HHS Public Access

Author manuscript

Atherosclerosis. Author manuscript; available in PMC 2015 August 07.

Published in final edited form as:

Atherosclerosis. 2012 October ; 224(2): 401–410. doi:10.1016/j.atherosclerosis.2012.08.012.

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

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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.

Keywords

PCSK9; LDLR; SREBP; HNF1α; PPARα; LXR; LDL-cholesterol; fasting; golden Syrian hamsters

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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 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 transactiving 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 9:00AM, 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 5,000 rpm for 10 min. The liquid phase was washed with 0.2 volume of 0.9% saline. The mixture was centrifuged again at 2,000 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 (Catalogue 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, primers were designed according to that sequence. If golden hamster mRNA sequence is not available, primers were designed according to the homologous part between the mouse (Mus musculus) and Chinese hamster (Cricetulus griseus) mRNA sequences. Primer sequences of hamster genes used in real-time PCR are listed in Table 1. The correct size of PCR product and the specificity of each primer pair were further validated by examination of PCR products on agarose gel.

2.5. Western blot analysis of LDLR in liver tissues

Approximately 90-100 mg of frozen liver tissue from individual hamster were homogenized in 1 ml RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4) containing 1 mM PMSF and protease inhibitor cocktail (Roche). After protein quantitation using BCA™ protein assay reagent (PIERCE), aliquots of lysate containing equal amounts of protein were used for SDS-PAGE and Western blotting. LDLR protein was detected by immunoblotting using a rabbit anti-LDLR antibody (Biovision, Mountain View, CA). Membranes were reprobed with an anti-β-actin antibody (Santa Cruz Biotechnology). Immunoreactive bands of predicted molecular mass were visualized using an ECL plus kit (GE Healthcare life Sciences, Piscataway, NJ) and quantified with the KODAK Molecular Imaging Software (Kodak, New Haven, CT). The amount of LDLR protein in individual hamster livers was normalized with actin signals.

2.6. Nuclear protein extract preparation

Nuclear extracts of liver tissue were prepared using method previously described [33]. Anti-HNF1α (sc-6547x) and anti-HDAC-1 (sc-7872) antibodies were obtained from Santa Cruz Biotechnology for detections of their target proteins in nuclear extracts, and anti-SREBP1 (sc-8984, Santa Cruz) was used to detect SREBP1 precursor form in total cell lysates by Western blotting. Anti-LXRα (catalog No. PP-K8607-00) and anti-LXRβ (catalog No. PP-K8917-00) were obtained from R&D System.

2.7. Detection of hamster PCSK9 in serum

Detection of PCSK9 of hamster serum samples was conducted by immuoprecipitation (IP), followed by Western blotting using a rabbit antibody that recognizes the C-terminal end of hamster PCSK9 (CRNRPSAKASWVHQ) as we previously described [34]. For each precipitation, equal amounts of serum sample from two hamsters of the same fasting group were pooled and used in the IP reaction.

2.8. Hamster primary hepatocyte preparation

Primary hepatocytes were prepared from male Golden Syrian hamsters using procedures previously described [35]. Preparations with higher than 90% cell viability were used for further experiments. Viable hepatocytes were plated at 2×10^6 cells/well in six-well BD Falcon Primaria Tissue Culture Plates in attaching medium and incubated at 37°C in a CO_2 tissue culture incubator for 3 h. Unattached cells were washed out and culture medium was replaced with HepatoZYME-SFM medium (Invitrogen, CA) overnight before the drug treatment of 24 h.

2.9. Statistical analysis

For lipid analysis and real-time qPCR assays of hepatic gene expression, significant differences between fed and fasted groups were assessed by One-way ANOVA with Bonferroni posttest. Two-tailed Student's *t*-test was used to analyze difference in hepatic LDLR protein expressions and serum PCSK9 levels between fed and each fasted group. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Fasting lowers serum LDL-C and PCSK9

Removing food for up to two days, while allowing free access to water, resulted in a continuously decline of serum LDL-C and TG levels (Fig. 1A). The LDL-C was lowered by ~60% ($p<0.001$) after 8 h of fasting and reached a nadir of ~68% ($p<0.001$) below that of fed state by one and two days of fasting. TG level dropped significantly by 4 h ($p<0.01$) of food withdraw and further declined to 20% ($p<0.001$) of control by two days of fasting. In contrast, serum cholesterol and HDL-C were only slightly decreased by ~30% ($p>0.05$) at the end of 48 h of fast. Analysis of hepatic lipids of corresponding liver samples showed that hepatic cholesterol levels maintained stable during fasting while hepatic TG levels increased about two-fold ($p<0.05$) of the value of non fasted livers (Fig. 1B). The increase in hepatic TG content by fasting was similar to the observation in mice after a 24 h fast [36]. The body weights of hamsters were not demonstrably decreased by two days of fasting (data not shown).

To detect changes in serum PCSK9 levels during fasting, we developed an IP assay using a rabbit antibody that specifically recognizes the C-terminus of hamster PCSK9 [34]. The immunoprecipiates were denatured and analyzed by SDS-PAGE and immunoblotting using the anti-PCSK9 antibody. To validate the IP method, we first examined PCSK9 levels from serum samples of another cohort of hamsters that were untreated or treated with 10 mg/kg and 20 mg/kg of RSV for 7 days [33]. Fig. 2A shows that the amount of serum PCSK9 was increased to 1.5-fold of control by 10 mg/kg RSV ($p<0.05$) and to 1.7-fold of control by 20 mg/kg RSV ($p<0.05$). These results were in agreement with our earlier demonstration of elevated PCSK9 mRNA expressions in liver samples of RSV-treated hamsters [33], and were in line with previously published human clinical studies [20,37]. After this validation, we conducted the IP assays to examine PCSK9 levels in non-fasted and fasted serum samples.

Fig. 2B shows that serum PCSK9 levels were not changed during the first 24 h of fasting, but declined by ~30% after 36 h. After 2 days of starvation the serum level of PCSK9 was ~70% ($p<0.01$) lower than that measured in the fed state. The decline in serum PCSK9 levels by fasting in hamsters was similar to the previously published results of human fasting studies [27,28], despite exhibiting a relatively slower kinetics. In the reported human fasting studies, plasma levels of PCSK9 declined readily after 8 h of fasting and reached a nadir by 36 h.

3.2. Fasting differentially regulates PCSK9 and LDLR mRNA expressions in hamster liver

Transcriptions of PCSK9 and LDLR genes are both modulated by sterol regulatory element binding proteins (SREBPs) through SRE motifs embedded in their proximal promoters [8,9,38]. To correlate changes of serum PCSK9 and LDL-C levels with alterations of relevant hepatic gene expression, utilizing real-time qPCR method, we compared mRNA levels of LDLR and PCSK9 in fed liver samples with that of fasted groups (Fig. 3A). The level of PCSK9 mRNA was unchanged at 8 h postprandial, but declined to 40% ($p<0.001$) of the value of fed state by 24 h of fasting. It was further lowered to ~12% of control at 48 h

of fasting. In contrast to PCSK9 mRNA, we detected a 2.3-fold ($p<0.001$) increase in LDLR mRNA levels after 8 h of fasting, which returned to the level of fed state by 24 h and stayed at the baseline. These data showed that gene expressions of LDLR and PCSK9 in hamster liver tissue were differentially regulated by nutrient deprivation. To confirm these findings, we analyzed another cohort of hamsters that were fed or fasted for 24 h [39]. The results were almost identical in that PCSK9 mRNA was markedly reduced in fasted group compared to the control group while LDLR mRNA barely changed after 24 h of fasting (Fig. 3B).

3.3. Fasting increases hepatic LDLR protein abundance

The fasting associated reduction of serum LDL-C could be caused by enhanced removal or reduced production of the lipoprotein particles by liver. The fact that plasma PCSK9 was lower in fasted animals was suggestive of increased uptake of LDL-C via elevated expression of LDLR on the hepatocytes of fasted hamsters. We examined LDLR protein abundance in liver samples of different groups by Western blot analysis using an antibody recognizing the mature form of hamster LDLR. The results shown in Fig. 3C were Western blots of individual liver samples. Quantitative analyses of the results are presented in Fig. 3D. Unlike the transient increase in LDLR mRNA levels by fasting, the amount of LDLR protein in liver increased 50% ($p<0.01$) after 8 h of fasting, further inclined to 2-fold by 24 h ($p<0.05$), and reached a peak at 36 h with a 3.5-fold ($p<0.01$) increase over control. At the end of two days of fasting, the level of LDLR protein was 3-fold ($p<0.01$) higher than that of fed hamsters. We repeated the Western blot analysis using pooled liver protein samples, and we obtained similar results (data not shown).

3.4. Suppression of SREBP1 expression and its transactivating activity by fasting is a primary underlying mechanism for the compromised PCSK9 transcription

Utilizing real-time qPCR we evaluated fasting induced changes in gene expression of SREBP2 and its target genes that are involved in cholesterol biosynthetic pathway including HMG CoA reductase (HMGCR) and HMG CoA synthetase 1 (HMGCS1) (Fig. 4A) as well as SREBP1 and its target lipogenic genes including fatty acid synthetase (FAS), acetyl-coenzyme A carboxylase alpha and beta (ACACA, ACACB) (Fig. 4B). The changes of SREBP2 mRNA during fasting exhibited a similar pattern as LDLR mRNA, which was elevated during the short time of fast and returned to the baseline by 36 h and 48 h. We observed an approximate 50% reduction of mRNA levels of HMGCR after 24 h of fast. Fasting led to an early induction without subsequent attenuation on mRNA expression of HMGCS1, which was nearly identical to the changes seen in LDLR mRNA levels.

In contrast to SREBP2, the mRNA levels of SREBP1 mRNA were rapidly reduced to 50% of control by 8 h of fast, and remained at the lower levels up to 36 h. Afterwards it returned to the level of fed state by 48 h of fast. The mRNA levels of FAS was not changed at 8 h but decreased drastically at 24 h, a kinetics similar to PCSK9. Interestingly, the gene expressions of ACACA and ACACB showed a transient induction by 8 h of fasting and subsequent reduction. The decline in the expression of these lipogenic genes reflected the attenuated SREBP1 transactivation activity, which was in line with previously reported fasting results conducted in mice [36]. To seek further evidence we attempted to examine

the protein abundance of the precursor and mature forms of SREBP1 in hamster liver samples of whole protein extracts and nuclear extracts by Western blotting. While we could not obtain specific signals of SREBP1 processed mature form in nuclear extracts by using three different commercially available anti-SREBP1 antibodies, we were able to detect the precursor form of SREBP1 in whole liver lysates. Fig. 4C clearly demonstrated that fasting induced a rapid decline in SREBP1 protein abundance. The level of SREBP1 protein declined to 51% ($p<0.001$) of the value of fed state by 8 h of fasting. It was further lowered to ~36% of control at 36 h of fasting, consistent with the reduction of SREBP1 mRNA levels by fasting. By 48 h of fasting it was barely detectable. The fasting-induced decline of SREBP protein abundance in hamster liver was consistent with the results reported in a previous study that examined SREBP1 protein abundances in livers of mice subjected to fasting and refeeding [40].

It has been shown that PCSK9 transcription in mice was positively regulated by insulin via a mechanism involving SREBP1 [17]. It is possible that the reduction of transcription of PCSK9 and other SREBP1-target genes was the consequence of insulin deprivation. We measured the insulin levels of fed and fasted serum samples and found that indeed it was reduced by 65% after one and two days of fasting (Fig. 4D).

In addition to SREBPs, it has been demonstrated that PCSK9 transcription can be modulated by PPARα as a negative regulator[41,42]. Since it has been reported that fasting increases PPARα expression in rodent liver[43], we investigated the possible role of PPARα in the fasting induced repression of PCSK9 transcription. The results of real-time qPCR in Fig. 5A attested the induction of mRNA expressions of PPARα as well as its target gene CPT1α in liver of hamster by fasting. To directly examine the function of PPARα in hepatic expression of PCSK9 in hamsters, we treated freshly isolated hamster primary hepatocytes with different doses of PPARα agonist WY14643 for 24 h. Fig.5B shows that activation of PPARα led to a dose-dependent increase in CPT1α mRNA levels, however, the mRNA levels of PCSK9 were unchanged. The lack of inhibitory effect of PPARα agonist on PCSK9 expression was further corroborated by Western blot analysis of PCSK9 protein abundance in these hepatocytes (Fig. 5C). Altogether, these new data suggested that PPARα might not be involved in fasting-induced repression of PCSK9 transcription.

LXRs are known to activate PCSK9 transcription through a SREBP1C-mediated mechanism, and direct activations of LXR with its agonists increase PCSK9 expression [17,44]. We were interested in knowing how fasting affects LXR expression and its downstream signaling pathways in regulation of hepatic metabolic genes. Fig. 6A shows that LXRα and LXRβ mRNA levels were not increased during the 24 h fasting period but they were increased approximately 1.5-fold by 36 h and ~2-fold by 48 h fasting compared to that of fed state. Likewise, we detected modest increases of LXRα and LXRβ protein abundances in nuclear extracts of pooled liver samples (Fig. 6B). We further examined the effect of fasting on LXR direct target genes that are involved in cholesterol transport (ABCA1, ABCG1) and bile acids metabolism (ABCG5 and ABCG8). The changes of mRNA expressions of these genes exhibited nearly identical kinetics as LXRs and were induced by fasting (Fig. 6C), particularly prominent at the later stage of fasting (36-48 h).

These results exclude LXR as a direct contributing factor for the fasting-induced repression of PCSK9 transcription.

3.5. Fasting downregulates HNF1α protein abundance

Our previous in vitro and in vivo studies have demonstrated HNF1α as a critical transactivator for PCSK9 gene expression in liver cells. It binds to a highly conserved HNF1 motif located 28 base pairs upstream from SRE-1 in both human and hamster PCSK9 promoters [26,33, 45]. To determine whether HNF1α expression could be altered by fasting, we measured HNF1α mRNA levels of liver samples by qPCR, and examined its protein amounts in nuclear extracts of fed and fasted liver tissues by Western blot analysis. Fasting did not alter the mRNA expression of HNF1α at all (Fig. 7A). However, the abundance of HNF1α protein was greatly affected by prolonged fasting. After two days of food deprivation, the amount of HNF1α protein in nuclear extract was reduced by 75% ($p<0.001$) as compared to that of fed livers (Fig. 7B and C). In contrast, fasting did not change the abundance of nuclear protein histone deacetylase 1 (HDAC1) in liver tissue. These results suggest that the attenuated expression of HNF1α protein at the later stage of fasting led to a further reduction of PCSK9 gene transcription.

4. Discussion

Numerous studies have demonstrated a positive correlation of plasma LDL-C and levels of circulating PCSK9 under conditions of therapeutic interventions including anti-PCSK9 antibodies [22]. However, whether this correlation occurs under physiological regulations is questionable and is challenged by the recent reports of human fasting studies of PCSK9 [27,28]. Results of the current study provide a glimpse of the interactive relationship between circulating PCSK9 and LDL-C level, and hepatic LDLR mRNA and protein expressions during the prolonged fasting in hamster species. The primary findings of our study were that fasting increased hepatic LDLR protein abundance, which occurred concurrently with the decline in serum PCSK9 and LDL-C levels. In addition, we showed that fasting differently modulated the gene expressions of LDLR and PCSK9 with insignificant inhibitory effect on former and a strong suppression of latter. Our further investigations of the underlying mechanisms responsible for the marked decrease in PCSK9 mRNA levels provided several pieces of evidence that ruled out the involvements of PPARα and LXR in fasting induced down regulation of PCSK9 and pointed to SREBP1 and HNF1α being major players in this physiological regulation.

It has been well known that the effects of fasting on lipid homeostasis vary among different species and among different study populations of humans. In the present study, we observed that fasting slightly reduced serum cholesterol levels but strongly lowered serum TG levels in hamsters. These phenomena were similar to observations in fasted rats [46], and were also in line with the results of human fasting studies [27,28], but they differed from a mouse fasting study in which no changes in serum TC and TG were observed after 24 h of starvation [41]. In rabbits, it has been shown that prolonged fasting is associated with an increase in serum cholesterol levels [47]. The lack of changes in HDL-C levels during fasting in the current study was consistent with previously published results of fasting studies in other animal models [46] and in human healthy subjects.

With regard to serum LDL-C levels, we observed a continuous decline during the fasting, and we demonstrated a time-dependent reduction of the circulating levels of PCSK9 and a concomitant increase in hepatic LDLR abundance in fasted hamsters. Hepatic cholesterol levels were not changed during the period of 48 h of fasting. Interestingly, we detected an elevation in LXR mRNA and protein expression, which led to a stimulated mRNA expression of ABCG5 and ABCG8 which gene products are involved in bile acids excretion by the liver into bile. In addition, we also found that fasting did not reduce liver mRNA expression levels of cholesteryl ester transfer protein (CETP) (data not shown). Considering all these measurable factors that participate in cholesterol uptake and excretion pathways, it is plausible to conclude that in hamsters fasting promotes LDL-C uptake through increased LDLR abundance on the surface of hepatocytes. The increased endocytosis of LDL-C into cells stimulated bile acid metabolic pathway mediated through LXR to enhance cholesterol excretion possibly via conversion to bile acids.

Different from most fasting studies of PCSK9 in animal models that were conducted by a single time point, namely 24 h, our experimental design of collecting serum and liver samples at different time points of fasting allowed us to capture the temporal changes in gene expression of liver tissue. We observed that while PCSK9 mRNA levels markedly declined after 24 h of fasting, the mRNA levels of LDLR showed an early induction by fasting and returned to baseline level at 24 h and maintained at the basal level up to 48 h of fasting. This kinetics was nearly mirrored by changes in SREBP2 as well as HMGCS1 mRNA levels. Because livers of 8 h-fasted hamsters were harvested at 5:00 PM whereas fed animals and 1 day- and two day-fasted groups were all killed at 9:00 AM, we could not exclude the possibility that the higher expression levels of LDLR and SREBP2 mRNA after 8 h of fasting could reflect a diurnal effect in their gene expression.

In cell culture studies and rodent models, it has been shown that PCSK9 transcription is activated by both SREBP1 and SREBP2 [8,17,48]. In the present study, we showed a strong correlation of reduction of PCSK9 gene expression with serum insulin, which is a critical factor regulating SREBP1 transcription and activity. SREBP1 mRNA levels were rapidly reduced as early as 8 h and maintained at the low level to 36 h of fasting (Fig. 4B). The reduction of SREBP1 protein by fasting was clearly seen at 8 h of fast and through the entire fasting period. Although we could not detect the mature form of SREBP1, the diminution on SREBP1 transactivating activity by fasting was clearly demonstrated by the strong reduction of gene expression of FAS, a bona fad SREBP1-target gene. Thus, our data suggest that fasting-induced down regulation of PCSK9 is primarily mediated through the attenuation of SREBP1-mediated transcription of PCSK9. A previous study has shown that administration of glucagon into Sprague Dawley rats led to a rapid decline of hepatic PCSK9 mRNA levels [49]. Since fasting is known to increase glucagon amounts in blood [50], it is possible that in addition to the decline of insulin, elevated glucagon levels in circulation contributed to the early reduction in PCSK9 gene expression.

In addition to SREBPs as the master regulator that relay the transcription of PCSK9 with changes in cellular sterol levels, PCSK9 transcription is shown to be negatively regulated by PPARα in mice and in human immortalized hepatocytes in culture [41,42]. In this study, we have clearly demonstrated that fasting activated PPARα pathway, evidenced by the time-

dependent increase in hepatic expression levels of PPARα and CPT1α. However, direct treatment of hamster primary hepatocytes with PPARα agonist did not reduce PCSK9 mRNA or protein levels. Taken together, these results suggest that PPARα does not play an active role in mediating the strong suppression of PCSK9 transcription in fasted hamster liver. Further study to apply PPARα agonist to hamsters in vivo will be required to conclusively define the role of PPARα in PCSK9 gene expression in hamster species. In addition to PPARα we also evaluated the possible role of LXR signaling pathway in mediating PCSK9 gene expression under fasting conditions. The observations that fasting stimulated the LXR signaling pathway concurrently with the suppression of PCSK9 expression largely excluded its role of transactivator in modulating PCSK9 transcription under this physiological regulation.

It has been recently reported that in mice, alterations in insulin signaling pathway through AKT/mTOR1 cascade affect PCSK9 expression via a HNF1α-mediated mechanism [51]. It was shown that treating mice with rapamycin, an inhibitor of mTORC1 activity, increased HNF1α mRNA and protein expression, consequently led to an elevated PCSK9 expression and reduced LDLR protein abundance in mouse liver. In this current study we have demonstrated that prolonged fasting has a strong effect in downregulation of HNF1α protein abundance in the absence of changes in HNF1α mRNA levels. It is quite possible that the mechanisms underlying the fasting induced reduction of HNF1α protein expression in hamster liver are distinct from the action mechanism of insulin in mice. Further studies to elucidate the underlying mechanisms of reduction of HNF1α protein levels by fasting and by BBR treatment are undergoing in our laboratory. Nevertheless, to the best of our knowledge, this was the first demonstration of reduction of hepatic HNF1α protein expression by nutrient deprivation in vivo. This new finding suggests that prolonged fasting triggers the down regulation of HNF1α protein abundance, thereby leading to a further decline in PCSK9 gene expression.

5. Conclusion

This is the first study to examine the dynamic interactive relationship between plasma PCSK9, LDL-C and hepatic LDLR expression during fasting in the hamster species. Our findings of reduced serum LDL-C and PCSK9 with concurrent increases in liver LDLR protein abundance provide new in vivo evidence to support the established role of PCSK9 in the control of plasma LDL-C metabolism under physiological regulations. In addition, 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.

Acknowledgments

This study was supported by the Department of Veterans Affairs (Office of Research and Development, Medical Research Service) and by grants (1RO1 AT002543-01A1, 1R21AT003195-01A2) from National Center of Complementary and Alternative Medicine.

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Highlights

- Fasting lowered PCSK9 and LDL-C levels and increased liver LDLR protein abundance
- Fasting reduced PCSK9 transcription by attenuating SREBP1 activity
- Prolonged fasting decreased nuclear HNF1a protein abundance in hamster liver

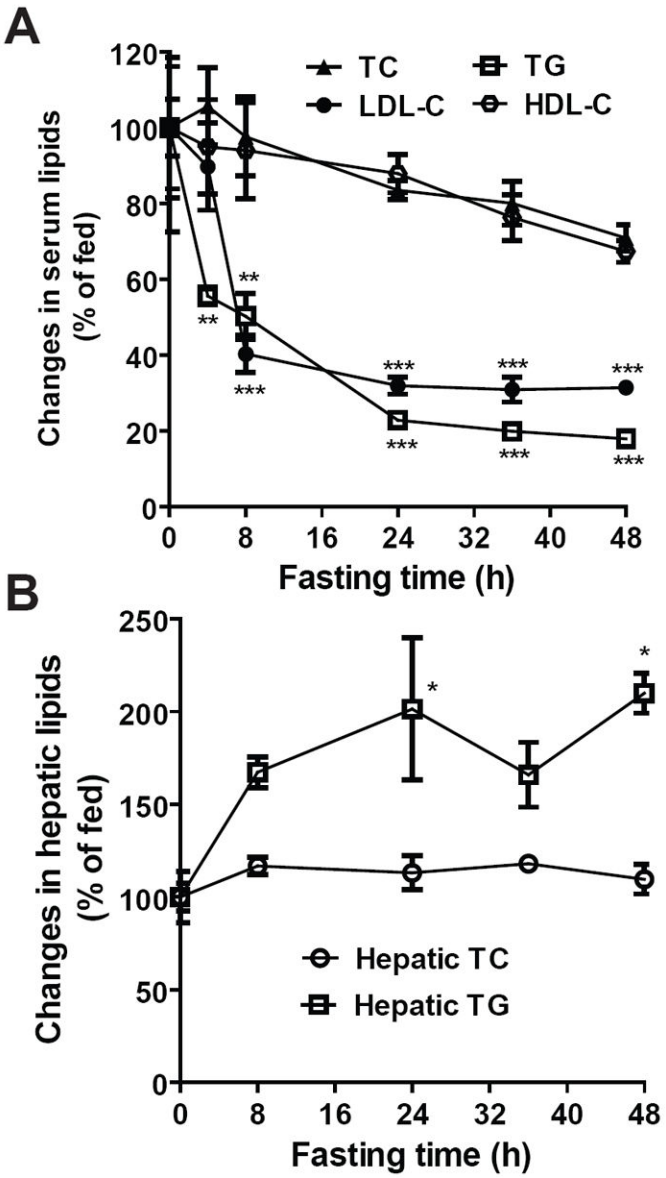

Figure 1.

Changes of serum and hepatic lipid levels of male hamsters during fasting up to 48 h. Lipid levels in serum (A) and liver (B) were measured at indicated fasting time. Data represent means ± SE; n=4 (fed) or 6 (fasted). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. fed group.

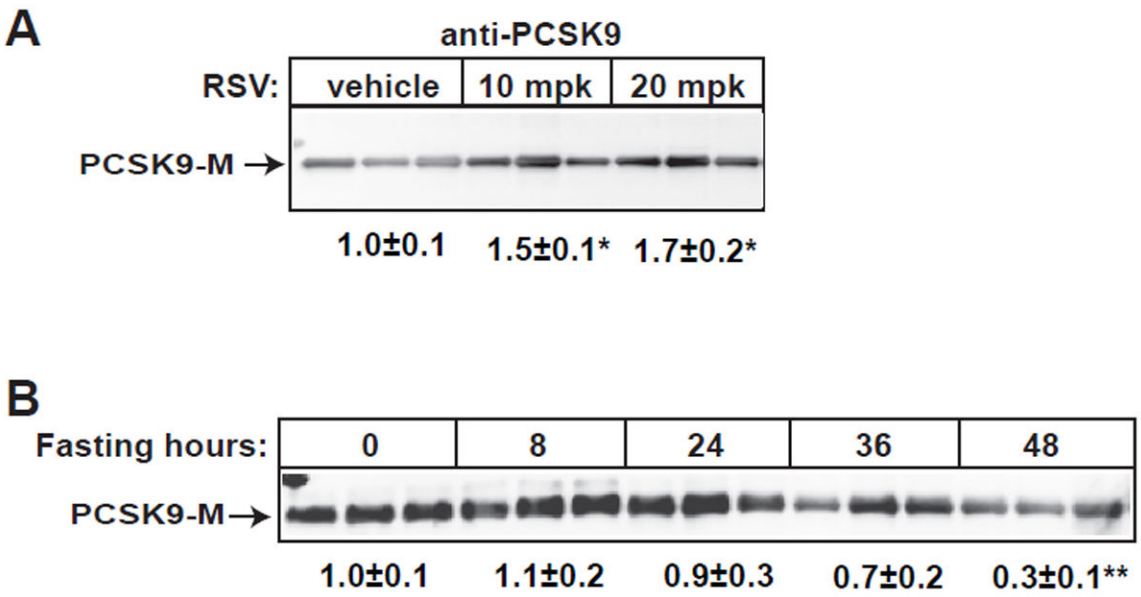

Figure 2. Detection of serum PCSK9

(A) Hamsters (9 hamsters per group) were fed a fructose-enriched diet for 3 weeks before administration of vehicle, 10 mg/kg of RSV and 20 mg/kg RSV for 7 days [33]. Serum samples were collected at the end of treatment and after an overnight fasting. Three serum samples from each treatment group were pooled, and 20 μL of pooled serum was used for conducting PCSK9 immunoprecipitation and Western blotting. *$p < 0.05$ vs. vehicle group. **(B)** Fifty-μL of individual serum samples of fed hamsters and pooled serum samples of fasted groups were used to conduct IP and immunoblotting with anti-hamster PCSK9 antibody. **$p < 0.01$ vs. fed group.

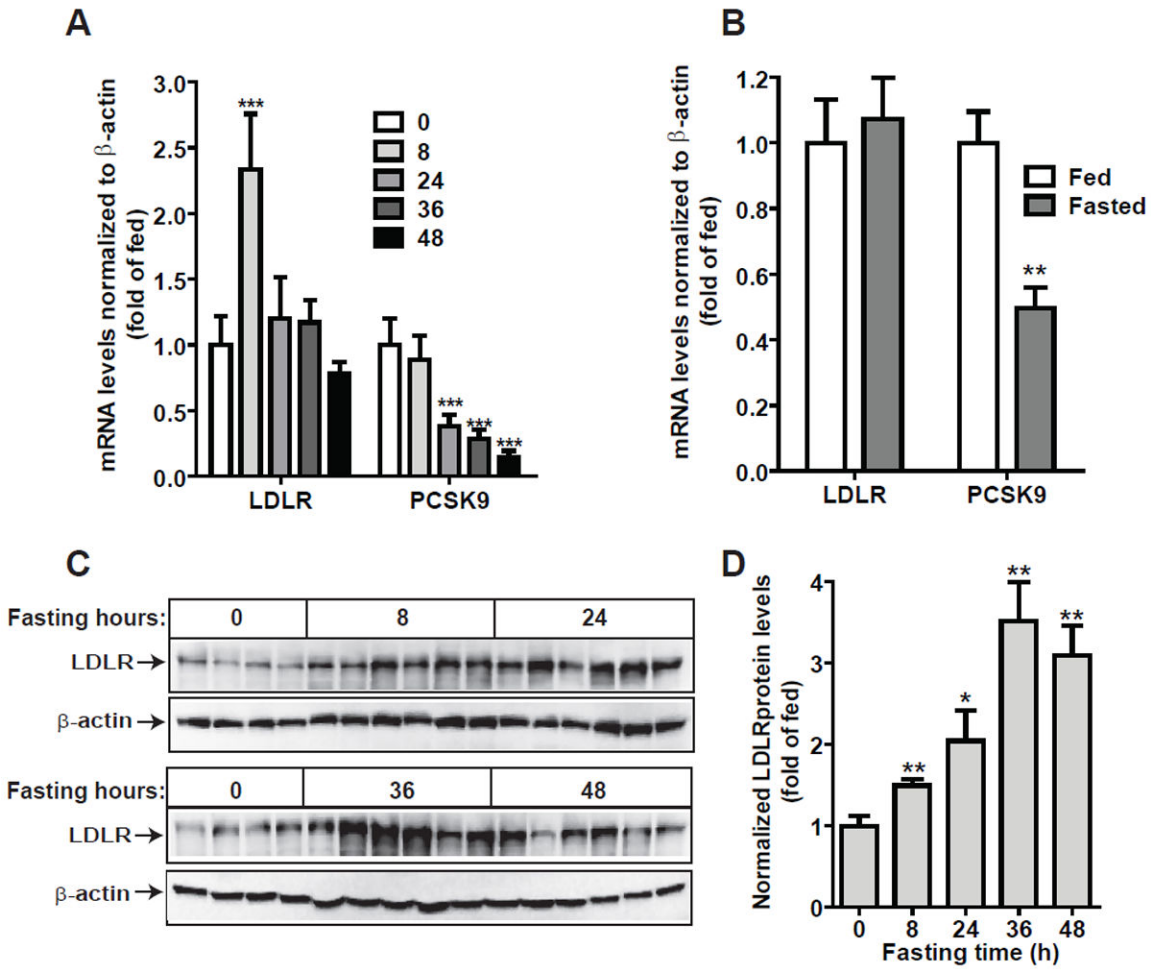

Figure 3. Fasting reduces PCSK9 gene expression and increases liver LDLR protein abundance

(A) At indicated fasting time, animals were euthanized and liver total RNA was isolated. Individual levels of PCSK9 and LDLR mRNAs were assessed by real-time qPCR using hamster specific PCR primers as described in the methods section. After normalization with β-actin mRNA levels, the relative levels are presented, and the results are means ± SE of 4-6 animals per group with duplicate measurement of each cDNA sample. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the fed control group.

(B) Liver samples of fed and 24-h fasted male hamsters were collected at 9-10:00 AM after sacrificing the animals [40]. Expressions of PCSK9 and LDLR mRNAs were analyzed as in A. n=6 per group.

(C) Individual liver protein extracts were prepared and protein concentrations were determined. Fifty μg of homogenate proteins of individual liver samples were resolved by SDS-PAGE and LDLR protein was detected by immunoblotting using a rabbit anti-LDLR antibody. The membrane was reprobed with an anti-β-actin antibody.

(D) The expression levels of LDLR were quantified with the KODAK Molecular Imaging Software with normalization by signals of β-actin. Values are mean ± SEM of 4-6 samples per group. *$p < 0.05$ and **$p < 0.01$ compared to the fed group.

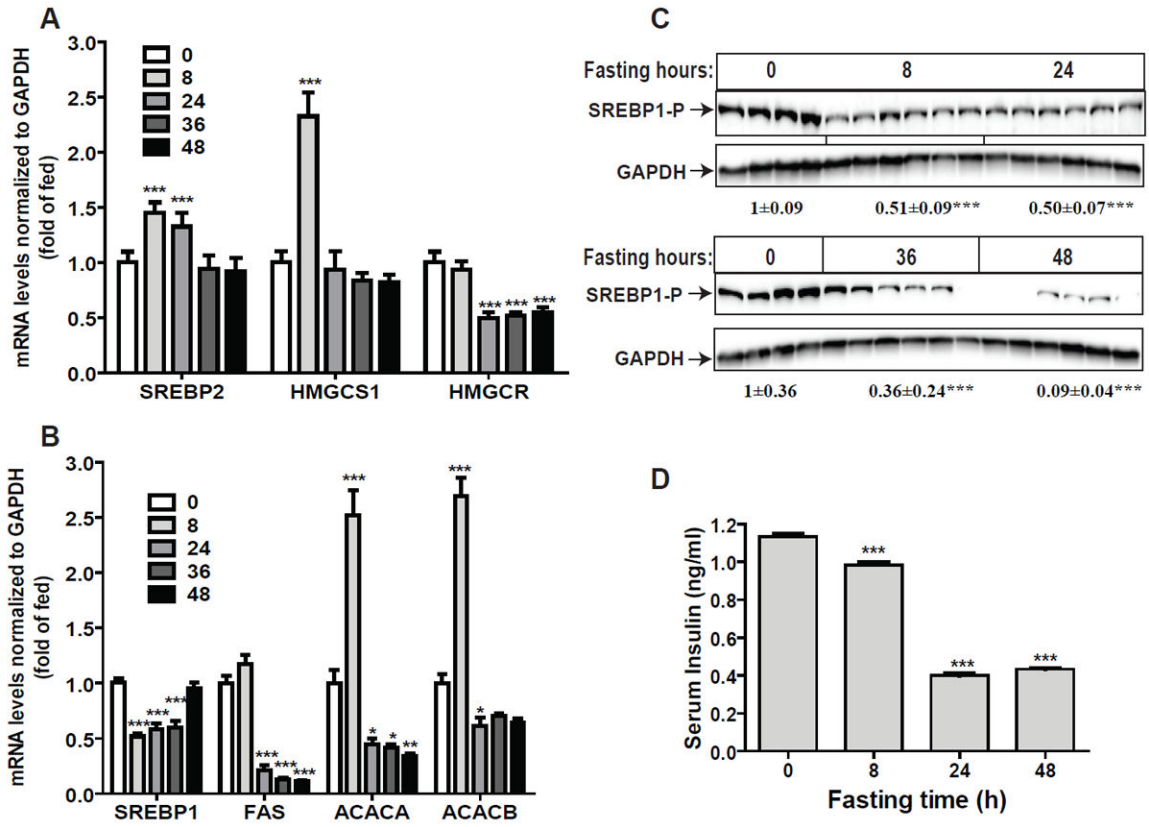

Figure 4. Evaluation of the effects of fasting on SREBP signaling pathway

(A) Real-time qPCR analysis of SREBP2, HMGCS1 and HMGCR mRNA levels of fed and fasted liver samples.

(B) Real-time qPCR analysis of SREBP1, FAS, ACACA and ACACB mRNA levels of fed and fasted liver samples.

(C) One hundred μg of homogenate proteins of individual liver samples were resolved by SDS-PAGE and SREBP1 precursor was detected by immunoblotting using a rabbit anti-SREBP1 antibody. The lower part of membrane was probed with an anti-GAPDH antibody. The specific SREBP1 signals were quantified with the Alpha View Software version 3.3 with normalization by signals of GAPDH.

(D) Serum insulin levels of fed and fasted samples of indicated time were measured. The results are means ± SE of 4-6 animals per group with duplicate measurement of each serum sample. In A-D, *$p < 0.05$ and ***$p < 0.001$ compared to the fed group.

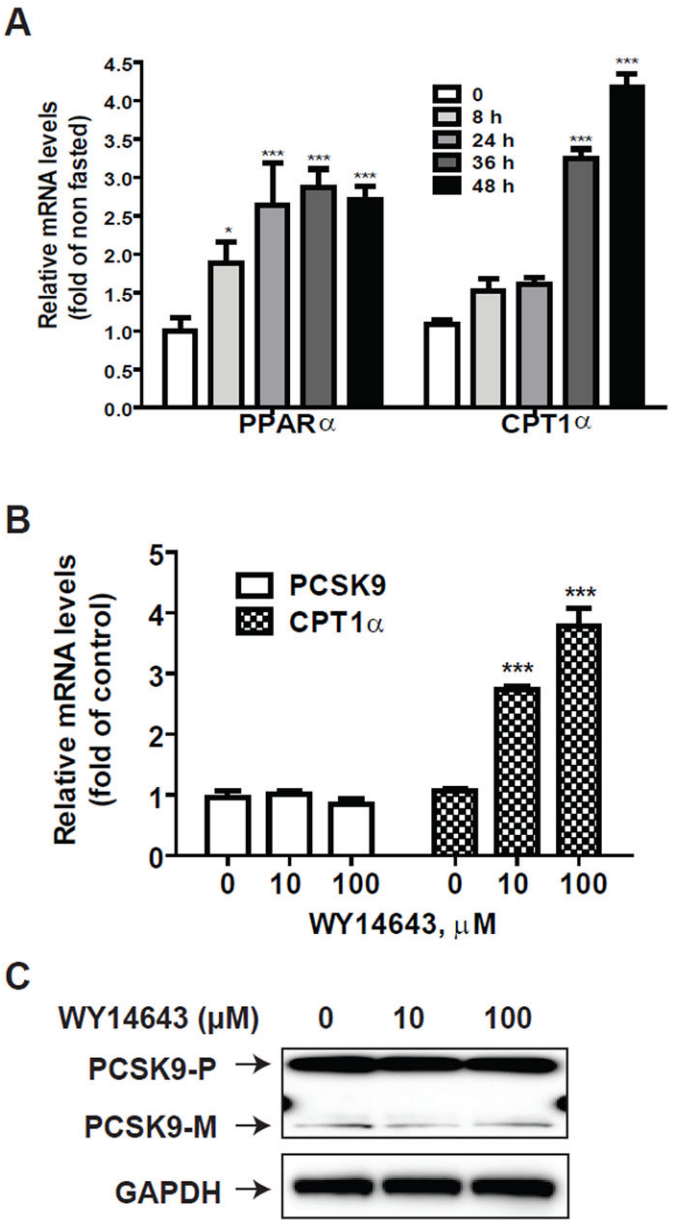

Figure 5. Examination of the role of PPARα on modulation of PCSK9 expression in hamster liver tissue and primary hepatocytes

In A, real-time qPCR assays were conducted to examine PPARα and CPT1α mRNA levels of fed and fasted liver samples. In B and C, hamster primary rat hepatocytes were cultured in HepatoZYME-SFM medium overnight. WY14643 at 1 μM and 10 μM concentrations were added to the cells for 24 h before the isolation of total RNA (B) and protein (C) for Western blotting of PCSK9 with a rabbit anti-hamster PCSK9 antibody.

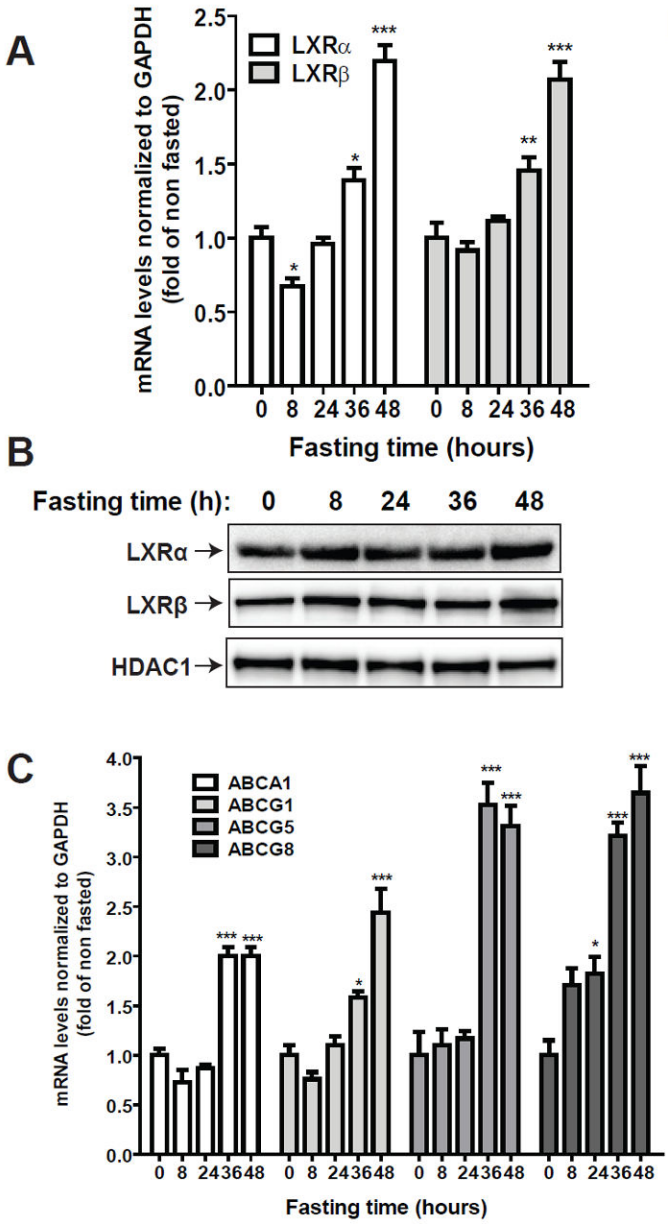

Figure 6. Evaluation of fasting induced changes in LXR signaling pathway

(A) Real-time qPCR assays were conducted to examine LXRα and LXRβ mRNA levels of fed and fasted liver samples.

(B) Individual nuclear extracts of equal amount from each group were pooled and 30 μg of pooled nuclear extracts were analyzed by Western blot for the protein abundances of LXRα and LXRβ. The membrane was reprobed with anti-HDAC1 antibody as a control of equal nuclear protein loading.

(C) Real-time qPCR assays were conducted to examine the mRNA expressions of LXR direct target genes ABCA1, ABCG1, ABCG5 and ABCG8. In A and C, *$p < 0.05$ and ***$p < 0.001$ compared to the fed group.

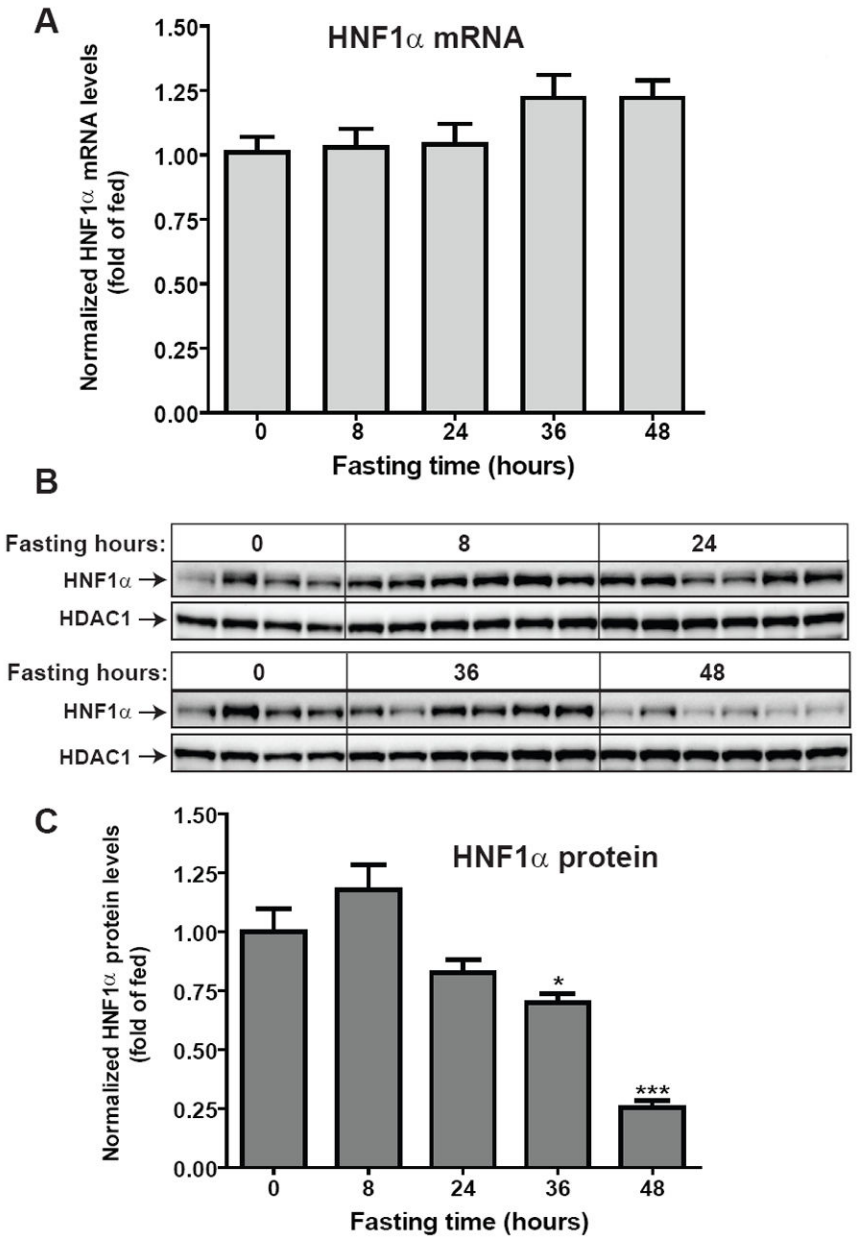

Figure 7. Examination of hepatic HNF1α mRNA and protein expressions

(A) Individual levels of hepatic HNF1α mRNA were assessed by real-time qPCR using hamster specific PCR primers as described in Figure 3A.

(B) Nuclear protein extracts were prepared from individual livers and protein concentrations were determined. Thirty μg of nuclear extracts per sample were resolved by SDS-PAGE and HNF1α protein was detected by immunoblotting using a rabbit anti-HNF1α antibody. The membrane was reprobed with anti-HDAC1 antibody.

(C) The protein abundances of HNF1α were quantified with the Alpha View Software with normalization by signals of HDAC1. Values are mean ± SEM of 4-6 samples per group. *$p < 0.05$ and ***$p < 0.001$ compared to the fed group.

Table 1

Hamster quantitative real-time PCR primer sequences.

Gene	Accession no.	Forward	Reverse
ABCA1	NM_013454, XM_003495857	AACAGTTTGTGGCCCTTTTG	AGTTCCAGGCTGGGGTACTT
ABCG1	NM_009593, XM_003504436	GAGGACCTTCCTCAGCATCA	AGGACCTTCTTGGCTTCGTT
ABCG5	NM_031884, XM_003496220	GCATGCTCAATGCTGTGAAT	GGATACAAGCCCAGAGTCCA
ABCG8	NM_026180, XM_003496233	GGAACCCAGGAATCCTCATT	AGATAGGGGTGCCAGATGTC
ACACA	NM_133360, AF356089	TGACACCATGTTGGGAGTTG	TGTGAGCAGGAAGGACTTGA
ACACB	AY762565	ACTATGAGGCCCAGCATGTC	TGACCCTATTGCCTCCAAAG
Actin, beta	DQ237887	TGACCGAGCGTGGCTACAG	CTTCTCTTTGATGTCACGCACAAT
CPT1α	AY762566	GGCCATCTGTGGGAGTATGT	ACTGTAGCCTGGTGGGTTTG
FAS	AF356086	AGTCCTTGTCCAGGTTCGTG	CCACCTAAGCCACCAGTGAT
GAPDH	DQ403055	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
HMGCR	M12705	GACGGTGACACTTACCATCTGT	GATGCACCGTGTTATGGTGA
HMGCS1	NM_145942, XM_003504950	TTTGATGCAGCTGTTTGAGG	CCACCTGTAGGTCTGGCATT
HNF1α	NM_009327, XM_003503514	GAGGTGGCTCAGCAATTCAC	CACTCCTCCACCAAGGTCTC
LDLR	NM_010700, NM_001246823	TTGGGTTGATTCCAAACTCC	GATTGGCACTGAAAATGGCT
LXRα	NM_013839, XM_003497307	GCAGGACCAGCTCCAAGTAG	ATTAGCATCCGTGGGAACAT
LXRβ	NM_009473, XM_003510892	CTTCCCCCACAAGTTCTCTG	GGCTCATCCTCTGGCTCTAA
PCSK9	NM_153565, XM_003495737	TGCTCCAGAGGTCATCACAG	GTCCCACTCTGTGACATGAAG
SREBP1	NM_001244003	GCACTTTTTGACACGTTTCTTC	CTGTACAGGCTCTCCTGTGG
SREBP2	NM_001244004	GAGAGCTGTGAATTTTCCAGTG	CTACAGATGATATCCGGACCAA