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Delayed liver regeneration after partial hepatectomy in adipose differentiation related protein-null mice

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Background & Aims: Adult hepatocytes undergo cell cycle progression and proliferation in response to partial hepatectomy (PH). Transient lipid accumulation within hepatocytes preceding the peak proliferative phase is a characteristic feature of regenerating livers. However, the molecular mediators and mechanisms responsible for lipid accumulation in regenerating livers are not well understood. Adipose differentiation related protein (ADRP; *Plin2*) regulates hepatic triglyceride storage and *Plin2*-deficient (*Plin2*$^{-/-}$) mice have significantly reduced triglyceride (TG) content in the liver. We sought to determine the functional significance of PLIN2 in liver regeneration in response to PH and toxic liver injury and examined whether absence of *Plin2* expression modulates hepatocyte proliferation and liver regeneration.
Methods: We subjected wild-type (WT) and *Plin2*$^{-/-}$ mice to 70% PH or acute carbon tetrachloride (CCL_4) treatment and examined the hepatic lipid content, the expression profile of lipid metabolism-related genes, the rate of cellular proliferation and the dynamics of liver regeneration in the treated animals.
Results: In response to PH, *Plin2*$^{-/-}$ mice showed decreased hepatic triglyceride accumulation and delayed cell cycle progression, which was associated with impaired liver regeneration. Fatty acid (FA) synthesis and lipid transfer gene expression profile were comparable between *Plin2*$^{-/-}$ and wild-type mice, while VLDL secretion rate was higher in the *Plin2*$^{-/-}$ mice. Downregulated β-oxidation and reduced cytosolic FA level in *Plin2*$^{-/-}$ mice may have contributed to the attenuation of the liver regeneration capacity in these animals. In parallel experiments, we also observed attenuated hepatic lipid accumulation and proliferation in response to CCl_4-mediated acute toxic liver injury in *Plin2*$^{-/-}$ mice.
Conclusions: We conclude that PLIN2-mediated lipid accumulation and utilization by the liver is important for efficient liver regeneration in response to PH and toxic liver injury.
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Introduction

The liver is a central metabolic organ that is involved in the synthesis, storage, metabolism, and repartitioning of the macronutrients carbohydrate, protein, and fat. It has a unique capacity to maintain its size for proper function, a property that is quickly activated upon loss of liver mass, as it happens after partial hepatectomy (PH) [1], a maneuver that has been used to study liver regeneration in rodents [2,3]. In this model, the left and medial lobes of the liver are removed, resulting in loss of 70% of the liver mass. As an acute response to PH, most remaining hepatocytes rapidly undergo proliferation, as they also start accumulating large amounts of lipids, including triglycerides (TG), fatty acids (FA), and cholesteryl esters, which are normally stored inside intracellular lipid droplets (LDs). The proliferative response is most marked within the first 3 days, and the original liver mass is restored within a week in rodents [4]. The hepatic LDs are thought to be critical for the process, as lipids stored in these droplets may be used as the source of new cell membrane formation as well as the energy source required for hepatocyte proliferation. However, the exact role of lipid accumulation and utilization in post-PH liver regeneration is poorly understood [5].

Obese patients with fatty livers tend to have poor outcome after liver resection or transplantation [6,7]. In rodents, pre-existing steatosis in high fat diet or genetic models, as well as in other models of augmented hepatic steatosis, are associated with impairment of liver regeneration after PH [8–11]. On the other hand, models such as liver-specific glucocorticoid receptor-inactivated [12] and caveolin 1 deficient mice [13], also display reduced hepatic TG and defective liver regeneration after PH. So, both insufficient and excessive liver fat may negatively impact the normal process of liver regeneration.

Keywords: *ADRP*; *ADFP*; PLIN2; Partial hepatectomy; Lipid oxidation; Triglyceride; Fatty acids.
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Adipose differentiation related protein (ADRP; *Plin2*) is a major LD protein readily detected in fatty liver. Interestingly, $Plin2^{-/-}$ mice display markedly reduced hepatic TG content [14,15]. In addition, they also exhibit evidence of abnormal lipid homeostasis in other tissues, e.g., reduced cholesteryl ester storage and increased cholesterol efflux in macrophages [16], modestly lowered milk fat [17], and compromised retinyl ester transport and storage in the retina [18]. The reduction of hepatic TG content in $Plin2^{-/-}$ mice can be accounted for, wholly or partly, by an increased output of very low density lipoprotein (VLDL) from the liver. Furthermore, in comparison with wild-type mice, the liver of $Plin2^{-/-}$ mice displays altered TG compartmentalization; the TG is markedly depleted in most sub-cellular compartments except the microsomes where the TG concentration is actually increased. To understand the role of *Plin2* and hepatic TG handling in regenerating livers, we studied the lipid dynamics and the regenerative response to 70% partial hepatectomy and carbon tetrachloride (CCl_4)-mediated toxic liver injury in $Plin2^{+/+}$ and $Plin2^{-/-}$ mice.

Materials and methods

Animals

We generated and maintained $Plin2^{-/-}$ mice in C57BL/6J background as described previously [14]; C57BL/6J mice purchased from Jackson Lab (Bar Harbor, Maine) served as wild-type (WT) controls. All experimental procedures were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were kept on 12 h dark-light cycle and maintained on standard chow and water before and after the surgery. We performed PH on 8–12 week old male mice by separately ligating and resecting the right upper, left upper (equivalent to median lobe), and left lower lobes (also known as left lateral lobe), which together make up about 70% of the liver [3]. The mortality rate is less than 5% for both the wild-type and $Plin2^{-/-}$ mice.

In order to evaluate liver regeneration in response to a different acute liver injury, we treated 8–12 weeks old $Plin2^{-/-}$ and wild-type male mice with a single intraperitoneal injection of carbon tetrachloride (CCl_4; 750 µl/kg BW in corn oil) and harvested the liver tissue after 48 and 72 h. BrdU (50 mg/kg BW) was administered intraperitoneally 2 h prior to tissue harvest, tissues were snap frozen in liquid nitrogen and stored in −80 °C for further analysis. For histological analysis, we fixed tissues in 10% formaldehyde and embedded in paraffin or used frozen tissues for further analysis.

Serum chemistry assays

We measured serum triglycerides (Infinity assay by Thermo Electron, Melbourne, Australia), non-esterified free-fatty acids (Wako Chemicals USA, Hercules, CA), Glycerol (Sigma, St. Louis, MO), β-hydroxybutyrate (Cayman Chemical, Ann Arbor, MI) by colorimetric assay. Serum IL-1β, IL-6, and TNF-α (eBioscience, San Diego, CA) were measured using an enzyme immunoassay kit according to the manufacturer's instructions.

Liver histology and immunofluorescence

We assessed liver morphology based on 5 µm hematoxylin and eosin-stained paraffin sections. The frequency of nuclear PCNA staining (anti-PCNA antibody; Cell Signaling Technology, Danvers, MA) was determined by examination of at least three random 200× fields and at least 300 cells and nuclei in each tissue section. We stained liver sections for BrdU positive nuclei with the BrdU labeling and detection kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Sections of liver frozen in OCT compound (Sakura Finetek, Torrance, CA) were stained with Oil Red O (Sigma, St. Louis, MO) for analysis of hepatic fat accumulation.

Liver lipid analysis

We homogenized 200 mg liver tissues in 2 ml of PBS, extracted lipids from these homogenates according to Bligh and Dyer [19] and fractioned different lipid species by one dimensional thin layer chromatography (silica Gel-60, Analtech, Newark, DE), using petroleum ether/ether/acetic-acid (85:25:1). Lipids were visualized by incubating the TLC plate in a saturated iodine chamber and quantified by comparing the optical density of samples with lipid standards using Image J software [14,15].

Determination of rate of very low density lipoprotein (VLDL) secretion in vivo

At 24 h after partial hepatectomy, we quantified the rate of VLDL secretion *in vivo* by injecting intraperitoneally Pluronic F-127 (2 mg/g BW in PBS, BASF Corporation, Florham Park, NJ), a lipoprotein lipase inhibitor, and monitored the serum triglyceride before, and 1, 2, 3, and 4 h after injection [14].

Quantitative reverse transcription PCR

We extracted total RNA from the liver by using the Absolutely RNA™ Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA was treated with DNase I, reverse transcribed using the Superscript III First Strand Synthesis System and used for quantitative PCR (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using the iQ™ SYBR Green Supermix (BIO-RAD, Hercules, CA) and an Mx3000P quantitative PCR machine (Stratagene, La Jolla, CA). *Gapdh*, *Hmbs*, and *Eef1g* were used as the housekeeping control genes.

Western blotting

We analyzed total liver protein extracts by 10% SDS-PAGE, and transferred the protein to nitrocellulose membrane (Bio-Rad) at 250 mA for 90 min. Membranes were blocked with 5% non-fat dry milk dissolved in TBST (Tris-buffered saline Tween) for 1 h at RT prior to incubation with antibodies specific for PCNA, cyclins D1 and A (Santa Cruz, CA), PLINs and MTTP (the latter two antibodies were developed in our laboratory [14,15]). To ensure equal loading, we stripped the membranes and reprobed them with anti-β-actin or anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis

We expressed all values as means ± S.D. with the exception of quantitative PCR analyses, which were expressed as means ± S.E. Difference between means was analyzed by using Student's *t*-test. A difference was considered significant when $p < 0.05$.

Results

PH induces PLIN2 expression

Partial hepatectomy (PH) is a well-established procedure for studying liver regeneration, and a large body of studies have established the chronological changes of hepatic lipids after PH [4,20,21]. However, in order to identify critical time points for comparative studies with the $Plin2^{-/-}$ mice, we first determined the chronology of biochemical and molecular parameters at various time points after PH in wild-type mice. Our initial characterization of plasma and hepatic lipid profiles and gene expression patterns after PH in the wild-type mice recapitulated previous studies (Supplementary Figs. 1 and 2).

Plin2 (Adrp) and *Plin3* (Tip47) are major LD proteins that are highly expressed in the liver. We examined the expression pattern of these LD proteins in response to PH and found that *Tip47* mRNA expression is not influenced by PH, whereas *Plin2* mRNA level doubled at 6 h and went up ~7-fold 12 h after PH,

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