AMP-kinase pathway is involved in tumor necrosis factor alpha-induced lipid accumulation in human hepatoma cells

Qiong Lv a, Qianna Zhen a, Lulu Liu a, Rufei Gao a,b, Shumin Yang a, Huang Zhou a, Richa Goswami a, Qifu Li a,b,⁎

a Department of Endocrinology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China
b Laboratory of Lipids and Glucose Metabolism, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

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Aim: It is well known that lipid accumulation and inflammation are two important steps in pathogenesis and progression of nonalcoholic fatty liver disease (NAFLD). However, fewer studies have explored the direct relationship between lipid accumulation and inflammation in early NAFLD. Tumor necrosis factor alpha (TNF-α) is one of the classical inflammatory cytokines. AMP-activated protein kinase (AMPK) is known as a critical regulator of energy homeostasis in metabolic processes. This study aims to investigate the role of TNF-α on lipid deposition of HepG2 cells and examine the modification of AMPK pathway.

Main methods: TNF-α was added in HepG2 cells and lipid accumulation was analyzed by Oil Red O staining and quantitative test of triglyceride (TG). The expressions of phosphorylated AMPK and its pathway (including mTOR and SREBP-1) were determined. Furthermore, an AMPK agonist (metformin or AICAR) or antagonist (compound C) was co-administered with TNF-α in HepG2 cells to investigate its effect on TNF-α induced lipid deposition.

Key findings: A significant increment of TG content in HepG2 cells was observed after TNF-α treatment. Meanwhile, substantially suppressed AMPK and ACC phosphorylation, enhanced mTOR and p70S6K phosphorylation, and increased protein expression of FAS and SREBP-1 were found. Co-treatment with metformin or AICAR decreased the TNF-α-induced intracellular TG accompanied by significantly enhanced AMPK and ACC phosphorylation, suppressed mTOR and p70S6K phosphorylation, and reduced SREBP-1 and FAS expressions. On the contrary, while co-incubated with compound C, AMPK and ACC phosphorylation were suppressed and the inhibitory effect of metformin on HepG2 cell lipid deposition was also attenuated.

Significance: Our results suggest that TNF-α directly induces lipid accumulation in HepG2 cells, at least in part, through the inhibition of AMPK/mTOR/SREBP-1 pathway.

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well supported that AMPK inhibits rate-limiting steps in lipogenesis, such as sterol regulatory element–binding protein–1 (SREBP-1) through mammalian target of rapamycin (mTOR) in hepatic cells, which leads to decreased lipid deposition [21]. Conversely, AMPK repression has been suggested to counter the above effects, accelerating fatty liver development. As one of the most widely used antidiabetic drug, metformin could improve hepatic steatosis by increasing AMPK phosphorylation [29]. 5-Amino-4-imidazole-carboxamidine riboside (AICAR), the pharmacological activator of AMPK, has been proven to exhibit similar effects. Previous studies have found that constitutively active AMPK could attenuate oleic acid-induced lipid deposition by inhibiting lipid synthesis gene SREBP-1 expression in rat hepatocytes [13].

However, it is still unknown whether AMPK is involved in the inflammation–induced lipid deposition process. Therefore, in this study, we examined the role of AMPK on TNF-α induced lipid deposition in human hepatoma HepG2 cells, and we found that TNF-α may promote lipid accumulation through AMPK/mTOR/SREBP-1 pathway.

2. Materials and methods

2.1. Culture and treatment of HepG2 cells

Human hepatoma cells (HepG2 cells), obtained from the American Type Culture Collection, were maintained in a RPMI-1640 medium (Gibco) with 10% FBS (Gibco) in a 5% CO2 atmosphere at 37 °C. We used the HepG2 cells at 70% confluence in 6-well plates and changed the medium to a serum-free RPMI-1640 containing 0.2% (wt./vol.) fat acid free BSA (Sigma-Aldrich) for 12 h. The cells were treated with human recombinant TNF-α (20 ng/mL, Novoprotein, Shanghai, China) for 24 h, combined with/without metformin (1 mmol/L, Sigma-Aldrich), AICAR (1 mmol/L, Sigma-Aldrich) or AMPK inhibitor compound C (40 μmol/L, Sigma-Aldrich). These drugs were added to the cell culture media 1 h before TNF-α treatment.

2.2. Oil Red O staining

Cells (5 × 10⁴) were cultured in a 24-well dish and treated according to the methods described above. After 24 h of incubation, cells were fixed with 4% paraformaldehyde for 1 h, stained using 5 mg/mL Oil Red O (Sigma-Aldrich) for 1 h, and hematoxylin for 2 min. After dyeing, the cells were washed with sterile water and observed under a microscope.

2.3. Quantitative measurement of intracellular triglycerides

Samples were collected from the 6-well plates and lipids were extracted by adding 1 mL solvents (n-hexane/isopropanol = 2/3.5). The lipid phase was collected and dried in vacuum. Intracellular triglyceride concentrations (mg/mL) were determined enzymatically with commercial kits (Dongou, Zhejiang, China) and normalized by protein content (mg/mL).

2.4. Real-time quantitative PCR analysis

Total RNA was extracted using the TRIzol reagent (Life Technologies, Maryland, USA), according to manufacturer’s instruction. RNA purity was assessed by measuring the OD at 260 nm and 280 nm with the standard of A260/A280 ≥ 1.8. The cDNAs were synthesized from 1 μg of the total RNA, using a PrimeScript RT reagent kit (TAKARA), according to the manufacturer’s instructions; the synthesis was done in two steps: 2 min at 42 °C, followed by 15 min at 37 °C and 5 s at 85 °C. The primer sequences used for PCR amplification are as follows: GAPDH, 5′-ACCAC TCTCCACCCCTCACT-3′ and 5′-TCCACCACCCCTGGTCCTTAG-3′; SREBP1, 5′-CAGAGGAGCATCAGACT-3′ and 5′-GCCAAGCTTTCTCTCCT-3′; FAS, 5′-GGGCGGACTAACATCC-3′ and 5′-GTTCTCCACCA CTATGCTCAG-3′. Real-time quantitative PCR was performed using SYBR Premix Ex Taq™ II (TAKARA). PCR conditions were as follows: initial denaturation at 95 °C for 30 s; 40 cycles in 5 s at 95 °C (denaturation), and 30 s at 60 °C (annealing and extension). The corresponding quantities of the target transcripts were calculated after normalization against the endogenous control, human GAPDH. Relative gene expression levels were calculated with the 2−ΔΔCT method.

2.5. Western blot analysis

Total cellular protein was obtained using the Total Protein Extraction Kit (Keygen, Nanjing, Jiangsu, China). Protein concentration was measured using the BCA protein assay kit (Beyotime, Jiangsu, China). The total cellular protein was separated by SDS-PAGE, transferred to PVDF membranes and incubated with primary antibodies, according to the manufacturer’s recommendations. Primary antibodies were detected using a HRP-conjugated secondary antibody from the appropriate species. Finally, immunoreactive proteins were visualized using enhanced chemiluminescence (ECL, Beyotime, China), and band intensities were quantified using Quantity One software (Bio-rad, USA). Primary antibodies against the following proteins were used: AMPKα1, Phospho-AMPKα (Thr172), and Phospho-p70S6K (Thr389/Ser424). Antibodies purchased from Cell Signaling Technology (Beverly, MA, USA); Phospho-mTOR [Ser2448] along with SREBP-1 antibodies purchased from Abcam (Hongkong, China); Phospho-ACC (Ser79), FAS and β-actin antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

3. Statistical analysis

Data from all experiments was evaluated for statistical significance by Student’s t test or one-way ANOVA using SPSS13.0. All data were used as mean ± SD. P value < 0.05 was considered statistically significant.

4. Results

4.1. Inflammatory cytokine TNF-α induced lipid deposition in HepG2 cells

TNF-α is an important cytokine that influences cellular homeostasis in the liver, hence, we used TNF-α treatment to induce inflammatory stress on HepG2 cells. Oil Red O staining showed more lipid droplets in HepG2 cells after TNF-α treatment, compared to the control group (Fig. 1A). Quantitative measurement also clarified that intracellular triglyceride content was significantly increased in the TNF-α group (P < 0.05, Fig. 1B).

4.2. AMPK phosphorylation was inhibited in HepG2 cells treated with TNF-α

To investigate whether AMPK activity was changed in an inflammatory state, we examined the AMPK and ACC phosphorylation levels in HepG2 cells. As shown in Fig. 2A, the p-AMPK and p-ACC levels obviously decreased in the TNF-α group (P < 0.05). SREBP-1 is an important transcription factor for regulating lipid metabolism that can activate hepatic lipogenesis genes, such as FAS. We found that the mRNA and protein expressions of SREBP-1 and FAS significantly increased in the TNF-α group (P < 0.05, Fig. 2C and D). It has been reported that AMPK phosphorylation inhibits SREBP1 expression through mTOR; hence, we examined the expressions of p-mTOR and p-p70S6K, and found that the phosphorylation of mTOR and p70S6K was subsequently enhanced in the TNF-α group, compared with the control group (P < 0.05, Fig. 2B).

4.3. Pharmacological activation of AMPK phosphorylation alleviated TNF-α-stimulated lipid deposition in HepG2 cells

Since the AMPK phosphorylation levels obviously decreased in HepG2 cells with enhanced lipid contents; we further investigated whether activation of AMPK phosphorylation by pharmacological...
agonists could alleviate lipid deposition in TNF-α treated HepG2 cells. Metformin or AICAR was administered in HepG2 cells, before TNF-α treatment. Oil Red O staining and the quantitative measurement of intracellular triglycerides showed that intracellular lipid deposition significantly decreased in both the metformin and AICAR groups (P < 0.05, Fig 3A and B), compared with the TNF-α group. In order to clarify the effect of metformin and AICAR in enhancing AMPK phosphorylation, we examined the p-AMPK and p-ACC levels. As expected, p-AMPK and p-ACC levels obviously increased in the metformin and AICAR groups (P < 0.05, Fig 4A); Higher levels of p-AMPK and p-ACC were accompanied by a significant suppression of mTOR and p70S6K phosphorylation with reduced levels of SREBP-1 and FAS mRNA and protein (P < 0.05, Fig 4B-D).

4.4. Inhibiting AMPK phosphorylation attenuated the effect of metformin on lipid deposition in TNF-α-treated HepG2 cells

To further test the effect of metformin, compound C (iAMPK), a classical AMPK inhibitor, was co-incubated with metformin (AMPK inhibition group) in TNF-α-treated HepG2 cells. The intracellular lipid contents were significantly increased in the compound C-treated group, compared with the metformin group (P < 0.05, Fig 3A and B). The p-AMPK and p-ACC levels were substantially suppressed by compound C, compared with the metformin group (P < 0.05, Fig 4A); p-mTOR and p-p70S6K levels along with mRNA and protein levels of SREBP-1 and FAS were considerably increased by compound C (P < 0.05, Fig 4B-D).

5. Discussion

Hepatic steatosis and inflammation are the two important features of NAFLD [6,7]. The traditional “two-hit” hypothesis suggested that insulin resistance is the central link of the “first hit”, promoting FFA intake and triglyceride deposition in the liver. The “second hit” which mainly refers to the inflammatory injury promotes the development of steatohepatitis and liver fibrosis. However, fewer studies have explored the direct relationship between lipid accumulation and inflammation in early stages of NAFLD. In this study, we described a novel finding that inflammatory cytokine TNF-α directly induced lipid accumulation in human hepatoma HepG2 cells, which is involved in the inhibition of AMPK, and subsequent activation of mTOR/SREBP-1 pathway. AICAR, the AMPK activator, reversed the effect of TNF-α on lipid deposition by activating AMPK phosphorylation. Previous animal studies indicated that macrophage-derived TNF-α contributed to the pattern and extent of fat accumulation and insulin resistance in diet-induced obesity [5,7]. In a study performed with TNF-α induced muscle cells in vitro and in vivo, Steinberg et al. also demonstrated that TNF-α-induced skeletal muscle insulin resistance was involved in AMPK signaling suppression [22], which was in line with our findings. Dysregulation of lipid synthesis is one of the main reasons for abnormal lipid accumulation in the liver. As a master transcriptional activator of lipogenesis, SREBP-1 mainly regulates the de novo lipid biosynthesis process by activating genes involved in triglyceride synthesis and fatty acids, such as FAS and SCD1 [12], through mammalian target of rapamycin (mTOR) in hepatic cells [21]. In the present study, the SREBP-1 and FAS expressions significantly increased in TNF-α-induced HepG2 cells. mTOR preferentially regulates cell growth and energy metabolism through the phosphorylation of S6K and the protein translation initiation factor binding protein, 4E-BP1. Previous studies demonstrated that the sustained activation of mTOR led to the increased mRNA and protein expressions of SREBP-1 in mouse embryonic fibroblast cells [10]. Furthermore, fatty acid synthesis significantly increased with the activation of mTOR–SREBP-1, while mTOR inhibition with rapamycin suppressed the SREBP-1 expression and fatty acid synthesis.
content. Meanwhile, the mTOR/p70S6K system was activated by their phosphorylation status (p-mTOR on Ser 2448 and p-p70S6K on Thr421/Ser424, respectively) [9,25], but inhibited by the LKB1/AMPK signaling pathway in cardiac myocyte cells. Jung et al. [13] found that activation of AMPK by AICAR reduced mTOR/p70S6K and IRS-1 phosphorylation, and ultimately improved orotic acid-induced hepatic insulin resistance. In accordance with those studies, our data revealed that the phosphorylation levels of p-mTOR on Ser 2448 and p-p70S6K on Thr421/Ser424 were dramatically upregulated in TNF-α-treated HepG2 cells; AICAR markedly decreased the phosphorylation levels of p-mTOR on Ser 2448 and p-p70S6K by promoting AMPK phosphorylation.

Metformin is the front-line, go-to treatment for diabetes. Several clinical researchers have reported some remarkable effects of metformin on ameliorating hepatic steatosis of NAFLD. In an open-label randomized trial, non-diabetic NAFLD patients were given metformin at 2 g/day for 12 months; biopsies showed a significant decrease in fatty liver, necroinflammation, and fibrosis [2]. In another open-labeled study, patients with histologically confirmed NAFLD were given metformin at 20 mg/kg/day for one year; liver biopsies revealed that hepatic steatosis improved in 33% of the patients [18]. In high-fat diet induced NAFLD of C57BL/6j mice, metformin treatment caused a significant decrease in liver weight and hepatic steatosis [26]. All these studies showed that metformin could effectively improve the manifestations of fatty liver. However, mechanisms underlying the effect of metformin are still not clear at present. It has been suggested that the significant impact of metformin may be attributable to increased fatty acid oxidation and suppressed lipogenesis, which is related to the activation of AMPK phosphorylation on Thr 172 within the catalytic α subunit [29].

In vitro, primary rat hepatocytes exposed to 0.5–2 mM of metformin induced AMPK phosphorylation and metformin-treated rats in vivo presented a reduced expression of SREBP-1 in the liver [28]. In this study, we also observed that 1 mM of metformin significantly increased AMPK phosphorylation in TNF-α-induced HepG2 cells. Some studies suggested that metformin may have other mechanisms independent of AMPK activation, such as antioxidant effects on endothelial cells [8]. To investigate whether AMPK activation mediates the effect of metformin in improving lipid deposition, compound C, the commercially available AMPK inhibitor, was used along with metformin in HepG2 cells. We observed that compound C
inhibited AMPK phosphorylation, and led to an increase in intracellular lipid content, indicating that the effect of metformin on improving TNF-α-induced lipid accumulation was dependent on AMPK activation. Similar results were observed by Kim et al., which revealed that exposing cultured bovine aortic endothelial cells to AICAR increased the phosphorylation levels of AMPK [15] and the activation was blocked by 40 μM of compound C [28]. Decreased levels of phosphorylated AMPK modulated a series of downstream events, such as increasing the expressions of SREBP-1 and FAS also enhancing the phosphorylated levels of mTOR and p70S6K.

6. Conclusion

In summary, we demonstrated that TNF-α induced HepG2 cell lipid accumulation by suppressing AMPK phosphorylation, which is closely associated with the enhanced phosphorylation of mTOR/p70S6K and increased SREBP-1 levels. Our study provides important information that could help better understand the role of the AMPK/mTOR/SREBP-1 pathway in TNF-α-induced fatty liver. However, our data is still limited to a single cell line and further experiments, for instance, using other sources of hepatocytes and some in vivo approaches, are needed to explore the mechanisms discussed above.

Conflict of interest statement

We declare no conflicts of interest.

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Fig. 4. The expression of AMPK, mTOR and SREBP-1 in TNF-α-stimulated HepG2 cells treated with metformin, AICAR and compound C. HepG2 cells were incubated in medium containing 20 ng/mL human recombinant TNF-α (TNF-α), or 20 ng/mL TNF-α plus 1 mM metformin (TNF-α + Met), or 20 ng/mL TNF-α plus 1 mM AICAR (TNF-α + AICAR), or 20 ng/mL TNF-α plus 1 mM metformin plus 40 μM compound C (TNF-α + Met + iAMPK). Western blotting was performed for the phosphorylation of AMPK (A), mTOR (B) and SREBP-1 (C) in the cells. Data are depicted as mean ± SD from three separate experiments. The mRNA expression of SREBP-1 in the cells (D) was determined by real-time PCR. Data are depicted as mean ± SD from at least three separate experiments. *P < 0.05 versus TNF-α group. #P < 0.05 versus metformin group. ▲P < 0.05 versus TNF-α group.

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