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Rapamycin-mediated CD36 translational suppression contributes to alleviation of hepatic steatosis

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ABSTRACT

Rapamycin, a mammalian target of rapamycin (mTOR)-specific inhibitor, has the effect of anti-lipid deposition on non-alcoholic fatty liver disease (NAFLD), but the mechanisms with which rapamycin alleviates hepatic steatosis are not fully disclosed. CD36 is known to facilitate long-chain fatty acid uptake and contribute to NAFLD progression. Hepatic CD36 expression is closely associated with hepatic steatosis, while mTOR pathway is involved in CD36 translational control. This study was undertaken to investigate whether rapamycin alleviates hepatic steatosis via the inhibition of mTOR pathway-dependent CD36 translation. Human hepatoblastoma HepG2 cells were treated with palmitate and C57BL/6J mice were fed with high fat diet (HFD) to induce hepatic steatosis. Hepatic CD36 protein expression was significantly increased with lipid accumulation in palmitate-treated HepG2 cells or HFD-fed C57BL/6J mice. Rapamycin reduced hepatic steatosis and CD36 protein expression, but it had no influence on CD36 mRNA expression. Rapamycin had no effect on CD36 protein stability, but it significantly decreased CD36 translational efficiency. We further confirmed that rapamycin inhibited the phosphorylation of mTOR and its downstream translational regulators including p70 ribosomal protein S6 kinase (p70S6K), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and eukaryotic initiation factor 4E (eIF4E). This study demonstrates that rapamycin inhibits hepatic CD36 translational efficiency through the mTOR pathway, resulting in reduction of CD36 protein expression and alleviation of hepatic steatosis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease in developed countries. It involves a series of liver diseases without significant alcohol consumption, ranging from non-alcoholic simple fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), hepatic fibrosis and cirrhosis.

The class B scavenger receptor CD36 is a transmembrane glycoprotein and binds many ligands including long-chain fatty acid, oxidised low-density lipoprotein, collagen, and thrombospondin-1 [1]. It has been implicated in various diseases such as inflammation, insulin resistance, atherosclerosis, and NAFLD [2,3]. Recent studies demonstrated that about 59% of liver fatty acids are derived from the circulation in NAFLD patients. The overexpression of CD36 exhibits significantly increased hepatic fatty acid trafficking and triglyceride accumulation in human hepatoblastoma cells, C57BL6 mice, and NAFLD patients [4–6]. This evidence demonstrates that hepatic steatosis is closely related to CD36 expression.

Rapamycin is a mammalian target of rapamycin (mTOR)-specific inhibitor that has been used as an immunosuppressant for the prophylaxis of transplant rejection in clinical practice. The important role of rapamycin in metabolic syndrome has been reported. It has been demonstrated that rapamycin attenuates atherosclerosis in human vascular smooth muscle cells [7] and apolipoprotein E-deficient mice [8]. Fraenkel showed that rapamycin induces type 2 diabetes by reducing beta-cell function and increasing insulin resistance [9]. The effect of rapamycin on hepatic steatosis has some controversy: Chaveroux indicated that rapamycin induces NAFLD in mice [10], Kenerson suggested that rapamycin is neither necessary nor sufficient to attenuate hepatic steatosis [11], but the vast majority of studies have demonstrated that

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rapamycin indeed alleviates hepatic lipid accumulation through down-regulating some important lipid metabolic enzymes including sterol regulatory element binding protein 1c (SREBP1c), SREBP2, fatty acid synthase (Fasn), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD1), and low-density lipoprotein receptor (LDLr), as described by our group [12] and others [13–15]. However, whether CD36 participates in the rapamycin-mediated alleviation of hepatic steatosis has not been reported.mTOR is a translational regulator that selectively regulates the expression of CD36 at translational level [16]. It has also been shown to be as a cellular nutrient sensor and plays a critical role in the pathogenesis of NAFLD [17]. Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70S6K), as two direct phosphorylation targets of mTOR, are responsible for ribosome recruitment to mRNA during the initiation phase of translation [18]. The phosphorylation of 4E-BP1 inhibits it binding to eukaryotic initiation factor 4E (eIF4E), which directs the 5′ cap structure containing 7-methylguanosine triphosphate to the 40S ribosomal subunit and enhances translation in the mRNA binding step [19]. The effect of p70S6K on translational control is through ribosomal protein S6 phosphorylation which recruits 5′ TOP mRNAs to ribosomes and increases mRNA translation [20]. However, the precise regulatory mechanism of mTOR pathway in hepatic CD36 translation remains unclear.

The present study was undertaken to investigate whether rapamycin alleviates hepatic steatosis and reduces CD36 protein expression via the inhibition of mTOR pathway-dependent CD36 translation in HepG2 cells and C57BL/6J mice.

2. Materials and methods

2.1. Cell culture

The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection and cultured with D-MEM (high glucose) medium containing 10% foetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. All experiments were carried out in serum-free D-MEM (high glucose) medium containing 0.2% bovine serum albumin (BSA), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were pre-incubated in serum-free medium for 24 h and then subjected to palmitate or 10 ng/mL rapamycin for another 24 h. Palmitate was obtained from Sigma–Aldrich (Poole, Dorset, UK). Rapamycin was obtained from Sangon Biotech (Shanghai, China).

2.2. Animal model

The animal care and experimental procedures were approved by the Animal Care Committee of Chongqing Medical University. Six- to eight-week-old male C57BL/6J mice were randomly assigned to be fed with normal chow diet (NCD, 10 kcal% Fat, Research Diets, New Brunswick, NJ, USA) or NCD plus subcutaneous injection of rapamycin (2 mg/kg body weight) or high fat diet (HFD, 60 kcal% Fat, Research Diets) or HFD plus subcutaneous injection of rapamycin (2 mg/kg body weight). The injections were done every other day and the mice were sacrificed at 14 weeks after the first injection.

2.3. Morphological examination

Lipid accumulation in the HepG2 cells and livers of C57BL/6J mice was evaluated by Oil Red O staining. The samples were fixed with 5% formalin solution for 30 min, stained with Oil Red O for 30 min, and counter-stained with haematoxylin for another 5 min. The results were examined by light microscopy.

2.4. Quantitative measurement of free fatty acid (FFA) and triglyceride (TG)

Intracellular FFA and TG levels of the HepG2 cells and livers of C57BL/6J mice were measured using an ELISA kit (CUSABIO, Wuhan, Hubei, China) and enzymatic assay (DONGOU, Wenzhou, Zhejiang, China), respectively. The samples were collected, and the lipids were extracted by the addition of 1 mL solvent (dipropylmethane/isopropanol = 2:3.5) followed by sonication for 2 min. The lipid phase was collected, dried under vacuum, and then dissolved in 0.15 mL solvent. The concentrations of FFA and TG were analysed using a standard curve and normalised to the total protein.

2.5. Total RNA isolation and real-time PCR

Total RNA was isolated from HepG2 cells and liver tissues using the RNAiso Kit (Takara, Dalian, Liaoning, China) according to the manufacturer's protocol. Real-time PCR was performed to amplify CD36 and β-Actin using a Bio-Rad CFX Connet Real-Time System (Bio-Rad, Hercules, California, USA) with SYBR Green dye and the following specific primers: human CD36 (forward) 5′-AAATAAACCTCCTTGGCCTGA-3′ and (reverse) 5′-GCAACAAACATCACCACACC-3′; mouse CD36 (forward) 5′-TTGAAGGCATTCCCACGTATC-3′ and (reverse) 5′-CGGACCCGTTGGCAAA-3′; human β-Actin (forward) 5′-CCTGGCACCCAGCACAAT-3′ and (reverse) 5′-GCCGATCCACACGGAGTA-3′; mouse β-Actin (forward) 5′-CGATGCCCTGAGGCTCTTT-3′ and (reverse) 5′-TGGATGCCACAGGATTCCAT-3′.

2.6. Western blotting

Total protein from HepG2 cells and liver tissue homogenates was extracted using the Total Protein Extraction Kit (Keygen, Nanjing, Jiangsu, China). Identical amounts of protein were separated by SDS–PAGE, transferred to PVDF membranes (Millipore, Bedford, MA, USA), and immunoblotted with primary antibodies according to the manufacturer's recommendations. The primary antibodies were detected using an HRP-conjugated secondary antibody from the appropriate species and then reacted with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Temecula, CA, USA). Primary antibodies against the following proteins were used: CD36, p-p70S6K (Thr 421/Ser 424), total p70S6K, p-4E-BP1 (Ser 65/Thr 70), total 4E-BP1, p-eIF4E (Ser 209), total eIF4E, and β-Actin (Santa Cruz, Dallas, TX, USA); p-mTOR (phospho S2448) (Abcam, Cambridge, UK); and total mTOR (Millipore, Temecula, CA, USA).

2.7. Protein degradation assay

HepG2 cells were pre-incubated in serum-free medium for 24 h and then incubated in serum-free medium containing 14 mg/L cycloheximide (CHX, Genview, Florida, USA), an inhibitor of protein synthesis, in the presence or absence of 0.08 mmol/L palmitate or 10 ng/mL rapamycin for the indicated time. The CD36 and β-Actin protein levels were detected by Western blotting.

2.8. Polysome analysis

HepG2 cells and liver tissues were harvested, and polysomes were extracted with 0.3 mL of low-salt buffer (LSB, 20 mM Tris–HCl, pH 7.4, containing 10 mM NaCl and 3 mM $MgCl_2$) prior to homogenization in 0.1 mL of LSB containing 1.2% (v/v) Triton X-100 and 0.2 M sucrose. The lysate was applied to a 7%–47% linear sucrose gradient and subjected to ultracentrifugation at 36,000*g* for 2 h at 4 °C. After ultracentrifugation, the absorbance at 254 nm was determined using a photometer and the gradients were fractionated into eight equal fractions. Total RNA was extracted from each fraction and analysed by semiquantitative PCR

Table 1
Primers for semiquantitative PCR.

Gene	Primer sequences
Human CD36	Forward: 5′-GAGAGAACTGTTATGGGGCTAT-3′ Reverse: 5′-TTCAACTGGAGAGGCAAAGG-3′
Human 28S rRNA	Forward: 5′-TTGAAAATCCGGGGGAGAG-3′ Reverse: 5′-ACATTGTTCCAACATGCCAG-3′
Human 18S rRNA	Forward: 5′-CAGCCACCCGAGATTGAGCA-3′ Reverse: 5′-TAGTAGCGACGGGCGGTGTG-3′
Human β-Actin	Forward: 5′-AGCGAGCATCCCCCAAAGTT-3′ Reverse: 5′GGGCACGAAGGCTCATCATT-3′
Mouse CD36	Forward: 5′-GAGCCATCTTTGAGCCTTCA-3′ Reverse: 5′-TCAGATCCGAACACAGCGTA-3′
Mouse 28S rRNA	Forward: 5′-TTGAAAATCCGGGGGAGAG-3′ Reverse: 5′-ACATTGTTCCAACATGCCAG-3′
Mouse 18S rRNA	Forward: 5′-AGGGGAGAGCGGGTAAGAGA-3′ Reverse: 5′-GGACAGGACTAGGCGGAACA-3′
Mouse β-Actin	Forward: 5′-GTCCCTCACCCTCCCAAAAG-3′ Reverse: 5′-GCTGCCTCAACACCTCAACCC-3′

for the CD36, 28S rRNA, 18S rRNA, and β-Actin genes using the primer sequences listed in Table 1.

2.9. Statistical analysis

All data are expressed as mean ± standard deviation. Comparison between groups was performed with one-way ANOVA followed by *Q*-test using SPSS17.0 software. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Palmitate or HFD increases hepatic CD36 protein expression, but has different effect on CD36 mRNA expression in vitro or in vivo

Our data demonstrated that hepatic CD36 protein expression was increased in palmitate-treated HepG2 cells in a dose-dependent manner (Fig. 1A) or HFD-fed C57BL/6J mice (Fig. 1B). Palmitate up-regulated CD36 mRNA expression in HepG2 cells when its concentration was greater than 0.16 mmol/L, but CD36 mRNA expression was not altered when HepG2 cells were treated with lower dose palmitate (⩽0.08 mmol/L) (Fig. 1C) or C57BL/6J mice were fed with HFD (Fig. 1D). We selected 0.08 mmol/L of palmitate for our further experiments because the change of CD36 protein and mRNA expression at this concentration in HepG2 cells was similar to that in C57BL/6J mice.

3.2. Rapamycin reduces hepatic lipid accumulation and CD36 protein expression

Oil Red O staining showed that the hepatic lipid droplet was significantly increased when HepG2 cells were treated with palmitate or C57BL/6J mice were fed with HFD, however, rapamycin alleviated the aggravating hepatic steatosis in palmitate-treated HepG2 cells (Fig. 2A) or HFD-fed C57BL/6J mice (Fig. 2B). A quantitative assay for FFA and TG (Fig. 2C and D) confirmed the results of the Oil Red O staining.

We found that rapamycin inhibited the CD36 protein expression in palmitate-treated HepG2 cells (Fig. 2E) or HFD-fed C57BL/

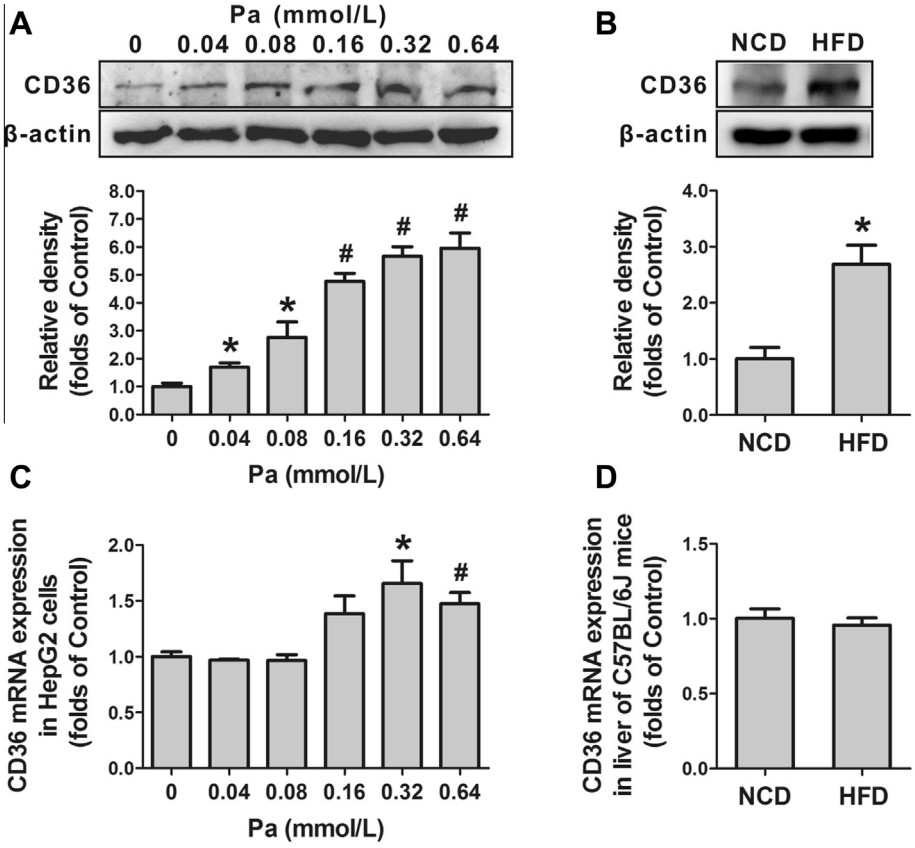

Fig. 1. Effect of palmitate or HFD on hepatic CD36 expression. HepG2 cells were incubated in serum-free medium containing different concentrations of palmitate (Pa). C57BL/6J mice were fed with normal chow diet (NCD) or high fat diet (HFD). The protein expression of CD36 in the cells (A) and mice (B) was examined by western blotting. The mRNA expression of CD36 in the cells (C) and mice (D) was determined by real-time PCR. Data are depicted as mean ± SD from at least three separate experiments. $^{*}P < 0.05$ and $^{\#}P < 0.01$ versus 0 mmol/L Pa or NCD group.

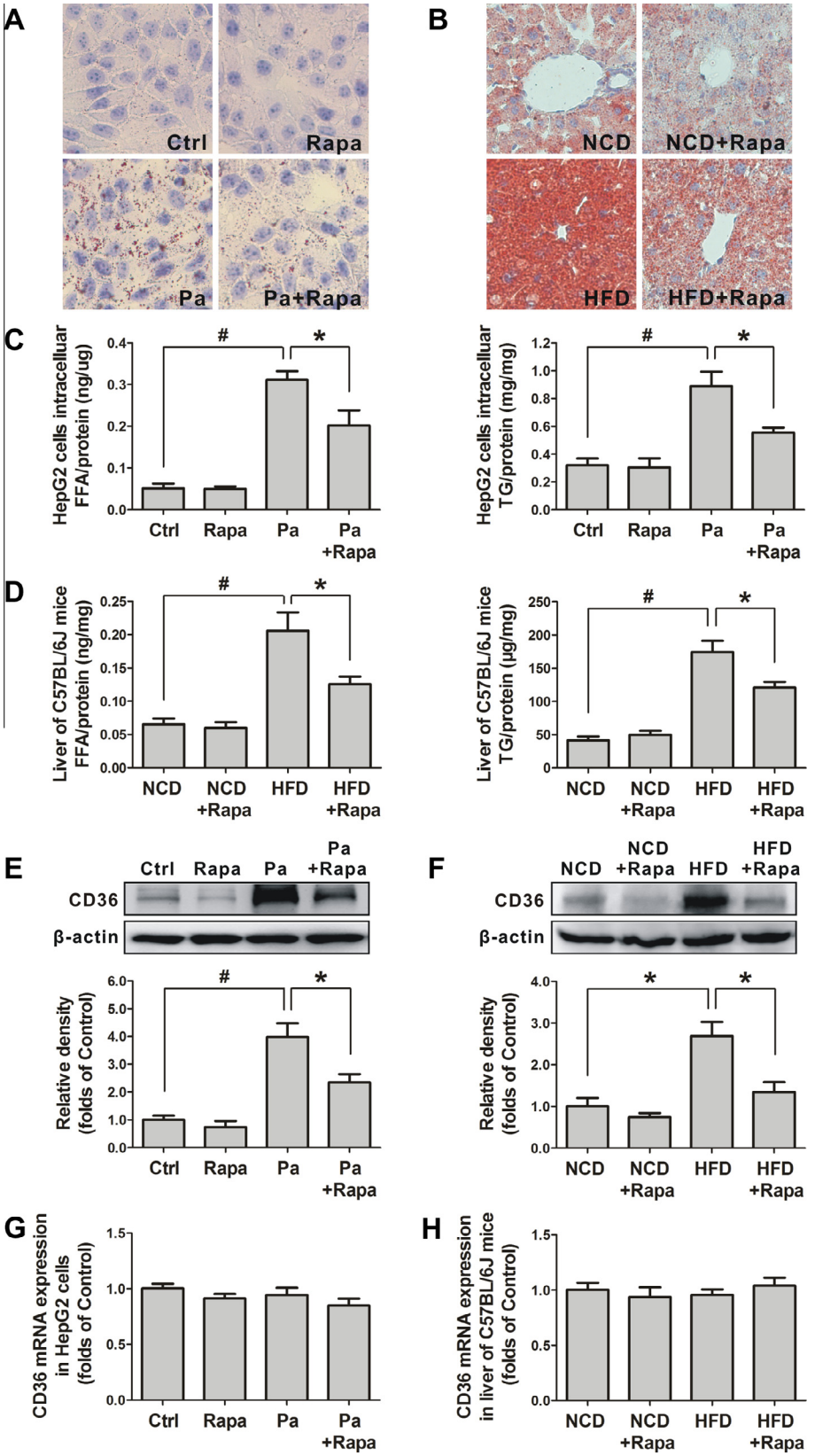

Fig. 2. Effect of rapamycin on hepatic lipid accumulation and CD36 expression. HepG2 cells were incubated in serum-free medium (Ctrl) or medium containing 10 ng/mL rapamycin (Rapa) or 0.08 mmol/L palmitate (Pa) or 0.08 mmol/L palmitate plus 10 ng/mL rapamycin (Pa + Rapa). C57BL/6J mice were fed with normal chow diet (NCD) or NCD plus rapamycin injection (NCD + Rapa) or high fat diet (HFD) or HFD plus rapamycin injection (HFD + Rapa). The hepatic lipid accumulation in the cells (A) and mice (B) was observed by Oil Red O staining (original magnification ×400). The concentrations of FFA and TG in the cells (C) and mice (D) were measured as described in the Materials and methods. The protein expression of CD36 in the cells (E) and mice (F) was examined by western blotting. The mRNA expression of CD36 in the cells (G) and mice (H) was determined by real-time PCR. Data are depicted as mean ± SD from at least three separate experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$.

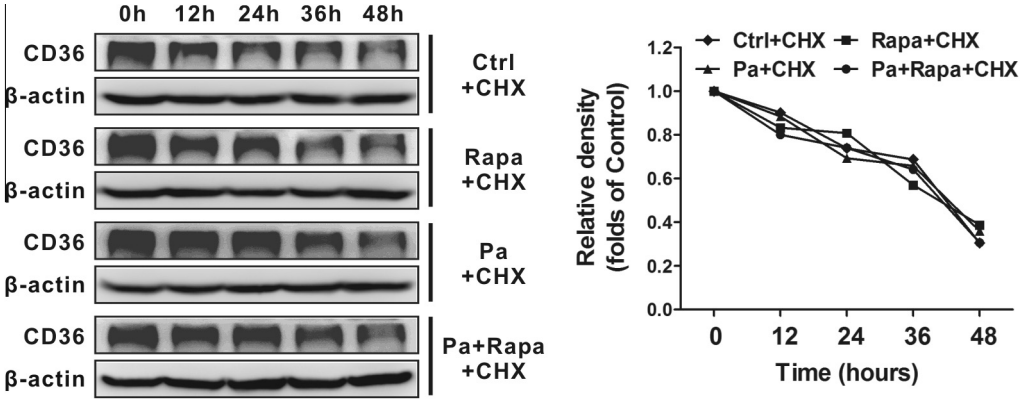

Fig. 3. Effect of rapamycin on hepatic CD36 protein stability. HepG2 cells were incubated in serum-free medium containing 14 mg/L CHX in the presence or absence of 0.08 mmol/L palmitate or 10 ng/mL rapamycin for the indicated time. The CD36 protein levels were analysed by western blotting and were normalised to β-Actin. Data are depicted as mean ± SD from three separate experiments. The statistical significance was set at $P < 0.05$.

6J mice (Fig. 2F), but there was no effect of rapamycin on CD36 mRNA expression (Fig. 2G and H). This suggests that rapamycin-mediated the down-regulation of CD36 protein expression occurred at the translational or post-translational level.

3.3. Rapamycin has no effect on hepatic CD36 protein stability

Since the post-transcriptional protein level is controlled by protein stability or translational efficiency, we investigated the effect of rapamycin on the degradation of hepatic CD36 protein using western blotting after 0–48 h of CHX treatment in the presence or absence of palmitate or rapamycin in HepG2 cells. There was no obvious difference in the CD36 protein half-life in the presence or absence of palmitate or rapamycin (Fig. 3), indicating that rapamycin had no influence on the degradation of hepatic CD36 protein.

3.4. Rapamycin suppresses hepatic CD36 translational efficiency through the inhibition of the mTOR pathway

To investigate whether hepatic CD36 translational efficiency is sensitive to rapamycin in HepG2 cells or C57BL/6J mice, we performed a polysome analysis for CD36 in the presence or absence of rapamycin. The data showed that the CD36 mRNA was shifted to heavier fractions when HepG2 cells were treated with palmitate or C57BL/6J mice were fed with HFD, whereas rapamycin shifted the CD36 mRNA to lighter fractions in palmitate-treated HepG2 cells (Fig. 4A) or HFD-fed C57BL/6J mice (Fig. 4B), indicating a decreased number of ribosomal loading and inactive translation of CD36. These findings suggest that rapamycin decreased hepatic CD36 protein expression via translational suppression of CD36.

We further examined the molecular mechanisms by which rapamycin inhibited hepatic CD36 translational efficiency. The data show that the phosphorylation of mTOR, p70S6K, 4E-BP1, and eIF4E was enhanced when HepG2 cells were treated with palmitate or C57BL/6J mice were fed with HFD, while rapamycin inhibited the enhanced phosphorylation of mTOR pathway in palmitate-treated HepG2 cells (Fig. 4C) or HFD-fed C57BL/6J mice (Fig. 4D), suggesting that mTOR pathway was involved in rapamycin-mediated CD36 translational suppression.

4. Discussion

Numerous studies have demonstrated that rapamycin has pleiotropic anti-lipid deposition effect on hepatic steatosis. In the present study, we used palmitate treatment in HepG2 cells or HFD feeding in C57BL/6J mice to induce hepatic steatosis. Our data demonstrate that palmitate or HFD significantly increased hepatic CD36 protein expression in HepG2 cells or C57BL/6J mice. It has been reported that CD36 is a transcriptional target of orphan nuclear receptors including liver X receptor (LXR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptor γ (PPARγ) in the promotion of hepatic steatosis [21], but we found that CD36 mRNA expression was not altered when HepG2 cells were treated with lower dose palmitate (⩽0.08 mmol/L) or C57BL/6J mice were fed with HFD. This may be because lower dose palmitate treatment or HFD feeding was not sufficient to activate these transcriptional regulators of CD36.

Using Oil Red O staining and a quantitative assay of FFA and TG, we confirmed that rapamycin significantly alleviated hepatic lipid accumulation in palmitate-treated HepG2 cells or HFD-fed C57BL/6J mice, suggesting that rapamycin provided a protective role in alleviation of hepatic steatosis.

Our data demonstrate that rapamycin inhibited hepatic CD36 protein expression, but had no effect on CD36 mRNA expression, suggesting that CD36 participates in the rapamycin-mediated alleviation of hepatic steatosis and rapamycin disrupts hepatic CD36 protein expression at a post-transcriptional level, rather than at a transcriptional level.

In order to determine whether the decreased hepatic CD36 protein expression results from reduced protein half-life, we detected the hepatic CD36 protein stability using a protein degradation assay. We found that CD36 protein degradation was not significantly different between the HepG2 cells treated with or without rapamycin, indicating that rapamycin did not accelerate the degradation of the CD36 protein *in vitro*.

Translational control is a key genetic regulatory mechanism in the development of different diseases. Arbini demonstrated that mitochondrial DNA depletion-induced translational repression of BRCA2 increases cancer cells sensitivity to PARP inhibitors [22]. Pan showed that translational inhibition of ER mannosidase I accelerates the progression of liver disease associated with alpha1-antitrypsin deficiency [23]. Wada demonstrated that microRNA-23a-induced translational suppression of MAFbx/atrogin-1 and MuRF1 integrates resistance to muscle atrophy [24]. We performed a polysome analysis to investigate whether rapamycin suppressed hepatic CD36 translational efficiency, which might be responsible for the reduction in the CD36 protein expression. The data show that the CD36 mRNA was shifted to heavier fractions in palmitate-treated HepG2 cells or HFD-fed C57BL/6J mice, whereas rapamycin shifted the CD36 mRNA to lighter fractions, corresponding to inhibited ribosome loading and translation of CD36. These data indicate that rapamycin-suppressed CD36 translational efficiency

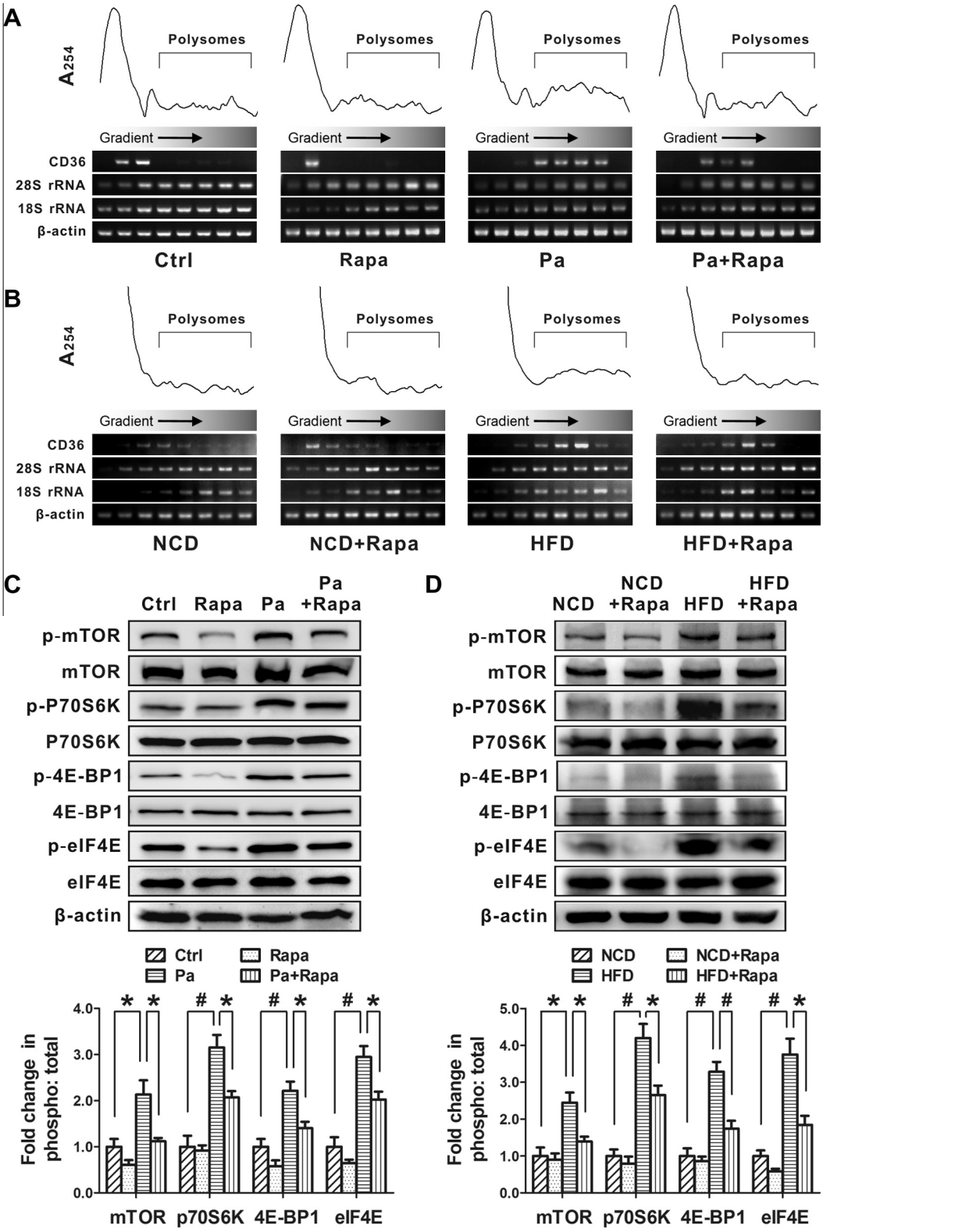

Fig. 4. Effect of rapamycin on hepatic CD36 translational efficiency and the phosphorylation of mTOR pathway. HepG2 cells were incubated in serum-free medium (Ctrl) or medium containing 10 ng/mL rapamycin (Rapa) or 0.08 mmol/L palmitate (Pa) or 0.08 mmol/L palmitate plus 10 ng/mL rapamycin (Pa + Rapa). C57BL/6J mice were fed with normal chow diet (NCD) or NCD plus rapamycin injection (NCD + Rapa) or high fat diet (HFD) or HFD plus rapamycin injection (HFD + Rapa). Polysomal analysis was performed in the cells (A) and mice (B). Western blotting was performed for the phosphorylation of mTOR pathway in the cells (C) and mice (D). Data are depicted as mean ± SD from three separate experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$.

was due to less ribosome encoding this mRNA, resulting in reduction of CD36 protein expression.

Furthermore, we investigated the regulatory mechanisms by which rapamycin suppressed the CD36 translational efficiency at the translational level. The results indicate that the phosphorylation of mTOR and its downstream translational regulators including p70S6K, 4E-BP1, and eIF4E was enhanced in palmitate-treated HepG2 cells or HFD-fed C57BL/6J mice, but rapamycin inhibited the phosphorylation of the mTOR pathway, and contributed to inhibit CD36 translation initiation. These findings demonstrate that rapamycin suppressed hepatic CD36 translation was mediated by the inhibition of the mTOR pathway.

In conclusion, our findings both *in vitro* and *in vivo* demonstrated that rapamycin decreased hepatic CD36 expression at the translational level, but not the transcriptional level. Rapamycin suppressed hepatic CD36 translational efficiency through the inhibition of the mTOR pathway, resulting in reduction of CD36 protein expression and alleviation of hepatic steatosis.

Acknowledgments

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