Rubicon deficiency exacerbates fasting-induced hepatic steatosis

Fan Dong, Xiao-Wen Hu, Shasha Zhang, Fan He, Amber Naz, Lin He, Hongxin Zhu*

Abstract

Objective: Rubicon is an inhibitory interacting protein of the autophagy-related protein Uvrag. We previously showed that Rubicon deficiency promotes autophagic flux in vivo and that autophagy can degrade lipid droplets. This study aimed to investigate the effects of Rubicon deficiency on fasting-induced hepatic steatosis.

Methods: Two-month-old wild-type (WT) and Rubicon-deficient mice were subjected to feeding or fasting for 24 hours to induce hepatic steatosis. The distribution of liver lipid droplets was revealed by oil red O staining. Hepatic and plasma triglyceride, non-esterified fatty acid (NEFA), and cholesterol levels were detected using commercially available kits. Real-time reverse transcriptase-polymerase chain reaction was performed to analyze the mRNA expression of genes related to lipid metabolism in the liver. Western blot was conducted to assess autophagy-related protein levels in the liver. The animal experiments were approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University, China.

Results: We showed that under fasting conditions, Rubicon-deficient mice had more lipid droplets in the liver than WT controls. Consistent with these results, the hepatic triglyceride, NEFA, and cholesterol levels in fasted Rubicon-deficient mice were significantly higher than those of fasted WT controls. The levels of SREBP-1, a key regulator of lipid synthesis, were significantly lower in livers from fasted WT mice than those of fed WT mice. However, the decrease in SREBP-1 in fasted mice was attenuated by Rubicon deficiency. Western blot analysis demonstrated that the fasting-induced increase in autophagic flux was amplified by Rubicon deficiency. Finally, we showed that Rubicon deficiency in mice led to elevated plasma triglyceride and NEFA acid levels under fasting conditions.

Conclusion: Rubicon deficiency exacerbates fasting-induced hepatic steatosis in mice.

Keywords: autophagy, fasting, hepatic steatosis, liver, Rubicon

Introduction

Steatosis refers to the accumulation of lipid droplets in non-adipocytes. Steatosis occurs in various organs such as the liver, heart, and kidney, and among these three presentations, hepatic steatosis is the most common. In the liver, non-esterified fatty acids (NEFAs) originate from food intake, endogenous synthesis, and plasma NEFAs that are released from peripheral tissues. These NEFAs are consumed by several pathways including metabolism by mitochondrial β-oxidation, esterification to triglycerides that are stored in lipid droplets, and attachment to apolipoprotein B to form very-low-density lipoproteins that are transported to the lysosome for degradation. Autophagy is a lysosomal pathway in which the cytoplasmic contents are engulfed by double-membrane autophagosomes that are transported to the lysosome for degradation. Autophagy has been shown to play an important role in regulating intracellular metabolism and cell survival.[1] An increasing amount of evidence has shown that a selective form of autophagy, called lipophagy,[4] can hydrolyze lipid droplets into NEFAs and released into the blood. Excessive NEFAs are transported to the liver and triglycerides are synthesized. When the rate at which liver cells synthesize triglycerides exceeds liver cell oxidation of NEFAs and the export of very-low-density lipoproteins, neutral fat (mainly triglycerides) gradually accumulates in the liver and cause hepatic steatosis. Hepatic steatosis is the main pathological change in non-alcoholic fatty liver disease.[2]

Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting protein) is an inhibitory interacting protein of the autophagy-related protein Uvrag.[11] Rubicon is mainly located in endosomes and lysosomes, and it is essential for the regulation of autophagosome–lysosome fusion and endocytic transport. Rubicon has been shown to inhibit autophagosome maturation by forming a complex with UVRAG-Vps34.[12] It previously showed that Rubicon deficiency in mice promotes autophagy.[13] Because lipophagy promotes hydrolysis of lipid droplets, we hypothesize that Rubicon deficiency attenuates fasting-induced hepatic steatosis.
Materials and methods

Animals

Mice on the FVB/NJ background were used in this study. All animal experiments were conducted in strict accordance with the regulations of the International Cooperation Committee of Animal Welfare and approved by the Institutional Animal Care and Use Committee at Shanghai Jiao Tong University, China. The Rubicon-deficient mice generated by piggyBac transposition were kindly provided by the Institute of Developmental Biology and Molecular Medicine of Fudan University.[13] All mice were housed in a specific pathogen-free (SPF) environment with a 12:12 hour light: dark cycle to simulate the natural environment. The mice had free access to standard rodent chow and drinking water. Rubicon heterozygous female and male mice (Rubicon+/−) were mated to obtain homozygous Rubicon-deficient mice (Rubicon−/−) and wild-type (WT) controls. Male WT and Rubicon-deficient mice at 2 months of age were used for this study. For the fasting experiment, mice were deprived of food for 24 hours but had free access to drinking water.

Oil red O staining

Frozen sections of mouse livers that were 8 μm thick were cut (Leica CM1950 rotary microtome) and stained with Oil red O (1320–06–5, Sigma-Aldrich, St. Louis, MO, USA). Briefly, the sections were washed in PBS, and then in 60% isopropanol for 5 minutes. The sections were incubated in the Oil red O staining solution for 15 minutes in the dark. The sections were then immersed in 60% isopropanol for 1 minute, and washed with distilled water, and the nuclei were counterstained with hematoxylin staining solution. A Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) was used to visualize the sections.

Total RNA extraction and real-time RT-PCR

Total RNA was extracted from mouse livers using TRIzol reagent (15596018, Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA integrity was detected using agarose gel electrophoresis. RNA concentration and purity were analyzed using Nanodrop spectrophotometers (Thermo Fisher Scientific, Inc.). cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser for real-time reverse transcriptase polymerase chain reaction (RT-PCR; Takara Biomedical Technology (Dalian) Co., Ltd., Dalian, China, RR047A). Expression of genes related to lipid metabolism in the liver was analyzed using FastStart Universal SYBR Green Master (Roxy) system (491385001, Roche Applied Science, Mannheim, Germany) and was normalized to Gapdh. RT-PCR was conducted in triplicate for each group. The sequences of primers that were used for the real-time RT-PCR are shown in Table 1.

Western blot

The liver Proteins were extracted using RIPA buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors and phosphatase inhibitors. The protein concentration was determined using the bicinchoninic acid (BCA) method. The Proteins were separated using sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and transferred to a nitrocellulose membrane (Millipore Corporation, MA, USA). The membrane was blocked for 1 hour, and then incubated with primary antibodies including rabbit anti-Rubicon polyclonal antibody (Cat# 8465, dilution 1:1000, Cell Signaling Technology, MA, USA), rabbit anti-Uvrag polyclonal antibody (Cat# 13115, dilution 1:1000), rabbit anti-Becn1 polyclonal antibody (Cat# PD017, dilution 1:1000, MBL, Nagoya, Japan), rabbit anti-ATG9 polyclonal antibody (Cat# ab108338, dilution 1:1000, Abcam, Cambridge, UK), rabbit anti-ATG7 polyclonal antibody (Cat# 8538, dilution 1:1000, Cell Signaling Technology), rat anti-LAMP-1 monoclonal antibody (Santa Cruz Biotechnology Inc., TX, USA, Cat# sc-20011, dilution 1:1000), rabbit anti-LC3 polyclonal antibody (Cat# PM036, dilution 1:500, MBL), rabbit anti-GAPDH polyclonal antibody (Cat# sc-365062, dilution 1:2000, Santa Cruz Biotechnology Inc), rabbit anti-Phospho-AMPKα polyclonal antibody (Cat# 2535, dilution 1:1000, Cell Signaling Technology), and rabbit anti-AMPKα polyclonal antibody (Cat# 5831, dilution 1:1000, Cell Signaling Technology) overnight at 4°C. After washing with TBST, the membrane was incubated with secondary antibodies for 1 hour at room temperature. After washing with TBST, the protein bands were detected using Immobilon™ Western Chemiluminescent HRP Substrate (WBKLS0050, Millipore Corporation). Quantitative western blot analysis was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurement of plasma insulin levels

An appropriate amount of sodium heparin and Avertin (Sigma-Aldrich) were injected intraperitoneally into the mice. The eyeballs were removed, which caused the mice to bleed, and the blood was collected in centrifuge tubes that were treated with sodium heparin anticoagulant, and centrifuged for 10 minutes at 3000 × g at 4°C to separate the plasma fraction. The plasma insulin levels were measured using a Rat/Mouse Insulin ELISA Kit (Millipore Corporation, Billerica, MA, USA).

Hepatic and plasma triglyceride, NEFA, and cholesterol levels

Triglyceride, NEFA, and cholesterol levels were detected using commercially available kits (LabAssay Triglyceride, LabAssay NEFA, and LabAssay Cholesterol, respectively; Nanjing Jiancheng, Nanjing, China) in accordance with the manufacturer’s instructions.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by table:

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5′–3′)</th>
<th>Primer sequences (5′–3′)</th>
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<tr>
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<td>CCG TGG CTC CGA TGG TCT AG</td>
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<td></td>
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<tr>
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<td>FGPa21</td>
<td>GCT GCT GGG GGA GGG TTA CA</td>
<td>CAC AGG TCC CCA GGA TGT TG</td>
</tr>
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</table>

RT-PCR= reverse transcription-polymerase chain reaction.
Bonferroni’s method was used for post hoc pairwise multiple comparisons (GraphPad Prism 9 Software, San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

**Results**

**Rubicon deficiency exacerbates fasting-induced hepatic steatosis**

We previously showed that *Rubicon* deficiency promotes autophagy,[13] which can hydrolyze lipid droplets. Therefore, we hypothesize that *Rubicon* deficiency alleviates fasting-induced hepatic steatosis. To test this hypothesis, WT and *Rubicon*-deficient mice at 2 months of age were subjected to feeding or fasting for 24 hours. Oil red O staining showed that the livers of fasted WT animals had more lipid droplets than fed animals (Fig. 1A). The increase in lipid levels in fasted animals was further amplified by *Rubicon* deficiency (Fig. 1A), suggesting that *Rubicon* deficiency promotes fasting-induced hepatic steatosis. We then assessed the triglyceride, NEFA, and total cholesterol levels in the liver of fed WT and *Rubicon*-deficient mice (Fig. 1B–D).

However, triglyceride, NEFA, and total cholesterol levels were significantly higher in livers from fasted *Rubicon*-deficient mice than the WT controls ($P < 0.05$; Fig. 1B–D). These results demonstrate that *Rubicon* deficiency exacerbates fasting-induced hepatic steatosis.

**The effect of Rubicon deficiency on the expression of genes related to lipid metabolism in the liver**

The main cause of lipid accumulation is the imbalance between NEFA intake and synthesis, and NEFA decomposition and consumption. Therefore, we examined the effect of *Rubicon* deficiency on the expression of genes related to hepatic lipid metabolism including CD36, SREBP-1, FAS, CPT-1, FGF21, and PPARα. Real-time RT-PCR showed that under fasting conditions, SREBP-1 and FAS expression was down-regulated (Fig. 2B and C), while CPT-1, PPARα, and FGF21 expression was up-regulated (Fig. 2D–F) in WT mice compared with the fed condition, which was consistent with the results of a previous study.[14] However, *Rubicon* deficiency did not affect CD36, FAS, CPT-1, PPARα, and FGF21 expression in livers from fasted animals (Fig. 2A, C–F). Additionally, the SREBP-1 levels were...
significantly higher in fasted Rubicon-deficient animals than WT controls (*P < 0.05; Fig. 2B). These results suggest that Rubicon deficiency promotes lipid synthesis in the liver under fasting conditions.

**The effect of Rubicon deficiency on liver AMPK activity**

AMP-activated protein kinase (AMPK) acts as a cell energy regulator, and it can sense changes in cell energy metabolism.[15] AMPK activity is closely related to hepatic steatosis.[16] Thus, we evaluated the effect of Rubicon deficiency on phosphorylated AMPK in mouse livers. Western blot showed that phosphorylated AMPK was more abundant in fed Rubicon-deficient mice than in WT controls (Fig. 3A). Under fasting conditions, phosphorylated AMPK was significantly higher in livers from WT controls than those from fed controls. However, no difference in the levels of phosphorylated AMPK was observed in livers from fasted Rubicon-deficient mice and WT controls (Fig. 3A and B), suggesting that increased lipid accumulation is caused by Rubicon deficiency under fasting conditions may not be due to changes in energy levels.

**The effect of Rubicon deficiency on liver autophagy**

Autophagy promotes lipid droplet hydrolysis. However, we showed that Rubicon deficiency exacerbates lipid accumulation in the liver. To determine if Rubicon deficiency enhances autophagy in the liver, we analyzed the expression of autophagy-related proteins. We first confirmed the absence of the Rubicon protein in the liver of Rubicon-deficient mice (Fig. 4A). We then detected the abundance of autophagy-related proteins including Uvrag, Beclin1, ATG9, ATG7, and LAMP-1, and LC3 II in the liver. No significant difference in Uvrag, Beclin1, ATG9, ATG7, and LAMP-1 levels was detected between WT and Rubicon-deficient mice under normal and fasting conditions (Fig. 4A–E and G). In addition, LC3 II levels were comparable between WT and Rubicon-deficient mice under fed conditions (Fig. 4A and F). However, fasting significantly increased LC3 II levels in the liver compared with fed animals (Fig. 4A and F).
which is consistent with a previous report.[13] Additionally, Rubicon deficiency markedly reduced LC3 II levels in livers from fasting animals (Fig. 4A and F), which is in agreement with previous data that Rubicon deficiency enhances autophagic flux.[13] Thus, these data suggest that Rubicon deficiency enhances autophagic flux in the liver under fasting conditions.

The effect of Rubicon deficiency on plasma lipid parameters

The observation that autophagic flux is enhanced in the liver of fasted Rubicon-deficient mice contradicts the observation that Rubicon deficiency exacerbates hepatic steatosis in fasted animals. Thus, we investigated whether uptake of NEFAs from the plasma is increased in the liver of Rubicon-deficient mice. Therefore, we assessed lipid parameters in the plasma. Under both fed and fasting conditions, the plasma triglyceride and NEFA levels were significantly higher in Rubicon-deficient mice than in WT controls ($P < 0.05$; Fig. 5A and B). No significant difference in plasma cholesterol and insulin levels was observed between WT and Rubicon-deficient mice under fed and fasting conditions (Fig. 5C and D). These data suggest that Rubicon deficiency elevates plasma triglyceride and NEFA levels, which may contribute to the exacerbation of hepatic steatosis.

Discussion

In this study, we found that Rubicon deficiency exacerbates fasting-induced hepatic steatosis in mice, which is accompanied by increased plasma NEFA levels. Rubicon forms a complex with Uvrag-Vps34 and inhibits Uvrag-Vps34 activity, leading to autophagy suppression.[17] We previously showed that under starvation conditions, Rubicon deficiency enhances autophagic flux in the heart by accelerating autophagosome degradation.[13] In the present study, our results showed that Rubicon deficiency markedly reduced liver LC3 II levels that were increased by fasting, which is consistent with a previous report that Rubicon deficiency increased autophagic flux.[13]

Because lipid droplets can be degraded by lipophagy, we hypothesize that Rubicon deficiency alleviates hepatic steatosis. However, we showed that Rubicon deficiency exacerbates fasting-induced hepatic steatosis. The lipid metabolism in the liver is regulated by lipid uptake, endogenous synthesis, hydrolysis, and consumption. We analyzed the expression of a set of key genes that are related to lipid metabolism including CD36, SREBP-1, FAS, CPT-1, PPARa, and FGF21. CD36 is a gene that is related to fatty acid uptake and transport.[18] SREBP-1 is a key transcription factor that regulates lipid synthesis.[19] FAS is an important fatty acid synthase.[20] CPT-1 is a key enzyme that regulates fatty acid oxidation.[21] PPARa is a
transcription factor involved in fatty acid oxidation. **FGF 21** is a secretory factor that controls liver lipid metabolism.\(^{[22]}\) We found that **CD36**, **FAS**, **CPT-1**, **PPAR**\(_a\), and **FGF21** expression was not significantly different in livers from fasted WT and Rubicon-deficient mice. The unaltered AMPK activity in fasted WT and Rubicon-deficient mice is consistent with the findings that the expression of genes regulating fatty acid oxidation was comparable between fasted WT and Rubicon-deficient mice. However, we found that **SREBP-1** expression, the key regulator of lipid synthesis, was different in livers from fasted WT and Rubicon-deficient mice, and that the fasting-induced reduction in liver SREBP-1 was attenuated in Rubicon-deficient mice. Thus, lipid droplet synthesis in the liver may be enhanced by Rubicon deficiency under fasting conditions.

Plasma NEFAs are the main source of NEFA for the synthesis of lipid droplets in the liver. Thus, increased lipid accumulation in the liver of fasted Rubicon-deficient mice may be due to increased uptake of plasma NEFA. We showed that NEFA levels were significantly elevated in fasted Rubicon-deficient mice. Under fasting conditions, the plasma NEFAs are mainly derived from adipocytes, which hydrolyze lipid droplets and release the NEFAs into the blood. Because the mice we used were global Rubicon-deficient mice, and it can promote hydrolysis of lipid droplets in adipocytes through enhanced lipophagy. A recent study using mice with adipocyte-specific Rubicon deletion showed that Rubicon is essential for proper maintenance of lipid metabolism in aging adipocytes. Adipocyte-specific Rubicon deficiency disrupts adipogenesis, leading to hepatic steatosis.\(^{[23]}\) Our data suggest that Rubicon deficiency enhances autophagic flux in adipocytes and increase the release of NEFA and triglycerides into the blood, leading to increased uptake of NEFA by hepatocytes and lipid droplet formation. If the rate of lipid droplet formation exceeds that of lipid droplet degradation by lipophagy, this could lead to lipid accumulation and hepatic steatosis.

**Limitations**

A methodological limitation in this study is that the Rubicon-deficient mice used were global knockout mice. Thus, lipid metabolism in organs other than the liver may be affected, which contributes to the elevated lipid parameters in the plasma and
hepatic steatosis. Tissue-specific Rubicon-deficient mice are useful to further investigate the cause of exacerbated hepatic steatosis that was observed in this study.

Conclusion

Rubicon deficiency exacerbates fasting-induced hepatic steatosis in mice.

Acknowledgments

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Author contributions

FD, XwH, SZ, FH, and AN contributed to experiment execution and data analysis. FD and XwH contributed to manuscript drafting. HZ contributed to manuscript revision. HZ and LH contributed to study conception. All the authors have read and approved the final manuscript.

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Institutional review board statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, China.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Editorial note: LH is an Editorial Board member of Journal of Bio-X research. He was blinded from reviewing or making decisions on the manuscript. This article was subject to the journal’s standard procedures, with peer review handled independently of this Editorial Board member and their research groups.

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