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Lipoprotein Mutations in Pigs Are Associated with Elevated Plasma Cholesterol and Atherosclerosis

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A strain of pigs bearing three immunogenetically defined lipoprotein-associated markers (allotypes), designated Lpb5, Lpr1, and Lpu1, has marked hypercholesterolemia on a low fat, cholesterol-free diet. Unlike individuals with familial hypercholesterolemia or WHHL rabbits, the affected pigs have normal low density lipoprotein receptor activity. The animals, by 7 months of age, have extensive atherosclerotic lesions in all three coronary arteries. This strain of pig represents an animal model for atherosclerosis and hypercholesterolemia associated with mutations affecting the structures of plasma lipoproteins. One of the variant apolipoproteins, Lpb5, is apolipoprotein-B. A second variant apolipoprotein (Lpr1), termed apo-R, is a 23-kilodalton protein present in both the very low density ($d < 1.006$ g/ml) and the very high density ($d > 1.21$ g/ml) fractions of pig plasma. Isoforms of this protein correlate with two *Lpr* alleles, Lpr^1 and Lpr^2. The *Lpr* genes segregate independently of the Lpb^5 and Lpu^1 alleles. The Lpu1 allotype is a component of low density lipoprotein and is genetically linked to Lpb^5.

CORONARY HEART DISEASE IS THE leading cause of death in the United States as well as in some other countries. Although epidemiological studies and studies in research animals have revealed as causes a number of environmental risk factors, they also disclose a strong genetic contribution to the risk of developing the disease (*1*). Several mutations predisposing individuals to atherosclerosis have been identified. For example, familial hypercholesterolemia (FH) is a disease caused by mutations affecting the receptor for low density lipoprotein (LDL) (*2*). The WHHL rabbit also has an inborn deficiency of LDL receptor activity and develops premature atherosclerosis (*3*). FH is the most common known inherited metabolic disease associated with premature atherosclerosis in humans, but FH and the other known mutations are rare relative to the high incidence of atherosclerosis in the human population. It is therefore likely that other mutations may exist which lead to premature atherosclerosis. We now report an association between mutations in plasma lipoproteins and atherosclerosis in a strain of pigs.

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The principal bloodborne transporter of cholesterol in many animal species, including human and pig, is LDL. Progress in the biochemical identification of mutations in the protein moiety of LDL has been hindered by its unusual physical properties (*4*). Apo-B, which makes up more than 90 percent of LDL protein, is difficult to solubilize by conventional detergent solubilization techniques used to study membrane proteins. The protein has a molecular size of 514 kD (*4, 5*).

Although apo-B has been resistant to biochemical analysis, valuable information has been obtained through immunogenetic investigation. Polyclonal alloimmune antibodies have been used to identify polymorphism in cattle, carp, chicken, human, mink, pig, rabbit, rhesus monkey, and sheep (*6*). Monoclonal antibodies identified three human apo-B markers encoded by three alleles (*7*) but no discernible clinical phenotypic differences were found between individuals bearing these different alleles.

In our studies lipoproteins obtained from pigs of various breed origins were used as alloantigens to obtain immune sera exhibiting different patterns of precipitation reactions with randomly selected pig sera. Genetic studies demonstrated that most of the epitopes associated with the LDL particles are inherited in groups of eight (*6, 8*). This led to the conclusion that the set of eight epitopes is encoded by a single codominant allelic apo-B gene. A total of eight different sets has been found. Each set has seven epitopes in common and differ from the other sets by one distinctive characterizing epitope. Thus there are a total of 16 epitopes, each defined by an alloantiserum. For clarity we have omitted the names of the

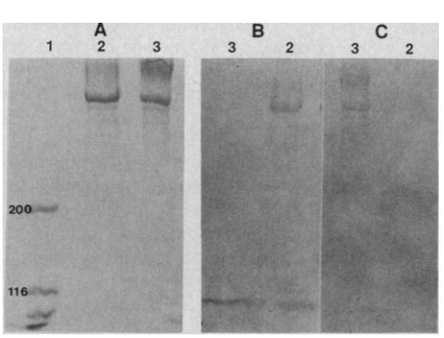

Fig. 1. Immunoblot of LDL's with antibodies to Lpb3 and antibodies to Lpb5. (Lane 1) Molecular size markers in kilodaltons; lane 2, LDL from an $Lpb^{1/3}$ pig; lane 3, LDL from an $Lpb^{5/5}$ pig. (A) Coomassie blue stain. (B) Immunoblot with antibodies to Lpb3. (C) Immunoblot with antibodies to Lpb5 (*20*).

Table 1. Summary of pig genotypes and corresponding cholesterol phenotypes (*19*).

Genotype	Plasma cholesterol* (mg/dl)
$Lpb^{x/x}$, $Lpr^{2/2}$, $Lpu^{2/2}$†	81.3 ± 12.7 (n = 136)‡
$Lpb^{5/5}$, $Lpr^{2/2}$, $Lpu^{2/2}$	103.0 ± 13.88 (n = 105)‡
$Lpb^{5/5}$, $Lpr^{1/-}$, $Lpu^{1/-}$	176.5 ± 63.3 (n = 175)‡

*All pairwise comparisons were judged significantly different with the Bonferroni method; $\alpha = 0.05$. †x represents alleles other than 5 and 8. ‡$P < 0.008$ for comparisons among groups.

common epitopes and refer only to those that are characteristic of the product of each allelic gene.

The epitopes of apo-B and the corresponding genes were designated originally Lpp and subsequently renamed Lpb [L, lipoprotein; p, pig; b, apo-B or apo-B locus (*6*)]. Numerals 1 to 8 refer to specific allotypes, allelic genes, or their products. For example, an animal with the genotype $Lpb^{5/5}$ has the phenotype Lpb5.

Antisera specific for the Lpb5 and the Lpb3 epitopes were used in an immunoblotting experiment with LDL from animals of genotype $Lpb^{5/5}$ or $Lpb^{1/3}$ (Fig. 1). The antiserum reacted only with apo-B100, specifically with the Lpb5 epitope or the Lpb3 epitope.

Altogether, we screened more than 14,000 pigs older than 3 months of age. All of the pigs with elevated cholesterol in the plasma (above 125 mg/dl) had the Lpb^5 allele (*8*). Animals homozygous for Lpb^5 did not always exhibit the same degree of hypercholesterolemia. The heterogeneity of animals carrying the Lpb^5 allele was investigated by preparing alloantisera in the two groups of animals. The serum lipoprotein fraction of $Lpb^{5/5}$ animals with a moderate increase in plasma cholesterol was injected into $Lpb^{5/5}$ animals that showed a much more pronounced increase in plasma cholesterol. The converse experiment was also performed. Recipient animals produced antibodies that reacted with two additional lipoprotein-borne markers. The loci corresponding to these markers were named *Lpu* and *Lpr* (*6, 8*). (There is no particular significance to the letters u and r.) Two alleles at each of these loci were found. Animals with the most pronounced hypercholesterolemia have at least one copy of Lpu^1 and Lpr^1. Both genes are very rare in the tested pig population.

Plasma cholesterol concentration in 4-month-old pigs fell into three distinct groups (Table 1). Pigs with the Lpb^5 apo-B mutation had a mean increase in plasma cholesterol of 22 mg/dl compared to that in pigs carrying the alleles other than Lpb^5. Pigs with the Lpb^5 apo-B mutation carrying Lpu1 and Lpr1 had a further increase in mean plasma cholesterol of 73 mg/dl. The statistical differences in plasma cholesterol levels between these three groups of animals were highly significant (*9*), suggesting that these three gene loci encode proteins or are linked to genes which affect plasma cholesterol levels.

Pedigreed pigs of defined lipoprotein genotypes were used to obtain progeny for estimating linkage relations between the three apoprotein loci. Genetic analysis of the segregation data of the progeny indicated tight linkage between *Lpu* and *Lpb*. No recombination event was observed in 244 progeny. Thus far the Lpu1 antigen has only been found in animals bearing Lpb5, but animals expressing Lpb5 can also express Lpu2. Our data therefore do not exclude the possibility that the *Lpb* and *Lpu* genes are located at one complex locus.

The *Lpr* locus, by contrast, is not linked with the *Lpb* and *Lpu* loci. This conclusion follows from the results of 27 matings; none of the matings, producing 192 progeny, showed a significant departure (*10*) from independent assortment of alleles at the two loci.

Animals with the genotype, $Lpb^{5/5}$, $Lpu^{1/X}$, $Lpr^{1/X}$ (where X can be either 1 or 2) fed a standard low-fat pig diet (3 percent fat, 0 percent cholesterol, University of Wisconsin gestation diet) show a two- to threefold elevation in total plasma cholesterol (Table 1). The cholesterol elevation is primarily due to an LDL elevation. The mutant pigs also show a dramatic 30-fold increase in IDL (intermediate density lipoprotein) cholesterol but this plasma fraction contributes only modestly to total plasma cholesterol. We therefore refer to these animals as hypercholesterolemic mutants. It should be empha-

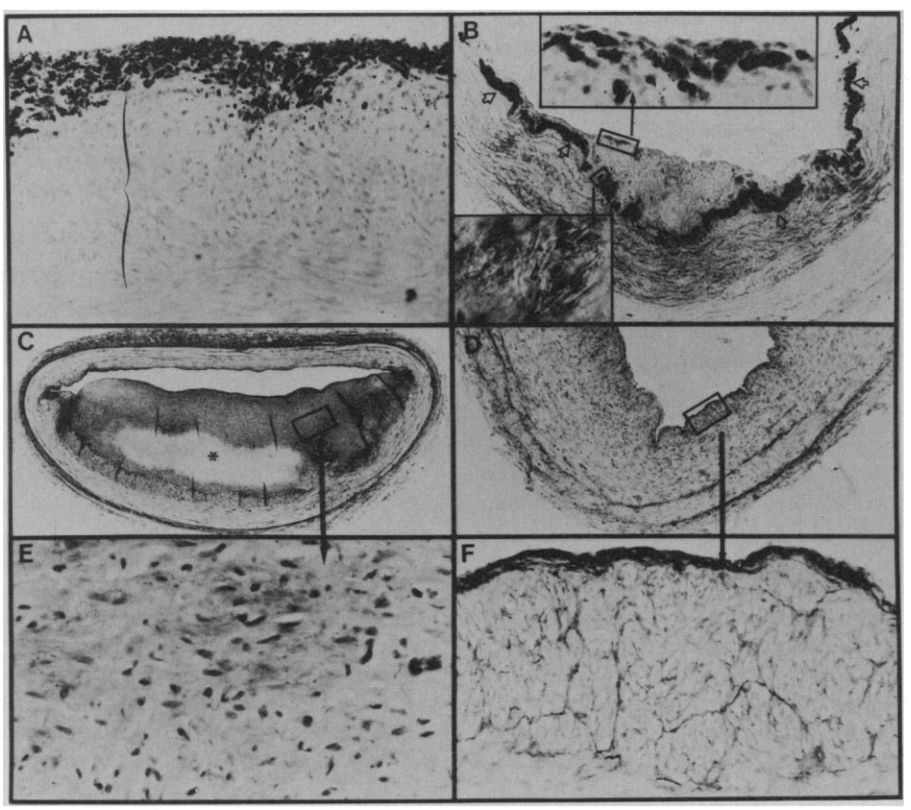

Fig. 2. Lesions in the right coronary artery of the $Lpb^{5/5}$, $Lpr^{1/1}$, $Lpu^{1/1}$ mutants compared with controls. (A) A typical fatty streak lesion from the right coronary artery of a 7-month-old mutant pig showing oil red O–positive neutral lipid contained within the cells. These lesions were composed of macrophage-like foam cells overlying an area of moderate intimal smooth muscle cell hyperplasia (bracket). (B) From mutant pig at 21 months of age. Lipid is predominantly extracellular in the form of crystals 18 to 26 μm in length (lower inset), in the intima. Foam cells are a prominent feature of even the most advanced lesions (upper inset). (C) Distal portion of the same right coronary artery, stained with GTAF, shows a stenosing lesion with a necrotic core (asterisk) and a thin fibrous cap (graded ++++ in Table 2). (D) Right coronary artery in a control pig matched for age, sex, and heart weight showing the absence of lesions (stained with GTAF). (E) Cellular hyperplasia in the intimal compartment. This is abnormal, especially when it is associated with lipid infiltration, fibro-histiocytic cell populations, and loss of fascicular architecture. (F) Higher magnification of (D) showing moderate medial hyperplasia consisting of longitudinally oriented medial smooth muscle fascicles located immediately below the internal elastic lamina. Moderate smooth muscle cell hyperplasia in the inner media is considered normal in pigs.

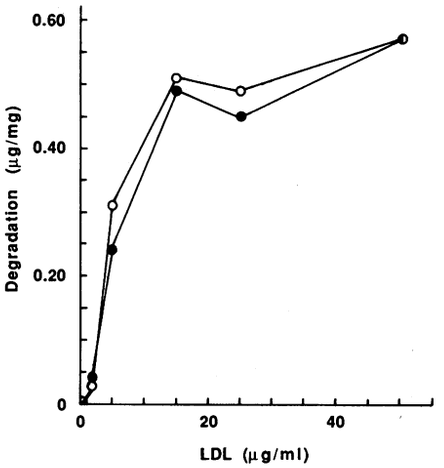

Fig. 3. Degradation of LDL by skin fibroblasts from a normal (●-●) and a mutant (O-O) pig. The experiment was performed as described (*21*).

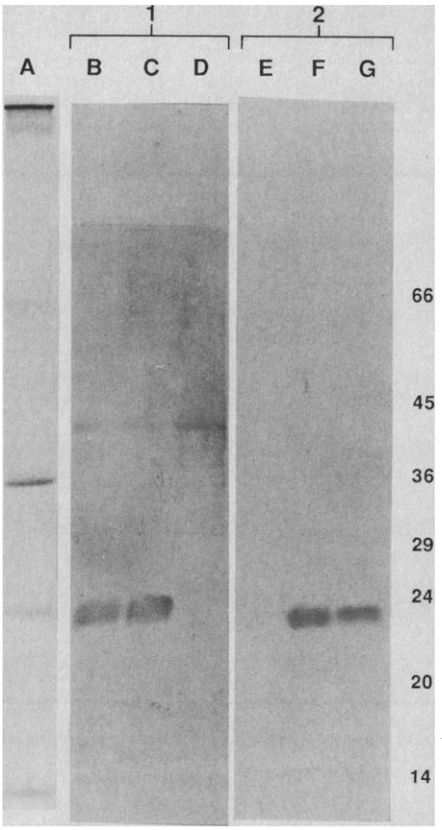

Fig. 4. Immunoblot of VLDL reacted with alloantiserum (*12*) against Lpr1 and Lpr2 antigens. (Lanes B to D) Reaction with alloantibodies to Lprl; (lanes E to G) reaction with antibodies to Lpr2; (lanes B and E) VLDL from an $Lpr^{1/1}$ pig; (lanes C and F) VLDL from an $Lpr^{1/2}$ pig; (lanes D and G) VLDL from an $Lpr^{2/2}$ pig. Lane A is a companion lane stained for protein. Molecular markers, in kilodaltons, are shown on the right.

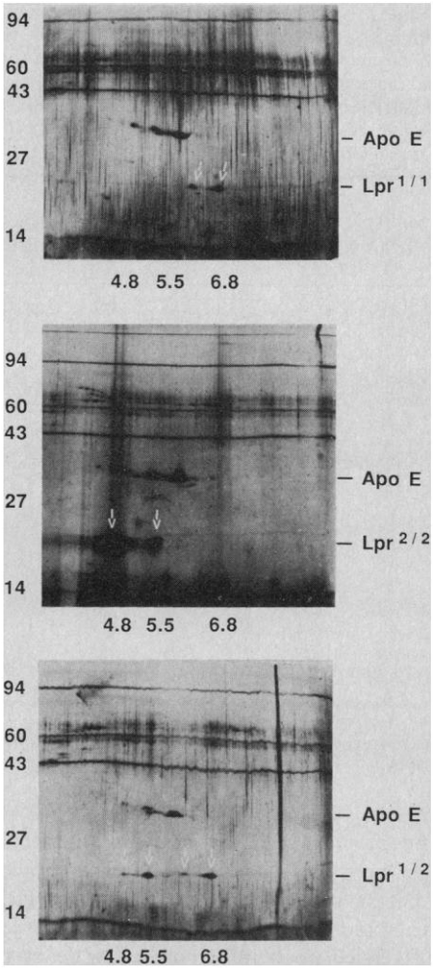

Fig. 5. Two-dimensional electrophoresis of VLDL from an $Lpr^{1/1}$ pig (top), VLDL from an $Lpr^{2/2}$ pig (middle), and VLDL from an $Lpr^{1/2}$ pig (bottom). Electrophoresis was performed by the method of Kendrick *et al.* (*22*). Molecular size markers (in kilodaltons) are shown at the left. The numbers at the bottom of each gel indicate the *p*H.

sized that every animal with the genotype $Lpb^{5/5}$, $Lpu^{1/X}$, $Lpr^{1/X}$ had high lipoprotein cholesterol levels.

Hearts from 26 number-coded pigs were prepared for histological examination of the three main coronary arteries. Seventeen of the pigs were hypercholesterolemic mutants ($Lpb^{5/5}$, $Lpu^{1/1}$, $Lpr^{1/-}$). The remaining nine pigs were normolipidemics ($Lpb^{non\text{-}5}$, $Lpu^{2/2}$, and were either $Lpr^{1/1}$, $Lpr^{2/2}$, or $Lpr^{1/2}$ genotypes at the *Lpr* locus). The animals were of both sexes and ranged in age from full-term fetuses to 62 months. In the 7-month-old mutant pigs, fatty streak or foam cell lesions were extensive (Fig. 2A), and at 21 months of age complex lesions were common (Fig. 2B) and large enough to restrict blood flow (Fig. 2, C and E). The right coronary artery from a 21-month-old control pig had moderate smooth muscle cell hyperplasia in the media immediately below the internal elastic lamina (Fig. 2, D and F), a normal finding (*11*).

In mutant pigs (Table 2) the earliest lesions developed throughout the right coronary artery. Between 7 and 14 months, the mutants developed extensive lesions in all three main coronary arteries. The severity became more pronounced with age. Mutant pigs studied over the past 8 years typically died before reaching 4 years of age (less than one-third of their normal life expectancy). Mutant pigs were matched for age, gender, and heart weight to nine pigs with $Lpb^{non\text{-}5}$, $Lpu^{2/2}$ genotypes. Control pigs, maintained on the same diet, were 2 to 62 months of age, with a mean serum cholesterol value of 78 ± 27 mg/dl. Only one of the nine animals showed moderate lipid infiltration. However, older animals did not show lipid infiltration or any other cellular changes associated with lesions.

Coronary atherosclerosis in the pigs resembled human disease in several respects: complex lesions were preceded by fatty streaks. Lesions were eccentrically placed and localized at branchpoints, and they developed first in the right coronary artery and become most severe in the epicardial portions of the right coronary and left anterior descending coronary artery. The complex lesions had a fibrous cap and a necrotic core. Although pigs often develop atherosclerosis between 6 and 8 years of age, the mutants deviated markedly from the normal animals in developing severe disease within 2 years.

Two well-studied models for inherited hypercholesterolemia and atherosclerosis—FH in humans and the WHHL rabbit—have defects in LDL receptor activity (*2, 3*). These defects fully account for the elevation in plasma LDL because they lead to inefficient catabolism of LDL. We excluded the possibility that the hypercholesterolemia in our mutant pigs is due to genetic defects in LDL receptor activity in the following way. Skin fibroblasts were obtained from normal and mutant pigs in order to measure LDL degradation in vitro. Since binding to the LDL receptor is rate-limiting for LDL degradation, the latter measurement is a sensitive measure of LDL receptor activity (*2*). The rate of degradation of LDL by both types of fibroblasts was the same (Fig. 3). This result supports the conclusion that the hypercholesterolemia in the mutants is due to the mutations of the structural genes for the apolipoproteins and is not in the LDL receptor gene.

Analysis of the plasma fraction of $d < 1.006$ g/ml (taken from overnight fasted animals) by immunoblotting revealed a 23-kD protein that reacted with the alloantisera to *Lpr* (Fig. 4). Antisera to Lpr1 was specific for the Lpr1 antigen and antisera to Lpr2 likewise only reacted with the Lpr2 antigen. Further analysis of very low density

Table 2. Severity of coronary artery lesions in pigs of defined lipoprotein genotype. Microscopic grading was performed on the three main coronary arteries from 17 pigs bearing the $Lpb^{5/5}$, $Lpu^{1/-}$, $Lpr^{1/-}$ genotypes at the indicated ages. One tissue section from each site was stained for connective tissue (Gomorri trichrome acid fuchsin, GTAF) and the other was stained for lipid (oil red O). The 240 slides were assigned random numbers and graded (with a repeatable accuracy of $P < 0.001$) for lipid infiltration, fibro-histiocytic intimal hyperplasia, focal edema, and loss of fascicular organization of cells and matrix. Lipid infiltration occurred in the absence of any of the other lesion components in both mutants and controls, and therefore minimal infiltration was graded as non-lesion (NL); histopathologic changes were graded (±), (+), (++), (+++), and (++++) reflecting degree of severity of lesions consisting of two or more of the components listed.

Sample No.	Genotype			Age (months)	Cholesterol (mg/dl)		Coronary artery lesions*				
	Lpb	*Lpr*	*Lpu*		At 3 to 5 months	At death	LAD-1	LAD-2	L Cor	R Cor-1	R Cor-2
18	5/5	1/1	1/1	Fetus			−	−	−	−	−
19	5/5	1/1	1/1	Fetus			−	−	−	−	−
101-5	5/5	1/1	1/1	2		191	NL	−	−	NL	±
101-2	5/5	1/2	1/1	2		249	++	−	+	+++	++++
9069	5/5	1/1	1/1	6		360	NL	−	−	NL	+
101-11	5/5	1/2	1/1	7	267	265	++	++	++	+++	+++
9236	5/5	1/1	1/1	8	352	400	−	++	+	NL	+++
9237	5/5	1/1	1/2	13	291	224	±	−	−	+	+
9235	5/5	1/1	1/1	14	313	320	+++	++++	++	+	++++
91-4	5/5	1/2	1/1	15		178	+++	+	++	++	++
9013	5/5	1/1	1/1	16	230	250	±	−	−	+	+
88-8?	5/5	1/1	1/1	16	183		++	−	+	++	+++
9012	5/5	1/1	1/2	19	173	236	+	−	+	+++	++++
101-9	5/5	1/1	1/1	27	296		+	+++	++++	++++	++++
23-3	5/5	1/1	1/1	27	210	138	++++	++++	+++	++	−
104-8	5/5	1/2	1/2	38	369	209	+++	++++	++	++++	++++
93-8	5/5	1/1	1/1	41	190	167	++++	++++	++++	++++	++++

*LAD-1, left anterior descending artery sampled at the orifice; LAD-2, left anterior descending artery sampled halfway between the orifice and the end of the epicardial portion; L Cor, left coronary artery sampled at the first branchpoint; R Cor-1, right coronary artery sampled at the orifice; R Cor-2, right coronary artery sampled halfway between the orifice and the end of the epicardial portion.

lipoprotein (VLDL) by two-dimensional polyacrylamide gel electrophoresis showed that the two forms differed in their isoelectric points (pI) and that the form observed depended upon the animal's *Lpr* genotype (Fig. 5). Thus $Lpr^{1/1}$ animals synthesized 23-kD proteins with pI's of 6.4 and 6.8 (Fig. 5, top), while $Lpr^{2/2}$ animals produced proteins with pI's of 4.8 and 5.5 (Fig. 5, middle). Heterozygous pigs had all isoforms of the protein (Fig. 5, bottom). All pigs had either the 5.5 or 6.8 isoforms of the protein, but some pigs lacked both the 4.8 and 6.4 forms. We have provisionally named this protein "apo-R." Partial characterization of apo-R (*12*) showed that less than 15 percent of the protein is associated with the plasma fraction of $d < 1.006$ g/ml. The remainder is in the fraction of pig plasma with a density greater than 1.21 g/ml.

We do not yet know the function of apo-R. Bradley *et al.* described a protein of similar molecular mass associated with VLDL from patients with hypertriglyceridemia (*13*). They demonstrated that this protein arises from a thrombin-catalyzed cleavage reaction of apo-E, a major apolipoprotein in VLDL. However, antibodies to pig apo-E (*14*) reacted with apo-E, but not with apo-R, in an immunoblot. Furthermore, apo-R is stable, in that it remains with the VLDL in preparations stored for months, while the apo-E fragment from hypertriglyceridemic VLDL is lost from VLDL upon storage or recentrifugation (*15*). Thus apo-R apparently does not appear to be a cleavage fragment of apo-E.

Antiserum to the *Lpu* gene product produced a precipitation reaction with LDL by single and double immunodiffusion. However, it did not react with any pig protein in an immunoblot. Thus we have not yet been successful in identifying the *Lpu* gene product.

The simplest explanation for our findings is that the mutations in the apolipoproteins that we have described lead to hypercholesterolemia and accelerated atherosclerosis, which is independent of dietary fat intake. Whether Lpu1 and Lpr1 proteins contribute to hypercholesterolemia in Lpb^5 pigs remains to be determined. The concentration of apo-R in $Lpr^{1/1}$ pigs is higher than in $Lpr^{2/2}$ pigs (*12*). Heterozygotes have intermediate concentrations of apo-R. However, the association between Lpb^5 and elevation in plasma cholesterol is firmly established because our pigs $Lpb^{5/5}$, $Lpr^{2/2}$, $Lpu^{2/2}$ were derived from six breeds of not closely related animals analyzed throughout several generations.

The effect of two *Lpb* genotypes on hypercholesterolemia in response to an atherogenic diet (25 percent fat, 2 percent cholesterol) was evaluated. Statistical analysis of data from 57 pigs of different Lpb genotypes showed evidence of diet-genotype interaction on fatty streaking; Lpb^5 pigs had a greater tendency ($P < 0.0003$) to develop fatty streaks than pigs of other Lpb genotypes (*16*). $Lpb^{3/3}$ pigs showed no elevation in plasma cholesterol while $Lpb^{3/5}$ pigs exhibited a twofold increase in plasma cholesterol (*6*). Preliminary studies with LDL from an animal with yet another apo-B allele, Lpb^7, show that this lipoprotein is much more efficiently metabolized by macrophages in vitro than Lpb^5 LDL (*17*). Thus in this instance, LDL function in vitro correlates with its apo-B genotype. The $Lpb^{7/-}$ pigs also appear to develop early coronary lesions (*18*).

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19. Blood samples from fasted animals were collected in EDTA (2 mg/ml, final concentration). Sodium azide (0.01 percent), glutathione (0.05 percent), chloramphenicol (20 μg/ml), and phenylmethylsulfonyl chloride (0.5 m*M*) were added to the plasma. Lipoproteins were isolated by sequential ultracentrifugation [V. N. Schumaker and D. L. Puppione, *Methods Enzymol.* **128**, 155 (1986)]. For tissue culture studies, lipoproteins were centrifuged again at the upper density, then dialyzed against phosphate-buffered saline. LDL density range for the pig was 1.019 to 1.073 g/ml. Total cholesterol was determined enzymatically (Sigma No. 351).
20. Antiserum was prepared as follows. A Hampshire pig of the genotype $Lpb^{3/4}$ was immunized by eight injections with LDL and seven injections with apoB100 from Lpb^{5} Chester white pigs. The 15 injections were given at 2- to 3-week intervals (*6, 8*). Antiserum to Lpb3 was produced by injecting an $Lpb^{1/5}$ pig with $Lpb^{3/3}$ LDL (six injections at 2- to 3-week intervals). Intact LDL from an $Lpb^{1/3}$ and from an $Lpb^{5/5}$ pig was subjected to discontinuous gel electrophoresis [U. Laemmli, *Nature (London)* **227**, 680 (1970)] with a 3 percent stacking gel and a 3.6 percent resolving gel. The proteins were transferred (10 A-hour) to nitrocellulose paper by a modification of the method of H. Towbin *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. The paper was first incubated with pig antiserum to Lpb5 or antiserum to Lpb3, and then with antiserum to pig immunoglobulin G conjugated to horseradish peroxidase (Cappel). Protein bands were visualized with 4-chloro-1-naphthol (Bio-Rad). An identical set of lanes was stained with Coomassie R250.
21. LDL from a normal pig was iodinated [M. A. K. Markwell and C. F. Fox, *Biochemistry* **17**, 4807 (1978)] and dialyzed exhaustively against phosphate-buffered saline containing EDTA. Degradation of LDL by cultured pig skin fibroblasts was determined by the method of J. L. Goldstein *et al.* [*Methods Enzymol.* **98**, 241 (1983)] modified as follows: Cells were grown in F-10 media, pig lipoprotein-deficient plasma was used, and silver nitrate was used to precipitate free iodide.
22. N. C. Kendrick, C. W. Bishop, H. F. DeLuca, *J. Biol. Chem.* **259**, 12691 (1984).
23. We thank W. Bradley, A. Chapman, J. F. Crow, R. A. Davis, J. E. Dahlberg, S. Glagov, C. E. Hayes, D. L. Nelson, R. C. Pittman, O. Smithies, and D. Steinberg for advice regarding the interpretation of our data and the preparation of the manuscript. Supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, NIA AG05-856; HL30594, the Wisconsin Pork Producers; the Milwaukee Foundation, the National Livestock and Meat Board, the March of Dimes Birth Defects Foundation, and the Harry and Evelyn Steenbock Foundation. This is paper number 2880 from the Department of Genetics.

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Lipid Domains in Fluid Membranes: A Quick-Freeze Differential Scanning Calorimetry Study

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The application of rapid-freezing techniques to differential scanning calorimetry (DSC) provides a new approach for understanding the organization of lipids in biomembranes. Use of quick-freeze DSC on membranes of mixed lipid composition supports the existence of nonrandom distributions of lipids (domains) in fluid bilayers. In addition to allowing investigations on the organization of lipids in fluid bilayers, the quick-freeze technique now allows calorimetric studies to be carried out on mammalian membranes which, because of their high cholesterol content, have not been previously amenable to study by DSC.

ONE OF THE MOST IMPORTANT problems in membrane structure is the possible existence of lipid domains—that is, regions of bilayer that differ from one another in lipid composition (*1, 2*). Such domains might have different cholesterol content, fatty acid composition, lipid class, and so forth. Since the activities of intrinsic membrane enzymes are dependent on the types of lipids surrounding them (*3*), the activity of a membrane enzyme would vary depending on which domains they occupy in the bilayer.

Under physiological conditions, biomembranes are in a fluid state. Membrane domains would thus not be expected to be static structures but to be time-averaged. While membrane regions of nonrandom lipid distribution might be brought about by the interactions of lipids with membrane proteins, domains are postulated to be a consequence of the intrinsic mixing properties of lipids. Lipid domains have been shown to exist in crystalline membranes (*4*). Some indication of fluid-fluid immiscibility in at least one artificial lipid mixture has been indicated (*5*). Because of the lack of suitable methodology, it has not been possible until now to establish, let alone to study in depth, the existence of lipid domains in the physiologically relevant fluid bilayer state. Findings establishing the existence of domains in fluid bilayers were obtained with a novel approach that allows investigations of lipid-lipid associations in fluid bilayers by the use of differential scanning calorimetry.

Many of the methods that have most profitably been used to study lipid-lipid interactions use strategies in which the conversion of a bilayer from a crystalline to a fluid state is monitored as a function of temperature. For example, differential scanning calorimetry follows heat absorption as a function of temperature, and many spectroscopic approaches follow alterations in the signals of probe molecules either embedded in or partitioning into the bilayer during changes in temperature. Much valuable knowledge on the interaction of bilayer lipids with one another and with membrane proteins has been obtained in this fashion. This approach has its limitations, however, since information on molecular associations in the fluid state is deduced from observations on the melting of the crystalline state. For example, materials that do not cocrystallize in a crystalline state can be homogeneously mixed in a fluid state. An example of this is seen in the distribution of intramembranous particles as visualized by freeze-fracture electron microscopy (*4*).

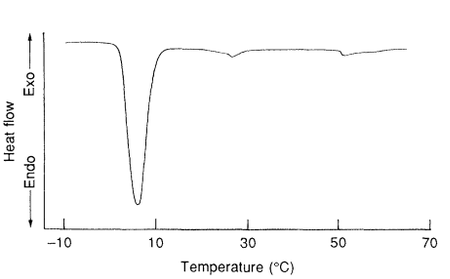

Fig. 1. Thermogram of a quick-frozen sample of homogeneous lipid bilayers in excess water formed from a 1:1 molar mixture of DMPC and DSPC. The sample was quick-frozen from 85°C, loaded as described (*11*), brought up to −30°C in the differential scanning calorimeter, and scanned. The large endotherm is the melting of water. The endotherms resulting from the melting of the lipid bilayers are seen at this sensitivity as slight high-temperature undulations. These endotherms are seen at higher sensitivity in Fig. 2C.

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