JCI The Journal of Clinical Investigation

Identical Structural and Receptor Binding Defects in Apolipoprotein E2 in Hypo-, Normo-, and Hypercholesterolemic Dysbetalipoproteinemia

Stanley C. Rall Jr., … , Robert W. Mahley, Gerd Assmann

J Clin Invest. 1983;71(4):1023-1031. https://doi.org/10.1172/JCI110829.

Research Article

Apolipoprotein E (apoprotein E or apo-E) from type III hyperlipoproteinemic subjects with the E2/2 homozygous phenotype displays both structural and receptor binding heterogeneity. The apo-E from all subjects thus far studied, however, has been functionally defective, though to different degrees. Although nearly every type III hyperlipoproteinemic subject has the E2/2 phenotype, 95-99% of the people with this same phenotype do not display type III hyperlipoproteinemia, nor do they have elevated plasma cholesterol levels. Consequently, it became important to determine whether the apo-E2 from hypo- and normocholesterolemic individuals with the E2/2 phenotype is also functionally abnormal. To do this, apo-E2 was isolated from two hypo-, two normo- and two hypercholesterolemic homozygous E2/2 subjects. The apo-E2 was recombined with vesicles and tested for its ability to displace ^{125}I-low density lipoproteins (LDL) from apo-B,E (LDL) receptors on human fibroblasts. The apo-E2 from all six subjects was found to be severely defective in receptor binding (<2% of the binding activity of normal apo-E3). In all cases, the binding activity of the apo-E2 was increased 10- to 20-fold by treating the apoproteins with cysteamine, a reagent that converts cysteine residues to positively charged lysine analogues. The cysteine content of each apo-E was determined by monitoring the change in the isoelectric focusing position of the cysteamine-treated apo-E2. Using this method, it was found that the apo-E2 […]

Identical Structural and Receptor Binding Defects in Apolipoprotein E2 in Hypo-, Normo-, and Hypercholesterolemic Dysbetalipoproteinemia

STANLEY C. RALL, JR., KARL H. WEISGRABER, THOMAS L. INNERARITY, and ROBERT W. MAHLEY, *The Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute, Departments of Pathology and Medicine, University of California, San Francisco, San Francisco, California 94140*

GERD ASSMANN, *Zentrallaboratorium der Medizinischen Einrichtungen der Westfälischen Wilhelms-Universität, Münster, West Germany*

ABSTRACT Apolipoprotein E (apoprotein E or apo-E) from type III hyperlipoproteinemic subjects with the E2/2 homozygous phenotype displays both structural and receptor binding heterogeneity. The apo-E from all subjects thus far studied, however, has been functionally defective, though to different degrees. Although nearly every type III hyperlipoproteinemic subject has the E2/2 phenotype, 95–99% of the people with this same phenotype do not display type III hyperlipoproteinemia, nor do they have elevated plasma cholesterol levels. Consequently, it became important to determine whether the apo-E2 from hypo- and normocholesterolemic individuals with the E2/2 phenotype is also functionally abnormal. To do this, apo-E2 was isolated from two hypo-, two normo-, and two hypercholesterolemic homozygous E2/2 subjects. The apo-E2 was recombined with phospholipid vesicles and tested for its ability to displace ^{125}I-low density lipoproteins (LDL) from apo-B,E (LDL) receptors on human fibroblasts. The apo-E2 from all six subjects was found to be severely defective in receptor binding (<2% of the binding activity of normal apo-E3). In all cases, the binding activity of the apo-E2 was increased 10- to 20-fold by treating the apoproteins with cysteamine, a reagent that converts cysteine residues to positively charged lysine analogues. The cysteine content of each apo-E was determined by monitoring the change in the isoelectric focusing position of the cysteamine-treated apo-E2. Using this method, it was found that the apo-E2 from each subject contained two cysteine residues per mole. A partial sequence analysis of the cysteine-containing regions of the apo-E from three of the six subjects indicated that the two cysteine residues were at residues 112 and 158 in the amino acid sequence. The cysteine at residue 158 has previously been implicated in the severe binding defect of the apo-E2 from a type III hyperlipoproteinemic subject. Since the apo-E2 of the hypo-, normo-, and hypercholesterolemic subjects in this study all displayed a severe functional abnormality, it is apparent that factors in addition to the defective receptor binding activity of the apo-E2 are necessary for the manifestation of type III hyperlipoproteinemia.

INTRODUCTION

The complex isoform pattern displayed by human apolipoprotein E (apoprotein E or apo-E)[1] is due to primary structural differences and posttranslational modification. Utermann and his colleagues (1–3) were the first to demonstrate the genetic polymorphism of the apo-E isoforms. Since then, evidence has been presented to show that this genetic polymorphism results from the presence of multiple alleles at a single genetic

Address correspondence to Dr. Mahley.
Received for publication 22 October 1982.

[1] *Abbreviations used in this paper*: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine.

locus and the posttranslational sialylation of apo-E (4, 5). Recently, we found that the genetically determined, major (unsialylated) isoforms of apo-E (E2, E3, and E4) differ from one another in primary structure (6, 7). This indicates that the genetic control is at the level of the structural gene for apo-E and confirms the prediction of the Zannis-Breslow hypothesis (4, 5).

Utermann et al. (8) demonstrated that a specific apo-E phenotype is associated with the lipid disorder type III hyperlipoproteinemia (primary dysbetalipoproteinemia), an observation confirmed by others (9, 10). Individuals with this disorder are homozygous for E2 (8) and have a hyperlipoproteinemia characterized by the accumulation of apo-E-enriched chylomicron and very low density lipoproteins (VLDL) remnants (of both hepatic and intestinal origin) in the plasma (11). Because apo-E appears to be responsible for the receptor-mediated uptake of remnant lipoproteins by the liver (12–16), and the mutant form of apo-E (E2) interacts poorly with lipoprotein receptors (17, 18), it is likely that this mutant apo-E2 is responsible for the defective lipoprotein clearance in type III hyperlipoproteinemic subjects and the resulting dyslipoproteinemia in certain patients (13, 17–19).

Population studies (1, 2, 5) have revealed a much higher frequency of the E2/2 homozygous phenotype (~1%) than would be expected from a specific association with type III hyperlipoproteinemia, which occurs in ~0.01–0.04% of the population (20). It has been found that most individuals homozygous for this phenotype display varying signs of dyslipoproteinemia without having the other clinical symptoms of the type III disorder, which is characterized by grossly elevated levels of plasma cholesterol and triglycerides, the presence of abnormal lipoproteins (the cholesteryl ester-rich, β-migrating VLDL [β-VLDL]), the occurrence of planar and tuberous xanthomas, and an increased risk of premature atherosclerosis (1, 2, 21, 22).[2] Observations from kindred studies have led to the suggestion that factors in addition to E2 homozygosity (the E2/2 phenotype), such as independently heritable hyperlipidemias, are necessary for the expression of type III hyperlipoproteinemia (2, 23).

In light of recent findings that apo-E from different type III hyperlipoproteinemic subjects homozygous for E2 displays a heterogeneity in receptor binding activity (17, 18), and that genotypic heterozygosity exists within the E2/2 phenotype (24), it seemed important to understand the structural and receptor binding characteristics of the apo-E of hypo- and normocholesterolemic individuals with the E2/2 phenotype. In the present study, we will report evidence to show that apo-E2 from four hypo- and normocholesterolemic subjects appears to be structurally and functionally identical to the apo-E2 from severely hypercholesterolemic type III patients.

METHODS

Lipoprotein and apoprotein isolation and characterization. Lipoprotein density fractions were prepared from plasma by sequential ultracentrifugation (25). Protein was determined by the method of Lowry et al. (26). Total cholesterol and triglyceride levels were determined by using enzymatic procedures (Biodynamics/bmc, Boehringer Mannheim Corp., Indianapolis, IN). Phospholipid content of lipoproteins or apo-E·phospholipid complexes was determined from the phosphorus content (27). The presence of β-VLDL was ascertained by its electrophoretic migration on paper electrophoretograms (28).

The $d < 1.006$ lipoproteins used for the preparation of apo-E were isolated from the plasma of subjects by ultracentrifugation for 16 h at 50,000 rpm in a 60-Ti rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) and washed one time by recentrifugation. The $d < 1.006$ lipoproteins were dialyzed against 0.01% EDTA (pH 7.4), lyophilized, and then delipidated with 2:1 (vol/vol) $CHCl_3$:CH_3OH. The moist apoprotein pellet was solubilized in 6 M guanidine, 0.1 M Tris, and 0.01% EDTA (pH 7.4), and then reduced with 1% β-mercaptoethanol and fractionated on a 2.5 × 300-cm column of either Sephadex G-200 or Sephacryl S-300 (Pharmacia Diagnostics, Div. of Pharmacia, Inc., Piscataway, NJ) equilibrated with 4 M guanidine, 0.1 M Tris, 0.01% EDTA, and 0.1% β-mercaptoethanol (pH 7.4). The apo-E fractions were combined, dialyzed against 5 mM NH_4HCO_3, and lyophilized.

Analytical isoelectric focusing was performed on 6-cm, 5% polyacrylamide gels containing 8 M urea and 2% Pharmalyte (pH 4–6) (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.), as previously described (6). In some instances, the $d < 1.006$ lipoproteins were chemically modified with β-mercaptoethylamine (cysteamine, Sigma Chemical Co., St. Louis, MO) by adding 1.0 mg of cysteamine to 150 μg of $d < 1.006$ lipoprotein protein in 0.1 M NH_4HCO_3 and incubating the mixture for 4 h at 37°C. The mixture was then lyophilized and delipidated before isoelectric focusing. Control samples were incubated in parallel with the modified samples and were reduced with β-mercaptoethanol before isoelectric focusing.

Amino acid and sequence analyses. Samples for amino acid analyses were hydrolyzed for 20 h at 110°C in 6 N HCl in sealed, evacuated tubes. Samples were dried in vacuo at 40°C, dissolved in 0.2 N sodium citrate, pH 2.2, and analyzed on a Beckman 121MB Analyzer equipped with a Model 126 Data System (Beckman Instruments, Inc.). Lyophilized apo-E for cyanogen bromide digestion was dissolved in 70% HCOOH at 5 mg/ml and digested with a 30-fold excess (wt/wt) of CNBr (Pierce Chemical Co., Rockford, IL) for 24 h at room temperature. After lyophilization, the digests were dissolved in 2.5 ml of 20% HCOOH and fractionated on a 2.5 × 190-cm column of Sephadex G-50 (fine beads, Pharmacia Diagnostics) in 0.02 N HCl at room temperature (flow

[2] For the purposes of this discussion, type III hyperlipoproteinemia is used to indicate the lipid disorder characterized by hypercholesterolemia, hypertriglyceridemia, the E2/2 phenotype, the presence of β-VLDL, and the clinical sequelae, including xanthomatosis and/or vascular disease. Dysbetalipoproteinemia is used to refer to subjects with the E2/2 phenotype and β-VLDL, unaccompanied by hyperlipidemia and vascular disease.

rate, 18 ml/h). Pooled fractions were lyophilized and redissolved in 0.5 ml of 50% acetic acid for sequencing and amino acid analysis (24).

Peptides from the G-50 chromatography were subjected to sequence analysis, without further purification, in the presence of 2 mg of polybrene (Sigma Chemical Co.) on a Beckman 890C Sequencer using a 0.1 M Quadrol program (no. 122974) and standard Beckman reagents. Methods of analysis and criteria for identification of the phenylthiohydantoin amino acids by high performance liquid chromatography were as described (6, 24).

Fibroblast binding studies. Dimyristoylphosphatidylcholine (DMPC, Sigma Chemical Co.) vesicles were prepared by sonication (29). The apo-E was treated with cysteamine or β-mercaptoethanol before apo-E·DMPC complex formation. Cysteamine modification was performed by treating 200 μg (1.0–1.5 mg of protein/ml) of apo-E in 0.1 M NH_4HCO_3 with 20 μl of a cysteamine solution (100 mg/ml). The mixture was incubated overnight at room temperature or for 4 h at 37°C. Unmodified samples were treated with 20 μl of β-mercaptoethanol solution (1:10 dilution with H_2O) and incubated in parallel with the cysteamine samples.

The apo-E·DMPC complexes were prepared by incubating apo-E (150 μg of protein in 150–300 μl of 0.1 M NH_4HCO_3) with 56 μl of DMPC (10 mg/ml) for 1 h at 25°C. Complexes were isolated by ultracentrifugation at 55,000 rpm (SW 55 rotor) for 20 h at 15°C on a gradient of KBr (d = 1.006–1.21) (18). The ability of the apo-E·DMPC complexes to bind to the apo-B,E receptors of cultured fibroblasts was determined in a 4°C competitive binding assay using ^{125}I-LDL. The methodology used for the maintenance of the cells and the conditions of the assay have been described (18, 29).

RESULTS

Classification of subjects. Subjects were identified as part of a study on apo-E phenotypes in the German population (30), and six subjects were selected for more detailed analysis on the basis of their apo-E phenotypes and plasma cholesterol levels. All six subjects had the E2/2 homozygous phenotype as determined by one-dimensional isoelectric focusing (Fig. 1). (Other selected data on these individuals are summarized in Table I.) Of the six subjects, two were classified as hypocholesterolemic, based on plasma cholesterol values below the 5th percentile as defined by the Lipid Research Clinics Prevalence Study (31). Two subjects were normocholesterolemic, and two were hypercholesterolemic. The latter two had plasma cholesterol values that exceeded the 95th percentile (31). Of the hypercholesterolemic subjects, one (H.-O.K.) was clearly a type III hyperlipoproteinemic by all of the usual criteria. He had xanthomatosis and both coronary and peripheral vascular disease. The other subject (W.H.) had symptoms of type III hyperlipoproteinemia in some respects, i.e., a borderline VLDL-cholesterol/plasma triglyceride ratio and greatly elevated VLDL-cholesterol and VLDL-triglyceride levels. His relatively young age might have been a factor in his failure to display all of the clinical criteria of the disorder. All of the subjects showed some evidence of dyslipoproteinemia (i.e., β-VLDL in the plasma), which has previously been demonstrated by other investigators to be prevalent within the E2/2 phenotype (22, 23). The subjects also had reduced LDL levels (as judged by LDL-cholesterol values); this is also consistent with previous findings for this phenotype (22).

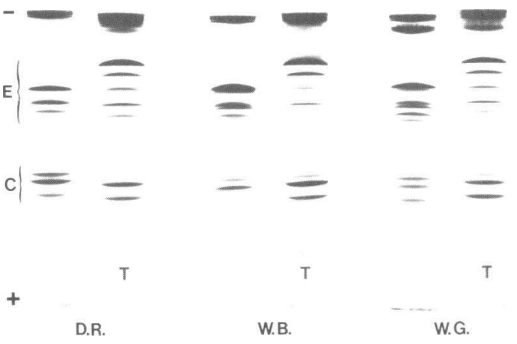

FIGURE 1 Isoelectric focusing on polyacrylamide gels (pH 4–6) of control and cysteamine-treated (T) VLDL from subjects with the E2/2 phenotype. The isoform pattern for the VLDL of D.R., who served as the source of apo-E2 reported previously (7), is shown for comparison. Brackets indicate the positions of the E isoforms and C apoproteins.

Characterization of the apo-E2 from the six subjects. The mutant forms of apo-E2 that have thus far been described differ from apo-E3 by a single amino acid substitution of a cysteine residue for an arginine within the amino acid sequence (7, 24). Apo-E3 contains a single residue of cysteine, whereas the apo-E2 mutants have two residues of cysteine per mole of protein. The number of cysteine residues per mole of apo-E can be rapidly determined by reacting the apo-E with the reagent cysteamine (6, 18, 24). For each cysteine residue present, cysteamine treatment adds a single positive charge to the apo-E, and by isoelectric focusing, the cysteamine-treated apo-E assumes a pI differing from the untreated apo-E by one unit of positive charge per cysteine. Thus, the mobility of cysteamine-treated apo-E3 is altered by one positive charge unit, and the mobility of cysteamine-treated apo-E2, which contains two cysteine residues, is altered by two positive charge units. As shown in Fig. 1, for two of the subjects, the mobility of the apo-E2 was altered by two units of charge after cysteamine treatment. The same result was observed for the apo-E2 of the other four subjects (data not shown). The cysteine content of the apo-E2 from three of the subjects was determined by direct sequence analysis, and these results confirmed that two residues of cysteine were present (see results described below).

Receptor binding of the apo-E2. The apo-E from

TABLE I
Clinical Data on Subjects

	E.G.	K.-H.M.	W.B.	W.G.	W.H.	H.-O.K.
Sex	F	M	M	M	M	M
Age	50	48	59	40	28	40
Plasma cholesterol, *mg/dl*	138*	128*	187	181	274‡	326‡
Plasma triglycerides, *mg/dl*	87	73	127	199	657‡	522‡
Apo-E phenotype	E2/2	E2/2	E2/2	E2/2	E2/2	E2/2
β-VLDL	+	+	+	+	+	+
$d < 1.006$ cholesterol, *mg/dl*	19	11	24	35	170	192
$d < 1.006$ triglycerides, *mg/dl*	ND	32	56	117	505	381
$d = 1.006$–1.02 cholesterol, *mg/dl*	ND	2	8	16	9	12
$d = 1.02$–1.063 cholesterol, *mg/dl*	51	41	77	63	19	33
$d = 1.063$–1.21 cholesterol, *mg/dl*	57	41	34	16	9	20
VLDL-cholesterol/plasma triglycerides	0.22	0.15	0.19	0.18	0.26	0.37

* Plasma cholesterol values below the 5th percentile, Lipid Research Clinics Prevalence Study (31).
‡ Plasma cholesterol values above the 95th percentile, Lipid Research Clinics Prevalence Study (31).
ND, no data.

each subject was recombined with DMPC and tested for its ability to bind to the apo-B,E receptors of human fibroblasts. In addition, apo-E from each subject was treated with cysteamine, and its binding activity was compared with that of untreated apo-E. A representative competition experiment is shown in Fig. 2, and a summary of all binding experiments is presented in Table II. In all cases, the receptor binding of apo-E2 was severely defective compared with that of normal-binding apo-E3, which served as the control. Treatment of the apo-E of the subjects with cysteamine invariably resulted in a large increase in binding activity. Markedly defective binding (<2% of the binding activity of normal apo-E3) and a large increase in binding after cysteamine treatment (10- to 20-fold) have previously been demonstrated (18, 24) for the apo-E2 of a classical type III hyperlipoproteinemic subject (D.R.), whose apo-E2 amino acid sequence has been reported (7, 24).

The similarity in binding activity of the apo-E from the six subjects in this study and from subject D.R. of a previous study suggested a structural similarity in the apo-E of these subjects regardless of whether they were hypo-, normo-, or hypercholesterolemic. Recently, it has been established that there is a structural heterogeneity in the apo-E2 from type III hyperlipoproteinemic individuals of the E2/2 homozygous phenotype (24). This structural heterogeneity is due to differences in the location of one of the two cysteine residues that occur in apo-E2. The structural heterogeneity has been directly correlated with the functional heterogeneity, i.e., the severity of the binding defect, which has previously been documented in certain type III hyperlipoproteinemic subjects (17, 18). Therefore, it seemed necessary to determine, where possible, the location of the cysteine residues in the apo-E of the German subjects.

A partial sequence analysis of the cysteine-containing region of the apo-E2 was undertaken as previously described (6, 24). Limitations in the availability of sufficient apo-E for this type of analysis meant that apo-E from only three of the six subjects (W.G., W.H., and H.-O.K.) could be studied. Column-purified apo-E was digested with CNBr and the peptides were separated by gel chromatography. Cysteine-containing peptides (Fig. 3) were subjected to amino acid and sequence analyses (Tables III and IV). Both the composition and sequence of the small peptide, CB4 (Fig. 3), were identical for the apo-E from subjects W.G., W.H., and H.-O.K. Furthermore, the peptide was identical to the comparable peptide of the apo-E2 from other type III subjects and of the apo-E3 from subjects without type III hyperlipoproteinemia (6, 7, 24). This peptide, corresponding to residues 109–125 in the apo-E sequence (7), had the following structure: Glu-Asp-Val-Cys-Gly-Arg-Leu-Val-Gln-Tyr-Arg-Gly-Glu-Val-Gln-Ala-Met. It has previously been shown that the cysteine residue of this peptide (position 112 in the sequence) is not involved in the interaction of apo-E3 with receptors (18) and, as could be expected, there was no effect on binding when this cysteine residue was replaced by arginine, as occurs in apo-E4 (6, 18, 24).

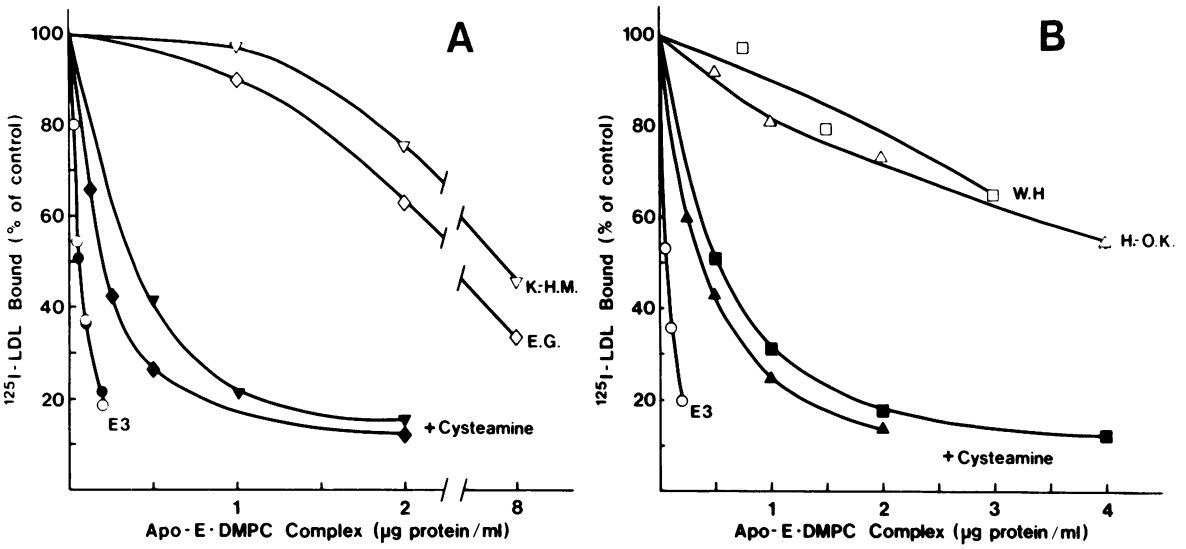

FIGURE 2 Ability of apo-E2·DMPC from hypolipidemic (A; E.G. and K.-H.M.) and hyperlipidemic (B; W.H. and H.-O.K.) subjects to compete with human ^{125}I-LDL for binding to receptors on cultured human fibroblasts. The binding activity of the apo-E2 (▽, ◇, □, △) was markedly enhanced after cysteamine treatment of the apo-E2 (▼, ◆, ■, ▲). Also shown for comparison is normal apo-E3·DMPC with (○) or without (●) treatment with cysteamine. The fibroblast monolayers were incubated for 48 h before the experiment with Dulbecco's modified Eagle's medium containing 10% human lipoprotein-deficient medium. Then 1 ml of Dulbecco's modified Eagle's medium containing 0.25 mM Hepes, pH 7.4, was added to the cells with 2 μg of ^{125}I-LDL and the indicated concentrations of protein·phospholipid complexes. After a 2-h incubation on ice, the cells on the 35-mm petri dishes were extensively washed, and the amount of ^{125}I-LDL that bound to the cells was determined. Both panels are from the same experiment and are plotted separately for clarity. The 100% control value with no added apo-E·DMPC complexes was 45 ng of ^{125}I-LDL protein bound/mg of cellular protein. Each point is the average of duplicate dishes.

The large, cysteine-containing peptide, CB5 (Fig. 3), from subjects W.G., W.H., and H.-O.K. (Table III) was not significantly different from the comparable peptide of subject D.R. (7). Partial sequence analyses of the CB5 of W.G., W.H., and H.-O.K. revealed that the cysteine residue occurred at cycle 33 (residue 158) in the apo-E sequence. This is the same site at which cysteine occurred in the apo-E2 of subject D.R. (7). It is this cysteine residue that has been shown to have a profound effect on the receptor binding ability of apo-E2 (18, 24). (Table IV compares data from W.H. and W.G.)

DISCUSSION

The structural and receptor binding studies on the apo-E2 from hypo-, normo-, and hypercholesterolemic E2/2 subjects indicated that the apo-E from these individuals was identical. A partial amino acid sequence analysis of the apo-E2 from three of these E2 homozygous subjects revealed that the cysteine-containing segment of the polypeptide chain (corresponding to residues 109–177) was also identical to that of the apo-E from the type III hyperlipoproteinemic subject D.R. (7). This is the region of the apo-E molecule in which all of the known amino acid substitutions occur (24) and is also the region of the molecule that has been implicated in receptor binding (32). A cysteine for arginine substitution was evident in the apo-E2 of subjects H.-O.K., W.H., and W.G. in this critical region of the molecule (residue 158), as well as in that of subject D.R. (7). Furthermore, the apo-E2 from all of these subjects was severely defective in receptor binding activity (<2% of the binding activity of normal apo-E3) and demonstrated a marked increase in binding after cysteamine treatment.

Recently, the structure of the apo-E2 from an additional type III hyperlipoproteinemic subject (W.M.) was shown to differ from normal apo-E3 by a cysteine/arginine interchange at residue 145 (24). This mutant form of apo-E2 was much less defective than the other mutant apo-E2 in respect to binding activity (demonstrating ~50% of the activity of normal apo-E). This along with other evidence indicates that binding heterogeneity correlates with structural heterogeneity (24, 32). Therefore, the similarity in binding activity

TABLE II

Summary of Receptor Binding Activity of Apo-E·DMPC from E2/2 Homozygous Subjects

Subject	Concentration of apo-E·DMPC at which 50% ^{125}I-LDL was displaced from fibroblasts*		Cysteamine activation (control/treated)
	Control	Cysteamine-treated	
	μg protein/ml		
"Normal" E3/3	0.057	0.051	1.1
Dysbetalipoproteinemic E2/2			
Hypocholesterolemic			
E.G.	3.5	0.21	16.7
K.-H.M.	6.2	0.37	16.7
Normocholesterolemic			
W.B.	5.0	0.35	14.3
W.G.	4.6	0.23	19.9
Hypercholesterolemic			
W.H.	5.5	0.44	12.5
H.-O.K.	5.0	0.38	13.2

* Compiled from competitive binding experiments as described in the legend to Fig. 2. A logit-logit plot of binding data was used to determine the 50% competition point.

TABLE III

*Amino Acid Compositions of the Cysteine-containing CNBr Peptides of Apo-E2**

	W.G.		W.H.		H.-O.K.	
	CB4	CB5	CB4	CB5	CB4	CB5
Asp	1.1 (1)	3.7	1.0 (1)	3.3	1.1 (1)	3.5
Thr	0.2	2.3	0.2	2.1	0.2	2.2
Ser	0.3	3.8	0.3	3.6	0.3	3.8
Glu	4.4 (4)	15.8	4.2 (4)	16.1	4.5 (4)	15.6
Pro	0.2	2.4	—	2.2	—	2.2
Gly	1.9 (2)	8.5	1.9 (2)	8.8	2.0 (2)	8.7
Ala	1.2 (1)	11.8	1.1 (1)	12.7	1.1 (1)	12.4
Cys‡	0.8 (1)	1.0	0.8 (1)	1.0	0.7 (1)	1.2
Val	2.8 (3)	5.5	2.9 (3)	6.0	3.1 (3)	5.7
Met§	0.3 (1)	0.3	0.4 (1)	0.3	0.3 (1)	0.4
Ile	0.1	1.0	—	1.0	—	0.9
Leu	1.5 (1)	14.5	1.2 (1)	14.7	1.4 (1)	14.7
Tyr	0.8 (1)	0.9	0.8 (1)	1.0	0.7 (1)	1.0
Phe	0.1	—	—	—	—	—
Lys	0.2	3.3	0.1	3.1	0.1	3.2
His	—	1.0	—	1.0	—	1.0
Arg	2.0 (2)	14.5	1.8 (2)	15.4	1.9 (2)	15.0

* Compositions are given in residues per mole; numbers in parentheses for CB4 are residues determined from sequence.
‡ Determined as cysteic acid after performic acid oxidation.
§ Determined as homoserine lactone.

and the presence of two residues of cysteine (as determined by partial sequence analyses or cysteamine treatment) in the apo-E of all six E2/2 subjects in the present study suggest that these proteins were structurally identical. One must conclude that the presence of receptor-defective apo-E2, as observed in these hypo-, normo-, and hypercholesterolemic E2/2 subjects, may not be sufficient for the expression of type III hyperlipoproteinemia.

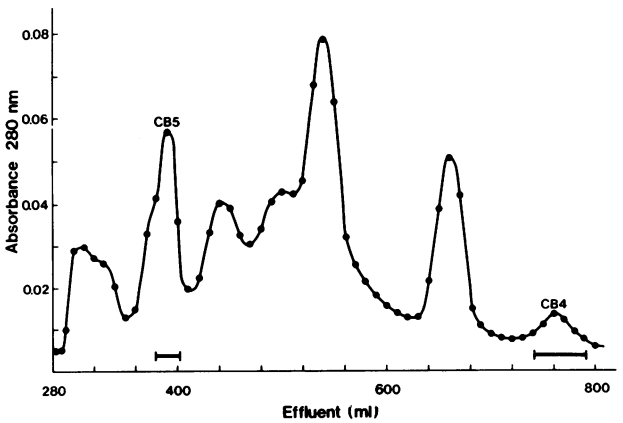

FIGURE 3 Sephadex G-50 chromatography of the CNBr digest of 6.5 mg of apo-E2 from subject W.H. Peptide fractions CB5 and CB4 were pooled as indicated by the horizontal bars.

The development of severe type III hyperlipoproteinemia (with hypercholesterolemia and hypertriglyceridemia) may require the existence of genetic or metabolic abnormalities in addition to the presence of a functionally defective form of apo-E. One is struck by the variety of environmental and hormonal factors that exacerbate or modulate the severity of the expression of the type III disorder, including age, sex, diet, obesity, hypothyroidism, and diabetes (21). It has recently been shown that the β-VLDL of type III hyperlipoproteinemic subjects actually represent two distinct lipoprotein classes, one of intestinal origin (chylomicron remnants) and a second apparently of hepatic origin (cholesterol-enriched VLDL) (11). Thus, the abnormalities of type III hyperlipoproteinemia may result from factors that impair the catabolism (hepatic uptake) of chylomicron remnants or that increase the production of hepatic VLDL, or both.

It is easy to envision how subjects with defective apo-E might develop hyperlipoproteinemia. The removal of chylomicron remnants from the plasma is carried out by the liver via receptor-mediated endocytosis (12–16), a process that appears to be mediated

TABLE IV

*Partial Sequence of Peptide CB5 of Apo-E2 from a Hypercholesterolemic (W.H.) and Normocholesterolemic (W.G.) Subject**

Residue number‡	Cycle number	Amino acid identified	Subject W.H.	Subject W.G.
			nmol	
—	0	—	57	81
126	1	Leu	19.1	19.4
127	2	Gly	26.2	26.7
128	3	Gln	11.6	6.9
129	4	Ser	9.7	11.9
130	5	Thr	10.8	13.3
131	6	Glu	25.0	10.9
132	7	Glu	25.8	13.4
133	8	Leu	25.7	21.0
134	9	Arg	6.7	2.4
135	10	Val	30.2	23.5
136	11	Arg	6.6	4.3
137	12	Leu	23.6	18.1
138	13	Ala	27.6	20.6
139	14	Ser	7.5	6.0
140	15	His	3.2	2.0
141	16	Leu	16.8	17.1
142	17	Arg	6.5	2.1
143	18	Lys	2.9	3.0
144	19	Leu	18.4	16.5
145	20	Arg	5.0	2.2
146	21	Lys	2.4	2.2
147	22	Arg	6.1	2.6
148	23	Leu	17.7	14.9
149	24	Leu	17.9	17.7
150	25	Arg	4.5	2.1
151	26	Asp	6.6	3.2
152	27	Ala	19.6	13.8
153	28	Asp	6.2	3.0
154	29	Asp	8.6	2.8
155	30	Leu	15.3	10.9
156	31	Gln	5.1	3.2
157	32	Lys	2.7	1.7
158	33	Cys	1.8	1.7
159	34	Leu	11.1	9.5
160	35	Ala	13.4	9.9
161	36	Val	11.2	7.6
162	37	Tyr	6.3	3.1
163	38	Gln	3.6	2.4
164	39	Ala	10.9	7.7
165	40	Gly	6.4	4.9
166	41	Ala	10.5	8.1
167	42	Arg	2.3	1.3
168	43	Glu	7.4	3.1
169	44	Gly	7.5	5.3
170	45	Ala	8.8	6.2
171	46	Glu	6.2	2.9
172	47	Arg	1.9	0.8
173	48	Gly	7.3	4.9
174	49	Leu	4.9	4.1
175	50	Ser	1.7	>0
176	51	Ala	6.7	4.9
177	52	Ile	3.9	2.7

* The same 52-residue sequence was also obtained for CB5 of apo-E2 from the type III hyperlipoproteinemic subject H.-O.K. (data not shown). The entire sequence of apo-E2 from the type III hyperlipoproteinemic subject D.R. has been reported (7).

‡ From apo-E2 of subject D.R. (6, 7, 24).

primarily or exclusively by apo-E. The presence of a mutant form of apo-E that is deficient in receptor binding capabilities would certainly disrupt this process. However, it is more difficult to understand why hyperlipidemia does not develop in all subjects in which an abnormal form of apo-E exists. In the present study, the apo-E of the hypo- and normocholesterolemic subjects was just as defective in respect to receptor binding as that of the hypercholesterolemic individuals.

It is reasonable to speculate that the chylomicron remnants of normolipidemic E2 homozygous subjects may be cleared by some other mechanism. It is possible that under these conditions the apo-B in chylomicron remnants of these subjects mediates the uptake of the remnants by the hepatic lipoprotein receptors. The efficiency of this process would depend on the level of the expression of the hepatic apo-B,E receptors. The expression of these receptors would be critical in determining the extent of the lipoprotein abnormality in subjects with defective apo-E. Furthermore, it should be recalled that, even though all six of these subjects had severely defective apo-E, the apo-E did display a low level of binding activity, which may account for some clearance of lipoproteins by either the apo-B,E or apo-E receptors.

These two distinct hepatic lipoprotein receptors, the apo-B,E (LDL) receptor and the apo-E receptor, are the primary lipoprotein receptors in the livers of man and certain animals. They are both capable of interacting with chylomicron remnants in vitro (15, 16). The hepatic apo-E receptor is relatively refractory to changes in expression that are brought about by metabolic perturbations (15, 16). However, the expression of hepatic apo-B,E receptors is rapidly and efficiently modulated by diet, drugs, hormones, and varying plasma concentrations of lipoproteins and bile acids (14–16, 33). In addition, the expression of the hepatic apo-B,E receptor is age-dependent. Young immature animals reveal very high levels of hepatic apo-B,E receptors, whereas adult animals express very low levels of hepatic apo-B,E receptors. (The hepatic apo-E receptors are not affected by age). Adult human livers, like those of adult animals, reveal very low levels of apo-B,E receptors (16). This is significant when considering that the manifestations of type III hyperlipoproteinemia are very age-dependent: they rarely, if ever, occur before adulthood (21). It is reasonable to speculate that the hyperlipidemia may result at this time because of the inability of the lower level of hepatic apo-B,E receptors to clear the chylomicron remnants.

The sensitivity of this lipid disorder to diet, drugs, and hormonal balance further suggests a role for the expression, or lack of expression, of the hepatic apo-

B,E receptors. For example, type III hyperlipoproteinemia in women most often occurs after menopause (21), and in these individuals the disorder is particularly responsive to estrogen therapy (34–36). The induction of hepatic apo-B,E receptors in animals given estrogen, though at nonpharmacologic doses, has previously been reported (37, 38). The role of hypothyroidism in the exacerbation of type III hyperlipoproteinemia (21) and the effects of thyroid hormone on receptor-mediated lipoprotein catabolism (39) further suggest that a modulation of hepatic apo-B,E receptors may be involved in the type III disorder. Those subjects homozygous for the E2/2 phenotype who do not have hyperlipoproteinemia may be capable of maintaining high levels of hepatic apo-B,E receptor activity and are therefore able to clear certain lipoproteins from the plasma (e.g., chylomicron remnants, which contain the defective apo-E).

The development of type III hyperlipoproteinemia may also result, at least in part, from an overproduction of hepatic cholesterol and/or hepatic VLDL. A primary cause for the overproduction of hepatic VLDL could be the impaired clearance of chylomicron remnants, which results in a deficiency in the delivery of cholesterol to the liver. As a result, hepatic cholesterol biosynthesis, and possibly hepatic VLDL production, could be stimulated. These newly secreted hepatic VLDL, either because of the presence of defective apo-E or because of an abnormal particle composition, may not be metabolized normally and thus could accumulate in the plasma. Furthermore, there may be several secondary causes for hepatic overproduction of VLDL and cholesterol. Various conditions that are known to exacerbate type III hyperlipoproteinemia, e.g., obesity, obesity with hypothyroidism, diabetes and age, have been shown to stimulate hepatic synthesis of VLDL and/or cholesterol (for review, see ref. 40). In addition, Utermann et al. (2) and Hazzard et al. (23) have presented compelling evidence from kindred studies that the development of type III hyperlipoproteinemia is expressed in some E2 homozygous subjects in combination with a second heritable hyperlipidemia, such as familial combined hyperlipoproteinemia. The defect in those individuals with familial combined hyperlipoproteinemia may well be a hepatic overproduction of cholesterol and VLDL (41).

It is clear that the expression of type III hyperlipoproteinemia is complex. The description of the abnormalities in the apo-E2 of these subjects provides a basis for understanding this lipoprotein disorder; however, other factors clearly interact to modulate its expression. The considerations discussed represent testable hypotheses.

ACKNOWLEDGMENTS

We thank Reed Harris, Kay Arnold, David Begert, and Jana Seymour for excellent technical assistance. We also thank Russell Levine for editorial assistance and Gwen Watson for graphic arts.

REFERENCES

1. Utermann, G., M. Hees, and A. Steinmetz. 1977. Polymorphism of apolipoprotein E and occurrence of dysbetalipoproteinaemia in man. *Nature (Lond.).* **269:** 604–607.
2. Utermann, G., K. H. Vogelberg, A. Steinmetz, W. Schoenborn, N. Pruin, M. Jaeschke, M. Hees, and H. Canzler. 1979. Polymorphism of apolipoprotein E. II. Genetics of hyperlipoproteinemia type III. *Clin. Genet.* **15:** 37–62.
3. Utermann, G., U. Langenbeck, U. Beisiegel, and W. Weber. 1980. Genetics of the apolipoprotein E system in man. *Am. J. Hum. Genet.* **32:** 339–347.
4. Zannis, V. I., and J. L. Breslow. 1981. Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and post-translational modification. *Biochemistry.* **20:** 1033–1041.
5. Zannis, V. I., P. W. Just, and J. L. Breslow. 1981. Human apolipoprotein E isoprotein subclasses are genetically determined. *Am. J. Hum. Genet.* **33:** 11–24.
6. Weisgraber, K. H., S. C. Rall, Jr., and R. W. Mahley. 1981. Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. *J. Biol. Chem.* **256:** 9077–9083.
7. Rall, S. C., Jr., K. H. Weisgraber, and R. W. Mahley. 1982. Human apolipoprotein E. The complete amino acid sequence. *J. Biol. Chem.* **257:** 4171–4178.
8. Utermann, G., M. Jaeschke, and J. Menzel. 1975. Familial hyperlipoproteinemia type III: deficiency of a specific apolipoprotein (apo-E-III) in the very-low-density lipoproteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **56:** 352–355.
9. Pagnan, A., R. J. Havel, J. P. Kane, and L. Kotite. 1977. Characterization of human very low density lipoproteins containing two electrophoretic populations: double prebeta lipoproteinemia and primary dysbetalipoproteinemia. *J. Lipid Res.* **18:** 613–622.
10. Zannis, V. I., and J. L. Breslow. 1980. Characterization of a unique human apolipoprotein E variant associated with type III hyperlipoproteinemia. *J. Biol. Chem.* **255:** 1759–1762.
11. Fainaru, M., R. W. Mahley, R. L. Hamilton, and T. L. Innerarity. 1982. Structural and metabolic heterogeneity of β-very low density lipoproteins from cholesterol-fed dogs and from humans with Type III hyperlipoproteinemia. *J. Lipid Res.* **23:** 702–714.
12. Sherrill, B. C., T. L. Innerarity, and R. W. Mahley. 1980. Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. Identity with the chylomicron remnant transport process. *J. Biol. Chem.* **255:** 1804–1807.
13. Havel, R. J., Y.-S. Chao, E. E. Windler, L. Kotite, and L. S. S. Guo. 1980. Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia. *Proc. Natl. Acad. Sci. USA.* **77:** 4349–4353.
14. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981.

Regulation of plasma cholesterol by lipoprotein receptors. *Science (Wash. DC).* **212:** 628–635.
15. Hui, D. Y., T. L. Innerarity, and R. W. Mahley. 1981. Lipoprotein binding to canine hepatic membranes: metabolically distinct apo-E and apo-B,E receptors. *J. Biol. Chem.* **256:** 5646–5655.
16. Mahley, R. W., D. Y. Hui, T. L. Innerarity, and K. H. Weisgraber. 1981. Two independent lipoprotein receptors on hepatic membranes of dog, swine, and man. Apo-B,E and apo-E receptors. *J. Clin. Invest.* **68:** 1197–1206.
17. Schneider, W. J., P. T. Kovanen, M. S. Brown, J. L. Goldstein, G. Utermann, W. Weber, R. J. Havel, L. Kotite, J. P. Kane, T. L. Innerarity, and R. W. Mahley. 1981. Familial dysbetalipoproteinemia. Abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits, and cows. *J. Clin. Invest.* **68:** 1075–1085.
18. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J. Biol. Chem.* **257:** 2518–2521.
19. Gregg, R. E., L. A. Zech, E. J. Schaefer, and H. B. Brewer, Jr. 1981. Type III hyperlipoproteinemia: defective metabolism of an abnormal apolipoprotein E. *Science (Wash. DC).* **211:** 584–586.
20. Hazzard, W. R., T. F. O'Donnell, and Y. L. Lee. 1975. Broad-beta disease (type III hyperlipoproteinemia) in a large kindred. Evidence for a monogenic mechanism. *Ann. Intern. Med.* **82:** 141–149.
21. Fredrickson, D. S., J. L. Goldstein, and M. S. Brown. 1978. The familial hyperlipoproteinemias. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. 4th edition). McGraw-Hill, Inc., New York. 604–655.
22. Utermann, G., N. Pruin, and A. Steinmetz. 1979. Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus on plasma lipid levels in man. *Clin. Genet.* **15:** 63–72.
23. Hazzard, W. R., G. R. Warnick, G. Utermann, and J. J. Albers. 1981. Genetic transmission of isoapolipoprotein E phenotypes in a large kindred: relationship to dysbetalipoproteinemia and hyperlipidemia. *Metab. Clin. Exp.* **30:** 79–88.
24. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4700.
25. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
28. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
29. Innerarity, T. L., R. E. Pitas, and R. W. Mahley. 1979. Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J. Biol. Chem.* **254:** 4186–4190.
30. Menzel, H. J., R. G. Kladetzky, and G. Assmann. 1983. Apolipoprotein E polymorphism and coronary artery disease. *Arteriosclerosis.* In press.
31. Heiss, G., I. Mebane, and I. Tamir. 1980. The Lipid Research Clinics Population Studies Data Book. I. The Prevalence Study. (NIH Publication No. 80-1527), Bethesda, MD. 1–136.
32. Mahley, R. W., S. C. Rall, Jr., T. L. Innerarity, and K. H. Weisgraber. 1983. Apolipoprotein E and cholesterol metabolism. *In* Atherosclerosis VI. In press.
33. Angelin, B., C. Raviola, T. L. Innerarity, and R. W. Mahley. 1983. Regulation of hepatic lipoprotein receptors in the dog. Rapid regulation of apolipoprotein B,E receptors, but not of apolipoprotein E receptors, by intestinal lipoproteins and bile acids. *J. Clin. Invest.* **71:** 816–831.
34. Chait, A., J. D. Brunzell, J. J. Albers, and W. R. Hazzard. 1977. Type-III hyperlipoproteinaemia ("remnant removal disease"). Insight into the pathogenic mechanism. *Lancet.* **I:** 1176–1178.
35. Kushwaha, R. S., W. R. Hazzard, C. Gagne, A. Chait, and J. J. Albers. 1977. Type III hyperlipoproteinemia: paradoxical hypolipidemic response to estrogen. *Ann. Intern. Med.* **87:** 517–525.
36. Falko, J. M., G. Schonfeld, J. L. Witzum, J. Kolar, and S. W. Weidman. 1979. Effects of estrogen therapy on apolipoprotein E in type III hyperlipoproteinemia. *Metab. Clin. Exp.* **28:** 1171–1177.
37. Windler, E. E., P. T. Kovanen, Y.-S. Chao, M. S. Brown, R. J. Havel, and J. L. Goldstein. 1980. The estradiol-stimulated lipoprotein receptor of rat liver. A binding site that mediates the uptake of rat lipoproteins containing apoproteins B and E. *J. Biol. Chem.* **255:** 10464–10471.
38. Kovanen, P. T., M. S. Brown, and J. L. Goldstein. 1979. Increased binding of low density lipoprotein to liver membranes from rats treated with 17 alpha-ethinyl estradiol. *J. Biol. Chem.* **254:** 11367–11373.
39. Thompson, G. R., A. K. Soutar, F. A. Spengel, A. Jadhav, S. J. P. Gavigan, and N. B. Myant. 1981. Defects of receptor-mediated low density lipoprotein catabolism in homozygous familial hypercholesterolemia and hypothyroidism *in vivo*. *Proc. Natl. Acad. Sci. USA.* **78:** 2591–2595.
40. Grundy, S. M. 1982. Hypertriglyceridemia: mechanisms, clinical significance, and treatment. *In* The Medical Clinics of North America: Lipid Disorders. R. J. Havel, editor. W. B. Saunders Company, Philadelphia. **66:** 519–535.
41. Chait, A., J. J. Albers, and J. D. Brunzell. 1980. Very low density lipoprotein overproduction in genetic forms of hypertriglyceridaemia. *Eur. J. Clin. Invest.* **10:** 17–22.