A natural apolipoprotein A-I variant, apoA-I(L141R)$_{Pisa}$, interferes with the formation of α-high density lipoproteins (HDL) but not with the formation of preβ_1-HDL and influences efflux of cholesterol into plasma

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Abstract ApoA-I(L141R)$_{Pisa}$ is a naturally occurring apolipoprotein A-I variant that causes virtual absence of HDL in hemizygotes and hypoalphalipoproteinemia with half-normal levels of HDL-cholesterol in heterozygotes. In this study we analyzed the distribution of HDL subclasses in plasmas of four hemizygotes for this mutation. We also investigated the abilities of these plasmas to esterify cholesterol and to promote cholesterol efflux. Residual apoA-I-containing lipoproteins in plasmas of hemizygotes for apoA-I(L141R)$_{Pisa}$ correspond to preβ_1-LpA-I and small α-LpA-I. Unlike normal preβ_1-LpA-I, preβ_1-LpA-I of apoA-I(L141R)$_{Pisa}$ hemizygotes was not converted into a larger α-migrating particle. Plasmas of apoA-I(L-141R)$_{Pisa}$ hemizygotes were significantly reduced in their activity to esterify cholesterol in either endogenous or exogenous lipoproteins. Cholesterol efflux capacity was significantly lower than that of normal plasma. Efflux of [^{3}H]cholesterol from radiolabeled fibroblasts into apoB-depleted plasma of normal probands was biphasic with fast cholesterol efflux occurring in the first minute. Thereafter, cholesterol efflux was slow and unsaturable. After incubation with radiolabeled fibroblasts, efflux values of [^{3}H]cholesterol into apoB-depleted plasma from normal controls and from apoA-I(L141R)$_{Pisa}$ hemizygotes were indistinguishable at 1 min. Longer incubations with apoB-free plasma from apoA-I(L141R)$_{Pisa}$ hemizygotes did not, however, lead to the unsaturable increase in cholesterol efflux that was observed during incubations with apoB-free plasma of normolipidemic probands. Preβ_1-LpA-I of apoA-I(L141R)$_{Pisa}$ hemizygotes took up significantly less cell-derived [^{3}H]cholesterol than preβ_1-LpA-I of normal donors. We conclude that apoA-I(L141R)$_{Pisa}$ interferes with the formation of lipid-rich α-HDL but not with that of lipid-poor preβ_1-LpA-I. Very low concentrations of α-HDL in plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes combined with reduced LCAT activity cause a decrease of the slow, unspecific, and LCAT-dependent components of cholesterol efflux into plasma.—**Miccoli, R., Y. Zhu, U. Daum, J. Wessling, Y. Huang, R. Navalesi, G. Assmann, and A. von Eckardstein.** A natural apolipoprotein A-I variant, apoA-I(L141R)$_{Pisa}$ interferes with the formation of α-high density lipoproteins (HDL) but not with the formation of preβ_1-HDL and influences efflux of cholesterol into plasma. *J. Lipid Res.* 1997. **38:** 1242–1253.

Supplementary key words apoA-I variants • reverse cholesterol transport • HDL subclasses • familial hypoalphalipoproteinemia • Tangier disease

It is widely assumed that high density lipoproteins (HDL) exert their antiatherogenic role by contributing to the reverse transport of excess cholesterol from peripheral cells to the liver and steroidogenic organs (reviewed in ref. 1). Apolipoprotein (apo) A-I, the predominant protein of HDL, plays a pivotal role in this process. ApoA-I activates the cholesterol esterifying enzyme lecithin:cholesterol acyltransferase (LCAT) and promotes cholesterol efflux from cells (1, 2). The latter process involves several mechanisms. *i)* In the presence of LCAT, lipid-rich HDL lead to a slow and unsaturable

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Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; IEF, isoelectric focusing; LDL, low density lipoprotein; LpA-I, lipoproteins containing apoA-I; VLDL, very low density lipoprotein; 2D-PAGGE, two-dimensional nondenaturing polyacrylamide gradient gel electrophoresis.

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cholesterol efflux from all cell types investigated so far (1, 3–6). Because of their electrophoretic alpha-mobility, these apoA-I-containing particles have been termed α-LpA-I. In normal plasma they represent the bulk of HDL (1, 3, 6, 7). *ii*) However, apoA-I also promotes fast, saturable, and LCAT-independent cholesterol efflux from fibroblasts, macrophages, and smooth muscle cell-derived foam cells, either as a lipid-free protein or as part of lipid-poor HDL (1, 3, 4, 6, 8–10). Because of their electrophoretic prebeta-mobility, such particles have been termed preβ_1-LpA-I (1, 7, 11). *iii*) This fast and specific cholesterol efflux pathway is sensitive to the treatment of cells with proteases (3, 10). Moreover, activation of protein kinase C enhances cholesterol efflux by lipid-free apoA-I while inhibition of protein kinase C inhibits cholesterol efflux (10, 12, 13). For these reasons and because apoA-I binds to cell membrane proteins, it has been hypothesized that apoA-I binds to a signal-transducing cell-surface receptor and that this binding facilitates the translocation of cholesterol from intracellular compartments to the plasma membrane (1, 6, 12, 13).

The structural requirements that allow apoA-I to fulfill its functions are unknown. Eight anti-parallel and amphiphilic alpha-helices constitute the largest proportion of the molecule and had been thought for a long time to mediate lipid binding and LCAT-activating properties of apoA-I (reviewed in ref. 2). More recent studies with recombinant apoA-I variants, however, suggest that the carboxyterminal proportion of apoA-I is of special importance for lipid binding (14, 15) and that a domain located between amino acids 90 and 120 is important for LCAT activation (14, 16). Contradictory data have also been reported on the structural features of apoA-I that are required for cholesterol efflux. Synthetic amphipathic peptides without any sequence homology to apoA-I stimulated cholesterol efflux from fibroblasts and macrophages (9, 17). However, monoclonal antibodies have been described that specifically inhibited cholesterol efflux by plasma, native and reconstituted HDLs, or lipid-free apoA-I (18–21). Epitopes of most antibodies resided between amino acid residues 121 and 186 (18–21). Moreover, our laboratory has previously shown that a naturally occurring apoA-I variant, apoA-I(P165R), forms reconstituted HDL that are impaired in their ability to promote cholesterol efflux from murine adipocytes or macrophages (22).

To gain insight into the structural features of apoA-I that are required for the formation of distinct HDL-subclasses, for the esterification of cholesterol, and for promotion of cholesterol efflux, we investigated the plasmas of four patients who were compound heterozygotes for an apoA-I null allele and a structural apoA-I variant, namely apoA-I(L141R)$_{Pisa}$. The plasmas of these patients were deficient in HDL-cholesterol and contained the variant protein at very low concentrations (23, 24). We used two-dimensional nondenaturing polyacrylamide gradient gel electrophoresis (2D-PAGGE) (7) in combination with anti-apoA-I immunoblotting to analyze the quality of apoA-I-containing particles. We also measured the cholesterol efflux capacity of these plasmas by incubating them with radiolabeled fibroblasts. To differentiate quantitative from qualitative effects of apoA-I on particle formation and cholesterol efflux capacity, we compared the data not only with plasmas of four normolipidemic controls but also with those of three patients with Tangier disease (25) as in both conditions apoA-I levels are decreased to similar degree.

MATERIALS AND METHODS

Subjects

Four normolipidemic probands, four compound heterozygotes for an apoA-I null allele and a structural apoA-I variant, apoA-I(L141R)$_{Pisa}$, and three patients with Tangier disease (TD) participated in this study. **Table 1** summarizes characteristics of their lipid metabolism. The hemizygotes for apoA-I(L141R)$_{Pisa}$ have been described by us previously (23, 24). Briefly, these four Italian siblings, three men and one woman, were affected by massive corneal clouding. The three brothers suffered from premature coronary heart disease. All of them were deficient in HDL-cholesterol. In some experiments, plasmas of relatives who were heterozygous for the structural apoA-I defect were also analyzed. They had half-normal concentrations of HDL-cholesterol as compared to unaffected family members and the Italian population (24). Two German siblings and a patient from Pakistan with TD have been described previously (26, 27). For some conversion experiments (see below) we used the plasma of an Italian woman who was HDL- and apoA-I-deficient because of homozygosity for a premature truncation of apoA-I at residue 32 (28).

Blood samples

Blood samples were taken after the subjects had fasted overnight. The blood was immediately placed on ice. Plasmas and sera were obtained by centrifugation at 4°C (800 *g*, 15 min), aliquoted and frozen at −70°C. Samples were shipped from Pisa to Münster on dry ice. Serum was used for the quantification of lipids. LCAT activity and cholesterol esterification rates were deter-

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TABLE 1. Characteristics of lipid metabolism in plasmas from hemizygotes for apoA-I $(L141R)_{Pisa}$, patients with Tangier disease, and normoalphalipoproteinemic individuals

Variables	ApoA-I $(L141R)_{Pisa}$ Hemizygotes (3M/1F)	Tangier Disease (2M/1F)	Normolipidemic Controls (3M/1F)
Total cholesterol (mg/dl)	226 ± 37^{ad}	75 ± 27^{b}	167 ± 19
Triglycerides (mg/dl)	133 ± 70	221 ± 76^{b}	69 ± 12
HDL-cholesterol (mg/dl)	0^{c}	0^{c}	58 ± 6
ApoA-I (mg/dl)	4 ± 1^{c}	3 ± 1^{c}	153 ± 15
ApoA-II (mg/dl)	11 ± 2^{c}	8 ± 5^{c}	46 ± 3
ApoB (mg/dl)	114 ± 24^{a}	75 ± 24	78 ± 11
LpA-I (mg/dl)	1^{c}	1^{c}	67 ± 5
LpA-I/A-II (mg/dl)	3 ± 1^{c}	2 ± 1^{c}	86 ± 11
LCAT activity (nmol/h/ml)	5.3 ± 0.1^{c}	5.8 ± 2.7^{c}	21.6 ± 2.9
CER (%/h/l)	3.9 ± 0.5^{b}	4.9 ± 0.7	7.7 ± 2.0

M, male; F, female; CER, cholesterol esterification rate.

$^{a}P < 0.01$; $^{b}P < 0.01$; $^{c}P < 0.001$: Level of statistical significance for the differences in mean values and standard deviations between normal controls and hemizygotes for apoA-I $(L141R)_{Pisa}$ or Tangier disease patients as calculated by Student's *t*-tests.

$^{d}P < 0.001$; Level of statistical significance for the differences in mean values and standard deviations between hemizygotes for apoA-I $(L141R)_{Pisa}$ and Tangier disease patients as calculated by Student's *t*-test.

mined in EDTA-plasma. EDTA plasma was also used for electrophoretic analyses. For experiments in which plasma was incubated with cells, streptokinase (Sigma) was used as the anticoagulant at a final concentration of 150 units/ml.

Quantification of lipids, apolipoproteins, and cholesterol esterification activities

Serum concentrations of triglycerides and cholesterol were quantified with an autoanalyzer (Hitachi/Boehringer, Mannheim, Germany). HDL-cholesterol concentrations were measured after precipitation of apoB-containing lipoproteins with phosphotungstic acid/$MgCl_2$ (Boehringer, Mannheim). Concentrations of apoA-I and apoB were determined with a modified, commercially available turbidimetric assay (Boehringer Mannheim) (29). Plasma LCAT activities were assayed either as the esterification of [^{3}H]cholesterol (New England Nuclear, Boston, MA) which was incorporated into reconstituted, apoA-I-containing particles (i.e., LCAT-activity, 30, 31) or after equilibration of [^{3}H]cholesterol with endogenous lipoproteins of plasma (i.e., plasma cholesterol esterification rate (CER, 32).

Nondenaturing two-dimensional electrophoresis

The distribution of apoA-I-containing lipoproteins (LpA-I) was analyzed by nondenaturing two-dimensional polyacrylamide gradient gel electrophoresis (2D-PAGGE) of plasma (33–35). Briefly, in the first dimension, 10–30 μl plasma was separated by electrophoresis at 4°C in a 0.75% agarose gel using a 50 mM merbital buffer (44.3 g Tris/19.2 g Merbital (Serva, Heidelberg, Germany)/0.5 g calcium lactate/1 g NaN_3/1000 ml distilled water, pH 8.7). Bromphenol blue was added to a standard sample to visualize albumin in the native gel. The electrophoresis was stopped when the albumin/bromphenol blue marker had migrated 6 cm. Agarose gel strips containing the preseparated lipoproteins were then transferred to a 3–20% polyacrylamide gradient gel. Separation in the second dimension was performed at 40 mA for 4–5 h at 10°C. During this time, endogenous plasma albumin, which was visible in the native gel as a faint blue band because of bromphenol blue added to the cathodic buffer (300 μl per liter buffer), had migrated 10 cm. The proteins separated in the PAGGE gel were electroblotted onto a nitrocellulose membrane. ApoA-I-containing lipoproteins were immunodetected by the use of sheep antibodies against human apoA-I (Boehringer Mannheim). Antigen–antibody compexes were visualized by the use of horseradish peroxidase-conjugated antibodies from rabbit to sheep-IgG (DAKO, Denmark).

IEF of apoA-I in preβ_1-LpA-I, α-$LpAI_2$, and α-$LpA\text{-}I_3$

Nondenaturing 2D-PAGGE was modified as a three-step-electrophoresis in the sequence agarose gel electrophoresis → PAGGE → IEF to determine the relative concentration of normal and variant apoA-I in HDL-subclasses. A schematic description of this procedure is presented in **Fig. 1.** Twenty μl plasma of two heterozygotes for the structural apoA-I variant as well as several standard plasmas were separated by agarose gel electrophoresis as described previously. Thereafter, one lane with the separated lipoproteins of a standard sample was removed and stained with oil-red. This gel strip was then used to localize preβ- and α-migrating lipoproteins in the unstained gel. The proportions of the unstained

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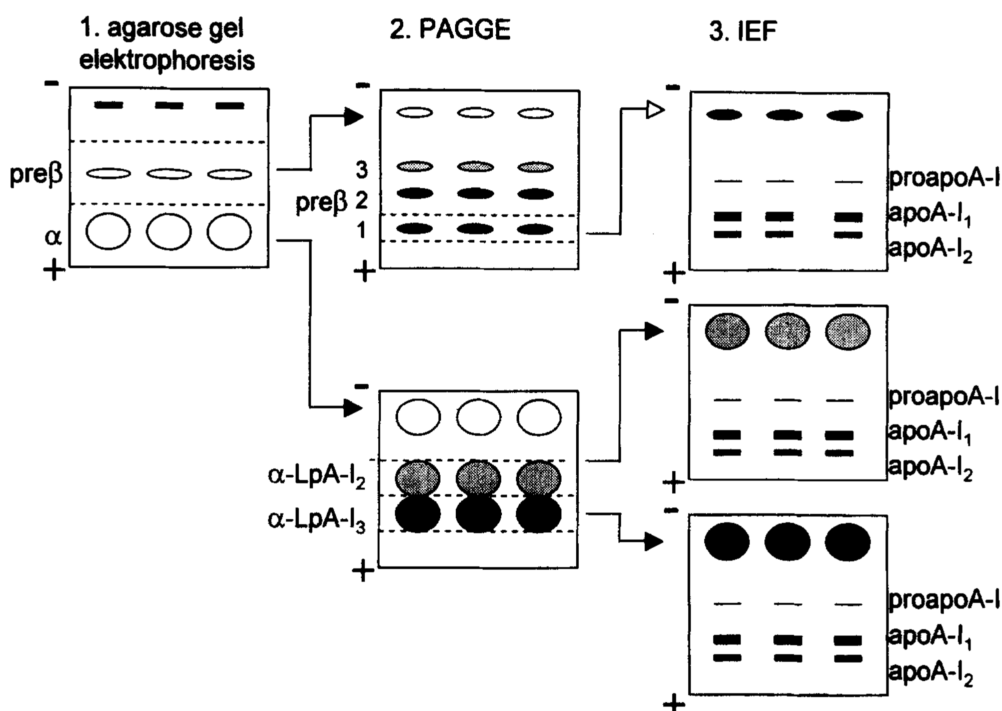

Fig. 1. Principle of a three-step electrophoresis in the sequence agarose gel electrophoresis → nondenaturing polyacrylamide gradient gel electrophoresis → nondenaturing isoelectric focusing. Plasmas were separated by agarose gel electrophoresis. Proportions of the unstained gel containing either the preβ- or the α-migrating lipoproteins of adjacent samples were cut perpendicular to the electrophoretic axis and removed. Each strip was transferred to a separate PAGGE-gel for electrophoresis. After completion of PAGGE, gel strips containing preβ$_1$-LpA-I, α-LpA-I$_2$ and α-LpA-I$_3$ of adjacent samples were excised perpendicular to the running axis in the respective gels and transferred into a buffer with urea to denaturate apoA-I. Thereafter the strips were transferred to IEF gels for the separation of apoA-I isoforms. After IEF, proteins were electroblotted onto nitrocellulose sheets for detection of apoA-I isoforms with anti-apoA-I antibodies.

gel containing either the preβ- or the α-migrating lipoproteins of all adjacent samples were cut perpendicular to the electrophoretic axis and removed. Each strip was transferred to a PAGGE-gel for electrophoresis as previously described. After completion of PAGGE, gel strips containing preβ-migrating and α-migrating proteins of a single standard sample were removed. Their proteins were electroblotted onto nitrocellulose sheets for subsequent visualization of preβ$_1$-LpA-I and α-LpA-I with anti-apoA-I-antibodies (see above). These immunoblots were then used to localize preβ$_1$-LpA-I, α-LpA-I$_2$, and α-LpA-I$_3$ in the native gels, which meanwhile had been stored at 4°C. Gel strips containing preβ$_1$-LpA-I, α-LpA-I$_2$, and α-LpA-I$_3$ of adjacent samples were excised perpendicular to the running axis in the respective gels and transferred to a solution with 8 M urea to denaturate apoA-I. After 1 h incubation the strips were transferred to IEF gels for the separation of apoA-I isoforms (36). After IEF, proteins were electroblotted onto nitrocellulose sheets for detection of apoA-I isoforms with anti-apoA-I antibodies (see above). The staining intensity of the bands was measured by scanning densitometry.

Conversion of preβ$_1$-LpA-I into α-LpA-I

In normal plasma preβ$_1$-LpA-I is converted to α-LpA-I during maturation. We have previously demonstrated that in TD plasma this conversion is interrupted and that this conversion defect can be corrected by the addition of apoA-I-deficient plasma to TD plasma (37). To test whether preβ$_1$-LpA-I-containing apoA-I(L141R) can be converted to α-LpA-I, we coincubated 50 µl plasma of a completely apoA-I-deficient patient (28) at 37°C for 18 h either with 50 µl plasma from an apoA-I(L141R) hemizygote or with 50 µl plasma from a TD patient. Incubations were halted by placing the samples on ice. The samples were then stored in a freezer at −70°C until analysis by 2D-PAGGE. 2D-PAGGE was performed on 20 µl as described before. After electrotransfer to nitrocellulose membranes, apoA-I-containing lipoproteins were immunodetected by biotinyl-

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ated anti-apoA-I antibodies and strepavidin-horseradish peroxidase also as described previously (37).

Determination of [^{3}H]cholesterol efflux from fibroblasts into plasma or lipoproteins

To measure cholesterol efflux capacity of plasma and apoA-Icontaining lipoproteins, plasmas were incubated with radiolabeled fibroblasts. Normal human skin fibroblasts (3×10^5) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in either single dishes of 3.5 cm diameter or in plates with 12 wells of 2.5 cm diameter. At the state of near confluence, cells were labeled in the presence of fetal calf serum with 0.5 mCi [1,2-^{3}H]cholesterol (3.5-cm dishes) or with 0.2 mCi [1,2-^{3}H]cholesterol (2.5-cm wells) (^{3}H-UC, New England Nuclear, Boston, MA); 51.7 Ci/mmol) for 72 h at 37°C. After washing six times with phosphate-buffered saline (PBS) pH 7.4, the specific radioactivity in the cells was $5.7 \pm 2.1 \times 10^8$ cpm/mg protein (33–35).

Multiwell plates were used to measure cholesterol efflux into total or apoB-depleted plasmas. ApoB-free plasma was obtained by incubation of plasma with phosphotungstic acid/$MgCl_2$ as recommended by the manufacturer (Boehringer Mannheim). The apoB-free supernatant was subsequently dialyzed against PBS (pH 7.4) at 4°C for 24 h. Complete removal of apoB-containing lipoproteins was ascertained by immunoblotting. Aliquots with 100 μl native or apoB-depleted plasmas were diluted with 900 μl DMEM and incubated for increasing time intervals with the radiolabeled fibroblasts. Thereafter, the media were removed into microliter tubes. Any cell debris in the medium was pelleted by centrifugation at 15,000 rpm for 15 min at 4°C in a microliter centrifuge. After removal of the supernatant, an aliquot of 50 μl was added to 5 ml scintillation buffer (Instant Scint Gel PL, Packard Instruments BV, Groningen, The Netherlands). Cells were lysed with 1.5 ml 0.5 M NaOH and their lipids were extracted by incubation with hexane–isopropanol 3:2. Radioactivity was then measured by scintillation spectrometry. Fractional cholesterol efflux was calculated as $cpm_{medium}/(cpm_{medium} + cpm_{cells}) \times 100\%$.

To measure the uptake of cell-derived ^{3}H-UC into preβ_1-LpA-I, radiolabeled cells of 3.5-cm dishes were incubated for 1 min with 1 ml undiluted plasma (33–35). After incubation, the medium was removed and cell debris was sedimented by centrifugation at 4°C (see above). Thereafter, aliquots of 30 μl plasma were separated by 2D-PAGGE as described above. 2D-PAGGE was performed in parallel on a radiolabeled and a non-radiolabeled sample. After separation, one half of the PAGGE gel containing the radiolabeled sample was stored at 4°C. The other half of the gel containing the nonradiolabeled sample was electroblotted onto a nitrocellulose membrane to identify apoA-I-containing lipoproteins. The immunoblot was then used as a template to localize the corresponding lipoproteins in the other half of the gel. These lipoproteins were cut out, and their lipids were extracted by chloroform–methanol 2:1, (v/v) for 72 h. After evaporation of the organic solvent, lipids were solubilized in 5 ml scintillation buffer to measure the radioactivity by scintillation spectrometry.

Statistics

All data are presented either as medians or means ± standard deviations. The levels of significance for differences between patients and controls were calculated by ANOVA. All calculations were done with Excel (Microsoft) and an add-in program for Excel (Astute, 1993 DDU Software, The University of Leeds).

RESULTS

(Table 1) summarizes data on lipid metabolism of four hemizygotes for apoA-I(L141R)$_{Pisa}$, three patients with TD, and four normolipidemic controls. Compared to normal plasmas, plasmas of either apoA-I(L141R) hemizygotes or TD patients were characterized by markedly reduced levels of HDL-cholesterol, apoA-I, and HDL-subclass LpA-I. Moreover, the ability of plasma to esterify cholesterol either in exogenous or endogenous substrates was significantly decreased. It is noteworthy that plasmas of patients with TD and apoA-I(L141R)$_{Pisa}$ did not differ from one another with respect to these properties. By contrast, TD plasmas had significantly reduced concentrations of total cholesterol and apoB.

Characterization of apoA-I–containing particles

2D-PAGGE of plasma and subsequent anti-apoA-I immunoblotting identified preβ_1-LpA-I in plasmas of all patients (**Figs. 2A and 2B**) and controls (fig. 2C). By contrast, the bulk of HDL, which has alpha-mobility in normal plasma (i.e., α-LpA-I, Fig. 2C) was completely absent from TD plasma (Fig. 2B) and was present in plasmas of apoA-I(L141R) hemizygotes at very low concentrations (Fig. 2A). These residual amounts corresponded to small particles, i.e., α-LpA-I_3. Moreover, preβ_2-LpA-I was present in plasmas of normolipidemic controls (Fig. 2C) but absent from plasmas of apoA-I(L141R) hemizygotes and TD patients (Fig. 2A and 2B). To investigate whether the lack of large α-LpA-I (i.e., α-LpA-I_2) in plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes reflects the failure of the variant isoform to associate with or form larger particles, we analyzed plasmas

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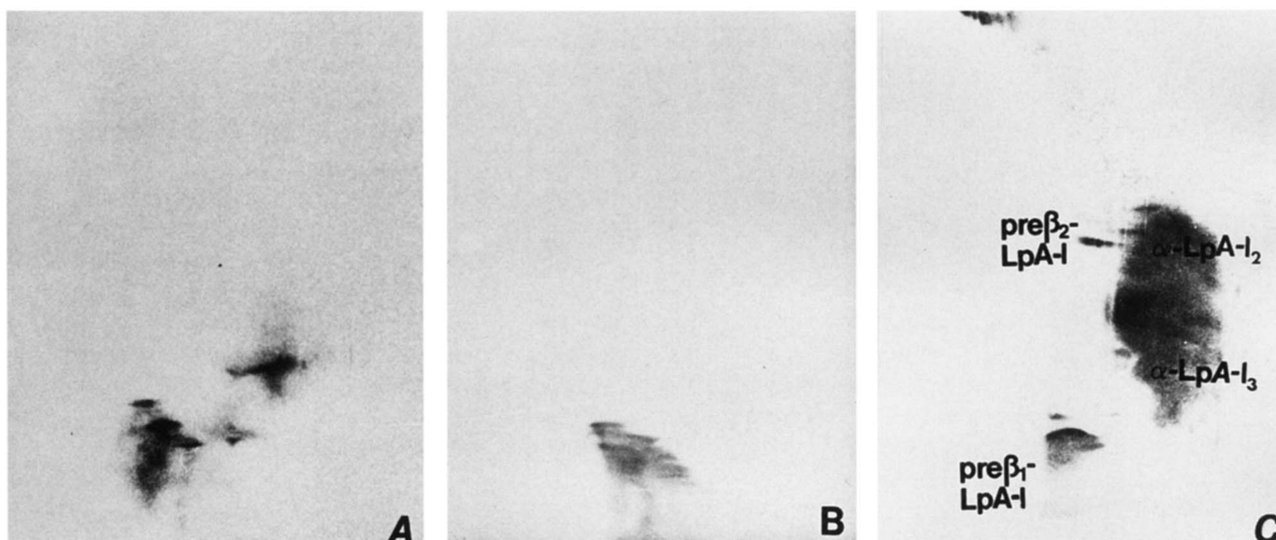

Fig. 2. 2D-PAGGE and immunoblotting of apoA-I-containing lipoproteins in plasmas of a hemizygote for apoA-I(L141R)$_{Pisa}$ (A), a patient with Tangier disease (B), and a normoalphalipoproteinemic individual (C). Nondenaturing 2D-electrophoresis was performed in the sequence agarose gel electrophoresis → nondenaturing polyacrylamide gradient gel electrophoresis. After electroblotting onto nitrocellulose membranes, apoA-I-containing lipoproteins were detected using polyclonal sheep antibodies against human apoA-I. Note the presence of preβ$_1$-LpA-I in all patient and control plasmas (A–C). By contrast, α-LpA-I was completely lacking in the plasma of the TD patient (B) and was present in greatly reduced amounts in plasmas of hemizygotes for apoA-I(L141R)$_{Pisa}$ (A). These residual amounts of α-LpA-I consisted of small particles.

of two heterozygotes for apoA-I(L141R)$_{Pisa}$ by a three-step electrophoresis in the sequence agarose gel electrophoresis → PAGGE → IEF (**Fig. 3**). This analysis revealed that α-LpA-I$_2$ of apoA-I(L141R) heterozygotes contain only normal apoA-I. Only 14 ± 4% of apoA-I in small α-LpA-I$_3$ corresponded to the variant isoform instead of expected 50% ($P < 0.05$ chi-square test). Preβ$_1$-LpA-I contained normal and variant apoA-I isoforms at approximately equal amounts (54 ± 10%, Fig. 3).

We have previously demonstrated that preβ$_1$-LpA-I is converted by normal or apoA-I-deficient plasma into α-LpA-I (37). To investigate whether apoA-I(L141R) interferes with the conversion of preβ$_1$-LpA-I into α-LpA-I, we co-incubated the plasma of an apoA-I(L141R) hemizygote with either PBS (**Fig. 4A**) or with the plasma of a completely apoA-I-deficient patient (28) (Fig. 4B). After 18 h incubation the mixtures were separated by 2D-PAGGE and apoA-I-containing lipoproteins were detected by immunoblotting. In control experiments, plasma of a TD patient, which contains preβ$_1$-LpA-I as the only apoA-I-containing particle (cf. Fig. 2B) and normal plasma were also incubated with PBS or the completely apoA-I-deficient plasma (Fig. 4C and 4D). As expected, incubation of normal plasma with PBS led to a great decrease in the concentration of preβ$_1$-LpA-I (Fig. 4E). As previously described (37), α-LpA-I was not generated after incubation of TD plasma with PBS (Fig. 4C). Co-incubation of TD plasma with apoA-I-deficient plasma, however, restored the conversion defect and

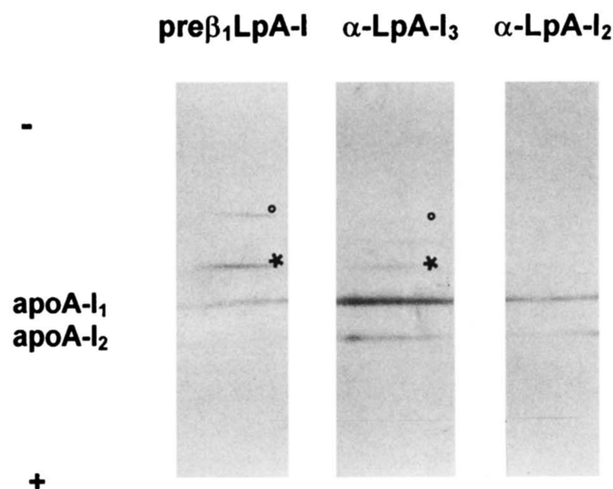

Fig. 3. Demonstration of apoA-I in HDL subfractions of a hemizygote for apoA-I(L141R)$_{Pisa}$ by a three-step electrophoresis in the sequence agarose gel electrophoresis → PAGGE → IEF. Twenty μl plasma from a heterozygote for apoA-I(L141R)$_{Pisa}$ was separated by agarose gel electrophoresis. Two proportions of the gel containing preβ- and α-migrating lipoproteins were removed from the unstained gel and transferred to two PAGGE-gels for electrophoresis. Three gel strips containing preβ$_1$-LpA-I, α-LpA-I$_2$, and α-LpA-I$_3$, respectively, were excised perpendicular to the running axis in the respective gels and transferred to IEF gels for the separation of apoA-I isoforms. After IEF, proteins were electroblotted onto nitrocellulose sheets for detection of apoA-I isoforms with anti-apoA-I antibodies. For details see Materials and Methods as well as Fig. 1. The positions of normal mature apoA-I isoforms are denoted apoA-I$_1$, and apoA-I$_2$. The position of the variant apoA-I$_1$ isoform is indicated by an asterisk (*); the position of the variant proapoA-I$_1$ is indicated by a kringle (°). Note that preβ$_1$-LpA-I contains normal and variant apoA-I isoforms at approximately equal concentrations. ApoA-I(L141R)$_{Pisa}$ was present in α-LpA-I$_3$ at severely reduced concentrations and was lacking in α-LpA-I$_2$.

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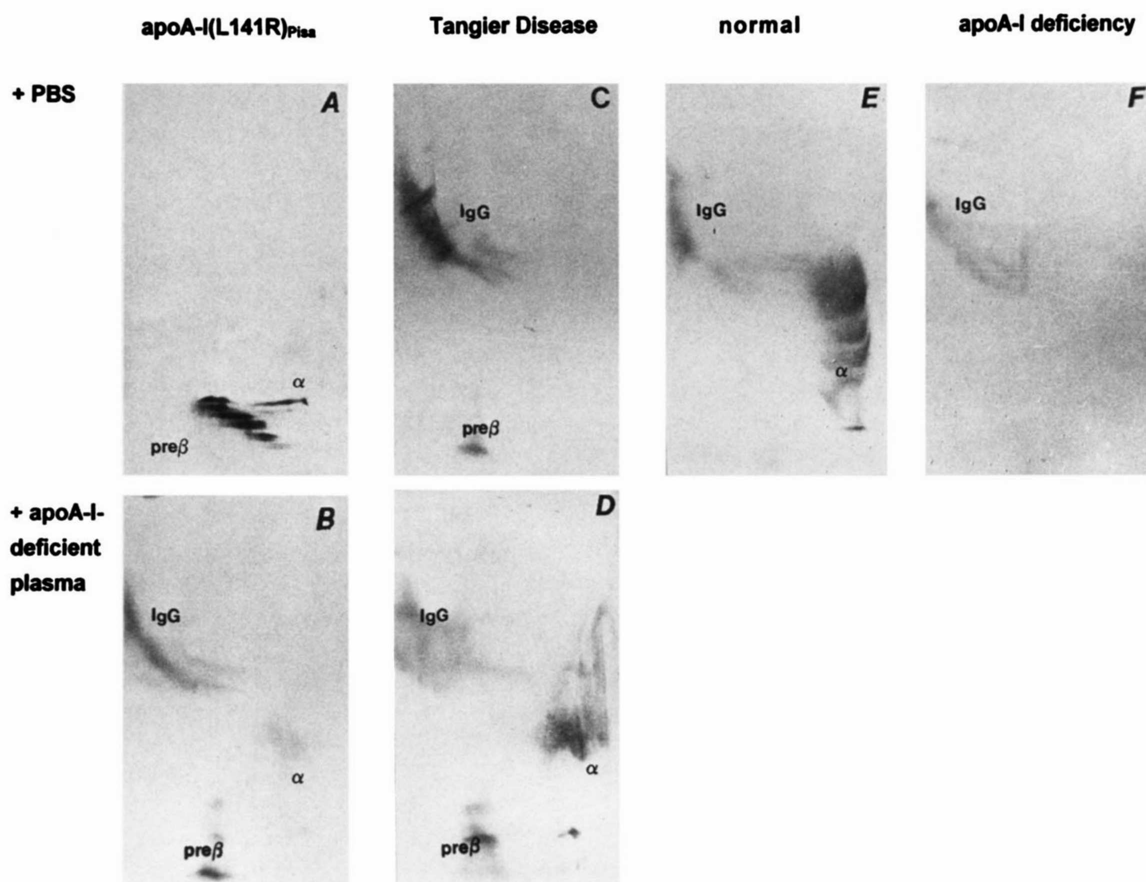

Fig. 4. Conversion of preβ$_1$-LpA-I and generation of α-LpA-I in plasmas of a hemizygote for apoA-I(L141R)$_{Pisa}$, a patient with Tangier disease, and normal plasma. Fifty μl PBS (A, C, E) or 50 μl plasma of a completely apoA-I-deficient patient (28) (B, D) were co-incubated at 37°C for 18 h either with 50 μl plasma of an apoA-I(L141R) hemizygote (A, B) or with 50 μl plasma of a TD patient (C, D). Thereafter, the electrophoretic distribution of apoA-I-containing particles was analyzed by 2D-PAGGE and anti-apoA-I immunoblotting. F demonstrates the absence of apoA-I in the apoAI-deficient sample. Incubation of normal plasma with PBS led to the expected decrease of preβ$_1$-LpA-I ((E, cf. Fig. 2C). Note the decrease in the concentration of preβ$_1$-LpA-I and the appearance of an α-migrating apoA-I-containing particle after incubation of TD plasma with apoA-I-deficient plasma (D) which was not generated in the presence of PBS (C) and which was lacking in the original sample (cf. Fig. 2b). After incubation of plasma from an apoA-I(L141R) hemizygote with either PBS (A) or apoA-I-deficient plasma (B), a smaller α-migrating particle was detectable that was already present in the original sample (cf. Fig. 2A). Moreover, the concentration of preβ$_1$-LpA-I did not decrease as was the case in TD-plasma.

led to a decrease in the concentration of preβ$_1$-LpA-I and to the occurrence of an α-migrating particle (Fig. 4D). By contrast, incubation of plasma from apoA-I(L141R)$_{Pisa}$ hemizygotes with PBS or apoA-I-deficient plasma did not reduce the concentration of preβ$_1$-LpA-I or increase the concentration of α-LpA-I (Fig. 4A and 4B). These data suggest that preβ$_1$-LpA-I containing apoA-I(L141R)$_{Pisa}$ are resistant towards conversion into α-LpA-I.

Characterization of cholesterol efflux capacity

To determine their cholesterol efflux capacities, normal and HDL-deficient plasmas were incubated for various time intervals with human skin fibroblasts that were radiolabeled with [^{3}H]cholesterol. At all time points, plasmas of either apoA-I(L141R) hemizygotes or TD patients showed significantly reduced ability to release [^{3}H]cholesterol from cells compared to plasmas from normolipidemic persons (**Table 2**). During incubations of 1 min or 1 h the cholesterol efflux capacity of plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes was significantly higher than that of TD plasmas ($P < 0.05$) (Table 2).

To eliminate the unspecific effects of apoB-containing lipoproteins on cholesterol efflux, the experiments were also performed on plasmas that had been depleted of apoB. After incubating for 1 min with radiolabeled fibroblasts, fractional efflux of [^{3}H]cholesterol into apoB-depleted plasmas from either HDL-deficient or normolipidemic patients did not differ significantly (**Table 3**). Whereas prolonged incubation of complete plasma produced a linear increase in [^{3}H]cholesterol efflux (compare Table 2), incubations longer than 1 min caused small increases of [^{3}H]cholesterol efflux into apoB-depleted plasma of normolipidemic pro-

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TABLE 2. Fractional cholesterol efflux from [^{3}H]cholesterol-labeled fibroblasts into plasma from hemizygotes for apoA-I $(L141R)_{Pisa}$, patients with Tangier's disease, and normoalphalipoproteinemic individuals

Subjects	Cholesterol Efflux			
	1 min	5 min	15 min	60 min
ApoA-I $(L141R)_{Pisa}$				
1	3.8 ± 0.1	6.6 ± 3.9	14.4 ± 4.2	30.0 ± 0.1
2	3.2 ± 0.3	6.0 ± 1.6	16.0 ± 6.2	36.9 ± 8.8
3	3.5 ± 1.2	6.5 ± 2.7	10.5 ± 1.3	17.0 ± 0.2
4	4.0 ± 0.8	5.6 ± 1.6	9.4 ± 1.5	19.3 ± 2.7
Mean ± SD	3.6 ± 0.7	6.2 ± 2.0	12.6 ± 4.1	25.8 ± 9.3
Tangier disease				
1	2.4 ± 0.1	6.6 ± 0.5	16.1 ± 2.8	24.0 ± 10.4
2	2.1 ± 1.0	6.6 ± 1.4	11.7 ± 1.2	15.9 ± 1.2
3	2.5 ± 0.6	5.8 ± 0.8	13.6 ± 0.1	15.9 ± 1.1
Mean ± SD	2.3 ± 0.6	6.3 ± 0.9	13.8 ± 2.4	18.6 ± 6.3
Control				
1	4.8 ± 2.3	7.3 ± 0.7	28.9 ± 1.8	42.3 ± 0.5
2	6.4 ± 2.0	8.1 ± 1.6	18.4 ± 7.9	43.9 ± 2.0
3	6.1 ± 0.2	8.1 ± 0.2	23.0 ± 2.7	41.1 ± 0.8
4	6.4 ± 0.1	8.9 ± 0.1	19.8 ± 10.1	50.0 ± 3.0
Mean ± SD	5.9 ± 1.3	8.1 ± 0.9	22.5 ± 6.6	44.3 ± 3.9
P values				
Control vs. apoA-I (Pisa)	0.0001	0.004	0.006	0.004
Normal vs. Tangier disease	0.0001	0.001	0.008	0.0001
Tangier disease vs. apoA-I (Pisa)	0.03	ns	ns	0.05

One ml of 10% plasma was incubated for indicated time intervals with [^{3}H]cholesterol-labeled fibroblasts. Lipids were extracted from both the medium and the cells for radioactivity measurements. Data give fractional cholesterol efflux rates which were calculated as $cpm_{medium}/cpm_{medium} + cpm_{cells} \times 100\%$. Each plasma was analyzed in two independent series of experiments. *P* values were calculated by ANOVA, comparing data of experiments performed on plasmas from patients versus those of controls.

bands and no increase of [^{3}H]cholesterol efflux into apoB-depleted plasmas of either TD patients or apoA-I$(L141R)_{Pisa}$ hemizygotes. Thus, after 5- or 60-min incubations, cholesterol efflux into apoB-depleted plasmas of either apoA-I$(L141R)_{Pisa}$ hemizygotes or TD patients was significantly lower than into apoB-depleted normolipidemic plasmas (Table 3).

To compare the uptake of cell-derived [^{3}H]cholesterol by preβ_1-LpA-I of apoA-I$(L141R)_{Pisa}$ hemizygotes, TD patients, and normolipidemic probands, incubations of plasma with [^{3}H]cholesterol-labeled fibroblasts were followed by 2D-PAGGE (**Table 4**). Whereas preβ_1-LpA-I of normal plasmas and TD plasmas did not differ in their content of radioactivity, significantly less radioactivity was found in preβ_1-LpA-I of apoA-I$(L141R)_{Pisa}$ hemizygotes (Table 4).

TABLE 3. Fractional cholesterol efflux from [^{3}H]cholesterol-labeled fibroblasts into apo-B-depleted plasma from hemizygotes for apoA-I $(L141R)_{Pisa}$, patients with Tangier's disease, and normoalphalipoproteinemic individuals

Subjects	Cholesterol Efflux		
	1 min	5 min	60 min
ApoA-I $(L141R)_{Pisa}$	1.6 ± 0.1	1.5 ± 0.6	1.6 ± 0.3
Tangier disease (n = 3)	1.3 ± 0.2	0.9 ± 0.5	1.1 ± 0.2
Controls (n = 4)	1.7 ± 0.3	1.9 ± 0.7	2.8 ± 1.0
P values			
Control vs. apoA-I (Pisa)	ns	0.02	0.0033
Control vs. Tangier disease	ns	0.001	0.0002
Tangier disease vs. apoA-I (Pisa)	ns	0.03	0.03

ApoB-containing lipoproteins were precipitated with phosphotungstic acid. The supernatant was dialyzed against PBS and diluted with DMEM to give a final concentration equivalent to 10% plasma. One ml of this solution was incubated for increasing time intervals with [^{3}H]cholesterol-labeled fibroblasts. Lipids were extracted from both the medium and the cells for radioactivity measurements. Data give fractional cholesterol efflux rates which were calculated as $cpm_{medium}/cpm_{medium} + cpm_{cells} \times 100\%$. Every plasma was analyzed in duplicate in two independent series of experiments. *P* values were calculated by ANOVA, comparing data of experiments performed on plasmas from patients with those of controls.

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TABLE 4. Efflux of [^{3}H]cholesterol from radiolabeled fibroblasts into preβ_1-LpA-I of plasma from hemizygotes for apoA-I (L141R)$_{Pisa}$, patients with Tangier disease, and normoalphalipoproteinemic individuals

Subjects	Radioactivity in Preβ_1-LpA-I
	cpm
ApoA-I (L141R)$_{Pisa}$	
1 (n = 1)	56
2 (n = 2)	70 (33–107)
3 (n = 2)	77 (64–89)
4 (n = 3)	42 (26–74)
Median	60
Tangier disease	
1 (n = 2)	173 (165–181)
Control	
1 (n = 4)	136 (64–198)
2 (n = 1)	168
3 (n = 1)	161
4 (n = 1)	98
Median	165

One ml of undiluted plasma was incubated for 1 min with [^{3}H]cholesterol-labeled fibroblasts. Thereafter, 30 μl of the medium was separated by 2D-PAGGE as described in Methods. Proportions of the gel containing pre β_1-LpA-I were removed. Lipids were extracted to count radioactivity. Presented are cpm values either as results of single experiments or medians (and ranges) of several experiments; n = number of experiments performed on the respective sample. Radioactivity taken up by pre β_1-LpA-1 of hemizygotes for apoA-I (L141R)$_{Pisa}$ was significantly lower than that taken up by preβ_1-LpA-I of Tangier disease patients and normoalphalipoproteinemic individuals ($P < 0.001$, ANOVA).

DISCUSSION

We have previously demonstrated that apoA-I(L141R)$_{Pisa}$ causes a virtually complete lack of HDL in hemizygotes and hypoalphalipoproteinemia with half-normal levels of HDL-cholesterol in heterozygotes (24). In this study we have shown that most of the residual apoA-I in plasmas of hemizygotes for apoA-I(L141R)$_{Pisa}$ resides in the lipid-poor HDL-precursor preβ_1-LpA-I (Fig. 2A). Moreover, in preβ_1-LpA-I of heterozygotes for apoA-I(L141R)$_{Pisa}$ variant and normal apoA-I isoforms occurred at equal concentrations (Fig. 3). This suggests that apoA-I(L141R)$_{Pisa}$ does not interfere with the formation or stability of nascent, lipid-poor particles. By contrast, plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes contained only trace amounts of small α-migrating particles (Fig. 3A). In plasmas of heterozygotes for apoA-I(L141R)$_{Pisa}$ the variant protein was undetectable in large α-LpA-I$_2$ and detectable only at very low levels in small α-LpA-I$_3$ (Fig. 3). As preβ_1-LpA-I of TD plasma (which contains normal apoA-I) but not preβ_1-LpA-I containing apoA-I(L141R)$_{Pisa}$ could be converted into α-LpA-I, we conclude that the arginine for leucine substitution at residue 141 of apoA-I interferes with the formation of lipid-rich and mature α-LpA-I rather than with the stability of pre-formed α-LpA-I. This situation is similar to that of an apoA-I variant previously described by us where the substitution of a leucine for an arginine at residue 160 leads to a lack of large α-LpA-I$_2$ (38).

It has been previously demonstrated that the phospholipid-binding domains of apoA-I are dispersed throughout the entire molecule and that the presence of anti-parallel amphipathic alpha-helices is the most important structural characteristic required for this ability (2). This appears to apply particularly to reconstituted HDL which are discoidal and contain phospholipids as the only lipids (39, 40). In agreement with this, apoA-I(L141R)$_{Pisa}$ (this study) and apoA-I(R160L)$_{Oslo}$ (38) do not interfere with the generation of preβ_1-LpA-I which is poor in lipids. By contrast, the absence or greatly reduced concentration of these apoA-I variants in α-LpA-I, which are spherical and contain cholesterol and cholesteryl esters in addition to phospholipids, demonstrates the essential role of normal apoA-I structure for the formation of mature HDL. Both of these single amino acid substitutions probably interfere with the conformation of one or perhaps two adjacent alpha-helices but are unlikely to disrupt more than that number. Thus, the presence of amphipathic alpha-helices appears to be insufficient for the formation of α-LpA-I.

Our data do not allow any conclusion to be drawn as to whether apoA-I(L141R)$_{Pisa}$ interferes directly or indirectly with the formation of α-LpA-I. A direct defect might be a resistance of apoA-I(L141R)$_{Pisa}$ to the conformational changes that are necessary for the molecule to be accomodated within a spherical particle (2, 41). In addition to the conformation of apoA-I, phosphatidylinositol and cholesterol have previously been shown to contribute to the negative charge of α-migrating HDL (42, 43). Thus it is possible that a binding defect towards specific phospholipids interferes with the formation of α-LpA-I. An indirect mechanism might be a defect in the interaction with LCAT that is necessary to fill the core of HDL with cholesteryl esters (2,44). This hypothesis is supported by the observation that LCAT activity was significantly reduced in hemizygotes for apoA-I(L141R)$_{Pisa}$. However, as LCAT is associated with HDL, decreased esterification activity may also be secondary due to a reduction in the number of particles and hence in the level of LCAT (45).

The absence of α-LpA-I in plasmas of TD patients and its very low level in apoA-I(L141R)$_{Pisa}$ hemizygotes was associated with a decreased capacity for cholesterol efflux (Table 2). Despite the very low concentration of HDL, the uptake of radioactivity from fibroblasts by plasmas from TD patients and apoA-I(L141R)$_{Pisa}$ did not reach saturation during incubations up to 1 h, probably due to the unspecific bidirectional flux of radiolabeled cholesterol between cells and non-HDL particles (3–6).

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In agreement with this interpretation, cholesterol efflux into apoB-depleted plasma of TD patients and apoA-I(L141R)$_{Pisa}$ hemizygotes was already saturated after 1 min of incubation (Table 3). At this time point, cholesterol efflux capacities of normal and HDL-deficient apoB-depleted plasmas did not differ significantly. By contrast, cholesterol efflux into apoB-depleted plasmas of normoalphalipoproteinemic donors slowly increased after this time and was not saturable. Thus, at later times, the capacity for cholesterol efflux was increased significantly relative to samples from HDL-deficient donors. Cholesterol efflux from fibroblasts into apoB-free plasma consists of two components. The fast saturable component is observed in both HDL-deficient and normolaphalipoproteinemic samples. The slow unsaturable component is only found in apoB-free plasmas of normolaphalipoproteinemic donors. These observations agree with previous measurements of cholesterol efflux from radiolabeled fibroblasts onto lipid-free apolipoproteins, native or reconstituted HDL, and protein-free phospholipid vesicles (4, 5, 8–10). In these experiments, lipid-free apoA-I or lipid-poor particles caused fast, saturable, and LCAT-independent cholesterol efflux whereas lipid-rich HDL (and LDL) and phospholipid vesicles caused slow, unsaturable, and LCAT-dependent cholesterol efflux (4, 5). The presence of the fast and saturable cholesterol efflux component in plasmas of both TD patients and apoA-I(L141R)$_{Pisa}$ hemizygotes indicates that these plasmas contain active particles, for example, preβ_1-LpA-I.

After a 1-min pulse incubation, preβ_1-LpA-I in plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes accumulated significantly less radioactivity than plasmas of either normolipidemic individuals or TD patients (Table 4). As the concentration of small preβ_1-LpA-I did not differ among these plasmas (Fig. 2) it is possible that the defect in apoA-I(L141R)$_{Pisa}$ directly interferes with cholesterol efflux from cells. Although this question must be addressed in cholesterol efflux experiments using isolated apoA-1(L141R)$_{Pisa}$, a crucial role for leucine 141 in promotion of cholesterol efflux promotion is supported by the findings of Fielding and colleagues (19) that a monoclonal antiobody directed against an epitope formed by amino acid residues 139 to 144 inhibited cholesterol efflux by plasma. The discrepancy between reduced uptake of cellular [^{3}H]cholesterol by preβ_1-LpA-I but normal initial cholesterol efflux capacity of apoB-free plasma from apoA-I(L141R)$_{Pisa}$ hemizygotes indirectly supports our previous proposition that apoA-I-free particles such as γ-LpE or LpA-IV contribute to the cholesterol efflux into plasma (33–35, 46, 47). These particles are present in plasmas of apoA-I(L141R)$_{Pisa}$ hemizygotes (not shown).

In conclusion, we have demonstrated that the arginine for leucine substitution at residue 141 of apoA-I interferes with the maturation of α-LpA-I but not with the formation of preβ_1LpA-I. The near absence of α-LpA-I interferes with the slow and unsaturable component of plasma capacity to promote cholesterol efflux. The presence of preβ_1-LpA-I helps to maintain the fast, saturable component of cholesterol efflux into plasma.

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