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Is there a link between proprotein convertase PC7 activity and human lipid homeostasis?

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ABSTRACT

A genome-wide association study suggested that a R504H mutation in the proprotein convertase PC7 is associated with increased circulating levels of HDL and reduced triglycerides in black Africans. Our present results show that PC7 and PC7-R504H exhibit similar processing of transferrin receptor-1, proSortilin, and apolipoprotein-F. Plasma analyses revealed no change in the lipid profiles, insulin or glucose of wild type and PC7 KO mice. Thus, the R504H mutation does not modify the proteolytic activity of PC7. The mechanisms behind the implication of PC7 in the regulation of human HDL, triglycerides and in modifying the levels of atherogenic small dense LDL remain to be elucidated.

1. Introduction

Many secretory proteins are synthesized as precursors that are subsequently cleaved at specific sites to become activated, e.g., receptors, hormones, growth factors, adhesion molecules, secretory membrane-bound transcription factors, enzymes, and surface proteins. Most of these cleavages are achieved by the proprotein convertases (PCs), a family of mammalian serine proteases related to bacterial subtilisin and yeast kexin (genes *PCSK1* to *PCSK9*) [1]. PCs are secretory enzymes and their substrates are proteins that are cleaved and activated or inactivated [2] along their secretory route (lumen of the *trans* Golgi network – TGN, secretory vesicles, endosomes) and/or at the cell surface. The first 7 members of the PC family (PC1, PC2, Furin, PC4, PC5, PACE4 and PC7) are basic amino acid (aa)-specific proteases that cleave their substrates predominantly after **Arg** residues in the general consensus motif **[R/K]**-$X_{0,2,4}$-**R**↓, which shows that the cleavage sites are often organized in doublets and/or contain, P4 or P6 **Arg** or **Lys** (4th or 6th residue upstream of the P1 Arg cleavage site) [3,4]. The properties of PCs, the phenotypes of their knockout (KO) mice, their clinical importance and potential translational applications were recently reviewed [2,5].

PC7, the most ancient and conserved basic aa-specific PC-family member, is a type-I membrane-bound protease that is ubiquitously expressed, but highly enriched in liver [6]. In the ER, it undergoes an autocatalytic cleavage at **RAKR**$_{141}$↓ [7,8]. The site of separation of the inhibitory N-terminal prosegment from mature PC7 (and hence activation of PC7) is not clear, as this is thought to occur either in the TGN, at the cell surface or in endosomes, without requiring a secondary cleavage. The prosegment is secreted intact into the medium, leaving an active membrane-bound PC7 in the cell [9]. The active enzyme is composed of a catalytic domain, followed by a P-domain that stabilizes the catalytic pocket of the enzyme [10], and then a transmembrane domain (TMD) and a cytosolic tail (CT). Assays using the luminal/extracellular domain of PC7 (soluble PC7) and fluorogenic substrates indicated that this Ca^{2+}-dependent enzyme has an *in vitro* neutral pH optimum, and a cleavage specificity for **Arg**↓ similar to Furin [3,11]. Unlike Furin, PC7 is not secreted as a soluble/shed form, restricting its activity to intracellular endosomes or to the cell surface [9].

In 2010, a single nucleotide polymorphism (SNP) analysis using GWAS [12] established a strong link between high plasma levels of

Abbreviations: ANGPTL3, angiopoietin-like 3; ANGPTL4, angiopoietin-like 4; ApoF, apolipoprotein-F; GOF, gain of function; GWAS, genome-wide association study; HDL, high-density lipoprotein; hTfR1, human PC7-substrates: transferrin receptor 1; KO, knockout; LDL, low-density lipoprotein; PCs, proprotein convertases; SNP, single nucleotide polymorphism; TGN, *trans* Golgi network; TMD, transmembrane domain; VLDL, very low-density lipoprotein

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the soluble human TfR1 (sol.TfR1) and an intronic SNP *rs236918* within the *PCSK7* gene (frequency 11.5%), suggesting that this SNP results in a gain-of-function (GOF) with higher levels of PC7. Indeed, we showed that PC7 is the only convertase that can shed within endosomes the type-II membrane-bound human TfR1 into sol.TfR1 by cleavage at the unusual **K**TECE**R**$_{100}\downarrow$ motif containing basic residues at the P1 and P6 positions and a Cys at P3 [13]. TfR1 constitutes the only PC7-specific substrate known so far, allowing the examination of the specific enzymatic properties of the enzyme in cells. While PC7 cleaves human and rat TfR1, it cannot shed mouse TfR1 that exhibits a P1 Lys$_{100}$ instead of an Arg$_{100}\downarrow$ [13]. We also showed that only membrane-bound full length PC7$_{1\text{-}785}$ can shed TfR1, but not its soluble form [13], as was the case for proBDNF [14].

Data from GWAS provided spectacular advances in identifying genes associated with dyslipidemia and coronary heart disease [15,16]. Very recent GWAS analyses have revealed that:

A human PC7 variant R504H (gene *PCSK7* [MIM 604874]) variant *rs142953140* (c.1511G>A; p.Arg504His) is associated with a very significant ~40% *increase* in the levels of circulating HDL and a ~30% *decrease* of TG ($p = 3 \times 10^{-20}$) [17], likely resulting from an increased HDL/LDL ratio [18,19]. This is the first evidence that reveals physiological functions of PC7 in relation to cardiovascular homeostasis and vascular calcification. It is noteworthy that the heterozygote R504H mutation was detected in a cohort of 14,330 black African Americans, occurring at a frequency of ~0.2% (1:500 individuals), but not at all in a cohort of 42,208 white Caucasians [17]. This variant could thus be an ancient mutation that was not exported frequently out of Africa during the first migration of humans out of this continent, as was reported for some PCSK9 variants [20].

Accordingly, the authors showed that over-expression of human *PCSK7* in the liver of mice using a recombinant Adeno-associated virus (AAV8) resulted in a ~15% reduction of HDLc in the first week and ~45% increased TG in the second week. This suggested that the *rs142953140* variant may result in a decreased PC7 level and/or activity, which may rationalize the associated enhanced HDLc and decreased TG levels in black African Americans [17]. It is interesting to note that Arg$_{504}$ is in the critical P-domain of PC7, the latter thought to be important for the folding of the protein, and is replaced by His in the rat and mouse and by Asn in *Xenopus laevis* homologues (Fig 1A). Therefore, it was surprising that this variant was associated with high HDL and low TG levels, though it is coincident with the lower ratio of HDL/LDL found in humans *versus* rodents. Rodents exhibit a high HDL/LDL ratio with low LDL in the circulation, thought to be due to the absence of cholesterol ester transfer protein in mouse, which exchanges cholesterol esters and TG from HDL to VLDL/LDL. However, PC7 exerts species-specific functions, evidenced by its inability to shed mouse TfR1 [13].

2. Materials and methods

2.1. Cellular transfections

HuH7 and HEK293 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% foetal bovine serum (FBS, Invitrogen). All cells were maintained at 37 °C under 5% CO_2. Using Lipofectamine (Invitrogen) HuH7 cells were co-transfected with 800 ng of hTfR1-V5 (V5-tagged) and either 800 ng or 8 ng of empty vector control, human PC7 wild type (WT) or its R504H mutant or 800 ng of rat PC7. HEK293 cells were co-transfected with myc-tagged human Sortilin [14] and either WT PC7 or its R504H mutant with Effectene (Qiagen) according to the manufacturer's instructions. At 24 h post-transfection, cells were washed, followed by incubation for an additional 20 h in serum-free medium. Cells and conditioned media were then collected for Western blot analysis. The separated proteins were revealed using mAb V5-HRP, PC7 or β-actin antibodies and a rabbit HRP-conjugated secondary antibody [13].

2.2. Isolation, culture, and transfection of mouse primary hepatocytes

Mouse primary hepatocytes were isolated from 8- to 10-week-old male livers using a two-step collagenase perfusion method [13]. In 3.5-cm Petri dishes coated with fibronectin (0.5 mg/ml, Sigma), 5×10^5 cells were seeded in Williams' medium E (Invitrogen) with 10% FBS. After 2 h, the medium was replaced with hepatozyme medium (Invitrogen) for 12 h prior to transfection. We first generated cDNAs of human and mouse Apolipoprotein F (ApoF) tagged with V5 at their C-termini. These were co-transfected in mouse primary hepatocytes with cDNAs coding for either an empty vector control, WT PC7 or its R504H mutant. Transfections were performed with Effectene using a total of 4 μg of cDNA, following the manufacturer's instructions. Cell lysates and media were collected 48 h post-transfection and subjected to SDS–PAGE separation (14% Tris-Tricine) followed by Western blot analysis.

2.3. In situ hybridization histochemistry of ApoF and PC7 mRNAs in mouse liver

Cryosections (10 μm thick) were prepared from livers of 3-month-old WT mice, fixed in 4% formaldehyde, and hybridized as previously described [14] with mouse sense (negative control) and antisense cRNA probes. The latter probes corresponded to coding regions of mouse PC7 (residues 1–213) or ApoF (full length), and were synthesized using ^{35}S-UTP (PerkinElmer).

3. Results and discussion

In this work we took advantage of our complete *Pcsk7* knockout (KO) mouse model [14] to evaluate the plasma lipid profiles in the presence and absence of PC7. Surprisingly, our results indicated no significant changes of TG, total cholesterol and HDLc in 3-month old *Pcsk7* KO mice compared to WT mice for either males or females (Table 1), also confirmed by FPLC analyses *(not shown)*. Furthermore, plasma analyses by ELISA revealed no difference in the levels of circulating PCSK9 [21] in WT and *Pcsk7* KO mice (*not shown*). This result suggests that under a normal Chow diet, and contrary to human *PCSK7* overexpression, the lack of expression of mouse *Pcsk7* is not significantly involved in regulating HDLc TG, or PCSK9. However, it was still plausible that the *PCSK7 rs142953140* variant could result in lower levels of PC7 expression, or represent a loss-of-function (LOF) mutation resulting in a reduced PC7 enzymatic activity.

Since overexpression of PC7 does not cause ER stress to cells [9], we sought to determine whether an Arg$_{504}$ to His mutation (R504H) may influence the cellular proteolytic activity of PC7. Accordingly, we evaluated *ex vivo* the cleavage of two reported human PC7-substrates: transferrin receptor 1 (hTfR1) [13] and proSortilin [14]. We also analyzed the processing of apolipoprotein F (apoF) and angiopoietin-like 4 (ANGPTL4) implicated in HDL and/or triglyceride metabolism [22,23]. (a) In human hepatoma HuH7 cells, the R504H mutant is just as active as WT PC7 in shedding hTfR1 into its secreted soluble form (s-hTfR1; Fig. 1B). However, when the cells expressed 100-fold less cDNA levels of PC7 WT or its mutant compared to its substrate hTfR1 we noticed a trend towards lower (−15%) activity of the R504H mutant (Fig. 1C). Finally, we also observed that rat PC7 (Fig. 1A) sheds equally well the hTfR1 *versus* human PC7 (Fig. 1D). (b) Similarly, in human

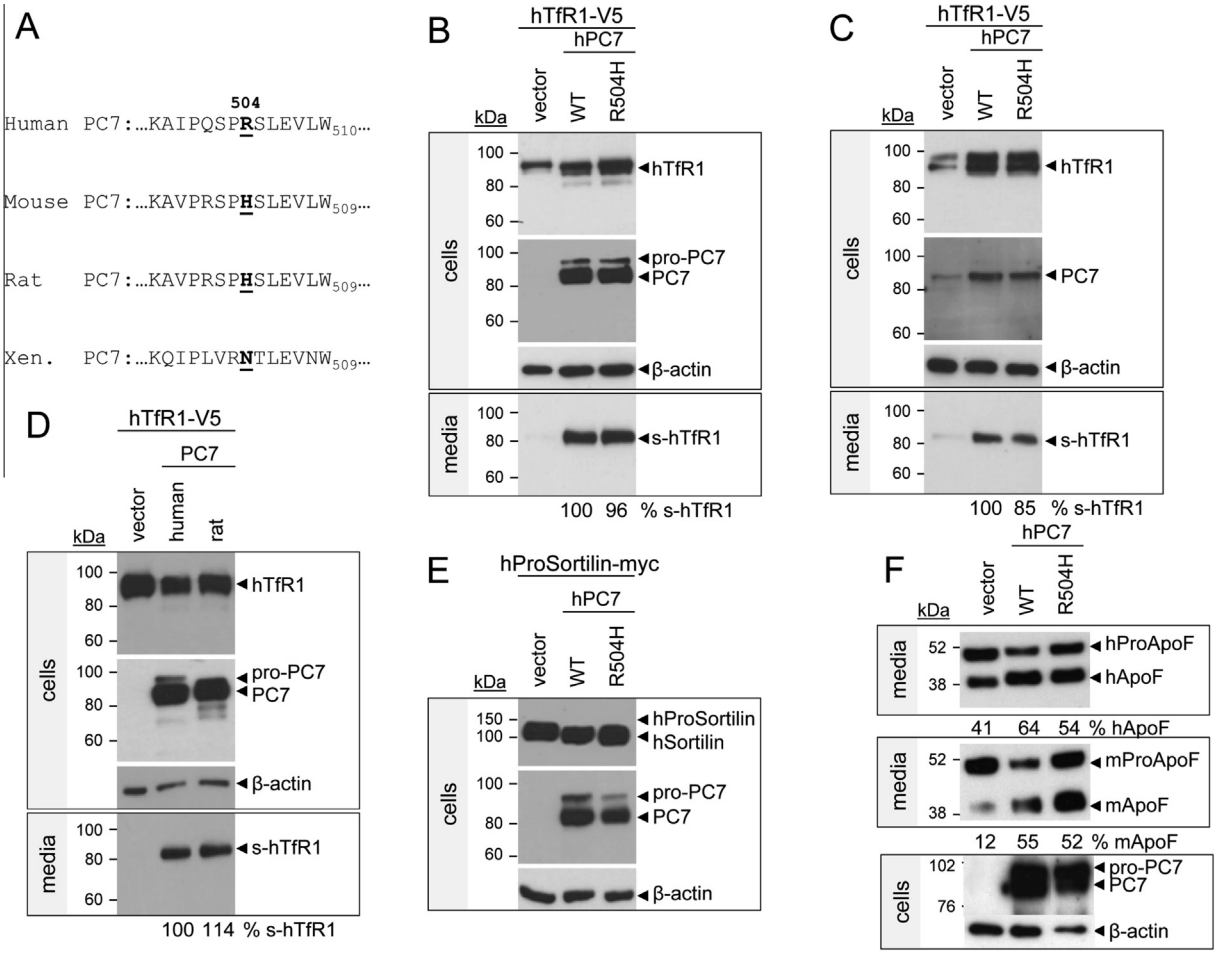

Fig. 1. Processing of PC7 substrates. Cleavage of human transferrin receptor 1 (hTfR1), human proSortilin and mouse proApolipoprotein F (proApoF) by wild type (WT) human PC7 and its R504H mutant or by rat PC7. (A) Comparison of human, mouse, rat and xenopus (Xen.) PC7 sequences. (B–D) Western blot analysis of the lysates and 20 h conditioned media of HuH7 cells co-transfected with 800 ng of hTfR1-V5 and either 800 ng (B, D) or 8 ng (C) of empty vector, human PC7 WT or its R504H mutant or rat PC7. The separated proteins were revealed using mAb V5-HRP, PC7 or β-actin antibodies and a rabbit HRP-conjugated secondary antibody. (E) Western blot analysis of cell lysates from HEK293 cells transiently transfected with human proSortilin-c-Myc and either empty vector, human PC7 WT or its R504H mutant. Proteins were revealed using mouse c-Myc, rabbit PC7 or rabbit β-actin antibodies and the corresponding HRP-conjugated secondary antibody. (F) Western blot analysis of lysates and 48 h conditioned media from mouse primary hepatocytes co-transfected with human or mouse proApoF-V5 and either empty vector, human PC7 WT or its R504H mutant. The separated proteins were revealed using mAb V5-HRP, PC7 or β-actin antibodies and a rabbit HRP-conjugated secondary antibody.

Table 1
Plasma triglycerides, total and HDL cholesterol, glucose and insulin in *Pcsk7*$^{+/+}$ and *Pcsk7*$^{-/-}$ mice.

	Male		Female	
	Pcsk7$^{+/+}$	*Pcsk7*$^{-/-}$	*Pcsk7*$^{+/+}$	*Pcsk7*$^{-/-}$
Triglycerides (mg/dL)	43.2 ± 3.3	45.1 ± 3.6	27.8 ± 3.6	25.1 ± 2.9
Total cholesterol (mg/dL)	112.7 ± 5.1	113.6 ± 5.9	94.4 ± 5.2	95.4 ± 1.6
HDL cholesterol (mg/dL)	99.6 ± 3.9	104.7 ± 5.2	84.6 ± 5.0	89.1 ± 1.7
Glucose (mmol/L)	8.3 ± 0.5	8.1 ± 0.6	7.7 ± 0.4	8.8 ± 0.6
Insulin (ng/mL)	1.5 ± 0.3	1.3 ± 0.2	0.9 ± 0.2	0.8 ± 0.1

Values are means ± S.E.M. Plasma samples were collected after 4 h fasting from 3- to 4-month-old C57BL/6J *Pcsk7*$^{+/+}$ or *Pcsk7*$^{-/-}$ mice. Triglycerides were determined using the Wako kit (n = 18–19 mice/group for male and n = 8 mice/group for female). Total and HDL cholesterol measurements were performed on a Roche Cobas Integra 400 plus analyzer (n = 6 mice/group for male and n = 5 mice/group for female). Glucose levels were determined using an automatic glucometer and insulin levels were measured using the Mouse Insulin Ultrasensitive ELISA Kit from Alpco Diagnostics (n = 12–14 mice/group for male and n = 6 mice/group for female). *Abbreviation:* HDL, High density lipoprotein.

embryonic kidney HEK293 cells, co-transfection of human proSortilin with WT PC7 or its R504H mutant revealed that both forms can completely process proSortilin into Sortilin (Fig. 1E). Altogether, these results indicated that the presence of the His_{504} did not appreciably affect the ability of PC7 to be autocatalytically activated (ratio of PC7 to proPC7 is unchanged) or process hTfR1 and proSortilin.

While Sortilin has been genetically linked to LDL, likely due to its enhanced degradation of apolipoprotein B in lysosomes [24], it has not been correlated to HDL or TG. Therefore, we sought to examine the possibility that PC7 or its mutant can differentially process ApoF, a potential substrate that is found at the periphery of HDL particles [22]. *In situ* hybridization histochemistry analysis [14] revealed that the mRNAs of PC7 and ApoF co-localize in liver (Fig. 2), supporting the possibility that apoF may be a substrate of PC7. Indeed, our data showed that overexpression of PC7 in mouse primary hepatocytes can process mouse and less so human pro-ApoF into ApoF (Fig. 1F) likely at **RAKR**$_{165}$↓ and **R**VG**R**$_{164}$↓, respectively, as previously hinted [22]. However, like for hTfR1 and proSortilin, WT PC7 or its R504H mutant process equally mouse and human proApoF (Fig. 1F). Such results revealed that proApoF constitutes a novel PC7 substrate, and indicated that the presence of the His_{504} did not affect the ability of PC7 to cleave it. Furthermore, as previously reported for angiopoietin-like 3 (ANGPTL3) [25], Furin and PC5/6A are the best convertases for ANGPTL4, and

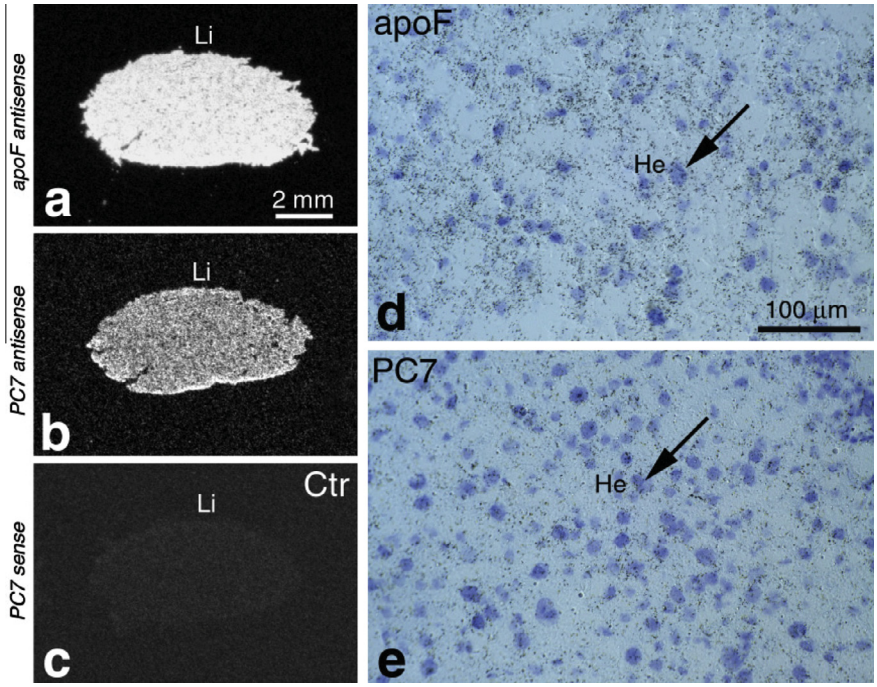

Fig. 2. Comparative localization of apoF and PC7 mRNA in mouse liver. *In situ* hybridization localization of ApoF and PC7 mRNA in the mouse liver at anatomical level. (a)X-ray film autoradiography showing apoF mRNA antisense labeling ubiquitously distributed throughout the liver (Li); dark field illumination; 5-day exposure time. (b) Adjacent section with PC7 mRNA labeling in the liver (Li); 5-day exposure time. (c) Control (Ctr) PC7 sense mRNA labeling; 5-day exposure time. (d) Emulsion autoradiography showing apoF mRNA labeling seen as dark silver grains under light field illumination. Labeling is mostly concentrated in hepatocytes (He); 2-day exposure time. (e) PC7 mRNA labeling (arrow) within liver hepatocytes (He); 10-day exposure time. Magnifications: (a–c) 6.4× and (d and e) 225×.

PC7 or its R504H mutant do not cleave these substrates (*not shown*).

It was suggested that atherogenic small dense LDL particles (sdLDL) contribute to increased cardiovascular disease and metabolic syndrome even when LDLc levels are within the normal range [26]. Very recently, it was reported that the levels of sdLDL were correlated with the *PCSK7* SNP *rs508487* [27] located in the 3' non-coding sequence of PC7 mRNA. The authors report that each copy of the minor allele at this SNP raise sdLDL by ∼4 mg/dl and triglycerides by ∼20 mg/dl, and is significantly associated with coronary heart disease. Furthermore, rare variants of *PCSK7* were also associated with increased circulating levels of sdLDLc by ∼7.5 mg/dl. We have tested these variants as well as the D186G, R316C and the frame-shifted sequence at L506P. Our data showed that the latter result in complete loss of function (LOF) of PC7 activity on the processing of hTfR1 (*not shown*). In that context, the authors suggest that LOF of *PCSK7* is associated with increased risk of cardiovascular disease.

However, our data on *Pcsk7* KO mice did not reveal any significant changes in either LDL, HDL, or triglycerides levels, under a Chow diet (Table 1). From plasma FPLC analyses [25] the lack of changes in lipids in the absence of PC7 was also confirmed (*not shown*). However, at the present time we cannot exclude that under a high fat or cholesterol diet the absence of PC7 may influence the lipid profiles. Furthermore, we also did not detect any changes between genotypes in either circulating insulin or glucose levels (Table 1).

In conclusion, our data associated with the study of Peloso et al. [17] suggested that the presence of the Arg_{504} does not modify the proteolytic activity of PC7, even though it is implicated in the regulation by PC7 of human cardiovascular homeostasis by an as yet undefined mechanism. Since no change in enzymatic activity of PC7 was detected on four substrates for the R504H mutant, we presume that the implication of PC7 R504H in lipid homeostasis may either be due to a difference between human and mouse lipid metabolism, or that it does not require the enzymatic activity of PC7. It will however be important in the future to figure out whether the R504H mutation is really involved with altering HDL levels, or if this mutation is associated with another mutation that is the direct cause for observed changes in HDL.

Conflict of interest

The authors declare no financial conflict of interest.

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