APOLIPOPROTEIN AV: GENE EXPRESSION, PHYSIOLOGICAL ROLE IN LIPID METABOLISM AND CLINICAL RELEVANCE

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ABBREVIATIONS

aa : Amino acid

Apo: apolipoprotein

CDCA: chenodeoxycholic acid

DR: direct repeat

FXR: farnesoid X-activated receptor

HDL: high-density lipoproteins

HNF-4α : hepatocyte nuclear factor-4α

HSPG: heparan sulfate proteoglycan

LDL: low-density lipoproteins

LDLr: low-density lipoprotein receptor

LRP: LDLr-related protein

L-FABP: liver fatty acid-binding protein

LPL: lipoprotein lipase

LXR: liver X receptor

nt: nucleotide(s)

PGC-1α : peroxisome proliferator-activated receptor-γ coactivator-1α

PPARα: peroxisome proliferator-activated receptor α

RXR : retinoid X receptor

siRNA: small interference RNA

SNP: single nucleotide polymorphism

SREBP: sterol regulatory element binding protein

T3: 3,5,3'-triiodo-L-thyronine

TG: triglyceride

TR: thyroid hormone receptor

TRL: triglyceride-rich lipoproteins

VLDL: very low-density lipoproteins

Key words : Apolipoprotein ; hypertriglyceridemia ; Lipoprotein lipase ; TG-rich lipoproteins ; nuclear receptor ; regulation of gene expression ; fibrate ; thyroid hormone

ABSTRACT

The apolipoprotein *APOA5* gene, a member of the gene cluster on chromosome 11q23 which includes *APOA1*, *APOC3* and *APOA4,* has gained considerable interest as it encodes apoAV, a key determinant of circulating levels of potentially atherogenic triglyceride-rich lipoproteins (TRL). Indeed, strong associations between genetic variants of the *APOA5* gene sequence and elevated triglyceride (TG) levels have been established. This apolipoprotein may potentiate lipolysis of TRL through facilitation of lipoprotein interaction with lipoprotein lipase. In addition, apoAV may enhance clearance of remnant lipoproteins by mediating their interaction with the LDL receptor-related protein (LRP1). The implication of apoAV in intravascular TRL metabolism is further supported by studies that have demonstrated up-regulation of *APOA5* gene expression by nuclear receptors (PPARα, FXR, HNF4α) and hormones (thyroxine) involved in hypotriglyceridemic pathways. *APOA5* expression may equally be modulated by nutritional status, and more specifically, by stimulation of lipogenesis through transcriptional regulation mediated by insulin and SREBP-1c.

However, despite the fact that studies in mice have clearly revealed that plasma levels of apoAV are inversely correlated with plasma TG levels, the relationship between apoAV and metabolism of TRL remains controversial in man. Indeed, positive correlations between apoAV and TG levels have recently been observed in patients with hypertriglyceridemia and type 2 diabetes. The question as to whether apoAV is a key determinant of TG levels in man therefore remains conjectural.

Introduction

The *APOA5* gene encoding (apo) apolipoprotein AV was recently identified and recognized as a potential determinant of plasma triglyceride (TG) levels in mice (1, 2). Indeed, overexpression of human apoAV in mice leads to reduction in circulating TG concentrations, whereas the lack of apoAV is associated with hypertriglyceridemia (1). In human subjects, polymorphisms in the *APOA5* gene sequence have been correlated with elevated serum TG levels in several populations (1, 3-5). By contrast, recent reports in man have documented either no correlation or alternatively a positive correlation between plasma apoAV and TG levels in hypertriglyceridemic states (6-8). It therefore remains unclear as to whether apoAV is a major modulator of circulating levels of TRL in man.

In vitro and *in vivo* experiments in mice support a role for apoAV in the acceleration of lipoprotein lipase (LPL)-mediated lipolysis of TG-rich lipoproteins (9-11). It is well-known that LPL is a central player in determining plasma TG levels, as LPL catalyses the hydrolysis of the hydrophobic core of TRL, including chylomicrons and VLDL. The discovery of a new plasma protein potentially implicated in the regulation of intravascular LPL activity, and probably more specifically in the postprandial state, is therefore of major significance.

Since hypertriglyceridemia is an independent risk factor for premature coronary disease (12-14), and is equally a key feature of the metabolic syndrome, further understanding of the factors that regulate circulating levels of TRL may provide new opportunities for therapeutic intervention in atherogenic dyslipidemia. Indeed, apoAV is potentially implicated in the dyslipidemia of metabolic disease and, more specifically, in the hypertriglyceridemia of insulin-resistance, type 2 diabetes and metabolic syndrome.

In this review, we discuss new insights from mouse and human studies into the physiological role of apoAV, with focus on the role of this apolipoprotein in the intravascular metabolism of TRL, on the mechanisms involved in the regulation of *APOA5* gene expression, and on the clinical relevance of apoAV.

Identification of apoAV
- **Discovery of apoAV in 2001 as a new apolipoprotein by sequence comparison, but equally as an acute phase protein in rat.**
- **Biochemical property: ApoAV is a highly hydrophobic apolipoprotein.**

- **Tissue distribution and synthesis of apoAV: In humans and rodents, apoA5 is produced exclusively by the liver and circulates in plasma at very low levels in association with lipoproteins.**

In 2001, two groups identified the sequence of a new member of the plasma apolipoprotein family using distinct methodological approaches. By sequence comparison of human and mouse genomes, Pennacchio et al. (1) identified a conserved genomic sequence (71% homology) that contained a putative apolipoprotein gene which was situated 30kb downstream from the *APOA1/C3/A4* cluster on human chromosome 11q23. In view of its homology with the *APOA4* gene sequence (24% identity and 49% similarity), the predicted 368 amino acid (aa) protein was termed apolipoprotein AV (1). At the same time, van der Vliet et al (2) identified apoAV in studies aimed at detection of new genes involved in liver regeneration in the rat following partial hepatectomy. They found that apoAV consisted of a cleavable signal peptide of 20 aa and a mature protein of 348 aa; an antibody raised against the mature protein allowed its identification as a plasma protein, which was mainly associated with HDL in the fasting state in mice.

Bioinformatic prediction and structural studies of the apoAV recombinant protein revealed a more highly hydrophobic apolipoprotein than apoAI and apoAIV, with high affinity for the lipid phase (15). In addition, apoAV presented low solubility between the pH limits of 3.5 and 9, a feature that may be attributed to a pI of 6.2 and a low percentage of charged residues (16). Secondary structure obtained by far-UV circular dichroism analysis revealed a large proportion of β−sheet, which is rather unusual for an apolipoprotein. These structural characteristics suggest tight binding of apoAV to lipoprotein lipid components. Recently, Beckstead et al (17) identified a tetraproline motif (293-296) in the C-terminal region of apoAV which modulates apoAV lipid-binding activity.

In humans and in rodents, gene expression of *APOA5* appears to be hepatospecific (2, 18). However, this does not appear to be the case in all species, as Dichlberger et al (19) have recently reported that apoAV is also expressed in the brain, kidney, small intestine and ovarian follicles of the chicken. In mice, apoAV is secreted by the liver and circulates in the plasma in association with HDL during fasting, but shifts to VLDL in the postprandial state (9). The specific binding of apoAV to lipoprotein particles has now been confirmed in human subjects in studies involving specific immunoprecipitation of distinct lipoprotein classes, and which have demonstrated an association of apoAV with VLDL and also HDL (20). Moreover, whereas apoAV does not appear to be expressed in the intestine (2, 18), this apolipoprotein

has equally been detected in chylomicrons in humans, suggesting that this association occurs in the circulation (20). Levels of apoAV in plasma are very low (~ 157 μg/l) and some ten-thousand fold less than that of apoAI, the major protein component of HDL (100-150 mg/dl) (20).

Implication of apoAV in triglyceride metabolism: insights from in vitro studies and mouse models

- **The *APOA5* KO mouse is hypertriglycerdemic whereas overexpression of apoAV reduces TG levels in murine models.**

- **In vivo and in vitro, apoAV induces increase in LPL activity.**

- **ApoAV is involved in lipoprotein uptake through its interaction with LRP1 and soRLA.**

- **A potential role for apoAV in hepatocyte.**

The earliest data concerning the potential physiological function of apoAV revealed its implication in intravascular triglyceride metabolism in mice. Indeed, transgenic mice that overexpressed human *APOA5* (1), as well as mice exhibiting adenoviral overexpression of apoAV (21), displayed marked reduction in plasma TG levels. This is in contrast to mice in which the *APOA5* gene had been deleted. ApoAV-deficient mice are highly hypertriglyceridemic, with fourfold greater levels of plasma TG as compared to their wild-type littermates (1). Interestingly, the lipid profiles described above are the exact opposite of those obtained in *APOC3* KO (22) and in apoCIII-overexpressing transgenic mice (23), which are respectively hypo- and hypertriglyceridemic. Indeed, apoCIII overexpression attenuates lipolysis of TRL via inhibition of LPL activity (24). These data suggest that apoAV and apoCIII, two members of the *APOA1/C3/A4/A5* cluster, play antagonistic roles in the metabolism of TRL, at least in mice.

Several studies have attempted to elucidate the mechanism(s) underlying the hypotriglyceridemic effect of apoAV. Schaap et al (10) reported that adenoviral overexpression of apoAV in mice dose-dependently attenuates postprandial hypertriglyceridemia, thereby suggesting that apoAV stimulates LPL-dependent TRL clearance. Similarly, in human *APOA5* transgenic mice, LPL-mediated hydrolysis was accelerated (9). Interestingly, Merkel et al (11) investigated the interplay between apoAV and LPL *in vivo*. Cross-breeding of *APOA5* transgenic mice with hyperTG LPL+/- mice revealed

that human apoAV diminished TG levels only moderately when LPL expression was reduced. This finding supported the notion that the effect of apoAV on plasma levels of TRL was dependent on LPL activity. Post-natal lethality of LPL$^{-/-}$ mice prevented investigation of the impact of hapoAV expression in the context of LPL deficiency.

In vitro, Fruchart-Najib et al (9) showed that recombinant human apoAV induces increase in LPL activity. Nonetheless, the amount of apoAV needed to activate LPL in these studies (600 μg/ml) was some 1000-fold higher than that of the physiological human plasma concentration. Indeed, Merkel et al (11) demonstrated that recombinant human apoAV increased heparan sulfate proteoglycan (HSPG)-bound LPL activity *in vitro*, but had no effect on free LPL. These data suggested that apoAV accelerates TG-rich lipoprotein lipolysis by facilitating lipoprotein interaction with proteoglycan-bound LPL, leading the authors to propose that apoAV enhances the function of the natural lipolytic system as an allosteric LPL activator at the endothelial wall. Reinforcing this hypothesis, the binding of apoAV to heparin may promote the interaction of TRL with cell surface HSPG (25). Recently, it has been shown that the domain encompassing residues 192-238 of apoAV is essential for LPL activation (26). Interestingly, apoAV has been reported to interact with glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) *in vitro*, a protein which plays a critical role in the lipolytic processing of chylomicrons. It is therefore tempting to hypothesize that apoAV could contribute to the binding of chylomicrons to GPIHBP1 (27). Taken together, *in vitro* and *in vivo* data lend support to the hypothesis that interaction between apoAV and LPL-bound HSPG is critical to enhanced LPL-mediated lipolysis of TRL (28) (Figure 1A).

In mice, apoAV deficiency resulted in attenuated removal of TG-rich lipoprotein remnants (29). Thus, VLDL particles isolated from *APOA5$^{-/-}$* mice exhibited a lower affinity for the LDL receptor as compared to wild type VLDL. Recently, Nilsson et al (30) have characterized the interaction between apoAV and two members of the LDL receptor family, the LDLR-related protein 1 (LRP1) and the mosaic type-1 receptor (SorLA). These interactions involve the R210 and K211 residues of the apoAV sequence, which are equally part of the heparin binding region. The identification of these residues supports a role of apoAV in recognition of VLDL particles by members of the LDL-receptor family and thus in clearance of these particles (Figure 1A).

Taken together, the association of apoAV with heparin and proteoglycans on the one hand, and the association with LRP1 and SorLA on the other, suggest that hepatic apoAV may be secreted, after which it directly associates with HSPG on the extra-cellular surface of the

hepatocyte plasma membrane. Therefore, we can hypothesize that apoAV may interact with other factors implicated in intravascular lipoprotein metabolism such as hepatic lipase, which is known to be cell-membrane associated. This hypothesis is of potential relevance as apoAV is partially associated with HDL during the post-prandial state, and HDL-TG are a substrate of hepatic lipase.

In view of its implication in TRL hydrolysis and clearance, it is tempting to speculate on a potential role for apoAV in the postprandial phase. Fruchart-Najib et al. (9) showed that in fasted apo*A5*xapo*C3* transgenic mice, apoAV is preferentially associated with HDL and that in the postprandial state after a lipid bolus, apoAV shifted from HDL to VLDL. Further investigation is however required in order to provide additional insight into the potential role of apoAV in the postprandial state.

In addition to its role in TRL clearance, Schaap et al (10) suggested that apoAV may contribute to regulation of VLDL production rate. Indeed, analysis of VLDL isolated from mice 2 hours after treatment with Triton WR1339, an inhibitor of lipolysis, showed that apoAV overexpression led to reduction in both the particle size and TG content (-26%) of VLDL. However the action of apoAV on VLDL secretion remains controversial. For instance, apoAV KO mice display a 30% increase in VLDL particle size but no change in production rate (29). Furthermore, in the human hepatic cell line Hep3B, apoAV overexpression had no effect on apoB secretion nor on the density distribution of apoB- and apoAV-containing lipoproteins (31).

Finally, the potential role of intracellular apoAV has been addressed in *in vitro* cell culture studies. Thus, Weinberg et al. (15) have shown that apoB6.6F and human serum albumin are both secreted from transfected Cos-1 cells, but not apoAV, leading the authors to propose an intracellular function for this protein. Interestingly, confocal fluorescence microscopy revealed that apoAV and apoB displayed a distinct subcellular localization pattern and a unique association of apoAV with lipid droplets in the rat hepatoma cell line, McA-RH7777 (31). These observations bring a new perspective to the potential role of apoAV in the storage and/or mobilization of intrahepatic lipids. Future studies to confirm the potential intra-cellular role of apoAV and its effect on plasma TG levels are awaited.

In summary, both *in vitro* and in murine models, it has been established that apoAV-associated TRL enhances TG hydrolysis through enhanced LPL activity, and that such action appears to involve binding of apoAV to HSPG at the vascular endothelium. In addition, apoAV has been proposed to enhance VLDL remnant removal by facilitating particle binding to the LDL receptor and interaction of these particles with LRP1 and SorLA. Finally, the low

circulating levels of apoAV considered together with data from *in vitro* studies support an intracellular role for apoAV. Nonetheless future investigations will be critical to our understanding of the precise role(s) of apoAV in lipoprotein metabolism by the hepatocyte.

Genetic variations of *APOA5* and impact on TRL metabolism

- **Common polymorphisms of the *APOA5* sequence are associated with high TG levels.**
- **Characterization of three rare point mutations in *APOA5* which are associated with hypertriglyceridemia in man.**

Two main types of genetic studies have been conducted on apoAV in man: firstly, those focusing on common genetic variants (single nucleotide polymorphisms) and secondly, those reporting clinical cases of patients displaying rare mutations.

With respect to common polymorphisms, several studies have demonstrated significant association between several single nucleotide polymorphisms (SNPs) in the *APOA5* gene sequence and elevated serum TG levels. Among them, two common polymorphisms have been widely studied (i.e. -1131T>C and c.56C>G(S19W)) (1, 3), (reviewed in (32-34). ApoAV levels were not associated with the -1131T>C SNP (7, 35), but have been positively correlated with elevated plasma levels of apoCIII (35, 36). In addition, a strong linkage disequilibrium between -1131T>C and a variant for *APOC3* (37) has been established. Finally, *in vitro* (38) and *in vivo* (39) studies have revealed that this SNP does not affect apoAV expression, thereby supporting the hypothesis that its association with TG levels is most probably due to the effect of the *APOC3* variant with which it is in linkage disequilibrium.

The c.56C>G(S19W) polymorphism, which leads to an amino acid change in the region coding for the signal peptide, has been positively correlated with TG levels and, surprisingly, with apoAV levels in patients with type 2 diabetes and equally in healthy men (7). Similarly, Henneman et al. (34) have shown that there is an increased allele frequency of the minor allele of the *APOA5* S19W SNP and a positive correlation with plasma apoAV and TG levels in patients with severe hypertriglyceridemia. These results seem to contradict *in vitro* functional studies, which suggest that the S19W polymorphism leads to a 50% decrease in apoAV secretion (38). In addition, the S19W change is associated with diminished apoAV plasma concentrations in a transgenic mouse model (39). These findings support the hypothesis that the W19 variant reduces translocation of apoAV across the endoplasmic

reticulum as predicted by molecular modeling. This hypothesis is not however consistent with the elevated apoAV plasma levels found in W19 carriers. Additional functional characterization appears necessary therefore in order to explain the paradox in the relationship between S19W and apoAV plasma levels. Finally, the W19 polymorphism has been positively associated with carotid intima media thickness (40) and with progression of atherosclerosis (4), findings which clearly identify our incomplete understanding of the functional impact of the 19W polymorphism.

Among other polymorphisms in the *APOA5* sequence, the G182C variant has been associated with high TG levels in both diabetic and non diabetic cohorts (41, 42). Interestingly, a significant interaction was found between G182C and fasting glucose in the diabetic group only (42), a finding which suggests that apoAV may be implicated in the expression of a complex and multifactorial type 2 diabetic phenotype.

The functional characterization of rare *APOA5* mutants has provided new insight into the physiological role of human apoAV (see ref.(43) for a review). Extrapolating from mouse models and from *in vitro* studies, rare mutations for *APOA5* would be expected to affect TG levels through loss in LPL activity. The sequencing of DNA from 10 patients with severe hypertriglyceridemia, and in whom mutations in *APOC2* or *LPL* were absent, led to the identification of a patient homozygous for a Q145X mutation in the *APOA5* gene (renamed Q148X) (44). This mutation predicted a truncated apoAV protein devoid of key functional domains. Significantly, LPL activity in this patient was 40% lower as compared to controls; in the family, 10 individuals were heterozygous for Q148X, of whom five displayed mild hypertriglyceridemia, whereas the other five were normolipidemic. The five hypertriglyceridemic Q148X heterozygotes were also heterozygous for the S19W variant, and the homozygous patient was equally homozygous for the W19 allele. In contrast, the Q148X normolipidemic carriers displayed the S19 allele. These findings suggest that the presence of the W19 allele is required for the expression of a hypertriglyceridemic phenotype.

Marçais et al. (45) identified a heterozygous *APOA5* Q139X mutation leading to production of a 15 kDa truncated peptide of apoAV. Two families were identified: in family A, the two carriers displayed hypertriglyceridemia, and in family B, 2 carriers (of 6) were hypertriglyceridemic. Three of the hypertriglyceridemic carriers equally exhibited a marked decrease in the catabolic rate of VLDL-apoB. In addition, the hypertriglyceridemic carriers, in contrast to the normolipidemic carriers, displayed substantial impairment of LPL activity and reduction in post-heparin LPL mass. Taken together, these results lend support to the hypothesis that apoAV regulates LPL activity. Nonetheless, it should also be mentioned that

the hypertriglyceridemic carriers in family B are heterozygous for the coding SNP S19W, whereas the normolipidemic carriers are S19 homozygotes, thereby suggesting that the W19 variant is a crucial determinant of dyslipidemia in heterozygous Q139X carriers.

Recently, Priore Oliva *et al.* (46) reported the case of a 17-year-old male with hypertriglyceridemia and low HDL-cholesterol levels but normal post-heparin LPL activity. The patient was homozygous for a point mutation (c.289 C>T) in the *APOAV* gene which resulted in transformation of a glutamine codon at position 97 into a termination codon (Q97X); apoAV was undetectable in plasma. These findings suggest that lack of apoAV has a dramatic effect on TG level without affecting post-heparin LPL activity, and support the notion that apoAV may influence LPL activity by an indirect mechanism, possibly involving complex formation with other elements such as HSPG and which are not implicated in post-heparin LPL activity.

Altogether, the functional characterization of these rare mutations supports the notion that apoAV acts as an activator of TRL hydrolysis mediated by LPL. However, as remarked by Talmud et al. (43) for Q148X and Q139X mutations, the penetrance of these mutations is low and that the co-inheritance of a polymorphism in *APOA5* which raises TG levels is crucial for the expression of hypertriglyceridemia.

ApoAV in lipid and lipoprotein metabolism in human subjects: clinical studies
- **ApoAV and TG levels are positively correlated.**
- **ApoAV levels are elevated in type 2 diabetic patients in the post-prandial state.**

Clinical studies have been conducted to evaluate the potential relationship between plasma levels of apoAV and TG in different pathophysiological contexts.

A positive correlation between apoAV and TG levels in the dyslipidemia of type 2 diabetes has been consistently observed. Thus, Dallinga-Thie et al. (8) found that apoAV levels, like those of apoCIII, were positively correlated with triglyceride concentrations in type 2 diabetic subjects and that the decrease in plasma TG levels following atorvastatin treatment in those subjects was correlated with reduction in apoAV. Similarly, apoAV levels were elevated in the postprandial state in type 2 diabetic subjects in contrast to healthy subjects ; in addition, apoAV levels were higher in type 2 diabetic subjects in the fasting state (47). Furthermore, Schaap et al (6) documented a 5-fold increase in apoAV levels in a small population of hypertriglyceridemic patients, and observed a positive correlation between apoAV and apoCIII levels, thereby providing evidence for a complex relationship between apoAV and

apoCIII in this phenotype. It is of note that apoAV and TG levels were not correlated in healthy subjects (6). Indeed, the authors indicated that it could be argued whether the positive correlation between TG and apoAV in type 2 diabetes was mainly due to apoCIII, as apoAV was no longer positively correlated with TG concentration after adjustment for apoCIII levels. The positive correlation between apoAV levels and TG levels is therefore established in dyslipidemic type 2 diabetic patients. To date however, there is insufficient data to ascertain as to whether increase in apoAV is a consequence of type 2 diabetes and/or hypertriglyceridemia, or whether it could be implicated in the initiation of these metabolic disorders. Additional understanding of both apoAV function and of the regulation of *APOA5* expression in man is essential in this respect. Genetically-engineered human *APOA5* transgenic mice could be valuable in this regard. Indeed, mice in which the *APOA5* gene had been deleted, but which overexpressed the human *APOA5* transgene, have been recently shown to display a positive correlation between TG and human apoAV levels (48).

A second aspect of the positive association between apoAV and TG levels involves a potential role for apoAV in the post-prandial state. As mentioned previously, apoAV levels are elevated in the post-prandial state in diabetic subjects (47). Furthermore, levels of both apoAV and apoCIII closely paralleled the postprandial triglyceride response, particularly in the VLDL1 fraction, but in contrast, did not correlate with post-heparin LPL activity (49). Finally, it has been reported that in addition to high TG levels, carriers of the -1131T>C and S19W polymorphisms display modifications in post-prandial lipoprotein metabolism which includes a higher post-prandial TG level (50, 51). Taken together, these results clearly establish that apoAV levels are elevated after a fat load, and are positively correlated with TG levels; the physiological significance of such elevation remains indeterminate however. Future studies in the post-prandial state should be designed to further our understanding of the contribution of apoAV to post-fat load hypertriglyceridemia.

APOA5 gene regulation: implication of nuclear receptors in lipid homeostasis

- *APOA5* gene expression is upregulated by PPARα agonists.
- FXR, HNF-4 and RORα are implicated in the regulation of *APOA5* gene expression.
- ApoAV is increased by T3 and decreased in hypothyroid rats.
- *APOA5* is downregulated by LXR and insulin, two activators of the expression of lipogenic genes.

It is well established that pharmacological activation of the nuclear receptor PPARα lowers plasma levels of TG-rich lipoproteins and especially those of VLDL (52-54). PPARα mediates the hypotriglyceridemic effect of fibrates through activation of the transcription of genes implicated in fatty acid (FA) catabolism (55-58) and TRL clearance (59, 60). This latter effect has been attributed partly to stimulation of LPL activity via activation of LPL expression itself (61), but equally to a decrease in hepatic apoCIII expression (60, 62).

In human hepatic cells, the activation of PPARα by specific agonists leads to increase in *APOA5* mRNA levels (18, 63). In addition, 5'deletion, mutagenesis and gel-shift analyses suggest that human *APOA5* gene expression is regulated by PPARα through its binding to a DR1 PPARα response element at –271 nt from the transcription start site. These studies demonstrated that the human *APOA5* gene is a de facto PPARα target gene (Figure 2). More recently, a 14-day treatment of cynomolgus monkeys with 0.3 mg/kg/day of the PPARα agonist LY570977 L-lysine revealed a 2-fold increase in apoAV plasma concentration and a 50% reduction in plasma TG levels (64). These findings demonstrate that pharmacological activation of PPARα can lead to increase in plasma apoAV levels, thereby shedding new light on the molecular mechanisms which may underlie the reduction in plasma TG levels induced by PPARα agonists. It can therefore be proposed that apoAV contributes to the hypotriglyceridemic effect of PPARα agonists by enhancing LPL activity upon PPARα activation. On the other hand, Dorfmeister et al (65) have recently demonstrated that *APOA5* mRNA levels are not increased by PPARα and PPARγ agonists in rat hepatocytes, an observation that may be explained by the lack of a functional PPAR response element in the *APOA5* promoter in this species.

Recently, Lai et al. (66) compared the effects of fenofibrate treatment on *APOA5* c.56G (W19) carriers and non-carriers. As previously mentioned, *APOA5* *c.*56G carriers display higher TG levels than non-carriers before treatment; such TG levels are positively associated with apoAV concentrations. Interestingly, fenofibrate treatment enhanced both the reduction in TG and the elevation in HDL-C concentrations in the fasting state in 56G carriers versus non-carriers. In addition, differences in plasma TG and HDL-C levels observed during the postprandial response to a fat load between 56C>G genotypes before treatment disappeared after fenofibrate intervention. The greater response of 56G carriers to fenofibrate could not however be explained by fenofibrate-induced *APOA5* mRNA expression. Indeed, Lai et al (66) hypothesize that, by an unknown mechanism, fenofibrate may enhance TG/cholesterol exchange between VLDL and HDL, thereby leading to decrease in the TG load carried by

VLDL. The authors therefore suggested that under conditions of fenofibrate-mediated reduction in TG levels, both the 19W and 19S apoAV variants might exert similar activities. These findings provide potentially new and interesting insight into the mechanism of action of fibrates.

The nuclear receptor FXR is equally implicated in TG metabolism. Even though FXR has been primarily identified as a bile acid-activated nuclear receptor involved in bile acid metabolism, it is equally a key regulator of plasma TG homeostasis. In mice, pharmacological activation of FXR by the synthetic FXR activator GW4064 or one of its natural ligands, cholic acid, led to a 50% reduction in plasma TG levels (67). We reported that human *APOA5* promoter activity is increased by FXR when activated by CDCA or GW4064 (18). Site-directed mutagenesis and gel-shift analysis demonstrated that the *APOA5* promoter responds to bile acids and FXR via a novel element consisting of an inverted repeat separated by 8 nt (IR8) (Figure 2). Furthermore, the *APOA5* IR8 element could confer responsiveness to heterologous promoters. However, no significant induction of *APOA5* mRNA levels was observed by either CDCA or GW4064 in human hepatocytes. This paradox might be explained by the effect of other response elements which were not present in the 5' flanking region of the *APOA5* gene (-2455 to +18) selected for the study, or alternatively by post-transcriptional mechanisms. Nevertheless, Stedman et al. (68) have recently shown that bile duct ligation in mice, which raises endogenous bile acid levels, equally increases *APOA5* mRNA levels in wild-type mice, whereas it decreases apoAV expression 6-fold in FXR KO mice. Moreover, FXR activation by elevated bile acid levels promotes liver regeneration (69). Given the appreciable up-regulation of *APOA5* gene expression after partial hepatectomy (2), study of the potential effect of bile acid signaling on apoAV expression during liver regeneration would be of considerable interest.

HNF-4α is a nuclear receptor which controls the expression of *APOA1*, *APOC3* and *APOA4* mainly through a common enhancer located 590 to 790 nucleotides upstream of the *APOC3* gene (70). Similarly, HNF-4 appears to regulate human *APOA5* expression through the DR1 and the IR8 elements present in the gene promoter (71). Inhibition of HNF-4α expression by small interfering RNA resulted in down-regulation of apoAV (71). In addition, the peroxisome proliferator-activated receptor-γ coactivator-1α is capable of stimulating HNF-4α-dependent transactivation of the *APOA5* promoter. Furthermore, both AMP-activated protein kinase and the MAPK signaling pathway, which are well known regulators of HNF-4 protein levels, also modulate human *APOA5* expression. These findings support a major role

of HNF-4 in the regulation of the expression of the *APOA1/C3/A4/A5* gene cluster. In addition to HNF-4, an additional nuclear receptor, RORα, can regulate *APOA5* gene expression through the DR1 motif (72, 73) (Figure2). Such observations suggest a new mechanism by which RORα may be implicated in the regulation of apolipoprotein gene expression and as a consequence, lipid metabolism.

The expression level of apoAV is upregulated by hypotriglyceridemic pathways, but it is also repressed by lipogenic pathways. Indeed, Jakel et al (74) demonstrated that a synthetic LXR agonist, T0901317, decreased *APOA5* mRNA levels in HepG2 and HuH7 cells. In addition, promoter analysis showed that *APOA5* promoter activity is directly regulated by SREBP1-c, a target of LXR, but not by LXR itself. SREBP-1c is a basic-helix-loop-helix-leucine zipper transcription factor involved in TG metabolism that activates lipogenic enzymes such as fatty acid synthase, SCD-1, and acyl-CoA synthase in the liver (74-76). Jakel et al. (74) identified two E-box response elements for SREBP at +10/+15 and at −76/−81 in the *APOA5* gene (Figure 2). These authors showed that SREBP-1c represses apoAV transcription through binding to the +10 E-box of the human *APOA5* gene sequence. When treated with T0901317, *APOA5* transgenic mice displayed an increase in TG levels and a reduction in *APOA5* mRNA concentrations. These findings allowed the authors to formulate the hypothesis that repression of apoAV expression by SREBP-1c could contribute to the hypertriglyceridemic effect of SREBP-1c activation. Furthermore, as SREBP-1c activation is modulated by nutritional status, then repression of apoAV expression by SREBP-1c could provide a link between apoAV level and nutritional state.

Insulin, another factor implicated in the response to nutritional status and a stimulus of lipogenesis, has also been reported to repress apoAV expression. Nowack et al (77) showed that insulin reduced both *APOA5* mRNA levels and promoter activity in human primary hepatocytes and HepG2 cells. Moreover, these authors identified a molecular mechanism involving the upstream stimulatory factors 1 and 2 (USF1/USF2), which bind to an E-box located −76 nt in the *APOA5* promoter. Insulin action subsequently leads to the activation of PI3K, which in turn phosphorylates USF1/USF2 and reduces binding to the apoAV E-box; this concerted mechanism leads to a decrease in apoAV transcription levels (Figure 2). The implication of USF1/USF2 in *APOA5* gene expression is particularly interesting as USF corresponds to the gene on human chromosome 1q21-23 that is associated with familial combined hyperlipidemia and especially with elevated TG levels in men (78, 79). Finally, insulin infusion in human subjects led to reduction in circulating levels of apoAV protein (77). Thus, investigation of the regulation of *APOA5* gene expression by insulin could help to

understand the mechanistic basis of the elevated apoAV levels in patients with Type 2 diabetes. Indeed, in the context of hepatic insulin resistance, the inhibition of *APOA5* expression by insulin may be altered, resulting in elevation of apoAV levels.

Thyroid hormones (thyroxine,T4; tri-iodothyronine, T3) are intimately involved in the metabolism of triglyceride-rich lipoproteins (80-83). Elevation of plasma TG concentration is associated with hypothyroidism in obese patients, who are characterized by attenuated rates of VLDL-TG clearance relative to those in obese euthyroid subjects (84). Such elevation in TG levels has been attributed to either low LPL (85) or to low hepatic lipase activities (86, 87) . The molecular mechanisms involved in the regulation of VLDL clearance by T4 remain to be defined however. Classically, T3 exerts its biological actions through binding to the nuclear thyroid hormone receptors α and β (TRα and TRβ). Recently, we have demonstrated that T3 treatment of human primary hepatocytes increases both *APOA5* mRNA and protein levels (88). Furthermore, T3 regulates apoAV expression at the transcriptional level through the binding of TR to a DR4 element located at −113 nt of the *APOA5* gene (Figure 2). In addition, we found that USF cooperates with TR, resulting in synergistic activation of the apoAV promoter in a ligand-dependent manner via an adjacent E-box motif. On the other hand, hypothyroid rats displayed low circulating levels of apoAV protein relative to controls, while administration of T3 restored apoAV protein abundance. In addition, one week of treatment with a single daily dose of the thyromimetic CGS-23425 increased hepatic levels of apoAV in fat-fed rats. Finally, this TRβ selective agonist induced a dramatic decrease in both VLDL-TG and total TG levels. Considered together, these results provide new insight into the low rates of VLDL-TG clearance and low LPL activity observed in hypothyroid patients. Furthermore, they emphasize the physiological role of TR in the regulation of genes involved in TG metabolism and confirm that TRβ may represent a potential pharmacological target for the treatment of hypertriglyceridemia.

In conclusion, the finding that apoAV is up-regulated by agonists of PPARα, FXR and TR, which together constitute three distinct classes of hypotriglyceridemic ligands (Figure 1B), support the contention that apoAV plays a central role in the metabolism of VLDL and TRL.

Conclusion

In vitro structure-function studies, in vivo evaluation of apoAV function in genetically-modified mouse models, together with characterization of clinical cases of patients carrying dysfunctional mutations, strongly support the contention that apoAV is a critical player in the

regulation of plasma TG levels and in turn, of intravascular TRL metabolism. Nevertheless, further studies are needed not only to evaluate the role of apoAV in chylomicron and VLDL catabolism during the post-prandial phase, but also to assess the physiological relevance of apoAV transfer from HDL to VLDL postprandially. The positive association between plasma TG and apoAV levels observed in human subjects suggests however that there is a complex relationship between apoAV and TRL metabolism. Such a positive correlation was observed in dyslipidemic and in type 2 diabetic patients; by contrast, data in healthy subjects are contradictory. A positive correlation between plasma apoAV and TG concentrations has equally been reported recently in mice genetically deficient in apoAV, but which overexpress the human apoAV transgene. These findings highlight the possibility that *APOA5* gene expression may respond to its metabolic environment, with potential upregulation in response to elevated TG levels. Upregulation of *APOA5* expression by nuclear receptors such as PPARs, FXR, TR or alternatively its repression by lipogenic pathways mediated by insulin and SREBP1-C, suggests that complex transcriptional or translational regulations may occur under metabolic conditions in which elevated TG levels arise either independently of insulin resistance, or in association with it. Future studies should therefore focus on furthering our understanding as to whether elevation in circulating apoAV levels is a cause or a consequence of hypertriglyceridemia and/or of an insulin resistant state. Finally, the action of apoAV in the lipolysis of TRL *in vivo* under normal physiological conditions remains indeterminate. The answers to these topical questions will ultimately enable us to evaluate the potential of apoAV and apoAV-like peptides as therapeutic agents in hypertriglyceridemic states of either genetic and/or lifestyle and nutritional origin.

Future perspective

APOA5 gene expression is up-regulated by hypotriglyceridemic ligands, and even if incompletely characterized, the potential role of apoAV in TG metabolism appears to be unique. In mice, overexpression of apoCII, an obligatory co-activator of LPL, inhibits LPL activity as VLDL particles are less accessible to hydrolysis. In contrast, overexpression of apoAV in mice increases LPL activity. Several experimental findings tend to support the contention that apoAV does not modify post-heparin LPL activity and that its role in LPL-mediated TRL hydrolysis is more complex, involving other factors. A better understanding of the implication of apoAV in TG hydrolysis is clearly a priority in future investigations of lipid and lipoprotein metabolism.

ApoAV is an apolipoprotein of low abundance (~ 150μg/l) and its secretion is less efficient as compared to other apolipoproteins. ApoAV may therefore possess intra-cellular functions. The identification of an association of apoAV with lipid droplets in hepatic cells represents such a possibility. In addition, the association of apoAV with heparin and proteoglycans suggests that hepatic apoAV is secreted and then directly associates with the extra-cellular surface of the hepatocyte plasma membrane. Indeed, earlier studies showing an impairment of apoAV secretion involved analyses of the whole cell lysate, thereby precluding determination of intra-cellular and membrane-associated apoAV. Therefore, as suggested above, apoAV could be associated to LRP and SorLA and/or may interact with other factors implicated in lipoprotein metabolism such as hepatic lipase, which is known to be cell-membrane associated. Future studies that focus on the functional domains of apoAV implicated in its intra-cellular and cell-associated actions remain crucial to the overall understanding of the role of apoAV in lipid and lipoprotein metabolism. Indeed, such knowledge may open new horizons for the therapeutic normalization of metabolic diseases involving moderate to marked hypertriglyceridemia, and which are associated with accelerated atherosclerosis, such as the mixed hyperlipidemias of type 2 diabetes and the metabolic syndrome.

Acknowledgments

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Figure Legends

Figure 1: Mechanism of action of apoAV on TG levels. (1A) TG-rich lipoproteins are hydrolyzed by LPL under the influence of specific apolipoproteins. ApoCII is an obligatory cofactor and apoCIII inhibits LPL activity. Recently, apoAV has been shown to positively regulate HSPG-bound LPL either by direct interaction or through interaction with proteoglycans. In addition, apoAV, by interacting with the LDL receptor-related protein (LRP1) and the mosaic type-1 receptor (SorLA), facilitates TRL remnant uptake.

In figure 1B, pharmacological regulation of apoAV expression is represented and directly linked to LPL activity. Fibrates inversely regulate apoAV and apoCIII expression levels and lead to enhanced LPL activity.

Figure 2: Schematic representation of the human *APOA5* promoter region and transcription factor binding sites. Homodimers and heterodimers of transcription factors bind to these motifs to modulate human *APOA5* gene expression.
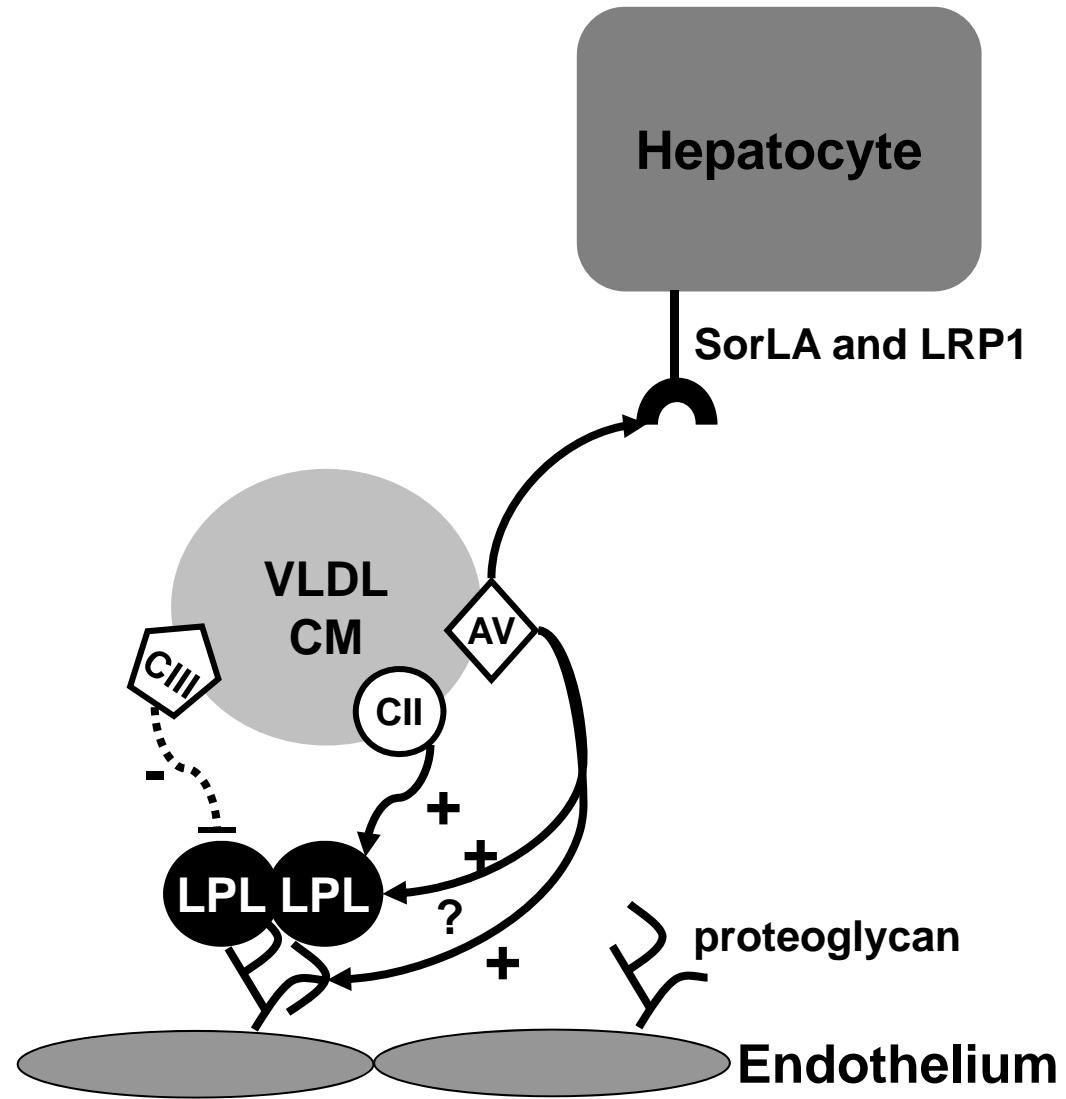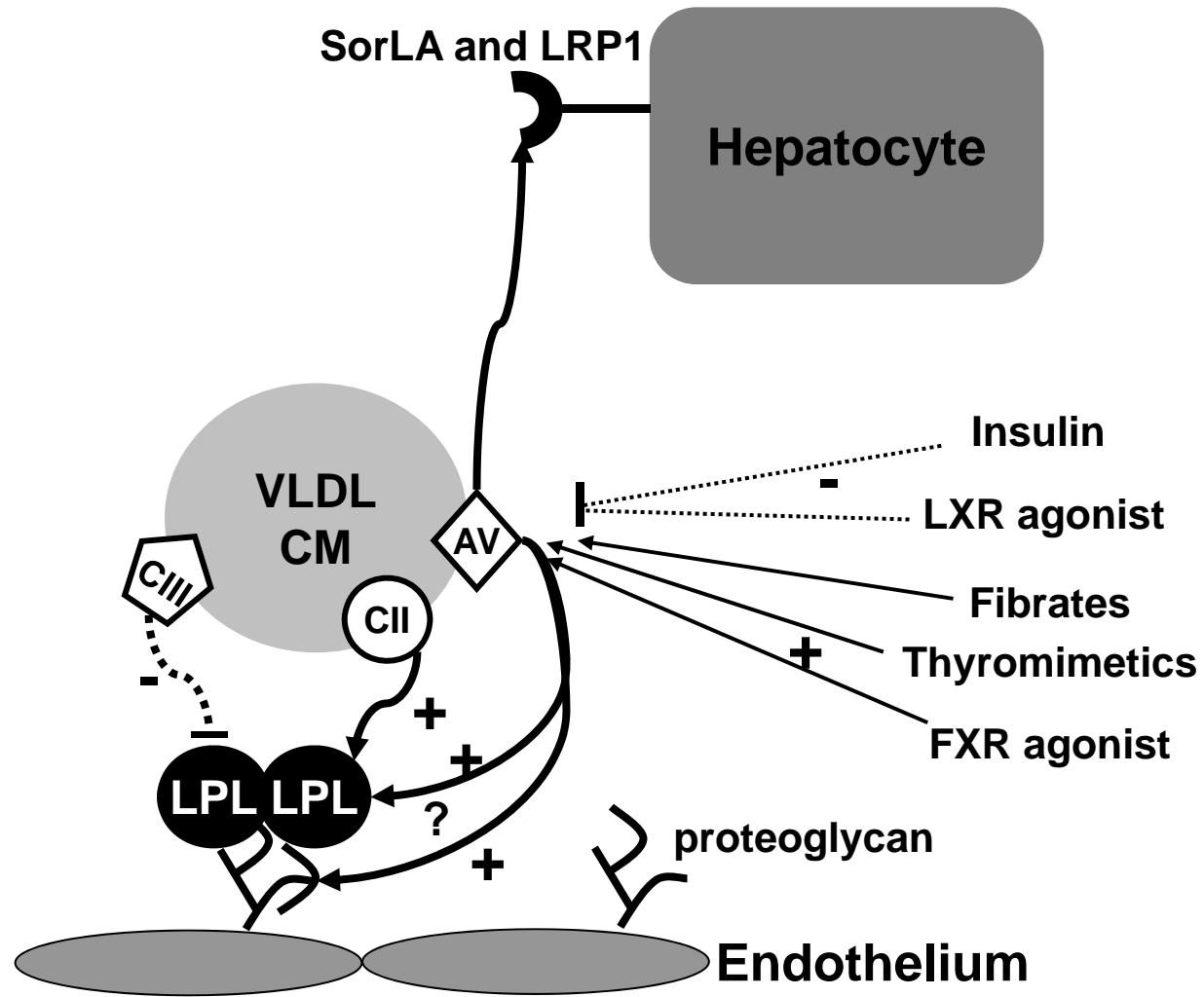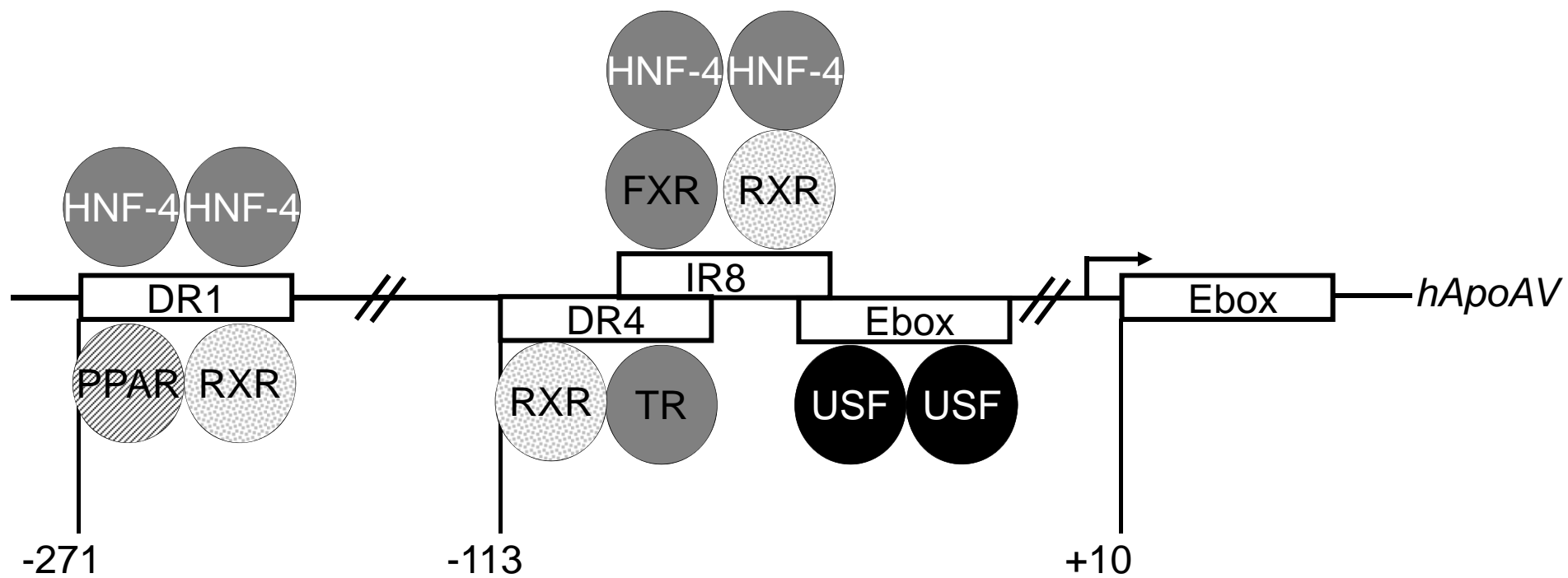
Figure 1A

Figure 1B

Figure 2