Journal of Biochemistry and Molecular Biology, Vol. 33, No. 2, March 2000, pp. 148-154

JOURNAL OF
Biochemistry and Molecular Biology

Lipoprotein Lipase-Mediated Uptake of Glycated LDL

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Received 26 November 1999, Accepted 12 January 2000

The glycation process plays an important role in accelerated atherosclerosis in diabetes, and the uptake of atherogenic lipoproteins by macrophage in the intima of the vessel wall leads to foam cell formation, an early sign of atherosclerosis. Besides the lipolytic action on the plasma triglyceride component, lipoprotein lipase (LPL) has been reported to enhance the cholesterol uptake by arterial wall cells. In this study, some properties of LPL-mediated low-density lipoprotein (LDL) uptake and the effect of LDL glycation were investigated in RAW 264.7 cells, a murine macrophage cell line. In the presence of LPL, ^{125}I-LDL binding to RAW 264.7 cells was increased in a dose-dependent manner. At concentrations greater than 20 μg/ml of LPL, LPL-mediated LDL binding was increased about 17-fold, achieving saturation. Without LPL, both very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) were ineffective in blocking the binding of ^{125}I-LDL to cells. However, LPL-enhanced LDL binding was inhibited about 50% by the presence of VLDL, while no significant effect was observed with HDL. Heat inactivation of LPL caused a 30% decrease of LDL binding. In the presence of LPL, the cells took up 40% of cell-bound native LDL. No significant difference was observed in cell binding between native and glycated LDL. However, the uptake of glycated LDL was significantly greater than that of native LDL, reaching to 70% of the total cell bound glycated LDL. These results indicate that LPL can cause the significant enhancement of LDL uptake by RAW 264.7 cells and the enhanced uptake of glycated LDL in the presence of LPL might play an important role in the accelerated atherogenesis in diabetic patients.

Keywords: Glycation, LDL, Lipoprotein lipase

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Introduction

Atherosclerosis is the cause of death in more than 50% of people in western societies. It results in significant cardiac morbidity, such as anginal syndromes, myocardial infarction, ischemic cardiomyopathy and sudden cardiac death. It also results in noncardiac morbidity, such as cerebrovascular accidents and peripheral vascular disease (O'Brien and Chait, 1994).

Epidemiological studies demonstrated an association of increased total plasma cholesterol with an increased risk of atherosclerotic events (Anderson *et al.*, 1987). The hallmarks of atherosclerosis are the deposition of lipid in the arterial intima, recruitment of inflammatory cells (predominantly monocytes and T lymphocytes) into the intima, smooth muscle cell accumulation and the elaboration of collagen and matrix proteins by smooth muscle cells (Raines and Ross, 1993).

For the cellular low-density lipoprotein (LDL) uptake, the LDL receptor expressed ubiquitously in most cell types plays the most important role (O'Brien and Chait, 1994). Brown and Goldstein (1983) reported that acetylated LDL was taken up by another cell surface receptor, called 'scavenger receptor'. Oxidized LDL also can be taken up by macrophage scavenger receptors leading to foam cell formation (Witztum, 1993). Other potential mechanisms of cellular cholesterol uptake include phagocytic uptake of aggregated LDL by macrophages (Vijayagopal *et al.*, 1993), or immune complexes of lipoproteins via the Fc receptor of macrophages (Klimov *et al.*, 1985).

Lipoprotein lipase (LPL, EC 3.1.1.34) is an enzyme that catalyzes the hydrolysis of the triglyceride component in circulating chylomicron and very low-density lipoprotein (VLDL) into two fatty acids and monoacylglycerol (Bensadoun, 1991). The major function of LPL is to direct the influx of plasma triglyceride in forms of fatty acids into the peripheral tissues for storage or fuel.

In addition to its lipolytic action, it is reported that LPL may also enhance the cholesterol uptake by arterial wall cells *in*

vitro (Rutledge and Goldberg, 1994; Beisiegel, 1996). Already in 1975, Felts *et al.* (1975) proposed that LPL might be attached to the remnants and serve as a recognition signal for the liver. Interest in this hypothesis was revived when Beisiegel *et al.* (1991) found that LPL was a ligand for the α2-macroglobulin receptor/LDL receptor-related protein (LRP) in hepatocytes and fibroblasts. Subsequently, it was shown in tissue culture that direct LPL-receptor interactions could enhance binding and cellular uptake of lipoproteins (Beisiegel, 1996). In the intima of the vessel wall, LPL may serve as an atherogenic protein by stimulating the uptake of atherogenic lipoproteins by smooth muscle cells and macrophages, leading to foam cell formation.

Diabetes is associated with accelerated atherosclerosis and the incidence of cardiovascular disease in diabetic patients is three to four times higher than that of nondiabetic individuals (Kannel and McGee, 1979). Attention has focused on potential cardiovascular risk factors that are specific to the diabetic metabolic milieu, and considerable evidence now exists to suggest that the process of oxidation and glycation are of major importance (O'Brien and Timmins, 1994). There is evidence to suggest that glycation of lipoproteins can occur *in vitro* and *in vivo* (Bucala *et al.*, 1993; O'Brien and Timmins, 1994).

The uptake of atherogenic lipoproteins by macrophage in the intima of the vessel wall leads to foam cell formation (Vijayagopal *et al.*, 1993), an early sign of atherosclerosis. Macrophages are known to be present mainly in intimal regions (Yla-Herttuala *et al.*, 1991) and synthesize LPL (O'Brien *et al.*, 1992). In this study, some properties of LPL-mediated LDL uptake and the effects of LDL glycation were investigated in RAW 264.7 cells, a murine macrophage cell line.

Materials and Methods

Purification of bovine milk LPL LPL was purified from fresh bovine milk using heparin-Sepharose chromatography (Bengtsson-Olivecrona and Olivecrona, 1991). After centrifugation of chilled milk for 15 min at 3,500×g, the skimmed milk was filtered through glass wool and adjusted to 0.34 M NaCl. Swollen heparin-Sepharose (Sigma Chemical Co., St. Louis, USA) was equilibrated with 0.34 M NaCl in 10 mM Tris, pH 6.5 and added to the milk. The mixture was shaken gently for 3 h at 4°C. After being washed with 0.5 M NaCl and subsequently with 0.85 M NaCl in Tris buffer, the mixture was applied to a column and washed with 0.9 M NaCl in Tris buffer. Thereafter, 1.5 M NaCl in Tris buffer was used as an elution buffer. The LPL-containing fractions were pooled, desalted using CentriPrep 30 (Amicon Inc. Beverly, USA), and applied to a second round of heparin-Sepharose column chromatography. After application of LPL fractions to heparin-Sepharose column, the column was washed with 0.9 M NaCl in Tris buffer, and eluted by a gradient of Tris buffer with 0.9 to 2.0 M NaCl. Fractions showing LPL activity were pooled, concentrated using Centriprep 30, and stored at -70°C with 50% glycerol. Purified LPL showed a single band of 57 kDa on SDS-PAGE (Fig. 1).

Assay of LPL activity Lipolytic activity of plasma was measured as described by Park *et al.* (1997). A stock triacylglycerol emulsion containing 5 mCi of tri[9,10(n)-^{3}H]oleoylglycerol (0.3 nmole, Amersham, Buckinghamshire, UK), 1.13 mmole of trioleoylglycerol, 60 mg of 1-α-phosphatidylcholine (bovine liver) and 9 ml of glycerol was prepared according to the method of Nilsson-Ehle and Schotz (1976). Before the assay, 1 vol. of the stock emulsion, 19 vol. of 3% bovine serum albumin (BSA) in 0.2 M Tris/HCl buffer (pH 8.1) and 5 vol. of heat-inactivated serum from fasted rats (heated at 60°C for 30 min) were mixed and incubated for 15-30 min. For the assay, 100 μl of this activated substrate mixture was added to a same amount of enzyme solution, and incubated at 37°C for 60 min. Released fatty acids were extracted and its radioactivity was measured. One unit of lipolytic activity represents the release of 1 mmole of fatty acid/min.

Preparation of lipoproteins Serum was obtained from the blood of normal healthy human adults by centrifugation at 1,200×*g* for 10 min. Lipoproteins were obtained by sequential preparative ultra centrifugation of serum in the presence of EDTA (0.04%), PMSF (0.015%) and NaN_3 (0.05%) as preservatives (Mackness and Durrington, 1992). After the centrifugation of serum at 100,000×*g* for 20 h at 15°C in a swinging bucket rotor, the top layer of VLDL was removed with a Pasteur pipette. The pellets in the infranatant were resuspended and adjusted to the density of 1.063 g/ml by NaBr. The LDL fraction was obtained by centrifugation of infranatant at 100,000×*g* for 24 h at 15°C. High-density lipoprotein (HDL) was obtained after the recentrifugation of this infranatant at the same condition for 48 h after the adjustment of density to 1.21 g/ml. The remaining infranatant fraction was used as a lipoprotein-deficient serum (LPDS). Lipoproteins and LPDS were extensively dialyzed against PBS.

Preparation of glycated LDL and iodination The glycation of native LDL was performed by incubation of native LDL with 20 mM glucose in 10 mM PBS with EDTA (pH 7.4) at 37°C for 7 days (Kobayashi *et al.*, 1995). After the incubation, glycated LDL was extensively dialyzed against 150 mM NaCl with 0.26 mM EDTA (pH 7.4).

LDL samples were iodinated using Iodo-Beads (Pierce, Rockford, USA) according to the manufacturers instructions (Markwell, 1982). The specific activity ranged from 60 to 360 cpm per ng of protein. After iodination, the LDL samples were dialyzed extensively against PBS, stored at 4°C and used within 2 weeks.

Cell culture and LDL binding and uptake RAW 264.7 (ATCC, Rockville, USA) cells were cultured in 75 cm^2 flasks in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. The cells were incubated at 37°C in an atmosphere containing 5% CO_2.

For LDL binding experiments (Arnold *et al.*, 1992), 2×10^6 cells were plated in 24-well plates, and incubated 24 h with DMEM containing 5% LPDS instead of fetal bovine serum. The binding

of ^{125}I-LDL to the cell in the presence and absence of LPL was determined after a 3 h incubation at 4°C with 20 μg/ml of ^{125}I-LDL. After removal of the medium, the cells were washed four times with ice-cold PBS containing 0.1% (w/v) BSA, and subsequently with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 M NaOH, and the radioactivity of an aliquot was determined. To calculate the specific cell binding, the amount of labeled LDL that was cell-bound after incubation in the presence of a 20-fold excess of unlabelled LDL was subtracted from the total cpm.

The LDL uptake experiments were performed as previously described for the binding studies, except that the 3 h incubation was performed at 37°C instead of 4°C (Arnold *et al.*, 1992). After the incubation, cells were placed on ice for 30 min in the presence of 10 mg/ml of heparin in order to remove the cell surface-bound LDL. Cells were then extensively washed as above, and the radioactivity of cell lysate was determined.

Protein determination Protein concentrations were determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as reference standard.

Results

To assess the LPL-mediated LDL uptake, LPL was purified from fresh bovine milk using heparin-Sepharose chromatography (Bengtsson-Olivecrona and Olivecrona, 1991). After two rounds of salt gradient elution of LPL from heparin-Sepharose column, purified LPL showed a single band of 57 kDa on SDS-PAGE (Fig. 1).

The binding of ^{125}I-LDL by RAW 264.7 cells, a murine macrophage cell line, was examined in the absence and presence of purified LPL (Fig. 2). Without LPL, cellular LDL binding was 0.60±0.24 μg per 2×10^6 cells, and increased with added LPL in a dose-dependent manner. At the concentrations over 15 μg/ml of LPL, LPL-mediated LDL binding was increased about 17-fold, achieving saturation. The concentration of 20 μg/ml LPL was used in later experiments.

The effects of VLDL and HDL on the binding of LDL were examined in the presence and absence of LPL (Fig. 3). In the absence of LPL, both VLDL and HDL were ineffective in

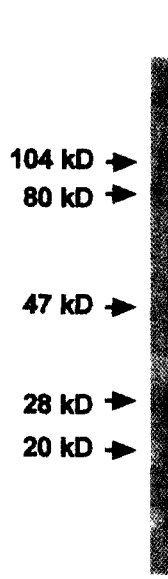

Fig. 1. SDS-PAGE of purified LPL from bovine milk. SDS-polyacrylamide gel electrophoresis was performed on 10% resolving and 3% stacking gel and stained with 0.3% Coomassie blue. Lane 1, molecular weight markers; lane 2, purified bovine milk LPL.

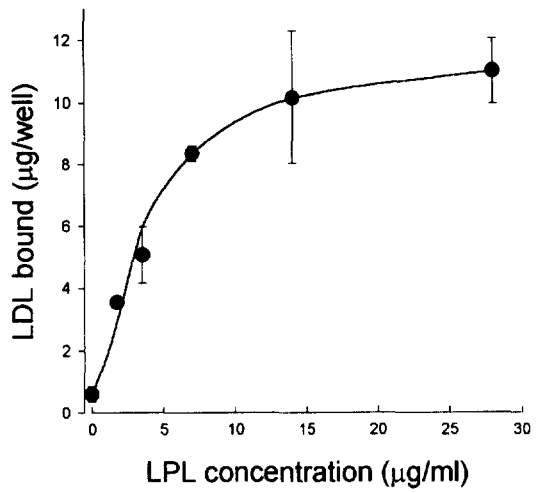

Fig. 2. Effect of LPL on the binding of LDL. The binding experiment was performed with RAW 264.7 cells (2×10^6 cells/well) preincubated with DMEM containing 5% lipoprotein deficient serum for 24 h. The binding of ^{125}I-LDL was determined after 3 h of incubation with 20 μg/ml of ^{125}I-LDL at 4°C in the presence or absence of LPL, as described under Materials and Methods. Data were presented as mean±S.D. of three measurements.

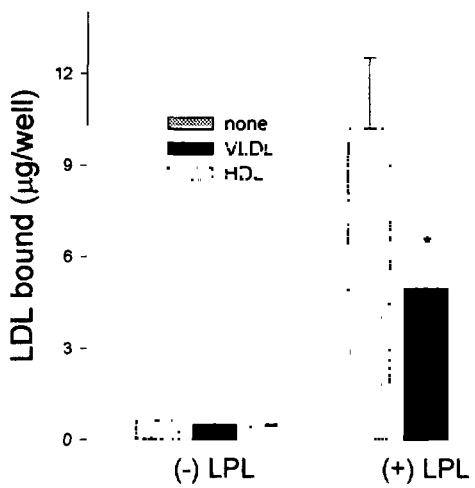

Fig. 3. Competition of LPL-mediated LDL binding by VLDL and HDL. The binding of ^{125}I-LDL was determined as described in the legend of Fig. 2. During the 3 h of incubation with of ^{125}I-LDL (20 μg/ml) and LPL (20 μg/ml) at 4°C, VLDL or HDL (20 μg/ml each) was co-incubated. Data were presented as mean±S.D. of three measurements. Difference was significant at $p<0.05$.

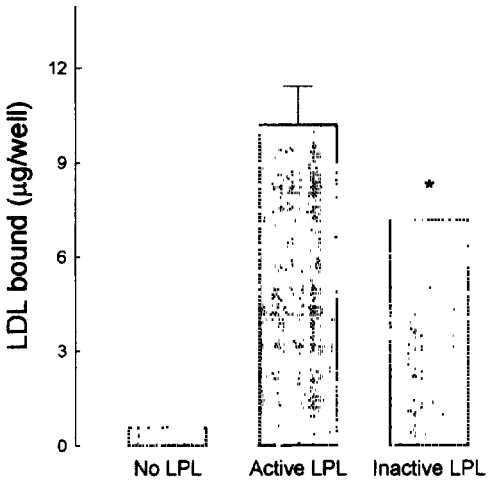

Fig. 4. Effect of the heat inactivation of LPL on LDL binding. The binding of ^{125}I-LDL was determined as described in the legend of Fig. 2. During incubation, 20 μg/ml of native LPL or heat inactivated LPL (65°C, 5 min) were co-incubated. Data were presented as mean±S.D. of three measurements. Difference was significant at $p<0.05$.

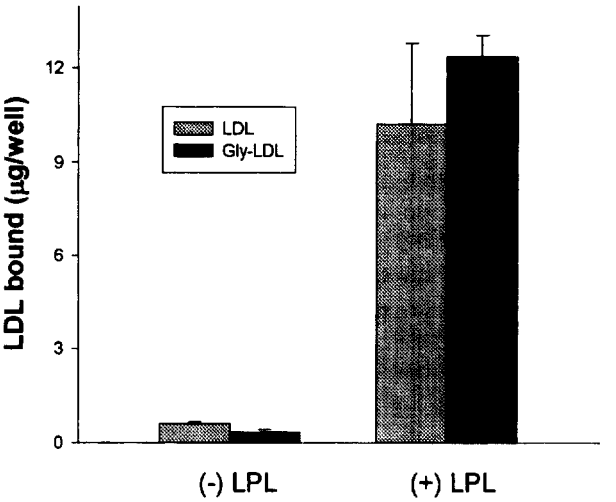

Fig. 5. Effect of LDL glycation on LPL-mediated LDL binding. The binding of ^{125}I-LDL or ^{125}I-glycated LDL was determined as described in the legend of Fig. 2. Incubation was performed in the presence or absence of 20 μg/ml LPL. Data were presented as mean±S.D. of three measurements.

blocking the binding of LDL to cells. However, the increase of LDL binding in the presence of LPL was inhibited about 50% by the presence of VLDL, while no significant effect was observed with HDL. When LPL was inactivated by heat treatment at 65°C for 5 min, there was about a 30% decrease of LDL binding, suggesting that the three dimensional structure of LPL has only a partial influence on the LPL-mediated LDL binding to RAW 264.7 cells (Fig. 4).

In order to determine the effect of glycation of LDL on LPL-mediated LDL binding and uptake, cells were incubated with glycated LDL and its binding and uptake were compared with those of native LDL. In the absence of LPL, both the binding and uptake of LDL, or glycated LDL, were very low and no difference was observed between the two lipoproteins (Fig. 5, 6). However, in the presence of LPL, the bindings and uptake of both lipoproteins were greatly increased. About 40% of the cell-bound native LDL was taken up by the cells (Fig. 6). No significant difference was observed in cell binding between native and glycated LDL (Fig. 5). However, the uptake of glycated LDL was significantly greater than that of native LDL, reaching to 70% of the total cell bound glycated LDL (Fig. 6).

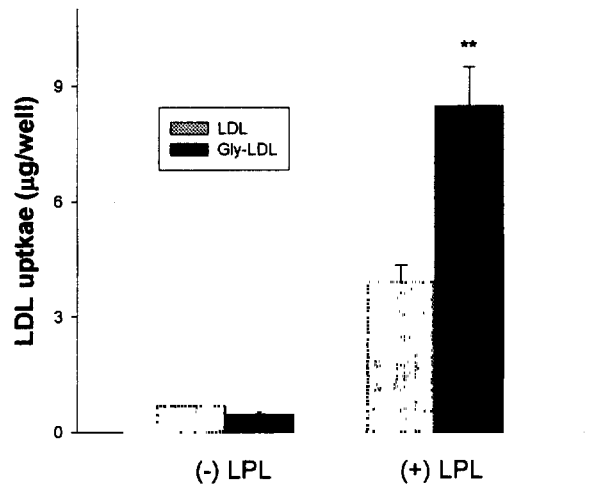

Fig. 6. Effect of LDL glycation on LPL-mediated LDL uptake. The uptake experiment was performed with RAW 264.7 cells (2×10^6 cells/well) preincubated with DMEM containing 5% lipoprotein deficient serum for 24 h. Cells were incubated with 20 μg/ml of ^{125}I-LDL or ^{125}I-glycated LDL for 3 h at 37°C, and then reincubated in the presence of 10 mg/ml heparin at 4°C for 30 min. Cell-associated radioactivity was determined as described under "Materials and Methods". Data were presented as mean±S.D. of three measurements. Difference was significant at $p<0.01$.

Discussion

The LPL-mediated increase in lipoprotein uptake may occur by several different mechanisms. Lipolysis-mediated structural changes in the conformation, or the amount of apoproteins on the lipoprotein surface, could lead to increased uptake via classical lipoprotein receptors (Aviram *et al.*, 1988). LPL binds to cell surface heparan sulfate proteoglycans (HSPG) (Saxena *et al.*, 1991). The cellular uptake of lipoproteins anchored to the LPL-HSPG complex could also occur during the normal turnover of cell surface molecules (Mulder *et al.*, 1992). The third possibility is that LPL concentrates the lipoproteins on the cell surface. The LPL-LDL complexes are in close proximity to the cell surface that enables them to efficiently interact with cell surface receptors. These two molecules bind together while on the cell surface and then are internalized and degraded by a process involving

the LDL receptor related protein (LRP)/α_2-macroglobulin (α_2M) receptor (Chappell *et al.*, 1994), GP330/LRP-2 (Kounnas *et al.*, 1993), and VLDL receptors (Argraves *et al.*, 1995). The relative importance of LPL-mediated LDL internalization and uptake differs between cells (Obunike *et al.*, 1994). The types of cell surface receptors for lipoproteins, as well as turnover rate of cell surface molecules, may be factors affecting the LPL-mediated lipoprotein degradation processes.

In RAW 264.7 cells, the LDL binding was increased about 17-fold by the presence of LPL, and about 40% of bound LDL were taken up by the cells. The binding of LPL to the lipoprotein surface is an obvious prerequisite for catalytic action of the enzyme. In our cell system, the LPL-mediated LDL binding was competitively inhibited by VLDL, a TG-rich lipoprotein, but not by HDL. Carrero *et al.* (1996) reported that LPL could bind to lipid emulsion droplets containing no apolipoproteins, indicating that the binding was dependent on the lipid content of the particle rather than on its apolipoprotein composition.

LPL binding to either HSPG or receptors can be separated from its lipolytic activity, as indicated by studies with LPL-C, the carboxyl-terminal non-catalytic domain of LPL (Chappell *et al.*, 1994). LPL binding to lipoproteins can also be separated from its lipolytic activity (Kobayashi *et al.*, 1989). With RAW 264.7 cells, heat inactivation of LPL protein decreased about 30% of LPL-mediated LDL binding. Heat inactivation of LPL can cause the conformational changes that may influence the lipoprotein binding and cell surface binding functions of LPL, in addition to the loss of enzyme activity. Huff *et al.* (1997) reported that catalytic activity of LPL was required for the enhanced interaction of VLDL and HepG2 cells. However, our results indicate that only a part of the binding is influenced by the native three-dimensional structure of LPL in RAW 264.7 cells.

Glucose can react with amino acids or nucleic acids in a process known as nonenzymatic glycation (Brownlee, 1992). The initial products of the reaction are Schiff bases, which rearrange to form more stable Amidori products. Amidori products are gradually degraded into reactive carbonyl compounds such as 3-deoxyglucosone, which can further react with free amino groups to form advanced glycation end products (AGE). The process of advanced glycation can lead to marked changes in the structure and function of proteins (Brownlee *et al.*, 1988; Charonis and Tsilbary, 1992).

In vitro and *in vivo* glycation of human apolipoproteins has been reported (Curtiss and Witztum, 1985). When LDL is incubated with glucose *in vitro*, AGE is detectable within 3 days, linked to both the apo-B and the phospholipid components of the lipoproteins (Bucala *et al.*, 1993). The glycation process plays an important role in the accelerated atherosclerosis in diabetes (O'Brien and Timmins, 1994), and LDL glycation in diabetic subjects has been shown to correlate with the degree of glycemic control (Lyons *et al.*, 1986). However, there has been little attention given to the effect of lipoprotein glycation on LPL-mediated lipoprotein uptake.

When RAW 264.7 cells were incubated with glycated LDL in the presence of LPL, no significant difference was observed in binding between native and glycated LDL. However, the uptake of glycated LDL was significantly greater, reaching to 70% of total bound LDL compared to 40% for native LDL. At present, the mechanism for the enhanced uptake of glycated LDL by macrophage is unclear. In macrophages, the rapid turnover of cell surface HSPG has been reported to play the major role in LPL-mediated uptake of native LDL, in contrast to the LRP-related mechanism of fibroblast (Obunike *et al.*, 1994). Scavenger receptors, or other less clearly defined receptors, might act on the glycated LDL that are concentrated on the cell surface through the binding with LPL.

Macrophages in atherosclerotic plaques synthesize LPL (O'Brien *et al.*, 1992) and HSPG (Owens and Wagner, 1991). Goldberg *et al.* (1988) showed that, in addition to synthesis and secretion of LPL, monocyte-derived macrophages have LPL attached to their cell membranes. In atherosclerotic lesions, the LPL protein is especially high in macrophage-rich intimal regions (Yla-Herttuala *et al.*, 1991). This suggests that the local concentration of LPL in atherosclerotic lesions may be much higher than the LPL concentrations found in plasma after heparin injection. In the intima of the vessel wall, LPL may serve as an atherogenic protein by stimulating the uptake of atherogenic lipoproteins by smooth muscle cells and macrophages, leading to foam cell formation (Vijayagopal *et al.*, 1993), an early sign of atherosclerosis. Our results suggest that the enhanced uptake of glycated LDL in the presence of LPL plays an important role in the accelerated atherogenesis of diabetic patients.

Acknowledgments This work was supported by a grant from the Korean Science and Engineering Foundation (KOSEF 971-0708-062-1).

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