Ethyl 2-[2,3,4-Trimethoxy-6-(1-Octanoyl)Phenyl] Acetate (TMPA) Ameliorates Lipid Accumulation by Disturbing the Combination of LKB1 with Nur77 and Activating the AMPK Pathway in HepG2 Cells and Mice Primary Hepatocytes

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Background: The AMP-activated protein kinase alpha (AMPKα) pathway has widely been considered a key factor in energy metabolism. Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate (TMPA) is a novel AMPK agonist, which influences the stability of Nuclear Receptor Subfamily 4, Group A, Member 1 (Nur77)-serine-threonine kinase 11 (LKB1) in the nucleus. A recent study has determined that TMPA can ameliorate the reduction of insulin resistance in type II db/db mice. However, the role of TMPA in hepatocyte lipid metabolism has not been elucidated.

Objective: To investigate whether TMPA could ameliorate liver lipid accumulation under the stimulation of free fatty acids (FFAs) in vitro.

Methods: We evaluated differences of Nur77 and AMPK pathway in mice fed a high-fat diet and those fed a normal diet. In vitro, TMPA was added to HepG2 cells and primary hepatocytes before FFAs stimulation. Oil red O staining, Nile red staining were used to evaluate lipid deposition. Western blot and immunofluorescence were used to quantify related proteins.

Results: Nur77, AMPKα, LKB1, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), acetyl-CoA carboxylase phosphorylation (p-ACC), and carnitine palmitoyltransferase 1 (CPT1A) showed significant differences in vivo. Under the intervention of TMPA, HepG2 cells and primary hepatocytes showed considerable amelioration of lipid deposition and improved the expression of phosphorylated (p)-AMPKα (p-AMPKα), p-LKB1, p-ACC, and CPT1A. Furthermore, Western blotting and immunofluorescence studies indicated that LKB1 dramatically increased expression in the cytoplasm but decreased in the nucleus. Further, AMPKα phosphorylation (p-AMPKα) also showed a higher expression in cytoplasm instead of the nucleus.

Conclusion: TMPA ameliorated lipid accumulation by influencing the stability of Nur77-LKB1 in vitro.

Keywords: ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate, Nur77, AMPK pathway, HepG2 cells, primary hepatocytes

Introduction
Metabolic associated fatty liver disease (MAFLD) is an umbrella term for a comprehensive range of liver diseases, including nonalcoholic fatty liver disease (NAFLD), and the more severe nonalcoholic steatohepatitis (NASH). MAFLD is...
the most common cause of chronic liver disease in the world, and increasing evidence shows that MAFLD is a multi-systemic disease, affecting extra-hepatic organs and regulatory pathways. Many factors can generate the occurrence and progression of this disease, such as type 2 diabetes mellitus (T2DM), cardiovascular (CVD), cardiac diseases, and chronic kidney disease (CKD).\(^2\) As MAFLD progresses, many patients will progress to liver cirrhosis and end-stage hepatocellular carcinoma. The increase in prevalence of this chronic disease has caused a growing economic burden and reduced the quality of life for many elderly individuals.\(^3\)

Nuclear Receptor Subfamily 4, Group A, Member 1 (Nur77, also called TR3, NGFIB, and NR4A1), is an orphan member of the nuclear receptor superfamily and plays a critical role in apoptosis, fibrosis, glucose metabolism, inflammation, lipid metabolism, and in the regeneration and injury of cells.\(^4\)-\(^9\) More recently, Nur77 has been shown to be involved in glucose and lipid metabolism,\(^10\),\(^11\) especially through the serine-threonine kinase 11/AMP-activated protein kinase (LKB1/AMPK) pathway.

The LKB1/AMPK axis is considered a crucial metabolic sensor in cellular homeostasis especially for glucose and lipid metabolism.\(^12\),\(^13\) The AMPK pathway is altered following exposure to a mixture of free fatty acids (FFAs) in HepG2 cells.\(^14\) During lipid metabolism in the liver, activated AMPK\(^\alpha\) can phosphorylate 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) at Ser872\(^15\) and acetyl-CoA carboxylase (ACC) at S79, and subsequently inhibits the activation of HMGCR and ACC, which is crucial for lipid production.\(^12\),\(^16\) In addition, ACC is a potent allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1A), which imports long chain fatty acyl-CoA into the mitochondria and promotes fatty acid β-oxidation. Therefore, the suppression of ACC promotes the activation of CPT1A and then reduces the deposition of FFAs.\(^12\) Hence, the AMPK pathway has been considered a useful approach to treat diseases associated with fat metabolism.

Zhan et al\(^17\) confirmed that Nur77-specific binding of liver kinase B1 (LKB1) in the nucleus prevented the translocation of LKB1 from the nucleus to the cytosol. Similar results showing that Nur77 stimulates FceRI signaling by counteracting the LKB1/AMPK axis have been reported in anaphylactic responses.\(^18\) Altogether, this evidence implies that the inhibition of Nur77 and LKB1 binding may improve hepatic steatosis. Recent studies have shown that ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate (TMPA), an Nur77 antagonist, prevents the binding of Nur77 and LKB1 as well as stimulates LKB1 transport to the cytosol and its phosphorylation (p-LKB1).\(^17\)-\(^19\) Activation of AMPK\(^\alpha\) by phosphorylated LKB1 activates downstream molecules related to lipid metabolism. Therefore, TMPA is also considered a potential target treatment for diabetes and obesity.\(^17\)

In this study, we investigated the effects of TMPA on lipid metabolism in HepG2 and primary liver cells. The results showed that TMPA facilitates LKB1 transport to the cytosol and its phosphorylation (p-LKB1). Furthermore, p-LKB1 subsequently promoted AMPK\(^\alpha\) phosphorylation (p-AMPK\(^\alpha\)) and activated downstream molecules like phosphorylated (p) ACC (p-ACC) and CPT1A. The results of the present study indicate that TMPA could prevent fatty accumulation in HepG2 cells and primary hepatocytes.

**Methods**

**Reagents**

Palmitic acid was purchased from MCE (NJ, USA). Oleic acid was purchased from Selleck Company (Shanghai, China). Antibodies against phosphorylated (p)-LKB1 (Ser428, 63473, 138386), p-ACC (S79, 68191), ACC (109368, 45174), Nur77 (109180), AMPK\(^\alpha\) (32047), HMGCR (174830), ADFP (108323), SREBP-1 (28481) and CPT1A (220789, 234111) were purchased from Abcam (UK). Antibodies against β-actin (#3700), and p-AMPK\(^\alpha\) (T172, #50081) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against LBKI (#3050)(A2122) was bought from Cell Signaling Technology (Danvers, MA, USA) and ABclonal (Wuhan, China). Antibodies against PCNA (10205-2-AP) were purchased from Proteintech (Wuhan, China). TMPA was purchased from MCE (NJ, USA), and subsequently dissolved in dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) to prepare a 40mM stock solution.

**Cell Culture**

The HepG2 cell line was purchased from Procell Life Science & Technology Co. (China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, China), supplemented with 10% fetal bovine serum (FBS; Gibco, New Zealand). The trypsin enzyme-digestion technique was used to passage the cells and the trypsin enzyme was produced by Gibco (USA).
Primary Hepatocytes Extraction and Culture
C57BL/6J mice were used as the source of mice primary hepatocytes. Mice livers were first perfused with Hank’s Solution (Solarbio, Beijing, China), followed by perfusion with IV collagenase via the portal vein. The digested liver tissue was then shaken with sterile forceps to exfoliate the cells on the ultra-clean table. Afterwards, the primary cells were centrifuged in a 15mL sterile centrifuge tube for 3 times, 1000 revolutions per time, 4min. After each centrifugation, discard the waste solution and wash with Hank’s solution. Finally, the primary cells were implanted in a dish covered with rat tail collagen and cultured in William’s E Medium (Gibco, England).

Induction of Fat-Overload in HepG2 Cells and Primary Hepatocytes
The cells were incubated with 0.25mM FFAs mixture (OA:PA = 2:1) for 24h. The FFAs (1.5mL, 0.5mL and 0.1mL) were added to the 6well, 24well and 96well plates, respectively. The FFAs were prepared in culture medium containing DMEM with no bovine serum albumin.

Hematoxylin-Eosin Staining and Oil Red O Staining
The tissue of liver in mice was isolated and washed with saline solution. The tissue was dehydrated and immersed in wax to be embedded. The samples were cut into sections with a thickness of 4–5μm. Subsequently, all sections underwent alcohol dehydration, xylene penetration, H&E staining, dehydration and permeabilization, and resinene sealing. Oil red O staining kit (Solarbio, Beijing, China) was used to assess lipid accumulation in cells cultured. HepG2 cells and primary hepatocytes were washed with phosphate-buffered saline (PBS) (Biosharp, China), and then fixed with cell Fixative for about 25min. We then used 60% isopropanol to wash the cells for 5min, after which the cells were subjected to oil red O staining for 20min. The nuclei were stained by hematoxylin for about 1min.

Nuclear and Cytoplasmic Separation
The Nucleoplasmic separation kit was bought from Boster company (Wuhan, China). After the cells were scraped in PBS, the RIPA Lysis Buffers in the kit were added successively. Protease inhibitor and phosphatase inhibitor were added to the lysate of each part in a ratio of 100:1:1. Each step was operated on ice. Extracted nucleoprotein and plasma protein were frozen in the −80°C refrigerator.

Protein Extraction and Western Blot Analysis
Proteins were extracted from HepG2 cells and primary liver cells using RIPA lysis buffer (Solarbio, Beijing, China) and protein phosphatase inhibitor (100:1:1) mixture. Cell lysates were centrifuged at 12,000rpm for about 20min. After centrifugation, middle-level clarified liquid was aspirated. Bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) was used to measure the protein concentration. All protein samples were stored at −80°C. Extracted protein was added to SDS-PAGE on a 10% resolving gel with 120V constant voltage and then transferred to a nitrocellulose (NC) filter membrane with 220V constant current. After blocking nonspecific binding with 5% BSA solution, the membranes were incubated with primary antibodies overnight at 4°C. The membranes were washed three times with TBST (Trisbuffered saline and 0.1% Tween 20) and then incubated with the HRP-conjugated secondary antibody which diluted to 1/5000 (Proteintech, Wuhan, China) at room temperature for 1h.

Nile Red Staining
Nile red staining was used to identify the intracellular lipid content in HepG2 cells and mice primary hepatocytes. Cells were washed three times by PBS first. They were then incubated with Nile red fluorescent stain (10μg/mL in PBS, Solarbio, Beijing, China) for 15min in 37°C. They were then incubated with DAPI (Solarbio, Beijing, China) for 7min at common temperature for nuclear localization. Inverted confocal fluorescence microscope is a tool for observing the fluorescence. Excitation wavelength of Nile red stain was 530nm.

Immunofluorescence Assay
Cells on the 24-wells were fixed in 4% paraformaldehyde for 25min at room temperature. After that, the paraformaldehyde was removed, and the cells were washed with PBS three times for 5min. Then, cells were permeabilized by 0.5% Triton X-100 for 40min at room temperature. Next, we used PBS to wash three times for 5min each. Goat serum was used to block for 1h at 37°C. First antibody (anti-LKB, ABclonal, WuHan, China) was diluted in goat serum to 1:100 and added to all wells in 300ul, incubating overnight at 4°C. The second antibody (SA00013-4 Proteintech, Wuhan, China) diluted in PBS to 1:200 and
incubated for 1h at room temperature. Finally, discarding the secondary antibody and wash with PBS three times in 5min. The results were photographed by an inverted confocal fluorescence microscope.

Experimental Animals and Diet
C57BL/6J male mice were purchased from the Charles River Laboratories (Beijing, China) at 6–7 weeks of age. All mice were fed adaptively for one week. Animal experiments were conducted according to the Principles of Laboratory Animal Care established by the National Institutes of Health. All experiments were approved by the Animal Research committee of Shandong Provincial Hospital. All mice were randomly divided into two groups: a normal diet group (NC) and a high-fat diet group (HFD). NC group was fed a standard mice chow diet (10% calories from fat, XieTong Organism Inc.) and the HFD group was fed a high-fat diet (60% calories from fat, XieTong Organism) over 8 weeks.

Biochemical Evaluation
Levels of alanine transaminase (ALT), aspartate transaminase (AST), triglyceride, total cholesterol, total bile acid in serum were determined to evaluate liver injury using RAYTO Chemray 800 automatic biochemical analyzer (Shenzhen, China) and commercial reagent kit (RAYTO, Shenzhen, China, S03030, S03040, S03074, S03027, S03042).

Statistical Analysis
All assays were performed on 3 separate occasions. Data are expressed as means ± SD. The Image J software (NIH) was used to quantify oil red O staining and Western blotting findings. The statistical data obtained from Image J quantitative data were processed using GraphPad Prism 6.0. Data were analyzed using one-way analysis of variance. A P-value <0.05 was considered statistically significant.

Table 1 Nonalcoholic Fatty Liver Disease (NAFLD) Activity Score (NAS)

| Group | n  | Steatosis       | Ballooning      | Lobular Inflammation | NAS       |
|-------|----|----------------|----------------|----------------------|-----------|
| NC    | 3  | 0.222±0.111    | 0.333±0.192    | 0.333±0.192          | 0.889±0.484 |
| HFD   | 3  | 2.667±0        | 2.667±0.192    | 2.000±0              | 7.333±0.192 |

Notes: All values are mean ± SEM. *Defined as NASH. The NAS represents the sum of steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning scores obtained by historical analysis (0–2), with a score of 5–8 points defined as NASH. Each H&E stain will be scored by three pathologists. The group are NC (10% calories from fat) and HFD (60% calories from fat).

Results
Expression of Nur77 and Related Proteins Differed Between MAFLD Mice and Normal Mice
Significant differences in weight were observed between the two groups after 4 weeks of feeding. These differences became more pronounced as time progressed. Oil red O staining revealed many fat droplets, which indicated severe fat deposition in the HFD group (Figure 1A) compared to the NC group. Hematoxylin and eosin (H&E) staining showed that the HFD group had more fat vacuoles with irregular hepatocyte structure compared with NC groups (Figure 1B). The NAS score indicated that mice fed with HFD could be defined as NASH (Table 1). HFD group exhibited significant changes in aspartate transaminase (AST), total bile acid (TBA), and cholesterol levels, but no obvious differences in glutamic-pyruvic transaminase (ALT) and triglyceride (TG) (Figure 1D). Next, we investigated differences in the expression of lipid-related proteins between the two groups. Western blotting analysis showed high expression of Nur77, p-ACC, CPT1A, and HMGCR and low expression of LKB1 and AMPKα in the HFD group. Interestingly, ACC expression showed no significant differences in the NC group (Figure 1E and F).

TMPA Reduced Lipid Accumulation Following FFAs Stimulation in HepG2 Cells and Primary Hepatocytes
TMPA (Figure 2A) can interfere with LKB1 and Nur77 binding. Oil red O staining was used to evaluate lipid accumulation induced by FFAs (0.25mM) in HepG2 cells and primary hepatocytes. Oil red O stain showed that under stimulation of FFAs (oleic acid and palmitic acid in a 2:1 ratio) (0.25mM), HepG2 cells (Figure 3A and B) and primary hepatocytes (Figure 3C and D) showed apparent lipid accumulation and fat vacuoles. However, the DMSO control group showed no significant differences compared with the FFAs-stimulated group. Furthermore,
Figure 1 Changes physiological and Nur77-AMPK related proteins levels in mice fed a normal diet (NC) and a high-fat diet (HFD). (A) Oil O red staining (40× magnification); and (B) hematoxylin and eosin (H&E) staining (40× magnification) of liver tissue of mice fed NC and HFD. Left, mice fed a NC (10% calories from fat). Right, mice fed a HFD (60% calories from fat). Sections of the liver of all mice were prepared after they were fed the respective diets for 8 weeks. (C) Weekly weight change in mice of the NC and HFD groups. (D) Indicators of serum associated liver injury in mice. Results of the t-test showed statistical differences AST, TBA, cholesterol. ALT and TG showed no significant changes. (E) Expression of metabolism-associated proteins in NC and HFD groups. (F) Results of the t-test showed statistical differences in the expression of relevant indicators. Nur77, HMGCR, p-ACC, and CPT1A were evaluated. A decline in expression was observed in LKB1, AMPKα. Acetyl-CoA carboxylase (ACC) showed no significant change. *P < 0.05, **P < 0.01, ***P < 0.001, t-test.
we attenuated TMPA in FBS to 10µm and added the solution to HepG2 cells and primary hepatocytes, which were incubated for about 6h before stimulation with FFAs. Lipid accumulation and fat vacuoles were both reduced by TMPA (Figure 3A and B). We also used Nile red staining to evaluate the lipid content in these cells. The cells in the FFAs group showed stronger fluorescence intensity and more lipid droplets. However, the TMPA treatment group had the lower fluorescence intensity compared with the FFAs-treated group (Figure 3E and F). Furthermore, the expression of ADFP (also known as Plin2) which was associated with hepatic lipid accumulation20 also increased under FFAs and the intervention of TMPA ameliorated the content of ADFP, compared with the FFAs-treated group and FFAs+DMSO group (Figure 3G and 3H). All the data indicated that TMPA ameliorated hepatic steatosis by FFAs.

**LKB1 Translocated from the Nucleus to the Cytosol Under the Influence of TMPA**

The binding of TMPA has been reported to interfere with the binding stability between Nur77 and LKB1 in the nucleus, and thereby facilitated the interruption of the interface region, based on atomistic molecular simulations.19 Therefore, we investigated whether Nur77 was involved in the subcellular localization of LKB1 following treatment with TMPA (10µm), and whether TMPA could destabilize the translocation of LKB1 from the nucleus to the cytosol, following AMPKα phosphorylation in HepG2 cells and primary hepatocytes under the stimulation of FFAs.

LKB1 exhibited marked translocation between cytosol and nucleus in HepG2 cells and primary hepatocytes (Figure 5A–H) under FFAs stimulation. Next, we compared cells subjected to FFAs stimulation alone with those in the TMPA treatment group. As expected, LKB1 levels were reduced in the nucleus (Figure 5C, D, G, H) and were increased in the cytosol (Figure 5A, B, E, F). Furthermore, p-AMPKα showed a significant increase in cytosolic levels (Figure 5A, B, E and F), but not in the nucleus (Figure 4C, D, G and H) in HepG2 cells and primary hepatocytes. In addition, AMPKα in both the nucleus and cytosol showed no apparent changes. Immunofluorescence staining of LKB1 indicated that the treatment of TMPA leads to a fluorescence intensity decline in the nucleus and an increased intensity in the cytoplasm of HepG2 cells and mice primary hepatocytes (Figure 5I and J). Our results confirmed that the phosphorylation of AMPKα in the TMPA group was influenced by the interaction between LKB1 and Nur77. Therefore, intervention by TMPA could decrease the stability of LKB1 and Nur77 in the nucleus and in turn, lead to the increased phosphorylation of LKB1 in the cytosol and activation of AMPKα.
Figure 3  Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate (TMPA) ameliorated lipid deposition under the influence of free fatty acids (FFAs) (0.25mM) stimulation. (A) Oil red O staining (40× magnification) exhibited lipid deposition in HepG2 cells. Left, HepG2 cells exposed to FFAs (0.25mM) for 24h. DMSO medium was added to HepG2 cells and left for 6h, after which the cells were exposed to FFAs for 24h (DMSO + FFAs). Right, TMPA with HepG2 cells were incubated for 6h before exposure to FFAs for 24h (TMPA + FFAs). (B) No significant differences were observed in the cells exposed to FFAs or DMSO + FFAs. In contrast, TMPA significantly ameliorated lipid accumulation. (C) The same treatments were added to primary hepatocytes (40× magnification). (D) Oil red O stain quantification showed that primary hepatocyte exhibited the same results observed in HepG2 cells. (E) HepG2 cells were treated with FFAs or FFAs combined with TMPA. Cellular lipids were stained with Nile red stain, and cells were observed with a fluorescence microscope (40× magnification). (F) Primary hepatocytes isolated from the same treatment groups in mice were exposed to Nile red stain (40× magnification) was used to detected the deposition of lipid. (G) Western blotting analysis detected protein expression of ADFP in HepG2 cells. The intervention of FFAs increased the expression of ADFP. No significant differences were observed in cells exposed to FFAs or DMSO + FFAs. However, TMPA reduced the content of ADFP. (H) Mice primary hepatocytes from the same treatment groups as in (G) exhibited the same results. *P < 0.05, **P < 0.01, ***P < 0.001, t-test.
Ethyl 2-[3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate (TMPA, 10µm) ameliorated lipid deposition via the AMP-activated protein kinase (AMPK) pathway. (A and B) Nur77, p-AMPK, CPT1A expression changed under stimulation by FFAs vs control group. TMPA increased p-LKB1, p-AMPKα, p-ACC and CPT1A. No significant changes were observed in Nur77, LKB1, or AMPKα, while downstream pathway proteins of AMPKα were increased, like p-ACC and CPT1A, which are directly related to lipid metabolism. ACC, HMGCR and cleaved-SREBP-1 showed no significant change in HepG2 cells. (C and D) The same treatments applied to in primary hepatocytes produced the same results. *P < 0.05, **P < 0.01, t-test.
The orphan nuclear receptor 4 subfamily 1 (NR4A1, Nur77) is considered a significant metabolic regulatory protein that can directly combine with LKB1 in the nucleus. In addition, Nur77 inhibition by TMPA affects glucose metabolism and the progression of diabetes. However, whether TMPA is involved in hepatic lipid metabolism or is associated with AMPK-related downstream proteins of lipid metabolism has not been confirmed. A lipid accumulation model in vitro was used to investigate the effects of TMPA on LKB1-Nur77 binding and the translocation of LKB1 to the cytosol. FFAs influenced decreased LKB1 expression in the cytoplasm, and TMPA increased the levels of LKB1 in the cytosol and nucleus. Immunofluorescence (40× magnification) was used to detect the location of LKB1 in HepG2 cells and mice primary hepatocytes, which showed a similar pattern as the HepG2 cells. *P < 0.05, **P < 0.01, t-test.

**Discussion**

The orphan nuclear receptor 4 subfamily 1 (NR4A1, Nur77) is considered a significant metabolic regulatory protein that can directly combine with LKB1 in the nucleus. In addition, Nur77 inhibition by TMPA affects glucose metabolism and the progression of diabetes. However, whether TMPA is involved in hepatic lipid metabolism or is associated with AMPK-related downstream proteins of lipid metabolism has not been confirmed. A lipid accumulation model in vitro was used to investigate the effects of TMPA on LKB1-Nur77 binding and the translocation of LKB1 to the cytosol. FFAs influenced decreased LKB1 expression in the cytoplasm, and TMPA increased the levels of LKB1 in the cytosol and nucleus. Immunofluorescence (40× magnification) was used to detect the location of LKB1 in HepG2 cells and mice primary hepatocytes, which showed a similar pattern as the HepG2 cells. *P < 0.05, **P < 0.01, t-test.
constructed by adding saturated FFAs and unsaturated FFAs. The AMPK pathway was closely associated with lipid metabolism in the liver. Thus, we investigated differences in protein expression between mice fed a regular chow diet and those fed a HFD. It has been demonstrated that male mice are often used to establish the model of MAFLD. The results showed remarkable differences in expression of proteins involved in the AMPK signaling pathway in livers with marked steatosis. Furthermore, marked increases in the expression of FFAs Oxidation.

**Figure 6** Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate (TMPA) interfered with FFAs stimulation in HepG2 cells and primary hepatocytes. The combination of Nur77 with LKB1 in the nucleus inhibited the phosphorylation of LKB1 and AMPKα. The stability of LKB1-Nur77 was compromised under the influence of TMPA. LKB1 was subsequently translocated to the cytosol from the nucleus and activated AMPKα. The phosphorylation of AMPKα suppressed ACC by phosphorylation and activated CPT1A, the rate-limiting enzyme of lipid oxidation.
p-ACC, and CPT1A also indicated the existence of some compensatory mechanisms in these mice.

The Nur77-LKB1 interaction is complex and attenuate the phosphorylation of LKB1.\textsuperscript{17,19} Under stimulation of FFAs, both HepG2 cells and primary hepatocytes exhibited changes in Nur77, p-AMPK\(\alpha\), and CPT1A levels, which showed that hepatic steatosis was associated with the AMPK signaling pathway. Following the addition of TMPA, lipid deposition was considerably improved under FFAs stimulation. A previous study confirmed that LKB1 is a major kinase that activates phosphorylation of AMPK\(\alpha\) at Thr172, while the phosphorylation of LKB1 at Ser428 is essential for the activation of AMPK\(\alpha\) by metformin.\textsuperscript{23} Thus, we hypothesized that the mechanism of TMPA activity in liver lipid metabolism is associated with AMPK and activation of relevant signaling proteins.

As we expect, the intervention of TMPA increased the expression of p-LKB1 and p-AMPK\(\alpha\) in these cells. Furthermore, the downstream proteins of the AMPK pathway were also increased, such as phosphorylated ACC and CPT1A. But no significant changes in HMGCR or SREBP-1 levels, which are strongly associated with cholesterol metabolism, were observed. It is generally accepted that ACC is a central enzyme that is crucial as source of fatty acids from nonlipid precursors by de novo lipogenesis.\textsuperscript{16} CPT1A is an essential rate-limiting enzyme involved in the breakdown of fatty acids (\(\beta\)-oxidation), and effectively translocates fatty acids into the mitochondria. The regulation of CPT1A is closely associated with malonyl-CoA, downstream of ACC, which negatively regulates CPT1A expression and may influence the process of hepatic steatosis. As expected, TMPA also antagonized LKB1-Nur77 interactions in the nucleus and promoted the translocation of additional LKB1 into the cytosol. Further study elucidated the mechanisms through which TMPA ameliorated hepatic steatosis involved the phosphorylation of the excitatory AMPK pathway through the nucleoplasmic shuttle LKB1. Our findings were consistent with previous reports that showed no significant changes in the levels of AMPK\(\alpha\), but instead we observed a marked increase in AMPK\(\alpha\) phosphorylation after exposure to TMPA. Differences in nucleoprotein and cytoplasmic protein expression indicated that the activation of LKB1 and AMPK\(\alpha\) may also be a consequence of reduced Nur77 and LKB1 binding, and translocation of LKB1 from the nucleus to the cytoplasm.

Zhan et al\textsuperscript{17} confirmed that TMPA could reduce blood glucose levels in mice, and predicted that this could represent a potential target treatment for diabetes. Importantly, MAFLD is also associated with diabetes and fat intake.\textsuperscript{36} Based on the results of the present study, TMPA influenced FFAs stimulation by affecting the formation of LKB1-Nur77 in the nucleus,
and phosphorylating LKB1 and AMPKα in the cytosol. Consequently, the effects of CPT1A, the rate-limiting enzyme of fatty acid oxidation, improved following activation of AMPK (Figure 6). Therefore, we propose TMPA as a potential target drug for use in the treatment of MAFLD, as it can inhibit the nuclear stability of LKB1 and Nur77.

**Abbreviations**

TMPA, Ethyl 2-[2,3,4-trimethoxy-6-(1-octanoyl)phenyl] acetate; MAFLD, Metabolic associated fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, MAFLD activity score; H&E stain, hematoxylin-eosin staining; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; TRIG, Triglyceride; Nur77, Orphan nuclear receptor 4 subfamily A; ADFP, adipose differentiation-related protein; CPT1A, carnitine palmitoyltransferase 1; LKB1, liver kinase B1; AMPKα, AMP-activated protein kinase alpha; FFAs, free fatty acids; ACC, acetyl-CoA carboxylase; CPT1A, carnitine palmitoyltransferase 1; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ADFP, adipose differentiation-related protein; SREBP-1, Sterol Regulatory Element Binding Protein 1.

**Funding**

This study was supported by grants from the National Natural Science Foundation of China (No. 81373172 and No. 81770646).

**Disclosure**

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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