Research Paper

Piceatannol attenuates fat accumulation and oxidative stress in steatosis-induced HepG2 cells

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD), which affects over 20% of the adult population, is the most common liver disease worldwide and can progress to inflammatory hepatitis, cirrhosis and liver cancer. The need to alleviate NAFLD is imperative, but there are limited pharmacological therapies available. Based on previous reports that piceatannol, a stilbenoid metabolite of resveratrol, exhibits anti-obesity, antioxidant and anti-inflammatory effects, the goal of this study was to determine the efficacy of piceatannol on prevention and/or treatment of NAFLD. The results showed that piceatannol significantly decreased fat accumulation and suppressed lipogenesis and fatty acids (FAs) uptake by decreasing sterol regulatory element-binding protein 1 (SREBP1) and cluster of differentiation 36 (CD36) in steatosis-induced HepG2 hepatocytes. Piceatannol treatment also promoted FAs β-oxidation by increasing farnesoid X receptor (FXR), peroxisome proliferator-activated receptor α (PPARα), and carnitine palmitoyltransferase 1α (CPT1α) under steatosis conditions. Moreover, piceatannol significantly suppressed FA-induced oxidative stress and inhibited phosphorylation of c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinases 1/2 (ERK1/2). Overall, it is suggested that piceatannol reduced fat accumulation in steatosis-induced HepG2 cells by suppressing lipogenesis (SREBP1 and ACC) and FA uptake (CD36), and promoting FAs oxidation (FXR, PPARα and CPT1α).

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide, affecting over 20–25% of the adult population. NAFLD can be benign in early stages, with simple hepatic fat accumulation, and progress to inflammatory hepatitis, irreversible fibrosis, cirrhosis and eventually liver cancer. However, there is no specific pharmacological treatment for NAFLD as of now, leaving diet restriction and weight loss as the most common options for NAFLD treatment (Tilg and Moschen, 2010; Romero-Gómez et al., 2017). There are several plant-originated phytochemicals that have been suggested to have anti-obesity and anti-inflammatory effects that could be considered potential candidates for the treatment of NAFLD.

Hepatic lipogenesis and fatty acid (FA) β-oxidation are two critical pathways determining fat deposits in the liver; these are associated with oxidative stress and the severity of NAFLD (Spahis et al., 2017). Previously, resveratrol has been suggested to improve NAFLD in mice (Shang et al., 2008), along with anti-inflammation (Das and Das, 2007), antioxidant (de la Lastra and Villegas, 2007), anti-cancer (Jang et al., 1997) and anti-obesity effects (Kim et al., 2011). However, a clinical trial has shown that resveratrol may only have minimal effect on improving NAFLD in humans due to its limited bioavailability (Zhang et al., 2016). Piceatannol found in grapes, whites tea, passion fruit and Japanese knotweed (Gehlert et al., 1990) is a hydroxylated stilbene analog and metabolite of resveratrol originally synthesized by the plant as phytoalexin in response to fungi infection. To compare, piceatannol was previously suggested to have higher bioavailability than resveratrol (Lin et al., 2010), while possessing similar bioactivities as a resveratrol analog: anti-inflammatory (Ashikawa et al., 2002), anti-oxidative (Lee et al., 1998), anti-cancer (Ku et al., 2005), anti-aging (Shen et al., 2017a) and anti-obesity effects (Shen et al., 2017b). Thus, the goal of this study was to determine the efficacy of piceatannol on lipid metabolism using HepG2 human hepatoma cells to gain insights into the potential use of piceatannol for prevention and/or treatment of NAFLD.

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2. Materials and methods

2.1. Materials and chemicals

Piceatannol (purity > 98.0%) was purchased from Tokyo Chemical Industry (Portland, OR). HepG2 human hepatoma cells were obtained from American Type Culture Collection (Manassas, VA). Rabbit antibodies for acetyl-CoA carboxylase (ACC), phosphorylated ACC, extracellular signal-regulated kinases 1/2 (ERK1/2), phosphorylated ERK1/2, c-Jun N-terminal kinase (JNK), phosphorylated JNK and peroxisome proliferator-activated receptor alpha (PPARα) were purchased from Cell Signaling Technology (Danvers, MA). Mouse antibodies for peroxisome proliferator-activated receptor gamma (PPARγ), and secondary antibodies of horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were obtained from Santa Cruz Biotechnology (Dallas, TX). Fetal bovine serum and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin was purchased from GE Healthcare (Marlborough, MA). All other chemicals were obtained with either analytical grade or cell-culture grade from Fisher Scientific (Waltham, MA).

2.2. Cell culture and treatment

HepG2 human hepatoma cells were maintained in Dulbecco's modified eagle media (DMEM) with 10% fetal bovine serum, 10,000 U/mL penicillin and 10 mg/mL streptomycin. Cells were kept with 5% CO_2 and 95% air in 37 °C incubator and subcultured at the confluence of 80–90%, or every 72–96 h. Piceatannol was pre-dissolved in dimethyl sulfoxide (DMSO), and a final concentration of 0.1% v/v DMSO was used in all treatment groups.

2.3. In vitro steatosis induction

Fatty acid (FA) was prepared as FA-BSA complex as previously described, with the ratio of oleic acid (OA): palmitic acid (PA) = 2:1 (Cousin et al., 2001). In brief, OA and PA were dissolved in 1M KOH and complexed with 10% BSA pre-dissolved in phosphate-buffered saline (PBS) by stirring overnight at room temperature. The complex was then neutralized to pH 7.2, filtered and adjusted as 20x stock containing 10% BSA (molar ratio of FA: BSA = 8:1). *In vitro* steatosis was induced by 600 μM of FA for 24 or 48 h. The concentration of BSA was controlled at 0.5% v/v in all treatment groups. In oxidative stress-induced triglycerides (TG) accumulation, HepG2 cells were treated with 500 μM of H_2O_2 for 3 h and then kept in DMEM for 24 or 48 h. TG contents were measured by Infinity Triglycerides reagent kit (Thermo Fisher Scientific, Waltham, MA, US) according to the manufacturer's instructions. Protein contents were measured by Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA).

2.4. Cell viabilities

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (Cousin et al., 2001). HepG2 cells were treated with different concentrations of piceatannol for 24 h and were then subject to MTT assay. The relative formation of formazan from MTT was measured by absorbance at 570 nm (SpectraMax i3, Molecular Devices LLC, San Jose, CA). The results showed that up to 600 μM of FA combined with up to 100 μM of piceatannol did not affect cell viabilities compared with the control (Sup. Fig. S1). Thus, 600 μM of FA and 100 μM of piceatannol were used for further experiments.

2.5. Immunoblotting

Immunoblotting to determine protein expression was performed with modification as previously described (Kim et al., 2014). Whole cell proteins were extracted and subject to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (10% acrylamide) for separation. Proteins were then blotted on polyvinylidene fluoride (PVDF) membrane, where target proteins are detected by corresponding primary antibodies. The protein-antibody complexes were visualized by applying HRP-conjugated secondary antibodies and enhanced chemiluminescence substrate and exposed by Image Station 4000 MM (Kodak, Rochester, NY). Protein expressions were then quantified by ImageJ (NIH, Bethesda, MD).

2.6. Real Time-PCR assay

Expressions of mRNA were performed by real-time polymerase chain reaction (RT-PCR) as previously described (Kim et al., 2014). TRIzol Reagent kit was used to extract cellular mRNA (Thermo Fisher Scientific, Waltham, MA) according to the suggested protocol. Extracted mRNA was then converted into cDNA with a reverse-transcription kit (Applied Biosystems, Foster City, CA) and stored at −20 °C before use. Taqman assay primers including 18s RNA housekeeping gene (catalog No. 4319413E) for control, acetyl-CoA carboxylase (ACC, Hs01046047_m1), cluster of differentiation 36 (CD36, Hs00354519_m1), carnitine palmitoyl-transferase 1α (CPT1α, Hs00912671_m1), fatty acid synthase (FAS, Hs00236330_m1), farnesoid X receptor (FXR, Hs01026590_m1), peroxisome proliferator-activated receptor α (PPARα, Hs00947536_m1) and sterol regulatory element-binding protein 1 (SREBP1, Hs01088679_g1) were purchased from Applied Biosystems (Foster City, CA).

In preliminary experiments, FA treatment suppressed the mRNA expression of SREBP1 (36% reduction with $P < 0.0001$ compared with the control) and promoted mRNA expression CPT1α (42% increase with $P = 0.0186$ compared with the control), without effects on other lipogenesis (FAS and ACC), FA oxidation (FXR and PPARα), or FA uptake marker (CD36) tested (Sup. Fig. S2). This finding is consistent with previous studies reporting that unsaturated FA, such as OA, may inhibit the transcription of SREBP1 (Ou et al., 2001; Chen et al., 2018). The treatment with piceatannol alone decreased CD36 (61% decrease with $P = 0.0113$ compared with the control), but did not influence other lipid metabolism markers tested (Sup. Fig. S3).

2.7. Measurement of intracellular reactive oxygen species (ROS)

HepG2 cells were subjected to fluorescence dye 2′,7′-dichloro-fluorescin diacetate (DCFDA) assay as described (Lebel et al., 1992) with modifications to measure intracellular ROS. DCFDA stock was prepared in DMSO at 50 mM and freshly diluted in 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES) buffer (20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 13.8 mmol/L glucose, pH 7.4) before use. During measurement, cells were pre-incubated with 25 μM DCFDA for 45 min at 37 °C, which was replaced by FA and piceatannol treatment and continued to incubate at 37 °C. ROS level was determined by measuring fluorescence at excitation 485 nm and emission 535 nm at 1, 2, 3, 4, 6, 12, and 24 h after treatment (SpectraMax i3, Molecular Devices LLC, San Jose, CA).

2.8. Statistical analysis

Data were obtained as quadruplets or octuplicates as biological replicates from an experiment and were analyzed by Student's t-test or one-way ANOVA followed by Tukey's multiple comparison test using Graphpad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). The *P*-value of less than 0.05 was considered statistically significant between groups.

3. Results

3.1. Piceatannol reduced FA-induced TG accumulation in HepG2 cells

The increased fat deposit in the hepatocyte is one of the classic symptoms of NAFLD, and the levels of hepatic fat accumulation also

correlate with the severity of the disease (Liu et al., 2010). 600 μM of FA treatment successfully induced TG accumulation (383% increase at 12 h and 580% increase at 24 h with both $P < 0.0001$ compared with the respective controls, Sup. Fig. S4), while 100 μM of piceatannol decreased TG accumulation (21% decrease at 12h and 20% decrease at 24h) in non-steatosis HepG2 cells (Sup. Fig. S5). However, pre-treatment of piceatannol for 24h did not have any effects on FA-induced TG accumulation in HepG2 cells (Sup. Fig. S6). Co-treatment of piceatannol (100 μM) and FA (600 μM) significantly reduced TG accumulation after 12 and 24 h of treatment (21% reduction with $P = 0.0007$ at 12 h and 19% reduction with $P = 0.0012$ at 24 h compared with FA, Fig. 1). Thus, co-treatments of piceatannol and FA were used for the following experiments.

3.2. Effects of piceatannol on lipogenesis and FA uptake in steatosis-induced HepG2 hepatocytes

In NAFLD, accumulative intracellular TG in the hepatocytes can result from lipogenesis. Acetyl-CoA carboxylase (ACC) is one of the rate-limiting steps in lipogenesis (Bechmann et al., 2012), and the activity of ACC can be regulated post-transcriptionally (Kim et al., 1989). In steatosis-induced HepG2 hepatocytes, 100 μM of piceatannol treatment increased the level of phosphorylated ACC (inactive form, 67% increase compared with FA, Fig. 2A and D) without influencing the expression of ACC (active form, Fig. 2B and D), resulting in an increased ratio of p-ACC/ACC (109% increase with $P = 0.0003$ compared with FA, Fig. 2C). These findings suggest that piceatannol suppressed the FA-induced ACC activity in steatosis-induced HepG2 hepatocytes.

Sterol regulatory element-binding protein 1 (SREBP1) regulates lipogenesis by promoting ACC and fatty acid synthase (FAS) (Shimano et al., 1999). In steatosis-induced HepG2 hepatocytes, treatment of piceatannol (100 μM) suppressed the mRNA expression of SREBP1 (27% reduction with $P = 0.0055$ compared with FA, Fig. 3A) and ACC (20% reduction with $P = 0.0346$ compared with FA, Fig. 3B), but did not alter the expression of FAS (Fig. 3C). SREBP1 also activates peroxisome proliferator-activated receptor gamma (PPARγ), another transcriptional factor regulating lipogenesis (Schadinger et al., 2005). Piceatannol significantly suppressed PPARγ protein expression in steatosis-induced cells (68% reduction with $P = 0.0076$ compared with FA, Fig. 3D and E). Thus, it was suggested that piceatannol reduced TG accumulation in part via regulating lipogenesis in steatosis-induced HepG2 hepatocyte.

FA uptake mediated through fatty acid binding proteins (FABPs), such as cluster of differentiation 36 (CD36), is an alternative fat source of hepatic TG accumulation in NAFLD. 100 μM of piceatannol reduced the expression of CD36 in steatosis-induced HepG2 cells (59% reduction with $P < 0.0001$ compared with FA, Fig. 3F), which is also observed when piceatannol was treated in normal HepG2 cells (Sup. Fig. S3). Taken
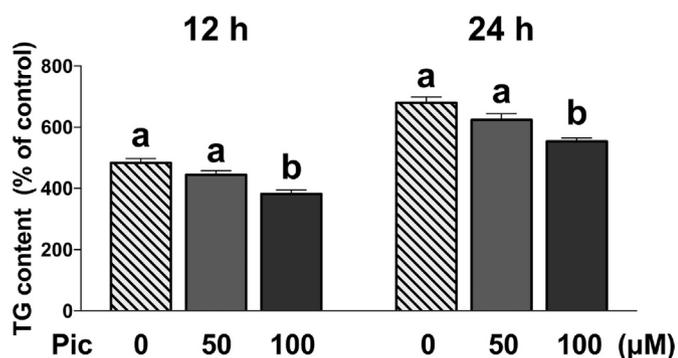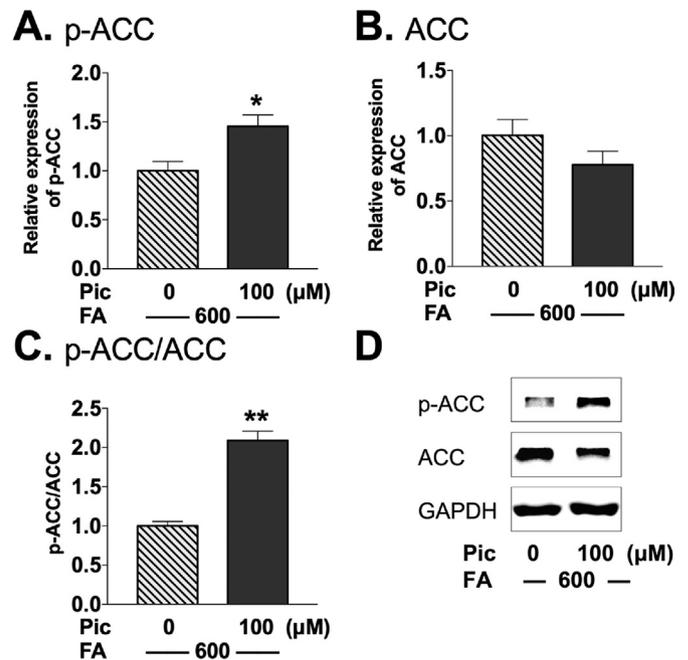
Fig. 2. Piceatannol inhibited ACC in steatosis-induced HepG2 cells. p-ACC (phosphorylated acetyl-CoA carboxylase (A), ACC (B), ratio of p-ACC/ACC (C), and representative pictures of p-ACC and ACC (D) with FA and piceatannol. HepG2 cells were treated with 600 μM of FA, with or without 100 μM of piceatannol for 24 h. Protein expression was measured by western blot. Numbers are mean ± S.E.M. n = 4. *Statistically different compared with the control at $P < 0.05$; ** at $P < 0.01$. Pic: piceatannol.

together, piceatannol suppressed lipogenesis via SREBP1 and ACC and suppressed FA uptake via CD36, contributing to lower TG accumulation in steatosis-induced HepG2 cells.

3.3. Effects of piceatannol on FA β-oxidation pathways in steatosis-induced HepG2 hepatocytes

The FA β-oxidation pathways were examined to determine if hepatic FA β-oxidation is involved in the piceatannol-induced fat reduction in HepG2 hepatocytes. In the liver, one of the critical transcriptional factors that modulate FA β-oxidation is the peroxisome proliferator-activated receptor alpha (PPARα), which can be regulated by farnesoid X receptor (FXR), the bile acids receptor (Kalaany and Mangelsdorf, 2006). PPARα promotes carnitine palmitoyltransferase 1α (CPT1α), an enzyme that facilitates long-chain FA to enter the mitochondria, the primary site of FA β-oxidation (Muoio et al., 2002).

In steatosis-induced HepG2 hepatocytes, 100 μM of piceatannol promoted the mRNA expression of FXR (41% increase with $P < 0.0001$ compared with the control, Fig. 4A). In addition, it was found that piceatannol increased the mRNA expression of PPARα (50% increase with $P = 0.0006$ compared with the control, Fig. 4B), and the protein expression of PPARα (76% increase with $P = 0.0048$ compared with the control, Fig. 4D and E). Moreover, 100 μM of piceatannol increased the mRNA expression CPT1α in the steatosis-induced HepG2 hepatocytes (133% increase with $P < 0.0001$ compared with FA, Fig. 4C). The results here suggest that increased fatty acid β-oxidation by piceatannol treatment may also contribute to its effect on TG accumulation in steatosis-induced HepG2 hepatocytes.

3.4. Effects of piceatannol on hepatic oxidative stress

During FA overload, mitochondrial FA β-oxidation can be switched to peroxisomal β-oxidation and endoplasmic reticulum ω-oxidation, which are not only less efficient but also generate more reactive oxygen species
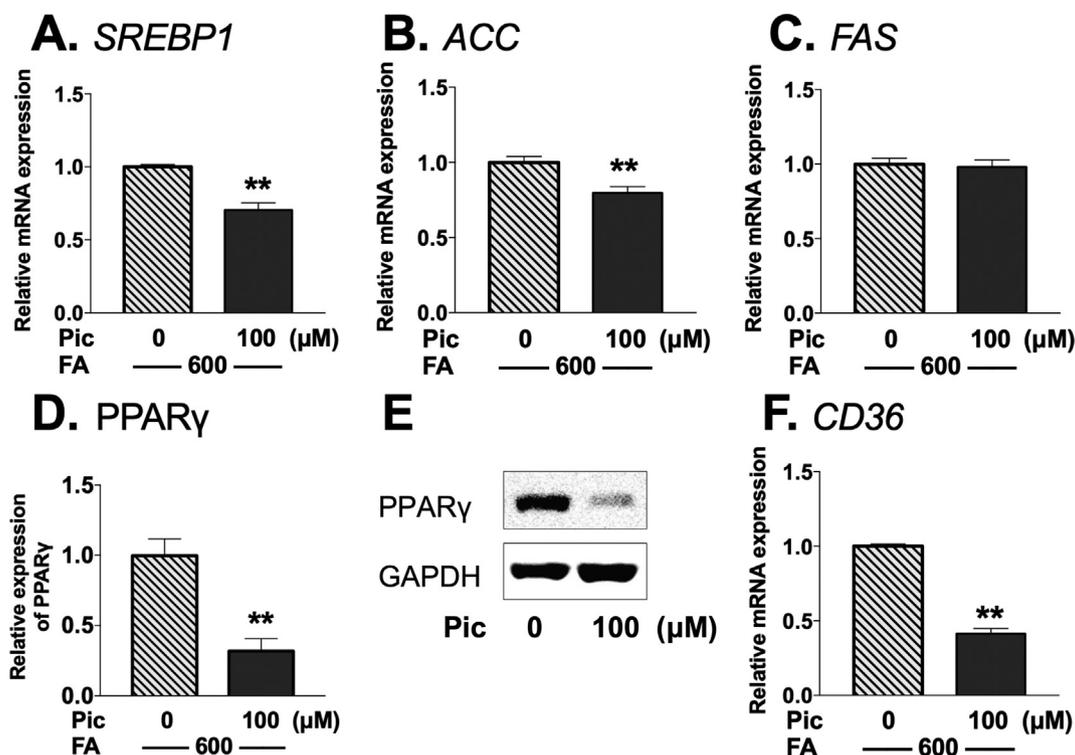
Fig. 1. Piceatannol reduced TG accumulation in steatosis-induced HepG2 cells. HepG2 cells were incubated with 600 μM of FA and 0 (steatosis control), 50, and 100 μM of piceatannol for 12 and 24 h. TG accumulation were present as % TG of HepG2 cells without FA and piceatannol (control), Numbers are mean ± S.E.M. n = 4. Means with different letters are statistically different at $P < 0.05$. TG: triglycerides.

Fig. 3. Effects of piceatannol on lipogenesis and fatty acid uptake pathway. mRNA expression of SREBP1 (sterol regulatory element-binding protein 1, A), ACC (acetyl-CoA carboxylase, B), and FAS (fatty acid synthase, C). Protein expression of PPARγ (peroxisome proliferator-activated receptor γ, D) and representative picture of PPARγ (E). mRNA expression of CD36 (cluster of differentiation 36, F). HepG2 cells were treated with 600 μM of FA with or without 100 μM of piceatannol for 24 h. mRNA analyzed by RT-qPCR. Protein expression was measured by western blot. Numbers are mean ± S.E.M. n = 4. **Statistically different compared with the control at *P* < 0.01. Pic: piceatannol.
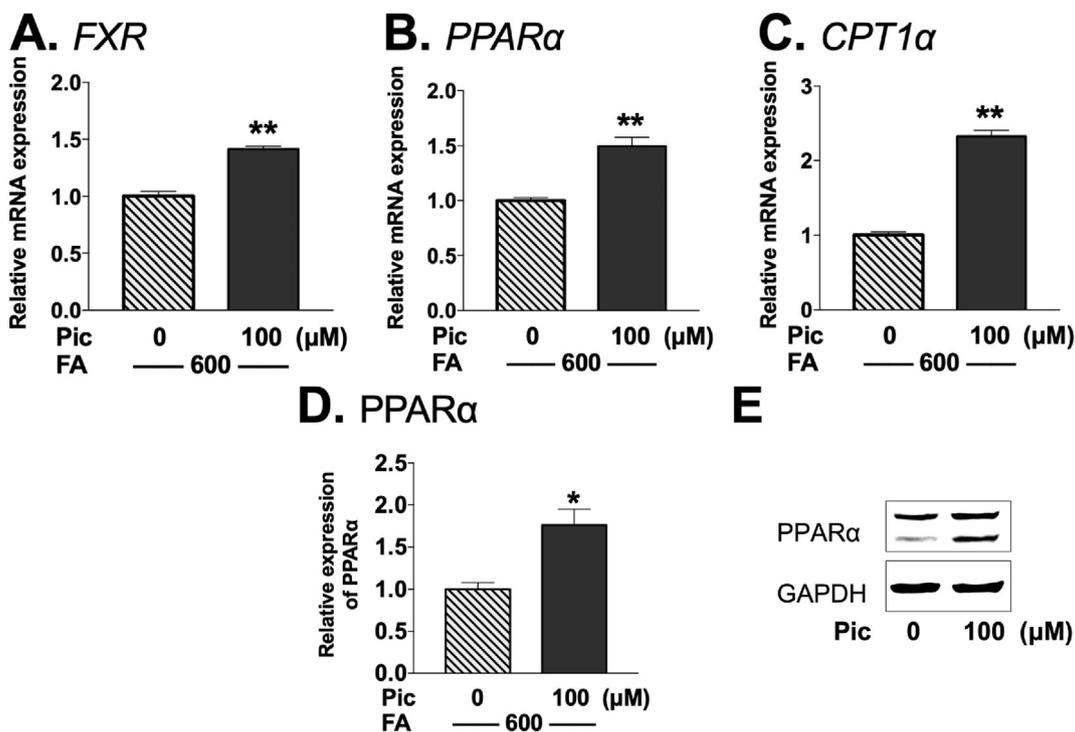

Fig. 4. Effects of piceatannol on fatty acid oxidation pathway. mRNA expression of FXR (farnesoid X receptor, A), PPARα (peroxisome proliferator-activated receptor α, B) and CPT1α (carnitine palmitoyltransferase 1α, C) with FA and piceatannol. Protein expression of PPARα (D) and representative picture of PPARα (E). HepG2 cells were treated with 600 μM of FA, with or without 100 μM of piceatannol for 24 h. Protein expression was measured by western blot. mRNA expressions analyzed by RT-qPCR. Numbers are mean ± S.E.M. n = 4. *Statistically different compared with the control at *P* < 0.05; ** at *P* < 0.01. Pic: piceatannol.
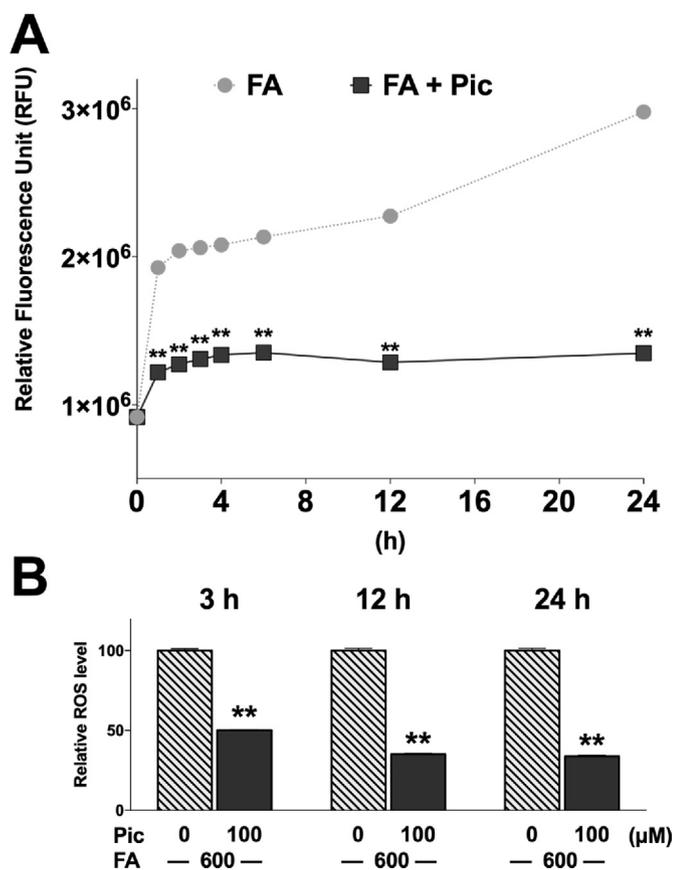
Fig. 5. Piceatannol suppressed FA-induced ROS production. (A) Kinetic plot of fluorescence reading reflecting ROS production. (B) Effects of piceatannol on FA-induced ROS production. HepG2 cells were pre-incubated with 25 μM DCFDA as ROS probe and treated with 600 μM of FA and 100 μM of piceatannol and ROS levels were measured at 0, 1, 2, 3, 4, 6, 12, and 24 h. Numbers are mean ± S.E.M. n = 8. **Statistically different compared with the respective controls at $P < 0.01$. ROS: reactive oxygen species; DCFDA: 2′,7′-dichlor-odihydrofluorescein diacetate; Pic: piceatannol.

(ROS) than mitochondrial FA β-oxidation (Reddy, 2001), causing oxidative stress. It is known that oxidative stress is a critical factor in NAFLD pathogenesis and disease progression (Rolo et al., 2012; Albano et al., 2005). It was found that 600 μM of FA significantly increased ROS production after 1 h, which lasted up to 24 h (Fig. 5A). The FA-induced ROS production was reduced by 100 μM of piceatannol (42% reduction at 3h, 65% reduction at 12h, and 66% reduction at 24h with all $P < 0.0001$, respectively compared with the respective controls, Fig. 5B).

3.5. Piceatannol suppressed p-JNK, ERK1/2, and oxidative stress-induced TG accumulation

It has been recognized that the activation of the mitogen-activated protein kinases (MAPKs) pathway, including c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinases 1/2 (ERK1/2), is associated with oxidative stress (Martindale and Holbrook, 2002) and hepatic lipogenesis (Wang et al., 2015). It was found that 100 μM of piceatannol suppressed the phosphorylation of JNK (73% reduction with $P = 0.0005$ compared with FA, Fig. 6A and G) without influencing the expression of JNK (Fig. 6B and G), leading to significant reduction on the ratio of p-JNK/JNK (70% reduction with $P = 0.0008$ compared with FA, Fig. 6C) in steatosis-induced HepG2 cells. Additionally, 100 μM of piceatannol suppressed the phosphorylation of ERK (33% reduction with $P = 0.0164$ compared with FA, Fig. 6D and G), while the expression of ERK was unchanged with piceatannol (Fig. 6E and G),

leading to significant suppression on the ratio of p-ERK/ERK by 100 μM of piceatannol (34% reduction with $P = 0.0024$ compared with FA, Fig. 6F).

To further determine if the antioxidant effect of piceatannol can contribute to its effect on reduced TG accumulation, hydrogen peroxide (H_2O_2) was used to induce oxidative stress in HepG2 hepatocytes. In this model, the TG content in HepG2 hepatocytes with 3 h of pre-treatment of 500 μM of H_2O_2 was not changed at 24 h but was significantly elevated at 48 h after the H_2O_2 was removed (42% increase with $P = 0.0005$ compared with the control, Fig. 6H). To compare with the H_2O_2 group, the TG content was significantly reduced by 100 μM of piceatannol at 48 h after the H_2O_2 treatment (31% reduction with $P = 0.0057$).

4. Discussions

Piceatannol has been considered to pose similar bioactivity when compared to its analog, resveratrol, but there is limited knowledge of its bioactivity. In the present study, we have demonstrated that piceatannol mitigated the FA-induced TG accumulation in HepG2 hepatocytes. The current results further suggest that piceatannol suppressed lipogenesis and FA uptake, while it promoted fatty acid β-oxidation to contribute to decreased TG accumulation in steatosis-induced HepG2 cells. Additionally, the results suggest that piceatannol inhibited FA-induced ROS production, as well as the phosphorylation of JNK and ERK1/2, which are known to be associated with obesity and insulin resistance, the risk factors of NAFLD.

Several *in vivo* and *in vitro* studies have shown that resveratrol ameliorated hepatic steatosis, it lowered oleic acid and alcohol-induced intracellular TG accumulation through the suppression of the activity of ACC and protein expression of SREBP1-c isoform in HepG2 cells (Tang et al., 2015). It was also indicated that administration of resveratrol ameliorated high-fat diet-induced NAFLD by reducing hepatic mRNA expression of SREBP1, FAS, ACC, and PPARγ in rodents (Shang et al., 2008; Andrade et al., 2014). Furthermore, resveratrol was found to promote the expression of FXR (Yang et al., 2016), a novel therapeutic target of developing therapeutic drugs for NAFLD treatment (Liu et al., 2014; Yang et al., 2010). However, in a human study, resveratrol supplementation only slightly improved NAFLD symptoms (Zhang et al., 2016). Similar to resveratrol, piceatannol exerted anti-obesity in high-fat diet-fed mice partly through modulating adipogenic proteins in adipose tissue, as well as hepatic ACC and PPARγ (Tung et al., 2016). Our results also showed that piceatannol suppressed SREBP1, ACC, and promoted the expression of FXR and subsequential FA β-oxidation. It has been found that activated hepatic 5′ AMP-activated protein kinase α, an energy modulator that regulates the activity of ACC, may be involved in the improvement of NAFLD by resveratrol and piceatannol as well (Shang et al., 2008; Tung et al., 2016). Thus, previous studies and current findings suggest that piceatannol and resveratrol may share a similar mechanism on hepatic lipid metabolism. Based on the previous reports that piceatannol is more bioavailable and has a stronger inhibitory effect on serum TG and hepatic lipogenesis over those of resveratrol (Tung et al., 2016; Setoguchi et al., 2014), piceatannol may have a greater potential to be used for the improvement of NAFLD. However, direct comparisons may be needed to draw any conclusions.

Oxidative stress and inflammatory response are critical factors for the pathogenesis and progression of NAFLD (Spahis et al., 2017). Oxidative stress can result from lipotoxicity, which is characterized as accumulation of lipid intermediates induced by excessive free fatty acids (FFAs) (Alkhouri et al., 2009). It was also known that oxidative stress could further promote TG accumulation in the HepG2 hepatocytes (Sekiya et al., 2008), thereby suggesting the reciprocal role of oxidative stress in NAFLD. Previous studies have demonstrated that resveratrol and piceatannol exerted radical scavenging activity (Cordova-Gomez et al., 2013). Similarly, resveratrol and piceatannol have been shown to exhibit antioxidant properties and prevent DNA damage in lymphoma cell (Livingston et al., 2015), and suppressing H_2O_2 release in human adipose tissue
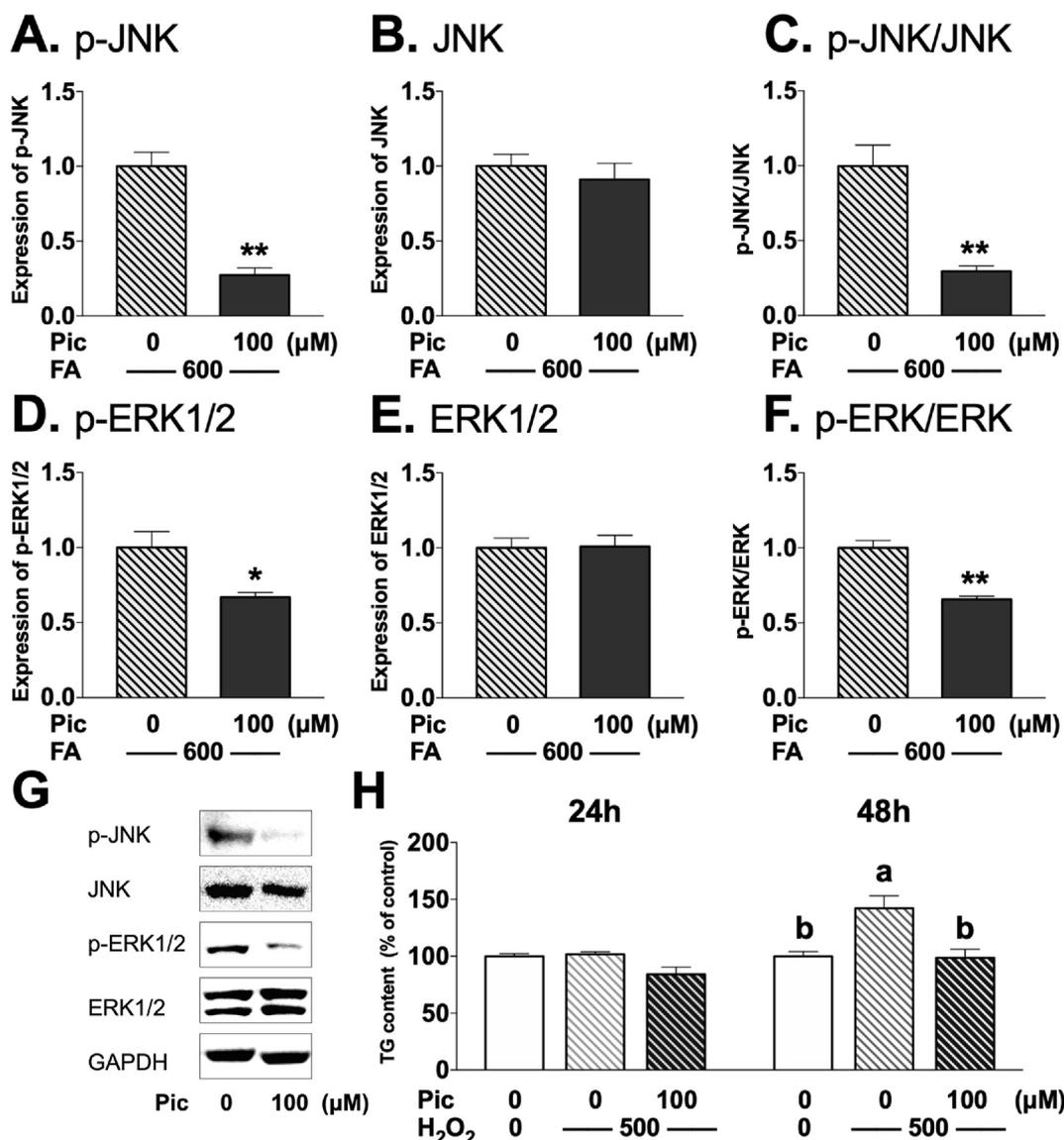
Fig. 6. Effects of piceatannol on ERK1/2, JNK and oxidative stress-induced TG accumulation. p-JNK (phosphorylated c-Jun N-terminal kinase) (A), JNK (B), and ratio of p-JNK/JNK (C) with FA and piceatannol. P-ERK1/2 (phosphorylated extracellular signal–regulated kinase 1/2) (D), ERK1/2 (E), and ratio of p-ERK/ERK (F) with FA and piceatannol. Representative pictures of p-JNK, JNK, p-ERK1/2, and ERK1/2 (G). Effects of piceatannol on H_2O_2-induced TG accumulation (H). HepG2 cells were treated with 600 μM of FA with or without 100 μM of piceatannol for 24 h (A–G). HepG2 cells were pre-incubated with 500 μM of H_2O_2 for 3 h, and then treated with or without 100 μM of piceatannol for 24 and 48 h. Protein expression was measured by western blot. Numbers are mean ± S.E.M. n = 4 for A-G and n = 3–4 for H. *Statistically different compared with the control at $P < 0.05$; ** at $P < 0.01$. Means with different letters are statistically different at $P < 0.05$. Pic: piceatannol; TG: triglycerides.

(Les et al., 2016). Piceatannol also reduced ROS level in *Caenorhabditis elegans* (Shen et al., 2017a). In the current study, piceatannol suppressed FA-induced TG accumulation and ROS production in the first 24h, while it abrogated H_2O_2-induced TG accumulation after 48h. Although further studies are needed to confirm the role of piceatannol in NAFLD pathogenesis, the current results suggest that the antioxidative effect of piceatannol may be related to its mitigation of TG accumulation in steatosis-induced HepG2 hepatocytes.

The activation of the MAPK family, which includes JNK and ERK1/2, is not only known to mediate oxidative stress, but also known to be correlated with obesity and insulin resistance, principal risk factors of metabolic syndrome and NAFLD (Sun et al., 2016; Bost et al., 2005). Previously, the activation ERK1/2 has been found in the liver of high fat diet-fed mice (Jiao et al., 2013), and it is also known to be associated with metabolic disease, such as obesity and type 2 diabetes (Bost et al., 2005).

In accordance, the current study is the first to demonstrate piceatannol suppressed the phosphorylation of JNK and ERK1/2 in HepG2 hepatocytes. In this regard, it is suggested that piceatannol inhibited oxidative stress, and the JNK and ERK1/2 pathway may play a role in protecting the pathogenesis of NAFLD.

The concentrations of piceatannol used in this study were based on the cell viability assay and previous studies (Tang et al., 2015; Kwon et al., 2012). It has been shown that 25 and 50 μM of piceatannol decreased fat accumulation in 3T3-L1 adipocytes without causing cytotoxicity (Kwon et al., 2012), while our group reported that 50 and 100 μM of piceatannol reduced fat accumulation in *Caenorhabditis elegans* without affecting the physiological conditions of the nematodes, such as pumping rate and moving speed (Shen et al., 2017b). In the current study, 100 μM of piceatannol resulted in significant TG reduction and regulation of lipid metabolism in steatosis-induced HepG2 hepatocytes

without causing cytotoxicity. The dosages used in the current study were determined based on cell viability assay, and it is comparable with another *in vitro* study (Kwon et al., 2012) and one *in vivo* study of the effects of piceatannol on regulation lipid metabolism in *Caenorhabditis elegans* (Shen et al., 2017b). However, the relevance of doses used along with physiological concentrations of piceatannol needs to be evaluated further.

In this study, the induction of *in vitro* steatosis can be achieved by applying excessive FAs consisting of OA and PA, the two most abundant fatty acids present in human serum (Baylin et al., 2002). The FA-induced *in vitro* steatosis in HepG2 human hepatoma cells has been used to determine the therapeutic effects of bioactives on the development of NAFLD (Gomez-Lechon et al., 2007). In addition, FA with the 2:1 ratio of OA:PA has been reported to stimulate moderate TG accumulation without causing apoptosis compared with OA or PA alone (Ricchi et al., 2009), which represents the early reversible stage of NAFLD (Buzzetti et al., 2016). By measuring the TG contents in steatosis-induced HepG2 cells, we were able to determine the potential efficacy of piceatannol on NAFLD. Although it was known that HepG2 human hepatoma cells exhibit most of the genotypic features and function of normal liver cells, including lipid metabolism (Sassa et al., 1987), the HepG2 cells still vary from the normal cells in some aspects, such as drug metabolism (Gerets et al., 2012). Moreover, the pathogenesis of NAFLD is a symptom complex resulted from imbalanced energy homeostasis, including lipid and glucose metabolism, which can occur in multiple organs. Therefore, further investigation with other models may enhance the significance of this current study, while *in vivo* studies are particularly needed to further confirm the efficacy and mechanisms of piceatannol on the development of NAFLD.

5. Conclusions

The current results suggest that piceatannol reduced fat accumulation by suppressing lipogenesis (SREBP1 and ACC) and FA uptake (CD36), and promoting FA oxidation (FXR, PPARα and CPT1α) in steatosis-induced HepG2 cells. Additionally, it was identified that anti-oxidative mediated pathways may be involved in the effect of piceatannol on ameliorating steatosis in this model. To conclude, our findings in the current study determined the efficacy of piceatannol in mitigating the development of NAFLD and the potentially involved mechanisms, which expands current knowledge on how to develop future NAFLD treatment using plant-originated phytochemicals as therapeutic agents.

Declaration of Competing Interest

There is no pending conflict of interest to disclose for all authors.

CRediT authorship contribution statement

Jason Szuhao Yang: Methodology, Investigation, Formal analysis, Writing - original draft. **Jozxelle Tongson:** Investigation, Formal analysis. **Kee-Hong Kim:** Conceptualization. **Yeonhwa Park:** Conceptualization, Resources, Validation, Supervision, Writing - review & editing, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2020.03.008.

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