



# Treating Human Hepatocyte-like C3A Cells with Estrogen, Thyroid Hormone, and Insulin Reduced the Ability of PCSK9 to Form Complexes with the LDL Receptor

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## Abstract

Atherosclerotic-related diseases resulting from hypercholesterolemia kills more people than any other cause of death worldwide. Proprotein convertase subtilisin/kexin 9 (PCSK9) and the low-density lipoprotein (LDL) receptor are two genes that when mutated lead to hypercholesterolemia. The LDL receptor removes the bad cholesterol from the bloodstream, while PCSK9 controls the expression of the LDL receptor at the plasma membrane. The studies performed here in demonstrated that hormonal treatment (estradiol, thyroid hormone & insulin, simultaneously) of hepatic C3A cells grown in a cholesterol rich, hormone-free medium enhanced the expression of PCSK9 and the LDL receptor. However, the ability of PCSK9 to form complexes with the LDL receptor was reduced under the same conditions. These results suggest that these hormones protect against cardiovascular diseases by preventing the PCSK9-dependent degradation of the LDL receptor which could result in reduced serum LDL levels.

**Keywords:** Hypercholesterolemia; Thyroid hormone; LDL receptor

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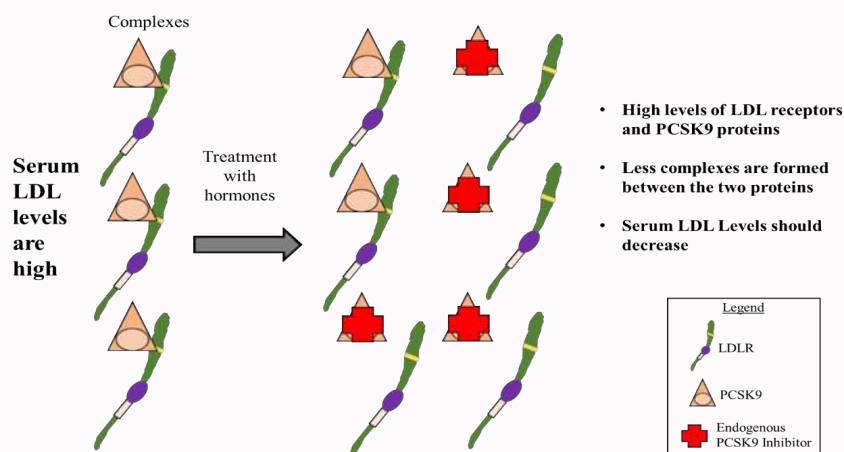
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## Introduction

Hypercholesterolemia is the leading cause of cardiovascular diseases, especially coronary artery disease [1,2]. Hypercholesterolemia is usually caused by mutations in genes encoding the low density lipoprotein (LDL) receptor, apolipoprotein B (Apo B), and proprotein convertase subtilisin/kexin type 9 (PCSK9) [1,3-5]. The LDL receptor is strategically located at the cell surface where it can bind LDL particles and transports it into the cell through receptor-mediated endocytosis [6-8]. In the endosome, LDL is released from the receptor triggered by the low pH; the LDL receptor returns to the cell surface to continue the process [6-8]. PCSK9 regulates plasma LDL levels by binding to and promoting intracellular degradation of LDL receptors [9-11]. The increased degradation of LDL receptor due to PCSK9 leads to reduced clearance of LDL, which accumulates in the plasma [9-11]. A direct correlation between plasma PCSK9 levels and LDL-cholesterol has been demonstrated [10,12-15].

It has been previously reported that PCSK9 protein levels could be elevated but still not able to bind the LDL receptor [16]. This was achieved by exposing hepatocyte-like C3A cells to a medium enriched with hormones and growth factors (supplemented with BDTM MITO+ serum extender) [16]. Under these conditions, cells synthesized and secreted high levels of PCSK9 protein, but the levels of PCSK9/LDL receptor complexes were significantly reduced as compared to cells grown in medium containing 10% FBS or delipidated serum [16]. Here, Figure 1 shows a schematic representation of this proposed mechanism. Herein, we decided to investigate whether three hormones present in the enriched medium, insulin, thyroid hormone, and estrogen, were responsible for the effects in the enriched medium. All three hormones are known to protect against coronary artery disease [17-19]. If fact, insulin, thyroid hormone, and estrogen have been identified as regulators of PCSK9 expression [20-29]. Estrogen and thyroid hormone appear to down-regulate the expression of PCSK9 [20-25]. Other factors might be involved in the regulation of PCSK9 by estrogen since males have 10% less circulating PCSK9 than females [20,21]. Insulin seems to upregulate the expression of PCSK9 [26-29]. Additional studies are required to determine the exact mechanism(s) for the hormonal regulation of PCSK9. The purpose of this project was to investigate some of these mechanisms.



**Figure 1:** Hypothetical model of the effects of hormones on the expression of the LDL receptor and PCSK9 and on the ability of these two proteins to form complexes. Hormonal treatment leads to higher levels of PCSK9 and LDL receptor proteins and lower levels of PCSK9/LDL receptor complexes [16]. This model suggests the secretion of an endogenous inhibitor of PCSK9 that prevents the interaction of PCSK9 with the LDL receptor [16]. This could result in lower levels of LDL particles in the serum of patients.

## Experimental Procedures

### Preparation of hormone deficient serum (THD)

The serum deficient in thyroid hormone, insulin, and estrogen (THD) was prepared as a modification of a previously published method [30]. In this approach, charcoal (Fisher Scientific; Pittsburgh, PA) was used to remove insulin and estrogen, whereas the anionic exchanger Dowex resin (Fisher Scientific) was employed to remove thyroid hormone [30]. Aliquots collected before and after the stripping process were tested for cholesterol and hormone levels. Cholesterol levels (measured using the Pointe Scientific Cholesterol Oxidase Assay; Fisher Scientific) were only slightly decreased from 15.17 mg/dL to 13.1 mg/dL after the processing. Hormone levels were reduced under detectable levels as confirmed using ELISA kits to detect total triiodothyronine (T3; Abnova, Walnut, CA), estrogen (E2; R&D Systems; Minneapolis, MN), and insulin (R&D Systems) (data not shown).

### Human hepatocyte-like C3A cell culture

C3A cells (American Type Culture Collection; Manassas, VA) were maintained between experiments in low-glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (maintaining medium), at 37°C with humidified atmosphere and 5% CO<sub>2</sub>. For experiments, cells were plated in maintaining medium. Twenty-four hrs later, the maintaining medium was replaced with THD medium (medium where FBS was replaced with THD serum) followed by another 24 hours incubation. Fresh THD media supplemented with and without hormones (10 nM E2, 50 mU/mL insulin and 3 nM T3, combined; Fisher Scientific) were added to the cells. These hormones were added together to determine whether the effects of the enriched medium previously reported [16] could be replicated with only these three hormones. Likewise, the concentration of each hormone was based on similarities to their concentrations in the enriched medium as determined using ELISA (data not shown). Furthermore, these concentrations are within the range previously used in cell culture experiments [31-33]. For cells not receiving hormones, the medium was supplemented with the same amount of vehicle (water for insulin, ethanol for E2, and NaOH for T3). Incubation with the treatments was carried out for an overall of 72 h. This time point was selected based on preliminary studies

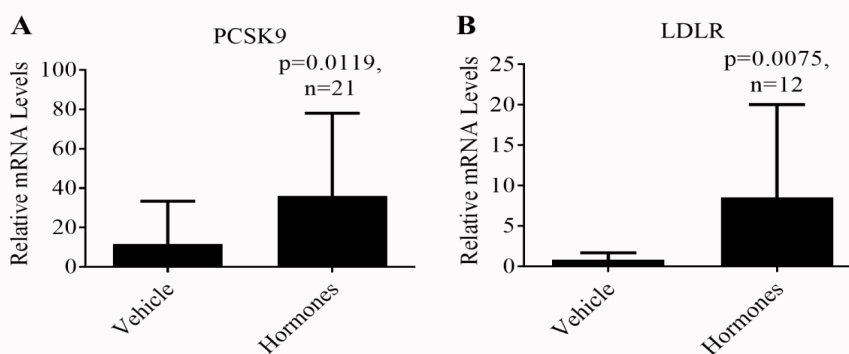
indicating that maximum levels of expression of PCSK9 and the LDL receptor, both mRNA and protein, are obtained and maintained after treating for 72 h. Conditioned medium samples were collected at the end of the incubation time for analysis of total PCSK9 levels. Cells were used for the preparation of RNA or protein (RIPA or non-denaturing, depending on the experiment) according to the protocols described below.

### RNA Preparation and quantitative real-time PCR (qRT-PCR) analysis

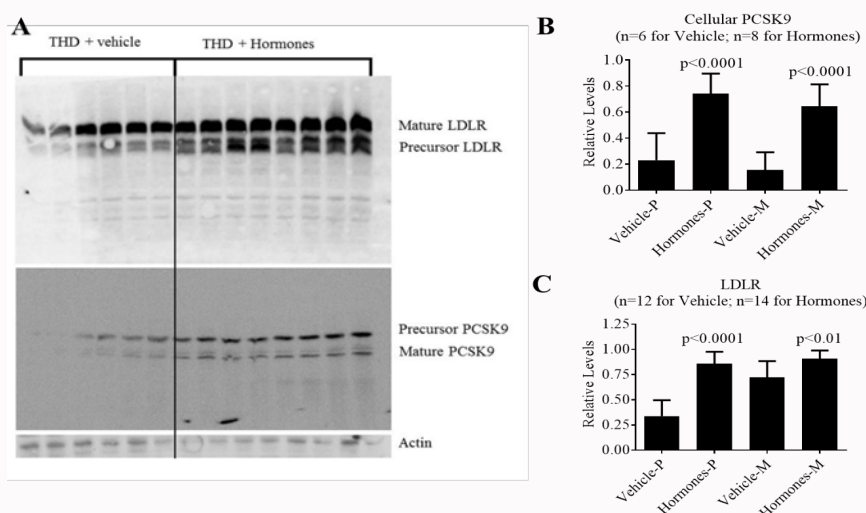
Isolation of RNA samples was carried out using the TRI reagent method as previously described [26]. The concentration of the RNA samples and the purity of the RNA preparation were determined using the Nanodrop 2000 (Wilmington, DE). The integrity of the RNA was confirmed using RNA electrophoresis (data not shown). DNase I treatment (Turbo DNA-free kit) of the RNA samples and preparation of ssDNA by reverse transcription (AB Reverse transcriptase system and random primers) were carried out using the protocols provided in each respective kit. The qRT-PCR reactions were performed essentially as previously described [34]. Primers specific for human 18s rRNA were utilized as the internal control for these studies. Quantitation of the result was done using a comparative CT method as previously done [34].

### RIPA Protein preparation and Western blotting analysis

Lysates prepared with cold RIPA buffer, electrophoresis, and Western blotting analysis were performed as previously described [35]. Equal protein loading was verified by staining membranes with 0.1% Ponceau S (in 5% acetic acid) and destained in water before they were used in the Western blotting. Western blotting analysis for the LDL receptor (diluted 1:2000; see Acknowledgments) and PCSK9 (dilution 1:1000; Cayman Chemicals; Ann Arbor, MI) was done by diluting each protein-specific antibodies in 5% non-fat milk-TTBS. Probing with an actin-specific antibody (Santa Cruz Biotechnology; Santa Cruz, CA) was used as the control for this experiment. All the secondary HRP-labeled antibodies were from Santa Cruz Biotechnology. Immunoreactive proteins were visualized using the Super Signal West Pico Chemiluminescence Substrate (Pierce Thermo Scientific; Rockford, IL). Images acquisition and quantitation of the protein signals were done as previously described [34].



**Figure 2:** Effects of hormones (estrogen or E<sub>2</sub>; triiodothyronine T<sub>3</sub> & Insulin) and vehicle on mRNA levels of PCSK9 **A**, and LDL receptor (LDLR) **B**. As determined by qRT-PCR. RNA preparation and qRT-PCR were done as described in Experimental Procedures. Primers specific for 18s rRNA were used for calculations using the CT method. P values and the number of samples considered for each condition have been indicated. The standard error of the means (SEM) were, for PCSK9 (**A**, 95% CI= 5.663 – 43.35), 4.2 for the vehicle and 9.158 for the hormone-treated group, and for the LDL receptor (**B**, 95% CI= 2.241 – 13.27), 0.2037 for the vehicle and 3.313 for the hormone treated group.



**Figure 3:** Effect of hormones (E<sub>2</sub>, T<sub>3</sub> & Insulin) and vehicle on protein levels of PCSK9 and the LDL receptor (LDLR) as determined by Western blotting analysis. Protein preparation, electrophoresis, and Western blotting analysis were done as described in Experimental Procedures. Actin was used as the internal control for these experiments. (**A**, A typical blot for each protein is shown. (**B**, Quantitated results of the signals from the Western blots. P values (obtained by comparing hormones versus vehicle for the same protein type) and the number of samples considered for each condition have been indicated. "M" refers to "mature." "P" refers to "precursor."

### PCSK9/LDLR complex ELISA

Non-denaturing lysates from cells and PCSK9/LDLR complexes prepared *in vitro*, with recombinant proteins, to be used for ELISA standards were prepared as previously described [16]. The PCSK9/LDLR protein complex ELISA was carried out using the Human PCSK9/LDLR DuoSet ELISA kit from R&D Systems (Minneapolis, MN) and conditions previously established [16]. After stopping the final assay reaction by adding 2 M sulfuric acid to each well, the color intensity in the plate was read at 450 and 540 nm using a plate reader.

### Total PCSK9 assay

To detect total PCSK9 protein levels in medium samples, a sandwich ELISA was performed using the Human PCSK9 DuoSet ELISA kit from R&D Systems and instructions previously published [16]. Again, after stopping the end reaction by adding sulfuric acid, the color intensity in each well was read in a plate reader at 450 and 540 nm.

### Statistical analysis

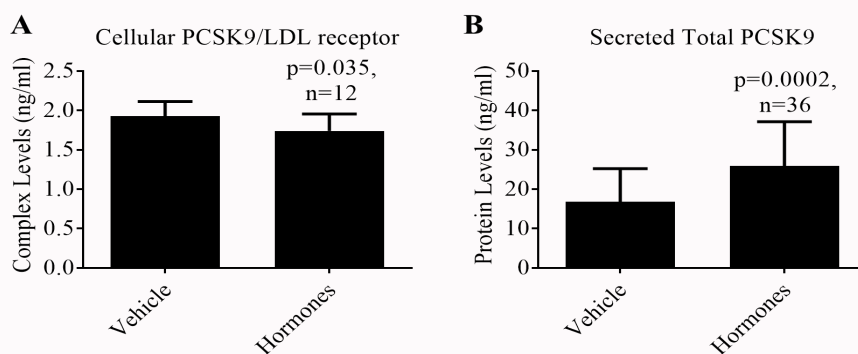
The GraphPad Prism 6 (Graph Pad Software, Inc., La Jolla, CA)

was used for statistical analysis. Two groups were compared using unpaired t-test. Analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used when comparing three or more groups. The significance level for the statistical tests was set at  $\alpha = 0.05$ .

## Results

First, the effects of treating cells grown in THD medium with 10 nM E<sub>2</sub>, 50 mU/mL insulin and 3 nM T<sub>3</sub>, combined, on the mRNA expression of the LDL receptor and PCSK9 were measured. Control cells received the vehicles in the same volumes added with the hormones. RNA preparations and qRT-PCR were done as described in Experimental Procedures. As shown in (Figure 2A & 2B), PCSK9 and the LDL receptor mRNA expression levels were significantly increased (3.12 and 10.7 fold, respectively) by treating with the hormone (E<sub>2</sub>, T<sub>3</sub> & Insulin) containing medium.

Next, the effects of these treatments on protein expression were examined. RIPA protein preparation, electrophoresis, and Western blotting analysis were done as described above. As illustrated in (Figure 3A), apparent increases in the protein expression levels of the



**Figure 4:** Effects of hormones (E2, T<sub>3</sub> & Insulin) and vehicle on cellular PCSK9/LDLR complex formation (A) and secreted total PCSK9 levels (B) using ELISA. (A. Preparation of non-denaturing proteins and complex standards was done as described above and utilized in the complex ELISA. (B. Medium samples employed in the assay were collected from treated cells. In both (A. and (B. p values and the number of samples considered for each condition have been indicated. The SEMs were, for complexes (A. 95% CI= -0.3553 – -0.0147), 0.054 for the vehicle and 0.062 for the hormone-treated group, and for secreted PCSK9 (B. 95% CI= 4.46 – 13.78), 1.404 for the vehicle and 1.866 for the hormone treated group.

LDL receptor and PCSK9 were seen in the blots. These increases were confirmed when quantitation of the protein signals was performed, and the results are shown in (Figure 3B & 3C). Precursor PCSK9 (before self-activation; [3]) was significantly increased 3.21 fold by treating with hormones, whereas mature PCSK9 (after self-activation; [3]) was significantly increased 4.13 fold by treating with hormones. Precursor LDL receptor (before glycosylation; [36]) was significantly increased 2.54 fold, whereas mature LDL receptor (after glycosylation; [36]) was significantly increased by 1.26-fold.

The following step was to determine whether these treatments affected the ability of PCSK9 to form complexes with the LDL receptor. For this, non-denaturing proteins were prepared and analyzed using the complex ELISA assay described above. As illustrated in (Figure 4A), even though the intracellular levels of both proteins were significantly increased see (Figure 3), the levels of PCSK9/LDL receptor complexes in the cells were reduced by 9% (p=0.035). The levels of total PCSK9 protein secreted into the media were also determined using ELISA. Figure 4B shows that treating with hormones also increased the levels of secreted PCSK9 by 1.54 fold (p=0.0002). Thus, treating with hormones reduced the percentage of PCSK9 forming complexes with the LDL receptor, compared to the secreted levels, by 42% (12% of PCSK9 formed complexes in samples treated with vehicle versus 7% of PCSK9 formed complexes in samples treated with hormone).

## Discussion

The studies reported here demonstrated for the first time that hormonal treatment (E2, T<sub>3</sub> & insulin, in combination) of hepatic C3A cells in a cholesterol rich-medium enhanced PCSK9 and LDL receptor expression, both at the mRNA and protein levels. However, the ability of PCSK9 to form complexes with the LDL receptor was slightly reduced under the same conditions. This suggests that these hormones enhanced the internalization of LDL particles by increasing the number of LDL receptor molecules present at the plasma membrane. The increase in the number of LDL receptor molecules could be the result of increased synthesis of the receptors as shown by the qRT-PCR and Western blotting analysis and a decrease in PCSK9 activity as demonstrated by the complex ELISA. This is critical because it could explain why females have more PCSK9 than males, but females are usually protected against cardiovascular diseases [37,38].

Currently, there is no evidence to explain the reduction in the ability of PCSK9 to form complexes with the LDL receptor in response to hormonal treatment. The enzymatic activity of PCSK9 is only necessary to self-activate the convertase [39]. Once, PCSK9 is self-activated the prodomain stays on the active side of PCSK9 preventing any further enzymatic activity by this convertase [39]. The only function that PCSK9 possess at that point is the ability to form complexes with the LDL receptor leading to the degradation of the LDL receptor in the lysosome [39]. As a result, fewer LDL receptors would be available to bind and remove LDL from the circulation causing an accumulation of this lipoprotein particle in the bloodstream [39].

One possibility could be that an endogenous inhibitor is upregulated by the treatment [16], and this inhibitor prevents the formation of complexes between PCSK9 and the LDL receptor. The upregulation of an endogenous inhibitor of PCSK9 in response to hormones such as estrogen could explain that males are less protected against cardiovascular diseases than females despite having 10% less circulating PCSK9 than females [20,21]. In fact, we have identified using affinity chromatography and mass spectrometry a 42 kDa protein that interacts with PCSK9 in the medium of cells incubated with the enriched medium (Lopez's unpublished observations). Another possibility is that PCSK9 suffers a post-translational modification that affects its LDL receptor-binding activity. Protein modifications reported for PCSK9 are phosphorylation at S47 and S688, glycosylation at N533, and sulfation at Y38 [40]. Of these, only phosphorylation has been shown to be necessary for the activity of PCSK9 because it prevents the proteolysis of the propeptide [41]. PCSK9 also undergoes alternative splicing, but the spliced variant, which has decreased self-activation, does not get secreted [42], so it is unlikely that this variant is responsible for the lower activity of secreted PCSK9. A third possibility is furin-cleavage of PCSK9 which results in a 2-fold less active PCSK9 [43,44]. However, no differences in the levels of wild-type and furin-cleaved PCSK9 in medium samples treated with hormones and vehicle were seen using Western blotting analysis (data not shown). Thus, further investigation is required to determine the exact mechanism for reducing PCSK9 activity in the presence of hormones.

Another important consideration is that the reduction levels (9%) in complex formation obtained herein, with a medium rich in cholesterol, insulin, E2, and T<sub>3</sub>, are not as significant as the reduction



levels (34%) seen in the previous study [16] using a medium poor in cholesterol, but rich in these three hormones are other growth factors. One possibility for this difference could be the presence of cholesterol in the medium used herein versus no cholesterol in the medium previously used. Cholesterol is not only a down-regulator of PCSK9 and the LDL receptor transcription [45,46], but it could also affect the synthesis of the endogenous inhibitor of PCSK9 suggested in the previous report [16]. Another possibility for the difference could be related to the lack of other hormones such as progesterone and testosterone, which are also removed using charcoal stripping [47,48], in the medium used in the current study versus the previous report [16]. There is no sufficient information to indicate a direct regulation of PCSK9 by progesterone except that PCSK9 levels are elevated at the time of parturition [22]. However, these effects have been attributed to differences in estrogen levels rather than changes in progesterone levels [22]. Testosterone, on the other hand, does not appear to affect the levels of circulating PCSK9 [37]. Nonetheless, testosterone deficiency in pigs has been related to an increase in PCSK9 expression and a corresponding increase in LDL levels [49]. Thus, the effects of progesterone and testosterone on PCSK9 expression are unclear and require further analysis.

The information reported herein could be used to develop diagnostic tools to measure the activity of PCSK9, which if it is decreased, it could lead to a reduction in plasma LDL levels, and consequently, it will reduce the risk of developing cardiovascular diseases. These results can also lead to the development of a novel therapeutic agent to treat patients with hypercholesterolemia.

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