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Defective Catabolism and Abnormal Composition of Low-Density Lipoproteins from Mutant Pigs with Hypercholesterolemia†

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ABSTRACT: Metabolic and chemical properties of low-density lipoproteins (LDLs) were studied in a strain of pigs carrying a specific apo-B allele associated with hypercholesterolemia and premature atherosclerosis. LDL mass was significantly greater in mutant than in control pigs (400 ± 55 mg/dL vs 103 ± 26 mg/dL), as was LDL cholesterol. When normal and mutant LDLs were injected into the bloodstream of normal pigs, the fractional catabolic rate (FCR) of mutant LDL was about 30% lower than that of control LDL. In mutant pigs, the mean FCRs of mutant and control LDL were similar, although they were much lower than the corresponding FCRs observed in normal pigs. The density profile of LDL particles differed in control and mutant pigs; the peak LDL flotation rate was shifted from $S_f^0 = 5.3 \pm 1.9$ in controls to a more buoyant 7.4 ± 0.5 in mutants. The elevation of LDL in the mutants was restricted to the most buoyant LDL subspecies. This subpopulation of mutant LDL was enriched with cholesteryl ester (47% vs 37%) and depleted of triglyceride, relative to LDL of similar density and size in controls. The lipid compositions of the denser LDL subpopulations ($\rho > 1.043$ g/mL) were similar in mutants and controls. We conclude that the hypercholesterolemia of these mutant pigs is accounted for by defective catabolism of LDL. The buoyant cholesterol ester enriched LDL subspecies that accumulate in plasma may contribute to the accelerated atherogenesis that occurs in these animals.

Hypercholesterolemia is a major risk factor for coronary heart disease. Population genetic studies have estimated a heritability coefficient for hypercholesterolemia of 0.5–0.6 (Berg, 1983; Goldbourt & Neufeld, 1986). Despite the apparently high genetic contribution in determining cholesterol levels, the molecular basis underlying the genetic contribution is unknown for the vast majority of individuals.

Hypercholesterolemia in humans usually involves an elevation in the plasma concentration of low-density lipoprotein (LDL). Elevated LDL concentrations result from increased production and/or defective clearance of LDL (Grundy et al., 1985). In some instances, hypercholesterolemia is clearly related to a decrease in receptor-mediated LDL clearance. The best understood genetic cause of impaired receptor-mediated catabolism of LDL is familial hypercholesterolemia, which results from mutations in the gene encoding the LDL receptor (Brown & Goldstein, 1986). Diminished activity of the LDL receptor leads to a marked elevation in LDL levels in two ways: (1) LDL is inefficiently removed from the bloodstream, and (2) hepatic receptor-mediated clearance of intermediate density lipoprotein (IDL), the metabolic precursor of LDL, is decreased, leading to increased conversion of IDL to LDL (Bilheimer et al., 1982). Although mutations in the gene for LDL receptors are common relative to other known human mutations, the frequency of these mutations, 0.002, is considerably less than the frequency of hypercholesterolemia

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(approximately 0.1) in the American population. Thus, it is likely that other mutations contribute to the high prevalence of hypercholesterolemia in our society.

Defective catabolism of LDL due to a defect in the LDL particle might also underlie some cases of hypercholesterolemia. Apolipoprotein B (apo-B), the predominant protein in the LDL particle, has been shown to be highly polymorphic by immunological and genetic methods (Rapacz, 1978; Breslow, 1985; Butler & Morganti, 1974); thus, clinically relevant mutations may be relatively common. In one study aimed at identifying polymorphic proteins by immunizing pigs with whole plasma, apo-B was shown to be among the most polymorphic of the plasma proteins (Rapacz & Hasler, 1970). Conceivably, the unusual degree of polymorphism of apo-B might contribute to the high prevalence of hypercholesterolemia in the human population.

In a previous paper (Rapacz et al., 1986), we documented the presence, in a strain of pigs, of a unique allele for apo-B and two additional lipoprotein-borne antigens. These pigs have hypercholesterolemia and develop advanced lesions in their coronary arteries before 2 years of age. Unlike patients with familial hypercholesterolemia (Brown & Goldstein, 1986) and WHHL rabbits (Goldstein et al., 1983), the mutant pigs have normal LDL receptor activity (Rapacz et al., 1986). We now report that LDL from the mutant pigs is catabolized abnormally in vivo and that the catabolic defect accounts for the hypercholesterolemia of these animals. In addition, our characterization of the lipoprotein particles in these animals has revealed that the elevation in LDL occurs primarily in one buoyant subspecies of lipoprotein particles.

Materials and Methods

Animals. The selection of mutant pigs was based upon the presence of three immunologically defined lipoprotein-associated markers, Lpb, Lpr, and Lpu (Rapacz et al., 1986), and hypercholesterolemia. The basis for categorizing certain pigs as control animals was that they did not exhibit an elevation in the buoyant subspecies of LDL. Table I lists the animals used in this study and each animal's age at the time of analysis. Mature male breeders were excluded from this study because they show significant fluctuations in plasma cholesterol levels. Pigs were fed ad libitum a diet containing 0% cholesterol and 5% fat (University of Wisconsin gestation diet). Blood was collected aseptically from overnight fasted animals into a phosphate-buffered ethylenediaminetetraacetic acid (EDTA)/sodium azide cocktail (final concentration: 8 mM sodium and potassium phosphates; 2 mg/mL EDTA; 0.01% sodium azide; pH 7.4). After centrifugation, the plasma was combined with the following (final concentrations): phenylmethanesulfonyl fluoride, 0.5 mM; chloramphenicol, 20 μg/mL; glutathione, 0.05%. The plasma from all pigs was tested for the following 22 allotypes by a double immunodiffusion test, with allotype-specific polyclonal antibodies (Rapacz et al., 1976; Rapacz, 1978): Lpb 1–8, Lpb 11–18, Lpr 1 and Lpr 2, and Lpu 1–2. Lipoprotein genotypes were determined through analysis of allotypic phenotype, pedigree, segregation pattern, and mode of inheritance of these allotypes (Rapacz et al., 1976, 1986; Rapacz, 1978). In some cases, plasma samples were shipped on wet ice to the Donner Laboratory, University of California, Berkeley, where some of the lipoprotein isolations and analyses were performed. All lipoprotein isolations were performed within 2 weeks of the blood collection. Lipoprotein isolation for the metabolic experiments was begun immediately after blood collection.

Lipoprotein Subfractionation and Analysis. Very low density lipoproteins (VLDL; $\rho < 1.006$ g/mL), intermediate-density lipoproteins (IDL; $\rho = 1.006$–1.019 g/mL), and LDL ($\rho = 1.019$–1.063 g/mL) were isolated by preparative ultracentrifugation (Lindgren et al., 1972). Subfractions of LDL were isolated from a subset of animals by subjecting the whole LDL fraction to equilibrium density gradient ultracentrifugation (Shen et al., 1981). In brief, 2 mL of LDL was dialyzed to $\rho = 1.040$ g/mL against NaBr and was layered between 2.5 mL of NaBr, $\rho = 1.054$ g/mL, and 2.5 mL of NaBr, $\rho = 1.027$ g/mL, in a 7-mL tube. The contents of the tube were subjected to ultracentrifugation until equilibrium was reached (40000 rpm for 40 h at 17 °C). Fractions of 0.5 or 1.0 mL were removed by pipetting. Diameters of the major particles in each subfraction were estimated by nondenaturing gradient gel electrophoresis (below). Protein concentration was determined in each fraction (Markwell et al., 1978). Total cholesterol and triglyceride concentrations were determined with enzymatic reagents on a Gilford 3500 analyzer. Free cholesterol was determined by the enzymatic method of Allain et al. (1974), and phospholipid was determined by the method of Bartlett (1959).

Table I: Genotypes of Animals Used in This Study[a]

ID no.	animal	sex	Lpb	Lpr	Lpu	age (months)
controls						
1-1	XN-8	F	8/8	2/2	2/2	12
1-2	X24-2	F	3/4	1/2	2/2	5
1-3	T-7351	F	5/5	2/2	2/2	12
1-4	T-2022	F	5/5	2/2	2/2	12
1-5	T-7352	F	5/5	2/2	2/2	12
1-6	X24-1	F	2/4	1/2	2/2	7
1-7	X24-4	M	4/4	1/2	2/2	7
1-8	X24-7	M	2/4	1/1	2/2	7
1-9	X100-1	M	1/7	1/1	2/2	26
1-10	X83-9	F	7/7	2/2	2/2	53
1-11	XTC2-1	F	ND			18
1-12	XTC2-4	F	ND			18
1-13	XTC2-5	F	ND			18
1-14	X40-28	M	ND			4
1-15	X38-6	M	2/7	1/2	2/2	5
1-17	X9118	M	3/6	2/2	2/2	20
1-26	X38-7	M	2/2	2/2	2/2	13
mutants						
2-1	X88-3	F	5/5	1/1	1/1	30
2-2	X9396	F	5/5	1/2	1/–	14
2-3	X9061	F	5/5	1/1	1/1	26
2-4	X102-6	M	5/5	1/1	1/2	24
2-5	X90-3	F	5/8	1/1	1/2	37
2-6	X102-5	F	5/5	1/1	1/2	25
2-7	X37-9	M	5/5	1/2	1/–	5
2-10	X37-2	F	5/5	1/1	1/–	5
2-11	X6-9	M	5/5	1/–	1/–	3

[a] ND, not determined. All animals without a determined genotype were normocholesterolemic.

Analytical Ultracentrifugation. The $\rho < 1.063$ g/mL fraction, isolated by preparative ultracentrifugation, was analyzed by analytical ultracentrifugation in a Spinco Model E instrument with schlieren optics (Lindgren et al., 1972). Centrifugations were performed at 26 °C and 52640 rpm with a salt density of 1.061 g/mL. Photographs of the schlieren patterns were traced and entered into a computerized program for calculation of mass in the following ranges of flotation rate (S_f^0): 0–12, 12–20, 20–400 (Lindgren et al., 1972).

Other investigators have found that LDL in some strains of pig can be found beyond the density range of human LDL (Janado et al., 1966; Fidge & Smith, 1975). In the present study, analytical ultracentrifugation revealed no differences in LDL mass in lipoproteins isolated at $\rho < 1.063$ g/mL vs $\rho < 1.21$ g/mL.

Gradient Gel Electrophoresis. Nondenaturing gel electrophoresis was performed as described previously (Krauss &

Burke, 1982) on 2–16% polyacrylamide gels (Pharmacia). Latex beads (Duke Scientific Corp., Palo Alto, CA) were included with the high molecular weight standards (Pharmacia), to extend the range beyond the size of LDL (Krauss & Burke, 1982). Gels with whole plasma or IDL were stained with oil red 0, and those with LDL (ρ < 1.09–1.063 g/mL) were stained with Coomassie R-250 (Bio-Rad, Richmond, CA) and destained in a methanol–acetic acid mixture.

Lipid Concentration in Lipoprotein Fractions. For the analysis of the distribution of cholesterol and triglyceride among lipoprotein fractions, VLDL (ρ < 1.006 g/mL), IDL (ρ = 1.006–1.019 g/mL), and LDL (ρ = 1.019–1.073 g/mL) were isolated from 50–100 mL of plasma by sequential ultracentrifugation in a Beckman Ti 50.2 rotor for 24 h at 40000 rpm, 15 °C. HDL (ρ = 1.073–1.21 g/mL) was isolated by ultracentrifugation for 30 h under the same conditions. For these studies, cholesterol and triglyceride concentrations were determined by enzymatic assays (Sigma No. 351 and Sigma No. 320-UV, respectively).

Preparation and Radioiodination of LDL for in Vivo Turnover Studies. After initial isolation from plasma by sequential preparative ultracentrifugation (described in the previous section), those LDL solutions to be used for in vivo turnover studies were subjected to the following purification. The densities of the LDLs were changed to approximately 1.09 g/mL with solid NaBr. Approximately 4 mL of LDL solution was layered beneath 8 mL of a NaBr solution (ρ = 1.073 g/mL; 1 mM EDTA) and centrifuged in a Beckman Ti SW41 rotor, 30 h, 40000 rpm, 15 °C. The LDLs were collected from the top of the tube.

The choice of radionuclide for each LDL preparation was assigned randomly. LDL preparations were dialyzed overnight at 4 °C against phosphate-buffered saline (PBS: 0.9% NaCl; 8 mM sodium and potassium phosphates, pH 7.4; 1 mM EDTA). Insoluble material was pelleted by centrifugation at 10000 rpm, 30 min, 4 °C. Briefly, $Na^{125}I$ (Du Pont) or $Na^{131}I$ (ICN) (1 mCi/2.5 mg of protein) was injected into a septum-sealed vial containing 30 μg of the solid-phase catalyst 1,3,4,6-tetrachloro-6α,6α-diphenylglycoluril (Iodogen, Pierce) (Markwell & Fox, 1978). LDLs (3–10 mg of protein/mL, pH 9.5) were added to the vial. The reaction was allowed to proceed for 15 min at room temperature. LDL was then transferred to a second vial containing the catalyst and the incubation continued for 15 min. LDL was exhaustively dialyzed against PBS at 4 °C until the amount of free radioiodide was <1% of the total activity. The insoluble material was removed by centrifugation. Finally, iodinated LDLs were passed through a sterile 0.2-μm filter (Gelman Acrodisc) and stored at 4 °C. Labeled LDL preparations were used within 1 week. More than 98% of the radioactivity was precipitable by 10% trichloroacetic acid and more than 96% was protein-bound. Typical iodinated LDL preparations were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels, stained with Coomassie Blue R-250, revealed that >95% of the protein in LDL was apo-B100. Apo-E was not observed. By autoradiography and through analysis by a high-resolution imaging detector (Bioscan System 200, Washington, DC), we estimated that >95% of the ^{125}I radioactivity was associated with apo-B. Before filter sterilization, all LDL preparations were screened for bacterial contamination by streaking on agar plates. Relative electrophoretic mobility on agarose gels (Beckman Paragon lipoprotien gels) was checked before and after iodination. Preparations with anomalous electrophoretic mobility were discarded. The specific activities of radioiodinated preparations were 50–400 cpm/ng.

In Vivo Turnover Studies. Total LDL cholesterol was estimated just prior to the initiation of each experiment. Five animals (four animals, 20–45 kg; one animal, approximately 125 kg) were injected 1 or 2 times. A total of nine turnover studies were conducted.

The experimental protocol was approved by the campus Research Animal Resources Center, the Animal Committee of the School of Veterinary Medicine, and the University of Wisconsin Safety Committee. Under anesthesia (Innovar/Ketamine), three jugular catheters were surgically implanted into each pig. The animals were allowed to recover from the anesthesia (5–24 h) prior to injection of the tracers. Animals were maintained with a continuous intravenous infusion of lactated Ringers solution (0.3 mL/min) to prevent blood from clotting in the catheters and were given free access to water and feed (UW gestation diet). Nonautologous and autologous LDLs were simultaneously injected (2 μCi of tracer/kg of body weight) through one catheter, and blood samples (6 mL) were collected from a second catheter into tubes containing EDTA (final concentration 1 mg/mL). Ordinarily, 14–18 samples were collected. Samples were typically collected at 15, 30, and 45 min; 1.25, 1.75, 2.75, and 3.75 h; 7, 12, 18, 24, and 30 h; and then every 12 h until the study was completed. With the exception of experiment 1 (Table VI), all experiments lasted 72–96 h. Plasma samples were precipitated in trichloroacetic acid (final concentration 10% w/v; 4 °C), and ^{125}I and ^{131}I radioactivities in the pellet were determined in a Packard γ counter.

The plasma disappearance curves for each LDL preparation were analyzed by a two-pool model, and kinetic parameters (slope and intercept for each exponential) were estimated with the DEXP program (developed and kindly supplied by W. W. Cleland; available upon request) on an IBM PC. This program uses an interactive least-squares procedure by the Gauss–Newton method (Cleland, 1979).

LDL apo-B production rates were estimated by assuming that the entire LDL fraction behaved as a kinetically homogeneous pool, although this assumption has not yet been experimentally tested. LDL protein concentrations were estimated by multiplying the LDL protein to LDL cholesterol ratios of the purified LDL preparation by the total plasma LDL cholesterol concentration.

Statistical Analysis. Student's *t* test (Snedecor & Cochran, 1980) was used for comparisons of plasma lipids, lipoproteins, and analytical ultracentrifuge data between mutant and control pigs. Repeated measures analysis of variance (Winer, 1971) was used for overall comparisons of size and composition of LDL subfractions between groups and between subfractions. Individual comparisons of these data and the lipoprotein concentration data were made with the 1-tailed Student's *t* test. The probability threshold was adjusted with Bonferroni's inequality (Snedecor & Cochran, 1980). Plasma disappearance curves were compared with the Fisher *F* test (Beck & Arnold, 1977). This test compares two models of each paired set of turnover curves: a full model of both curves taken separately and a reduced model of the two curves combined. If the two models are significantly different (p < 0.05), then the two paired curves are different.

Results

Distribution of Triglyceride and Cholesterol in Plasma. Mutant pig plasma had approximately a threefold elevation in cholesterol, almost entirely in LDL (Table II). The mutant pigs in this study also exhibited a 9-fold increase in IDL cholesterol and an almost 2-fold increase in VLDL triglyceride.

Table II: Plasma Lipid Distribution in Control and Mutant Pigs[a]

cholesterol (mg/dL)	control (n = 7)	mutant (n = 8)
plasma	82.7 ± 22.6	247.7 ± 56.3[b]
VLDL	2.0 ± 1.2	4.3 ± 1.5[c]
IDL	0.5 ± 0.4	4.8 ± 4.4
LDL	41.7 ± 16.8	185.7 ± 44.1[d]
HDL	30.5 ± 7.0	20.9 ± 6.3[e]
triglyceride (mg/dL)	**control (n = 6)**	**mutant (n = 5)**
plasma	44.9 ± 17.6	68.5 ± 15.7
VLDL	21.0 ± 11.3	41.3 ± 10.41[f]
IDL	5.4 ± 2.7	4.5 ± 3.2
LDL	7.6 ± 2.6	8.5 ± 2.7
HDL	5.6 ± 2.1	3.3 ± 2.8

[a] Mean ± SD. p values were calculated with correction for Bonferroni's inequality. n denotes number of animals. Animals. Cholesterol: (control) 1-1, 1-2, 1-7, 1-8, 1-14, 1-15, and 1-17; (mutant) 2-1, 2-2, 2-3, 2-4, 2-5, 2-6, 2-7, and 2-10. Triglyceride: (control) 1-1, 1-2, 1-8, 1-10, 1-15, and 1-17; (mutant) 2-1, 2-2, 2-3, 2-4, 1-15, and 2-10. [b] Mutant value significantly different from control value, $p = 0.00014$. [c] Mutant value significantly different from control value, $p = 0.016$. [d] Mutant value significantly different from control value, $p = 0.00006$. [e] Mutant value significantly different from control value, $p = 0.038$. [f] Mutant value significantly different from control value, $p = 0.034$.

Table III: Peak Flotation Rates and Concentration of S_f^0 = 20–400 Lipoproteins in Control and Mutant Pigs[a]

			total mass (mg/dL)		
	n	peak S_f^0	S_f^0 = 0–12	S_f^0 = 12–20	S_f^0 = 2–400
control	13	5.3 ± 1.9	103 ± 26	0.5 ± 1.0	3.6 ± 4.8
mutant	5	7.4 ± 0.5[b]	400 ± 55[b]	62 ± 23[b]	17 ± 21

[a] Control animals: 1-1, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, and 1-13. Mutant animals: 2-1, 2-2, 2-3, 2-4, and 2-5. n denotes number of animals. [b] $p < 0.005$.

Characterization of the Lipoprotein Particles. There were striking differences in mass, size distribution, and chemical composition of lipoproteins of S_f^0 = 0–20 between control and mutant pigs, as demonstrated by analytical ultracentrifugation (Figure 1). The patterns from control pigs revealed little material in the region of S_f^0 = 12–20 and a moderate mass of lipoprotein of S_f^0 = 0–12. In contrast, the patterns from mutant pigs showed significant increases in mass in both regions, especially in the larger, more buoyant LDL of S_f^0 = 5–12. Mean peak S_f^0 rates and total mass of $\rho < 1.063$ g/mL lipoproteins (S_f^0 = 0–400) are shown in Table III. It is clear that the mutant pigs have substantially more LDL and IDL mass and that the peak amount of this mass has a higher flotation rate than is seen in the control pigs.

Protein concentrations of LDL density subfractions in three mutant and eight control animals are shown in Figure 2. The three mutant animals had profiles remarkably similar to each other and distinctly different from the profiles of control animals. The elevation of LDL protein in mutant pigs occurred specifically in the most buoyant density region. Protein concentrations of the more dense LDL subfractions were not different between mutants and controls.

Chemical compositions of LDL subfractions are shown in Table IV. Composition was not computed for the first fraction (ρ = 1.022 g/mL) because the LDL mass was below detectable levels in six out of the eight control animals. In all remaining LDL subfractions, the relative protein content was similar for mutants and controls. Mutant LDL had higher cholesterol ester content across all density fractions (p = 0.068), with the greatest differences occurring in the most buoyant LDL particles. Mutant LDL had lower percent triglyceride across all density fractions (p = 0.061), with the greatest differences also occurring in the most buoyant LDL subfractions. Unesterified cholesterol content did not vary between mutants and controls in any density range.

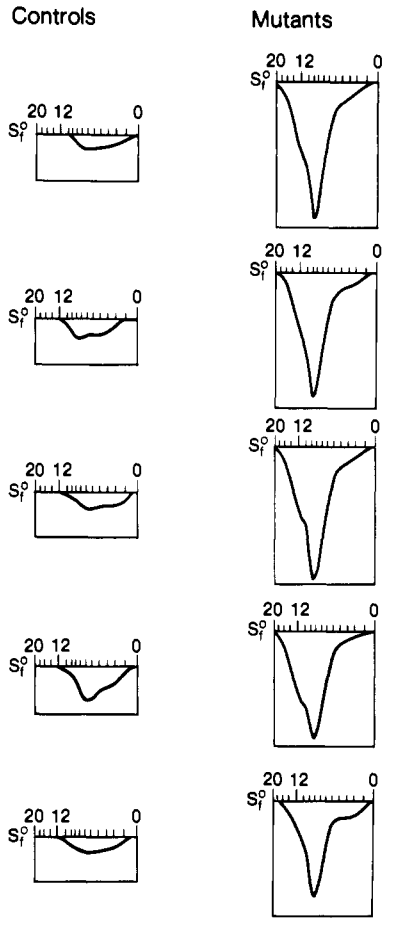

FIGURE 1: Schlieren patterns reflecting analytical ultracentrifugation of LDL and IDL (S_f^0 = 0–20) from five control and and five mutant pigs. Centrifugation speed was 52 640 rpm at 26 °C with a NaBr solution of density 1.061 g/mL. Control animals (from top): 1-4; 1-6; 1-7; 1-9; 1-10. Mutant animals (from top): 2-1; 2-2; 2-3; 2-4; 2-5.

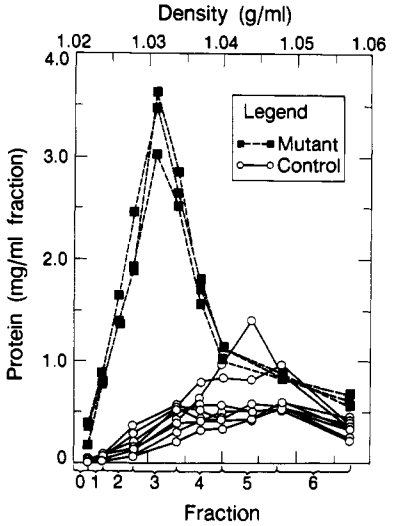

FIGURE 2: Distribution of protein following density gradient ultracentrifugation of LDL from control and mutant pigs. Centrifugation proceeded until equilibrium was reached (40 000 rpm for 40 h at 17 °C). Control animals: 1-1; 1-2; 1-3; 1-4; 1-5; 1-6; 1-7; 1-8. Mutant animals: 2-3; 2-4; 2-5.

Nondenaturing gradient gel electrophoresis revealed considerable heterogeneity within lipoproteins of ρ = 1.063 g/mL in both control and mutant pigs. Gel scans from whole plasma and $\rho < 1.019$ g/mL and ρ = 1.019–1.063 g/mL plasma

Table IV: Composition of Pig LDL Subfractions[a]

fraction	group[b]	% composition				
		protein	triglyceride	chol ester	cholesterol	phospholipid
1	control	15.9 ± 5.2	21.3 ± 17.2	23.4 ± 26.1	14.8 ± 12.9	24.5 ± 2.9
	mutant	16.1 ± 1.4	2.2 ± 0.2	48.1 ± 0.5	11.4 ± 0.9	22.2 ± 0.5
2	control	19.8 ± 2.2	8.4 ± 5.0	37.7 ± 6.1	10.5 ± 3.0	23.6 ± 1.6
	mutant	18.3 ± 0.6	2.0 ± 0.3[c]	48.0 ± 1.0[c]	10.5 ± 0.2	21.3 ± 0.9
3	control	21.0 ± 1.1	4.1 ± 1.5	42.7 ± 2.3	9.4 ± 1.0	22.7 ± 0.7
	mutant	19.9 ± 0.8	1.9 ± 0.4	47.8 ± 1.3[c]	9.7 ± 0.2	20.7 ± 1.0[c]
4	control	22.3 ± 2.0	4.0 ± 0.7	43.1 ± 1.9	8.6 ± 1.1	22.0 ± 1.1
	mutant	22.0 ± 0.5	2.5 ± 0.2[c]	45.1 ± 0.8[c]	9.3 ± 0.4	20.0 ± 0.5
5	control	26.0 ± 2.3	4.8 ± 0.7	41.6 ± 2.6	7.4 ± 1.4	20.2 ± 1.6
	mutant	25.9 ± 2.0	3.8 ± 0.6	42.6 ± 1.2	7.8 ± 0.5	19.9 ± 0.7
6	control	29.6 ± 1.5	6.1 ± 0.7	37.7 ± 4.7	7.4 ± 2.7	19.2 ± 1.8
	mutant	29.8 ± 1.6	4.7 ± 0.8	39.8 ± 1.2	7.3 ± 0.8	18.4 ± 0.8.

[a] Mutant and control animals were the same as for Figure 2. For controls, $n = 8$; for mutants, $n = 3$. n denotes number of animals, the values are mean ± SD. [b] Data from fraction 0 was not analyzed due to insufficient lipoprotein mass concentration in this fraction. [c] Mutant value significantly different from control value, $p < 0.05$ after correction with Bonferroni's inequality.

Table V: Diameter of Major Particles in Pig LDL Subfraction[a]

	fraction						
	0	1	2	3	4	5	6
density (g/mL)	1.022	1.024	1.028	1.033	1.039	1.047	1.057
particle diameters (Å)							
controls ($n = 8$)	301 ± 4[b]	291 ± 3	278 ± 3	268 ± 2	259 ± 2[c]	248 ± 3	242 ± 3
					253 ± 4	243 ± 3	
mutants ($n = 3$)	301 ± 2[b]	291 ± 1	284 ± 2	272 ± 2	265 ± 2	248 ± 3	241 ± 4
			278 ± 3	271 ± 2	257 ± 3		

[a] Mutant and control animals were the same as for Figure 2. n denotes number of animals. [b] Mean ± SD. [c] One-half-milliliter fractions were taken in the range of greatest LDL mass. When two numbers are shown for a given density, they represent average diameters for each of the two 0.5-mL fractions.

Table VI: Plasma Turnover of Mutant and Control LDL in Control Pigs[a]

animal	LDL protein (mg/dL)	experiment	FCR (pools/h)		synthetic rate (mg dL^{-1} h^{-1})	F test[b]
			control	mutant		
1-2 (2-6)	41.0	1[c]	0.0478	0.0313	1.96	$p < 0.01$
		2	0.0388	0.0263	1.59	$p < 0.005$
1-14 (2-7)	39.5	3	0.0423	0.0310	1.67	$p < 0.005$
		4	0.0458	0.0324	1.81	$p < 0.01$
1-15 (2-10)	ND	5	ND	0.0289	ND	NA
			0.0437[d]	0.0300[d]	1.76[d]	NA[d]

[a] Samples were collected for 72–96 h except where noted. Nonautologous donor is noted in parentheses. ND, not determined. NA, not applicable. [b] Fisher F test was used to compare plasma disappearance curves for mutant versus control LDL. [c] A 44-h time course. [d] Mean.

Table VII: Plasma Turnover of Mutant and Control LDL in Mutant Pigs[a]

animal	LDL protein (mg/dL)	experiment	FCR (pools/h)		synthetic rate (mg dL^{-1} h^{-1})	F test[b]
			mutant	control		
2-7 (1-15)	84.2	6	0.0168	ND	1.42	NA
		7	0.0173	0.0138	1.46	$p < 0.05$
2-6 (1-17)	100.1	8	0.0157	0.0182	1.57	NS
		9	0.0174	ND	1.74	NA
			0.0168[c]	0.0160[c]	1.55[c]	NA[c]

[a] Samples were collected for 72–96 h. Nonautologous donor is noted in parentheses. NS, not significantly different ($p > 0.05$). ND, not determined. NA, not applicable. [b] Fisher F test was used to compare plasma disappearance curves for mutant versus control LDL. [c] Mean.

fractions from representative control and mutant animals are shown in Figure 3. In control pigs, three discrete peaks were seen in whole plasma, with diameters of 268, 257, and 244 Å. Particles of similar diameter were observed in LDL (ρ = 1.019–1.063 g/mL). Mutant whole plasma contained particles of similar size to those of controls but also was comprised of LDLs with modal diameters of 299 and 282 Å. The particles of 299 Å were in the plasma fraction corresponding to IDL ($\rho < 1.019$ g/mL), while the 282-Å lipoproteins coincided with the most abundant particles in the LDL fraction of mutants.

Diameters of LDL particles corresponding to specific densities along the gradient are shown in Table V. In general, particle diameter decreased with increasing density. The relationship between particle diameter and density was the same for control and mutant pigs.

In Vivo Catabolism of Control and Mutant Pig LDL. Plasma disappearance curves for control and mutant LDL in control and mutant pigs are shown in Figure 4. In control pigs, the fractional catabolic rate (FCR) of mutant LDL was about 30% lower than the FCR for control LDL (Table VI). Two major differences were observed when results obtained in mutants were compared with those obtained in control pigs (Table VII): (1) In mutant pigs the FCRs of both control and mutant pig LDLs were substantially lower than rates seen in control pigs, and (2) the FCRs of the two types of LDL were similar.

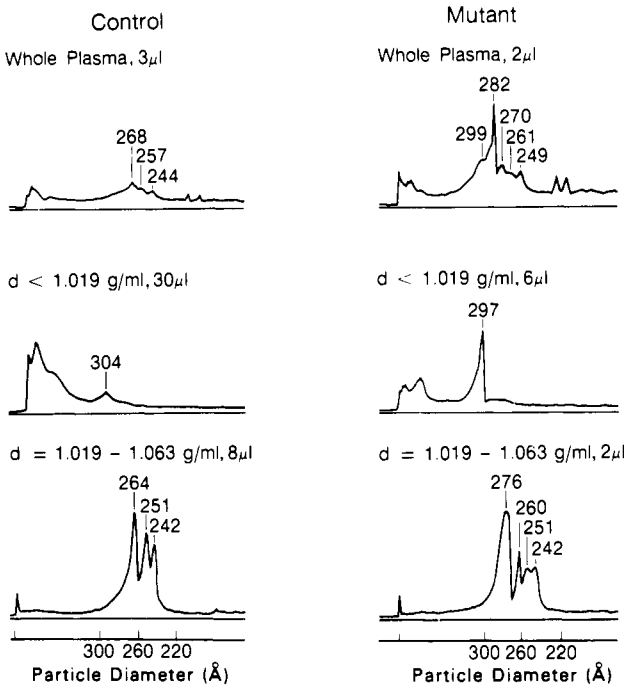

FIGURE 3: Scans of nondenaturing gradient gels performed for whole plasma, IDL (ρ < 1.019 g/mL), and LDL (ρ = 1.019–1.063 g/mL) from representative control and mutant pigs. Particle diameters (Å) are shown above major peaks. Volumes shown are volumes of each sample applied to the gel. Acrylamide gradients extended from 2 to 16%. The gels were stained with either oil red 0 or Coomassie R-250. One control and one mutant animal were used. Control animal: 1-8. Mutant animal: 2-4.

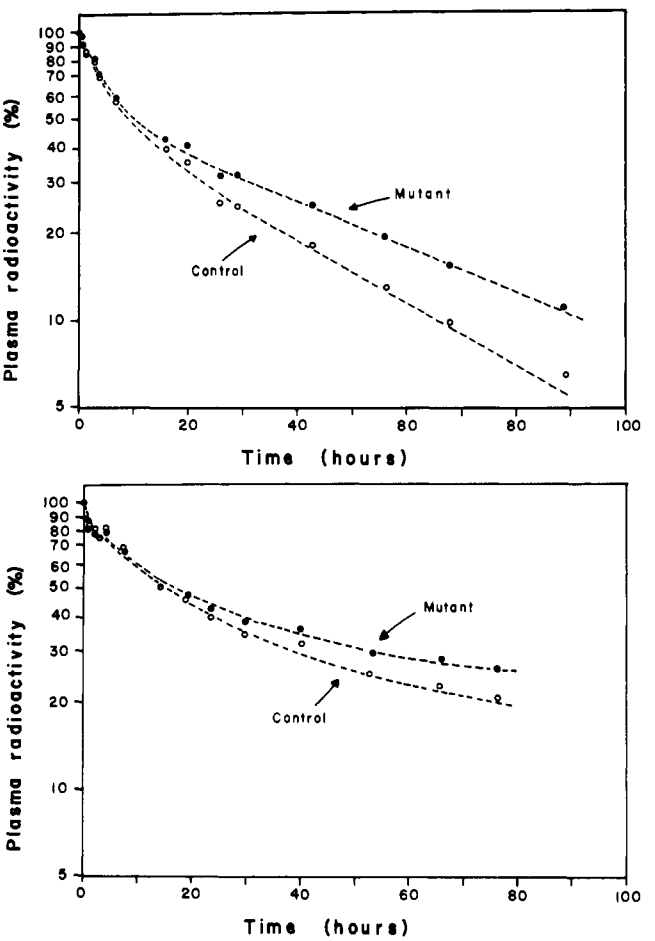

FIGURE 4: Representative plasma disappearance curves of control and mutant LDLs in a control pig (top; Table VI, experiment 3) and a mutant pig (bottom; Table VII, experiment 8). Each animal was simultaneously injected with ^{125}I- and ^{131}I-labeled mutant and control pig LDL.

Since the size of the LDL pool was elevated approximately twofold in the mutants used in these studies, we calculated a synthetic rate for LDL that was similar in control and mutant pigs (Tables VI and VII). The FCRs in our control pigs were similar to those previously reported (Calvert et al., 1975; Sniderman et al., 1975; Carew et al., 1976; Attie et al., 1979; Pittman et al., 1979).

Discussion

In recent years, many of the human apolipoproteins have been shown to be polymorphic (Breslow, 1985). Immunological and genetic studies (Young et al., 1986; Marcel et al., 1985) have also identified polymorphisms in human apo-B. Recently, an *Xba*I restriction fragment length polymorphism was found in the apo-B genes of individuals, from certain populations, with increased incidence of myocardial infarction (Hegele et al., 1986). Since these individuals had normal plasma cholesterol levels, it was not clear how the mutation in apo-B might be causally related to the increased risk of myocardial infarction.

Increased concentrations of LDL and plasma apo-B levels are correlated with an increased risk of coronary artery disease (Brunzell et al., 1984). Consequently, mutations in apo-B that affect LDL catabolism might place an individual at risk for heart disease if these mutations lead to an increase in the plasma LDL concentration. Given the prevalence of hypercholesterolemia in the human population, such mutations might be relatively common. Supporting this possibility is a recent report describing a series of patients with primary hypercholesterolemia, possessing LDL more slowly catabolized in vivo than simultaneously injected LDL from normocholesterolemic individuals (Vega & Grundy, 1986).

Since pigs, like humans, spontaneously develop atherosclerosis, they provide a good model for studying the possible role of apolipoprotein mutations in coronary artery disease. Furthermore, we have found, as reported by other investigators (Fidge & Smith, 1975; Calvert et al., 1975), that pig and human LDL have similar ultracentrifuge flotation characteristics. In previous studies (Janado et al., 1966; Calvert et al., 1975; Jackson et al., 1976) two major pig LDL subpopulations were described. In this paper, LDL heterogeneity was further characterized. Specifically, four distinct subpopulations were identified which have homology to major LDL subspecies in humans (Krauss & Burke, 1982). Three of these species were similarly represented in mutants and controls. The fourth, the largest and most buoyant, was present in substantially greater concentration in the mutant animals.

In addition to a predominance of buoyant LDL in mutant pigs, there were differences in composition of mutant and control LDL subspecies in the most buoyant density range (ρ = 1.019–1.043 g/mL). Although the differences were not significant at $p < 0.05$, mutant LDLs were enriched in cholesterol ester and depleted in triglyceride relative to controls. Because of these differences in particle composition, our in vivo turnover studies assessed particles that differed in two ways: (1) they had different core lipid compositions, and (2) they were comprised of apo-B molecules encoded by different apo-B alleles.

Cholesterol feeding of normal pigs induces changes in LDL subpopulations similar to those seen in our mutant pigs, even though the mutant pigs were maintained on a 0% cholesterol diet. Miniature pigs fed a high-fat, 3% cholesterol diet developed hypercholesterolemia, increased LDL mass, and a shift

to increased buoyancy in the predominant LDL species (Hill et al., 1975), as well as increases in IDL and β-VLDL. Another group (Mahley & Weisgraber, 1974) also found cholesterol feeding to be associated with increased LDL mass in miniature pigs. Therefore, the cholesterol-fed animals and the spontaneously hypercholesterolemic animals may have similar abnormalities of LDL and IDL subspecies distribution, associated with delayed clearance.

We observed a relatively low FCR of mutant LDL in control pigs. This is most likely due to defective binding to the LDL receptor. This conclusion is supported by preliminary in vitro experiments in which mutant LDL was recognized more poorly than control LDL by the LDL receptor (unpublished observations). We cannot eliminate the possibility that defective LDL clearance may also be attributable to differences in LDL particle size or composition. For example, buoyant cholesterol-enriched LDL, regardless of the type of apo-B carried on the particle, might be catabolized more slowly than other forms of LDL, and the difference in turnover of the two types of LDL we studied might merely reflect the predominance of buoyant LDL in the plasma of mutant pigs. Indeed, Calvert et al. (1975) reported that in the pig the more buoyant LDL_1 (ρ = 1.035 g/mL) is cleared more slowly than LDL_2 (ρ = 1.050 g/mL). Preliminary studies from our laboratory show that the control buoyant LDL subspecies is degraded by cultured pig fibroblasts at a slower rate than the control dense LDL subspecies (unpublished observations).

Although the mutant pigs have normal LDL receptor activity (Rapacz et al., 1986), the FCR of normal LDL was much lower in these animals than in the controls. Since LDL in the mutant pigs is richer in cholesterol (cholesterol:protein ratio = 1.8 in mutants and 1.3 in controls), when expressed in terms of LDL cholesterol, mutant pigs are clearing approximately 40% more LDL cholesterol than control pigs. This increased turnover of LDL cholesterol might produce a downregulation of the LDL receptor. An additional possibility is that enhanced synthesis and removal of cholesterol-rich IDL that is not further processed to LDL might efficiently downregulate the hepatic LDL receptor. Although we did not study the turnover of VLDL or IDL, the increase in IDL pool size and the normal LDL synthetic rate in mutants pigs are both consistent with increased flux through IDL. Such phenomena would account for the slow catabolism of control LDL in mutant pigs. Our observations in the pigs differ from those described in hypercholesterolemic patients by Vega and Grundy (1986); their patients catabolized control LDL at a normal rate.

We were surprised to observe, in the mutant pigs, a similar FCR for control and mutant LDL. Our data do not rule out the possibility that control LDL is modified in the mutant pig in a manner affecting its rate of catabolism. The prolonged residence time of LDL in the circulation might subject it to more extensive modification, leading to the formation of a more buoyant lipoprotein particle. When control radiolabeled LDL was injected into a mutant pig, there was a pronounced broadening in the density profile to encompass the buoyant density range (unpublished observations). Kostner et al. (1985) have reported differences in the lipid composition of LDL isolated from plasma containing active or inactive lecithin:cholesterol acyltransferase. In their studies, LDL isolated from plasma with the active enzyme was cleared from the circulation at a significantly slower rate than LDL not exposed to active enzyme.

Recently, 2-year changes in plasma IDL concentration were shown to predict progression of coronary heart disease in hypercholesterolemic men (Krauss et al., 1987). Conceivably, cholesterol ester rich buoyant IDL and LDL are part of a family of particles that are especially atherogenic by virtue of their ability to deposit their lipid cargo in cells destined to become foam cells in the arterial wall.

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Association of Melittin with the Isolated Myosin Light Chains†

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ABSTRACT: Melittin is a 26-residue peptide which undergoes high-affinity calcium-dependent binding by calmodulin [Barnette, M. S., Daly, R., & Weiss, B. (1983) *Biochem. Pharmacol. 32*, 2929; Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269; Anderson, S. R., & Malencik, D. A. (1986) *Calcium Cell Funct. 6*, 1]. The results in this paper show that three different types of myosin light chain—the smooth muscle regulatory light chain, the smooth muscle essential light chain, and the skeletal muscle regulatory 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) light chain—also associate with melittin. The resulting complexes have dissociation constants ranging from 1.1 to 2.5 μM in the presence of 0.10 M NaCl and from ~50 to ~130 nM in solutions of 20 mM 3-(*N*-morpholino)propanesulfonic acid alone. The regulatory smooth muscle myosin light chain exhibits two equivalent melittin binding sites while each of the others displays only one. The myosin light chains evidently contain elements of structure related to the macromolecular interaction sites present in calmodulin and troponin C but not in parvalbumin. The association of melittin and other peptides with the light chains requires consideration whenever assays of the calmodulin-dependent activity of myosin light chain kinase are used to determine peptide binding by *calmodulin*. The binding measurements performed on the DTNB light chain and melittin necessitated derivation of the equation relating complex formation to the observed fluorescence anisotropy of a solution containing three fluorescent components. This analysis is generally applicable to equilibria involving the association of two fluorescent molecules emitting in the same wavelength range.

X-ray crystallography and amino acid sequencing studies revealed a family of evolutionarily related calcium-binding proteins: calmodulin, the myosin light chains, troponin C, parvalbumin, the brain-specific S-100 protein, and the intestinal calcium-binding protein [cf. Kretsinger (1980)]. Calmodulin is probably the most generalized in function of the calcium-modulated proteins. The calcium–calmodulin complex is a well-recognized activator of cyclic nucleotide phosphodiesterase (Cheung, 1967), adenylate cyclase (Cheung et al., 1975; Brostrom et al., 1975), phosphorylase kinase (Grand et al., 1981), myosin light-chain kinase [cf. Stull (1980) and Small and Sobieszek (1980)], and calcineurin [cf. Tallant and Cheung (1986)]. Calmodulin also undergoes calcium-dependent interactions with small molecules. These include a number of peptides exhibiting common structural features—notably clusters of basic amino acid residues in close conjunction with hydrophobic sequences [cf. Anderson and Malencik (1986)]. Although most of the known peptide–calmodulin associations probably do not occur in vivo, they proved to be useful models for protein binding by calmodulin. The prediction that sequences similar to those of the peptides occur in calmodulin-dependent enzymes (Malencik & Anderson, 1982) was borne out by the structures of high-affinity calmodulin-binding fragments prepared from skeletal muscle and smooth muscle myosin light-chain kinases (Blumenthal et al., 1985; Lukas et al., 1986).

Melittin, a 26-residue peptide from honey bee venom, is one of the more widely publicized calmodulin-binding peptides. Its potent inhibition of the calmodulin-dependent activity of cyclic nucleotide phosphodiesterase was first described by

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