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Delineation of molecular pathways that regulate hepatic PCSK9 and LDL receptor expression during fasting in normolipidemic hamsters

Minhao Wu [1], Bin Dong [1], Aiqin Cao, Hai Li, Jingwen Liu*

Department of Veterans Affairs, Palo Alto Health Care System, 3801 Miranda Avenue, Palo Alto, CA 94304, USA

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ABSTRACT

Background: PCSK9 has emerged as a key regulator of serum LDL-C metabolism by promoting the degradation of hepatic LDL receptor (LDLR). In this study, we investigated the effect of fasting on serum PCSK9, LDL-C, and hepatic LDLR expression in hamsters and further delineated the molecular pathways involved in fasting-induced repression of PCSK9 transcription.
Results: Fasting had insignificant effects on serum total cholesterol and HDL-C levels, but reduced LDL-C, triglyceride and insulin levels. The decrease in serum LDL-C was accompanied by marked reductions of hepatic PCSK9 mRNA and serum PCSK9 protein levels with concomitant increases of hepatic LDLR protein amounts. Fasting produced a profound impact on SREBP1 expression and its transactivating activity, while having modest effects on mRNA expressions of SREBP2 target genes in hamster liver. Although PPARα mRNA levels in hamster liver were elevated by fasting, ligand-induced activation of PPARα with WY14643 compound in hamster primary hepatocytes did not affect PCSK9 mRNA or protein expressions. Further investigation on HNF1α, a critical transactivator of PCSK9, revealed that fasting did not alter its mRNA expression, however, the protein abundance of HNF1α in nuclear extracts of hamster liver was markedly reduced by prolonged fasting.
Conclusion: Fasting lowered serum LDL-C in hamsters by increasing hepatic LDLR protein amounts via reductions of serum PCSK9 levels. Importantly, our results suggest that attenuation of SREBP1 transactivating activity owing to decreased insulin levels during fasting is primarily responsible for compromised PCSK9 gene transcription, which was further suppressed after prolonged fasting by a reduction of nuclear HNF1α protein abundance.

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1. Background

The concentration of low density lipoprotein-cholesterol (LDL-C) is recognized as one of the most important predictors of atherosclerosis and coronary heart disease (CHD) [1–3]. There is abundant evidence indicating that the reduction of LDL-C lowers morbidity and mortality from CHD [4]. In humans, the majority of LDL particles in blood is removed and metabolized in liver through LDL receptors (LDLR) that are expressed on the surface of hepatocytes. Hepatic LDLR mediates the uptake of LDL particles from the circulation and delivers the receptor-bound LDL to the endosomal system for degradation while the receptor returns to the cell surface. Thus, the expression level of hepatic LDLR becomes a crucial factor that determines the concentration of LDL-C in the blood [5–7].

Recent studies have identified proprotein convertase subtilisin/kexin type 9 (PCSK9) as a new player in LDL metabolism [8–10] through its interaction with hepatic LDLR. PCSK9, a member of the subtilisin family of serine proteases, is highly expressed in adult liver hepatocytes and in small intestinal enterocytes [11]. It is synthesized as a 72 kDa zymogen that undergoes autocatalytic cleavage in the endoplasmic reticulum into a heterodimer of a prosegment (122 amino acids) and a 60 kDa active form, associated together noncovalently. The processed PCSK9 is rapidly and efficiently secreted from liver into plasma where it binds to the EGF-A extracellular domain of LDLR. Subsequent to the binding, the PCSK9-LDLR protein complex is endocytosed, and traveled to the lysosome compartment for degradation within hepatocytes [12,13]. Thus, PCSK9 plasma levels directly influence the level of circulating LDL-C [14,15].

The discovery of PCSK9 as a natural LDLR degrader and the subsequent observations that PCSK9 mutations can profoundly affect LDL-C levels and the risk of CHD have galvanized great interest in understanding the in vivo regulation of plasma PCSK9

* Corresponding author. Tel.: +1 650 493 5000x64411; fax: 1 650 496 2505.
E-mail address: Jingwen.Liu@va.gov (J. Liu).
[1] These authors contributed equally to this work.

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and its correlation with LDL-C levels. Thus far, most studies conducted in animal models [16–18] and humans [19–21] have been focused on pharmacological interventions by applying cholesterol lowering drugs such as statins and fibrate class of drugs. In addition, various approaches to block PCSK9-LDLR interactions by neutralizing antibodies [22], PCSK9 small interference RNAs [23] and antisense RNAs [24] as well as small molecule inhibitors of PCSK9 gene expression [25,26] have been applied to lower circulating LDL levels via the diminution of PCSK9-mediated LDLR degradation. In contrast to the wealth of knowledge in understanding the impact of pharmacological interventions on plasma PCSK9, limited studies have been conducted to examine the effects of physiological and nonpharmaceutical interventions on plasma PCSK9 levels and the correlation with hepatic LDLR abundances.

Recently, two investigations have explored this area of PCSK9 research by examining the effects of fasting on plasma PCSK9 in healthy humans [27,28]. It was found that fasting strongly reduced circulating PCSK9, which occurred concomitantly with suppressed hepatic cholesterol synthesis. Somewhat unexpected was that reductions of plasma PCSK9 under fasting conditions did not correlate with changes in plasma LDL-C levels. In one study the plasma LDL-C level was increased during the fasting [28] while it was unchanged in another report [27]. Inasmuch as the liver samples were not accessible in both studies, it was unknown of how hepatic levels of LDLR protein were affected during fasting by the drastic reduction of plasma PCSK9 in those healthy individuals.

In order to gain a better understanding of the interactive relationship between circulating PCSK9 and LDL-C level and hepatic LDLR protein expressions under physiological regulations, we utilized a normolipidemic hamster model to simulate the human fasting studies. The golden Syrian hamster has been used with increasing frequency in recent years to study lipoprotein metabolism and atherosclerosis, because hamsters share more lipid metabolism characteristics with humans than mouse or rat [29–32]. Previously, we have utilized a dyslipidemic hamster model to demonstrate a tight correlation of serum LDL-C levels with changes in hepatic gene expressions of PCSK9 and LDLR in response to treatments of rosuvastatin (RSV) and a natural cholesterol lowering alkaloid berberine [33]. In that study, we have shown that the LDL-C level in dyslipidemic hamsters was increased by RSV treatment, which was accompanied by a marked increase in PCSK9 mRNA levels and a reduced abundance of LDLR protein in hamster liver [33].

In the present study, we examined the time-dependent effects of fasting on hamster serum LDL-C and PCSK9 levels, and correlated that further with hepatic mRNA and protein expressions of LDLR and PCSK9. We observed that in hamsters fed a regular chow diet, fasting lowers serum LDL-C and PCSK9 with concomitant increases in LDLR protein levels in liver. We further investigated involvements of molecular pathways mediated by SREBP, peroxisome proliferator-activated receptor α (PPARα), liver X receptors (LXR) and hepatic nuclear factor 1α (HNF1α) in the strong suppression of PCSK9 transcription by fasting. Our results suggest that fasting exerts a strong impact on PCSK9 gene transcription via combined effects of attenuation of SREBP1 transactivating activity and reduction of HNF1α protein abundance in hamster liver. These findings shed new light on our current understanding of regulation of PCSK9 transcription under physiological conditions.

2. Materials and methods

2.1. Animals and diet

Animal use and experimental procedures were approved by the Institutional Animal Care and Use Committee of the VA Palo Alto Health Care System. Male golden Syrian hamsters were purchased from Harlan Sprague Dawley. Hamsters were housed (2 animals/cage) under controlled temperature (22 °C) and lighting (12 h light/dark cycle). Before initiation of fasting studies, hamsters were maintained on a standard laboratory rodent chow diet. For the fasting experiments, twenty-eight hamsters of 11–12 week-old were randomly divided into five groups (4 hamsters for non fasted, and 6 hamsters per group for fasted): non-fasted, fasted for 8, 24, 36, and 48 h. At the experimental termination, all animals were sacrificed and liver tissues were harvested. During the fasting course, hamsters had free access to water. The non-fasted group was fed *ad libitum* with chow diet and were killed on 9:00 AM of day 2. The fasting was started on day 1 at 9:00 AM, and serum and liver samples of fasted groups were collected at the following schedule:

Eight h-fasted: serum collection at day 1, 1:00 PM (4 h fast) and 5:00 PM (8 h fast); liver collection on day 1, 5:00 PM.

Twenty-four h-fasted: serum collection and termination on day 2, 9:00 AM.

Thirty-six h-fasted: serum collection and termination on day 2, 9:00 PM.

Forty-eight h-fasted: serum collection and termination on day 3, 9:00 AM.

At the time of dissection, body weight, liver weight, and the gross morphology of the liver were recorded. Livers were immediately removed, cut into small pieces, and stored at −80 °C for lipid analysis, RNA isolation and protein isolation.

2.2. Measurement of serum and hepatic lipids

Blood samples (0.2 ml) were collected from the retro-orbital plexus using heparinized capillary tubes under anesthesia. Serum was isolated at room temperature and stored at −80 °C. Standard enzymatic methods were used to determine TC, TG, LDL-C, and HDL-C with commercially available kits purchased from Stanbio Laboratory (Texas, USA). To measure hepatic cholesterol and TG levels, one hundred mg of frozen liver tissue were homogenized in 2 ml chloroform/methanol (2:1). After homogenization, lipids were further extracted by rocking samples for 1 h at room temperature, followed by centrifugation at 5000 rpm for 10 min. The liquid phase was washed with 0.2 volume of 0.9% saline. The mixture was centrifuged again at 2000 rpm for 5 min to separate the two phases. The lower phase containing lipids was evaporated and lipids were dissolved in 0.5 ml isopropanol containing 10% Triton X-100 for cholesterol and TG measurements.

2.3. Measurement of serum insulin

Insulin levels in fed and fasted serum samples were measured with a commercially available enzyme-linked immunosorbent assay kit (Catalog number 1730887; Millipore, Billerica, MA).

2.4. RNA isolation, cDNA generation and real-time quantitative PCR (qPCR)

Total RNA was isolated from flash-frozen hamster liver tissue using an RNeasy kit (Qiagen, CA). RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining. Two μg of total RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) using random primers. Real-time PCR was performed on the ABI PRISM® 7900HT Sequence Detection System with SYBR PCR master mix (Applied Biosystems). Each cDNA sample was run in duplicate.

For designing hamster real-time PCR primers, if golden Syrian hamster (*Mesocricetus auratus*) mRNA sequence is available,