Multiple dysfunctions of two apolipoprotein A-I variants, apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R), that are associated with hypoalphalipoproteinemia in heterozygous carriers

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Abstract **ApoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) are naturally occurring apolipoprotein (apo) A-I variants that are associated with low HDL-cholesterol in heterozygous carriers. We characterized the capacity of these variants to bind lipid, to activate lecithin:cholesterol acyltransferase (LCAT), and to promote efflux of biosynthetic cholesterol from porcine aortic smooth muscle cells (SMCs) or exogenous cholesterol from lipid-loaded mouse peritoneal macrophages. During cholate dialysis, normal apoA-I and both variants associated completely with dipalmitoylphosphatidylcholine (DPPC) and formed rLpA-I of identical size. However, both apoA-I(P165R) and apoA-I(R160L)$_{Oslo}$ showed a reduced capacity to clear a turbid emulsion of dimyristoylphosphatidylcholine (DMPC). Compared to normal apoA-I, the LCAT-cofactor activity of apoA-I(P165R) and apoA-I(R160L)$_{Oslo}$ as defined by the ratio of V_{max} to appK_m was reduced significantly by 62% and 29%, respectively (here and throughout the text, the apparent K_m is given as Michaelis-Menten kinetics do not take particle binding into account and therefore would result in errors with an interfacial enzyme such as LCAT; V_{max} estimates are not affected by this error). ApoA-I/DPPC complexes induced biphasic cholesterol efflux from SMCs with a fast and a slow efflux component. Compared to rLpA-I reconstituted with wild type apoA-I, rLpA-I with apoA-I(P165R) or apoA-I(R160L)$_{Oslo}$ were significantly less effective in promoting cholesterol efflux from SMCs in incubations of 10 min duration but equally effective in incubations of 6 h duration. Lipid-free apoA-I did not induce efflux of biosynthetic cholesterol from SMCs but induced hydrolysis of cholesteryl esters and cholesterol efflux from acetyl-LDL-loaded mouse peritoneal macrophages. In the lipid-free form, both apoA-I variants promoted normal cholesterol efflux from murine peritoneal macrophages. We conclude that amino acid residues arginine 160 and proline 165 of apoA-I contribute to the formation of a domain that is very important for initial lipid binding and contributes to LCAT-activation and promotion of initial cholesterol efflux but not to the stabilization of preformed rLpA-I.**—Daum, U., T. P. Leren, C. Langer, A. Chirazi, P. Cullen, P. H. Pritchard, G. Assmann, and A. von Eckardstein. **Multiple dysfunctions of two apolipoprotein A-I variants, apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R), that are associated with hypoalphalipoproteinemia in heterozygous carriers.** ***J. Lipid Res.*** **1999.** 40: **486–494.**

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The concentration of high density lipoprotein (HDL) cholesterol is inversely correlated with the risk of myocardial infarction (reviewed in ref. 1). Nonsynonymous mutations in the gene encoding apolipoprotein (apo) A-I are a rare cause of familial low HDL-cholesterol levels, i.e., hypoalphalipoproteinemia (reviewed in refs. 2, 3). Although structural apoA-I variants do not account for the common form of familial hypoalphalipoproteinemia seen in survivors of myocardial infarction (2), they are nevertheless invaluable tools in elucidating structure–function relation-

Abbreviations: apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's minimum essential medium; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; FAFA, fatty acid-free albumin; FER, fractional esterification rate; FCS, fetal calf serum; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; PBS, phosphate-buffered saline; rLpA-I, reconstituted lipoproteins containing apoA-I; SMC, smooth muscle cell.

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ships that help this multifunctional protein to play its pivotal role in reverse cholesterol transport.

ApoA-I binds phospholipids and is the predominant protein component of HDL. Moreover, it activates the cholesterol esterifying enzyme lecithin:cholesterol acyltransferase (LCAT), and promotes cholesterol efflux from cells (4, 5). The latter process involves several mechanisms. In the presence of LCAT, lipid-rich HDLs produce slow and non-saturable cholesterol efflux from all cell types investigated so far (6, 7). However, apoA-I also promotes a fast, saturable, and LCAT-independent variety of cholesterol efflux from fibroblasts, macrophages, and smooth muscle cell-derived foam cells, either as a lipid-free protein or as a component of lipid-poor HDL (5, 6). This fast cholesterol efflux pathway is sensitive to the treatment of cells with proteases (8). Moreover, activation of protein kinase C enhances cholesterol efflux by lipid-free apoA-I while inhibition of protein kinase C inhibits it (9–11). For these reasons, and because apoA-I binds to cell membrane proteins, it has been hypothesized that interaction of apoA-I with a signal-transducing cell-surface receptor facilitates the translocation of cholesterol from intracellular compartments to the plasma membrane (5, 6, 10, 11).

Incomplete and partially controversial information exists about which domains of apoA-I are required for lipid binding, formation of HDL, activation of LCAT, and promotion of cholesterol efflux from cells. Not only apoA-I but also the other large water-soluble apolipoproteins A-IV and E are also able to form lipoproteins, to activate LCAT, and to promote cholesterol efflux. It has therefore been argued that the essential feature of apoA-I allowing it to perform these functions is the presence of several antiparallel amphipathic alpha-helices (4, 12). However, results of studies, in which distinct alpha-helices were blocked in apoA-I with monoclonal antibodies (13–18) or deleted in recombinant apoA-I variants (19–22), indicated more specific structure–function relationships within apoA-I. In these studies, the alpha-helices formed by amino acid residues 122–186 and the carboxyterminus were important for initial lipid binding whereas the alpha-helix formed by amino acid residues 100–121 appeared to be more important for the stabilization of preformed particles (19, 20). LCAT cofactor activity of apoA-I has been assigned to the alpha-helices formed by amino acid residues 100–121 and amino acid residues 143–186 (13, 14, 21, 22). These domains and the carboxyterminus have been held responsible for effects of apoA-I in stimulating cholesterol efflux (15–18). However, these studies on the effects of large deletions or monoclonal antibodies, which may cause steric hindrances, do not fully rule out that the losses of functions in these recombinant apoA-I variants and apoA-I/antibody complexes, respectively, are due to quantitative rather than qualitative changes within apoA-I. It is therefore important to investigate the effects of small structural changes on the function of apoA-I. Naturally occurring apoA-I variants that are associated with low levels of HDL-cholesterol are likely to have altered functional properties. We therefore investigated the lipid-binding, LCAT-activating, and cholesterol efflux-promoting activities of two apoA-I variants, apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R), both of which are associated with low levels of HDL-cholesterol in heterozygous carriers (23, 24). One of these variants, apoA-I(P165R), has been previously investigated by us in cooperation with other laboratories (25, 26) and has been found to be associated with subtle losses in function compared to normal apoA-I. We included this variant here to ensure that we could reproduce these changes in independent experiments using different methods for the assessment of lipid binding, LCAT activation, and stimulation of cholesterol efflux.

MATERIALS AND METHODS

Isolation of lipoproteins and variant and normal apoA-I isoforms

Three hundred to 400 ml plasma was obtained by plasmapheresis from two individuals heterozygous for apoA-I(R160L)$_{Oslo}$ (24) or apoA-I(P165R) (23) or from normolipidemic controls with normal apoA-I. LDL and HDL were isolated from plasma by sequential ultracentrifugation (27). Normal and variant apoA-I isoforms were prepared from delipidated HDL by isoelectric focusing (IEF) in preparative gels with immobilized pH gradients and reversed phase HPLC as described previously (23, 25, 26). Contamination with other proteins was excluded by analytical IEF of aliquots with 20 μg apoA-I. Because of their identical isoelectric points, contamination of apoA-I(R160L)$_{Oslo}$ with deamidated isoforms of normal apoA-I and of normal apoA-I with deamidated isoforms of apoA-I(P165R) could not be prevented. Results from scanning densitometry of normal apoA-I preparations suggest that such contamination accounted for approximately 5% (25). The isolated proteins were lyophilized and stored as 500-μg aliquots at −70°C

Cells

Smooth muscle cells (SMCs) were isolated from porcine aorta. After removal of the endothelium, 1 cm long and 1 to 2 mm thick strips were removed from the media and transferred into 20 ml of DMEM solution which contained 1% antibiotic/antimycotic solution with 10,000 IU/ml penicillin G, 10 μg/ml streptomycin, 25 μg/ml amphotericin B (Sigma), and 0.4% collagenase (Sigma). After overnight cultivation at 37°C, the solution was centrifuged for 10 min at 200 *g*. The precipitated cells were resuspended and cultivated in DMEM with 10% FCS and 1% antibiotic/antimycotic solution. For each experiment, cells of the fifth passage were seeded into 35-mm culture dishes at a density of 35,000 cells per dish.

Monocyte-derived macrophages were obtained from C57BL6 mice by peritoneal lavage with a solution of 0.15 m NaCl and 1 IU heparin/ml. For every series of experiments, peritoneal fluid from 10 mice was pooled and washed three times with DMEM medium by centrifugation at 900 rpm for 15 min. The cells were then suspended in 20 ml DMEM medium containing 2 ml fetal calf serum (FCS), 2 mm glutamine, and 200 μl antibiotic/antimycotic solution. One-ml aliquots of this suspension (= cells of 1.5 mice) were dispensed into plastic petri dishes (diameter = 35 mm) and cultivated at 37°C and 5% CO_2. Non-adherent cells were removed after 2 h by washing with PBS. Cultivation was continued in DMEM/10% FCS for 24 h at 37°C and 5% CO_2.

Baby hamster kidney (BHK) cells transfected with the human LCAT gene (28) were cultivated in DMEM with 10% FCS and 1% antibiotic/antimycotic solution. Maximal LCAT activity was recorded in the medium after incubation of confluent BHK cells

for 6 h in FCS-free DMEM. After removal of cells and debris by centrifugation, the harvested medium was concentrated using Amicon-Centriprep 10 tubes. The LCAT preparation obtained in this fashion had an activity of approximately 20 nmol/h per ml and was stored as aliquots of 1 ml at −70°C.

Preparation of reconstituted HDL

Reconstituted HDL (rLpA-I) were obtained by the cholate dialysis method according to Jonas et al. (25) and Matz and Jonas (29). rLpA-I that were used for the electrophoretic characterization of size and homogeneity and for the investigation of cholesterol efflux from SMCs contained dipalmitoylphophatidylcholine (DPPC) and apoA-I in a molar ratio of 100:1. LCAT-cofactor properties were characterized by the use of rLpA-I that contained DPPC, cholesterol, and apoA-I in molar ratios of 100:5:1 as well as 1.56 μCi [^{3}H]cholesterol (Amersham) per 1 mmol cholesterol (25).

Electrophoretic characterization of reconstituted HDL

The size and homogeneity of rLpA-I were examined by nondenaturating polyacrylamide gradient gel electrophoresis (PAGGE) in 4–30% polyacrylamide gradient gels prepared in our laboratory (30). Electrophoresis was performed with 50 μg rLpA-I per lane and 50 μg high molecular weight marker mixture (Pharmacia) per lane at 40 mA for 4–5 h at 10°C. The proteins separated in the PAGGE gel were stained with Coomassie Blue. The Stokes diameter of the particles was calculated by regression analysis on the basis of relative electrophoretic mobilities of the particles and high molecular weight markers (25).

Clearance of phospholiposomes by apoA-I

The clearance of phospholiposomes by apoA-I was measured by kinetic turbidimetry as described by Holvoet et al. (19) and Pownall (31). Dimyristoylphosphatidylcholine (DMPC) and apoA-I were dissolved in 0.01 m Tris/HCl buffer, pH 7.4, containing 700 mm KBr, 0.01% sodium azide (w/v), and 270 μm EDTA to give final concentrations of 600 μm and 6 μm, respectively. After warming to 24°C in a water bath for at least 10 min, equal volumes of the two solutions with DMPC and apoA-I were mixed and the lipid–protein association was monitored as the rate of disappearance of liposomal turbidity at 325 nm in a tempered ultraviolet-photometer (Beckman). Statistical differences between the clearance curves were calculated by *t*-test.

Determination of LCAT cofactor activity

LCAT cofactor activity of normal apoA-I and apoA-I variants was determined using an assay basically described by Pritchard et al. (32). Increasing amounts of cholesterol- and [^{3}H]cholesterol-containing rLpA-I with normal or variant apoA-I isoforms were incubated for 30 min at 37°C in 140 μl assay buffer (10 mm Tris-HCl, pH 7.4, 5 mm EDTA, 0.15 mm NaCl). The assay was started by the addition of 15 μl BHK cell medium that contained LCAT. The reaction was stopped after 30-min incubation at 37°C by the addition of 4 ml chloroform–methanol 2:1 (v/v). Unesterified and esterified cholesterol in extracted lipids were separated by thin-layer chromatography. Radioactivity in cholesterol- and cholesteryl ester-containing fractions was monitored by liquid scintillation spectrometry. The fractional esterification rate (FER) was calculated as cpm [^{3}H]CE/(cpm [^{3}H]CE + cpm [^{3}H]UC). As each assay contained 3 mmol cholesterol and was performed over an incubation period of 30 min, LCAT activity was calculated as FER × 3 nmol cholesterol/0.5 h. The kinetic constants V_{max}, appK_m, and appV_{max}/K_m of dosage-dependent activation of LCAT by apoA-I were calculated as a function of apoA-I concentration using double reciprocal Lineweaver-Burk plots assuming normal Michaelis-Menten kinetics (25). The assays were performed in duplicate on every sample. The coefficient of variation was below 10%. Statistical differences in the kinetic constants were calculated by *t*-test on the basis of the slope and intercepts of the regression curve.

Cholesterol efflux from smooth muscle cells

SMCs of the fifth passage were cultivated in dishes of 3.5 cm diameter. At 70% to 80% confluence, SMCs were incubated for 6 h at 37°C with 1 ml of DMEM medium that contained 10% (w/v) fatty acid-free albumin (FAFA), 1% (v/v) antibiotic/antimycotic solution, 2.5 μg octimibate as the inhibitor of acyl:cholesterol acyltransferase (ACAT) (Nattermann, Cologne), 0.05 mm mevalonolactone, and 10 mCi RS-[2-^{14}C]mevalonate (Amersham) (33). Thereafter, cells were washed five times with PBS and incubated at 37°C for various time intervals varying between 0 and 360 min with 1 ml DMEM containing 1 mg FAFA, 10 μl antibiotic/antimycotic solution, and 20 μg rLpA-I. In a few experiments lipid-free apoA-I was used instead of rLpA-I.

Incubation with rLpA-I was stopped by placing the dishes with SMCs on ice and removal of the medium. Cells were washed with 1 ml ice-cold PBS which was then added to the medium. Cells and debris of the medium were precipitated by centrifugation for 10 min at 4°C and 200 *g*. Media were stored at −20°C until lipid extraction. Lipids were extracted by shaking for 10 min with 2 ml chloroform–methanol 2:1 (v/v). After phase separation by centrifugation for 10 min at 4°C, the aqueous phase was used to repeat this procedure. Thereafter the organic phases of the two extraction steps were pooled (34).

Cells were fixed in the dishes by incubation for 30 min at 4°C with ice-cold 1% glutardialdehyde solution (v/v). After removal of glutardialdehyde, cells were washed twice with 1 ml ice-cold PBS. Lipids were extracted from cells by incubation of dishes with 2 ml diethyl ether–hexane 3:2 (v/v) for 30 min at room temperature and subsequent washing with diethyl ether–hexane 3:2 (v/v). Organic phases were pooled. The organic solvent was evaporated under nitrogen (34).

Lipids extracted from media and cells were separated by thin-layer chromatography in silica gels G60 (Merck Darmstadt) using hexane–isopropanol 3:2 (v/v) as the mobile phase. [^{14}C]cholesterol and [^{14}C]cholesteryl oleate were separated in every gel as standards. Radioactivity associated with [^{14}C]cholesterol and [^{14}C] cholesteryl oleate in standards, media, and cells was recorded by photoimaging using imaging plates and a BAS-1500 photoimager (Fuji, Japan). The light intensity of the bands was proportional to the radioactivity of [^{14}C]cholesterol and [^{14}C] cholesteryl oleate and was evaluated with the TINA program (Raytest, Straubenhardt, Germany). Data on cholesterol effluxed into the medium is presented as fractional pools, where [^{14}C]UC in the medium + cellular [^{14}C]cholesterol + cellular [^{14}C]cholesteryl esters = 100%. Statistical differences between normal and variant apoA-I isoforms were calculated by *t*-test.

Cholesterol efflux from mouse peritoneal macrophages

After 48 h cultivation, mouse peritoneal macrophages were loaded with cholesterol by a 24-h incubation with 1 ml DMEM medium containing 30 mg FAFA, 10 μl antibiotic/antimycotic solution, and 100 μg acetyl LDL (35). After washing three times with PBS, the cells were incubated at 37°C for 18 h with 1 ml DMEM containing 1 mg fatty acid-free albumin, 10 μl antibiotic/antimycotic solution, and 20 μg lipid-free apoA-I. Procedures used for stopping the incubation and separation of cells and media were the same as described for SMCs. Extraction of lipids from cells and media as well as quantification of cholesterol and cholesteryl esters were performed as described previously by Cullen et al. (36). The separation of lipids by HPLC allowed specific quantifications of cholesterol, cholesteryl arachidonate, cho-

lesteryl linoleate, cholesteryl oleate, cholesteryl palmitoleate, and cholesteryl palmitate and correction for losses of cholesterol and cholesteryl esters during lipid extraction by inclusion of an internal standard. Statistical differences between normal and variant apoA-I isoforms were calculated by *t*-test.

RESULTS

Characterization of rLpA-I by nondenaturating PAGGE

Cholate dialysis of DPPC and apoA-I(R160L)$_{Oslo}$ or DPPC and apoA-I(P165R) generated rLpA-I that were homogenous as judged by nondenaturating PAGGE and did not differ in size from rLpA-I that were generated with wild type apoA-I (**Fig. 1**). The mean Stokes diameter of rLpA-I was 9.3 nm. Neither normal nor variant apoA-I isoforms were detectable as lipid-free proteins. These findings are in agreement with those reported previously by Jonas et al. (25) for normal apoA-I and apoA-I(P165R).

Clearance of a DMPC emulsion by apoA-I

Compared to normal apoA-I, both apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) had a significantly reduced capacity to clear an emulsion of DMPC at its transition temperature of 23.9°C ($P < 0.0001$, Student's *t*-test) (**Fig. 2**). As reported by others (19, 31), the DMPC emulsion was nearly completely cleared by normal apoA-I within 10 min. During this time the absorbance was reduced by less than 20% through apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R). ApoA-I (R160L)$_{Oslo}$ appeared to be even less effective than apoA-I(P165R) although the difference was not significant.

Activation of LCAT

To investigate the LCAT cofactor activity of normal and variant apoA-I isoforms, 0.625, 1.25, 2.5, 5, 10, 20, 40, or 80 μg rLpA-I with DPPC, cholesterol, [^{3}H]cholesterol, and apoA-I were incubated with LCAT-containing medium of BHK cells. Lineweaver-Burk curves were plotted (**Fig. 3**) and kinetic constants were calculated by linear regression (summarized in **Table 1**). Although we used the crude medium of an LCAT-producing transfected cell line and not purified enzyme, and although the incubation

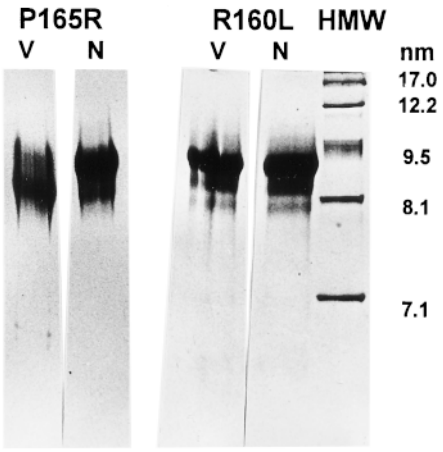

Fig. 1. Nondenaturating PAGGE of rLpA-I containing normal apoA-I, apoA-I(R160L)$_{Oslo}$, or apoA-I(P165R). rLpA-I containing apoA-I and DPPC were reconstituted at a molar ratio of 1:100 with the cholate dialysis method. Aliquots with 50 μg protein were separated in each lane. The gels were stained with Coomassie Blue.

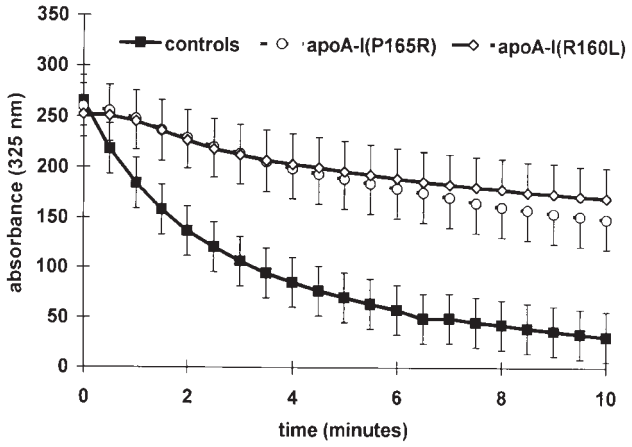

Fig. 2. Clearance of a DMPC emulsion by normal apoA-I, apoA-I(R160L)$_{Oslo}$, or apoA-I(P165R). Normal or variant apoA-I isoforms and DMPC were mixed at a molar ratio of 1:100 and incubated at 24°C in a photometer. The clearance of the turbid phospholipid emulsion was monitored at 325 nm. The dots and bars represent mean values of duplicate experiments that were performed on apoA-I(R160L)$_{Oslo}$ or apoA-I(P165R) and nine experiments that were performed on normal apoA-I. The differences between the curves for normal apoA-I and the two apoA-I variants are significant ($P < 0.0001$; Student's *t*-test).

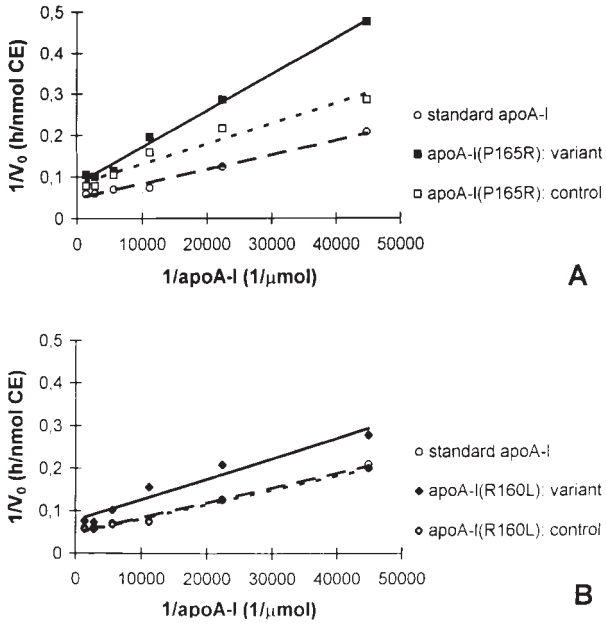

Fig. 3. Lineweaver-Burk plots of the LCAT reaction performed in the presence of rLpA-I with normal apoA-I, apoA-I(P165R) (A), or apoA-I(R160L)$_{Oslo}$ (B). rLpA-I with DPPC, cholesterol, [^{3}H]cholesterol, and 0.625, 1.25, 2.5, 5, 10, 20, 40, or 80 μg apoA-I were used as the substrate, medium of LCAT expressing BHK cells as the enzymatic activity. Symbols represent mean values from duplicate experiments.

TABLE 1. Kinetic constants of the LCAT reaction performed in the presence of rLpA-I with normal apoA-I, apoA-I(R160L)$_{Oslo}$, or apoA-I(P165R)

	Normal ApoA-I	ApoA-I(R160L)	ApoA-I(P165R)
Coefficient of correlation	0.994 ± 0.001	0.974	0.997
V_{max} (nmol CE/h)	21.16 ± 0.32	12.82[a]	12.07[b]
V_{max}/app. K_m (10^5 * nmol CE/h * μmol)	2.88 ± 0.13	2.08[a]	1.13[b]
App. K_m (10^5 * μmol)	7.36 ± 0.41	6.17[a]	10.70[b]

rLpA-I with DPPC, cholesterol, [^{3}H]cholesterol, and 0.625, 1.25, 2.5, 5, 10, 20, 40, or 80 μg apoA-I were used as the substrate, medium of LCAT expressing BHK cells as the enzymatic activity. Data are from duplicate experiments on apoA-I variants and from three duplicate experiments with normal rLpA-I. Kinetic constants were calculated by linear regression analysis of Lineweaver-Burk plots. All values represent mean ± SD.

[a] $P < 0.05$; [b] $P < 0.001$ (*t*-test that was performed on the regression equation of the Lineweaver-Burk regression lines depicted in Figs. 3A and 3B).

time of the assay was longer than in experiments reported by others, we observed saturation kinetics in which the fractional esterification of cholesterol increased with the concentration of rLpA-I. Reciprocal Lineweaver-Burk plots indicated that the kinetics of normal and variant apoA-I isoforms remained within linear ranges (Fig. 3). It is important to note, however, that the kinetic constants observed by us differ from those reported by Jonas et al. (25) by one order of magnitude. In agreement with previous data (25) we observed considerable variation in the LCAT activation kinetics of various normal apoA-I preparations. Slopes and intercepts of the Lineweaver-Burk curves of three normal apoA-I preparations as well as the deduced kinetic constants vary considerably. Nevertheless, the curves of both apoA-I(P165R) (Fig. 3A) and apoA-I(R160L)$_{Oslo}$ (Fig. 3B) had steeper slopes and larger intercepts than any normal apoA-I preparation. Compared to normal apoA-I, apoA-I(R160L)$_{Oslo}$ was characterized by a significantly increased affinity constant (appK_m) and significant decreases of the maximal velocity (V_{max}) and V_{max}/appK_m ratio (−29%). ApoA-I(P165R) had a significantly increased affinity constant appK_m and a 44% lower reactivity (V_{max}/appK_m). The lower (V_{max}) and V_{max}/appK_m ratio of apoA-I(P165R) are in agreement with our previous observations (25).

Cholesterol efflux from smooth muscle cells

In agreement with data from Li and Yokoyama (9) we found that biosynthetic cholesterol was released from SMCs in the presence of rLpA-I but not in the presence of lipid-free apoA-I (not shown). For this reason, we used only rLpA-I to investigate the effects of structural variation in apoA-I on cholesterol efflux from SMCs. Maximal cholesterol efflux was reached by incubation with at least 20 μg rLpA-I (not shown). The time kinetics of efflux of biosynthetic cholesterol were investigated by a 6-h incubation with DMEM/10% FAFA during which 20 μg rLpA-I was added at different time points. Efflux of biosynthetic [^{14}C]cholesterol increased rapidly during the initial 10-min incubation. Longer incubations led to a slow increase of radioactivity in the medium (**Fig. 4**). Because of the outcome of this pilot study, 10- and 360-min incubations with SMCs were chosen to test the cholesterol efflux capacity of rLpA-I reconstituted with apoA-I variants (**Table 2**). In contrast to rLpA-I with normal apoA-I, rLpA-I with apoA-I(R160L)$_{Oslo}$ or apoA-I(P165R) did not greatly enhance the efflux of biosynthetic cholesterol from SMCs taking place during the initial 10 min of incubation even in the absence of rLpA-I. Thus, initial cholesterol efflux was significantly decreased in the presence of apoA-I(R160L)$_{Oslo}$ or apoA-I(P165R). During incubation for 360 min, rLpA-I with normal and variant isoforms induced cholesterol efflux that was greater than efflux in the absence of rLpA-I. rLpA-I containing apoA-I(P165R) or normal apoA-I were equally effective. Cholesterol efflux in the presence of rLpA-I(R160L) was 17% lower than cholesterol efflux in the presence of normal rLpA-I. However, this difference was also not significant.

Cholesterol efflux from mouse peritoneal macrophages

As lipid-free apoA-I did not induce cholesterol efflux from SMCs, we investigated the effect of lipid-free apoA-I variants on the cholesterol homeostasis in mouse peritoneal macrophages. Kinetic dose-finding experiments indi-

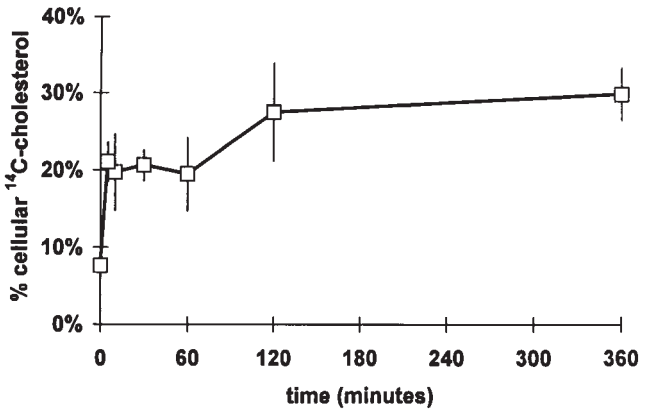

Fig. 4. Time-dependent mobilization and efflux of biosynthetic cholesterol from porcine smooth muscle cells by rLpA-I. Biosynthetic cholesterol of SMCs was radiolabeled by incubation with [^{14}C]mevalonic acid. Thereafter SMCs were in incubated for 6 h with DMEM/10% FAFA; 5, 10, 30, 60, 120, and 360 min before ending of this period 20 μg rLpA-I were added to the cells. Lipids extracted from cells and media was separated by TLC. The curve was deduced from mean values of two independent experiments and represent percentages of cellular [^{14}C]cholesterol that was found in the medium.

TABLE 2. Efflux and mobilization of biosynthetic cholesterol in smooth muscle cells by rLpA-I-containing normal apoA-I, apoA-I(P165R), or apoA-I(R160L)$_{Oslo}$

Series	Normal ApoA-I	ApoA-I (P165R)	ApoA-I (R160L)$_{Oslo}$
1: % Cellular [^{14}C]cholesterol			
10 min	18.4 ± 1.0	10.9 ± 3.5[a]	
360 min	34.1 ± 6.1	35.5 ± 0.7	
2: % Cellular [^{14}C]cholesterol			
10 min	28.8 ± 1.9		15.2 ± 2.2[a]
360 min	63.5 ± 2.3		52.9 ± 0.3

Biosynthetic cholesterol of smooth muscle cells was radiolabeled by incubation with [^{14}C]mevalonic acid. Thereafter, SMCs were incubated for 10 min or 6 h with DMEM/10% fatty acid-free albumin and 20 μg rLpA-I that contained either normal apoA-I, apoA-I(R160L)$_{Oslo}$, or apoA-I(P165R). The data are from duplicate experiments and represent the percentage of cellular [^{14}C]cholesterol that was found in the medium. All values represent mean ± SD.

[a] $P < 0.05$ (Student's *t*-test).

cated that incubation of mouse peritoneal macrophages with ⩾20 μg apoA-I per milliliter medium for 18 h induced a near maximal decrease in the cellular content of cholesteryl esters and a significant increase of cholesterol in the medium (**Fig. 5**). **Table 3** compares the effects of incubations for 18 h with normal apoA-I, apoA-I(R160L)$_{Oslo}$, and apoA-I(P165R) on the cellular content of cholesteryl esters and on the accumulation of cholesterol in the medium. The structural changes in apoA-I had no statistically significant effect on cholesterol efflux and the cellular content of unesterified and esterified cholesterol. It is, however, noteworthy that the percentage of cellular cholesterol released into the medium was always lower after incubation with the mutant proteins than after incubation with normal apoA-I isoforms. In parallel, the relative proportion of unesterified cholesterol but not of esterified cholesterol was increased in macrophages that were incubated with variant apoA-I isoforms.

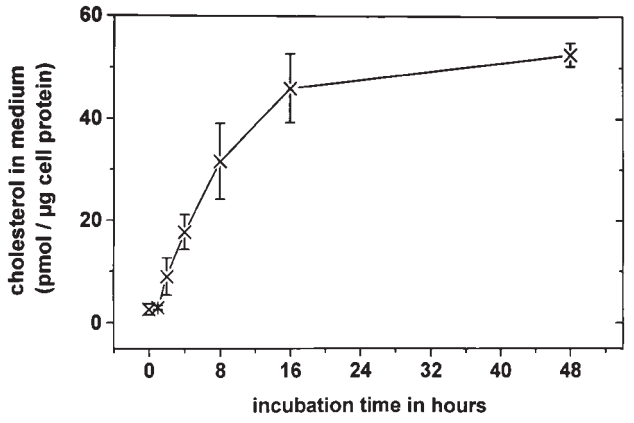

Fig. 5. Time-dependent cholesterol efflux from mouse peritoneal macrophages by lipid-free apoA-I. Mouse peritoneal macrophages were incubated for 24 h with 100 μg acetyl-LDL and were then incubated for increasing time intervals with 20 μg lipid-free apoA-I. Lipids of the medium were extracted in the presence of an internal standard and separated by HPLC. Data are from two independent experiments that were performed as triplicates.

TABLE 3. Distribution of cholesterol after incubation of mouse peritoneal macrophages with lipid-free normal apoA-I, apoA-I(P165R), or apoA-I(R160L)$_{Oslo}$

	pmol/μg cell protein (% of initial cellular cholesterol)[a]		
	Cholesterol in Media	Cellular Free Cholesterol	Cellular Cholesteryl Esters
ApoA-I(P165R)	41.0 ± 11.9 (34 ± 5)	58.1 ± 21.7 (52 ± 4)	12.0 ± 1.0 (14 ± 5)
Normal apoA-I	34.4 ± 12.6 (39 ± 1)	43.6 ± 16.2 (49 ± 2)	9.2 ± 0.2 (12 ± 2)
ApoA-I(R160L)$_{Oslo}$	47.4 ± 1.8 (41 ± 1)	61.2 ± 0.2 (52 ± 2)	8.3 ± 0.5 (7 ± 2)
Normal apoA-I	63.4 ± 10.4 (46 ± 6)	63.2 ± 9.3 (46 ± 8)	9.8 ± 3.2 (7 ± 0)

Mouse peritoneal macrophages were incubated for 24 h with acetyl LDL and were then incubated for 18 h with 20 μg lipid-free apoA-I isoforms. Lipids of the medium and cells were extracted in the presence of an internal standard and separated by HPLC. Cholesteryl esters refer to cholesteryl palmitate, cholesteryl oleate, cholesteryl linoleate, and cholesteryl arachidonate. Data are from triplicate experiments on apoA-I variants and the respective normal allele products. Differences between results obtained with normal and variant apoA-I isoforms are not significant. All values represent mean ± SD.

[a] Percentage of cellular cholesterol refers to the amount of cholesterol that was present in the cells before incubation, i.e., cellular free cholesterol + cellular cholesteryl esters + cholesterol in the medium equals 100%.

DISCUSSION

ApoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) are associated with about half-normal plasma levels of HDL-cholesterol and apoA-I in heterozygous carriers (23, 34). The metabolic basis of hypoalphalipoproteinemia is not known. In this study we have demonstrated that both apoA-I variants are defective in clearing a turbid emulsion of phospholipids. Moreover, although both variants form homogenous rLpA-I of regular size, rLpA-I containing either of these variants do not function normally as substrates of LCAT or in promoting initial efflux of biosynthetic cholesterol from SMCs. During longer incubations, however, both rLpA-I(R160L)$_{Oslo}$ and rLpA-I (P165R) did not differ significantly from normal rLpA-I in their ability to release biosynthetic cholesterol from SMCs. Likewise, during prolonged incubation with acetyl-LDL-loaded murine macrophages, lipid-free apoA-I variants induced regular hydrolysis of cholesteryl esters and cholesterol efflux. These data indicate that arginine residue 160 and proline residue 165 help to form a functional domain which is involved in initial lipid binding, LCAT activation, and promotion of at least initial cholesterol efflux by preformed rLpA-I. However, this domain does not appear to contribute to the stabilization of preformed rLpA-I or to the promotion of cholesterol efflux during prolonged incubation with either rLpA-I or lipid-free apoA-I.

Lipid binding and HDL formation by apoA-I

The lipid binding properties of apoA-I have frequently been attributed to the presence of several anti-parallel am-

phipathic α-helices rather than to specific amino acid residues within the molecule. Even deletion of one or several of the amphipathic α-helices encompassing amino acid residues 44–126, 100–143, 122–166, 139–170, 144–186, 190–243 in recombinant apoA-I variants did not interfere with the formation of rLpA-I by the cholate dialysis method although some of these particles were smaller than those produced with wild type apoA-I (19–22, 37–39). In contrast, the spontaneous association between apoA-I and DMPC which leads to the clearance of the turbid phospholipid emulsion was disturbed by substitutions of amino acid residues 160 and 165 (Fig. 2). It would therefore appear that single amino acid residues in apoA-I determine the kinetics of lipid association and are important for the initial lipid-binding properties of apoA-I rather than for the stabilization of preformed particles. In agreement with a central role of amino acid residues 160 and 165 for initial lipid binding, recombinant apoA-I deletion variants apoA-I(Δ122–165) and apoA-I(Δ144–186) but not apoA-I(Δ100–143) were found defective in clearing DMPC emulsions (20). It is, however, important to note that the carboxyterminal part of apoA-I was also shown to be involved in initial lipid binding (19).

LCAT activation by apoA-I

The mechanism by which apoA-I facilitates the esterification of cholesterol through LCAT is controversial. The amphipathic α-helices of apoA-I enable it to span the water/lipid barrier and thus render the substrates cholesterol and phosphatidylcholine accessible to LCAT (21). Accordingly, recombinant apoA-I variants in which one or two adjacent α-helices were deleted had reduced abilities to bind lipid and to activate LCAT (21, 37, 39). However, as apoA-I is a more potent activator of LCAT than other water-soluble and amphipathic apolipoproteins, it is also possible that apoA-I activates LCAT by means of direct protein/protein interactions. Two recombinant apoA-I chimeras, in which either the sequence of amino acid residues 143–164 was inverted or replaced with the sequence of amino acid residues 220–241 or in which amino acid residues 123–166 were replaced with amino acid residues 12–77 of apoA-II, had normal lipid-binding properties but reduced LCAT cofactor activity (22, 40). Moreover, in addition to apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) (Table 1 and ref. 25), other naturally occurring apoA-I variants, namely apoA-I(Δ107K), apoA-I(P143R)$_{Giessen}$, apoA-I(L159R)$_{Fin}$, and apoA-I(R173C)$_{Milano}$, have also been shown to be defective LCAT-activators (25, 36, 41–44). However, none of these defects completely prevented LCAT activation. Moreover, considerable variation has been observed already for the kinetic LCAT cofactor activity constants of various normal apoA-I preparations. Nevertheless, the clustering of defects with impaired LCAT cofactor activity between amino acid residues 143 and 173 suggests that the two α-helices formed by amino acid residues 143–186 contain a domain which plays a central role in activation of LCAT by apoA-I.

Cholesterol efflux stimulation by rLpA-I and apoA-I

Cholesterol efflux is a heterogenous process that involves the interaction of cells with either apolipoprotein/lipid complexes or lipid-free apolipoproteins (5, 6). Different cell types differ in terms of their cholesterol efflux kinetics and also in the availability of cholesterol for efflux induced by lipid-free apolipoproteins (5, 6). In this study and in agreement with data from other laboratories (9, 45, 46), we found that lipid-free apoA-I promotes cholesterol efflux from lipid-loaded mouse peritoneal macrophages but not from SMCs. rLpA-I (and native HDL_3, data not shown) induce biphasic mobilization and efflux of biosynthetic cholesterol from SMCs (Fig. 5). This phenomenon has also been described for cholesterol efflux from human skin fibroblasts as induced by native and reconstituted HDL (33). A first maximum of cholesterol efflux is reached during the initial 10 min of incubation with rLpA-I (Fig. 5) which is accompanied by a steep decrease in the cellular content of oxidizable cholesterol but not by changes in non-oxidizable cholesterol and cholesteryl esters (not shown). This suggests that cholesterol efflux occurs initially from the plasma membrane compartment. Longer incubations led to a slow cholesterol efflux (Fig. 5) that was associated with little changes in oxidizable plasma membrane cholesterol but with decreases in intracellular cholesterol and cholesteryl esters (not shown).

After 10 min of incubation, rLpA-I containing either apoA-I(R160L)$_{Oslo}$ or apoA-I(P165R) released significantly less biosynthetic cholesterol from SMCs than rLpA-I containing normal apoA-I (Table 2). After 360 min incubation, however, the differences in cholesterol efflux induced by normal rLpA-I, rLpA-I(P165R), and rLpA-I (R160L)$_{Oslo}$ were not statistically significant.

The defect in promotion of cholesterol efflux shown by rLpA-I(P165R) or rLpA-I(R160L)$_{Oslo}$ was, if anything, greater during short than long incubations and was paralleled by the degree of impairment these variants showed in clearing phospholiposomes. Nevertheless, both variants showed normal ability to form rLpA-I during cholate dialysis. It is therefore possible that the initial cholesterol efflux defect of these variants may involve a defect in the initial association with lipids.

The reduced cholesterol efflux capacity of rLpA-I with apoA-I(P165R) is in agreement with previous data from our laboratory which showed reduced cholesterol efflux from murine macrophages or adipocytes in the presence of apoA-I(P165R)/DMPC complexes. In those experiments, however, the cholesterol efflux defect of apoA-I(P165R) was also found after 360 min of incubation (26). In apoA-I(R160L)$_{Oslo}$ an amino acid exchange in this region is also associated with defective cholesterol efflux at least during a short incubation time. This suggests that the amphipathic helix containing an arginine at position 160 and a proline at position 165 forms a domain that contributes to the ability of apoA-I/lipid complexes to begin cholesterol efflux. In agreement with such a model, monoclonal antibodies that recognize epitopes located within amino acid residues 139–186 of apoA-I inhibited the fast phase of cholesterol efflux induced by plasma or HDL (15, 16). However, our data are in contrast to observations that complexes of DMPC with the recombinant apoA-I(Δ139–170) or with the natural mutant apoA-I(L159R)$_{Fin}$ produce regular efflux of choles-

terol from fibroblasts (38, 44). The discrepancy may originate from methodological differences, e.g., in the incubation times or radiolabeling procedures.

In the lipid-free form, both apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) did not differ from normal apoA-I in their capacity to promote cholesterol efflux from macrophages during 18 h of incubation. As apoA-I(Δ146→160)$_{Seattle}$ and amphipathic α-helical peptides without homology to apoA-I promote cholesterol efflux from CHO cells, fibroblasts, or macrophages as effectively as lipid-free apoA-I (46–48), we expected that single amino acid substitutions would have no effect on the ability of lipid-free apoA-I to induce cholesterol efflux. However, it must be emphasized that the percentage of cellular cholesterol released by the mutant apoA-I isoforms into the medium was always lower than the percentage of cellular cholesterol released by the respective normal apoA-I isoforms. Because the possibly defective recruitment of cellular lipids and the definitely defective clearance of phospholiposomes by lipid-free apoA-I(P165R) and apoA-I(R160L) may be exerted by similar mechanisms, additional work is necessary to rule out that these defects interfere with cholesterol efflux by lipid-free apoA-I. In this context it is noteworthy that particles formed by apoA-I$_{Seattle}$ or normal apoA-I in the presence of CHO cells differ in size and phospholipid composition (48).

The cell-specific differences in the availability of cholesterol for efflux by lipid-free apoA-I and rLpA-I, the different impacts of single amino acid exchanges on the efficacy of either initial versus prolonged cholesterol efflux or rLpA-I versus apoA-I-induced cholesterol efflux suggest that different domains in apoA-I are responsible for different types or phases of cholesterol efflux. It appears that at least initial cholesterol efflux by apoA-I/lipid complexes requires greater structural stringency than prolonged cholesterol efflux by lipid-free apoA-I or rLpA-I.

Conclusion

Both apoA-I(R160L)$_{Oslo}$ and apoA-I(P165R) are severely impaired in clearing a lipid emulsion but have a normal ability to form HDL-like particles during cholate dialysis. The abilities to activate LCAT and to stimulate cholesterol efflux appear to be moderately disturbed. These findings support the concept of a multi-domain model of apoA-I. In this model the alpha-helix formed by amino acid residues 143 to 165 of apoA-I appears to be of great importance for initial lipid binding but not for the stabilization of preformed particles. The same domain also appears to influence the ability of apoA-I to activate LCAT and to induce cholesterol efflux, although this structure–function relationship appears to be less stringent.

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