The Inverse Association of Plasma Lipoprotein(a) Concentrations with Apolipoprotein(a) Isoform Size Is Not Due to Differences in Lp(a) Catabolism but to Differences in Production Rate

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Abstract

Lipoprotein(a) (Lp[a]) is an atherogenic lipoprotein which is similar in structure to low density lipoproteins (LDL) but contains an additional protein called apolipoprotein(a) (apo[a]). Apo(a) is highly polymorphic in size, and there is a strong inverse association between the size of the apo(a) isoform and the plasma concentration of Lp(a). We directly compared the in vivo catabolism of Lp(a) particles containing different size apo(a) isoforms to establish whether there is an effect of apo(a) isoform size on the catabolic rate of Lp(a). In the first series of studies, four normal subjects were injected with radiolabeled S1-Lp(a) and S2-Lp(a) and another four subjects were injected with radiolabeled S2-Lp(a) and S4-Lp(a). No significant differences in fractional catabolic rate were found between Lp(a) particles containing different apo(a) isoforms. To confirm that apo(a) isoform size does not influence the rate of Lp(a) catabolism, three subjects heterozygous for apo(a) were selected for preparative isolation of both Lp(a) particles. The first was a B/S3-apo(a) subject, the second a S4/S6-apo(a) subject, and the third an F/S3-apo(a) subject. From each subject, both Lp(a) particles were preparatively isolated, radiolabeled, and injected into donor subjects and normal volunteers. In all cases, the catabolic rates of the two forms of Lp(a) were not significantly different. In contrast, the allele-specific apo(a) production rates were more than twice as great for the smaller apo(a) isoforms than for the larger apo(a) isoforms. In a total of 17 studies directly comparing Lp(a) particles of different apo(a) isoform size, the mean fractional catabolic rate of the Lp(a) with smaller size apo(a) was 0.329±0.090 day^{-1} and of the Lp(a) with the larger size apo(a) 0.306±0.079 day^{-1}, not significantly different. In summary, the inverse association of plasma Lp(a) concentrations with apo(a) isoform size is not due to differences in the catabolic rates of Lp(a) but rather to differences in Lp(a) production rates. (*J. Clin. Invest.* 1994. 93:2758–2763.) Key words: lipoprotein(a) • apolipoprotein(a) • atherosclerosis • lipoproteins • kinetics

Introduction

Lipoprotein(a) (Lp[a])[1] is an atherogenic lipoprotein particle in human plasma related in structure to low density lipoproteins (LDL) (1). Elevated plasma Lp(a) concentrations are associated with an increased risk of premature coronary heart disease (CHD) (2–6). Lp(a) levels are predictive of the extent of angiographically documented CHD independently of LDL cholesterol levels (7), although the relative risk of elevated Lp(a) concentrations is significantly increased in patients who also have high levels of LDL cholesterol (8, 9). One study estimated that the population attributable risk of CHD due to elevated Lp(a) levels was ~ 25% in men under 60 yr old (10). Family studies in a group of 102 probands with premature CHD indicated that Lp(a) excess was the most frequent familial lipoprotein disorder found in this cohort with premature CHD (11). It has been suggested that Lp(a) levels may account for most of the familial predisposition to premature CHD which cannot be accounted for by other known risk factors including LDL and HDL cholesterol levels (12).

Lp(a) is an LDL-like lipoprotein consisting of lipids and apoB-100, but differs from LDL in that it contains an additional protein called apolipoprotein(a) (apo[a]). Apo(a) is thought to be covalently linked to apoB via a disulfide bridge (13, 14), but can also associate noncovalently with apoB (15, 16). Lp(a) concentrations are strongly genetically determined (17), with at least 90% of the variation determined by variation within the gene for apo(a) (18). One important factor determining plasma Lp(a) levels is the apo(a) isoprotein phenotype, with a strong inverse correlation between the size of the apo(a) isoprotein and the Lp(a) concentration (19). To establish the mechanism by which apo(a) isoform size affects plasma Lp(a) concentrations, we performed a series of in vivo kinetic studies in humans directly comparing the metabolism of Lp(a) particles containing apo(a) isoproteins of different sizes.

Methods

Study subjects. 17 subjects participated in the studies. All subjects had normal fasting plasma glucose levels, and normal thyroid, liver, and renal function. They were free of illness and were on no medications. All subjects gave informed consent and the study protocol was approved by the Institutional Review Board of the National Heart Lung and Blood Institute.

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Received for publication 15 October 1993 and in revised form 25 January 1994.

1. *Abbreviations used in this paper:* apo(a), apolipoprotein(a); CHD, coronary heart disease; Lp(a), lipoprotein(a).

Isolation and iodination of Lp(a). For the first series of studies, Lp(a) particles were isolated from the fasting plasma of healthy subjects with one identifiable apo(a) isoform in plasma. S1-Lp(a) particles were isolated from an individual with a plasma Lp(a) level of 53 mg/dl, S2-Lp(a) particles were isolated from an individual with a level of 91 mg/dl, and S4-Lp(a) particles were isolated from an individual with a level of 36 mg/dl. Plasma was obtained after a 12-h fast and NaEDTA (0.01%), sodium azide (0.05%), and DFP (1 mM) were added. Lp(a) was isolated according to the procedure described by Fless et al. (13). Plasma was adjusted to a density of 1.21 g/ml using solid NaBr and ultracentrifuged for 48 h to isolate total plasma lipoproteins. The $d < 1.21$ g/ml fraction was adjusted to $d = 1.4$ g/ml with NaBr and ultracentrifuged on a 0–30% NaBr density gradient in order to remove HDL. The fraction containing Lp(a) was then adjusted to a concentration of 7.5% CsCl and ultracentrifuged for 30 h to separate Lp(a) from VLDL and LDL. Residual LDL was eliminated by chromatofocusing on a PBE94 column (Pharmacia, Uppsala, Sweden) within a pH range of 7.0 to 4.0.

In the second series of studies, three subjects who each had two clearly identifiable plasma apo(a) isoforms were used for preparative Lp(a) isolation. Plasma was adjusted to a density of 1.21 g/ml using solid NaBr and ultracentrifuged for 48 h to isolate total plasma lipoproteins. Lipoproteins were dialyzed vs. 0.1 M phosphate buffer, pH 7.4, with 0.01% Na_2EDTA, 0.01% NaN_3, and 1 mM benzamidine and then passed over a lysine-sepharose 4B column (Sigma Chemical Co., St. Louis, MO). The column was washed with the same phosphate buffer and then bound material was eluted with 20 mM epsilon amino caproic acid (EACA) in the same phosphate buffer. Eluted material was adjusted to a concentration of 7.5% CsCl and ultracentrifuged for 30 h at 45,000 rpm to separate the two classes of Lp(a) particles containing the two different apo(a) isoforms. The apo(a) isoform affects the density of the Lp(a) particle, as previously shown by us (20) and others (21), allowing effective separation of particles by density. Isolated Lp(a) was analyzed for purity by reducing and nonreducing SDS-PAGE and by 0.6% agarose electrophoresis (Helena Laboratories, Beaumont, TX).

All Lp(a) samples were extensively dialyzed against PBS with 0.01% EDTA before iodination using a modification of the iodine monochloride method (22). Briefly, an equal volume of a 1 M glycine buffer was added to the Lp(a) sample. 5 mCi ^{125}I or ^{131}I were then added, followed by ICI. Approximately one mole of iodine was incorporated per mole of Lp(a). Samples were dialyzed extensively against PBS/0.01% EDTA to remove free iodine. Human serum albumin was added to a final concentration of 5% (wt/vol), samples were sterile-filtered through a 0.22-μm filter and tested for pyrogens and sterility before injection. Iodinated Lp(a) particles were evaluated by SDS-PAGE and agarose electrophoresis.

Table I. Characteristics of Study Subjects

Subjects	Sex	Age	BMI	TG	Chol	LP(a)
		yr	*kg/m²*		*mg/dl*	
Series 1						
Normal subjects (n = 8)						
Mean		24	25.0	93	167	18
SD		3	3.1	29	28	30
Series 2						
Apo(a) heterozygotes						
1	F	72	26.2	80	214	197
2	M	76	22.2	77	199	153
3	F	65	24.6	202	270	133
Normal subjects (n = 6)						
Mean		21	23.6	77	170	15
SD		2	2.4	29	21	21

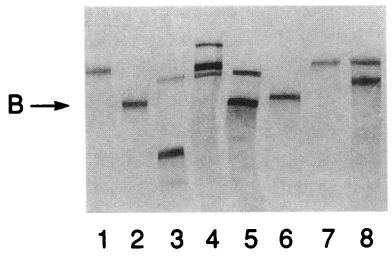

Figure 1. Immunoblot of plasma apo(a) from three heterozygous apo(a) subjects who served as donors for Lp(a) particle isolations and several apo(a) standards. Lane *1*, S3 standard; lane *2*, B standard; lane *3*, subject 3 (F/S3); lane *4*, subject 2 (S4/S6) lane *5*, subject 1 (B/S3); lane *6*, S1 standard; lane *7*, S4 standard; and lane *8*, S2/S4 standard. The *B* at left marks the position of apoB-100 and the apo(a)-B isoform.

Study protocol. Subjects were permitted to eat a normal diet but were instructed not to drink alcoholic beverages for 1 wk before and during the study. 1 d before injection, the subjects were given potassium iodide at a dose of 900 mg/d in divided doses and this was continued for the duration of the study. Radioiodinated Lp(a) was injected after a 12-h fast. Blood samples were obtained 10 min after injection and then at 1, 3, 6, 12, and 24 h, and at 2, 3, 4, 5, 7, 9, 11, and 14 d. Blood was drawn into tubes containing EDTA at a final concentration of 0.1%, immediately placed at 4°C, and plasma was separated by low-speed centrifugation in a refrigerated centrifuge. Sodium azide and aprotinin were added to plasma at a final concentration of 0.05% and 200 KIU/ml, respectively. Radioactivity in 4-ml plasma aliquots was quantitated in a Packard Cobra gamma counter (Packard Instrument Co., Downers Grove, IL). Plasma curves were constructed by dividing the plasma radioactivity at each time point by the plasma radioactivity at the initial 10-min time point. The fractional catabolic rates (FCRs) were obtained from the plasma radioactivity curves using a multi-exponential curve-fitting technique (23). Production rates (PR) were determined using the formula: PR = [(Lp(a) concentration) × (FCR) × (plasma volume)]/(body weight). Plasma volume was assumed to be 4% of body weight.

Apo(a) immunoblotting. Apo(a) isoform determination was performed on whole plasma using a sensitive immunoblotting technique previously described (20). Briefly, plasma samples were delipidated twice in chloroform-methanol 8:5 (vol/vol) and washed twice with phosphate buffered saline. Samples were reduced with 100 mM dithiothreitol in 8 M urea, incubated at 37°C for 30 min, and solubilized in 40 μl 0.02 M ethylmorpholine containing 10% SDS. Samples were applied to 7.5% polyacrylamide gel electrophoresis with 0.1% crosslinker (24) and run for ~ 4.5 h at 20 mA. After electrotransfer of the proteins to Immobilon PVDF transfer membranes (Millipore Corp., Bedford, MA), membranes were incubated with a 1:2,000 dilution of a monoclonal anti-apo(a) antibody (2D1; Cappel Laboratories, Durham, NC) and detected with the Vectastain ABC anti-mouse IgG test kit (Vector Laboratories, Burlingame, CA). Several plasma samples of known apo(a) isoform were used as calibration standards. In the apo(a) heterozygotes, the relative quantities of the two apo(a) isoforms were determined by laser scanning densitometry.

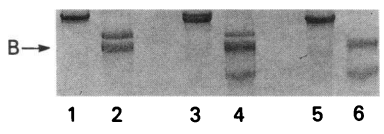

Figure 2. Nonreducing and reducing SDS-PAGE of purified Lp(a) particles from heterozygous apo(a)-F/S3 subject No. 3. Lanes *1*, *3*, and *5* are nonreducing; lanes *2*, *4*, and *6* are reducing. Lanes *1* and *2*, Lp(a) containing apo(a)-S3 isoform; lanes *5* and *6*, Lp(a) containing apo(a)-F isoform; lanes *3* and *4*, mixture of both purified Lp(a) particles. The *B* at left marks the position of apoB-100 and the apo(a)-B isoform.

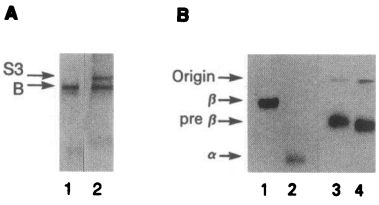

Figure 3. (*A*) Reducing SDS-PAGE of radioiodinated Lp(a) isolated from heterozygous subject 1: lane *1*, B-Lp(a) with single band in B position representing both B-apo(a) and apoB; lane *2*, S3-Lp(a) showing both labeled apo(a) (*upper band*) and labeled apoB (*lower band*). (*B*) Nondenaturing agarose electrophoresis of radioiodinated Lp(a) isolated from the same subject: lane *1*, LDL standard; lane *2*, HDL standard; lane *3*, B-Lp(a); lane *4*, S3-Lp(a).

Analytical methods. Plasma cholesterol and triglycerides were quantitated by automated enzymatic techniques on an Abbott VPSS analyzer (Abbott Laboratories, North Chicago, IL). Plasma apoB concentrations were determined by ELISA as previously described (25). Plasma Lp(a) concentrations were determined by a differential ELISA based on the method of Fless et al. (26), as previously described (22). Briefly, a monoclonal antibody against apo(a) (2D1; Cappel Laboratories) was used to coat microtiter plates at a concentration of 10 μg/ml. After blocking with 5% sucrose and 2% BSA, plasma samples at a 1:5,000 dilution were added to wells and incubated for 60 min at 37°C. A sheep polyclonal anti-apoB (Biodesign, Kennebunkport, ME) labeled with horseradish peroxidase was added to the wells at a 1:5,000 dilution and incubated for 60 min. Substrate was then added and absorbance read at 450 nm. The standard was a secondary plasma standard calibrated against two commercial Lp(a) standards (Terumo, Elkton, MD; and Immuno, Austria). Two controls were run with each assay. Intra- and interassay CV's were < 3% and < 10%, respectively.

Results

Characterization of study subjects. The clinical and lipid data on the study subjects are presented in Table I. Values are the mean of at least five fasting determinations made during the metabolic study. Immunoblots of plasma apo(a) in the three apo(a) heterozygotes who participated in these studies are shown in Fig. 1.

Analysis of Lp(a). Preparatively isolated Lp(a) was analyzed by reducing and nonreducing SDS-PAGE. A representative Coomassie blue–stained gel of the two Lp(a) particles isolated from one of the heterozygous subjects (apo[a]-F/S3) is illustrated in Fig. 2. The nonreduced lanes demonstrate the absence of apoB not complexed to apo(a). The reduced lanes demonstrate the purity of the isolations, each limited to only one apo(a) isoform plus the accompanying apoB. Electrotransfer and immunoblotting with an apo(a)-specific antibody confirmed the bands as apo(a). Radiolabeled Lp(a) particles were analyzed by SDS-PAGE and by agarose electrophoresis (Fig. 3). In all cases, radiolabeled apo(a) and apoB were intact and not degraded, and radiolabeled Lp(a) was found to migrate only in the prebeta region.

Metabolism of Lp(a). The first series of studies involved the isolation of Lp(a) particles from individuals with only one apo(a) isoform and direct comparison of the kinetics of Lp(a) particles containing different apo(a) isoforms isolated from different subjects. These studies indicated that the fractional catabolic rates of S1-Lp(a), S2-Lp(a), and S4-Lp(a) were not significantly different (Table II). However, the Lp(a) particles used for these studies were isolated from different individuals.

Table II. Fractional Catabolic Rates of Lp(a) Particles with Different Apo(a) Isoforms

	Apo(a) phenotype	F	B	S1	S2	S3	S4	S6	Diff*
					day^{-1}				
Series 1									
1	S2			0.420	0.394				0.026
2	S4			0.416	0.361				0.055
3	S4			0.461	0.393				0.068
4	S4			0.443	0.374				0.069
5	S2				0.244		0.213		0.031
6	S2				0.281		0.258		0.023
7	S4				0.242		0.222		0.020
8	S4				0.266		0.246		0.020
Series 2									
1‡	B/S3		0.251			0.266			−0.015
1-1	S3/S5		0.359			0.373			−0.014
1-2	S1/S5		0.360			0.382			−0.022
2‡	S4/S6						0.211	0.195	0.016
3‡	F/S3	0.257				0.303			−0.046
3-1	S5/S6	0.288				0.338			−0.050
3-2	S4/S5	0.289				0.322			−0.033
3-3	0	0.289				0.330			−0.041
3-4	S6	0.308				0.338			−0.030
Mean		0.286	0.323	0.435	0.319	0.332	0.230	0.195	0.005§
SD		0.018	0.063	0.021	0.067	0.037	0.021	—	0.039

In Series 1, three different homozygous individuals were the donors for the S1, S2, and S4-Lp(a) particles. * Difference: FCR of smaller apo(a) isoform minus FCR of larger apo(a) isoform. ‡ Heterozygous apo(a) subjects who were donors for isolated Lp(a) particles in Series 2. § Not different from zero by paired *t* test.

Therefore, in the second series of studies, we preparatively isolated both types of Lp(a) particles from three subjects who each had two identifiable apo(a) isoforms in plasma. In all cases, the smaller apo(a) isoform was in substantially higher plasma concentration than the larger apo(a) isoform by apo(a) immunoblotting (Fig. 1). The two Lp(a) particles isolated from the same individual were then radiolabeled and injected back into the same subject, and in some cases into other control subjects. The Lp(a) plasma curves in the three heterozygous apo(a) subjects whose autologous Lp(a) particles were isolated, labeled, and reinjected are illustrated in Fig. 4. In all cases, there was no difference in the catabolism of the two forms of Lp(a) in the three heterozygotes (Fig. 4) as well as in the control subjects. The fractional catabolic rates of Lp(a) for all studies are provided in Table II. The mean difference in FCR between the two Lp(a) particles of different apo(a) isoform was not significantly different from zero by paired t test. These results indicate that the size of the apo(a) isoform did not affect the in vivo catabolism of its associated Lp(a) particle.

In the three heterozygous subjects, the relative plasma concentrations of the two apo(a) isoforms were estimated by densitometric scanning of plasma apo(a) immunoblots and isoform-specific Lp(a) production rates were determined. In all three subjects, the production rate of the smaller apo(a) isoprotein was substantially greater than that of the larger apo(a) isoprotein (Table III).

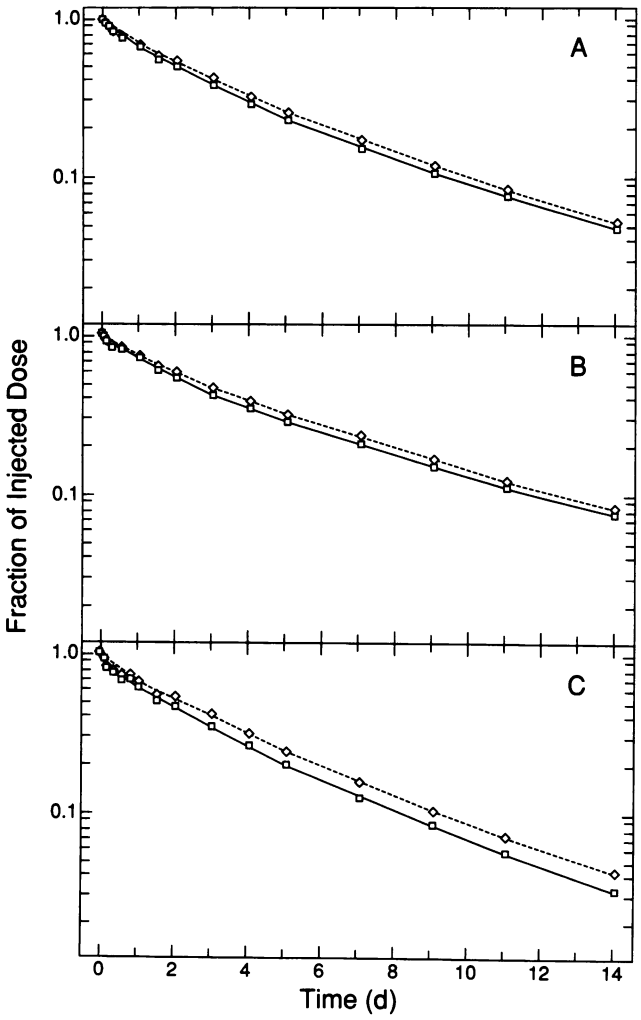

Figure 4. Plasma radioactivity curves of autologous radiolabeled Lp(a) particles isolated from heterozygous apo(a) subjects and injected back into the same subjects. (*A*) subject no. 1, (*B*) subject no. 2, (*C*) subject no. 3.

Table III. Lp(a) Kinetic Parameters in Heterozygous Apo(a) Subjects

Subject	Apo(a) isoform	Lp(a) conc	Lp(a) FCR	Lp(a) PR	PR ratio*
		mg/dl	*day^{-1}*	*mg/kg-d*	
1	B	134	0.251	13.4	2.0
	S3	62	0.266	6.6	
2	S4	106	0.211	8.9	2.4
	S6	47	0.195	3.7	
3	F	99	0.257	10.2	2.5
	S3	34	0.303	4.1	

* Ratio of the production rates of the smaller/larger apo(a) isoform.

Discussion

The inverse association of apo(a) isoprotein size and plasma Lp(a) concentration is well established (17–19). By apo(a) genotyping, the apo(a) size polymorphism has been estimated to account for ~ 70% of the variation in Lp(a) plasma levels (18). Because the physiologic basis for this inverse association has not been elucidated, we undertook a series of Lp(a) kinetic studies in humans to determine whether apo(a) isoform size affects Lp(a) catabolic or Lp(a) production rates.

In a series of eight normal subjects we established that S2-Lp(a) was not catabolized at a different rate than S1-Lp(a) or S4-Lp(a). To confirm that apo(a) isoform size does not affect Lp(a) catabolic rate, we then isolated Lp(a) from three subjects who had two distinct apo(a) bands on protein phenotyping. The size of the apo(a) isoprotein determines the density of the Lp(a) particle (20, 21), and this difference in density can be used to preparatively isolate Lp(a) particles containing different apo(a) isoproteins from the same heterozygous individual. In all cases, the catabolic rates of the two different Lp(a) particles were not significantly different, establishing that apo(a) protein size has no effect on the catabolic rate of its associated Lp(a) particle. Apo(a) isoform-specific production rates were determined in the three heterozygous subjects and were substantially higher for the smaller size apo(a) alleles. Therefore, the apo(a) size polymorphism does not affect catabolism of Lp(a) but rather is associated with variation in Lp(a) production.

Krempler et al. (27) reported the turnover of autologous Lp(a) in a series of nine subjects and found a correlation between Lp(a) level and production rate, but the study subjects were of undefined apo(a) phenotype. Knight et al. (28) reported the turnover of autologous Lp(a) in eight subjects of variable apo(a) phenotype and also found a correlation between Lp(a) level and production rate, but did not directly compare the metabolism of different apo(a) isoforms. We recently reported that differences in Lp(a) among individuals with the same apo(a) isoform are due to variation in Lp(a) production, not catabolism (22). These studies now establish

that the effect of apo(a) isoform size on Lp(a) concentration is also mediated through modulation of Lp(a) production, not catabolism.

A few potential limitations to the interpretation of these studies should be mentioned. First, only individuals with high plasma levels of Lp(a) were used as donors for the isolation of Lp(a) particles, in order to obtain enough pure Lp(a) to perform the kinetic studies. However, because these studies involved the direct comparison of two different apo(a) isoforms in the same individual, the fact that the Lp(a) particles were isolated from individuals with high levels is unlikely to have biased the results. Second, lysine–sepharose affinity chromatography was used to isolate the Lp(a) particles in the second series of studies, even though it has been demonstrated that not all plasma Lp(a) binds effectively to lysine–sepharose. However, this method of isolation is unlikely to have biased the conclusion, because the majority of the Lp(a) bound to the lysine–sepharose and because Lp(a) containing the two different isoforms were isolated in exactly the same fashion and injected back into the same individual. Third, radioiodination could theoretically modify the Lp(a) particles and obscure the ability to detect differences in catabolic rates among apo(a) isoforms. Although this possibility cannot be completely excluded, there is a variety of evidence to suggest that radiolabeling does not significantly affect Lp(a) kinetics. (*a*) There is no evidence by SDS-PAGE or agarose electrophoresis that Lp(a) is altered in its physical properties; (*b*) there are data from endogenous labeling of other apolipoproteins, such as apoA-I (29) and VLDL and LDL apoB (30) that radioiodination does not substantially affect their metabolism; and (*c*) Lp(a) turnover rates in several different laboratories using different iodination methods have been consistent with each other and are generally somewhat slower than LDL turnover rates. The final potential limitation is that laser densitometry of plasma apo(a) immunoblots may not be absolutely quantitative, and therefore the isoform-specific Lp(a) production rates in Table III may not be quantitatively accurate. Nevertheless, the basic conclusion—that a difference in Lp(a) production rates, not catabolic rates, is the basis for the difference in plasma concentrations among the apo(a) isoforms—is not changed.

The cellular mechanism of the inverse association of apo(a) size with rate of Lp(a) production remains to be established. The size polymorphism may be linked to other *cis*-acting factors which determine the rate of apo(a) gene transcription (31). Alternatively, the apo(a) size polymorphism could directly modulate apo(a) gene transcription, apo(a) mRNA stability, apo(a) protein translation, apo(a) posttranslational stability, or Lp(a) particle assembly and secretion. Azrolan et al. (32) reported that in a cynomolgus monkey model hepatic apo(a) mRNA transcript length did not correlate with hepatic apo(a) mRNA abundance. However, Wade et al. (33) reported that in human liver the size of the apo(a) mRNA transcripts was inversely correlated with apo(a) mRNA concentration. In both studies, variation in hepatic apo(a) mRNA abundance did not account for the majority of the variation in plasma Lp(a) concentrations. Recently, White et al. (34) reported that in baboon hepatocytes there was significant variation in apo(a) secretion rates among different apo(a) isoforms. Therefore, the size of the apo(a) isoprotein may affect its posttranslational intracellular metabolism before secretion.

In summary, the apo(a) size polymorphism does not substantially influence the plasma catabolic rate of Lp(a) in humans. This finding directs further investigation to the cellular basis of the inverse association between the apo(a) size polymorphism and the production rate of Lp(a).

Acknowledgments

We would like to thank Marie Kindt for excellent technical assistance, Betty Kuzmik and National Institutes of Health Clinical Center nursing staff for care of the study subjects, and the subjects who volunteered for these studies.

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