1 **AMPK α 1 overexpression alleviates the hepatocyte model of nonalcoholic fatty liver disease**
2 **via inactivating p38MAPK pathway**

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14 **Running title: Zhang et al. : AMPK α 1 overexpression alleviates NAFLD**

15 **Abbreviations¹**

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¹ NAFLD, nonalcoholic fatty liver disease; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyl transferase-1; PPAR- α , peroxisome proliferators-activated receptor alpha; ACC, acetyl CoA carboxylase; SREBP-1c, sterol regulatory element binding proteins-1c; FAS, fatty acid synthetase; IL, interleukin; TNF- α , tumor necrosis factor-alpha; p38MAPK, p38 mitogen-activated protein kinase.

Abstract

Nonalcoholic fatty liver disease (NAFLD) has a wide spectrum of liver damage with a worldwide prevalence of almost 20%. AMP-activated protein kinase $\alpha 1$ (AMPK $\alpha 1$) is an energy sensor that plays a key role in regulating lipid metabolism of the liver. This study explores the role of AMPK $\alpha 1$ overexpression in a steatotic hepatocyte model. The results displayed that the AMPK $\alpha 1$ overexpression suppressed lipid accumulation in the cytoplasm, decreased triglyceride levels, maintained the survival of steatotic hepatocyte model with decreased cell apoptosis and increased survival rate. Besides, AMPK $\alpha 1$ overexpression promoted the expression of lipid catabolism-related genes, reduced the level of anabolism-related genes, alleviated the inflammatory response by reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. Moreover, AMPK $\alpha 1$ overexpression could inhibit the activation of p38 mitogen-activated protein kinase (p38MAPK). Finally, Anisomycin, a frequently-used activator of p38MAPK, reversed the inhibitory effect of pc-AMPK $\alpha 1$ on the expression of p-p38MAPK, suggesting that AMPK $\alpha 1$ overexpression alleviates inflammatory response through the inactivation of p38MAPK. These results indicated that AMPK $\alpha 1$ may serve as a novel target for treatment of NAFLD.

Key words: NAFLD; AMPK $\alpha 1$; Metabolism; Inflammatory cytokines; p38MAPK

Introduction

Nonalcoholic fatty liver disease (NAFLD) refers to fat accumulation, mainly triglycerides, in hepatocytes, so that the fat exceeds 5% of the liver weight[1]. The clinical importance of NAFLD is due to its almost 20% prevalence worldwide and its wide spectrum of liver damage, ranging from simple steatosis to non-alcoholic steatohepatitis and even hepatocellular carcinoma[2]. Inflammation in NAFLD is believed to be the chief reason behind diseases and may cause the progression of fibrosis and subsequent cirrhosis[3]. To date, however, the precise molecular pathogenesis of NAFLD, especially in steatosis formation and regulation, is still unclear.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase composed of a catalytic α subunit and regulatory β and γ subunits[4]. Studies have shown that AMPK plays a key role in regulating lipid metabolism of the liver and adipose tissue[5]. Activated AMPK phosphorylation leads to the inhibitory expression of downstream lipid metabolism, which are targets of AMPK in the liver or adipose tissue, including peroxisome proliferator activated receptor γ (PPAR γ), sterol regulatory element binding proteins (SREBPs), fatty acid synthase (FAS) and so on[6]. AMPK activation requires the phosphorylation of threonine 172 in the activation loop of α subunit[7]. Besides, crystal structure studies have indicated that elevated AMP is bound to the γ subunit; the inhibitory domain of the $\alpha 1$ subunit is released from the kinase domain, resulting in an active conformation of AMPK[8]. These reports indicate that AMPK $\alpha 1$ plays a critical role in activating AMPK. AMPK activation is vital for blocking the accumulation of liver fat[9]. AMPK activation results in weight gain and liver fat accumulation in mice with genetically engineered chronic liver disease and high-fat diets[10]. However, AMPK activation was suppressed in NAFLD, and rats fed a high sucrose diet developed NAFLD in association with

reduced AMPK[11], thus hinting that the overexpression of AMPK, especially AMPK α 1, may alleviate NAFLD. p38 mitogen-activated protein kinases (p38MAPK) are a class of evolutionarily conserved serine/threonine MAPKs that link extracellular signals to the intracellular machinery and regulate a plethora of cellular processes[12]. This pathway is frequently activated by a wide range of environmental stresses and cytokines to induce inflammation, a key process in the host defense system[13]. Excessive inflammation is a crucial factor in the pathogenesis of several different human diseases, making the MAPK pathway, and in particular p38MAPK, potential targets for development of anti-inflammatory therapeutics[14]. A report indicated that free fatty acids induced hepatic insulin resistance through p38MAPK pathway[15], and an increased p38 phosphorylation level was observed in the livers of transgenic mice with a high fat diet[16].

In this study, human hepatocyte L02 cells were treated with saturated fatty acids to establish steatotic hepatocyte model. We found that AMPK α 1 overexpression maintains the survival and metabolism homeostasis, and alleviates inflammatory response through the inactivation of p38MAPK in the steatotic hepatocyte model. These results may provide a novel target into the mechanism and treatment of NAFLD.

Material and methods

Cell line and culture

The human hepatic cell line L02 was obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). L02 cells were cultured in RPMI-1640 medium in a humidified atmosphere at 37°C with 5% CO₂. All media were supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 lg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA).

Steatotic hepatocyte model

The Free Fatty Acid (FFA) with bovine serum albumin (BSA) solution was prepared as described previously[17]. Briefly, 18.4% fatty acid-free BSA (Sigma, St. Louis, MO, USA) was dissolved in RPMI-1640 through gentle agitation at room temperature for 3 h. Sodium palmitate (9 mM) was added into the BSA-containing cell culture medium and then the mixture was agitated 6 h or overnight at 37°C. The pH value of the medium was adjusted to 7.4 followed by sterile filtering. It was then aliquoted and stored at -20°C until used. Cells were grown until 80–85% confluent and treated with the FFA/BSA mixture for up to 48 h.

Constructs and transfection

Human AMPK α 1 expression plasmid was constructed by inserting human AMPK α 1 cDNA (purchased from Beyotime, Shanghai, China) into the pcDNA3-EGFP (Invitrogen, Shanghai, China) expressing vector. The plasmid was amplified with Top 10 bacteria (Invitrogen), extracted, and purified by Plasmid Midi Kit (Qiagen, Shanghai, China). For transfection, the steatotic hepatocyte model was cultured in antibiotic- and serum-free α -MEM medium (with 50–60% confluence). AMPK α 1 plasmid (2 μ g/well) or the vector (2 μ g/well) was transfected into steatotic

hepatocyte model with lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. After transfection for 48 h, AMPK α 1 expression in transfected cells was examined by western blots.

Western blotting

Cells in each group were lysed in lysis buffer (Beyotime, China) supplemented with 1 mM Phenylmethanesulfonyl fluoride (PMSF). The protein concentration was determined using the BCA protein assay (Tiangen, China). Twenty micrograms of protein in each sample was separated by 12% SDS-PAGE and electro-transferred to Polyvinylidene Fluoride (PVDF) membranes (Millipore, Massachusetts, USA) for immunoblotting. The following primary antibodies were used: anti-carnitine palmitoyl transferase-1 (CPT-1), anti-PPAR- α , anti-acetyl CoA carboxylase (ACC), anti-SREBP-1c, anti-FAS, p38MAPK, p-p38MAPK and anti-GAPDH, which was used as the internal reference. After incubation with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibody, proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad, San Francisco, California, USA).

Oil red O staining

The Oil Red O stock solution consisted of 0.5% Oil Red O in isopropyl alcohol. The working Oil Red O solution was prepared by adding 4 volumes of water to 6 volumes of Oil Red O stock solution. After treatment with sodium palmitate as described above, the cells were washed twice in phosphate-buffered saline (PBS), fixed for 30 min with 4% formaldehyde, and then stained with freshly diluted Oil Red O solution for 40 min at room temperature. The cells were then washed with 60% isopropyl alcohol once and with water twice. The nuclei of the cells were counterstained with hematoxylin. The number of Oil Red O-positive cells was counted under a bright-field

microscope in 10 replicates of 10 fields at 209 magnification and averaged.

MTT Assay

Cell viability was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in 96-well plates at 6×10^3 cells/well. The surviving fractions were determined at 0, 24, 48, 72, 96 and 120 h. Thereafter, the old medium was discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon, Shanghai, China) was added and incubated for an additional 4 h. Then, dimethyl sulfoxide was used to dissolve the formazan and the absorbance at 490 nm was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed in triplicate.

Flow cytometric assay

Cell apoptosis was detected by the Annexin V Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, 24 h after the indicated treatment, the medium containing the floating cells was collected, and the attached cells were washed and trypsinized. The floating and trypsinized cells were collected in the same centrifuge tubes. After centrifugation and washing, the cell pellets were fixed in cold 70% ethanol. The fixed cells were stained with propidium iodide (PI) and PE-Annexin V. Cell apoptosis percentage was reflected by Annexin V/PI ratio, detected by a flow cytometry machine (BD Bioscience, Shanghai, China).

RT-PCR

The total RNA was extracted from TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For the mRNA detection of inflammatory cytokines, the cDNAs were generated using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA), and amplified, β -actin was used as the internal control of the mRNA.

137 **Statistical analysis**

138 The quantitative data were expressed as mean $\pm$ SD. Statistical analysis was carried out using
139 one-way analysis of variance (ANOVA) followed by Bonferroni test. Differences with a *P* value
140 less than 0.05 were regarded as statistically significant.

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Results

AMPK α 1 overexpression reduced lipid level in steatotic hepatocyte model

The pc-AMPK α 1 plasmid was transfected into a steatotic hepatocyte model. Western blot analysis exhibited that the expression of AMPK α 1 was significantly decreased in Palmitate group compared with Control group, no obvious difference of AMPK α 1 level was detected between Palmitate group and Palmitate + pcDNA group. However, the AMPK α 1 expression was strongly promoted in Palmitate + pc-AMPK α 1 group in comparison with Palmitate group ($P < 0.05$) (Figure 1A). The TG content in Palmitate group and Palmitate + pcDNA group was elevated significantly compared with Control group ($P < 0.05$), indicating that the steatotic hepatocyte model was established successfully. (Fig. 1B). In accordance with the former results, the accumulation of Oil Red O-positive droplets was increased in sodium palmitate-treated L02 cells compared with Control group, but significantly decreased in Palmitate + pc-AMPK α 1 group in comparison with Palmitate group (Fig. 1C). These results indicated that the AMPK α 1 expression was suppressed under palmitate induction, and the AMPK α 1 overexpression reduced the lipid level in the steatotic hepatocyte model.

AMPK α 1 overexpression maintains the survival of steatotic hepatocyte model

To investigate the impact of AMPK α 1 overexpression on steatotic hepatocyte model, we studied whether AMPK α 1 overexpression was capable of affecting cell apoptosis and cell survival. Flow cytometric analysis indicated that the apoptotic cells in Palmitate group were significantly increased compared with Control group, but that cell apoptosis was strongly decreased in Palmitate + pc-AMPK α 1 group compared with Palmitate group ($P < 0.05$) (Fig. 2A, 2B). Consistent with the apoptotic analysis, the survival rate of hepatocytes in Palmitate group was

decreased by about fifty percent. However, the Palmitate+pc-AMPK α 1 group exhibited a survival rate of $89.46 \pm 8.14\%$, which is higher than the rate in Palmitate group ($P < 0.05$) (Fig. 2C). These results suggested that AMPK α 1 overexpression maintains survival of the steatotic hepatocyte model.

AMPK α 1 overexpression sustains metabolism homeostasis in the steatotic hepatocyte model

The effect of AMPK α 1 overexpression on lipid metabolism-related genes expression were examined by Western blot, and a slight growth was detected in the expression of lipid catabolism-related genes (CPT-1, PPAR- α and ACC) in Palmitate group compared with Control group. In comparison with Palmitate group, the expressions of CPT-1, PPAR- α and ACC were strongly promoted in Palmitate+pc-AMPK α 1 group ($P < 0.05$) (Fig. 3A, 3B). The expression of lipid anabolism-related genes (SREBP-1c, FAS and PPAR- γ) was increased in Palmitate group in comparison with Control group ($P < 0.05$), but suppressed in Palmitate+pc-AMPK α 1 group compared to Palmitate group ($P < 0.05$) (Fig. 3C, 3D). These results indicate that AMPK α 1 overexpression sustains the metabolism homeostasis by promoting lipid catabolism and reducing anabolism in the steatotic hepatocyte model.

The anti-inflammatory role of AMPK α 1 is related to the inactivation of p38MAPK

The mRNA level of inflammatory cytokines in the steatotic hepatocyte model were detected by RT-PCR. The expression of pro-inflammatory cytokines (IL-6, IL-12 and TNF- α) was increased in Palmitate group compared with Control group, but decreased in Palmitate + pc-AMPK α 1 group compared to Palmitate group ($P < 0.05$) (Fig. 4A, 4B). The opposite behavior was observed on the expression of anti-inflammatory cytokines (IL-4, IL-10 and IL-13) (Fig. 4A, 4C). Cellular events of inflammatory responses are associated with MAPK signaling pathways[18]. We further

explored the expression and phosphorylation of p38MAPK. The results indicated that p38MAPK expression remain unchanged in each group, but the p-p38MAPK level was elevated in Palmitate group compared with Control group ($P < 0.05$), the transfection of pc-AMPK α 1 suppressed the p-p38MAPK expression significantly in comparison with Palmitate group ($P < 0.05$) (Fig. 4D). Moreover, an activator of p38MAPK, Anisomycin, was added in steatotic hepatocyte model cells. The results displayed that compared with Palmitate + AMPK α 1 + Anisomycin group, the p-p38MAPK expression was promoted in Palmitate + Anisomycin group, but strongly inhibited in Palmitate + pc-AMPK α 1 group ($P < 0.05$) (Fig. 4E). Opposite behavior was also measured in the expression of pro-inflammatory cytokines and anti-inflammatory cytokines ($P < 0.05$) (Fig. 4F). The results indicated that Anisomycin counteracted the inhibitory effect of pc-AMPK α 1 on the activation of p38MAPK, and AMPK α 1 overexpression alleviates inflammatory response may through the inactivation of p38MAPK.

Discussion

NAFLD is an emerging disease predominantly regulated by the interplay of genetic predisposition and environmental factors[19]. The AMPK system plays a major role in the regulation of hepatic glucose and lipid metabolism through its acute effect on energy metabolism and long-term effects on gene expression patterns in the liver[20]. AMPK activation plays a major role in mediating the effects of adiponectin in blocking accumulation of liver fat[9]. Considering that AMPK was inactivated in NAFLD, the overexpression of AMPK subunit in NAFLD was explored in this research.

A report indicated that AMPK α 1- and α 2-containing complexes each account for about half of the total AMPK activity in rodent liver, but that AMPK α 1-containing complexes are predominant in human hepatocytes[21]. This study overexpressed AMPK α 1 by transfecting pc-AMPK α 1 plasmid into steatotic hepatocyte model, which is consistent with the report of Lin et al.[22]. Oil Red O staining and triglyceride quantification revealed prominent lipid accumulation in the cytoplasm and increased triglyceride levels, respectively. Thus, we were able to determine the role of saturated fatty acid in liver cells and the molecular signaling. Saturated fatty acids has been reported to induce endoplasmic reticulum stress and apoptosis in human liver cells[17]. Consistent with their findings, increased cell apoptosis and a decreased survival rate was detected in Palmitate group in this research, but the AMPK α 1 overexpression reversed the effect, indicating that AMPK α 1 overexpression maintains the survival of the steatotic hepatocyte model.

AMPK phosphorylation inactivates a number of metabolic enzymes involved in lipid metabolism in order to maintain liver energy status[23]. Inhibition of ACC by AMPK leads to a decrease in fatty acid synthesis concomitant with an increase in β -oxidation[24]. A report indicated that TG

levels were strongly decreased in obesity rat liver with CPT-1 overexpression[25]. PPAR- α exerted its influence on hepatic lipid metabolism through activating genes encoding for fatty acid oxidation[26]. The expression of CPT-1 and PPAR- α and ACC was promoted by adding pc-AMPK α 1 in Palmitate group in the present study, indicating the lipid catabolism-promoting role of AMPK α 1 in the steatotic hepatocyte model. AMPK is capable of down-regulating lipogenic transcription factor SREBPs, which regulate the lipogenesis-associated genes, including FAS, FATP, FABP and so on [27]. The expression of SREBP-1c, FAS and PPAR- γ was strongly inhibited by pc-AMPK α 1 transfection, suggesting the lipid anabolism-suppressing role of AMPK α 1 in the steatotic hepatocyte model. As the significant increase of lipogenesis-associated genes results in promoting lipid synthesis and the abnormal accumulation of fat in the liver, which may refer to the fatty liver, AMPK α 1 overexpression may sustain the metabolism homeostasis to alleviate NAFLD.

Inflammation is one of the major risk factors associated with NAFLD[28]. A positive correlation has been found between IL-6 hepatic expression and the severity of NAFLD, thus IL-6 has been proposed as a potential mediator leading to NAFLD [29]. IL-12 could induce fatty liver and increased TNF- α level in NAFLD patients [30]. In contrast to IL-6, IL-12 or TNF- α , IL-4 and IL-13 play protective roles in diet-induced NAFLD[31], and IL-10 is mainly produced by the liver, which exhibited a beneficial role in hepatic steatosis[28]. Pro-inflammatory cytokines TNF- α and IL-6 has been reported to activate p38MAPK in liver injury or failure[32]. Emergent research suggested that AMPK is involved in anti-inflammatory signaling[33,34]. The transfection of pc-AMPK α 1 suppressed the p-p38MAPK expression significantly in comparison to Palmitate group in this study, suggesting that AMPK α 1 overexpression could inhibit the activation of p38

MAPK. Anisomycin, a frequently-used activator of p38MAPK[35], was added to the steatotic hepatocyte model. The results demonstrated that Anisomycin reversed the inhibitory effect of pc-AMPK α 1 on the expression of p-p38MAPK, thus suggesting that AMPK α 1 overexpression alleviates inflammatory response, possibly through the inactivation of p38MAPK.

In summary, this study established a steatotic hepatocyte model using human hepatocyte L02 cells induced by saturated fatty acids. We found that AMPK α 1 overexpression suppressed the lipid accumulation in the cytoplasm, decreased triglyceride levels, maintained the survival and metabolism homeostasis, and alleviated inflammatory response through the inactivation of p38MAPK in the steatotic hepatocyte model. These results indicated that AMPK α 1 may serve as a novel target for the treatment of NAFLD.

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Figure legends

Figure1 AMPK α 1 overexpression reduced lipid level in steatotic hepatocyte model.

Cells were divided into four groups. Control group: human hepatic cell line L02; Palmitate group: L02 induced by 9 mM sodium palmitate; Palmitate + pcDNA group: cells in Palmitate group were transfected with 2 μ g/well vector; and Palmitate + pc-AMPK α 1 group: cells in Palmitate group were transfected with 2 μ g/well AMPK α 1 plasmid. (A) The expression of AMPK α 1 in each group after transfection was measured by Western blotting; (B) The total cellular TG concentration was determined using the commercially available triglyceride assay kit (Roche Diagnostics); (C) Lipid accumulation in each group was detected by Oil Red O staining. Data are represented as the mean $\pm$ SD of three experiments. * P < 0.05 versus Control group, # P < 0.05 versus Palmitate group.

Fig. 2 AMPK α 1 overexpression maintains survival of the steatotic hepatocyte model.

(A) Cell apoptosis in each group was measured by flow cytometric analysis; (B) The histogram represents the statistical result of flow cytometric analysis; (C) The survival rate in each group was detected by MTT. Data are represented as the mean $\pm$ SD of three experiments. * P < 0.05 versus Control group, # P < 0.05 versus Palmitate group.

Fig.3 AMPK α 1 overexpression sustains metabolism homeostasis in the steatotic hepatocyte

model. The protein expression of lipid catabolism-related genes (CPT-1, PPAR- α and ACC) (A) and lipid anabolism-related genes (SREBP-1c, FAS and PPAR- γ) (C) in each group were detected by Western blotting; The relative protein expression of lipid catabolism-related genes (B) and lipid anabolism-related genes was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data are represented as the mean $\pm$ SD of three experiments. * P < 0.05 versus Control group, # P < 0.05 versus Palmitate group.

Fig. 4 The anti-inflammatory role of pc-AMPK α 1 is related to the inactivation of p38MAPK.

(A) The mRNA expressions of IL-6, IL-12, TNF- α , IL-4, IL-10 and IL-13 in each group were detected by RT-PCR and normalized to β -actin (B)(C); (D) p38MAPK and p-p38MAPK expression levels were measured by Western blotting; (E) Anisomycin was applied to activate p38MAPK, cells were then divided into five groups; Control group: human hepatic cell line L02; Palmitate group: L02 was induced by 9 mM sodium palmitate; Palmitate + pc-AMPK α 1 group: cells in Palmitate group was transfected with 2 μ g/well AMPK α 1 plasmid. Palmitate + Anisomycin group: cells in Palmitate group were incubated with 50 ng/mL Anisomycin; Palmitate + AMPK α 1 + Anisomycin group: cells in Palmitate group were transfected with 2 μ g/well AMPK α 1 plasmid and incubated with 50 ng/mL Anisomycin. Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to GAPDH; (F) The mRNA expressions of inflammatory cytokines in each group were measured by RT-PCR. Data are represented as the mean $\pm$ SD of three experiments. * $P < 0.05$ versus Control group, # $P < 0.05$ versus Palmitate group, & $P < 0.05$ versus Palmitate + pc-AMPK α 1 + Anisomycin group.

Figure 1

Figure 2

Figure 3

Figure 4