Effect of Low-Density Lipoproteins on Apolipoprotein AI Kinetics in Heterozygous Familial Hypercholesterolemia

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In patients with heterozygous familial hypercholesterolemia (FH), both synthetic and clearance rates of high-density lipoproteins (HDL) are increased compared with control subjects. According to in vitro data on hepatocytes, the expanded pool size of low-density lipoproteins (LDL) in FH could partly explain the enhanced HDL production. Therefore, we have tested the hypothesis that a reduction of LDL pool size, achieved by LDL-apheresis, is associated with a downregulation of HDL synthesis. We studied the kinetics of HDL by infusing [5,5,5-$^2H_3$]-leucine in 7 heterozygous FH patients before and after 3 biweekly LDL-apheresis using dextran sulfate columns. Both plasma and LDL-cholesterol levels were decreased after LDL-apheresis (169 ± 35 *v* 422 ± 27 mg/dL, *P* < .05, and 85 ± 19 *v* 327 ± 52 mg/dL, *P* < .05, respectively). Plasma triglyceride level was unaffected (162 ± 43 *v* 176 ± 35 mg/dL, not significant [NS]) and HDL composition remained stable (HDL-cholesterol 29 ± 6 *v* 37 ± 7 mg/dL, NS, and HDL-triglyceride 20 ± 6 *v* 19 ± 8 mg/dL, NS). Plasma apolipoprotein AI (apo AI) was also similar (122 ± 20 *v* 115 ± 18 mg/dL, NS). Mean HDL-apo AI fractional catabolic rate (FCR) was slightly higher (0.41 ± 0.07 *v* 0.36 ± 0.14 pool/d, NS), and absolute production rate (APR) was increased (22.1 ± 5.7 *v* 18.0 ± 5.7 mg/kg/d, *P* < .05) after LDL-apheresis. These human kinetic data suggest that LDL do not play a major role on HDL production in heterozygous FH patients.

HETEROZYGOUS FAMILIAL hypercholesterolemia (FH) is related to a genetic defect on the low-density lipoprotein (LDL)-receptor and is characterized by metabolic disturbances in the apolipoprotein (apo) B100 pathway.[1,2] We recently performed a kinetic study of high-density lipoprotein (HDL)-apo AI metabolic disturbances in 7 patients with heterozygous FH compared with 7 healthy subjects. We reported that the fractional catabolic rate (FCR) of HDL was 64% increased in FH,[3] as also previously observed in homozygous FH.[4] The enhanced FCR of HDL-apo AI was linked to changes in HDL composition (particularly a decrease of HDL-cholesterol level and an increase of both HDL-triglyceride and -apo E concentrations). However, there was no data linking apo AI gene expression or HDL production to hepatic cholesterol content and/or SREBP activity in humans. Only 1 study has shown that SREBP-1a induced HDL receptor scavenger receptor SR-Bl gene expression in rats. In our first study, we showed that unlike homozygous, patients with heterozygous FH adapted their HDL production (with a 61% increase) and thus maintained plasma apo AI level. This increased HDL-apo AI production in heterozygous FH was consistent with an in vitro assay performed in a human hepatocellular carcinoma model.[5] Monge et al[5] observed that LDL uptake by Hep G2 cells led to increased levels of apo AI mRNA in these cells. They concluded that LDL may play an important role in apo AI gene expression and regulation. To provide further insights in humans to these cellular findings, we have tested the hypothesis that a reduction of LDL pool size, achieved by LDL-apheresis, may be responsible for a decrease of HDL production in heterozygous FH. Therefore, we have explored in vivo HDL kinetics in 7 heterozygous FH before and after LDL-apheresis treatment by adsorption to dextran sulfate, using endogenous labelling of apo AI with stable isotope tracers.

MATERIALS AND METHODS

Subjects

Two kinetic studies of apo AI metabolism were performed in 7 heterozygous FH patients before and after 3 LDL apheresis. Some relevant clinical characteristics are shown in Table 1. None of the study subjects was taking any medication that could affect carbohydrates or lipids for at least 2 weeks before the first kinetic assay and were off these treatments through the study. FH patients had no diabetes mellitus, proteinuria, or hypothyroidism and were not regular cigarette smokers or alcohol consumers. FH was diagnosed according to the presence of an heterozygous mutation on the LDL-receptor gene[6] and also from characteristic clinical signs and an analysis of lipid parameters in family members (total plasma cholesterol >350 mg/dL, LDL-cholesterol >230 mg/dL, plasma triglycerides slightly increased and <250 mg/dL) showing a dominantly inherited hypercholesterolemia in the family from each proband (3 or more affected relatives including at least 1 first-degree relative). Patients were never treated with probucol. Study subjects were instructed by a dietician to eat a weight-maintenance diet composed of 50% of the usual daily caloric intake as carbohydrate, 35% as fat and 15% as protein, for at least 1 week before the study. The experimental protocol was approved by the Ethical Committee of Nantes University Hospital, and informed consent was obtained before the study was started.

Experimental Protocol

Infusion of stable isotope tracer. The first kinetic study was performed before the first apheresis procedure was carried out. The kinetic protocol has been described in a previous study.[3] Briefly, the endogenous labeling of apo AI was performed by administration of L-[5,5,5-$^2H_3$]-leucine (99.8 Atom% $^2H_3$; Cambridge Isotope Laboratories, Andover, MA) dissolved in a 0.9% saline solution and tested for sterility and absence of pyrogens before the study. All subjects fasted overnight

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Table 1. Clinical and Biological Characteristics of Study Subjects

Subject	Sex	Age (yr)	BMI (kg/m^2)	LDL-Receptor Mutation*	Apo E Isoform	Clinical Signs
FH 1	F	64	19.2	Phe105Ser, exon 4	E3/E3	GX, TX
FH 2	M	55	24.9	Frameshift, exon 4	E3/E3	CAD, GX, TX
FH 3	F	50	31.2	Trp66Gly, exon 3	E3/E3	CAD, GX, TX
FH 4	M	28	27.9	ND†	E3/E3	AG, GX, MI, TX
FH 5	F	39	32.9	−‡	E3/E3	AG, CAD, TX
FH 6	M	57	24.9	Ala370Thr, exon 8 Glu702STOP, exon 15§	E3/E3	CABG, GX, PA, TX
FH 7	M	41	25.2	Frameshift, exon 4	E3/E3	CAD, TX
FH mean	4/3	47.7	26.6			
(SD)	M/F	(12.4)	(4.6)			

Abbreviations: AG, angioplasty (PCTA, percutaneous coronary angioplasty); BMI, body mass index; CABG, coronary artery bypass grafting; CAD, coronary artery disease; GX, gerontoxon (corneal arcus); MI, myocardial infarction; PA, peripheral atherosclerosis; TX, tendon xanthoma; ND, not determined.

* All mutations were found heterozygous on the LDL-receptor gene.

† FH4 was dead. ‡ FH 5 was negative for LDL receptor mutation (candidate for a third gene mutation. § Both mutations supposedly on the same chromosome. FH patients (FH 4 not determined) were negative for the Arg3500Gln mutation of apo B100.

for 12 hours before the study and remained fasting during the entire protocol. Each subject received intravenously a prime of 10 mol · kg^{-1} of tracer, immediately followed by a constant tracer infusion (10 mol · kg^{-1} · h^{-1}) for 14 hours. Venous blood samples were withdrawn in EDTA tubes (Venoject, Paris, France) at baseline, every 15 minutes during the first hour, every 30 minutes during the next 2 hours, and then hourly until the end of the study. Plasma was immediately separated by centrifugation for 30 minutes at 4°C; sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montluçon, France), a protease inhibitor, were added to blood samples at a final concentration of 1.5 mmol/L and 0.5 mmol/L, respectively.

Design of LDL-apheresis. Study subjects underwent 3 LDL-apheresis sessions over 4 weeks. We used Kaneka MA-01 monitor (Kaneka Pharma Europe N.V., Brussels, Belgium) with a plasma separator (Sulflux FS-05; Kanegafuchi Chemical Industry, Osaka, Japan) and 2 dextran sulfate columns (Liposorber LA-15; Kanegafuchi Chemical Industry). As previously described,[7] the blood was continuously withdrawn from an antecubital arteriovenous fistula and anticoagulated with a bolus of heparin 0.8 mg/kg. Blood flux was maintained between 125 and 150 mL/min. Plasma flow rate was set at 35% the flow rate of the blood. The plasma was separated with a plasma filter and then passed through 1 of the dextran sulfate columns (DSC), which retains LDL and, to a lesser degree, the other subclasses of apo B100-containing particles (very–low-density lipoprotein [VLDL] and intermediate-density lipoprotein [IDL]).[8,9] The duration of a session was approximately 3 hours, while 3.8 ± 0.9 L plasma were treated. HDL, apo AI, and other serum proteins do not adsorb to DSC, and consequently their plasma concentration remain unchanged.[10,11] The second kinetic assay was performed the day after the last apheresis procedure.

Analytical Procedures

Measurement, isolation, and preparation of apo. VLDL ($d < 1.006$ g/mL), were isolated from 3 mL of plasma by a sequential ultracentrifugation using an angle rotor at 40,000 rpm · min^{-1} for 24 hours at 10°C (Himac CP70, Hitachi, Japan). HDL2 ($1.063 < d < 1.125$ g/mL) and HDL3 ($1.125 < d < 1.210$ g/mL) were than isolated by a density gradient ultracentrifugation modified method,[12] using a swinging bucket rotor at 40,000 rpm · min^{-1} for 24 hours at 10°C (Centrikon T 2060, Kontron Instruments, Japan). Plasma and HDL cholesterol and triglycerides levels were measured using enzymatic kits (Boehringer, Mannheim GmbH, Germany). Apo AI concentration was measured in plasma and HDL fractions by immunonephelometry (Behring, Rueil Malmaison, France). The apo AI pool size (mg · kg^{-1}) was calculated by multiplying the mean plasma apo AI concentration by 0.041 to 0.047 (L · kg^{-1}, assuming a plasma volume of 4.1% to 4.7% of body weight according to age, gender, and body weight of the patient.[13] The plasma apo AI concentration was taken to be the HDL apo AI concentration, with the assumption that > 90% of plasma apo AI resides in the HDL fraction.[14]

HDL-apo AI and VLDL-apo B100 were concentrated[15] and isolated from other apolipoproteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-5-10% discontinuous gradient. Apolipoproteins were identified by comparing migration distances with known molecular weight standards (Cross-linked phosphorylase b markers; Sigma, St Louis, MO and electrophoresis calibration kit, Pharmacia LKB, Biotechnology, Piscataway, NJ). Apolipoprotein bands were excised from polyacrylamide gels and dried in vacuum (RC 10-10 Jouan, Saint Herblain, France). The desiccated gel slices were hydrolyzed with 1 mL of 4 N HCl (Sigma, St Quentin Fallavier, France) at 110°C for 24 hours. Hydrolysates were then evaporated to dryness and the amino acids purified by cation exchange chromatography using a Temex 50W-X8 resin (Bio-Rad, Richmond, CA). Plasma amino acids were esterified with propanol/acetyl chloride and further derivatized using heptafluorobutyric anhydride (Fluka Chemie AG, Buchs, Switzerland) before analysis.

Determination of tracer-to-tracee ratios. Chromatographic separations were performed on a 30 m × 0.25 mm i.d. DB-5 capillary column (J&W Scientific, Rancho Cordova, CA). The column temperature program was as follows: initial temperature was held at 80°C, then increased at 10°C · min^{-1} to a final temperature of 180°C. Electron-impact gas chromatography-mass spectrometry was performed on a 5890 gas chromatograph connected with a 5971A quadrupole mass spectrometer (Hewlett Packard, Palo Alto, CA). The isotopic ratio was determined by selected ion-monitoring at *m/z* of 282 and 285. Calculations of apo AI kinetic parameters were based on the tracer-to-tracee mass ratio.[16]

Modeling

Kinetic analysis of tracer-to-tracee ratios was achieved by a computer software for simulation, analysis, and modeling (SAAM II v 1.0.1, Resource Facility for Kinetic Analysis, Department of Bioengineering, SAAM Institute, Seattle, WA). For HDL modeling, we used a 1-compartment model, as previously described.[3] VLDL apo B100 and HDL apo AI data were kinetically analyzed using a monoexponential function[16]: $A(t) = Ap[1-\exp(-k(t-d))]$, where A(t) is the tracer-to-tracee

ratio at time t, Ap, the tracer-to-tracee ratio at the plateau of the VLDL apo B100 curve, d, the delay between the beginning of the experiment and the appearance of tracer in the apolipoprotein, and k the fractional production rate (FPR) of the apolipoprotein. For the estimation of apo AI synthesis, we used the plateau of VLDL-apo B100 tracer-to-tracee ratio as precursor pool enrichment. It was assumed that this plateau value, obtained using a monoexpontential function, corresponded to the tracer-to-tracee ratio of the leucine precursor pool. This estimation is made on the assumption that apo B100 and the majority of apo AI are synthesized by the liver.[17] We estimated the FPR, ie, the proportion of apo AI entering the pool per unit time (d^{-1}) and the absolute production rate (APR), ie, the amount of apo AI entering the pool per unit time ($mg \cdot kg^{-1} \cdot d^{-1}$). Under our steady state conditions, FPR equals FCR. APR was the product of FCR multiplied by apo AI mass in HDL.

Statistical Analysis

We used StatView statistical Software package (BrainPower, Calabalas, CA) to design the statistical analysis. Data were reported as mean ± SD unless otherwise specified. The nonparametric Wilcoxon signed-rank paired test was used to determine significant differences before and after LDL-apheresis. A 2-tailed probability value of .05 was accepted as statistically significant.

RESULTS

Apolipoprotein and Lipid Concentrations

Individual data for plasma and HDL composition are shown in Table 2. Patients with FH showed characteristically lowered fasting plasma total cholesterol and LDL-cholesterol concentrations after LDL-apheresis (−60% and −74%, respectively, $P < .05$). The treatment did not significantly modify plasma triglycerides, HDL-triglyceride concentrations (not significant [NS]), or HDL-apo E levels (NS). Although a 19% decrease of HDL-cholesterol after apheresis was observed, the difference was not significant. Plasma apo AI levels remained unchanged after extracorporeal removal of LDL particles (NS). Furthermore, plasma apo AI concentrations were constant over the infusion period (Fig 1A). LDL-to-HDL-cholesterol ratio was significantly decreased (−69%, $P < .05$).

Kinetic Data

Enrichment in plasma-free leucine reached a plateau value after 30 minutes and remained stable to the end of the study (data not shown). The tracer-to-tracee ratio curves in VLDL and HDL are shown in Fig 1. A plateau of tracer-to-tracee ratio was reached after 10 hours for VLDL-apo B100 (Fig 1B), but not for HDL-apo AI (Fig 1C), meaning a slow rate of synthesis for this apolipoprotein. Kinetic parameters of apo AI are shown in Table 3. Mean HDL-apo AI catabolic and synthetic rates were increased after the treatment (+14%, NS, and +23%, $P < .05$, respectively).

Table 2. Mean Plasma and Lipoprotein Levels of Lipids and Apolipoproteins Before (base) and After (post) LDL-Apheresis in Heterozygous FH Patients

	Base	Post
Plasma total cholesterol	422 ± 27	169 ± 35*
LDL-cholesterol	327 ± 52	85 ± 19*
HDL-cholesterol	37 ± 7	30 ± 5
Plasma triglycerides	176 ± 35	162 ± 43
HDL-triglycerides	19 ± 8	20 ± 6
Plasma apo AI	115 ± 18	122 ± 20
LDL-C/HDL-C	9.0 ± 2.0	2.8 ± 0.4*
HDL-apo E	5.3 ± 0.8	6.0 ± 2.0

NOTE. Values represent the mean ± SD, mg/dL.
* $P < .05$ *v* post.

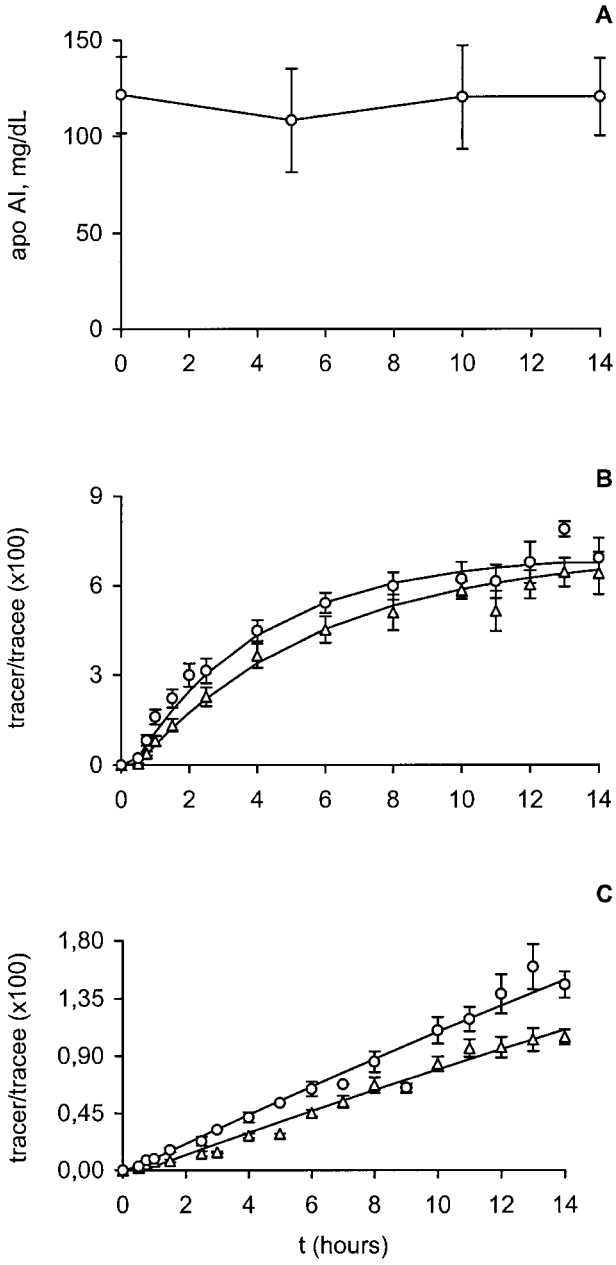

Fig 1. Plasma apo AI concentrations after LDL-apheresis (A) and mean tracer-to-tracee ratios for apo B100-VLDL (B) and for HDL-apo AI (C) in heterozygous FH patients before (△) and after (○) LDL-apheresis. Fits (lines) were calculated using monocompartmental analysis during a primed constant infusion of [$^2H_3$]-leucine. Data are shown as mean ± SEM.

DISCUSSION

This study is the first to document the effect of LDL apheresis on HDL-apo AI kinetics in humans. It was designed to test

Table 3. Apo AI Pool Size and Kinetic Parameters Before (base) and After (post) LDL-Apheresis in Heterozygous FH Patients

Subject	Apo AI Pool Size (mg/kg)	FCR HDL-apo AI (pool/d)	APR HDL-apo AI (mg/kg/d)
FH-1			
Base	52.7	0.32	17.0
Post	49.5	0.38	18.7
FH-2			
Base	43.4	0.34	14.9
Post	44.7	0.35	15.9
FH-3			
Base	39.1	0.24	9.5
Post	46.2	0.34	15.9
FH-4			
Base	51.7	0.59	30.6
Post	57.3	0.55	31.3
FH 5			
Base	54.8	0.34	18.9
Post	49.4	0.45	22.3
FH-6			
Base	61.2	0.16	10.1
Post	69.7	0.37	25.6
FH-7			
Base	50.5	0.49	24.9
Post	55.5	0.45	25.0
Mean			
Base	50.5	0.36	18.0
SD	7.3	0.14	7.7
Mean			
Post	53.2	0.41	22.1*
SD	8.6	0.07	5.7
Mean			
controls	53.5	0.22†	11.2†
SD	10.9	0.05	2.3

Abbreviations: FCR, fractional catabolic rate; APR, absolute production rate.

* $P < .05$ *v* post.

† $P < .05$ *v* base.

in vivo the hypothesis that a reduction of LDL pool size, achieved by extracorporeal cholesterol removal, is associated with a downregulation of HDL synthetic rate in heterozygous FH. We performed a kinetic study of HDL metabolism before and the day after a sequence of 3 biweekly apheresis by dextran sulfate adsorption. As expected, both plasma and LDL-cholesterol levels were lowered.[11,18-20] Plasma triglyceride concentration was unaffected. Plasma apo AI level and HDL composition remained stable before and after selective LDL-apheresis. Mean HDL-apo AI FCR was slightly higher after LDL-apheresis, and mean HDL-apo AI production rate was also increased. This increase was significant mainly due to 2 patients, even if other patients also showed a trend to increase. Nevertheless, a reduced LDL pool size was not followed by a downregulation of HDL production rate, and we have rejected our hypothesis that LDL could enhance HDL production in vivo.

HDL kinetics were evaluated using a 1-compartment model, allowing to study their overall catabolism.[3] In fact, our experimental enrichment data could not be adjusted on a 2-pool model because our study design with a constant infusion of tracer and our period of sampling do not allow the characterization of tracer exchanges with a second pool. As used in most previous apo AI kinetic studies,[21,22] we have taken VLDL-apo B100 enrichment at the plateau for apo AI leucine precursor pool, assuming that apo AI was mainly synthesized by the liver.[17] Unlike the LDL metabolic pathway, we postulated a steady state for HDL, because no significant differences were observed in plasma apo AI levels at 4 sampling times.

The effect of LDL-apheresis on plasma apo AI and HDL-cholesterol levels was similar to that observed in previous studies dealing with several cases of FH.[7,10,23,24] We did not report any significant difference on HDL composition by apheresis. Because we have previously shown a significant relationship between HDL-triglyceride level and HDL clearance,[25] the nonsignificant increase of HDL-apo AI FCR ($P = .13$) we now report is in agreement with the lack of clear-cut changes in HDL composition. In addition, the slight decrease of HDL-cholesterol level concurs with the moderate increase of HDL-apo AI clearance rate. However, because neither HDL-triglyceride nor HDL-apo E levels were changed after apheresis, we can hypothesize that HDL clearance is probably enhanced via the SR-BI receptor, a specific HDL removal pathway. This lack of clear-cut effect of apheresis on HDL composition or plasma apo AI level was also in good agreement with studies dealing with the effect of statin therapy on HDL concentration.[26,27]

Furthermore, several factors may contribute to an increase of HDL-apo AI production that we have observed.[28] The use of a bolus of heparin as anticoagulant during apheretic procedures causes an increased lipolysis of triglyceride-rich lipoparticles, and surface material released become substrate of HDL production. Furthermore, LDL-to-HDL ratio is greatly increased in hypercholesterolemia. Therefore, LDL-apheresis may stimulate physiologic mechanisms to restore LDL-to-HDL ratios to normal values. Particularly, the decrease of LDLC-to-HDL-C was in good agreement with the increase of HDL apo AI production rate. Finally, patients undergoing frequent LDL apheresis are subject to periodic removal of about 5% of total HDL-apolipoproteins, which might be sufficient to stimulate HDL-apo AI synthesis.

This current study provides new insights in vivo in the short-term effect of extracorporeal removal of LDL on HDL-apo AI metabolism. In patients with heterozygous FH, the defect in hepatic LDL uptake lead to a net increase of peripheral tissue cholesterol level. In response to this excessive storage, we previously reported that in vivo both production and clearance of HDL-apo AI, a surrogate of the reverse transport of cholesterol pathway, were increased compared with control subjects.[3] We hypothesized that increased LDL pool size induced the activation of HDL metabolic pathway. Such an hypothesis was built from in vitro data on a human hepatocellular carcinoma cell line.[5] Indeed, Monge et al[5] incubated Hep G2 cells with LDL and observed a significant increase of the expression of apo AI mRNA within these cells. They concluded that LDL may be of major importance in apo AI gene expression and regulation. This may involve a feedback pathway, depending on the LDL receptor, which regulates the hepatic expression of both apo AI and apo B100.[4] However, in this study, we observed that a reduced LDL pool size and a 46% increased FCR of LDL-apo B100 after LDL apheresis (0.35 ± 0.09 *v* 0.24 ± 0.08 pool $\cdot$ d^{-1}, $P = .067$, data not shown) do not

interfere in HDL pathway, because both HDL-apo AI clearance and production rates remained increased. This would suggest that LDL-receptor pathway may not play a direct role in the control of HDL-apo AI metabolism in humans. This appears in sharp contrast with in vitro findings[5] or at least may signify that if an LDL loading is associated with an enhanced apo AI production, in vitro[5] as well as in vivo,[3] the reverse relationship remains unverified. Such apparent discrepancy could be partly related to the experimental conditions, such as in vitro incubation time course. In addition, to explain these conflicting results, we suggest that the intracellular uptake of LDL could be still enhanced, despite the reduction of LDL pool size. In fact, extrapolating in vitro and in vivo data on animals models, we previously hypothesized that HDL catabolic rate could be enhanced via the hepatic SR-BI pathway in FH.[3] Because SR-BI display high-affinity binding for LDL,[29] the hepatic uptake of LDL and the expression of apo AI mRNA could be enhanced. Therefore, the SR-BI activity could become substantial in conditions of functional defects in the LDL receptor pathway.[30] Thus, investigations on SR-BI expression could provide insights about the increased production and clearance of HDL in FH.

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