (CPT-1) and hyper-activates fatty acid oxidation simultaneously [27]. Insulin signaling is initiated by insulin that interacts and activates the insulin receptor (IR), inducing auto-phosphorylation of IR's core tyrosine residues [35]. Moreover, tyrosine residues of the insulin receptor substrate (IRS) are subsequently phosphorylated by the IR kinase, which activates downstream of the PI3K-Akt signaling pathway transduction.

Piperine (1-piperoylpiperidine) (Fig. 1A), one of active alkaloid constituents in black pepper (*Piper nigrum*), long pepper (*P. longum*), and other Piper species (family: Piperaceae), is used as an effective remedy for gonorrhea, tuberculosis, menstrual pain, sleeping problems, respiratory-tract infections, chronic gut-related pain and several arthritic conditions [38]. Piperine affects various biological activities, in-

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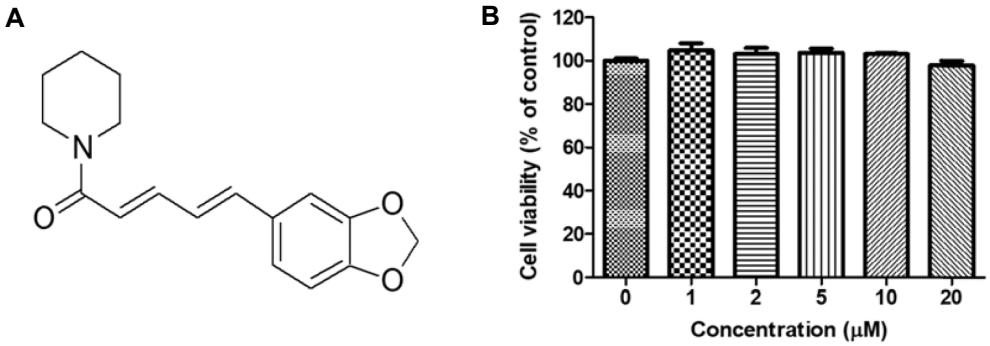

Fig. 1. Chemical structure of piperine (A) and cell viability of piperine on HepG2 cells (B). Cells (1×10^4 cells/well) were preincubated using various concentrations (up to 20 μM) of piperine for 24 hr. Cell viability was determined using the EZ-Cytox assay and expressed as the percentage of absorbance values relative to the control group. Data shown represents mean ± S.E.M. of triplicate experiments.

cluding anti-cancer [26, 36], anti-angiogenesis [14], anti-oxidant [29], anti-diabetic [3, 4], improved metabolic syndrome [13], and anti-inflammatory effects [39]. Previous research on piperine investigated the effects of hepatic steatosis and insulin resistance on high-fat diet-induced mice [23]. In spite of many previous studies, the underlying molecular mechanism by which piperine inhibits palmitate-treated lipid accumulation and insulin resistance has currently not been. Therefore, we explored the molecular mechanism of piperine on lipid accumulation and insulin resistance in palmitate-treated HepG2 cells.

Materials and Methods

Materials

Piperine, sodium palmitate and Oil red O staining solution were obtained from Sigma Aldrich, St. Louis, USA. PVDF membrane was obtained from Millipore Corp. (Billelica, MA, Germany) and the enhanced chemiluminescence detection system was obtained from Amersham Life Sciences, Inc. (Buckinghamshire, UK). Antibodies targeted toward SREBP-1c, FAS, p-ACC (Ser79), CPT-1, p-IRS (Ser307), p-IRS (Try632), IRS-1, p-Akt (Ser473), Akt, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Serum triglyceride content was determined enzymatically using commercial kits (Bio-Clinical System, Gyeonggi-do, Korea). All other chemicals were of the highest purity available from Junsei Chemical Co. (Tokyo, Japan).

Cell culture and treatments

Human HepG2 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Media (Welgene, Daegu, Korea) accompanied with 10% heat-inactivated fetal bovine serum, 100 mg/ml penicillin-streptomycin, and 0.25 μg/ml amphotericin B in an atmosphere of 5% CO_2. Piperine was dissolved in 100% DMSO. The final concentration of DMSO did not exceed 0.1%.

Cell viability assay

Cell viability was determined by the EZ-Cytox assay. Briefly, HepG2 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well, and incubated 37℃ for 24 hr. Media was replaced with fresh Dulbecco's Modified Eagle Media containing piperine (up to 20 μM) and incubated for 24 hr. After incubation, 10 μl of EZ-Cytox solution was added to each well, and cells were incubated for an additional 2-4 hr. The absorbance of each well was measured at 450 nm using the ELISA reader (TECAN, Salzburg, Austria). The percent inhibition due to piperine was obtained according to the formula: inhibition (%) = [(OD (sample) - OD (control)) / (OD (normal) - OD (control))] ×100. All assays were performed in triplicate and then averaged.

Preparation and treatment with sodium palmitate in HepG2 cells

Palmitate was conjugated with fatty acid-free BSA (Gen DEPOT, Barker, TX, USA) using a previously reported method [32]. Briefly, 250 mM stock solution was made by alternated heating and vortexing 69.6 mg of sodium palmitate dissolved in 1 ml of 0.1 N sodium hydroxide at 70℃. After dissolution of palmitate, the stock solution was immediately added to serum-free DMEM (containing 5% fatty acid-free BSA), to obtain 0.5 mM palmitate solution. The cells were

pre-treated for 24 hr with various concentrations of piperine (5 or 20 μM) prior their exposure to 500 μM palmitate for 24 hr. Cells cultured in a 5% BSA medium were used as the control.

Oil red O staining

HepG2 cells grown in 6-well plates were collected, washed with cold PBS and fixed with 4% paraformaldehyde for 10 min. Cells were soaked in 60% isopropanol for 5 min, stained with Oil red O solution for 1 hr, and rinsed with ddH_2O several times to remove excess stain. Pictures were taken using a microscope (Motic, CA, USA).

Measurements of intracellular triglyceride content

Intracellular triglyceride content were measured using enzymatic colorimetric assay kits after lysis of the HepG2 cells with 1% Triton X-100 in PBS. Protein concentrations were determined using the BCA method with BSA as the standard. Intracellular triglyceride levels were normalized to determine cellular protein content.

Western blotting

Western blotting was performed as described previously [19]. The cells were harvested, washed twice with ice-cold PBS and lysed in buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40 supplemented with protease and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride) for 1 hr on ice, vortexing every 5 min. Lysates were centrifuged at 12,000 rpm for 10 min to remove insoluble materials. Equal amounts of protein were separated on 8-10% SDS-PAGE gels. The separated proteins were subsequently transferred onto PVDF membranes via electro-blotting for 2 hr at 80 V. The membranes were blocked in a 5% non-fat milk solution in TBS with 0.5% Tween-20; and incubated with primary antibodies overnight at 4℃. The membranes were washed and incubated for 2 hr at room temperature with HRP-linked secondary antibodies. Pre-stained blue protein markers (Bio-Rad, Hercules, CA, USA) were used for molecular weight determination.

Statistical analysis

Statistical significance was analyzed using the one-way analysis of variance to determine differences within treatments followed by the Bonferroni test (GraphPad Prism 5 software, La Jolla, CA, USA) and was noted at $^{*}p<0.05$ was considered statistically significant. All experiments were carried out in triplicate, repeated on 3 individual days, and expressed as the mean ± S.E.M. (n=3).

Results

Piperine attenuated intracellular triglyceride contents in palmitate-treated HepG2 cells

To evaluate the cytotoxic effects of piperine on HepG2 cells, the cells were treated with several concentrations of piperine (0-20 μM) for 24 hr. Cell viability was not affected up to 20 μM after 24 hr, indicating that the tested concentrations of piperine did not affect cell growth and viability (Fig. 1B). Therefore, subsequent experiments were conducted using up to 20 μM piperine. To examine whether piperine inhibited intracellular lipid content in palmitate-treated HepG2 cells, Oil red O staining and an intracellular triglyceride content assay were performed. The cells were incubated in 500 μM palmitate and several concentrations of piperine for 24 hr. As shown in Fig. 2, palmitate-treated HepG2 cells presented higher Oil red O staining than control cells (Fig. 2A), indicating higher triglyceride content (Fig. 2B). These results indicated that piperine treatment may suppress palmitate-treated triglyceride content in HepG2 cells.

Piperine down-regulated SREBP-1c and FAS and up-regulated CPT-1 and phosphorylation of ACC in palmitate-treated HepG2 cells

SREBP-1c is an important transcription factor known to regulate the expression of lipid forming enzymes in the hepatic lipogenic pathway [37]; and plays a crucial role in the pathogenesis of NAFLD [2]. In fatty acid synthesis, acetyl-CoA is the initiator and is involved in the addition of two carbon units that elongate the fatty acid chain. ACC mediates the conversion of acetyl-CoA to malonyl-CoA; which is an essential rate-limiting step in lipogenesis [8]. FAS utilized acetyl-CoA, malonyl-CoA, and NADPH, and lengthens the fatty acid chains to generate palmitate [21]. Therefore, we investigated the effects of piperine on several lipogenic genes, including SREBP-1c, CPT-1, FAS, and ACC, and their resulting effects on lipogenesis and lipolysis in HepG2 cells using western blotting. As shown in Fig. 3A, palmitate enhanced the expression of SREBP-1c and FAS; however, the expression of SREBP-1c and FAS was significantly decreased in the palmitate-treated HepG2 cells in a dose-dependent

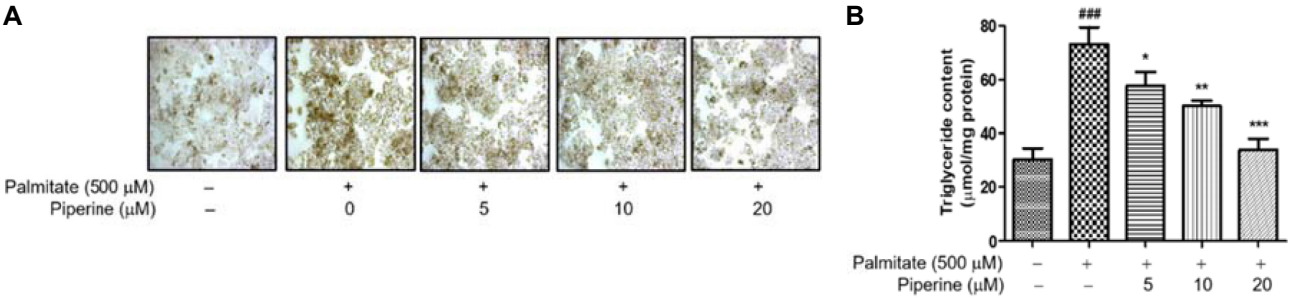

Fig. 2. Effects of piperine on palmitate-treated lipid content. HepG2 cells were left untreated or treated with piperine for 24 hr. Then, cells were used for Oil red O staining (A) and intracellular triglyceride assay (B). Values are mean ± S.E.M. of three experiments; $^{\#\#\#}p<0.001$ versus that of untreated cells; $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ versus that of the palmitate-treated cells.

manner upon piperine treatment (Fig. 3B, Fig. 3C).

Moreover, we observed the phosphorylation of ACC (Ser79) in the palmitate-treated HepG2 cells were down-regulated compared into control group (Fig. 3E). On the other hand, the expression of total ACC increased in the palmitate treatment. Next, we scrutinized whether piperine promoted fatty acid degradation via β-oxidation. We found that palmitate notably decreased the protein level of CPT-1 in HepG2 cells, while the protein level increase of CPT-1 was dose-dependent on piperine levels (Fig. 3C). Our results indicate that piperine activates the expression of CPT-1 and ACC phosphorylation, and selectively regulates the expression of lipid metabolism-related proteins. Therefore, the regulation of lipid regulatory proteins may facilitate the inhibitory effects of piperine on lipid accumulation.

Piperine improved palmitate-treated insulin resistance through up-regulated p-IRS-1/p-Akt signaling

To elucidate the prospective mechanism underlying the piperine ameliorating insulin resistance, the key proteins involved in insulin signaling pathway in the palmitate-treated HepG2 cells was investigated (Fig. 4). We first examined the changes in the phosphorylation of IRS-1 in palmitate-treated HepG2 cells to assess insulin resistance. IRS-1 was either stimulated or inhibited by the phosphorylation of specific tyrosine or serine resides [11]. Therefore, we confirmed that

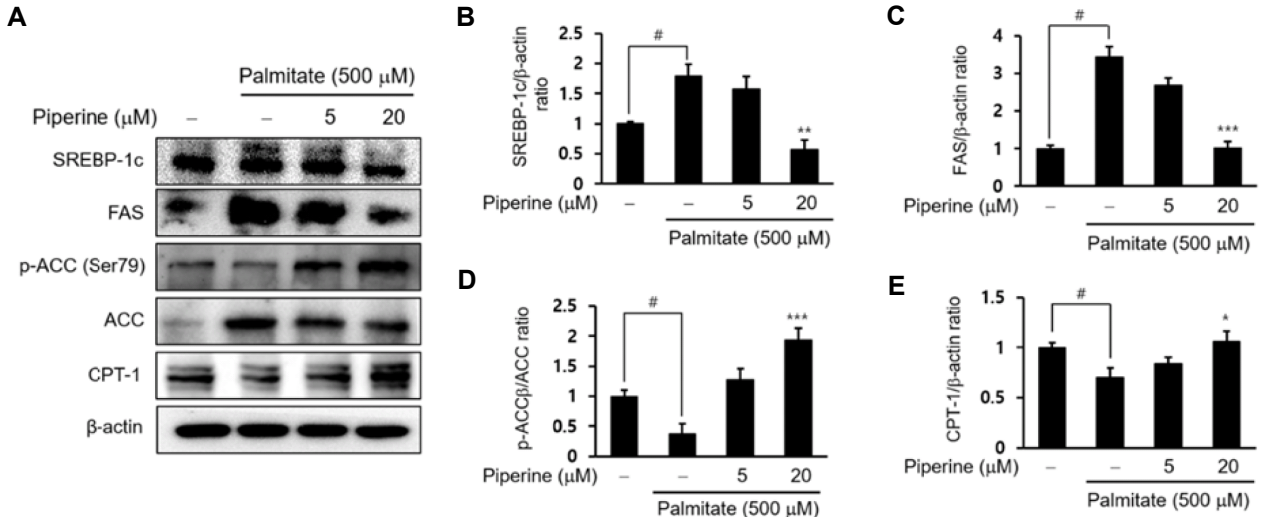

Fig. 3. Piperine suppressed lipid accumulation while enhancing fatty acid oxidation in palmitate-treated HepG2 cells. HepG2 cells were treated with piperine (5 or 20 μM) in serum free DMEM for 2 hr and changed to palmitate (500 μM) conjugated with fatty acid free-BSA for 24 hr. (A) Western blot was performed to detect SREBP-1c, FAS, p-ACC (Ser79), ACC, and CPT-1 protein levels. Levels were normalized to β-actin. (B-E) The protein levels of SREBP-1c, FAS, p-ACC (Ser79), ACC, and CPT-1 were quantified using CS analyzer software. A representation of three experiments that yielded similar results. One-factor ANOVA: $^{\#}p<0.05$ versus vehicle treated controls; $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ versus palmitate-treated cells. Bars indicate standard errors of means (S.E.M.).

palmitate induced IRS-1 phosphorylation at the Ser307 residue; an inhibition site for insulin signaling and deactivated IRS-1 phosphorylation at the Tyr632; a stimulation site for insulin signaling. As shown in Fig. 4B and 4C, IRS-1 (Ser307) phosphorylation via piperine treatment was attenuated, while IRS-1 (Tyr632) phosphorylation increased using the two concentrations of piperine treatment. In order to further confirm amelioration of insulin resistance in these cells, we examined the protein's expressions associated with insulin signaling Akt. As shown in Fig. 4D, the phosphorylation of Akt (Ser473) decreased in palmitate-treated HepG2 cell, but piperine treatment significantly alleviated the levels of Akt phosphorylation. Our findings demonstrate that piperine could increase the phosphorylated Akt and IRS-1 (Tyr632) phosphorylation, and improve insulin signaling in palmitate-treated HepG2 cells. Our results suggest that piperine is a modulator of palmitate-treated lipid accumulation and insulin resistance. We did not observe differences in the total amount of IRS and Akt protein (Fig. 4).

Discussion

Here, we demonstrated that piperine treatment alleviated lipid accumulation and insulin resistance induced by palmitate-treated HepG2 cells. As an active alkaloidal phenolic component of black pepper and long pepper [38], piperine can exert an antidiabetic effect and improve insulin resistance based on animal experiments with rats and mice [3, 10, 23, 33]. In addition, palmitate is the most common fatty acid in mammals. It composes 30-35% of the total free fatty acid in human plasma and directly attenuates insulin signaling in cultured hepatocytes [20, 42]. Our study revealed that palmitate could induce lipid accumulation (Fig. 2). This is consistent with previous reports indicating that palmitate can induce lipid accumulation in a variety of cell types including, cardiomyocytes, NIT-1 pancreatic β-cells, human adipose-derived stem cells, C2C12 myotubes, and L6 fibroblasts [1, 5, 26, 40, 42]. We investigated the inhibitory mechanism of piperine against hepatic lipid accumulation through improved insulin resistance using palmitate-treated HepG2 cells. Oil red O stains are well-known markers to detect intracellular lipid accumulation [12]. Consequently, our study clarified that piperine treatment suppresses palmitate-treated lipid accumulation and concomitantly enhances insulin signaling (Fig. 3, Fig. 4). Our results suggest that treatment with piperine plays a beneficial role in preventing intracellular lipid accumulation in palmitate-treated HepG2 cells.

SREBP-1c is a transcription factor that plays a role in the biosynthesis of cholesterol, fatty acid, and triglyceride [16, 18]. SREBP-1c functions through key regulators (FAS and ACC) involved in fatty acid biosynthesis that mediate fatty acid biosynthesis [24]. ACC is the critical rate-limiting en-

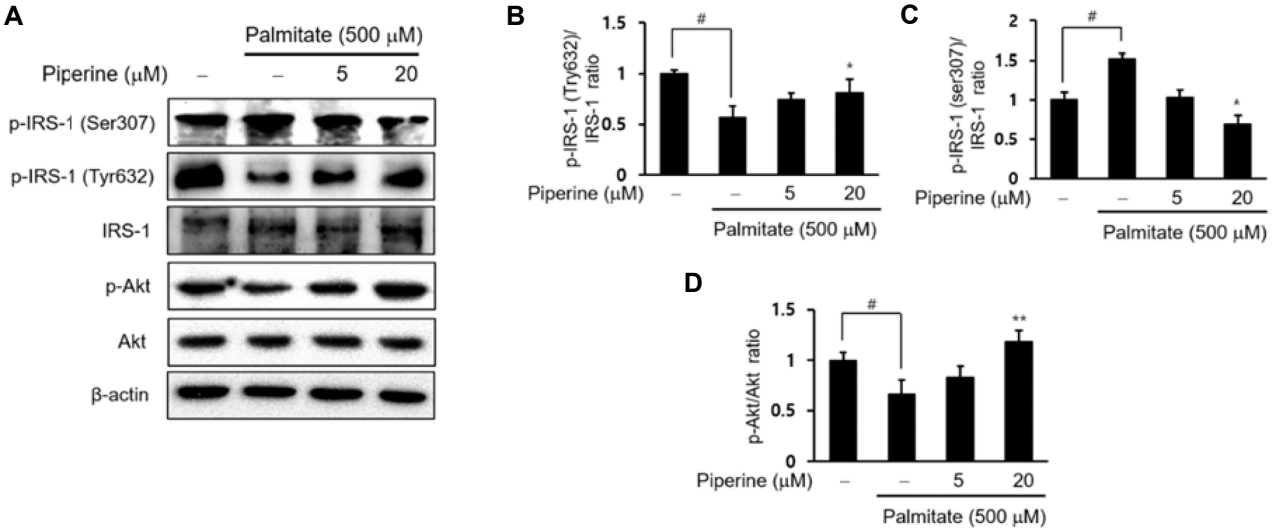

Fig. 4. Piperine attenuated palmitate-treated insulin resistance in HepG2 cells. HepG2 cells were treated with piperine (5 or 20 μM) in serum free DMEM for 2 hr and palmitate (500 μM) conjugated with fatty acid free-BSA for 24 hr. (A) Western blot was performed to detect p-IRS-1 (Ser307), p-IRS-1 (Tyr632), IRS-1, p-Akt, and Akt protein levels. Levels were normalized to β-actin. (B-D) The protein levels of p-IRS-1 (Ser307), p-IRS-1 (Tyr632), IRS-1, p-Akt, and Akt were quantified using CS analyzer software. A representation of three experiments that yielded similar results. One-factor ANOVA: $^{\#}p<0.05$ versus vehicle treated controls; $^{*}p<0.05$ and $^{**}p<0.01$ versus palmitate-treated cells. Bars indicate standard errors of means (S.E.M.).

zyme for malonyl-CoA synthesis that acts as a key substrate for fatty acid biosynthesis and is a potent inhibitor of fatty acid oxidation [45]. FAS is a major lipogenic enzyme in mammals; its concentration is carefully regulated by the nutritional and hormonal status of lipogenic tissues, such as adipose tissues and liver [7].

We examined the potential role of SREBP-1c in the lipid accumulation reduction of piperine. We found that piperine suppressed the expression of SREBP-1c, FAS, and ACC in palmitate-treated HepG2 cells. Moreover, upregulation of SREBP-1c significantly suppressed the alleviative effect of piperine against palmitate-mediated cellular insulin resistance and lipid accumulation in hepatocytes *in vitro*. Our results propose that SREBP-1c and its downstream target genes (including FAS and ACC) contributed to piperine-exhibited suppression of lipid accumulation and insulin resistance (Fig. 3). CPT-1 is an important rate-limiting enzyme that is involved in fatty acid oxidation [41], which can increase the ability of fatty acids to enter the mitochondria. Thus, it is reasonable to speculate that piperine alleviates lipid metabolism disorders by activating the ACC phosphorylation (Ser79)-CPT-1 pathway.

Palmitate-treated insulin resistance has been reported in other insulin sensitive cells, such as 3T3-L1 adipocytes [31] and skeletal muscle cells [9]. Together with these results, our results suggest that palmitate inhibits insulin signal transduction at the level of phosphorylation of IRS (Tyr632) and Akt, regardless of cell type. Our results support *in vitro* findings that palmitate triggers insulin resistance in hepatocytes, in which phosphorylation of IRS (Tyr632) and Akt decreases [30, 43].

In summary, piperine treatment is beneficial in reducing lipid contents in palmitate-treated HepG2 cells. Moreover, piperine affects the lipid metabolic regulatory system by activating the expression of SREBP-1c, FAS, and ACC and changing the activation of the phosphorylation of ACC (Ser79) and CPT-1; an enzyme involved in lipid metabolism regulation that promotes catabolism of fuel storage. In addition, we found that piperine treatment restored the levels of IRS (Tyr632) and Akt phosphorylation that were reduced by palmitate. The decline of hepatic lipid accumulation and insulin resistance by piperine might also contribute to its efficacy in suppressing hepatic metabolic disease (Fig. 5). This study helps to understand the mechanisms of insulin resistance, and provides a new therapeutic target for the treatment of obesity and metabolic disorders. Further research should focus on animal experimentation to confirm the metabolism-modulation activity of piperine *in vivo*.

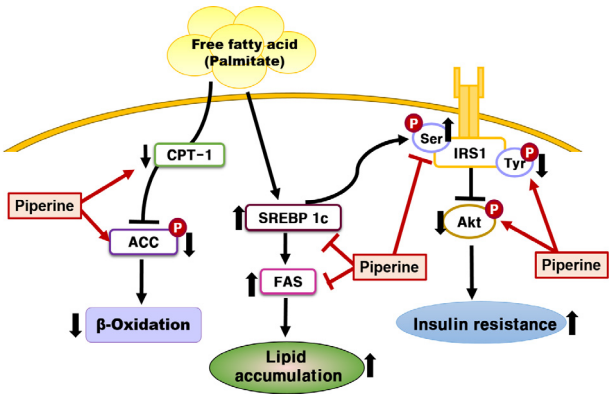

Fig. 5. Possible mechanism against lipid accumulation and insulin resistance of piperine.

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초록 : Palmitate처리된 인간 간세포주 HepG2 세포에서 piperine의 지질 축적과 인슐린 저항성 기전에 대한 연구

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간의 지질 축적과 인슐린 저항성은 비알콜성 지방간 환자에게서 증가한다. Piperine은 후추(*Piper nigrum*)와 필발(인도산 후추, *P. longum*)의 주요 성분으로 항암, 항비만, 항 당뇨병, 항염증 및 항산화 등의 생리활성이 보고되었다. 그러나 piperine의 인간 간세포 HepG2 세포에서 지질 축적과 인슐린 저항성의 억제제로서의 연구는 보고된 바가 없다. 본 연구의 목적은 지질 축적 및 인슐린 저항성에 대한 piperine의 효과를 palmitate처리된 HepG2 세포에서 잠재적인 분자 기전을 밝히는 것이다. 그 결과 piperine처리군은 지질 함량을 감소시켰고, 지방 형성 표적 유전자인 SREBP-1c와 FAS의 발현을 억제함으로써 palmitate처리된 세포내 지질 축적을 감소시켰다. 게다가 piperine처리군은 지방산 산화에 관련된 CPT-1과 인산화된 ACC 및 인산화된 IRS-1 (Tyr632)와 Akt의 레벨을 증가시켰다. 또한, piperine처리군은 인산화된 IRS-1 (Ser307)의 레벨을 감소시켰다. 결론적으로 palmitate처리된 HepG2 세포에서 piperine은 SREBP-1와 FAS발현의 감소 및 CPT-1과 ACC 인산화의 증가 및 인산화된 IRS-1 (Try632)와 Akt 신호전달 경로를 조절함으로써 지질 축적 및 인슐린 저항성을 개선함을 확인하였다. 따라서 piperine의 지질 축적 및 인슐린 저항성을 예방하는 약물로써 가능성이 제시되었다.