the refolding of the short-time denatured enzyme, but that a fraction of the prolines must be very rapidly buried during the initiation of the refolding process (P^B in Fig. 3) and thus become inaccessible to PPIase. It has been reported (*10–12*) that an initiation structure of carbonic anhydrase is rapidly formed during refolding. When the prolines in this core of the enzyme structure (P^B in Fig. 3) are *cis-trans* equilibrated by 1 hour of denaturation, the isomerization of these residues to the native conformers limits the rate of reactivation ($t_{1/2}$ = 4 min) in the presence of PPIase. However, when the prolines in this core are in correct conformation from the beginning of refolding and all the other prolines are *cis-trans* equilibrated by the action of PPIase only during the first 3 s of refolding, uncatalyzed isomerization of the PPIase-accessible prolines (P^A; Fig. 3) is rate determining ($t_{1/2}$ = 5 min) in the reactivation process.

Thus, the action of PPIase on the refolding HCA II reveals the presence of two classes of prolines (accessible and buried), as evidenced by the impact of PPIase on the refolding kinetics. For ribonuclease T_1, the structural context of prolines is important for the efficiency of catalysis (*13*). That PPIase acts as a classical chaperone is clearly demonstrated also by its independent effect on the kinetics and the yield of the refolding reaction. This effect is exemplified in Fig. 1: after 10 s of refolding, inhibition of PPIase still gives rise to a 100% yield and a relatively slow $t_{1/2}$ (9 min; chaperone), whereas addition of PPIase after 10 s of refolding does not increase the yield but does result in a shorter $t_{1/2}$ (4 min; isomerase).

Two mechanisms proposed for the action of PPIase are catalysis by distortion (*14*) and catalysis by desolvation (*15–17*). The latter mechanism was suggested because binding of a peptide segment into a hydrophobic environment, as in the binding site of PPIase (*18*), promotes *cis-trans* isomerization by decreasing the charge separation of the peptide bond and thus creating a peptide that has a more single-bond character. Hence, if this in fact is the mechanism for PPIase activity, then other chaperones that bind peptide chains to apolar sites (*19, 20*) might also possess isomerase activity. Because PPIase (which is also cyclophilin) is known to be involved in T cell activation, it is possible that the chaperone function of this protein is important for essential protein-folding processes in the immune response.

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Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E

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Apolipoprotein E (apoE) is a ligand for receptors that clear remnants of chylomicrons and very low density lipoproteins. Lack of apoE is, therefore, expected to cause accumulation in plasma of cholesterol-rich remnants whose prolonged circulation should be atherogenic. ApoE-deficient mice generated by gene targeting were used to test this hypothesis and to make a mouse model for spontaneous atherosclerosis. The mutant mice had five times normal plasma cholesterol, and developed foam cell–rich depositions in their proximal aortas by age 3 months. These spontaneous lesions progressed and caused severe occlusion of the coronary artery ostium by 8 months. The severe yet viable phenotype of the mutants should make them valuable for investigating genetic and environmental factors that modify the atherogenic process.

Atherosclerotic cardiovascular disease, the major cause of death in Western society, results from complex interactions among multiple genetic and environmental factors (*1*). Among the factors that have been identified to date are changes in the genes involved in lipid metabolism, including the gene encoding apolipoprotein E (apoE) (*2*). ApoE is a glycoprotein with a molecular size of approximately 34 kD that is synthesized in the liver, brain, and other tissues in both humans and mice (*3*); it is a structural component of all lipoprotein particles other than low density lipoprotein (LDL). One of its most important functions is to serve as a high affinity ligand for the apoB and apoE(LDL) receptor and for the chylomicron-remnant receptor, thereby allowing the specific uptake of apoE-containing particles by the liver.

A frequent genetic variant of human apoE, apoE-2, differs from the most common form, apoE-3, by having cysteine at position 158 in place of arginine. This amino acid substitution in the LDL receptor binding region reduces the binding ability of apoE-2 to less than 2% relative to that of apoE-3. Homozygosity for the gene $Apoe^2$ is associated with type III hyperlipoproteinemia, which is characterized by increased plasma triglyceride and cholesterol levels, yellow lipid-laden xanthomatous skin nodules, and the early development of atherosclerosis (*4*). The complexities of the pathogenesis of this disease are, however, illustrated by the fact that only about 2% of $Apoe^2/Apoe^2$ individuals develop hyperlipoproteinemia. The majority of individuals

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with this genotype have, in fact, lower than normal plasma cholesterol levels (5). Thus, other factors must be necessary for the expression of type III hyperlipoproteinemia associated with apoE-2.

Three human kindreds with inherited apoE deficiency have been reported (6). Individuals deficient in apoE, have tuberoeruptive xanthomatosis and type III hyperlipoproteinemia with elevated cholesterol and near normal triglyceride levels.

ApoE-deficient mice were generated by inactivating the *Apoe* gene by targeting (7). Mice homozygous or heterozygous for the disrupted *Apoe* gene appeared healthy. No difference in their body weights compared to normal mice was observed. Matings between homozygotes, between heterozygotes, and between homozygotes and heterozygotes all yield litters of normal size. No sex-versus-genotype bias was observed in the litters, indicating that the mutants had no apparent reproductive problems. However, significant phenotypic differences between normal animals and the homozygous mutants were observed in their lipid and lipoprotein profiles (Table 1). The apoE-deficient mice had markedly increased total plasma cholesterol levels (434 ± 129 mg/dl), which were five times those of normal litter mates (86 ± 20 mg/dl). These levels were unaffected by the age or sex of the animals. Although the total plasma cholesterol levels were greatly elevated in the mutants, the HDL (high density lipoprotein) cholesterol levels, measured after removing apolipoprotein B–containing particles, were only 45% the normal level [$P < 0.0005$ (8) by Student's t test for unpaired observations]. The triglyceride levels were 68% higher than those of normal animals ($P < 0.0005$). Marked increase in total cholesterol accompanied by near normal triglyceride levels was also observed in human patients with apoE deficiency (6). In mice heterozygous for the mutant allele, cholesterol levels were the same as normal animals, but triglyceride levels were increased by about 39% ($P < 0.005$).

Differences in the lipoprotein profiles of the homozygous mutants from those of normal and heterozygous animals were revealed by fractionating total lipoproteins from plasma by gel filtration chromatography (Fig. 1). HDL was the major cholesterol-carrying lipoprotein in the plasma of normal mice; lipoproteins with sizes that correspond to VLDL (very low density lipoprotein), IDL (intermediate density lipoprotein), and LDL were found in only trace amounts. In the mutants lacking apoE, the particles with sizes in the range of HDL were reduced, and nearly 80% of the total cholesterol was carried in particles having sizes that are similar to the lower density lipoproteins of normal humans. Confirmation of these results was obtained by agarose gel electrophoresis of whole plasma (8). In normal mouse plasma, HDL, which is the major lipoprotein, migrates in the same position as α globulins. VLDL and LDL, which migrate more slowly at the pre-β and β positions, respectively, are minor components (9). In the mutant plasma, particles that migrate at the α position were reduced while those migrating between the β and pre-β positions were markedly increased. Thus, the distribution of lipids in the plasma of the mutants was shifted toward the region where lipoproteins of lower density normally migrate.

To better characterize the molecular basis for the changes associated with the absence of apoE, we determined the apolipoprotein compositions of plasma lipoproteins. The lipoprotein particles were first

Table 1. Plasma cholesterol and triglyceride levels in apoE-deficient mice. Mice of both sexes, 2 to 5 months of age, were kept on regular mouse chow (ProLab formula 3000, 4.5% crude fat). After an overnight fast, blood (about 300 μl) was drawn by retro-orbital bleeding and EDTA, gentamicin sulfate, and aprotinin (Sigma) were added to final concentrations of 2 mM, 50 mg/ml (w/v) and 0.046 trypsin inhibitor unit per milliliter, respectively. Plasma was separated by centrifugation at 14,000*g* for 10 min. at 4°C. Cholesterol and triglyceride levels were determined by enzymatic reaction followed by spectrophotometric quantitation with 1 μl of plasma and 100 μl of commercially available reagents (cholesterol 20; triglyceride 10; Sigma). HDL cholesterol was measured after removing the apoB-containing lipoproteins by $MgCl_2$–dextran sulfate precipitation (*18*). For hyperlipidemic plasma, a 1:2 dilution was made with phosphate-buffered saline prior to precipitation for the HDL cholesterol test. When multiple measurements were done on a single animal, average values were used to derive the overall mean value. The numbers of animals tested are given in parentheses.

Animals	Total cholesterol in mg/dl ± SD (*n*)	HDL cholesterol in mg/dl ± SD (*n*)	Triglyceride in mg/dl ± SD (*n*)
Normal	86 ± 20 (46)	73 ± 28 (34)	73 ± 36 (32)
Heterozygous	88 ± 22 (47)	75 ± 18 (29)	102 ± 40 (41)
Homozygous	434 ± 129 (40)	33 ± 15 (28)	123 ± 51 (42)

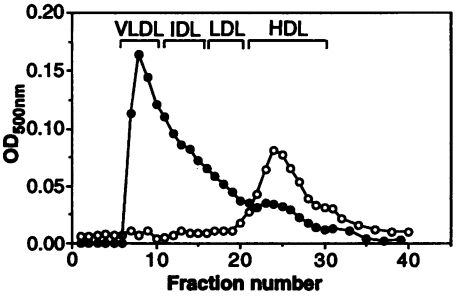

Fig. 1. Cholesterol concentrations in lipoprotein fractions of normal and apoE-deficient mouse after Superose 6 column chromatography of total lipoprotein. Total lipoproteins (density <1.21 g/ml) from 50 μl of fasting plasma were isolated by ultracentrifugation (*10*), applied to a Superose 6 (Sigma) column (1 cm by 30 cm), and eluted at a constant flow rate of 0.4 ml/min with 10 mM tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 0.01% (w/v) EDTA, and 0.02% (w/v) NaN_3. Fractions of 0.32 ml were collected. A portion of each fraction (0.05 ml) was dried completely, dissolved in 100 μl of cholesterol-testing reagent (Sigma), and incubated at 37°C for 5 min. Absorbance at 500 nm provides a measure of the relative amounts of cholesterol in each fraction. Open circles, normal mouse; closed circles, homozygous mutant. The approximate positions where the major human lipoprotein classes normally elute are indicated.

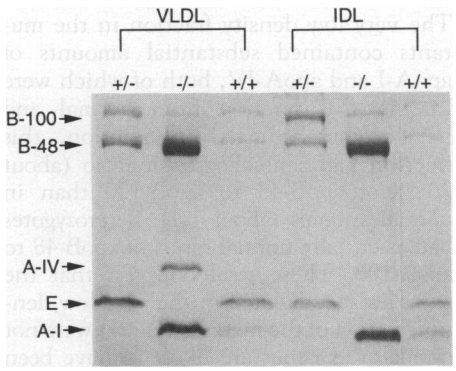

Fig. 2. SDS–polyacrylamide gel electrophoresis of VLDL and IDL fractions from fasting plasma from heterozygous mutants (+/−), homozygous mutants (−/−), and normal mice (+/+). Plasma (100 μl each) from six mice was pooled and lipoprotein fractions were separated by sequential flotation (*10*) in a TL-100 bench-top ultracentrifuge with a TLA 100.3 rotor (Beckman), at 100,000 rpm for 2 hours for VLDL, IDL, and LDL, and for 4 hours for HDL. Each fraction (500 μl) was concentrated and desalted by means of a Centricon-10 filter apparatus (Amicon). Samples equivalent to 200, 20, and 100 μl of original plasma from heterozygotes, homozygotes, and normal animals, respectively, were denatured and separated by electrophoresis on a gradient polyacrylamide gel (4 to 20%, Bio-Rad) at 150 V for 45 min. Proteins were stained with Coomassie brilliant blue. Apolipoprotein Cs and apolipoprotein AII were not retained in this gel, but there were no notable differences in these proteins in other gels.

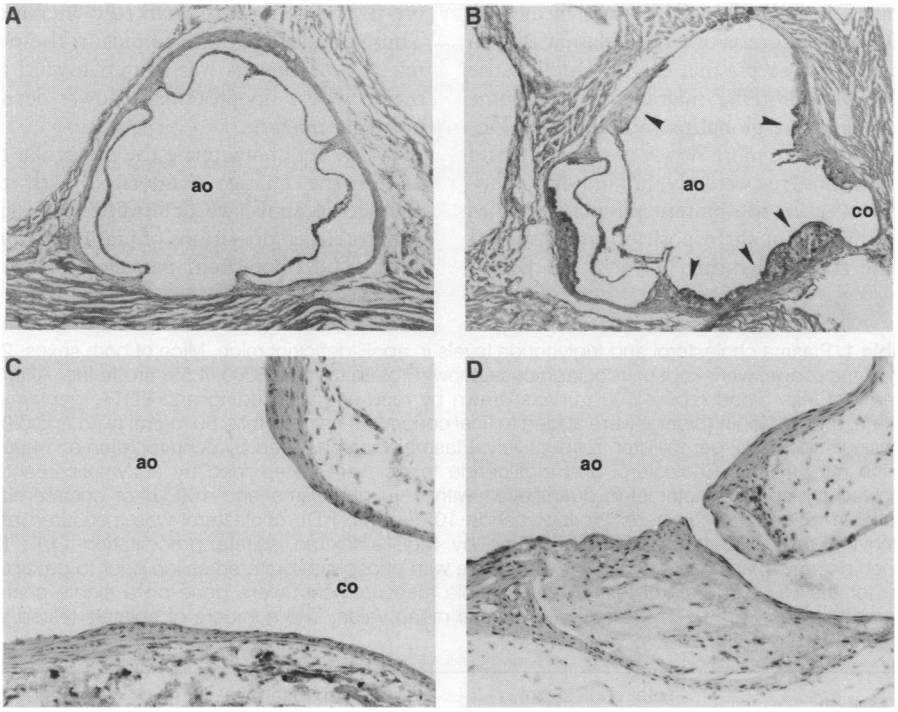

Fig. 3. Transverse frozen sections of the proximal aorta stained with Sudan IVB and counterstained with Harris hematoxylin. (**A**) A section of the aortic sinus of a 5-month-old male heterozygote, showing the valve attachment sites as outgrowths of smooth muscle cells from the vessel wall. Final magnification, ×26. (**B**) An equivalent section of a 5-month-old male homozygote, ×26. Small arrowheads indicate some of the multilayered intimal foam cell deposits. The entrance to a coronary artery can be seen as an evagination of the aortic wall. (**C**) A longitudinal section of the coronary artery of a 5-month-old normal male, ×260, showing normal architecture of the ostium. (**D**) An equivalent section of an 8-month-old female homozygote, ×260, showing near total occlusion at the ostium of a coronary artery. Abbreviations: ao, aorta; co, coronary artery.

separated by sequential density flotation into the four density ranges that would normally include VLDL, IDL, LDL, and HDL particles (*10*). The apolipoprotein compositions of these fractions were then investigated by SDS–polyacrylamide gel elec-electrophoresis (Fig. 2). Substantial changes were seen in the apolipoprotein contents of the VLDL and IDL fractions, while changes in the LDL and HDL fractions were minor. As expected, apoE was present in normal and heterozygous mice, but was absent in the homozygous mutants. The very low density fraction in the mutants contained substantial amounts of apoA-I and apoA-IV, both of which were low in this fraction from normal and heterozygous animals. In addition, this fraction had a much greater ratio (about 20:1) of apoB-48 to apoB-100 than in normal animals (about 1:1); heterozygotes had essentially normal ratios of apoB-48 to apoB-100. These results suggest that the particles accumulated in the very low density fraction of the mutants (Fig. 1) were not normal VLDL but are likely to have been remnants of chylomicron or VLDL. Remnants of these types in mice are expected to be rich in cholesterol and to have apoB-48 as a major component since mice, unlike humans, produce B-48 in the liver (*11*).

The significance of the presence of considerable amounts of apoA-I and apoA-IV in the particles with VLDL and IDL density ranges in the mutants remains to be determined; in normal humans these apolipoproteins are present in newly secreted chylomicron but are negligible in VLDL and chylomicron remnants (*3*). Our observation that the mutants have about half normal HDL cholesterol levels also requires comment. We note that the total amount of apoA-I in the plasma of mutant and normal mice is about the same (*8*) when evaluated by nonreducing SDS–polyacrylamide gel electrophoresis (*12*). Additionally, apoA-I mRNA levels in the liver and intestine of the mutants were approximately the same as in normal mice (*8*). Thus, it appears that the decreased level of HDL in the homozygotes is neither because of reduced synthesis nor because of accelerated catabolism of apoA-I. Perhaps the apoA-I is redistributed to remnant particles as a structural component in the absence of apoE, thereby limiting the amount of apoA-I available for HDL formation in the mutants. The reduced HDL levels may also reflect the absence of apoE-containing HDL particles.

Mice naturally have high levels of HDL and low levels of LDL, in contrast to humans who are high in LDL and low in HDL. In addition, mice apparently lack the cholesteryl ester transfer protein, an enzyme that transfers cholesterol ester from HDL to VLDL and LDL (*13*). Despite these differences, apoE-deficient mice have phenotypes remarkably similar to those of apoE-deficient humans. ApoE-deficient individuals in both species have hypercholesterolemia, near normal triglyceride levels, decreased HDL, a large accumulation of particles in the VLDL and IDL size ranges, and a typical broad β band on agarose gel electrophoresis. They both have an elevated percentage of apoB-48 in apoB-containing particles, and *apo*A-I and apoA-IV is found in particles in the VLDL density range.

Evidence of fatty streaks in the proximal aorta was found in a homozygous mutant mouse that was killed at 3 months of age. No pathological changes were noted in the distal aorta and proximal iliac vessels. Lipid deposition was found in the liver but not in kidney, lung, or spleen. To further document the development of the arterial lesions, a histological study of the proximal aortic region (*14*) of six sets of age-matched normal, heterozygous and homozygous animals was performed. These animals were 3 to 8 months of age and had been fed normal mouse chow (4.5% crude fat). Examination of sections of the proximal aorta showed no abnormalities in the normal and heterozygous animals. The luminal surface of the aorta in normal mice was smooth, and smooth muscle cells were orderly arranged (Fig. 3A). The coronary ostium was free of obstructions (Fig. 3C). In contrast, examination of the same region in all the female and male homozygous mutant animals revealed fatty streak formation. Intimal foam cell deposits were present diffusely throughout the area of the aortic sinus as illustrated in a section of a 5-month-old male (Fig. 3B). These deposits varied from small collections of lipid-filled cells to raised deposits within the intima. The small foam cell deposits were most often found adjacent to valve attachment sites, and frequently covered this area. Multilayered foam cell deposits were also present between the valve attachment sites, and were often in the form of continuous deposits. Foam cells were not found on the valve leaflets. Lipid was occasionally present in the superficial media. In some areas, the surface of the lesion was flattened, suggesting an early stage of fibrous cap formation. The lesions progressed with age, and near total occlusion at the entrance of a coronary artery was seen in the section of an 8-month-old female homozygote (Fig. 3D). At this stage, the lesions appeared to contain less lipid but more elongated cells, which represents a more progressed stage of lesions.

The presence of these pre-atherosclerotic lesions in the proximal aorta demonstrates that a simple lack of apoE is sufficient to initiate atherogenesis. Although different strains of mice exhibit heritable differences in the levels of their circulating lipids (*15*), no inbred strains are known to develop lesions spontaneously on a normal diet. However, they do show significant differences in their tendencies to form atherosclerotic plaques when placed on a high fat diet (*16*). Strain C57BL/6 is more susceptible to atherogenic diets than others that have been tested and strain 129/J is moderately susceptible (*16*). The apoE-deficient mice we studied here are mainly F2 animals that have a combination of the genetic backgrounds of both C57BL/6 and 129/J; the precise composition is unique for each individual animal (*17*). This genetic heterogeneity may cause some variability among different individuals in their plasma lipoprotein phenotypes and in the likelihood of their developing arterial lesions. However, all 40 mice lacking apoE had elevated total plasma cholesterol levels, and all six that we have studied histologically had lesions in their proximal aorta. None of these changes were observed in normal and heterozygous litter mates.

The early development of lesions in mice lacking apoE makes them of great practical value. The combined phenotype of the homozygotes (high cholesterol and early development of non-lethal lesions) provides a baseline against which either detrimental or protective genetic and environmental factors can be investigated. These mice should also be of value for use as an in vivo test system for studies of pharmacological or genetic treatments of hyperlipidemia.

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14. After overnight fast, mice were killed with an overdose of avertin (2,2,2-tribromoethanol) and the vascular tree was perfused via the heart with 4% (w/v) paraformaldehyde in 0.12 M phosphate buffer, pH 7.4. The heart and entire aorta, including the iliacs, were removed intact and immersed in fresh paraformaldehyde. Prior to cryostat sectioning, the heart and an approximately 0.5-cm length of ascending aorta was removed from the remainder of the aorta. The heart was divided into two halves by cutting transversely along a line parallel to the tip of the atria. The half with the attached aortic segment was then placed in OCT compound (Tissue-Tex), gently compressed to remove bubbles, positioned in semi-frozen OCT to allow cross sectioning of the aorta, and then frozen in OCT compound on a metal pedestal prior to sectioning. Eight micrometer-thick frozen sections at 24-μm intervals were made beginning with the ascending aorta and proceeding through the entire aortic sinus until the ventricular chamber was reached. Adequacy of the frozen sectioning process was monitored by light microscopic evaluation of unstained sections. The frozen sections were allowed to dry overnight at room temperature. Sections were stained to detect lipid with Sudan IVB followed by counter-staining with Harris hematoxylin.
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17. The embryonic stem (ES) cells we used for introducing a modification into *Apoe* were derived from strain 129/Ola. Chimeras made from these ES cells were crossed with strain C57BL/6J mice, and F1 offspring that were heterozygous for the modified gene were obtained. F2 mice homozygous for the mutation were then generated by crossing two F1 heterozygotes. The genetic background of an F2 animal is therefore a discrete blending of the two strains that is unique to each particular animal.
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Formation of a Gated Channel by a Ligand-Specific Transport Protein in the Bacterial Outer Membrane

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The ferric enterobactin receptor (FepA) is a high-affinity ligand-specific transport protein in the outer membrane of Gram-negative bacteria. Deletion of the cell-surface ligand-binding peptides of FepA generated mutant proteins that were incapable of high-affinity uptake but that instead formed nonspecific, passive channels in the outer membrane. Unlike native FepA, these pores acted independently of the accessory protein TonB, which suggests that FepA is a gated porin and that TonB acts as its gatekeeper by facilitating the entry of ligands into the FepA channel. The sequence homology among TonB-dependent proteins suggests that all ligand-specific outer membrane receptors may function by this gated-porin mechanism.

The cell envelope of Gram-negative bacteria contains high-affinity, ligand-specific outer membrane proteins that translocate substrates into the periplasm. Such transport systems are multifunctional—a single outer membrane protein usually serves as the surface receptor for several dissimilar ligands (*1*)—and multicomponent, requiring the participation of periplasmic and cytoplasmic membrane proteins (*2*). TonB is the most notable of these accessory proteins; it resides in the cytoplasmic membrane but is thought to project across the periplasmic space and facilitate the transport function of outer membrane receptors by direct, protein-to-protein interactions (*3*).

Ligand-specific receptor proteins have been considered to be distinct from porins, which form nonspecific, hydrophilic channels in the outer membrane (*4*). Porins contain amphiphilic β strands assembled in the form of a β barrel (*5*), which acts as a molecular sieve that equilibrates small molecules (<600 daltons) across the bilayer

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Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E

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