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Research Paper

AMPK Re-Activation Suppresses Hepatic Steatosis but its Downregulation Does Not Promote Fatty Liver Development

Nadia Boudaba [a,b,c], Allison Marion [a,b,c], Camille Huet [a,b,c], Rémi Pierre [a,b,c], Benoit Viollet [a,b,c], Marc Foretz [a,b,c,*]

[a] INSERM, U1016, Institut Cochin, Paris 75014, France
[b] CNRS, UMR8104, Paris 75014, France
[c] Université Paris Descartes, Sorbonne Paris Cité, Paris 75014, France

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ABSTRACT

Nonalcoholic fatty liver disease is a highly prevalent component of disorders associated with disrupted energy homeostasis. Although dysregulation of the energy sensor AMP-activated protein kinase (AMPK) is viewed as a pathogenic factor in the development of fatty liver its role has not been directly demonstrated. Unexpectedly, we show here that liver-specific AMPK KO mice display normal hepatic lipid homeostasis and are not prone to fatty liver development, indicating that the decreases in AMPK activity associated with hepatic steatosis may be a consequence, rather than a cause, of changes in hepatic metabolism. In contrast, we found that pharmacological re-activation of downregulated AMPK in fatty liver is sufficient to normalize hepatic lipid content. Mechanistically, AMPK activation reduces hepatic triglyceride content both by inhibiting lipid synthesis and by stimulating fatty acid oxidation in an LKB1-dependent manner, through a transcription-independent mechanism. Furthermore, the effect of the antidiabetic drug metformin on lipogenesis inhibition and fatty acid oxidation stimulation was enhanced by combination treatment with small-molecule AMPK activators in primary hepatocytes from mice and humans. Overall, these results demonstrate that AMPK downregulation is not a triggering factor in fatty liver development but in contrast, establish the therapeutic impact of pharmacological AMPK re-activation in the treatment of fatty liver disease.
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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease worldwide, increasing rapidly with the expanding burden of obesity and insulin resistance (Cohen et al., 2011). However, the precise mechanisms leading to the aberrant accumulation of triglycerides (TG) in the liver remain poorly understood, and therapeutic approaches are limited. In this context, the energy sensor AMP-activated protein kinase (AMPK) has recently garnered much attention for its ability to coordinate multiple metabolic pathways, including hepatic lipid metabolism (Viollet et al., 2009). AMPK inhibits lipogenesis by phosphorylation of acetyl-CoA carboxylase 1 (ACC1) at Ser79 and ACC2 at Ser212, key rate controlling enzymes in malonyl-CoA synthesis. Malonyl-CoA is both a critical precursor for fatty acid biosynthesis and a potent allosteric inhibitor of long-chain fatty acyl-CoA transport into mitochondria for β-oxidation during the carnitine palmitoyltransferase 1 (CPT1) step. AMPK-mediated ACC inhibition leads to a decrease in intracellular malonyl-CoA levels, relieving CPT1 inhibition, resulting in an increase in fatty acid oxidation. In addition to these short-term effects, AMPK inhibits the transcription of lipogenic genes by phosphorylating transcription factors, such as sterol regulatory element binding protein-1c (SREBP-1c) (Li et al., 2011) and carbohydrate-responsive element-binding protein (ChREBP) (Kawaguchi et al., 2002). Although AMPK has been implicated in the control of lipid partitioning between the biosynthetic and oxidative pathways in the liver in conditions of stress, its physiological relevance in normal conditions has not been formally investigated in vivo.

In light of the multiple effects of AMPK on lipid metabolism, it has been suggested that the impairment of hepatic AMPK activity is a key pathological event in the development of the insulin resistance and metabolic disorders associated with metabolic syndrome, including hepatic steatosis (Ruderman et al., 2013). This hypothesis is supported by the observation of low levels of hepatic AMPK activity in various rodent fatty liver models (Muse et al., 2004; Yu et al., 2004). The inhibition of AMPK may stimulate anabolic pathways, such as lipid synthesis, and attenuate catabolic pathways, such as β-oxidation. Interestingly, hepatic AMPK is downregulated by hyperglycemia, the saturated fatty acid palmitate, branched-chain amino acids, the adipocyte-derived cytokine resistin and insulin, and this downregulation is associated with nutrient overload and the development of insulin resistance (Kraegen et al., 2006; Li et al., 2010; Mankouri et al., 2010; Muse et al., 2004; Wu

* Corresponding author at: Institut Cochin, Inserm U1016, CNRS UMR8104, Université Paris Descartes, Department of Endocrinology, Metabolism and Diabetes, 24, rue du Faubourg Saint-Jacques, F-75014 Paris, France.
E-mail address: marc.foretz@inserm.fr (M. Foretz).

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et al., 2007). Furthermore, AMPK inhibition is also associated with the hepatic fat accumulation induced by chemical agents, such as ethanol, sidestream cigarette smoke and orotic acid (Jung et al., 2011; You et al., 2004; Yuan et al., 2009). Despite the recognized correlation between fatty liver and AMPK downregulation, it remains unknown whether AMPK plays a causal role in the excessive accumulation of TGs in the liver or whether its downregulation is secondary to the increase in lipid levels or associated complications.

Conversely, by inhibiting lipid synthesis and activating fatty acid oxidation, AMPK activation in the liver would be expected to decrease lipid accumulation. AMPK is, thus, a highly attractive target for hepatic steatosis management. Consistent with this notion, a large number of AMPK-activating compounds have been reported to have beneficial effects for fatty liver treatment (Smith et al., 2016b; Viollet et al., 2009). In particular, metformin, a widely used antidiabetic drug, markedly decreases hepatic steatosis in rodents (Lin et al., 2000), presumably by activating hepatic AMPK (Fullerton et al., 2013; Zhou et al., 2001). AMPK activation by AICAR (Bergeron et al., 2001; Yu et al., 2004), berberine (Kim et al., 2009) or the small-molecule direct AMPK activators A-769662 (Cool et al., 2006), C13 (Gomez-Galeno et al., 2010) and PF-249 and PF-739 (Cokorinos et al., 2017), has also been shown to decrease liver fat content in obese rodents, highlighting the potential of AMPK activation for therapeutic interventions in the liver. However, most of these AMPK agonists are known to display AMPK-independent effects and may therefore interfere with various biological pathways unrelated to AMPK (Benziane et al., 2009; Foretz et al., 2010; Guigas et al., 2006; Hasenour et al., 2014; Moreno et al., 2008). The extent to which specific hepatic AMPK activation can alleviate fatty liver thus remains unclear. Indeed, it cannot be excluded that at least some of the metabolic effects of AMPK-activating drugs in vivo could be mediated independently of AMPK activation in the liver. Hence, pharmacological studies should be combined with genetic studies examining the role of AMPK in mediating the observed pharmacological outcomes. To our knowledge, no study has employed genetic mouse models with specific AMPK deletion in the liver to investigate the effect of direct AMPK activators on NAFLD.

In this study, we generated liver AMPK-deficient mice, to investigate the consequences of AMPK loss on the development of fatty liver disease and to assess the AMPK-dependent action of indirect and direct AMPK activators in the reduction of hepatic steatosis. We found that a deficiency of AMPK in the liver was not sufficient to trigger hepatic lipid accumulation, indicating that AMPK dysfunction is not a causal factor leading to the development of fatty liver disease. We also obtained genetic evidence that pharmacological AMPK activation decreased hepatic TG content through direct effects on lipogenesis and β-oxidation rates, rather than changes in lipogenic gene expression profile. Finally, we showed that small-molecule AMPK activators efficiently inhibited lipogenic flux alone or in combination with metformin in human hepatocytes. Thus, our findings demonstrate the potential of pharmacological AMPK activation for therapeutic interventions in fatty liver disease.

2. Materials and Methods

2.1. Study Approvals

All animal studies were approved by the Paris Descartes University ethics committee (no. CEEA34.BV.157.12) and the Direction Départementale des Services Vétérinaires of the Préfecture de Police de Paris (authorization no. 75-886).

2.2. Animals

All mice were maintained in a barrier facility under a 12-h light/12-h dark cycle (8 am-8 pm) with free access to water and standard mouse diet (in terms of energy: 65% carbohydrate, 11% fat, 24% protein). AMPKα1 floxed mice and AMPKα1 total knockout mice were generated, as described below. AMPKα2 floxed and AMPKα2 total knockout mice have been described elsewhere (Viollet et al., 2003). The generation of AMPKγ1 total knockout mice has been described previously (Foretz et al., 2011). Liver double-knockout of AMPKα1 and AMPKα2 catalytic subunits was achieved by crossing AMPKα1$^{lox/lox}$ mice with AMPKα2$^{lox/lox}$ mice and then Alfp-Cre transgenic mice (kindly provided by François Tronche, Université Pierre et Marie Curie, Paris), all on the C57BL/6 background, to generate AMPKα1$^{lox/lox}$, α2$^{lox/lox}$ (control) and AMPKα1$^{lox/lox}$, α2$^{lox/lox}$-Alfp-Cre (liver AMPK-deficient mice). Liver-specific LKB1 knockout mice were generated by crossing LKB1-$^{lox/lox}$ mice (kindly provided by Ronald DePinho, Harvard University, USA) with tamoxifen-inducible albumin-Cre-ERT2 mice (kindly provided by Daniel Metzger, IGBMC, France). Twelve-week-old LKB1$^{lox/lox}$-Alb-Cre-ERT2 mice were treated with vehicle (sunflower oil containing 10% ethanol; control) or tamoxifen (Sigma) at a dose of 1 mg/mouse, injected intraperitoneally in a final volume of 100 μl, over five consecutive days. Mice were studied or used for primary hepatocyte isolation three weeks after the start of tamoxifen administration. aP2-nSREBP-1c transgenic mice (Shimomura et al., 1998) were purchased from the Jackson Laboratory. aP2-nSREBP-1c transgenic males (C57BL/6J × SJL background) were crossed with C57BL/6J females. We studied the aP2-nSREBP-1c mice and their control littermates generated from this cross. aP2-nSREBP-1c transgenic mice were crossed with AMPKα1$^{lox/lox}$, α2$^{lox/lox}$-Alfp-Cre mice to generate aP2-nSREBP-1c$^{tg/wt}$-AMPKα1$^{lox/lox}$, α2$^{lox/lox}$ and aP2-nSREBP-1c$^{tg/wt}$-AMPKα1$^{lox/lox}$, α2$^{lox/lox}$-Alfp-Cre mice. C57BL/6J mice were obtained from Harlan France. Eight-week-old control AMPKα1α2 floxed and (liver AMPK-deficient mice) were fed a high-fat diet (in terms of energy: 45% fat, 35% carbohydrate, 20% protein) (Research Diets, #D12451) for 5 months to induce a diet-induced obesity (DIO).

2.3. Gene Targeting and Generation of AMPKα1 (Prkaa1)-Knockout Mice

The *Prkaa1* targeting construct was generated from PCR products amplified from the DNA of 129/Sv ES cells. The 5′ and 3′ homology arms, which were 3.8 and 3.5 kb long, respectively, were inserted on either side of a PGK promoter-driven hygromycin selection cassette flanked by FRT sites and containing a 3′ loxP site, in the pL3-FRT-Hygro vector. A 2.1 kb fragment of genomic DNA bearing exons 4 and 5, partly encoding the catalytic domain, including the phosphorylation site Thr172 within the activation loop (corresponding to amino acids 113-190), with a 5′ loxP site, was introduced between the hygromycin resistance cassette and the 5′ homology arm (Supplemental Fig. S1A). Exponentially growing 129/SV CK35 embryonic stem cells were electroporated with the linearized target DNA construct and selected on plates containing hygromycin. The targeted clones were identified by PCR across both homology arms, with confirmation by Southern blot analysis. Cell populations expanded from the targeted clones were injected into C57BL/6 blastocysts, and animals displaying germline transmission were mated with C57BL/6 mice. The hygromycin resistance cassette flanked by FRT sites was excised by crossing AMPKα1$^{+/lox}$ mice with FLP-expressing mice. The resulting heterozygous offspring were backcrossed onto the C57BL/6J background for at least four generations. These mice were crossed to generate AMPKα1$^{lox/lox}$ mice. Exons 4 and 5, flanked by loxP sites, were disrupted by crossing AMPKα1$^{+/lox}$ mice with deleter EIIa-CRE transgenic mice to produce AMPKα1$^{+/-}$ mice. These mice were crossed to generate wild-type (control) and AMPKα1$^{-/-}$ mice. Routine genotyping was carried out by multiplex PCR with phire hot start II DNA polymerase (Thermo Scientific) on tail DNA with the P1 (5′-tattgctgccattaggctac-3′), P2 (5′-gacctgacagaataggatatgcccaacctc-3′) and P3 (5′-attaaacaccactaattggaaaacattccc-3′) primers, to yield amplification products of 586 bp (WT allele) and 682 bp (floxed allele) with P1/P2 and of 2365 bp (WT allele) and 348 bp (KO allele) with P3/P2 (Supplemental Fig. S1B).

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