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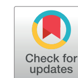
# Ablation of prolactin receptor increases hepatic triglyceride accumulation



Shanshan Shao [a, b, c], Zhenyu Yao [a, b, c], Jiayu Lu [a, b, c], Yongfeng Song [a, b, c], Zhao He [a, b, c], Chunxiao Yu [a, b, c], Xiaoming Zhou [a, b, c], Lifang Zhao [a, b, c], Jiajun Zhao [a, b, c], Ling Gao [b, c, d, *]

[a] Department of Endocrinology, Shandong Provincial Hospital Affiliated to Shandong University, China
[b] Shandong Provincial Key Laboratory of Endocrinology and Lipid Metabolism, China
[c] Institute of Endocrinology and Metabolism, Shandong Academy of Clinical Medicine, China
[d] Scientific Center, Shandong Provincial Hospital Affiliated to Shandong University, China

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ABSTRACT

Increasing prevalence of non-alcoholic fatty liver disease (NAFLD) worldwide has necessitated a more thorough understanding of it and expanded the scope of research in this field. Women are more resistant to NAFLD than men despite equal exposure to major risk factors, such as obesity or hyperlipidemia. Female resistance is hormone-dependent, as evidenced by the sharp increase in NAFLD incidence in post-menopausal women who do not take hormone replacement therapy. Here, we found that the estrogen-responsive pituitary hormone prolactin (PRL), through specific PRL receptor (PRLR), down-regulates hepatic triglyceride (TG) accumulation. PRL was demonstrated to significantly down-regulate hepatic TG accumulation in female mice and protect male mice from liver steatosis induced by high-fat diet. Interestingly, Ad-shPRLR injected mice, whose hepatic PRLR abundance was effectively decreased at the protein levels, exhibited significantly aggravated liver steatosis. PRL could decrease the expression of stearoyl–coenzyme A desaturase 1 (SCD1), the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids, in animal models and multiple hepatic cell lines. Following knockdown of PRLR, the changes to PRL-triggered SCD1 expression disappeared. Thus, PRL acted as a previously unrecognized master regulator of liver TG metabolism, indicating that modification of PRL via PRLR might serve as a potential therapeutic target for NAFLD.

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## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common etiology of chronic liver diseases worldwide [1]. The exact cause of NAFLD remains unknown. However, risk factors such as obesity, type 2 diabetes, malnutrition, drugs, and inborn errors of metabolism likely play an important role in its pathology [2].

The earliest stage of NAFLD is liver steatosis, which is characterized by the accumulation of triglyceride (TG) as lipid droplets within hepatocytes, and it can progress to nonalcoholic steatohepatitis and further, to fibrosis, cirrhosis and hepatocellular carcinomas [3,4]. Liver steatosis occurs when TG homeostasis is disrupted, caused by increased de novo lipogenesis and fatty acid uptake, decreased fatty acid β-oxidation and TG export [5–8].

Despite equal exposure to major risk factors, such as obesity or hyperlipidemia, men develop NAFLD much more than women [9]. Protection in women is hormone-dependent, as evidenced by the sharp rise in female NAFLD incidence following menopause [10]. Hormone replacement therapy in postmenopausal women restores NAFLD resistance [11]. Estrogen has been shown to contribute directly to liver steatosis inhibition through reduction of oxidative damage, inhibition of stellate cell activation or TG synthesis [12,13]. Prolactin (PRL), secreted mainly by lactotropes in the anterior pituitary gland, is an estrogen-responsive peptide hormone expressed at higher levels in females than males. The actions of PRL are mediated by its transmembrane receptor, PRLR, which is a member of the haematopoietic cytokine receptor superfamily, and expressed in most organs in mammals [14].

In fact, PRL is involved in several other physiological functions except for lactation, including osmoregulation, cell proliferation control, and adaptive stress response [15]. Studies related to the influence of PRL on metabolic processes have lately attracted

* Corresponding author. 324 Jing 5 Rd, Jinan, Shandong, 250021, China.
*E-mail address:* gaoling1@medmail.com.cn (L. Gao).

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attention. Hyperprolactinemia has been reported to be associated with the development of insulin resistance, reduced glucose tolerance, increased body mass, and the increase in cardiovascular and all-cause mortality [16–19]. Hypercholesterolemia and hypertriglyceridemia has also been found in population with hyperprolactinemia, with improvement after treatment with dopamine agonists [20,21]. PRL has also been found to impede hepatocellular carcinoma [22], a disease that is associated with liver steatosis [4]. These results suggest a possible link between PRL and liver steatosis. We sought to investigate this possibility and elucidate the underlying mechanisms.

We showed that PRL decreases hepatic TG accumulation *in vitro* and *in vivo* through PRLR, in which SCD1 down-regulation involves. Our study will provide important insights in targeting PRL or PRLR for the treatment of liver steatosis and NAFLD.

## 2. Materials and methods

### 2.1. Animals and treatment

All animals used in this study were obtained from Beijing Vital River Laboratory Animal Technology Company (China) and kept in a temperature-controlled room (22–24 °C) under a 12:12 h light: dark cycle and fed *ad libitum* with food and water. Our research was specifically approved by the Ethics Committee of Shandong Provincial Hospital (Jinan, China).

#### 2.1.1. Female mice

Eight-week-old female C57BL/6 mice were bilaterally ovariectomized and randomly divided into three groups: (1) vehicle group (vehicle), (2) 1 mg/kg/d oPRL intraperitoneally injected group (oPRL 1 mg/kg/d), and (3) 2 mg/kg/d oPRL intraperitoneally injected group (oPRL 2 mg/kg/d). Intraperitoneal injections of oPRL (Sigma, L6520) were given daily for 4 weeks until the mice were sacrificed. We weighed the mice each week and adjusted the oPRL dose according to body weight. For analgesia in the ovariectomy operation, the mice were given subcutaneous injections of Meloxicam (2 mg/kg of body weight; Aladdin, M129228) 1 h before and 24 h after the operation.

#### 2.1.2. High-fat diet-fed male mice

Eight-week-old male C57BL/6 mice were fed a diet containing 60% fat (Research Diets, D12492) and then divided into three groups the same as female mice described above.

#### 2.1.3. Injection of mice with PRLR shRNA

Eight-week-old male C57BL/6 mice were used in this study. The delivery of recombinant adenovirus expressing shRNA against PRLR (Shanghai Gene Pharma, China) or the negative control was performed via tail-vein injection using $10^9$ plaque-forming units/mouse. The adenovirus was given three times within a six-day interval. On the 19th day, all animals were sacrificed.

Mice were sacrificed after fasted for 6 h, and fasting blood was collected right before the mice were sacrificed. Tissues were rapidly obtained, freshly frozen in liquid nitrogen for immunoblot analysis or RNA extraction. A portion of tissues was frozen in OCT for Oil Red O Staining. The remaining portions were fixed in 4% paraformaldehyde for histological analysis.

### 2.2. Human and mouse hepatic cell lines, mouse primary hepatocyte isolation and cultures

HepG2 cells were routinely maintained in MEM supplemented with 10% FBS (Gibco) and 100 U/mL penicillin-streptomycin. AML12 hepatocytes were maintained in DMEM/F12 supplemented with 1% ITS Liquid Media Supplement (Sigma, I3146), 40 ng/mL dexamethasone, 10% FBS and 100 U/mL penicillin-streptomycin. All cell lines were cultured at 37 °C (95% relative humidity, 5% CO2).

Mouse primary hepatocytes were isolated using the two-step collagenase perfusion protocol as described before [23]. Briefly, C57BL/6 mice were anesthetized, and the portal vein was cannulated under aseptic conditions. The liver was perfused with 0.9% saline supplemented with 0.5 mM EDTA and DMEM-low glucose (HyClone) with 100 CDU/ml type I collagenase. The isolated mouse hepatocytes were then cultured at 80%–90% confluence in Williams' medium E (HyClone) containing 20% FBS. Cells were cultured at 37 °C (95% relative humidity, 5% CO2).

### 2.3. Silencing of genes using adenoviruses

Adenoviruses expressing shRNA specific for mouse PRLR gene was designed at Gene Pharma (Shanghai, China). The shRNA sequence for mouse PRLR is 5′-GCCACCTACCATAACTGATGT-3′, which targets both long- and short-form PRLR. Viruses were diluted in PBS and administered at a dose of $10^7$ plaque-forming units/well in 12-well plates or via tail-vein injection using $10^9$ plaque-forming units/mouse.

### 2.4. RNA isolation and real-time quantitative RT-PCR

Total RNA from mice liver tissue was isolated using RNAiso plus reagent (Takara), following the manufacturer's instructions. RT reaction was carried out using the PrimeScript RT reagent kit (TaKaRa). Real-time PCR reaction was performed using the Roche 480 detection system and SYBR Green (DBI) according to the manufacturer's protocol. β-actin gene was employed as an endogenous control. Relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, and the results are expressed as the fold change relative to the control. The PCR primers used were shown in Table 1.

### 2.5. Protein extraction and western blotting

Mouse liver tissue and hepatocytes were homogenized in RIPA buffer containing protease inhibitors. Protein concentration was determined using the BSA method. 80 μg total protein were mixed with SDS loading buffer and subjected to SDS/PAGE on a 10% gel. Proteins were electrotransferred onto a PVDF membrane (Milipore) and the blots were probed with the following primary antibodies overnight at 4 °C: Anti-PRLR antibody was from Santa Cruz, anti-SCD1 antibody was from Abcam and anti-GAPDH antibody was from Proteintech. The appropriate secondary antibodies conjugated to horseradish peroxidase (Amersham) were used at 1:5000. The bound primary antibodies were visualized using Alpha Q detection system.

### 2.6. Serological index analysis

Serum TG, total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), ALT and AST in all mice were measured using enzymatic methods with Mindray reagents and automated spectrophotometry performed on an Mindray system (Mindray Bio-medical Electronics co., LTD).

### 2.7. H&E staining

For histological analyses, liver tissue was fixed with 4% formaldehyde and embedded in paraffin blocks. Serial 5 μm sections were stained with haematoxylin-eosin (H&E) stain.

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