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The mutant Asn^{291}→Ser human lipoprotein lipase is associated with reduced catalytic activity and does not influence binding to heparin

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Abstract Lipoprotein lipase (LPL) plays a central role in triglyceride metabolism, regulating the catabolism of triglyceride-rich lipoprotein particles. LPL performs its hydrolytic action attached to heparan sulfate proteoglycans at the luminal surface of capillary endothelial cells. We have assessed the effect of the Asn^{291}→Ser (N291S) substitution found in LPL gene from a human hyperlipemic patient. Our results showed that both the wild-type (WT) and N291S hLPL expressed in COS1 cells were secreted to the extracellular medium, and presented similar intracellular distibution patterns. Furthermore, heparin-Sepharose affinity chromatography assays revealed normal heparin affinity of the N291S hLPL. In addition, both the mutant and the WT protein bound to the surface of human fibroblasts and untransfected COS1 cells. Interestingly, disminished LPL specific activity was observed in the extracellular medium from mutant expressing cells. Therefore the lack of normal LPL activity in patients harbouring such a mutation could be the cause of their hyperlipemic disorder.

Key words: Hypertriglyceridemia; Lipoprotein lipase; Mutation

1. Introduction

Lipoprotein lipase (LPL) is responsible for the hydrolysis of core triglycerides of circulating chylomicrons and very low density lipoproteins (VLDL). The active enzyme is a non-covalent homodimer [1], is synthesized and secreted in several parenchymal cells including the adipocyte, myocyte and those of lactating mammary gland [2–4]. After secretion LPL binds with high affinity to heparan sulfate proteoglycans at the surface of secretory cells [5] and it is anchored to similar receptors on the capillary endothelial cells, which do not themselves synthesize LPL [6,7].

LPL has high affinity for heparin, which is provided by several amino acid clusters in the enzyme that confer electrostatical potential. *Cluster 1:* Arg^{263}-Arg^{279}-Lys^{280}-Arg^{282} and *cluster 2:* Arg^{294}-Lys^{296}-Arg^{297}-Lys^{300} contain two hypothetical heparin-binding regions whose sequences form part of an irregular surface loop at the junction of the N-terminal and C-terminal domains. A third heparin binding domain is found between residues 148 and 152 (cluster 3). This region is contained in a surface loop located close to clusters 1 and 2, and could therefore contribute to heparin binding [8]. Recently, site-directed mutagenesis studies [9] have confirmed that the region including the residues from 220 to 330 in the N-terminal domain of LPL is crucial for heparin affinity. Other studies [10] have identified further heparin binding determinants in the protein. These studies suggest that both LPL tertiary structure and consensus sequences are essential to the heparin affinity of the enzyme.

Lipoprotein lipase deficiency is characterized by hyperchylomicronemia, which often develops to lipemia retinalis, pancreatitis, and eruptive xanthomas [11]. A number of genetic defects within the coding region of the LPL gene, leading to catalytically inactive protein, have been reported [12]. Many of the defects have been found in the middle portion of the LPL molecule, a highly conserved region among species and in the lipase gene family [13,14]. In a screening study of hypertriglyceridemic human patients we investigated the genetic background of a patient (J.K.) who presented Von Gerke's syndrome associated with a disorder in the glycogen metabolism. His plasma was milky and had half of normal LPL activity. Analysis of the coding sequence of the LPL gene of this patient using PCR-SSCP revealed the presence of a single base substitution, in heterozygous form, predicted to replace Asp^{291} by a serine in the mature LPL protein. Since the Asn^{291} is located between clusters 1 and 2, which are known to be involved in LPL heparin binding [8], we postulated that this mutation may reduce LPL affinity for heparin. Furthermore, Reymer et al. [15] and Gehrisch et al. [16] reported that this mutation (Asn^{291}→Ser) was found, with relatively high frequency, among patients with Familial Combined Hyperlipoproteinemia (FCH) or Familial Dyslipemia (FDL). Reymer et al. [17] also reported association of the allele N291S with premature atherosclerosis. It was thus suggested that this allele could be a polymorphism in some populations and might be one of the genetic factors involved in the development of FCH or FDL.

In order to determine the contribution of the Asn^{291} residue to the affinity of LPL for heparin and to its catalytic activity, we produced and characterized the N291S hLPL mutant protein by site-directed mutagenesis and expression in COS1 cells. We expressed the wild-type (WT hLPL) and the mutant cDNAs in COS1 cells by transient transfection, and we searched for differences between these two proteins.

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Abbreviations: LPL, lipoprotein lipase; hLPL, human lipoprotein lipase; ER, rough endoplasmic reticulum.

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2. Matherials and methods

2.1. LPL mutagenesis

The full-length human LPL (hLPL) cDNA clone was isolated by reverse transcription of RNA from THP-1 cells (ATCC TIB202) differentiated with phorbol esters and dexamethasone [18], followed by PCR amplification. It was sequenced in its entirely and shown to encode the wild-type enzyme. Site-directed mutagenesis of the full-length human LPL cDNA cloned into the EcoRI site of PTZ18U vector was carried out according to the method of Kunkel et al. [19] using a site-directed mutagenesis kit from Bio-Rad (Bio-Rad, Richmond, CA, USA). The oligonucleotide primer (5'GC TAT GAG ATC **TCC** AAA GTC A3') used for mutagenensis (Eurogentec, Belgium) contained the codon 291 substitution TCC (Asn) for AAT (Ser). This substitution created a *Bgl*II restriction site, which was confirmed by digestion with *Bgl*II (Pharmacia, Brussels, Belgium) and by sequencing. For expression in COS 1 cells, the wild type and the mutant cDNAs were cloned into the *Eco*RI site of the expression vector pCAGGS [20,21], which contains the β-actin promoter and the cytomegalovirus enhancer.

2.2. Cell culture and transfection

COS1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Whittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Whittaker), antibiotics and glutamine (2 mM) (Sigma, St. Louis, MO, USA). For immunofluorescence experiments, cells were cultured in 6 multi-well dishes containing glass coverslips. For blotting and other experiments, cells were cultured in 10 cm plates containing 10 ml of medium. Cells at 80% of confluence were transfected with 2.5 μg of DNA by the DEAE-Dextran method [22]. All cells were tested 48 h after transfection.

2.3. Heparin treatment and cell lysis

For heparin treatment transfected cells were incubated with medium containing 10 U/ml of heparin (Sigma) after transfection. To obtain the cell lysates, cells were washed (twice) in cold PBS 48 h after transfection and then lysed in the buffer A (1% Triton X-100, 1 mM PMSF, 5 μg/ml leupeptin, 2 mM EDTA, 0.5 μg/ml aprotinin (Sigma) in PBS). The lysates were scraped from the dishes and passed through a syringe (with a 22-gauge needle) and then being rapidly frozen in liquid nitrogen. Then they were sonicated for 30 s at maximum power and centrifuged at 12,000 $\times$ g for 10 min at 4°C in a Heraeus-Sepatech Biofuge. The supernatant was considered as the cell extract.

2.4. Western blotting assays

Cell extracts and media of transfected cells were removed from the culture dishes and loaded into SDS-polyacrylamide gels. Gels were blotted to nitrocellulose at 15 V for 1.5 h using the semi-dry system of Bio-Rad. The nitrocellulose membranes (Cellulosenitrat BA85 from Schleicher and Schuell, Dassel. Germany) were blocked with 3% powdered milk in PBS and LPL was detected with a monoclonal antibody against bovine LPL (5D2) (Oncogene, NY, USA) and a secondary peroxidase-conjugated anti-mouse antibody at dilution (1:2000) (Dakopatts, Glostrup. Denmark). The blot was developed with the ECL system from Amersham (Bucks, United Kingdom).

2.5. Heparin-Sepharose chromatography, ELISA and activity assays

Heparin-Sepharose affinity chromatography was performed as described by Östlund-Lindqvist and Boberg [23] using the FPLC system from Pharmacia. The column was equilibrated with Robinson buffer (10 mM Tris-HCl, pH 7.2, 20% w/v glycerol, 0.1% w/v Triton X-100) and chromatography was performed at a flow rate of 0.5 ml/min. 30 fractions of 1 ml were collected. LPL was eluted from the column using a linear gradient of NaCl.

LPL mass was measured in the fractions from heparin-Sepharose chromatography and media using solid-phase sandwich ELISA with polyclonal chicken antibodies (from Dr. Bengtsson-Olivecrona, Umeå, Sweden) for coating and the 5D2 monoclonal antibody for detection as described by Vilella et al. [24]. Absorbance at 492 nm was measured and used as a relative value to plot the heparin-Sepharose chromatograms and to calculate specific activities.

Lipoprotein lipase activity in medium was determined as described by Ramirez et al. [25]. One milliunit of lipolytic activity represents release of 1 nmol fatty acid/min.

2.6. Binding assays and immunofluorescence microscopy

LPL binding assays were performed by placing glass coverslips with a subconfluent monolayer of human fibroblasts in a 10 cm dish with COS1 cells transfected with either the WT hLPL or the N291S hLPL. The coverslips were placed immediately after the transfection process and both growing cell lines shared the same culture medium, in which the LPL secreted by the transfected cells could bind to the surface of the fibroblasts added. LPL binding was then detected by immunofluorescence.

For immunofluorescence labeling, cells grown on glass coverslips were rinsed briefly in PBS, fixed with methanol (−20°C) for 2 min, washed twice in PBS and processed. As primary antibody, we used the monoclonal antibody anti bovine LPL (5D2) (Oncogene). To visualize the primary antibody, we used a secondary RITC-conjugated goat anti-mouse (Boehringer-Mannheim, Mannheim, Germany). All antibodies were diluted in PBS containing 0.5% of BSA (Sigma). Primary antibodies were applied for 45 min at 37°C, followed by a 10 min wash in PBS, then 45 min incubation at 37°C with the secondary antibody, followed by a final wash of 10 min in PBS. Finally, coverslips were labeled with the nuclear stain Hoechst 33342 (Sigma) diluted in PBS. The coverslips were mounted with immunofluorescence medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) and viewed with 40 $\times$ or 100 $\times$ objective using a Reichert Jung Polyvar II microscope equipped with epifluorescence illumination. Photographic exposures were made on Kodak Tmax 400 film.

3. Results

To study the effects of the N291S substitution on LPL processing, affinity for heparin and catalytic activity, we expressed either the WT or the variant LPL cDNAs in COS1 cells by transient transfection using the DEAE-dextran chloroquine method. Assays were carried out 48 h following transfection. By RNA slot blot and hybridization with [^{32}P]hLPL labelled probe we found equal expression of both constructs (not shown). Intracellular LPL was detected by Western blotting of cell extracts, and in both cases (the WT and the N291S hLPL) a single band with the same molecular weight (58 kDa) similar to bovine LPL (55 kDa) was identified using the monoclonal 5D2 antibody (Fig. 1A). This result indicates that similar high levels of expression were accomplished when transfecting either with the wild-type or with the mutant protein. Western blot experiments also revealed high amounts of LPL in culture media upon transfection with either the variant or WT LPL, indicating that both proteins were equally well secreted to the medium (Fig. 1B).

The efficiency with which heparin released the N291S protein from cells was next determined. Cells transfected with either N291S or wild type were treated with 10 U/ml of heparin for 24 h (at 48 h after transfection). Slot blot assays of culture media using the 5D2 antibody showed similar increasess in LPL after treatment with heparin (Fig. 2A). We found increased presence of LPL in heparin-treated cells in both cases, indicating the same stimulating effect. Involvement of the Asn^{291} in the affinity of the LPL for heparin was investigated using heparin-sepharose affinity chromatography. Our results indicated that both the N291S and the WT hLPL presented the same heparin affinity (Fig. 2B). The molarity of LPL elution was around 0.6 M NaCl for the monomeric LPL and 1 M NaCl for the dimeric LPL in the case of both mutant and the WT hLPL. As expected, most of the LPL in the media was found in its monomeric form (in both the WT hLPL and the mutant hLPL) since it is known that LPL monomerizes after incubation at 37°C.

Immunofluorescence microscopy was used to determine the intracellular distribution pattern of LPL in mutant-expressing

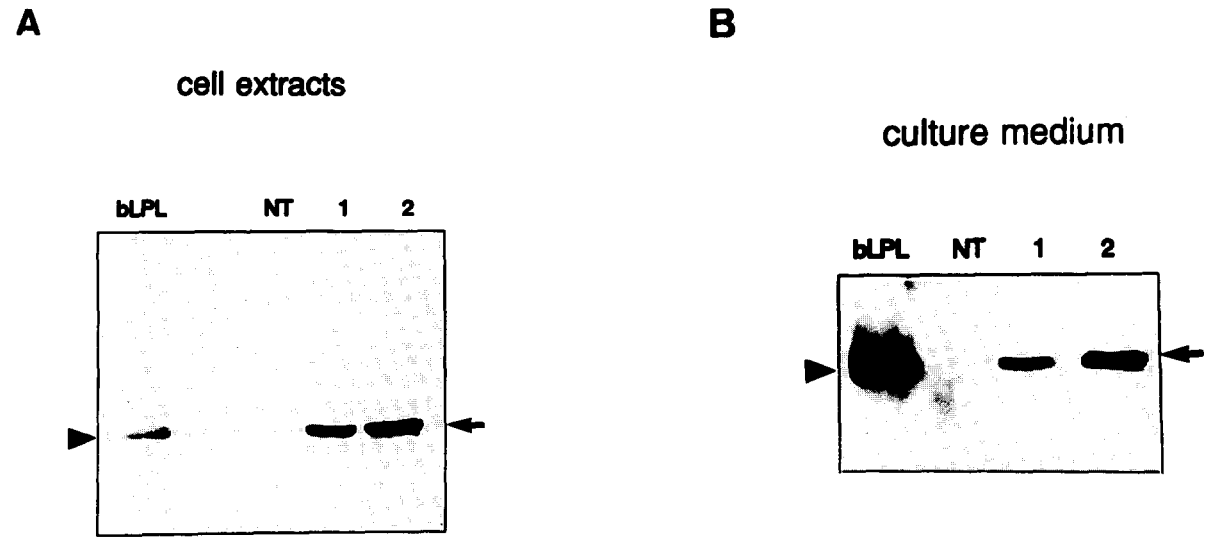

Fig. 1. Western blot analysis of LPL protein in cell extracts and media in wild-type and N291S COS1 transfected cells. Equal amounts of vector (NT), wild-type (lane 1) and N291S mutant hLPL expressing cell extracts (A) or medium (B) were separated by SDS-PAGE electrophoresis. 0.5 μg of bovine LPL (bLPL) was included as control (arrowhead, 55 kDa). The gels were blotted into nitrocellulose and LPL was detected using the 5D2 monoclonal antibody, revealing a single band of 58 kDa (arrow).

cells. As expected from the secretion studies, the intracellular LPL detected with the 5D2 antibody showed the same distribution pattern for both the wild type and the mutant expressed LPL. The immunoreactivity (Fig. 3A,B) seemed to be located mostly in the Golgi (see arrow) and spread throughout the cytoplasm in the ER but never showed an intracellular pattern of retention, which was reported previously for N43A expressed LPL mutant proteins [26].

Recently, it has been demonstrated that LPL binds with high affinity to heparan sulfate proteoglycans on the surface of skin human fibroblasts following an ordered regular distribution pattern. This pattern mostly reflects the arrangement of these cellular receptors on the cell surface [27]. With the purpose of determinating whether the mutant N291S hLPL was capable of binding to the cell surface of human skin fibroblasts and to untransfected COS1 cells, we performed binding assays by placing glass coverslips with a fibroblast culture into dishes of transfected COS1 cells (WT and mutant expressing cells). Immunofluorescence studies were then carried out by incubating coverslips with the 5D2 monoclonal antibody. The immunofluorescent images demonstrated that both the WT and the mutant hLPL proteins were clearly able to bind to the cell surface of both the human skin fibroblasts (Fig. 3C,D) and untransfected COS1 cells (Fig. 3E,F). This result indicated that the affinity for heparan sulfate receptors remained unaltered by the mutation N291S. Up to this point we had not been able to detect differences between the molecular behaviour of the WT and the N291S hLPL.

LPL activity and mass in media of N291S- and WT hLPL-expressing cells were measured. The results (Fig. 4) indicate that the specific activity of mutant LPL in the medium was approximately 60% of the secreted WT hLPL.

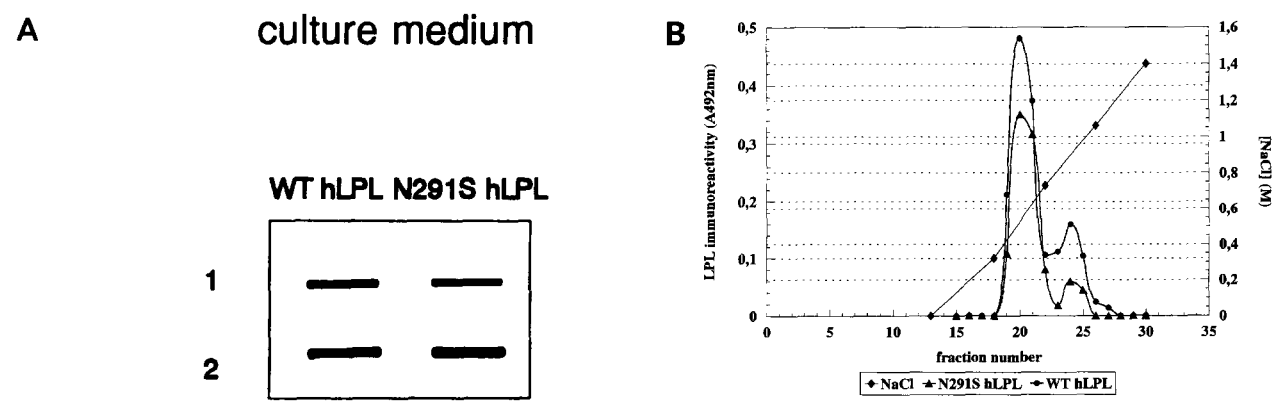

Fig. 2. Slot blot analysis of culture media from wild-type (WT) and N291S hLPL transfected cells upon heparin treatment (A). Transfected cells with wild-type or mutant cDNAs were incubated with (2) or without (1) medium containing 10 U/ml of heparin for 48 h. Media was collected and subjected to slot blot analysis using 5D2 monoclonal antibody. Heparin-Sepharose chromatography assay of culture media from WT and mutant expressing COS1 cells (B). Media from WT and N291S hLPL transfected cells were injected in a heparin Sepharose chromatography column to check the heparin affinity of both expressed proteins. The FPLC system from Pharmacia was used and 30 fractions of 1 ml were collected after elution with a NaCl gradient. The fractions were quantified for LPL using an ELISA assay with polyclonal rabbit immunoglobulins for coating and the 5D2 monoclonal antibody for detection.

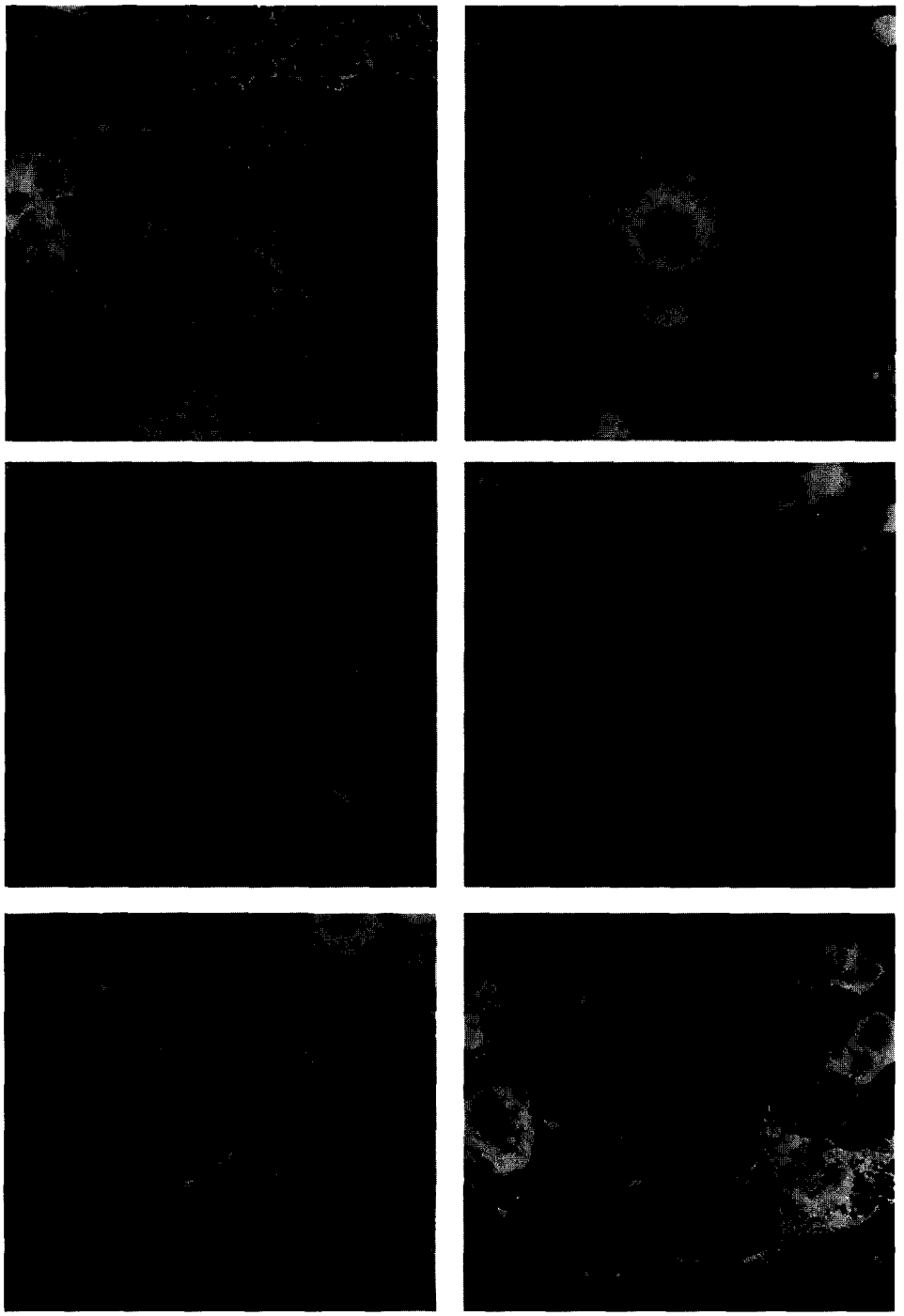

Fig. 3. Intracellular distribution of the wild-type (WT) and the N291 S hLPL in COS1 cells (A,B). Immunofluorescent detection of LPL on transfected COS1 cells grown on glass coverslips. (A) shows the LPL immunostaining of WT hLPL transfected cells; (B) shows the LPL distribution pattern of N291S hLPL expressing cells. The monoclonal 5D2 antibody was used as the primary antibody and anti-mouse FITC immunoglobulins as the secondary antibody. Immunofluorescent detection of the WT and mutant hLPL on the surface of human fibroblasts (C,D) and non-transfected COS1 cells from the same culture (E,F). Glass coverslips of cultured transfected COS1 cells as well as glass coverslips with subconfluent human skin fibroblasts placed in the transfected COS1 cells culture dish for 24 h were immunolabeled with the 5D2 monoclonal antibody and the secondary anti-mouse FITC-conjugated antibody. WT (C) and mutant (D) hLPL labeling on the surface of human fibroblast sharing culture medium with the transfected COS1 cells is shown. WT (E) and mutant hLPL (F) labeling (arrow) on surface of non-transfected COS1 cells. Bars = 30 μm.

4. Discussion

In this study we report effects of the mutation N291S in the human lipoprotein lipase molecule. We created the N291S mutation in the human LPL cDNA by site-directed mutagenesis and we transiently expressed the WT hLPL and the mutant hLPL in COS1 cells. We found out that the N291S hLPL was normally synthesized and distributed inside the cell, and nor-

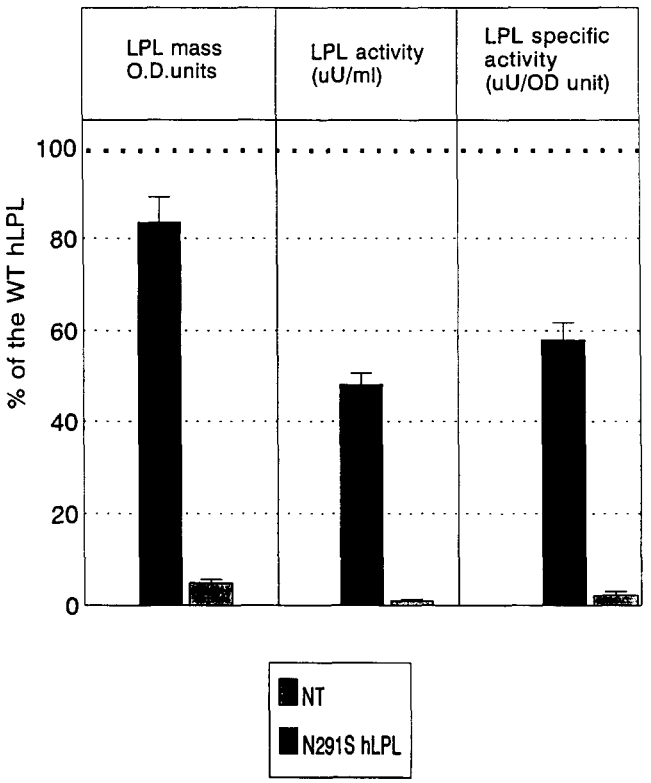

Fig. 4. LPL mass in medium (expressed in OD units at 492 nm) was measured in the case of the wild-type (WT) and mutant N291S expressing cells as described in section 2. LPL activity (expressed in mU/ml of medium) of the same medium was assayed, and LPL specific activity was expressed as mU of LPL lipolytic activity per OD unit at 492 nm. In all cases the activity and specific activity values of media from non-transfected COS1 cells (NT) and N291S hLPL expressing cells are referred to the level of the WThLPL considered as 100% ($n = 4$)

mally secreted to the extracellular medium. In addition we observed that it had normal affinity for heparin and was capable of binding to surface heparan sulfate receptors of untransfected COS-1 cells and human fibroblasts. However, the mutant enzyme presented 42% less specific catalytic activity than the WT hLPL.

Extracellular matrix glycosaminoglycans regulate a number of metabolic processses [28] and the binding of LPL on the extracellular matrix is well documented [29]. For example, LPL is bound to the luminal side of the endothelial cell wall through glycosaminoglycans. The electrostatic nature of this interaction is provided by the heavily negatively charged regions of heparin-like polysaccharides and the positively charged regions on the LPL surface. The maximum positive electrostatic potential in LPL is constituted by four positively charged clusters: Arg^{263}-Arg^{279}-Lys^{280}-Arg^{282} (cluster 1), Arg^{294}-Lys^{296}-Arg^{297}-Lys^{300} (cluster 2), residues from the 248 to the 152 (cluster 3), and Lys^{319}-Lys^{403}-Arg^{405}-Lys^{407}-Lys^{414}-Lys^{415} (cluster 4). Among them, only clusters 1 and 2 have been found to be involved in heparin binding [10]. The mutation of Asn^{291} to Ser which was found in J.K., a patient with the Von Gerke's disease, and that has been found with high frequency in FCH or FDL affected populations, is situated between these two clusters known to be crucial for LPL heparin binding. The aim of our work was to elucidate the potential role of Asn^{291} in LPL affinity for heparin. Our results indicate that this residue was not a determinant for heparin binding, since the N291S hLPL expressed in COS1 cells had normal affinity for this molecule. A tridimentional molecular model of LPL based on the homology with the pancreatic lipase X-ray structure was proposed by van Tilbeurgh et al. [8]. According to this model, the heparin binding consensus sequences found in clusters 1 and 2 are close to each other in the folded LPL molecule. These two clusters are part of an irregular surface loop at the junction of the N- and C-terminal domains of LPL. Since Asn^{291}, found between these two clusters, does not affect heparin binding, we hypothesize that this neutral residue might remain hidden and not taking part in the loop exposed to the surface.

After the completion of our studies, Reymer et al. [15] and also Gehrisch et al. [16] reported at the International Atherosclerosis Congress, Montreal (1994) that the frequency of the N291S substitution, in the heterozygous state, was almost five times higher in FCH patients than in controls (9.3% vs. 1.9%, and more than five times in FDL patients (9.7% vs. 1.9%). Reymer et al. [17] also described a high frequency of N291S in population with premature atherosclerosis (5.2% vs. 1.8% of controls) Reymer et al. [15] also performed in vitro mutagenesis experiments, which revealed that the mutant N291S hLPL presented normal heparin affinity but reduced LPL activity. Furthermore, Gehrisch et al. [16] also suggested that the N291S mutation influenced LPL activity. Heterozygosity for the $Asn^{291} \rightarrow Ser$ substitution may be one of the contributing factors in the development of Familial Combined Hyperlipidemia (FCHL). Recently, heterozygosity for a mutation in the LPL promoter was found to be associated with FCHL [30].

Many missense mutations in the LPL gene have been reported to disrupt the activity of the enzyme. Among them we find: $Gly^{142} \rightarrow Glu$ [31], $Asp^{156} \rightarrow Gly$ [32,33], $Ala^{194} \rightarrow Thr$ [34], $Arg^{243} \rightarrow His$ [35], $Ser^{244} \rightarrow Thr$ [36], $Asp^{250} \rightarrow Asn$ [37], $Tyr^{262} \rightarrow His$ [12,38]. Most of the residues involved in these mutations are known to take part in or to be adjacent to the catalytic domain of LPL, which consists of Ser^{132}, Asp^{156} and His^{241}. The mutation characterized in this paper ($Asn^{291} \rightarrow Ser$) is not associated with any residue or domain known to be involved in the hydrolytic capacity of the enzyme. This mutation was not observed among LPL deficient individuals since it does not completely abolish LPL activity. The reduction of activity could be due to instability of the LPL dimer in the culture medium. When LPL is converted from its dimeric form to a monomer, it loses catalytic activity [1]. We therefore suggest that the residue Asn^{291} could be involved in dimer formation. At present, very little is known about the residues and domains of LPL involved in dimerization. Further studies will be required to elucidate this aspect of LPL structure.

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