Gene-Environment Interaction in the Conversion of a Mild-to-Severe Phenotype in a Patient Homozygous for a Ser^{172}→Cys Mutation in the Lipoprotein Lipase Gene

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Abstract

Normal pregnancy is associated with a two- to threefold increase in plasma triglyceride levels, particularly in the third trimester, due both to the overproduction of VLDLs and to the possible suppression of lipoprotein lipase (LPL) activity. Numerous mutations in the human LPL gene causing complete LPL deficiency have been described, but naturally occurring mutations that result in defective LPL with partial activity have not yet been reported. Here we describe a 30-yr-old woman who was first diagnosed with LPL deficiency during pregnancy after she developed pancreatitis. Her plasma triglyceride levels remained mildly elevated at approximately 300 mg/dl (3.4 mmol/liter) after the first pregnancy but rose significantly after she became pregnant again (1800 to 2000 mg/dl) (20.2 to 22.5 mmol/liter). DNA sequence analysis of the LPL gene showed that the patient is homozygous for a Ser^{172}→Cys missense mutation in exon 5. In vitro mutagenesis revealed that the Ser^{172}→Cys mutation caused a mutant LPL protein that had residual activity higher than that seen in all eight other missense mutations in patients with LPL deficiency identified in our laboratory. We propose that some mutations in the LPL gene produce a defective LPL with partial activity, which usually leads to mild hypertriglyceridemia. (*J. Clin. Invest.* 1993. 91:1953–1958.) Key words: lipoprotein lipase • missense mutations • pregnancy • pancreatitis • hyperlipoproteinemia

Introduction

Normal pregnancy is associated with alterations in plasma lipoprotein and lipid levels (1, 2), including a mild increase in plasma cholesterol level and approximately a two- to threefold increase in triglyceride level, which reaches its highest level during the third trimester (2). This physiological hypertriglyceridemia is believed to be induced by an increased level of estrogen during pregnancy. The direct correlation between estrogen levels and hypertriglyceridemia has also been observed in premenopausal women using estrogen-containing oral contraceptives (3, 4). A similar increase in triglyceride levels have been seen in estrogen-fed rats (5) and in estrogen-fed chicks (6).

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Although the exact mechanisms of estrogen-induced hyperlipidemia remain unclear, estrogen appears to modulate lipid levels by affecting both the rate of production and the efficiency of the removal of plasma triglyceride-rich lipoproteins. Increased secretion of VLDL triglycerides has been described in pregnant women (2, 7, 8, 9), and to a lesser extent in women using oral contraceptives (3, 10). In addition, kinetic studies in estrogen-fed chicks have revealed that the estrogen-treated VLDL particles were found to differ from the normal VLDL with respect to size and apolipoprotein composition and were catabolized at a slower rate than control VLDL (6). Similarly in humans, under the influence of estrogens, lipoprotein lipase (LPL)[1] mediated triglyceride removal is also decreased resulting in hypertriglyceridemia (9–11).

LPL gene mutations causing chylomicronemia have been studied extensively in recent years since the cloning of the human LPL cDNA (12). More than 25 different mutations in the LPL gene have been identified that result in a completely catalytically inactive LPL protein (13). However, mutations in the LPL gene that result in defective LPL with partial activity have not yet been reported. Here we describe a 30-yr-old woman who was not diagnosed with LPL deficiency until she developed pancreatitis during pregnancy. The LPL deficiency in this patient is due to a missense mutation in the LPL gene that causes a defective LPL enzyme with partial activity. In the nonpregnant state, this is associated with modestly elevated plasma triglyceride levels that, increase markedly during pregnancy with the development of chylomicronemia.

Methods

Subject. The patient is an East Indian woman who was in good health with no history of abdominal pain prior to pregnancy. At 33 wk of gestation of her first pregnancy she developed abdominal pain. Pancreatitis was confirmed by raised serum amylase levels of 1014 and 1940 IU/liter (normal range: 23–85 IU/liter). The patient was discharged after a short stay in hospital. She continued to be clinically well until 37 wk of gestation, when she experienced recurrent severe right upper-quadrant pain associated with nausea and vomiting and was again hospitalized with recurrent pancreatitis with a serum amylase level of 1,920 IU/liter. Ultrasound examination showed a normal 37-wk-old fetus and a thickened pancreas with ascites. No fetal heartbeat could be identified, and an intrauterine fetal death was diagnosed. The patient recovered after being treated initially with intravenous rehydration and nil per mouth. Treatment with total parental nutrition without fat was initiated on day five after her admission. 3 d after delivery of the stillborn infant, the patient's triglyceride level decreased to below 600 mg/dl but continued to be moderately elevated until her discharge.

There was no history of alcohol abuse or oral contraceptive use.

1. *Abbreviations used in this paper:* HL, hepatic lipase; LPL, lipoprotein lipase.

Family history revealed that the parents of the patient were first cousins. None of her four siblings had a history of pancreatitis.

Lipid measurements. Plasma total triglyceride, total cholesterol, LDL and HDL levels were measured using methods previously described (14, 15).

Measurements of LPL mass and catalytic activity. Blood samples were collected from the proband after an overnight fast. An intravenous injection of heparin was given (60 U/kg body mass) and the postheparin blood samples were obtained 10 min after the injection. Plasma was separated after centrifugation at 3000 *g* for 10 min at 4°C. LPL mass in pre- and postheparin plasma samples was measured, using the 5D2 monoclonal antibody against purified bovine milk LPL (16). We have recently identified the location for the epitope for the 5D2 antibody to residue 400 of the mature LPL protein in the COOH-terminal domain (17). All the mutant proteins that we have tested in this study are located between residue 156 and 250 in the NH_2-terminal domain. The 5D2 antibody appears to bind normally to all these mutant LPL, both in patients' plasma and in transfected COS medium and cell homogenate.

LPL and hepatic lipase (HL) lipolytic activities were measured using a radiolabeled tri-*l*-^{14}C-oleate emulsion as previously described (18). The LPL in postheparin plasma was blocked with a polyclonal rabbit antibovine LPL antibody.

DNA analysis. Genomic DNA was isolated from white blood cells of the patient as previously described (19). DNA haplotypes of mutant LPL alleles were constructed using the HindIII, BamHI, and PvuII (20, 21) RFLP at the LPL locus.

Each of the 10 coding exons of LPL was individually amplified 0.5–1 μg of genomic DNA from the proband using the PCR as previously described (22, 23). Several independent PCR reactions were performed for each exon. The amplified exons were then purified and sequenced either directly or after cloning into a TA cloning vector (Invitrogen Inc. San Diego, CA).

In vitro site-directed mutagenesis of human LPL cDNA and transient expression in COS cells. A 1.6-kb DraI/EcoRI fragment containing the entire coding sequence was prepared from the full-length LPL cDNA clone (pLPL35). This fragment was cloned in the sense orientation into the phagemid vector CDM8 (24) which served as a dual-function vector for both mutagenesis and expression. In vitro site-directed mutagenesis was performed as previously described (25). The sequence of the oligonucleotide for mutagenesis is: 5′-CCGAGTCGTCTTTGTCCTGATGAT-3′. Mutant clones were identified by oligonucleotide hybridization and verified by DNA sequencing.

Expression phagemids were purified and introduced into the COS-1 cells by DEAE-Dextran transfection. Briefly, COS-1 cells were seeded in DME medium with 10% FCS (GIBCOBRL, Gaithersburg, MD) in 90 mm culture dishes at 25% confluence. Transfections were performed when the cells became 80% confluent. At this point, the medium in each plate was replaced with 5 ml of IMDM containing 10% Nuserum (Collaborative Research Inc., Lexington, MA), 400 μg/ml DEAE Dextran, and 100 μM chloroquine. 15 μg of purified phagemid DNA were added to each plate and mixed by swirling the medium. The plates were then incubated in a CO_2 incubator for 90 min and the medium was replaced with 5 ml of PBS with 10% DMSO (Sigma Immunochemicals, St. Louis, MO). After a 2-min incubation at room temperature, the PBS/DMSO solution was removed, the plates were rinsed once with 5 ml of PBS, and 10 ml of DME medium with 10% FCS was added to each plate. After a 24-h incubation, the medium was replaced with fresh medium containing 40 μg/ml of heparin, and the incubation continued for another 24–48 h. At this stage, the culture medium was removed and stored at −120°C. The cells were collected, counted, and the cell homogenate was prepared as previously described (23). LPL mass as detected by 5D2 monoclonal antibody and LPL activity against trioleate emulsion were measured in both COS cell medium and cell homogenate as described above for plasma samples.

Results

Biochemical measurements. The results of clinical laboratory investigations during the patient's first pregnancy revealed an elevated serum amylase level of 1,014 and 1,940 IU/liter and very high triglyceride levels of 2,134 and 2,464 mg/dl, respectively. Because of the history of consanguinity and a high serum triglyceride level, a genetic cause for her chylomicronemia was strongly suspected.

Lipoprotein lipase and hepatic lipase activities were measured (Table I) initially at 10 d following the delivery of the stillbirth female. LPL activity at that time was undetectable, and hepatic lipase activity was decreased to 81.7 nmol/min per ml. The results of LPL assays at 6 wk and 4, 6, 9 mo postpartum are shown in Table I. Although the LPL activity increased only slightly, from 0 to 33 nmol/min per ml, the plasma triglyceride levels decreased dramatically from 2464 mg/dl at the time of delivery to 262 mg/dl 9 mo postpartum.

The second pregnancy was followed prospectively. Dietary fat restriction was implemented with fat content of less than 10% of patient's diet. Serum triglyceride levels were decreased consequent to strict dietary restriction, but then increased with advancing gestational age. There were two episodes of abdominal pain at 26 and 31 wk without pancreatitis (Fig. 1). Labor was induced at 38 wk gestation, and a healthy male infant was delivered.

DNA analysis. DNA haplotyping indicated that the proband is homozygous for LPL haplotype H6. Together with the fact that there is consanguinity in the family, this strongly suggests a single mutation underlying LPL deficiency in this patient. The nomenclature for the haplotypes (total of 8) has previously been described (22, 23).

DNA sequence analysis was performed for all 10 exons and intron-exon junctions. Several DNA alterations were identified, and the patient was found to be homozygous for all substi-

Table I. Plasma LPL, HL, and Triglyceride Levels of the Patient

Time after delivery	LPL	HL	TG	Diet	Comments
	nmolFFA/min/ml		*mg/dl*		
0 day	ND	ND	2464	IV	In hospital
10 days	0	81.7	398	IV	In hospital
6 weeks	5.0	283.3	510	Low fat	In hospital
4 months	46.7	566.6	328	Low fat	Home
9 months	33.3	615.0	262	Low fat	Home
Normal control (n = 6)	280±51.7	581±250	< 200 mg/dl		

TG, triglyceride; ND, not done.

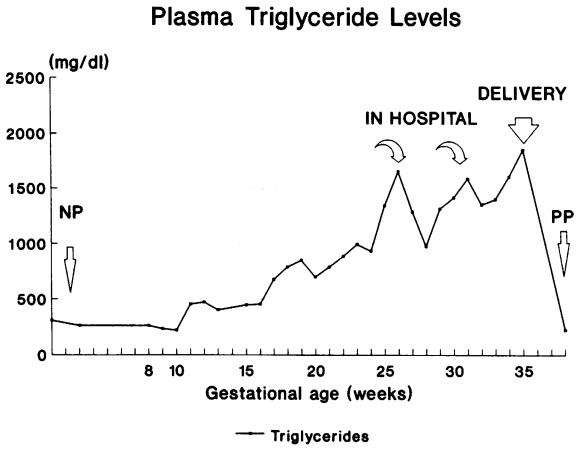

Figure 1. Triglyceride levels at different gestational ages. Triglyceride levels were measured weekly beginning at 8 wk of gestation of the patient's second pregnancy. Two separate lipid measurements during the nonpregnant (*NP*) state and one measurement 6 mo postpartum (*PP*) are also included.

tutions (Fig. 2). First, a C-to-G substitution at nucleotide 770 in exon 5 was identified, which resulted in an amino acid substitution of cysteine for serine at residue 172 (Fig. 2 *a*). Second, two adjacent substitutions from c to g and t to c, respectively, were identified in intron 5 at nucleotide 33 and 37 downstream to the exon5/intron 5 junction (Fig. 2*b*). Finally, a C-to-G transversion was identified which results in a premature stop codon at residue 447 in exon 9 (data not shown). Several independent PCR amplifications and subsequent DNA sequencing for both the coding and noncoding strand were performed to confirm the presence of these DNA substitutions. The DNA sequence of the eight remaining exons and the exon-intron junctions of the LPL gene were found to be normal.

The C-to-G transversion at residue 447 has previously been identified as a normal variant within the LPL gene (25–27). The nucleotide 33/37 substitutions are located in intron 5 and therefore do not alter the protein sequence of LPL. The t-to-c substitution at nucleotide 37 generates a new FokI restriction site 5′-*C*ATTC-3′. FokI digestion was performed following PCR amplifications of DNA from 29 normal controls. Five normal controls were found to be heterozygous and one homozygous for the substitution (Fig. 3). The individual homozygous for this substitution at nucleotide 37 did not have any evidence for LPL deficiency and had normal lipid levels, suggesting that this is a polymorphism in the LPL gene.

In vitro site-directed mutagenesis and expression. To determine whether the Ser^{172}→Cys missense substitution is responsible for the reduced catalytic activity of LPL in the patient, we introduced this mutation into cloned LPL cDNA by site-dir-

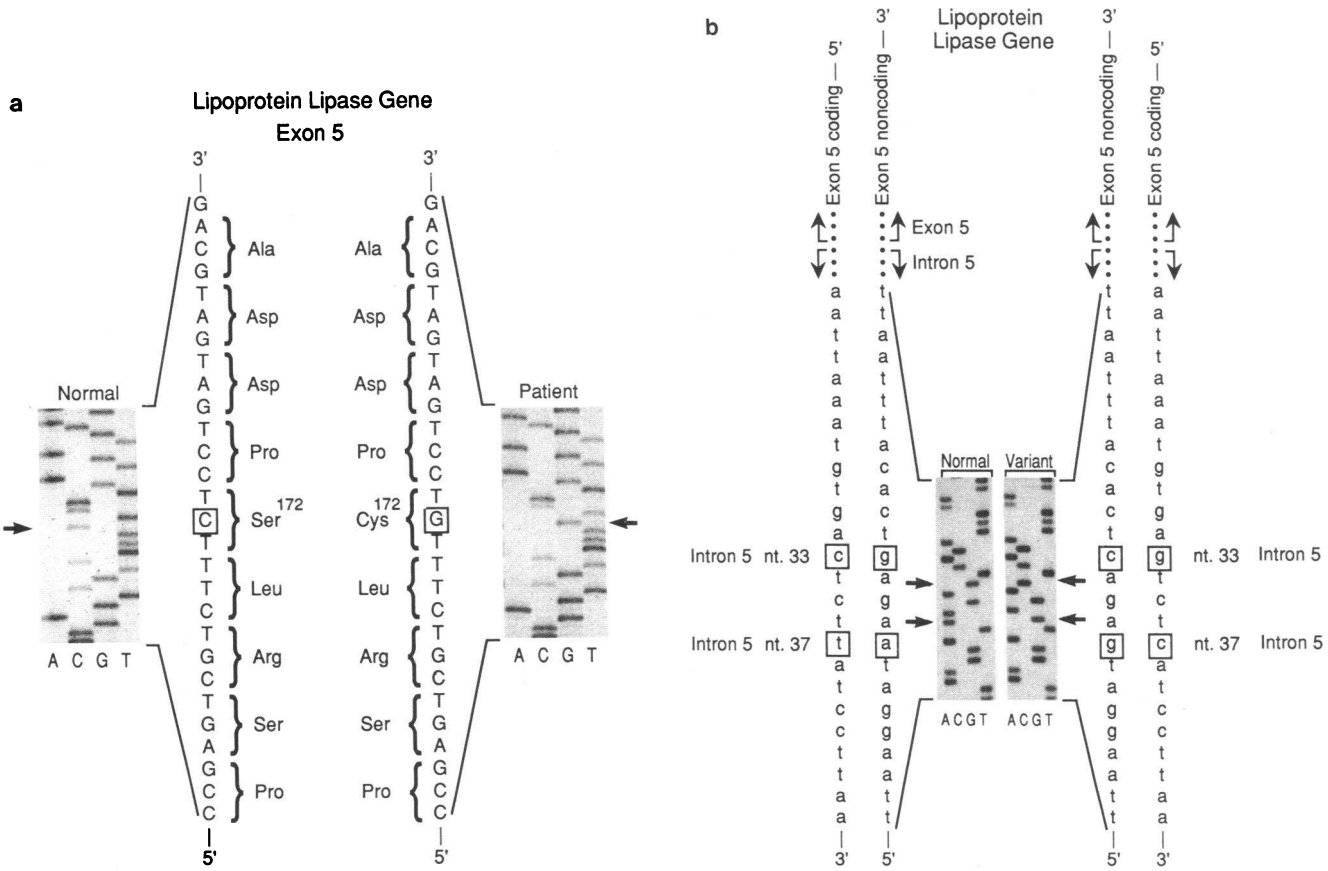

Figure 2. DNA sequence of the normal and mutant exon 5 spanning residue Ser^{172} and exon5-intron 5 junction. Exon 5 with the flanking intron sequence form the proband was PCR amplified and cloned into a TA vector. Plasmid DNA was individually purified from 8 positive clones for each exon and sequenced for both coding and noncoding strands. (*a*) Nucleotide sequence of the coding strand from exon 5 of the proband and a control normal subject. A C→G substitution resulting in a substitution of cysteine for serine at residue 172 is highlighted in the boxes. (*b*) DNA sequence of the coding strand from exon 5-intron 5 junction of the same patient. Two adjacent substitutions from c to g and t to c, at nucleotide 33 and 37 downstream to the exon/intron boundary, are indicated in the boxes.

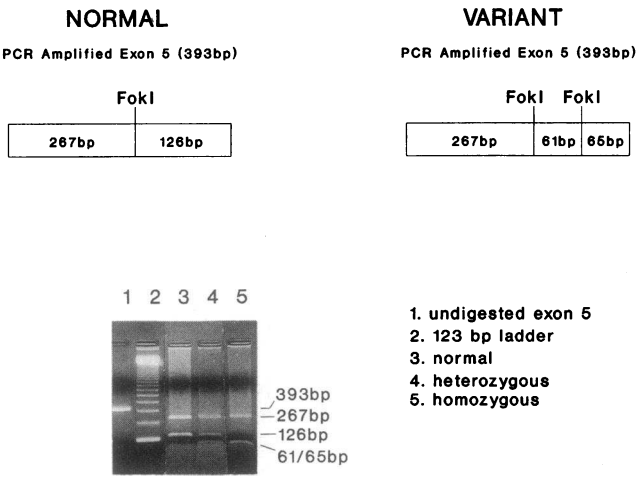

Figure 3. Schematic representation of the t-to-c substitution at nucleotide 37 in intron 5 generating a new FokI restriction site. Exon 5 DNA (393 bp in length) was amplified by PCR, digested with FokI, and separated on a 2% agarose gel. The proband (lane *5*) is homozygous for the 61 and 65 bp abnormal bands. Lane *3* is a normal control.

ected mutagenesis using a dual-function mutagenesis-expression vector. Mutant specific clones were identified by oligonucleotide hybridization. DNA from positive clones was purified and the substitution was confirmed by sequencing.

The transfection experiments were performed using DEAE-Dextran method. Transfection efficiency of this method is estimated to be ~10–20% using a reporter vector. In two separate experiments, equal amounts (15 μg) of wildtype LPL cDNA, mutant cDNA and negative control vector DNA were transfected in parallel into 3×10^6 COS-1 cells. LPL mass and activity were assayed in the transfected COS cell medium and the cell homogenate for the wildtype control and mutant cDNA. LPL protein was present in the medium of COS cells transfected with mutant Ser^{172}→Cys cDNA at mean levels of 78 and 51 ng/dish, respectively. These levels are ~ 20% of that seen in the medium of cells transfected with the normal LPL cDNA (Table II). However, in the cell homogenate, the mutant LPL mass levels were found to be higher than that of the wildtype control at 184% and 300%. This suggests either defective secretion of the mutant LPL protein or enhanced degradation of the secreted LPL within the medium. Cells transfected with vector only (negative control) showed no LPL mass both in medium and in cell homogenate.

The mean levels of the Ser^{172}→Cys mutant LPL activity in COS-1 medium were 16.4 and 17.2 nmol FFA/min per ml (Table III). These levels are ~ 5% of the wildtype control and are significantly ($P = 0.007$) higher than those seen in eight other LPL gene mutations that cause complete deficiency of LPL activity (Table III and IV) previously identified in our laboratory. The specific activity of the Ser^{172}→Cys mutant LPL in COS medium is approximately 20% of the wildtype level (Table V). In the COS cell homogenate, the specific activities for both wildtype LPL and mutant LPL are very low, at 0.07 and 0.008, respectively. The low specific activity for wildtype LPL is consistent with the mean level of 0.06 ± 0.02 based on the results from our previous transfection experiments. A possible reason for the low specific activity of wildtype LPL in the cells is that within cells LPL is likely to be at different stages of posttranslational modification. Even though LPL mass is recognized by the 5D2 antibody, the LPL may not be fully processed and therefore is likely to be inactive.

Table II. LPL Mass Levels in COS Medium and in Cell Homogenate

cDNA	Media	%	Cells	%
	ng/dish		*ng/dish*	
S172C Mutant				
wtLPL	384.0±8.4	100	155.0±7.1	100
S172C	78.0±18.0	20	285.0±5.6	184
wtLPL	198.0±12.0	100	100.0±8.5	100
S172C	51.0±18.0	26	300.0±14.1	300
8 Inactive mutants				
wtLPL	1125.2±163.1	100	574.2±59.5	100
I194T	419.3±3.8	37	796.0±126.6	139
wtLPL	1430.4±47.4	100	463.2±32.2	100
P207L	403.4±10.3	28	427.4±27.9	92
wtLPL	447.9±2.3	100	92.9±2.3	100
P157R	350.0±10.2	78	138.8±8.9	149
G188E	806.9±22.7	180	167.1±12.0	180
D250E	448.5±9.9	100	164.3±11.7	177
wtLPL	413.4±5.1	100	549.0±23.2	100
D156N	570.0±7.6	138	613.5±21.5	112
D156G	304.5±14.8	74	750.1±11.7	137
C216S	170.7±27.6	41	486.8±23.3	89

S172C, Ser^{172} → Cys; I194T, Ile^{194} → Thr; P207L, Pro^{207} → Leu; P157R, Pro^{157} → Arg; G188E, Gly^{188} → Glu; D250N, Asp^{250} → Asn; D156N, Asp^{156} → Asn; D156G, Asp^{156} → Gly; C216S, Cys^{216} → Ser.

Discussion

Increased plasma triglyceride levels are commonly observed in normal pregnant women, particularly during the third trimester (1–5). This physiological hypertriglyceridemia is likely to

Table III. LPL Activity Levels in COS Medium

cDNA	LPL activity	%
	nmol FFA/min/dish	
S172C Mutant		
wtLPL	389.4±5.1	100
S172C	16.4±4.5	4.2
wtLPL	282.2±4.1	100
S172C	17.2±2.3	6.1
8 Inactive mutants		
wtLPL	293.1±2.1	100
I194T	0±0	0
wtLPL	534.0±3.2	100
P207L	1.3±0.3	0.2
wtLPL	124.9±4.2	100
P157R	1.0±1.0	0.8
G188E	1.3±1.0	1.0
D250E	0±0	0
wtLPL	219.6±1.8	100
D156N	1.2±1.7	0.5
D156G	1.9±0.7	0.8
C216S	0±0	0

Table IV. Comparison of Mean LPL Activity Levels among Mutants

Mutant	Mean	Standard deviation
	nmol FFA/min/dish	
8 inactive mutants	0.878	0.998
$Ser^{172} \rightarrow Cys$	16.80	0.565

$P = 0.007$ (Student t test).

be due to the increased level of estrogen during pregnancy, which stimulates the secretion of triglyceride-rich VLDL and decreases the activity of hepatic triglyceride lipase, although the effects on LPL are less clear (7–11). The first occurrence of chylomicronemia and pancreatitis in pregnancy may identify a group of persons with partial defects in triglyceride hydrolysis due to mutations in the LPL gene that result in defective but not completely inactive LPL. These patients would manifest with mild hypertriglyceridemia in the nonpregnant state.

In this report, we have described a 30-yr-old woman who was diagnosed for the first time with LPL deficiency after she developed pancreatitis due to chylomicronemia during the third trimester of her first pregnancy. In the nonpregnant state, she had only mild hypertriglyceridemia. We have shown that the cause for the catalytically defective LPL is a $Ser^{172} \rightarrow Cys$ missense mutation in the LPL gene. In vitro mutagenesis studies have demonstrated that the small amount of mutant LPL that is secreted has reduced but some residual catalytic activity. The total activity of the $Ser^{172} \rightarrow Cys$ mutant LPL in COS medium is ~5% of the normal level. Also, this mutation results in a relative increase in intracellular LPL mass compared with that present in the medium that might be due either to a defect in secretion of the mutant LPL or to a rapid degradation of mutant LPL in the medium.

This missense mutation results in the substitution of serine for cysteine, which is a highly conserved residue in LPL, PL, and HL. In LPL and HL, of all species, there are 10 completely conserved cysteine residues, and they are all linked by disulfide bridges (28–30), which suggests that the cysteine residues are important in maintaining the secondary structure required for optimal function of the lipases. The $Ser^{172} \rightarrow Cys$ mutation generated an extra cysteine residue in the mutant LPL protein of the patient. This extra cysteine residue may be present in its reduced form or linked to 1 of the 10 other cysteine residues in LPL by a disulfide bond that would disrupt normal disulfide bridges and in turn may affect the stability of the LPL protein and the catalytic activity of the enzyme.

Table V. LPL Activity, Mass & Specific Activity in COS Medium and in Cell Homogenate

cDNA	Activity	Mass	Specific activity
	nmolFFA/min/dish	*ng/dish*	*nmol FFA/min/ng*
In COS medium			
wtLPL	282.2±4.1	198.0±12.0	1.43
S172C	17.2±2.3	51.0±18.0	0.34
In cell homogenate			
wtLPL	6.6±0.2	100.0±8.5	0.07
S172C	2.4±0.4	300.0±14.1	0.008

The serine residue at 172 is conserved in LPL and HL of all known species but is replaced with an aspartate residue in PL. In vitro mutagenesis of this serine residue has previously been reported (31, 32). When the serine at residue 172 is replaced by an aspartate, as seen in PL, the specific activity of the mutant LPL is 36% of normal. However, when the $serine^{172}$ is replaced by an alanine or threonine, the specific activities of the mutants are only 4% and 7% of normal, respectively. However, when glycine is substituted for $serine^{172}$, the mutant LPL is completely inactive. In this report, the specific activity of the $Ser^{172} \rightarrow Cys$ mutant LPL is approximately 20% of the wildtype values. Taken together, all these data suggest that the serine residue at 172 is important for LPL catalysis but that different substitutions might have different effects on LPL catalytic activity.

In the search for naturally occurring mutations in the LPL gene, the phenotype chosen for study has been chylomicronemia (16). This might lead to the conclusion that homozygous mutations in both LPL genes are always associated with chylomicronemia. However, this study shows that mild hypertriglyceridemia might also be associated with mutations in both alleles of the LPL gene. Different environments that either increase VLDL secretion and/or further decrease LPL activity may precipitate chylomicronemia in these patients. These environment triggers may include pregnancy, estrogen therapy, or possibly alcohol intake or diabetes.

Other genetic variations might also play a role in determining the response to different environmental challenges. For example, the presence of a single apoE4 allele has been found to be strongly associated with higher levels of triglyceride and VLDL in persons heterozygous for apo CII deficiency (33). In this study, the proband is homozygous for the normal apoE3 allele (data not shown). While DNA variation in the apoE gene cannot be invoked as predisposing this patient to a subsequent hypertriglyceridemic response to pregnancy, variations in other genes cannot be excluded as having some role in mediating this response.

Several patients with LPL deficiency in pregnancy have been reported (1, 34–38). For some the hypertriglyceridemia due to LPL deficiency was detected before pregnancy and therefore could be monitored and carefully controlled during pregnancy (1, 34, 38). For others, however, hypertriglyceridemia was not noticed until abdominal pain and pancreatitis developed late in pregnancy, a condition associated with an increased risk of death for both mother and fetus (37). The presence of mild hypertriglyceridemia in early pregnancy might alert the physician to remeasure triglyceride levels again during pregnancy to allow careful management to prevent chylomicronemia and pancreatitis in some patients.

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