INTRODUCTION

Diabetes mellitus is characterized by elevated blood glucose levels. The estimated number of people with diabetes worldwide in 2015 is 415 million, the number is expected to increase to 642 million by 2040(1). Type 2 diabetes (T2D) makes up about 90% of cases of diabetes(2), insulin resistance (IR) as one of the most important defect appears in various tissues (e.g., muscle, liver, and adipose)(3).

Recent research showed that increasing ectopic triglyceride (TG) accumulation in non-adipose tissues were associated with IR and T2D. Adipose tissue is well known lipid storage, but has a limited maximal capacity to store lipids. Once beyond this limitation, excess fatty acids would spill over into the blood, lead to their ectopic storage in the tissues which normally contain only small amount of fat, such as the liver, skeletal muscle, heart, and pancreas. Ectopic fat accumulation induce metabolic processes disruption and organ function impaired(4).

Plants are abundant in biologically active molecules that play critical roles in pharmacology(5). Most of the available therapeutic agents widely used for treating diabetes are associated with side effects(6). However, natural antidiabetic products which are believed to have minimal side effects have been highlighted for the treatment of diabetes(7,8). Citrus sudachi is an evergreen tree that was found mainly in Tokushima Prefecture in Japan(9, 10), and it has been demonstrated that Citrus sudachi had the capability of inhibiting the rising trend of blood glucose and fatty acid in human subjects, though its mechanism has not been clarified yet(11).

Methanol extraction fraction from Citrus Sudachi peel exerts lipid reducing effects in cultured cells

Wenting Xu1, Licht Miyamoto1*, Haruna Aihara1, Tomomi Yamaoka1, Naonobu Tanaka2, Yuki Tsuchihashi1, Yasumasa Ikeda1, Toshiaki Tamaki3, Yoshiki Kashiwada2, and Koichiro Tsuchiya1

1Department of Medical Pharmacology, Institute of Biomedical Sciences, Graduate School of Tokushima University, Tokushima, Japan; 2Department of Pharmacognosy, Institute of Biomedical Sciences, Graduate School of Tokushima University, Tokushima, Japan; 3Department of Pharmacology, Institute of Biomedical Sciences, Graduate School of Tokushima University, Tokushima, Japan

Abstract: Ectopic fat accumulation is associated with insulin resistance and type 2 diabetes mellitus. Citrus sudachi is an evergreen tree that is found mainly in Tokushima Prefecture in Japan. Previously, it was demonstrated that Citrus sudachi could inhibit the rising trend of blood glucose and fatty acid in human subjects. In the current study, we illustrated the function of methanol extracts from sudachi peel and investigated the mechanism of this effect. We got the five kinds of methanol extracts by using diaion HP-20, and those were named by their hydrophobicity from M-F1 to M-F5. Among the 5 kinds of sudachi methanol extracts, only M-F4 significantly decreased the intracellular triglyceride of C2C12 cells. It augmented the AMPK activity and increased the hydrophobicity from M-F1 to M-F5. Among the 5 kinds of sudachi methanol extracts, only M-F4 significantly decreased the intracellular triglyceride of C2C12 cells. It augmented the AMPK activity and increased the AMPK(AMP-activated protein kinase) is a fuel-sensing enzyme that plays a crucial role in response to starvation. AMPK is activated by phosphorylation of the threonine residue (Thr172) on its catalytic α-subunit, which initiates processes such as fatty acid oxidation that restore ATP, and inhibits ATP consumption such as triglyceride and protein synthesis, and cell proliferation(12).

Peroxisome proliferator-activated receptors (PPARs) belong to a subgroup in the family of nuclear hormone receptors and highly expressed in the most of metabolic tissues. PPAR-α was the first discovered PPARs and is known to contribute to oxidative processes including fatty acid metabolism(13). PPAR-α exists in skeletal muscles and liver, which is closely correlated with fatty acid oxidation(14). PPAR-α has been suggested as an important therapeutic target for metabolic diseases with hyperlipidemia(15).

METHODS AND MATERIALS

Materials

We purchased AICAR, sirtinol and Gw6471 from Sigma-Aldrich, Cosmo bio or Funakoshi Co., Ltd. (Tokyo, Japan), respectively. Other reagents were obtained from Kanto Chemical (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), or Tokyo Chemical Industry (Tokyo, Japan). The following commercially available antibodies were used: anti-AMPK (Sigma-Aldrich, St Louis, MO, USA); anti-sirt1 (Cell Signaling Technology Japan, K.K.); anti-Phospho-AMPKα (Thr172) (Cell Signaling Technology Japan, K. K.); anti-β-actin, as a loading control, from Cell Signaling Technology Japan, K.K.; anti-GAPDH, as a loading control, from Wako Pure Chemical Industries, Ltd.

The extract process of sudachi peel

The extract process of Citrus sudachi peel was shown in Figure 1. Sudachi peel dry powder were extracted with hexane at room temperature.
temperature for 24 hr, the residue was extracted with methanol again at the same condition, the hexane extract part and methanol extract part were separated. The methanol extract was concentrated in 90% methanol by portioned between hexane and 90% methanol. The fraction was chromatographed on Diaion HP 20 column, eluted with methanol and H₂O solution, and then gradient and removing the sugar dissolved in water. 5 kinds of fraction named by hydrophobicity from M-F1 to M-F5 were got at last.

In order to screen the function of Sudachi peel extractions, the extractions were dissolved in dimethyl sulfoxide (DMSO), made to different concentration.

Figure 1: The extract process of Citrus sudachi peel

**Cell culture and treatment**

Cell culture and differentiation was conducted as reported previously(16). C2C12 myoblasts, which are derived from mouse thigh muscle cell, were obtained from American Type Culture Collection. The C2C12 myoblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L D-(+)-Glucose (nacalai tesque), supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere with 5% CO₂, and grown in DMEM supplemented with 2% horse serum for 1 week to be induced differentiation into myotubes.

**Triglyceride and non-essential fatty acid measurement**

Following to the stimulation, lipids were extracted with 2-propanol at 4°C overnight, and concentrations of triglyceride (TG) and NEFA (non-essential fatty acid) were determined by Triglyceride E-test Kit and NEFA C-test Kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer’s instruction, respectively.

**Oil Red O staining**

Cells were fixed with 10% formalin for 1 h, rinsed with PBS for 2 times, and stained with 0.5% Oil Red O dye for 20 minutes. After washing again with PBS, cells were visually monitored by OLYMPUS microscopic observation.

**Western blotting**

After the stimulations, cells were washed with ice-cold PBS and lysed with lysis buffer (500 mM Tris-HCl, 10 mM NaCl, 1 mM MgCl₂, 250 mM sucrose, 0.5% NP-40, 0.1 mM EGTA) as previously reported (17-20). Protein expression and phosphorylation was detected by western blot analysis. Equal amounts of proteins were separated by 7.5% SDS-PAGE, and electrotransferred to PVDF membranes (Millipore, USA), and then were blocked with PBS solution with 1% Tween20 and 5% skim milk for 1 hr at room temperature. Membranes were incubated with appropriately diluted primary antibodies overnight at 4°C. Then incubated with the relevant secondary antibodies for 2 hr at room temperature. The immune complexes were visualized with the enhanced chemiluminescence (ECL) detection system according to the manufacturer’s instructions, in conjunction with ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) or β-actin was served as an internal control protein.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from cells, and was reverse-transcribed using the Superscript II™ First Strand Kit (Invitrogen). We evaluated the mRNA levels of peroxisome proliferator-activated receptor alpha (PPARα) , sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), carnitine palmitoyl trans- ferase 1b (CPT1b), acyl-coenzyme A oxidase (Ac0), medium chain acyl coenzyme A dehydrogenase (MCAD), mitochondrial transcription factor A (mtTFA), uncoupling protein 2 (UCP2), and housekeeping gene GAPDH, primer sequences are shown in Table1. Real-time PCR was performed on an ABI PRISM 7500 PCR System (Applied Biosystems, USA) using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). PCRs were carried out in a total of 20 μL as follows: one cycle at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 65°C for 30 sec and 72°C for 40 sec. The gene expression from each sample was analyze in duplicates and normalized against GAPDH. The results are expressed as relative

<table>
<thead>
<tr>
<th>GENE</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARA</td>
<td>AAGGCTGTAAAGGCTTTTTCG</td>
<td>GGCATTGTTGCTCGGGTCTTC</td>
</tr>
<tr>
<td>SREBP1</td>
<td>GCGTATATGCTGTAAGACATCCTA</td>
<td>ATCCAGGCGCTTGAAACCTC</td>
</tr>
<tr>
<td>PGC1A</td>
<td>TCCAACCAAGCAGAATGT</td>
<td>CGCTGTCGCTGAGGTATT</td>
</tr>
<tr>
<td>CPT1B</td>
<td>CCGAAGGACACCCAGGAATGA</td>
<td>GCCAAGCCTGGTTGGTGGAAT</td>
</tr>
<tr>
<td>ACO</td>
<td>GCCGTCGCAAATGCAAGACTA</td>
<td>TCTTACAGCCACCTGCTAAG</td>
</tr>
<tr>
<td>MCAD</td>
<td>GAGCTGGAGAAATGCTGTAAG</td>
<td>GCCAGGCGCAAGCCACTT</td>
</tr>
<tr>
<td>MTTFA</td>
<td>GAAGGGATGGGAAGAGTGA</td>
<td>AACAGCATGAGGAGCAGAT</td>
</tr>
<tr>
<td>UCP2</td>
<td>CTACAAGACATTTGACAGAGG</td>
<td>AGCTGTCTATGTTGACACAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AACACTGAGCATCCTCCCTCA</td>
<td>GTGGGTTGAGGCAACTT</td>
</tr>
</tbody>
</table>

Table 1: Sequences of the primers used in real-time PCR
gene expression using the ΔCt method.

Evaluation of viability

Cell viability was evaluated by MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (12, 19, 21).

Statistical Analysis

All the data are shown as the means± standard deviation (SD). Student’s t-test was performed to determine the difference between two groups, and one-way ANOVA and post-hoc Tukey-Kramer test were performed to evaluate multiple groups. *p < 0.05 was considered statistically significant.

RESULT

Crude sudachi peel and M-F4 reduce intracellular triglyceride level significantly in C2C12 cell.

In another paper, we demonstrated that Citrus sudachi had the capability of inhibiting the rising trend of blood glucose and fatty acid in human (11). We supposed that if the crude sudachi peel could reduce intracellular Triglyceride (TG) level in C2C12 cells. We try to find out which part of this crude extraction cause this result. C2C12 myoblast were stimulated by 5 kinds of sudachi peel methanol extractions (M-F1-M-F5) (100 μg/mL) for 24 hours, only M-F4 obviously down-regulated TG level, but M-F5 had the opposite effect (Fig 2A). The similar results were also observed from C2C12 myotubes treated with sudachi peel methanol extractions (Fig 2B). Consistent with TG level, the neutral lipids, cholesterol esters, and triglycerides, staining in C2C12 myotubes which treatment with M-F4 (Fig 2C-b) was lower than the control group (Fig 2C-a). We purposed to investigate the mechanism of this effect.

M-F4 increases AMPK phosphorylation but did not alter Sirt1 expression.

To elucidate whether M-F4 exert a TG-lowering effect via AMPK or Sirt1, C2C12 myotubes were treated with 100 μg/mL M-F4 for 24hr and the protein levels were examined by western blotting. As expected, M-F4 increased the phosphorylation of AMPK in C2C12 myotubes obviously (Fig 3A). 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), a specific AMPK activator, was reported tobe used as a positive control to increase fatty acid oxidation levels (22). Both AICAR and M-F4 increased phosphorylation level of AMPK, but had no further combination impact. (Fig 3D)

On the contrary, as shown in Figure 3C, the protein expression level of Sirt1 did not significantly increased by M-F4. Sirinol is a non-specific Sirt1 inhibitors. Same as the treatment by M-F4, sirinol (25 μM) induced a statistically insignificant trend toward reduced triglyceride (Fig 3E), which means that Sirt1 had no substantive role in reducing TG. Those datum were consistent with previous western blotting results. In summary, the M-F4 caused TG-lowering effect mediated by AMPK signaling pathway but not involved Sirt1.

PPARs and downstream targets were involved in the AMPK signaling pathway which caused the TG-lowering effect by M-F4.

As a master regulator of cellular energy homeostasis, AMPK has a lot of downstream substrates. PPARα and SREBP1 are two targets in lipid metabolism related to AMPK. Considering whether those two targets were involved in this triglyceride reducing effect, we used the real-time PCR to measure the mRNA level in myotubes after 100 μg/mL M-F4 treated for 24 hr. PPARα expression was increased by M-F4, while M-F4 did not change expression levels of SREBP1 (Fig 4A). This triglyceride reducing effect probably mediated by augment of lipid oxidation though PPARα.

In the PPARα signal pathway, a lot of downstream genes were concerned to be involved (Fig 4B). PPAR γ coactivator 1-alpha (PGC-1α) is a regulator of mitochondrial biogenesis and function, but in our experiment PGC-1α were not activated by M-F4. Carnitine palmitoyl transferase1 (CPT1b) which considered to be the gene that controlled fatty acid mitochondrial β-oxidation were mightily activated in 24hr by M-F4 stimulated. Considering the genes involved in lipid oxidation, the expression of Aco, MCAD and mtTFA were slightly increased following M-F4 treatment, but there were no significant differences. Mitochondrial uncoupling protein 2 (UCP2) gene expression was increased in myotubes of M-F4 treated with statistically significant.

PPARα was the downstream target of AMPK signaling pathway and involved in TG and NEFA reducing effect caused by M-F4.

According to some papers, the relationship between AMPK and PPARα was a kind of complicated. In most cases, AMPK was the upstream target of PPARα, but in some special cases PPARα was the upstream one. In order to confirm this relationship, we use Gw6471 as a PPARα inhibitor to treat cells. In the WB result, the level of p-AMPK were increased by M-F4, and this increasing trend did not change in the presence of Gw6471 (Fig 5A). In the triglyceride measurement, the reducing effect caused by M-F4 was relieve when treated with Gw6471 (Fig 5B).

M-F4 leaded the increasing trends of PPARα, CPT1b and UCP2 were mitigated when treated with Gw6471 (Fig 5C). This result was consistent with our expectation that CPT1b and UCP2 were the downstream target of PPARα to reduce triglyceride level.

CPT1b is a rate limiting enzyme on the mitochondrial outer membrane, governing long-chain fatty acid entry into mitochondria. UCPs increase the proton conductance of the mitochondrial inner membrane, eventually promote fatty acid oxidation in the
mitochondrial. Therefore we conjecture the lipid reducing effect of M-F4 not only reveals in triglyceride but also in fatty acid. In figure 5D, we measure the non-essential fatty acid (NEFA) in the myotubes, the result shown that M-F4 assuredly decreased the NEFA level and decreasing tendency alleviated by Gw6471 intervening.

The proposed scheme for M-F4-stimulated signaling pathway in C2C12 myotubes was shown in Figure 7. In the lipid metabolism process, M-F4 is primarily phosphorylated AMPK-Thr172, but does not involve Sirt1 pathway. Then mediated by PPARα and its downstream targets CPT-1b and UCP2, ultimately leading to improve lipid metabolism characterized by TG and NEFA reducing.

**DISCUSSION**

The aim of this research is to find the lipid lowering substance in *Citrus sudachi*, and investigate its mechanism. We found that the sudachi peel crude could reduce intracellular TG level of C2C12 cells. Moreover, M-F4, a kind of sudachi peel extractions, reduces triglyceride of C2C12 cells. In this lipid-lowering effect, the PPARα and its downstream targets were activated by AMPK, suggesting that M-F4 improved the lipid metabolism characterized by TG and NEFA reducing, via activated AMPK and mediated by PPARα and its downstream targets CPT-1b and UCP2.

Several reports have demonstrated that citrus played important roles in regulating glucose and lipid metabolic disorders. In the present research, we got five kinds of methanol sudachi peel extractions. Among the kinds of extracts, M-F4 had the strongest effect on triglyceride reducing in both myoblasts and myotubes. Because the C2C12 myotubes were differentiated cells and have mature cell function, we thought use the myotubes in the next experiments were more close to the skeletal muscle in vivo. It is interesting that the M-F5 was opposite to M-F4 in triglyceride measurement, but we have not found the answer yet.

In order to find out the proper condition for the mechanism research, we tested a series of varied concentration (1 μg/mL - 500 μg/mL) and duration (2 hr - 48 hr).
In the concentration-variable experiment, while the triglyceride reduction effect of M-F4 as 500μg/mL was most intensely, but considering the cell viability result we got in MTT assay, we chose concentration as 100 μg/mL for 24 hr stimulation in the next step of study.

It is known that AMPK have effect on carbohydrate and lipid metabolism, it is already clear that the system is a major player in the development and/or treatment of obesity, diabetes, and other metabolic syndrome. Several natural ingredients have been shown to exert antidiabetic effects, accompanied by AMPK activation. Thus, we first hypothesized that sudachi peel extraction reduce triglyceride effect is likely to be related to AMPK. In addition, Sirt1 always has a complicated relationship with AMPK in many metabolic regulate fields. But in our Western Blotting assay, only AMPK related proteins were activated obviously. In the following experiment, sirtinol and AICAR were used to intervene the expression of Sirt1 and AMPK, the results were consistent with previous ones. It means that AMPK should be involved in the TG reducing effect caused by M-F4, and this effect may depended on some other AMPK-related target but not Sirt1.

PPARs act as fatty acid sensors to control many metabolic programs that are essential for systemic energy homeostasis. Fig. 4A and 5B-D demonstrates that M-F4 exerts triglyceride reducing effect by activating PPARα. There also reported that naringenin treatment lowers triglyceride and cholesterol in plasma and liver with increasing PPARα expression in rat liver (23). PPARα null mice have decreased expression of cardiac fatty acid oxidation genes and decreased rates of fatty acid oxidation, but maintain normal cardiac function. PPARα/γ may exert the synergic inhibitory effects on fatty acid synthesis by controlling the expressions of the transcription factor SREBP-1c, though this does not correspond with our results.

The target genes regulated by PPARα and involved in lipid metabolism and obesity include PGC1α, CPT1b, MCAD, UCP2, ACO and mTFA. PGC1α has been implicated in increasing the oxidation of fatty acids via increasing mitochondrial capacity and function, making this co-factor a key candidate for the treatment of lipotoxicity recently. Unexpectedly, the expression level of PGC1α were declined by stimulation, which is probably because PGC1α activity is also altered via AMPK and Sirt1 pathways. Some reports showed that synergistic activation of CPT1b gene is promoted by heterodimer nuclear receptors PPARα-RXRα. Increase in CPT1 expression in muscle can recover insulin sensitivity and reduce lipid accumulation. Our current results exactly suggest that TG reducing effect is mediated by augment of lipid oxidation through PPARα-CPT1b pathway. UCP2 is expressed and functionally active in both central and peripheral tissues involved in glucose and lipid metabolism. It was reported that obesity could be treated by promoting the activity of native UCP. Tsutsumi also reported that UCP2 gene expression was significantly increased in skeletal muscle of mice which improved glucose and lipid metabolism (9). Molecular mechanisms how M-F4 exerts the lipid-lowering effects we propose is summarized in Fig. 7.

We considered if M-F4 improves lipid metabolism by reducing TG only, or it could effect on other lipid substance. Consistent with our expectation, M-F4 improved the lipid metabolism characterized by both TG and NEFA (Fig. 5D).

CONCLUSION

In summary, our study is the first to demonstrate that sudachi peel methanol extract reduces triglyceride and fatty acid level in skeletal muscle cell. Additionally, our findings suggest that M-F4 improves lipid metabolism through AMPK, PPARα and their downstream targets, CPT-1b and UCP2. Furthermore, these observations indicate that this extract may be useful for preventing obesity and diabetes related diseases.
CONTRIBUTIONS AND DISCLOSURES

L.M. designed and conducted the study, and contributed to manuscript preparation. X.W. performed most of the experiments, wrote the manuscript, and contributed to the study design. The others contributed to the experiments and/or discussion.

Disclosures: The authors declare no conflict of interest.

REFERENCES