

Salidroside-Mitigated Inflammatory Injury of Hepatocytes with Non-Alcoholic Fatty Liver Disease via Inhibition TRPM2 Ion Channel Activation

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Qi Feng
Chen Liu
Wei Gao
Xiao-ling Geng
Ning Dai 

Department of Gastroenterology, The First Affiliated Hospital of Dalian Medical University, Dalian 116011, Liaoning Province, People's Republic of China

Purpose: Oxidative stress plays an important role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). TRPM2 ion channel functions as a molecular sensor for oxidative stress. The aim of this study was to examine the protective effects of Salidroside, a powerful antioxidative plant, on TRPM2 in an established in vitro model of NAFLD.

Methods: NAFLD model was established by palmitic acid (PA) in hepatic L02 cell lines and was added to the media at a final concentration of 400 μM . Cells were used as normal group, PA group and PA receiving varied concentrations of Salidroside (75 $\mu\text{g}/\text{mL}$, 150 $\mu\text{g}/\text{mL}$, 300 $\mu\text{g}/\text{mL}$). After treating 24 hrs, MTT assay was used to detect cell viability, and ALT level was measured using an appropriate kit assay. Intracellular lipid accumulation was observed by Oil red O staining. Cytosolic Ca^{2+} concentrations were evaluated by flow cytometer with Fluo-3/AM. Quantitative RT-PCR was used to measure the mRNA expression of TRPM2, IL-1 β and IL-6, and the protein expressions of TRPM2, p-CaMKII and autophagy (LC3B, p62) were determined using Western blot.

Results: Treatment with Salidroside effectively restored liver injury and alleviated lipid droplet deposition in a dose-dependent manner, which was associated with inhibition of TRPM2/ Ca^{2+} /CaMKII pathway. Additionally, autophagic clearance was enhanced by intervention with Salidroside in a dose-dependent manner. Further investigation indicated that Salidroside down-regulated the mRNA expression of IL-1 β and IL-6-pro-inflammatory cytokines.

Conclusion: These results suggest that Salidroside could alleviate inflammatory injury and steatosis via autophagy activation mediated by downregulation of the TRPM2/ Ca^{2+} /CaMKII pathway. Targeting the TRPM2 ion channel is a novel treatment strategy for oxidative stress-induced liver in NAFLD.

Keywords: non-alcoholic fatty liver disease, salidroside, TRPM2 ion channel, autophagy, cytokines

Introduction

Non-alcoholic fatty liver disease (NAFLD) is regarded as the most significant chronic liver dysfunction in Western countries. NAFLD is characterized by fat accumulation in the liver in the absence of excessive alcohol consumption.¹ NAFLD is associated with insulin resistance, obesity, type II diabetes mellitus, and dyslipidemia. NAFLD is a spectrum of hepatic abnormalities extending from simple steatosis to steatohepatitis and steatofibrosis. Non-alcoholic steatohepatitis (NASH), the inflammatory condition of NAFLD characterized by neutrophil infiltration and hepatocyte ballooning, can eventually evolve to fibrosis, cirrhosis, and

Correspondence: Ning Dai
Department of Gastroenterology, The First Affiliated Hospital of Dalian Medical University, Zhongshan Road, Dalian, 116011, Liaoning Province, People's Republic of China
Tel +86 411 83635963
Fax +86 411 83635965
Email ning_dldn@outlook.com

hepatocellular carcinoma. Approximately one-third of people with NAFLD will develop NASH.² In addition to the elevated risk for liver disease, NAFLD is also associated with an increased risk of cardiovascular disease.³ Despite its severity and high prevalence, currently there is no pharmacological agent for the treatment of NAFLD/NASH.⁴

Progression from NAFLD to NASH has been modeled as a “double-hit” hypothesis.⁵ The first hit involves excess hepatic triglyceride and cholesterol accumulation (i.e., steatosis).⁶ This sensitizes the liver to the second hit, consisting of elevated hepatic oxidative stress and inflammation.⁷ According to the double-hit theory, oxidative stress is a major player triggering the progression of steatosis to NASH as a result of an imbalance between pro- and antioxidants that lead to liver cell damage. Although the precise mechanisms involved in the progression of NAFLD/NASH are poorly understood, blocking oxidative stress may be an effective treatment for NAFLD/NASH.²

Intracellular calcium ions (Ca^{2+}) regulate a diverse set of cellular processes in all cells and tissues of the body, including liver diseases.⁸ Recently, a subset of transient receptor potential (TRP) ion channels have attracted attention because of their permeability to Ca^{2+} . TRP channels are a superfamily of monovalent and divalent cation-permeable ion channels. Members of the melastatin subfamily, TRPM, have been shown to have important roles in cell proliferation and survival.⁹ TRPM2, the second member of this subfamily to be cloned, is a 1503-amino acid channel permeable to Ca^{2+} , Na^+ , and K^+ . Most members of the TRP family of ion channels have been shown to be present in hepatocytes.¹⁰ Arguably, the most important activator of TRPM2 channel is oxidative stress.¹¹ TRPM2 knockdown reduced acetaminophen-induced liver injury related to oxidative stress;¹² thus, it has emerged as a potential therapeutic target in fighting oxidative stress-related liver diseases.¹³

Salidroside (Sal), a phenylpropanoid glycoside compound [2-(4-hydroxyphenyl)-ethyl- β -D-glucopyranoside], is the active ingredient of *Rhodiola rosea*, a plant growing at high-altitude zones that has excellent antioxidant properties.¹⁴ Multiple lines of evidence suggested that Sal could effectively improve liver diseases induced by some chemical toxins.^{15–18} Studies also show that Sal markedly protected against inflammatory injury of hepatocytes with NAFLD through scavenging ROS production.¹⁹

Materials and Methods

Materials

Human L02 hepatic cell lines (Wanleibio, Shenyang, China), RPMI-1640 medium and fetal bovine serum (Gibco, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)(Sigma, USA), dimethyl sulfoxide (DMSO) (Sigma); Oil Red O (Sigma), fluo-3/AM kit (Beyotime Biotech, Shanghai, China), palmitic acid (Sigma), alanine aminotransferase (ALT) kit (Nanjing Jiancheng Bioengineering Institute, China), and Salidroside (purity >98%, Aladdin Biotech, Shanghai, China). The antibodies included anti-TRPM2 (Proteintech, Wuhan, China), anti-phospho-CaMKII (Proteintech), anti-LC3B (Wanleibio), anti-p62 (Wanleibio) and goat anti-rabbit secondary antibody (Wanleibio); Trizol reagent (Invitrogen, CA, USA); Reverse Transcription Kit (BioTeke, Beijing, China); Sybr Green (Solarbio, Beijing, China).

Cell Culture

Human L02 hepatic cell lines were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . At logarithm growth phase, L02 cells were selected to conduct follow-up experiments.

Cell Viability Assay

L02 cells were plated in 96-well plates at a density of 3×10^3 cells per well and cultured for 12 hrs. After treatment with palmitic acid (PA, 400 μM)²⁰ in the presence of different dosages of Sal (10, 30, 60, 75, 150, 300 $\mu\text{g}/\text{mL}$) for 24 hrs, 50 μL of MTT were added into the media for 4 hrs. The media were removed and DMSO was added to each well. The absorbance was measured in a microplate reader (BioTek, USA) at 570 nm, and the relative cell viability was recorded as a ratio with the normal control (untreated cells, fresh medium only). Five replicate wells were counted for each condition.

NAFLD Model in L02 Cells and Sal Intervention

NAFLD model was induced by PA and was added to the media at a final concentration of 400 μM in hepatic L02 cell lines.²⁰ Hepatic L02 cells ($3 \times 10^3/\text{well}$) were plated into 96-well plates and were divided into five groups: normal group (untreated cells, fresh medium only), PA group and PA plus Sal intervention groups (low dose group, middle dose group and high dose group). After 24

hrs of treatment, hepatocytes were harvested for following assays.

Determination of ALT Content in the Cell Supernatant

The cultured cell supernatant was collected, and ALT content were measured using commercial kits according to the manufacturer's protocol.

Oil Red O Staining

Cells were stained with Oil red O to examine the amount of lipid accumulation. The cultured L02 cells grown on cell plates were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 1 hr. Then, the fixed cells were washed with 60% isopropanol for 30 s and PBS three times. In darkness, the cells were stained with freshly diluted Oil Red O working solution for 1 hr at 37°C. The cells were washed with PBS two times and ultimately observed and photographed by an inverted phase-contrast microscope. The positive area for Oil Red O staining was measured with the ImagePlus 6.0 software (Media Cybernetics, Bethesda, Maryland).

Cytosolic Ca²⁺ Measurement

Cytosolic calcium ([Ca²⁺]_c) was evaluated by the cell-permeable calcium-sensitive fluorescent dye Fluo-3/AM. The cells were incubated with 5 μM Fluo-3/AM for 45 mins at 37°C to load fluo-3 AM into the cell. After loading, cells were washed twice with PBS, the fluorescence intensity of [Ca²⁺]_c was analyzed by flow cytometric analysis.

Quantitative Real-Time PCR Analysis

For the quantitative real-time PCR (qRT-PCR) analysis, total RNA was isolated from cultured cells with Trizol reagent according to the manufacturer's instructions. Reverse transcribed into cDNA using a Reverse Transcription Kit. Quantitative PCR assays were performed with Sybr Green and real-time PCR detection system (Applied Biosystem). Amplicon expression in each sample was normalized to that of β-actin, and after normalization, gene expression was quantified using the 2^{-ΔΔCt} method. The primer sequences are given as follows: TRPM2-forward, 5'- CCCAGAACTGCTC ACCC -3', TRPM2-reverse, 5'- ACACGCCACAGCC CATT -3'(GenBank ID:NM_015760); IL-1β-forward, 5'- CGAATCTCCGACCACCACTA -3', IL-1β-reverse, 5'-

GCACATAAGCCTCGTTATCCC -3'(GenBank ID: NM_00 0454); IL-6-forward, 5'- AATAACCACCCCTGACCCAA -3'; IL-6- reverse, 5'- CCAGAAGAAGGAATGCCATT (GenBank ID: NM_031459); β-actin-forward, 5'- GGCAC CCAGACAATGAA, β-actin- reverse, 5'- TAGAAGCATT TGCGGTGG-3'.

Western Blot Analysis

The cultured cells were lysed in cold RIPA buffer. The cells' total protein was separated by SDS-PAGE (Bio-Rad, CA, USA) and electro-transferred onto the polyvinylidene difluoride (PVDF) membranes. Furthermore, the PVDF membranes were incubated overnight at 4°C with following specific primary antibodies: TRPM2 (1:1000), p-CaMKII (1:1000), LC3B (1:500), p62 (1:500) and β-actin (1:1000). The appropriate secondary antibodies (1:5000) were used to tag primary antibody for 3 hrs and an enhanced chemiluminescence (ECL) detection system (Beyotime Biotech) was used to develop the immunoreactive bands. Finally, the protein band densities were quantified with Gel-Pro-Analyzer (Version 5.0; Media Cybernetics, Rockville, MD, USA). Targeted protein expression levels were quantitated relative to the β-actin level in the same sample and normalized to the control group, which was set as one-fold.

Statistical Analysis

All data are presented as the mean of at least three independent experiments ± S.D. Analysis of variance was conducted by one-way analysis of variance (ANOVA) using least significant difference method (LSD) on the SPSS statistical package (version 18.0 for Windows, SPSS Inc.). The level of statistical significance was set at *p*<0.05.

Results

Sal Alleviated PA-Induced Cell Injury and Lipid Accumulation in Hepatic L02 Cells

MTT results demonstrated that compared to the PA-treated cells, administration of 75 μg/mL Sal to the PA-treated cells could dramatically recover cell viability (Figure 1A). We proceeded to examine Sal at 75 μg/mL (low dose group), 150 μg/mL (middle dose group), and 300 μg/mL (high dose group). Alanine aminotransferase (ALT), a sensitive indicator of hepatocyte injury, was elevated in the PA group compared with normal controls (Figure 1B), indicating that cytotoxic damage occurred in response to PA treatment. Sal

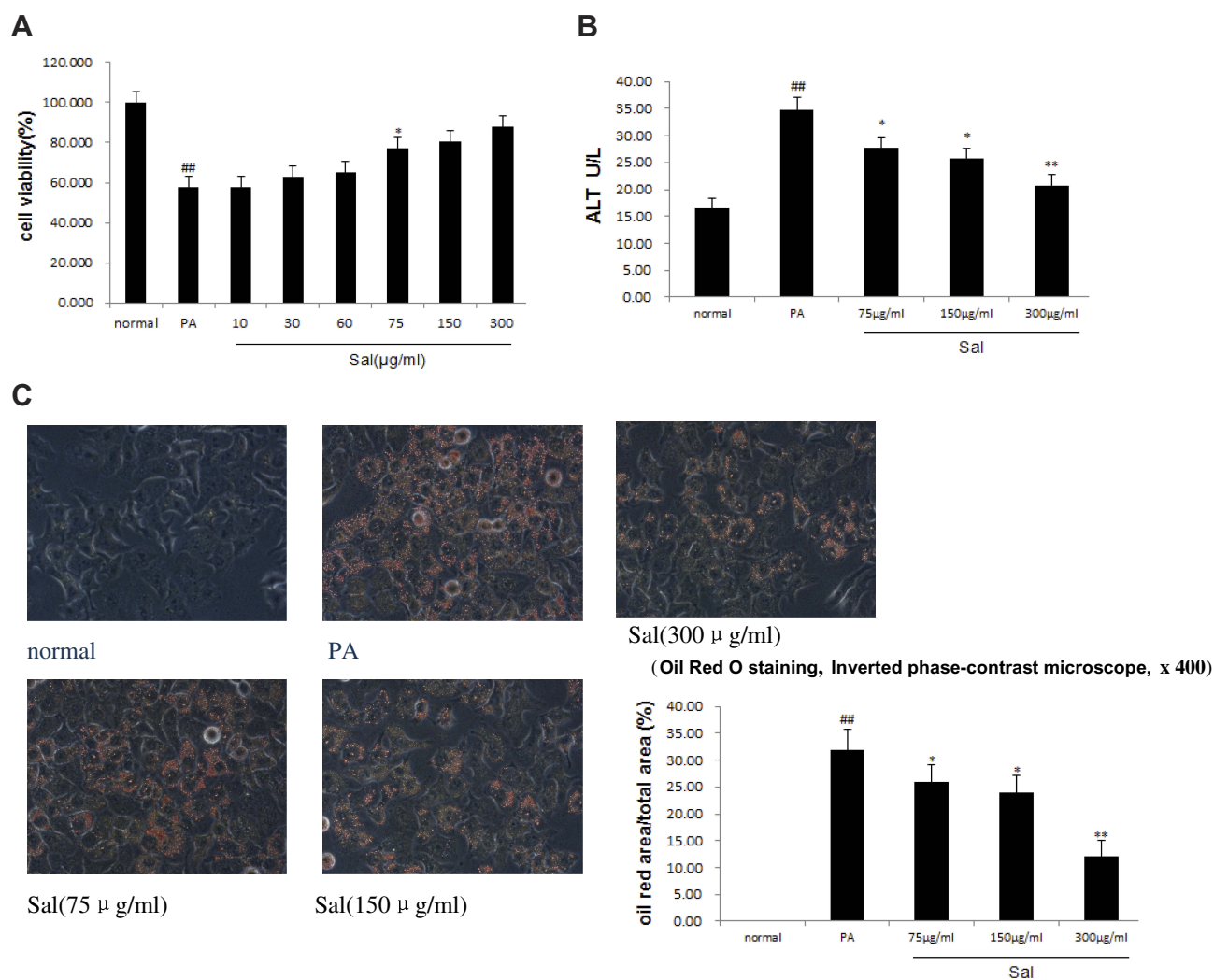


Figure 1 Sal dose-dependent alleviates PA-induced cell injury and lipid accumulation in hepatic L02 cells.

Notes: PA was added to the media at a final concentration of 400 μM in hepatic L02 cell lines and PA receiving varied concentrations of Salidroside (75 μg/mL, 150 μg/mL, 300 μg/mL). (A) Cell viability. (B) ALT. (C) Oil red O staining (inverted phase-contrast microscope) and the positive area for Oil Red O staining. Data were shown as mean ± SD (n=3), ^{##}P<0.01 vs normal group; ^{*}P<0.05, ^{**}P<0.01 vs PA group.

Abbreviations: PA, palmitic acid; ALT, alanine aminotransferase.

prevented the adverse effects of PA-induced cell injury in a dose-dependent manner. Furthermore, Oil red O staining showed that Sal was able to reduce lipid droplet massive deposition in PA-treated L02 cells in a dose-dependent manner (Figure 1C). These results suggest that Sal has protective effects against biological symptoms of NAFLD mimicked in vitro in the hepatic L02 cell line.

The Protective Effect of Sal Against NAFLD Involved Inhibition of TRPM2/Ca²⁺/CaMKII Pathway

TRPM2 ion channel is a Ca²⁺ permeable, non-selective cation channel. As shown in Figure 2, Western blot and

qPCR analysis showed that TRPM2 channel protein and mRNA expressions markedly increased in L02 cells treated with PA compared with normal controls (Figure 2A and B). Sal significantly inhibit the increased protein and mRNA expressions of TRPM2 induced by PA in a concentration-dependent manner. To explore whether TRPM2 activation is associated with increased [Ca²⁺]_c, we examined the fluorescence intensity of [Ca²⁺]_c by flow cytometric analysis using the fluorescent probe Fluo-3/AM (Figure 2C). The [Ca²⁺]_c was found to be increased about three fold in cells treated with PA. Sal was able to reduce the PA-induced [Ca²⁺]_c increase in a concentration-dependent manner. Simultaneously, p-CaMKII protein expression,

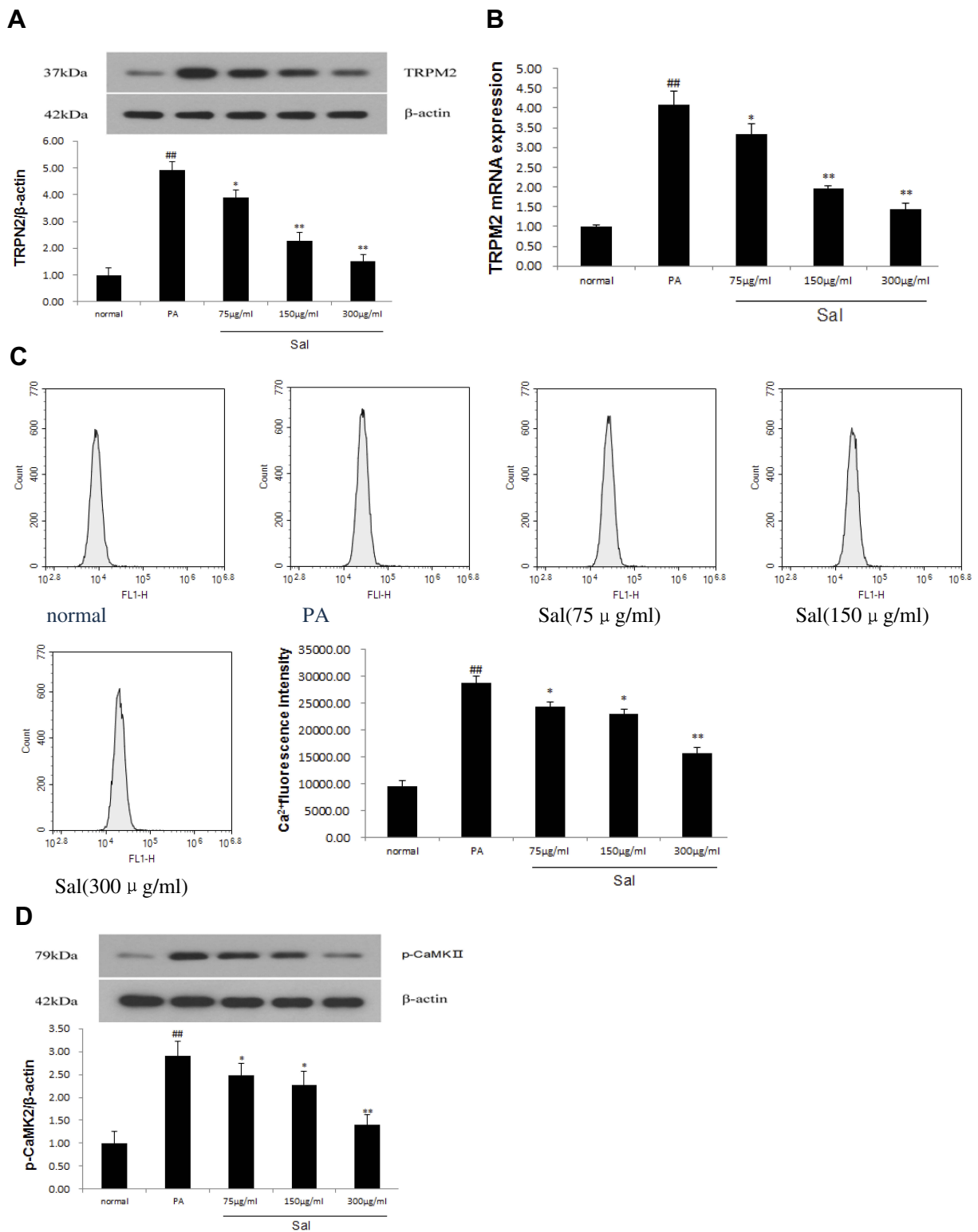


Figure 2 Sal dose-dependent inhibited TRPM2/Ca²⁺/CaMKII pathway in L02 cells treated with PA.

Notes: PA was added to the media at a final concentration of 400 μ M in hepatic L02 cell lines and PA receiving varied concentrations of Salidroside (75 μ g/mL, 150 μ g/mL, 300 μ g/mL). **(A)** Western blot analysis of TRPM2 protein. **(B)** qPCR analysis of TRPM2 mRNA. **(C)** Fluorescence intensity of [Ca²⁺]_i was detected by flow cytometer with Fluo-3/AM. **(D)** Western blot analysis of p-CaMKII protein. Data were shown as mean \pm SD (n=3), ^{##}P<0.01 vs normal group; ^{*}P<0.05, ^{**}P<0.01 vs PA group. **Abbreviations:** PA, palmitic acid; TRPM2, transient receptor potential melastatin 2; CaMKII, calmodulin-stimulated protein kinase II.

a downstream target of $[Ca^{2+}]_c$, was up-regulated in PA-treated L02 cells, but could be progressively ameliorated by increasing dosages of Sal (Figure 2D). Our results demonstrated that Sal significantly downregulated the phosphorylation of CaMKII, suggesting that TRPM2/ Ca^{2+} /CaMKII signaling pathway was involved in hepato-protective benefits of Sal against NAFLD.

Sal Enhanced Autophagy in L02 Cells Treated with PA

Previous studies reported that autophagy enhancement might be responsible for the protective role during NAFLD/NASH.²¹ As a result, we measured autophagy in hepatic cells after Sal treatment. LC3B, an autophagy indicator, which correlates with the content of autophagosomes reflects the activity of autophagosomes. We used Western blotting to examine the expression of LC3B in PA-stimulated L02 cells. Sal treatment of L02 cells increased the relative expression of LC3B-II and the LC3B-II/LC3B-I ratio compared to PA group in a dose-dependent manner (Figure 3A). To corroborate this finding, we measured another autophagy-related protein – p62. Protein degradation of p62 was increased in L02 cells treated with Sal (Figure 3B). These results confirm that Sal strengthened autophagic clearance in a cell line exogenously treated to mimic some of the cellular phenotypes of NAFLD.

Effect of Sal Intervention on the Expressions of IL-1 β , IL-6 mRNA in PA-Stimulated L02 Cells

Finally, we further investigated the mRNA expression of IL-1 β and IL-6 in hepatic L02 cells treated with PA. qPCR showed that the expression of IL-1 β and IL-6 mRNA was significantly up-regulated in hepatic L02 cells treated with PA. Sal intervention notably down-regulated the expressions of both IL-1 β and IL-6 mRNA in L02 cells (Figure 4A and B).

Discussion

Lipotoxicity refers to cellular toxicity in the presence of excessive free fatty acids. Fatty acid-induced lipotoxicity in hepatocytes plays an essential role in the pathogenesis of nonalcoholic fatty liver disease.²² Fatty acids are chemically classified as saturated and unsaturated, and their structure affects cell biological functions. Palmitic acid, a saturated fatty acid, is the most toxic lipid species.²³ In the current study, we investigated the direct effect of Sal on PA-induced hepato-lipotoxicity in vitro. Our data support the possibility that Sal could attenuate the progression of disease symptoms associated with NAFLD via regulation of the TRPM2/ Ca^{2+} /CaMKII pathway and inflammatory cytokines.

Excessive formation of ROS and subsequent oxidative stress occupy an important position in the pathogenesis of NAFLD/NASH.²⁴ Studies in vivo and in vitro showed higher

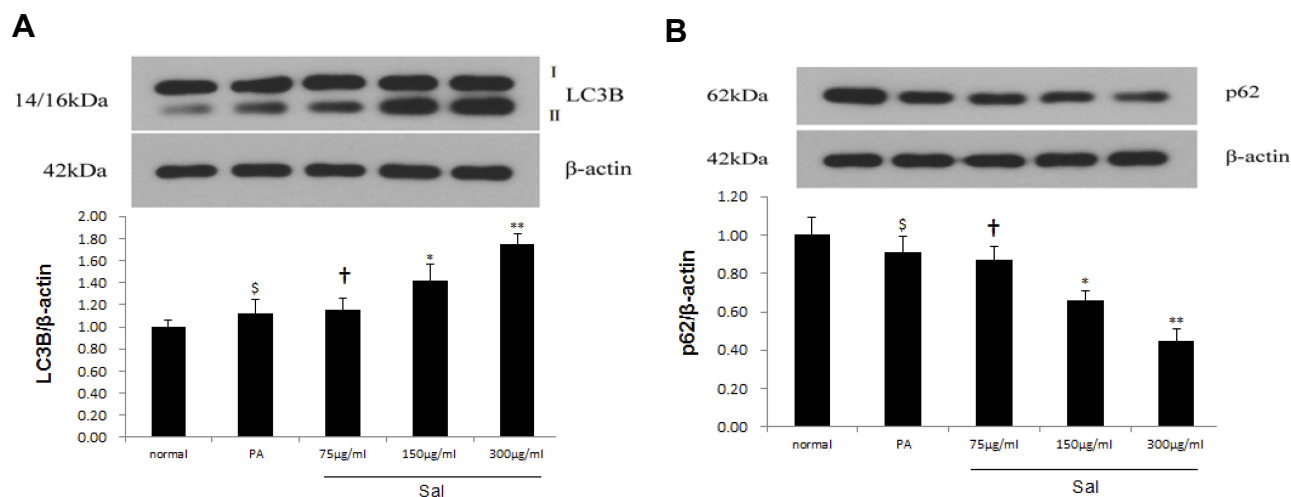


Figure 3 Sal reinforced autophagy in PA-stimulated hepatic L02 cells.

Notes: PA was added to the media at a final concentration of 400 μ M in hepatic L02 cell lines and PA receiving varied concentrations of Salidroside (75 μ g/mL, 150 μ g/mL, 300 μ g/mL). (A) Western blot analysis showed that the dose dependence of Sal induced upregulation of the LC3B-II expression and the ratio of LC3B-II to I. (B) Western blot analysis showed that the dose dependence of Sal downregulated p62 expression. Data were shown as mean \pm SD (n=3), $^{\$}p > 0.05$ vs normal group; $^{\dagger}p > 0.05$ vs PA group; $^*p < 0.05$, $^{**}p < 0.01$ vs PA group.

Abbreviations: PA, palmitic acid; LC3B, Light chain 3B.

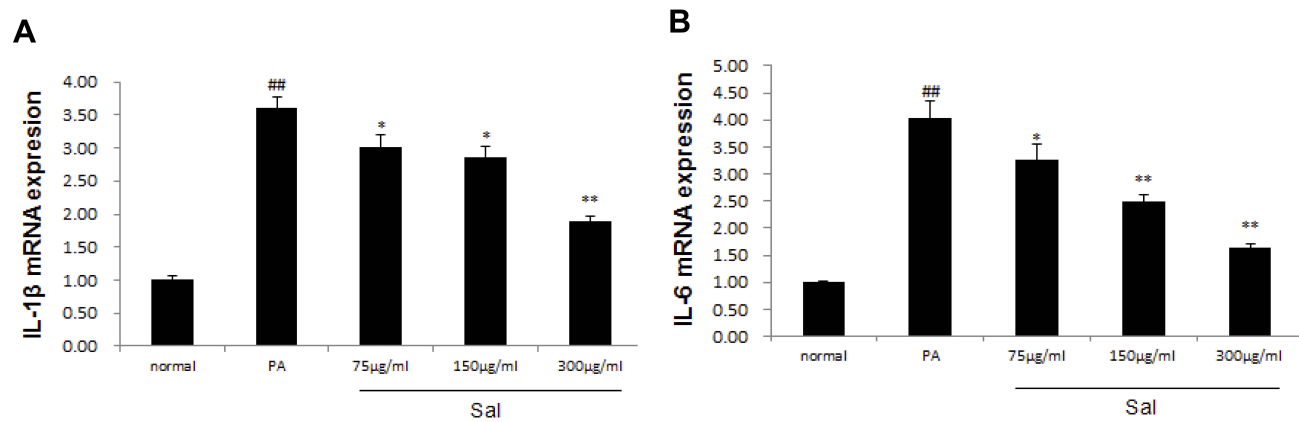


Figure 4 Sal down-regulated the expressions of IL-1 β , IL-6 in L02 cells treated with PA.

Notes: PA was added to the media at a final concentration of 400 μ M in hepatic L02 cell lines and PA receiving varied concentrations of Salidroside (75 μ g/mL, 150 μ g/mL, 300 μ g/mL). (A) qPCR analysis of IL-1 β mRNA. (B) qPCR analysis of IL-6 mRNA. Data were shown as mean \pm SD (n=3), ^{##}P<0.01 vs normal group; ^{*}P<0.05, ^{**}P<0.01 vs PA group.

Abbreviations: PA, palmitic acid; IL-1 β , Interleukin 1 β ; IL-6, Interleukin 6.

free radical activity, including superoxide and hydrogen peroxide (H₂O₂) production, as shown by mitochondrial dysfunction and CYP2E1 up-regulation.^{25,26} TRPM2 ion channel is a cellular sensor of oxidative stress and is widely recognized as an ion channel with an important role in cell survival in several physiological and pathological conditions.¹¹ H₂O₂ induces TRPM2 channel activation and subsequent increase of intracellular Ca²⁺ concentration in various cell types.²⁷ The mode of TRPM2 channel activation by H₂O₂ has long been debated. Accumulating evidence suggests that H₂O₂ can activate TRPM2 channel either directly or indirectly.²⁸ It has been postulated that the activation of TRPM2 channel by oxidative stress is triggered via ADP-ribose production (Citation). Mitochondria are a major source of ADP-ribose. In mitochondria, ADP-ribose is generated by the oxidative stress-induced hydrolysis of NAD⁺, leading to the production of nicotinamide and ADP-ribose.²⁹ Thus, the H₂O₂-induced TRPM2 channel activation is often explained by formation of ADPR. However, H₂O₂ may also directly activate TRPM2 channel. The TRPM2 with a deletion in the C-terminus (TRPM2 Δ C) is insensitive to ADPR, but still responds to H₂O₂, suggesting a direct effect of H₂O₂ on TRPM2.^{30,31} The activated TRPM2 ion channel further triggered Ca²⁺ influx of hepatocytes. Ca²⁺ is a critical second messenger involved in many important signaling pathways. TRPM2 channel knockout mice or pharmacological inhibition of the TRPM2 channel could protect the liver from acetaminophen toxicity.^{12,32} Indeed, the level of intracellular Ca²⁺ was increased dramatically during NAFLD stage and led to lipid accumulation, mitochondrial damage, and ER stress.³³ Ca²⁺/calmodulin-stimulated

protein kinaseII (CaMKII) is an important protein kinase in intracellular Ca²⁺ signaling.³⁴ An increase in cytosolic Ca²⁺ induces phosphorylation of CaMKII.³⁵ Nifedipine (NFD), a calcium channel blocker, attenuated hepatic steatosis through downregulation of p-CaMKII.³⁶ Together with the results above, our research demonstrated that Sal inhibited TRPM2 channel activation of NAFLD-like hepatocytes in a dose-dependent manner, consequently decreasing Ca²⁺ influx and down-regulating of p-CaMKII.

Autophagy is a highly conserved self-digestion process, bring dispensable or potentially dangerous cytoplasmic material, such as damaged organelles and misfolded proteins, to lysosomes for degradation. Autophagy is one of the major downstream events regulated by CaMKII. The increased Ca²⁺ influx via TRPM2 to activate CaMKII and p-CaMKII subsequently phosphorylates BECN1/Beclin1 to suppress autophagy. This sequence of events ultimately renders hepatocytes more vulnerable to damage.³⁷ Autophagy has been implicated as a key regulatory factor in the pathogenesis of NAFLD.²¹ Autophagy activation is considered one of the pathways in lipid clearance (lipophagy) and is associated with blocking of NASH.^{38,39} The expression of LC3-II and the autophagic adaptors p62 are commonly used to monitor autophagy influx. In this study, Sal stimulated LC3BII accumulation and promoted p62 protein degradation, which strengthened the initiation of autophagy. The results suggested that Sal was a reduced autophagy inducer and that autophagy activation could serve as a hepatoprotective mechanism in restoring NAFLD/NASH.

The excessive formation of ROS can drive pro-inflammatory cytokine production, which exacerbates NAFLD progression. Oxidative stress-induced TRPM2 siRNA knockdown attenuated IL-1 β and IL-6 secretion.^{40,41} Pro-inflammatory cytokine IL-1 β is regulated by NLRP3 inflammasome and Sal treatment dramatically suppressed IL-1 β expression in the liver with NAFLD.¹⁹ NASH patients have higher IL-6 levels in serum than simple steatosis.^{42,43} STAT3 phosphorylation induced by IL-6 could accelerate high-fat diet-induced nonalcoholic steatohepatitis in mice.⁴⁴ In this study, qPCR analysis showed that Sal significantly down-regulated the expression of IL-1 β and IL-6 mRNA. We deduce that the protective effect of Sal on NAFLD was at least partly due to its regulation on IL-1 β and IL-6 via TRPM2-mediated signaling pathway.

Conclusion

The results of this study demonstrate that inhibition of TRPM2 ion channel is a novel target that ameliorates oxidative stress-inflammatory injury in liver cells mimicking NAFLD. Sal dramatically attenuated liver injury and lipid accumulation via autophagy induction. Autophagy activation mediated by downregulation of TRPM2/Ca²⁺/CaMKII pathway could serve as an anti-inflammatory strategy in hepatocytes with NAFLD-like symptoms. The present results support further exploration of Sal for the treatment of NAFLD.

Abbreviations

NAFLD, non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; TRPM, transient receptor potential melastatin; Sal, Salidroside; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CaMKII, calmodulin-stimulated protein kinaseII; PA, palmitic acid; ALT, alanine aminotransferase; qRT-PCR, quantitative real-time PCR; LC3B, Light chain 3B; IL, interleukin; ROS, reactive oxygen species.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it

critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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