HFD-induced downregulation of TRPV2 mediates hepatic steatosis via p21 signaling

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Abstract

The global prevalence and incidence of nonalcoholic fatty liver disease (NAFLD) exhibit a growing trend. Although its underlying mechanism is still unknown, NAFLD is characterized by a significant accumulation of lipids. Here we report that high-fat diet (HFD) feeding HFD induced hepatic steatosis in mice, accompanied by a reduction in the expression and function of hepatic TRPV2. By conditional knockout TRPV2 in hepatocytes, we found that HFD-induced hepatic steatosis was exacerbated. In vitro model of NAFLD, we found TRPV2 regulated the lipid accumulation in HepG2 cells, and TRPV2 activation inhibited the expression of $p21$ and $p16$ which are cellular senescence markers. Finally, we found administration of probenecid, TRPV2 agonist, impaired HFD-induced hepatic steatosis and suppressed HFD-induced elevation in $p21$ and $p16$. Collectively, our findings imply that hepatic TRPV2 protects against the accumulation of lipids by modulating $p21$ signaling.

1. Introduction

The global prevalence and incidence of nonalcoholic fatty liver disease (NAFLD) have shown an increasing trend. It is an umbrella term that comprises a continuum of liver abnormalities from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and increases the risk of many chronic diseases such as liver cirrhosis, type 2 diabetes, cardiovascular and cerebrovascular diseases, and malignant tumors. It has been well documented that hepatocytes are not capable of metabolizing all energy substrates such as fatty acids resulting in the accumulation of toxic lipids\(^1\), which is one of the main drivers of this disease progression\(^1,2\). Over-accumulated lipids induce endoplasmic reticulum stress\(^3\) and activation of unfolded protein response\(^4\) which stimulate apoptotic pathways leading to hepatocellular death\(^5\).

In addition to apoptosis, recently growing evidence suggests that cellular senescence also modulates lipid accumulation in NAFLD patients\(^6,7\). Moreover, recent findings have connected $p21$, one of the most well-established senescence markers, with NAFLD as well\(^8\). $p21$ is a pleiotropic inhibitor of cyclin/cyclin-dependent kinase (CDK) complexes that mediate cell cycle progression, and it is reported that in ob/ob mice NAFLD is associated with increased expression of $p21$\(^8\). In human NAFLD liver samples, expression levels of $p21$ were significantly elevated compared with normal liver samples\(^9\). And in patients with NASH, the hepatic expression level of $p21$ is significantly higher than in patients with simple steatosis\(^9,10\). Aravinthan's study demonstrated that hepatic $p21$ expression predicted an adverse liver-related outcome in patients with NAFLD\(^11\). These findings imply that $p21$ might be a key modulator to regulate the development of hepatic steatosis.

Transient Receptor Potential (TRP) channels are a class of cationic channels that act as a signal transducer by altering intracellular calcium concentration. In mammalian systems, TRP channels comprise six related protein subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML\(^12\). They are expressed in many tissues and are famous in thermosensation\(^13\). Recently, it is also found that TRPV2 is
involved in energy metabolisms. Tominaga's group found TRPV2 was expressed in both WAT and BAT and primary TRPV2-deficient (TRPV2KO) adipocytes show decreased mRNA levels of multiple genes involved in mitochondrial oxidative metabolism. Importantly, they also found TRPV2KO mice had significant increases in body weight and adipose tissue upon a high-fat diet (HFD) treatment. These findings implied that TRPV2 might play an important role in the balance of intrahepatocellular lipids. However, it is not clear how TRPV2 modulates the lipid balance in hepatocytes. Recent studies found an association between TRP channels and age-related diseases and TRPC7, as a primary initiator of epidermal aging and skin tumorigenesis, contributes to mutations in the p53 gene family. These findings imply that TRP channels may be involved in the regulation of cellular senescence and p21.

In this study, we found that TRPV2 was functionally expressed in hepatocytes. Strikingly, HFD-induced hepatic steatosis was accompanied by a downregulation of TRPV2 expression in hepatocytes. By establishing hepatocyte-specific TRPV2 knock-out mice (TRPV2\textsuperscript{flox/flox};AlbCre\textsuperscript{+/−}), we found the increase in body weight, NAFLD score and steatosis in these mice were happened earlier and more severe than that in control mice. To further address the underlying mechanisms of TRPV2 in hepatic steatosis, in vitro experiments were conducted. A NAFLD cell model was established, and we found TRPV2 activation impaired lipid accumulation. Furtherly, we found that p21 and p16 were decreased and CDK2 was increased when TRPV2 was activated, compared to the TRPV2-inhibited group. Finally, HFD-induced an increase in hepatic steatosis was compromised when TRPV2 agonist was added to the feed, companies by reductions of p21 and p16 in the liver.

Taken together, we concluded that TRPV2 is functionally expressed in hepatocytes and its activation protects HFD-induced hepatic steatosis via p21 signaling. Our findings might provide a promising target to treat NAFLD in the future.

2. Results

2.1. HFD-induced hepatic steatosis in mice, accompanied by a reduction in the expression and function of TRPV2 in the liver tissue or hepatocytes.

At first, we established the NAFLD model in mice as described in the methods. We found the body weight increased significantly from the 8th week and lasted to the 12th week after the onset of HFD, compared with the control group (Fig. 1B, **<0.005, ***<0.001, two-way ANOVA, F (6, 84) = 7.442). Similarly, the liver weight (Fig. 1C, ****<0.0001, two-way ANOVA, F (1, 8) = 114.5) and the ratio of liver to body weight (Fig. 1D, ****<0.0001, two-way ANOVA, F (1, 8) = 91.88) were also elevated at the 12th week after the onset of HFD, comparing with the control group. We also tested the expression level of TG in the liver after the onset of HFD and found the amount of TG was enhanced at the 12th week (Fig. 1E, ****<0.0001, two-way ANOVA, F (1, 10) = 14.63), comparing with the control group. To determine the degree of NAFLD at the
12th week, we assessed the NAFLD score according to liver histology and the score in the HFD group was higher significantly than that in the control group (Fig. 1F, **<0.005, unpaired t-test).

It is very striking that the protein level of TRPV2 in the liver was dimmed at the 12th week after the onset of HFD (Fig. 1G-1H, *<0.05, two-way ANOVA, F (2, 8) = 6.258), by using western blot method. To verify this finding, we performed immunostaining in cultured hepatocytes from HFD-treated mice or control mice. Similarly, the immune response of anti-TRPV2 was decreased in HFD-treated mice vs control mice at the 12th week (Fig. 1I-J, **<0.005, unpaired t-test). Given TRPV2 displays high Ca\(^{2+}\) permeability, we screened the cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in the Fura2-loaded hepatocytes from HFD-treated mice or control mice by perfusion 2-aminoethoxydiphenyl borate (2-APB, 0.5mmol/L for 1 min), which is TRPV2 specific agonist\(^{14}\). 2-APB stimulated an increase in [Ca\(^{2+}\)]\(_i\) in cultured hepatocytes in both groups, but the response was higher in the HFD-treated group than that in the control group (Fig. 2A-2B, *<0.05, two-way ANOVA, F (30, 240) = 1.642).

### 2.2. Conditional knockout hepatic TRPV2 aggravated HFD-induced hepatic steatosis in mice

To investigate the roles of TRPV2 in hepatocytes, we specifically deleted Trpv2 from Albumin-Cre-expressing cells by breeding AlbCre\(^{+/−}\) mice with TRPV2\(^{floxed/floxed}\) mice (TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\), Fig. 3A). As the control group, AlbCre\(^{−/−}\) mice were crossed with TRPV2\(^{floxed/floxed}\) mice (Fig. 3A). By immunostaining against TRPV2 in cultured hepatocytes from both groups, TRPV2 was not observed in the TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) group but expressed in the control group (TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\), Fig. 3B). Next, we fed high fat and carbohydrate diet or a standard chow diet in each group. The increment in body weight in TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with HFD feeding was more serious than that in TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with chow feeding (Fig. 3C, ****<0.0001, two-way ANOVA, F (6, 60) = 41.25) or TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with HFD (Fig. 3C, ****<0.0001, two-way ANOVA, F (6, 60) = 11.51).

Moreover, the increase in body weight was happened earlier in TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with HFD than that in other groups (Fig. 3D, ***<0.0005 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with Chow, **<0.001 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with HFD, one-way ANOVA, F (3, 20) = 12.35). However, there was no difference in body weight between TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with HFD and Chow group at the 4th week (Fig. 3D, P > 0.05, one-way ANOVA, F (3, 20) = 12.35). In agreement with the findings in body weight, the amount of TG in liver (Fig. 3E, ***<0.0005 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with Chow, ***<0.0005 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with HFD, one-way ANOVA, F (3, 20) = 34.25) and NAFLD score (Fig. 3F-3G, ***<0.0005 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with Chow, **<0.001 vs TRPV2\(^{floxed/floxed}\), AlbCre\(^{−/−}\) mice with HFD, one-way ANOVA, F (3, 20) = 11.54) in TRPV2\(^{floxed/floxed}\), AlbCre\(^{+/−}\) mice with HFD were also increased significantly at the 4th week after HFD feeding.

### 2.3. Activation of TRPV2 in HepG2 cells protect the cells against Palmitic Acids-induced lipid accumulation
To further investigate the roles of TRPV2 in lipid metabolisms, HepG2 cells were used to establish in vitro NALFD model, as described in the methods. Since TRPV2 agonist 2-APB and antagonist SKF were used to observe the effects of TRPV2 on lipid accumulation, the cytotoxicity of them in different doses (from 0.5 µM to 100 µM) on HepG2 cells was measured using a CCK-8 assay. We found that cell viability was not affected by up to 100 µM 2-APB (Fig. 4B, P > 0.05, one-way ANOVA, F (6, 28) = 1.955) or SKF (Fig. 4B, P > 0.05, one-way ANOVA, F (6, 28) = 3.121) in HepG2 cells for 24 hours. As shown in Fig. 4C, the Oil-red O staining assay revealed that PA in induced lipid droplet accumulation in HepG2 cells. However, 2-APB in 100 µM inhibited lipid accumulation significantly and this inhibitory effect was blocked in the presence of TRPV2 antagonist SKF (Fig. 4C-D, ***<0.0005, ****<0.0001, one-way ANOVA, F (2, 12) = 49.94). Interestingly, we found that SKF exacerbated lipid accumulation in HepG2 cells with PA incubation (*<0.05, one-way ANOVA, F (2, 12) = 49.94). Furthermore, we also tested the level of TG expression in HepG2 cells with PA incubation. As expected, 2-APB reduced TG expression and SKF blocked 2-APB effects (Fig. 4E, *<0.05, **<0.001, one-way ANOVA, F (2, 12) = 12.33).

2.4. Activation of TRPV2 in HepG2 cells downregulated \( p21 \) signaling after Palmitic Acids treatment

Next, we measured the expression level of \( p21 \), CDK2 and \( p16 \) in HepG2 cells treated with PA only, PA & 2-APB and PA & 2-APB & SKF. By comparing to PA only group, we found \( p21 \) (Fig. 5B, ****<0.0001, one-way ANOVA, F (2, 12) = 44.72) and \( p16 \) (Fig. 5D, *<0.05, one-way ANOVA, F (2, 12) = 6.853) were decreased, and CDK2 (Fig. 5C, **<0.001, one-way ANOVA, F (2, 12) = 12.80) was increased in the PA & 2-APB group. However, in the presence of SKF, which is TRPV2 antagonist, the reduction in \( p21 \) (Fig. 5B, ****<0.0001, one-way ANOVA, F (2, 12) = 44.72) and \( p16 \) (Fig. 5D, *<0.05, one-way ANOVA, F (2, 12) = 6.853) and increase in CDK2 (Fig. 5C, **<0.001, one-way ANOVA, F (2, 12) = 12.80) were reversed.

2.5. Activation of TRPV2 \textit{in vivo} attenuated HFD-induced lipid accumulation, and a reduction in the expression of \( p21 \) and \( p16 \) in the liver tissue

To verify the protective role of TRPV2 against lipid accumulation in hepatocytes, probenecid-treated water (containing 5% sucrose) was administered via bottles in the animals’ cages. The volume was measured before and after the consumption to determine the approximate dose. The calculated daily probenecid dose was 103.6 ± 2.1 mg/kg/day. We found that the HFD-induced increase in body weight was compromised after probenecid administration, compared to the vehicle group (Fig. 6B, **<0.001, two-way ANOVA, F (6, 84) = 3.990). Moreover, the liver weight (Fig. 6C, **<0.001, unpaired t-test) and the ratio of liver to body weight (Fig. 6D, *<0.05, unpaired t-test), in the probenecid and HFD administrated group, were lower than these at the 12th week after the onset of HFD with vehicle administration. Next, the NAFLD score in the above two groups was measured at the 12th week after HFD, and found that probenecid administration reduced the NAFLD score significantly, compared to the vehicle group (Fig. 6E, *<0.05, unpaired t-test). We tested the expression of \( p21 \) and \( p16 \) and found that both were inhibited in
3. Discussion

In the present study, utilizing a combination of *in-vivo* and *in-vitro* approaches, we established a critical role of TRPV2 expressed in hepatocytes in the regulation of lipids accumulation and the development of fatty liver in mice. Firstly, we found that HFD promoted fatty liver, accompanied by a down-regulation of the expression and function of TRPV2 in hepatocytes. Secondly, conditional knockout TRPV2 in hepatocytes exacerbated HFD-induced hepatic steatosis. *In vitro* experiments, we also found that TRPV2 regulated the lipid accumulation in HepG2 cells, accompanied by a reduction in *p21* and *p16* and an increase in CDK2. Finally, the administration of TRPV2 agonist *in vivo* prevented HFD-induced hepatic steatosis and inhibited HFD-induced elevation in *p21* and *p16*. These findings provide evidence that hepatic TRPV2 may be a useful therapeutic target for fatty liver.

Nonalcoholic fatty liver disease (NAFLD), a continuum of liver abnormalities from fatty liver alone, which is referred to nonalcoholic fatty liver (NAFL). Although the mechanisms of NAFLD is complex and still controversial, a conceptual framework is that the hepatocytes’ capacity to handle the metabolic energy substrates is overwhelmed, leading to an accumulation of toxic lipid species. Accordingly, modulation of the fatty acid in hepatocytes is a rational therapeutic target for NAFLD. In this study, we found that TRPV2 was functionally expressed in hepatocytes, and was downregulated after HFD treatment. Barritt et al., has reviewed that many members of the TRP family are expressed in hepatocytes and the mRNA of *Trpv2* was also detected in HepG2 cells. Some of them are implicated in up- or down-regulating fatty acid metabolism. For example, *Trpm2* mRNA and protein levels significantly increased in PA-treated L02 cells. TRPV1 was detected in HepG2 cell lines and mouse liver tissues, and dietary its agonist reduced lipid accumulation and TG levels in the livers of HFD-treated mice. However, the role of TRPV2 and the downregulation of it whether results in lipid accumulation in hepatocytes are not well known. To address this question, we crossed *AlbCre*+/− mice with *TRPV2*\textsuperscript{floox/floox} mice to generate *TRPV2*\textsuperscript{floox/floox};*AlbCre*+/− mice. By using these mice, we established the HFD model and found that specific deletion of TRPV2 in hepatocytes exacerbated the increase in body weight and hepatic steatosis, indicating that TRPV2 expressed in hepatocytes plays a protective role against lipid accumulation. These findings are consistent with the reported results from global TRPV2 knockout mice (TRPV2GKO). Tominaga’s group found mice lacking TRPV2 increased the expression of genes related to lipid accumulation, such as lipoprotein lipase (LPL), and a cluster of differentiation 36 (CD36), and TRPV2GKO mice had significant increases in body weight and adipose tissues upon an HFD treatment.

Next, we further investigated the underlying mechanisms of the effects of TRPV2 on lipid accumulation in hepatocytes. Because HepG2 cells are expressed TRPV2\textsuperscript{20}, we conducted in vitro experiments to observe the effects of TRPV2 activation on lipid accumulation by applying a TRPV2 agonist or antagonist. Firstly, cell viability was determined by using the CCK8 kit when the TRPV2 agonist or its
antagonist at different dose was co-incubated for 24 hours and we found either agonist or antagonist did not alter cell viability from 0.5 µM to 100 µM. Subsequently, we observed the level of lipid accumulation and TG amount after PA treatment which is usually used to establish in vitro models of NAFLD\textsuperscript{24}. In agreement with the \textit{in vivo} findings, activation of TRPV2 significantly inhibited lipid accumulation and the amount of TG, and this inhibitory effect disappeared in the presence of a TRPV2 antagonist. Next, we tested the expression of \textit{p21}, cyclin-dependent kinase 2 (CDK2) and \textit{p16}. \textit{p21} is one of the most well-established senescence markers and it promotes cell cycle arrest by blocking progression through G1/S when associated with CDK2\textsuperscript{25–27}. And \textit{p16} is another common and reliable senescence marker\textsuperscript{28}. We found that the expression of \textit{p21} and \textit{p16} was significantly inhibited when TRPV2 was activated, and this activation was compromised when its antagonist was added. We also found CDK2 was up-regulated when TRPV2 was activated, and the TRPV2 antagonist blocked this up-regulation. CDK2 was reported has the ability to suppress senescence, and it interacts with Myc at promoters, where it regulates \textit{Bmi-1}, \textit{p16}, \textit{p21}, and \textit{hTERT}, which encode proteins known to control senescence\textsuperscript{29}. It is implied that the activation of hepatic TRPV2 might modulate cellular senescence. Indeed, recent studies have demonstrated senescence plays an important role in NAFLD\textsuperscript{7,30,31}. Finally, we examined HFD-induced body hepatic steatosis and the expression of \textit{p21} and \textit{p16} in livers when the TRPV2 agonist, probenecid, was administrated. In agreement with the \textit{in vitro} findings, the administration of probenecid significantly inhibited the increase of body weight, hepatic steatosis, and the expression of \textit{p21} and \textit{p16} in livers. According to the \textit{in vitro} and \textit{in vivo} findings, it is implied that TRPV2 activation exhibits its protective role against lipid accumulation via suppression of cellular senescence.

In summary, this study reveals a novel function of hepatic TRPV2 in regulating the development of diet-induced fatty liver in mice. Targeting TRPV2 expression and function in the liver may be a potential therapy for fatty liver and NAFLD.

4. Materials And Methods

4.1. Animals

All animal procedures were approved by the Animal Care and Use Committee of Health Science Center at Shenzhen University and complied with relevant ethical guidelines. Male C57BL/6 mice (8–12 weeks of age) were purchased from Guangdong Province Laboratory Animal Center (Guangzhou, China). \textit{Albumin}-\textit{Cre} mice (\#: 003574) were purchased from Jackson Laboratory. The \textit{Trpv2-Flox} (NM-CKO-00023) mice were purchased from Shanghai Model Organisms. The animals were housed in plastic cages (5 per cage) in a temperature-controlled environment on a 12 h/12 h light/dark cycle. Food and water were available \textit{ad libitum}.

4.2 Dietary interventions and the administration of TRPV2 agonist in vivo
Male mice were fed a high fat and carbohydrate diet (HFD) with 42% kcal from fat and containing 0.1% cholesterol with a high fructose-glucose solution (SW, 23.1 g/L d-fructose + 18.9 g/L d-glucose) for 12 weeks\(^32\). Control mice were fed a standard chow diet (Chow) with normal water (NW). The TRPV2 agonist, probenecid, was dissolved in water containing 5% sucrose at a concentration of 0.5 mg/mL. Both probenecid-added and sucrose water were administered via bottles in the animals’ home cages to limit intrusion and ensure that all treatment groups were receiving treatment simultaneously. Water was changed twice a week, and the volume was measured before and after the consumption to determine the approximate dose. The calculated daily probenecid dose was 103.6 ± 2.1 mg/kg/day.

4.3 Body and liver weight measurement

The body weight of mouse was recorded every week during the experiment period. After mice were sacrificed, livers were harvested and weighed.

4.4 Histochemical study

For histological examination, livers were dissected and fixed in 10% neutral buffered formalin. The samples were subsequently embedded in paraffin or OCT compound. For hematoxylin-eosin (H&E) staining, tissue sections were cut at 5 µm in thickness and then stained using a commercial kit (HHS123, Sigma-Aldrich, St. Louis, MO). Oil red O staining was performed using oil red O dye (Sigma, St. Louis, MO, United States). In brief, the HepG2 cells were fixed with 4% formalin and incubated at room temperature (RT) for at least 1 h. Subsequently, cells were washed twice with purified water and then washed with 60% isopropanol at RT for 5 min. The cells were dried completely, and oil red O solution was added and then incubated at RT for 10 min. Oil red O solution was removed by the addition of purified water, and the cells were washed 4 times with purified water. Images were acquired using a microscope (Olympus, Tokyo, Japan). For the determination of triglyceride levels, water was removed, and the cells were dried completely. Oil red O dye was eluted with 100% isopropanol and incubated with gentle shaking for 10 min. The OD values were measured at 490 nm using a spectrophotometer (Thermo Scientific, Waltham, MA, United States) with 100% isopropanol as a blank.

4.5 Primary hepatocyte, HepG2 cell culture and cellular NALFD model

Primary hepatocytes were isolated using a rapid two-step perfusion method and cultured as described previously\(^33\). The HepG2 cell line (Cell Bank, Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (DMEM 11965-09; Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotics (Biological Industries) and maintained at 37°C in a humidified 5% CO\(_2\) and 95% air atmosphere. A cell NALFD model was established by exposing cells to 200 µM Palmitic acid (PA, P0500; Sigma-Aldrich) conjugated to fatty acid-free bovine serum albumin (BSA) (A8806; Sigma-Aldrich) for 24 h, as previously described\(^34\). Cells treated with BSA were used as controls.

4.6 Measurement of triacyl glyceride (TG)
Tissue triglyceride was extracted using the Folch extraction method (J Biol Chem 226, 497–509. 1957), and the amount was then measured with a commercially available kit (Biovision, Triglyceride Quantification Colorimetric/Fluorometric Kit K622, Milpitas, CA), according to the manufacturer's instructions. Briefly, tissues in 5% NP40 solution were homogenized and then boiled for 5 min. Five microliters of extract obtained by centrifugation (10,000xg for 2 min) was diluted with 130 µL of TG assay buffer supplied from the kit to make TG solution. Fifty microliters of TG solution was reacted with 2 µL of lipase (kits) at room temperature for 20 min, and the digested TG solution was then mixed with 50 µL of TG reaction mix supplied from a kit. After incubation at room temperature for 60 min, the absorbance was measured at 570 nm by a microplate reader (BIO-RAD, Hercules, CA, USA). The amount of TG was calculated using a standard curve.

### 4.7 CCK8 assay

Cell viability was analyzed by Cell Counting Kit-8 (cat# C0037, Beyotime, Shanghai, China) according to the manufacturer's protocols. Cells were seeded at a density of 2000/well into 96-well microplates. After 24 h, 10 µl of CCK-8 reagent was added to each well and then cultured for 2 h. The absorbance was analyzed at 450 nm using wells without cells as blanks.

### 4.8 Western blot analysis

The liver tissues or cells were extracted by sonication in lysis buffer (Invitrogen, Carlsbad, CA) containing Tris, 2.3% sodium dodecyl sulfate (SDS) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The nuclear fraction was isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Pierce Biotechnology). The cell debris was eliminated and the supernatant was collected. Protein amounts were determined with a DCTM protein assay kit (Bio-Rad, Hercules, CA, USA) by using BSA as the standard by the Lowry method. Protein (15 µg) from each sample was mixed with SDS loading buffer, separated by SDS polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked via incubation overnight at 4°C in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 7% skim milk and then incubated with the primary antibody with shaking for 1 h at room temperature. After washing, the specific bands were visualized by further incubation with horseradish peroxidase (HRP)-conjugated second antibody followed by enhanced chemiluminescence detection using the ECL system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The PVDF membrane was photographed using a ChemiDocTM XRSC imaging system (Bio-Rad, United States) or the membranes were exposed to Fuji Medical X-ray film (Tokyo, Japan). The densities of protein blots were analyzed using ImageJ (National Institutes of Health, United States). The protein bands were detected with antibodies against TRPV2 and p16 (ab272862 and ab189034, Abcam, Cambridge, UK), p21 and CDK2 (64016 and 18048, Cell Signaling Technology, Beverly, MA, USA).

### 4.9 Intracellular free calcium measurement

Primary cultured hepatocytes were loaded with Fura2-AM (2 µmol/L, Invitrogen) for 60 min at room temperature in the Ca$^{2+}$ imaging buffer. Ca$^{2+}$ imaging was measured using the PTI Fluorescence Master
Systems (Photon Technology International, Birmingham, NJ, USA). Ca\(^{2+}\) imaging protocol was a ratio metric method with 340/380-nm wavelength light for dual excitation. Data were presented as DR/R0, determined as the fraction DR(Rt-R0) of the increase of a given ratio over baseline ratio (R0).

4.10 Immunofluorescence staining

Primary cultured hepatocytes from WT, \(\text{TRPV2}^{\text{flo}}x/\text{flo}x;\text{AlbCre}^{-/-}\) or \(\text{TRPV2}^{\text{flo}}x/\text{flo}x;\text{AlbCre}^{+/-}\) mice were fixed with 10% formalin at room temperature for 1 hour and then bathed in a 2% hydrogen peroxide methanol solution for 0.5 hours. The cells were incubated with TRPV2-specific antibodies (1/100; #ACC-039, Alomone, Israel) overnight at 4°C. After washing out, cells were incubated for 2 h with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG) at room temperature. Images were obtained with a TE2000-U Nikon eclipse microscope and analyzed with NIS-Elements imaging software (Nikon, Japan).

4.11 Statistical analysis

The data are expressed as means ± SEM and analyzed with a t-test or ANOVA. All statistical analyses were performed using GraphPad Prism 8.0. (GraphPad Inc., La Jolla, CA, USA). Significance was defined as P < 0.05.

Declarations

Author contributions: Conceptualization: Q.L.; Methodology: P.W., L.L., C.R., M.J., H.Z., K.Y., Y.W. and M.J.; Writing: H.P. and J.G.; Supervision: J.G. and Q.L. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: The authors declare no conflicts of interest.

References


**Figures**

Figure 1

A downregulation of TRPV2 expression in livers and hepatocytes of HFD-fed mice. (A) Schematic of the experimental design. The adult mice were fed with HFD or Chow (control) for 12 weeks. (B) The changes in body weight in the HFD-fed group (n=8) and control group (n=8) for 12 weeks. (C,D) The liver weight and the ratio of liver to body weight from the HFD-fed group (n=5) and the control group (n=5) at the 4th and 12th week after the onset of HFD or Chow. (E) The expression level of TG in the liver at the 4th and 12th week after the onset of HFD (n=6) or Chow (n=6). (F) Representative HE staining of liver sections (Left) and the NAFLD score (Right) at the 12th week after the onset of HFD. Scale bar, 100 μm. (G,H) The TRPV2 expression after HFD feeding at the 1st, 4th and 12th were examined by western blotting and the
quantification (H, n=3). (I) Immunostaining images of primary cultured hepatocytes from the HFD-fed group (n=5) and the control group (n=5) at the 12th week after the onset of HFD or Chow. The green is against albumin, the red is against TRPV2 and the blue is for dapi. Scale bar, 100 μm. (J) The ratio of TRPV2 to albumin. Data are presented as mean ± SEM. *<0.05, **<0.005, ***<0.001, ****<0.0001. B, C, D, F and H were determined by two-way ANOVA, F and J were determined by unpaired t-test. Data are representative of biologically independent replicates.

Figure 2

**An impairment of hepatic TRPV2-mediated calcium response in HFD-fed mice.** (A) Representative images of the hepatic calcium response to 2-APB (0.5mmol/L for 1 min) in the HFD-fed group or the control group. Scale bar, 50 μm. (B) Time course traces show the hepatic calcium response to 2-APB (0.5mmol/L for 1 min) in the HFD-fed group (n=5) or the control group (n=5). Data are presented as mean ± SEM. *<0.05, two-way ANOVA, Data are representative of biologically independent replicates.
Figure 3

Conditional knockout hepatic TRPV2 in hepatocyte aggravated HFD-induced hepatic steatosis. (A) Schematic of the experimental design. (B) Immunostaining images of primary cultured hepatocytes against TRPV2 from TRPV2<sup>flox/flox</sup>,Cre<sup>−/−</sup> or TRPV2<sup>flox/flox</sup>,Cre<sup>+/−</sup> mice. (C) The changes of body weight in TRPV2<sup>flox/flox</sup>,Cre<sup>−/−</sup> and TRPV2<sup>flox/flox</sup>,Cre<sup>+/−</sup> mice with Chow or HFD feeding for 12 weeks (n=6 in each group). (D) The body weight from each group at the 4th week after the onset of HFD or Chow feeding (n=6 in each group). (E) The expression level of TG in the liver from each group at the 4th week after the onset of HFD or Chow (n=6 in each group). (F, D) Representative HE staining (F) and the NAFLD score (G) of liver sections from each group at the 4th week after the onset of HFD or Chow. Scale bar, 100 μm. Data are presented as mean ± SEM. *<0.05, **<0.005, ***<0.001, ****<0.0001. C was determined by two-way ANOVA, D, E and G were determined by one-way ANOVA. Data are representative of biologically independent replicates.
Figure 4

**Effects of TRPV2 in HepG2 cells on PA-induced lipid accumulation.** (A) Schematic of the experimental design. (B) The cell viability of HepG2 cells incubated with 2-APB or SKF in different doses. (C) The representative images of Oil Red O staining of HepG2 cells treated with PA in the presence of 2-APB or 2-APB&SKF. Scale bar, 50 μm. (D) The OD value from each group (n=5 in each group). (E) The expression
level of TG from each group (n=5 in each group). Data are presented as mean ± SEM. *<0.05, **<0.005, ***<0.001, ****<0.0001. one-way ANOVA. Data are representative of biologically independent replicates.

**Figure 5**

Effects of TRPV2 in HepG2 cells on p21 signaling. (A) Schematic of the experimental design. (B-D) The expression of p21, CDK2 and p16 (n=5 in each group) in HepG2 cells treated with PA in the presence of 2-APB or 2-APB&SKF. Data are presented as mean ± SEM. *<0.05, **<0.005, ****<0.0001. one-way ANOVA. Data are representative of biologically independent replicates.

**Figure 6**

Effects of TRPV2 *in vivo* on HFD-induced lipid accumulation and the expression of p21 signaling in the liver tissue. (A) Schematic of the experimental design. (B) The changes of body weight with HFD feeding.
with probenecid or vehicle for 12 weeks. (C,D) The liver weight and the ratio of liver to body weight from the HFD-fed group (n=5) and the control group (n=5) at the 12th week after the onset of HFD. (E) Representative HE staining of liver sections (Left) and the NAFLD score (Right) at the 12th week after the onset of HFD with probenecid or vehicle. Scale bar, 100 μm. (F) The expression of p21 and p16 (n=5 in each group) in the liver tissue from the HFD-fed mice with probenecid or vehicle administration. Data are presented as mean ± SEM. *<0.05, **<0.005, ****<0.0001. B, B was determined by two-way ANOVA, D and E were determined by unpaired t-test. F was determined by one-way ANOVA. Data are representative of biologically independent replicates.