The nonenzymatic glycation of LDL is a naturally occurring chemical modification of apolipoprotein (apo)-B lysine residues by glucose. Once glycated, LDL is only poorly recognized by lipoprotein receptors including the LDL receptor (LDL-R), the LDL-R–related protein (LRP), and scavenger receptors. Glycated LDL (gLDL) is a preferred target for oxidative modifications. Additionally, its presence initiates different processes that can be considered “proatherogenic.” Thus, LDL glycation might contribute to the increased atherosclerotic risk of patients with diabetes and familial hypercholesterolemia. Here we investigate whether lipoprotein lipase (LPL) can mediate the cellular uptake of gLDL. The addition of exogenous LPL to the culture medium of human skin fibroblasts, porcine aortic endothelial cells, and mouse peritoneal macrophages enhanced the binding, uptake, and degradation of gLDL markedly, and the relative effect of LPL on lipoprotein uptake increased with the degree of apoB glycation. The efficiency of uptake of gLDL by LDL-R–deficient fibroblasts and LRP-deficient Chinese hamster ovary cells in the presence of LPL suggested a mechanism that was independent of the LDL-R and LRP. In macrophages, the uptake of gLDL was also correlated with their ability to produce LPL endogenously. Mouse peritoneal macrophages from genetically modified mice, which lacked LPL, exhibited a 75% reduction of gLDL uptake compared with normal macrophages. The LPL-mediated effect required the association of the enzyme with cell surface glycaminoglycans but was independent of its enzymatic activity. The uptake of gLDL in different cell types by an LPL-mediated process might have important implications for the cellular response after gLDL exposure as well as the removal of gLDL from the circulation. Diabetes 50: 1643–1653, 2001
lar system that produce LPL (macrophages) or bind large amounts of the enzyme (endothelial cells). We found that LPL markedly increased the capacity of cells to bind and internalize gLDL independently of the classic LDL-R pathway and LRP.

RESEARCH DESIGN AND METHODS

Lipoprotein preparation. Lipoproteins were prepared from plasma of fasted normolipidemic subjects by sequential ultracentrifugation using standard techniques (18) in the presence of EDTA (0.1%) and NaN3 (0.1%). The pooled plasma was stored in the presence of 0.6% sucrose and 0.1% EDTA at -70°C. Protein concentrations of lipoproteins were determined by the method of Lowry et al. (19) using bovine serum albumin as standard. Total cholesterol (TC) was determined using an enzymatic procedure (CHOD-PAP, Boehringer Mannheim).

Lipoprotein glycation and LDL acetylation. In vitro glycation of lipoproteins was performed in the absence and presence of NaBH4-CN as described previously (8). LDL at a concentration of 1 mg TC/ml was incubated in 10 mmol/l phosphate-buffered saline (PBS) (pH 7.4) containing 0.1% EDTA, 25 μmol/l butyrylhydroxyoxetene, 50 μmol/l diethylaminomaleic-acetic acid, 12 mg/ml NaBH4-CN, and different concentrations of n-glucose (2.5, 5, 10, 20, 40, and 80 mmol/l). Incubation of control LDL (cLDL) was performed without glucose. The mixture was filter-sterilized (0.22-μm filters), N2-purged, and incubated at 37°C for 6 days in the dark. HDL, VLDL, and albumin were glycated under identical conditions at a protein concentration of 0.025 mg/ml and in the presence of 40 mmol/l glucose. Glycation of LDL in the absence of NaBH4-CN was performed with 50 μmol/l glucose for 14 days at 37°C. The amount of glycation was estimated by the trinitrobenzenesulfonic acid (TNBS) assay (Pierce). TNBS reacts specifically with free lysines and NH2-terminal amino acid residues to form trinitrophenyl derivatives. The relative reduction of absorbance of gLDL compared with cLDL is a linear function of the non–trichloroacetic acid (TCA) precipitable radioactivity in the medium after precipitation of free iodine with AgNO3 (31). Medium (0.25 ml) was mixed with 50 μl BSA (30 mg/ml) and 0.5 ml ice-cold TCA (0.7 mol/l) and incubated for 30 min at 4°C. Subsequently, 125 μl AgNO3 (0.7 mol/l) was added. The mixture was vortexed and centrifuged for 15 min at 8,000 rpm at 4°C. The radioactivity in 0.5 ml of the supernatant was quantitated in a gamma counter. Cell-associated (nondegraded) radioactivity was measured after lysis of cells in 0.1 N NaOH, 0.1% SDS. Control experiments without cells were performed in parallel to determine the amounts of degradation not attributable to cellular effects.

The internalization of the Dil-labeled lipoproteins was measured after lysis of cells in 0.1 N NaOH, 0.1% SDS, for 4 h at room temperature as described by Teupser et al. (32). Fluorescence was measured on microwell plates in a microplate reader (1420 Multilabel Counter; Wallac), with excitation and emission wavelengths set at 520 and 572 nm, respectively. The fluorescence intensity of the Dil-labeled LDL was monitored (from 20 ng TC/ml to 1 μg TC/ml, r = 0.990). All fluorimetric data were corrected for the autofluorescence of cells incubated with medium alone. The concentration of protein in the cell lysate was measured by the method of Lowry et al. (19).

RESULTS

To study the effect of glycation on the LDL-R-mediated uptake of LDL in HSF, it was necessary to establish a reproducible glycation protocol and to determine the degree of LDL glycation. Incubation of LDL with D-glucose resulted in a dose-dependent glycation of the susceptible amino groups of apoB (Fig. 1A). In the presence of NaBH4-CN and at a glucose concentration of 40 mmol/l, 40% of all susceptible lysine residues in apoB were modified. This preparation is expected to contain both glucitolysine and fructoseosyl modifications (6) and was termed glycated LDL. Additionally, a glycation protocol without the use of NaBH4-CN was utilized that resulted in lower glycation efficiency (12% modification). This moderately glycated LDL (mgLDL) is expected to predominantly contain fructoseosyl modifications. The glycation procedures did not cause the aggregation of LDL, as demonstrated by the lack of changes in the dynamic and static light scattering behavior of the particles (Fig. 1B). Even at

trays. When cells were confluent, the medium was refreshed or switched to DMEM containing 10% LPDS 24 h before the experiment.

Endothelial cells were isolated from porcine aortas, as described by Graier et al. (26). Briefly, fresh porcine aortas were incubated at 37°C in DMEM containing 200 U/ml collagenase (type II), 2% DEEM essential amino acids (Life Technologies), 1% DMEM nonessential amino acids (Life Technologies), 1% DMEM vitamins (Life Technologies), and trypsin inhibitor (soybean type I, 1 mg/ml). Isolated cells were cultured in 10-cm Petri dishes in Opti-DMEM containing 3% FCS. For experiments, cells of passage 1 were trypsinized and seeded into 24-well trays. When cells were confluent, the medium was refreshed 24 h before the experiments.

Isolation of peritoneal mouse macrophages from mice was performed as described previously (29). Briefly, peritoneal macrophages from wild-type mice (wt-macrophages) were elicited by the intraperitoneal injection of 2 ml thioglycollate medium (3% in H2O; Life Technologies) and harvested 3 days after injection. Cells were plated on 24-well plates for 4 h. Subsequently, macrophages were washed three times with PBS (10 mmol/l, pH 7.4, 0.15 mol/l NaCl) and cultured overnight in DMEM containing 10% FCS. For the isolation of LDL-deficient peritoneal macrophages (ko-macrophages), the identical protocol was used starting with genetically modified mice that expressed the enzyme exclusively in muscle. Peritoneal macrophages from these mice have been shown previously to completely lack LPL expression (30). LPL-deficient peritoneal macrophages, and binding, uptake, and degradation studies. For determination of binding of lipoproteins, cells were placed on ice and incubated with the labeled lipoproteins for 4 h at 4°C under constant shaking. For determination of binding and internalization of lipoproteins at 37°C, cells were incubated with the labeled lipoproteins for 6 h under standard conditions. Cells were then placed on ice and washed twice with PBS containing 0.5% BSA and twice with PBS without BSA. Surface-bound lipoproteins were removed by incuba-
the highest degree of LDL glycation, no increase in particle diameter was observed. As shown previously (33), the in vitro glycation procedures did not lead to oxidative modifications of LDL.

The inhibitory effect of glycation on LDL-R–mediated uptake was analyzed in HSF cell cultures (Fig. 2A). Compared with nonglycated cLDL, a decrease of DiI-LDL uptake was observed when gLDL was used in cell uptake experiments. Decreased uptake correlated directly with the extent of LDL glycation. When the LDL-R was upregulated by preincubation of HSF with medium containing LPDS, a similar result was obtained. Although uptake of

FIG. 1. A: Glycation of LDL. LDL (1 mg TC/ml) was incubated with the indicated concentrations of D-glucose for 6 days at 37°C. The degree of glycation was estimated using the TNBS assay, as described in RESEARCH DESIGN AND METHODS. Data represent the means of duplicate determinations of one typical experiment. B: Static and dynamic light scattering analysis of cLDL and gLDL. LDL was glycated with the indicated concentrations of D-glucose. Experiments were performed on a laboratory-built goniometer at a LDL concentration of 1 mg TC/ml. The hydrodynamic radius ($R_H$) of cLDL and gLDL was obtained from the dynamic scattering experiments. The intensities were obtained from static scattering. Data represent the mean of 10 determinations for ± SD.

FIG. 2. Effect of glycation on LDL-R–mediated and LPL-mediated uptake of LDL in HSF. HSF were grown to confluency and incubated in DMEM containing either 10% LPDS (upregulated) or 10% FCS (nonupregulated) for 24 h. After this period, cells were incubated with DiI-labeled LDL (20 µg TC/ml) with different degrees of glycation for 6 h at 37°C in the absence (A) or in the presence (B) of 5 µg/ml LPL. Subsequently, the cells were washed and incubated with 100 U/ml heparin for 1 h at 4°C under constant shaking to remove cell-bound lipoproteins. Uptake was determined after lysis of cells in 0.1N NaOH/0.1% SDS. Data represent means ± SD from triplicate wells.
Data represent means ± SD from triplicate wells. 

To extend our studies to a cell system exhibiting more physiologically relevant features, lipoprotein binding, uptake, and degradation were determined in HSF, porcine aortic endothelial cells (PAECs), and mouse peritoneal macrophages. Endothelial cells are a particularly suitable model because, first, they represent the cell type to which LPL is bound in vivo and, second, they directly encounter increased levels of gLDL in diabetic individuals. Macrophages have been shown to produce endogenously high levels of LPL (35). Additionally, in these experiments, we compared the effect of LPL on the binding, uptake, and degradation of gLDL versus mgLDL. mgLDL was produced by a glycation protocol without the use of NaBH₄CN, which resulted in a lower glycation efficiency and the presumed absence of glucitollysine formation. In a dose-response experiment (Fig. 6), the addition of increasing amounts of LPL to the incubation medium led to a pronounced dose-dependent increase of both gLDL and mgLDL binding in all investigated cell types (Fig. 6A). Uptake and degradation of gLDL and mgLDL are shown in Fig. 6B and C, respectively. The addition of LPL caused a marked increase of both gLDL and mgLDL uptake and degradation, which was saturable between 1 and 2 μg LPL/ml in all cell types. The pronounced increase in the uptake and degradation of both gLDL and mgLDL indicated that the nature of lysisine modification did not affect LPL action. The consistently higher uptake and degradation rates of mgLDL in the absence and presence of LPL can be explained by the more moderate degree of glycation that did not completely abolish its binding to the LDL receptor.

To investigate the role of endogenously synthesized macrophage LPL in the uptake of gLDL, we used peritoneal macrophages from induced mutant mice that lack LPL (ko-macrophages) and compared them with macrophages from wild-type mice (wt-macrophages). Figure 7 shows the uptake of Dil-labeled gLDL and acetylated LDL.
(acLDL) in wt- and ko-macrophages. ko-Macrophages exhibited a marked decrease in their capacity to internalize gLDL. At a gLDL concentration of 40 μg/ml LDL (TC)/ml medium, gLDL uptake was 2.6-fold higher in macrophages that expressed LPL compared with ko-macrophages (Fig. 7A). In contrast, the uptake of acLDL, which is mediated
by scavenger receptors, was not affected by the presence or absence of LPL (Fig. 7B). Additionally, the amount of acLDL internalized by macrophages was more than 10-fold higher than the amount of gLDL taken up, confirming that LDL glycation did not transform the lipoprotein into a particle readily internalized by macrophages. Increased uptake also affected the lipoprotein degradation (Fig. 8). Degradation of 125I-labeled cLDL was increased by 28% in wt-macrophages compared with ko-macrophages, whereas degradation of labeled gLDL was increased sixfold. In the additional presence of exogenously added bovine LPL (1 μg/ml), lipoprotein degradation was further increased, and the difference between wt- and ko-macrophages was abolished.

To reveal whether the observed effects of LPL required an active enzyme that is bound to the cell surface by glycosaminoglycans, the degradation of gLDL was measured in the presence of inactivated LPL and in the presence of heparin. LPL inhibition was achieved by the reversible active site inhibitor tetrahydrolipstatin (THL) (Orlistat). At a concentration of 20 μg/ml, this serine hydrolase inhibitor completely abolished the activity of LPL in conventional LPL activity assays (not shown). As shown in Fig. 9, the addition of THL to the incubation medium did not significantly affect the degradation of gLDL. Enzymatically active LPL (1 μg/ml) increased the degradation 16-fold, and THL-inactivated LPL led to a 13-fold increase in gLDL degradation. In contrast, heparin treatment completely abolished the effect of LPL. These experiments indicated that an enzymatically inactive LPL is sufficient to enhance the uptake of gLDL, but the effect critically depends on the interaction of LPL with cell surface proteoglycans.

Finally, we examined the effect of LPL on the degradation of 125I-labeled glycated VLDL (gVLDL) and 125I-labeled glycated HDL (gHDL). In the absence of LPL, glycation of VLDL and HDL decreased the degradation of the particles by 44 and 72%, respectively, compared with nonglycated lipoproteins (Fig. 10). The addition of LPL enhanced the degradation of both control VLDL (sixfold) and gVLDL (ninefold) (Fig. 10A). The degradation of control HDL and gHDL was enhanced 1.4- and 3.5-fold, respectively (Fig. 10B). LPL had no measurable effect on the degradation of albumin or glycated albumin (not shown). These results indicated that the effect of LPL can also be observed in other lipoprotein classes.

**DISCUSSION**

ApoB glycation is a naturally occurring modification of LDL that might produce a particle of high atherogenic potential. Increased concentrations of gLDL have been measured in diabetic patients as well as individuals suffering from hypercholesterolemia (36). Once glycated, LDL becomes a preferential target for different oxidative modifications (11). Additionally, gLDL can enhance chemotaxis and the production of superoxide anions in macrophages (37). In endothelial cells, it prevents shear stress-mediated L-arginine uptake and nitric oxide formation (33) and causes increased production of plasminogen-activator inhibitor 1 (38) and prostaglandins (39), while inhibiting the expression of tissue plasminogen activator (38). All these processes can contribute to the development of atherosclerosis. Although little is known about how gLDL initiates these processes, it is reasonable to assume that gLDL must interact with the cell surface of target cells—namely endothelial cells and macrophages.

In contrast to native LDL, which is efficiently removed from plasma by the LDL-R, gLDL is only poorly recognized by lipoprotein receptors. Neither the LDL-R nor LR or macrophage scavenger receptors bind and internalize gLDL with high affinity. In search of a potential binding partner and uptake mechanism for gLDL, we investigated the ability of LPL to facilitate this process. LPL appeared to be
an attractive candidate because, first, it is expressed in or located on the surface of cells that exhibit the most pronounced biological response to gLDL (macrophages and endothelial cells) and, second, LPL has been shown to interact with native and modified LDL mediating their cellular uptake (40,41). Several basic mechanisms have

![Graphs showing binding, uptake, and degradation of 125I-labeled gLDL and mgLDL in HSF, PAEC, and mouse macrophages.](image)

**FIG. 6.** Binding, uptake, and degradation of 125I-labeled gLDL and mgLDL in HSF, PAEC, and mouse macrophages. Cells were incubated with LDL (10 μg TC/ml) glycated in the absence (12% glycation, mgLDL) or in the presence (40% glycation, gLDL) of NaBH3CN and with increasing concentrations of LPL for 6 h at 37°C. Subsequently, the cells were washed and the heparin-releasable binding was determined after incubating the cells with 100 U/ml heparin for 1 h at 4°C under constant shaking. Uptake represents cell-associated (nondegraded) LDL plus degraded LDL. Degradation of lipoproteins was measured as non-TCA precipitatable radioactivity in the medium. Data represent means ± SD from triplicate wells.
been shown to contribute to LPL-mediated lipoprotein uptake. Beisiegel et al. (12) showed in cross-linking experiments that LPL can directly bind to LRP (ligand function). Other authors (15,42) suggested that the increased binding of LDL to cell surface heparan sulfate proteoglycans in the presence of LPL facilitates LDL-R–mediated LDL uptake (bridging function), whereas Rumsey et al. (16) concluded from their experiments that LPL increases lipoprotein uptake by a non–LDL-R pathway. Finally, Fuki et al. (43) showed that in the presence of LPL, syndecan proteoglycans can mediate the uptake of lipoproteins, a process with characteristics distinct from classic receptor pathways.

The present study demonstrates that LPL can markedly enhance the binding, uptake, and degradation of gLDL by HSF, PAEC, and mouse peritoneal macrophages. LPL promoted the uptake of both “moderately glycated” mgLDL and “heavily glycated” gLDL. This suggested that the type of lysine modification (glucositollysine vs. fructoselysine) introduced by the presence or absence of NaBH₃CN in the glycation procedure did not affect the LPL-mediated process. A number of experimental observations indicated that the LPL-mediated binding and uptake of gLDL is independent of the LDL-R and LRP. First, and in accordance with previous studies (4), gLDL was not recognized by cells in the absence of LPL. Second, changes in LDL-R activity in HSF in response to LPDS stimulation did not affect gLDL binding or uptake. Third, LPL can mediate the...

FIG. 7. Uptake of DiI-labeled gLDL (A) and acLDL (B) in wt- and ko-macrophages. Cells were cultured in DMEM containing 10% FCS for 24 h. Subsequently, the cells were incubated with the indicated concentrations of DiI-labeled gLDL or acLDL for 6 h at 37°C. Uptake was determined after lysis of cells in 0.1 N NaOH/0.1% SDS. Data represent means ± SD of duplicate wells from macrophages of two completely independent experiments starting from different wild-type (wt) and knockout (ko) mice.

FIG. 8. Effect of endogenous (A) and exogenous (B) LPL on the degradation of ¹²⁵I-labeled cLDL and gLDL in wt- and ko-macrophages. Before the experiment, cells were cultured in DMEM containing 10% FCS for 24 h. After this period, cells were incubated with cLDL and gLDL (20 μg TC/ml) for 6 h at 37°C. LPL was added at a concentration of 1 μg/ml. Subsequently, degradation of lipoproteins was measured as non-TCA precipitable radioactivity in the medium. Data represent means ± SD from triplicate wells.
uptake of gLDL in FH-HSF and LRP-deficient CHO cells. From these results we conclude that cLDL and gLDL are taken up by different mechanisms. Whereas cLDL once bound to the cell surface via LPL is mainly internalized and degraded by the LDL-R pathway, gLDL is internalized by other LDL-R- and LRP-independent uptake mechanisms.

Fibroblasts are a commonly studied model in lipid metabolism. However, these cells do not produce LPL, and it is not likely that fibroblasts are exposed to high concentrations of LPL in vivo. The functional site of LPL action is the microvascular endothelium. Although endothelial cells themselves do not produce LPL, the enzyme is delivered from the surrounding LPL-producing parenchymal tissues and subsequently tightly bound to the luminal surface of endothelial cells (44). Similarly to the situation observed with fibroblasts, LPL markedly increased the binding, uptake, and degradation gLDL in endothelial cells in a dose-dependent manner. Because the vascular endothelium in muscle and adipose tissue contains large amounts of LPL on its luminal surface that come in contact with plasma lipoproteins, it is likely that LPL mediates the effects of gLDL on endothelial cell physiology. Additionally, endothelial cells might participate in the removal of gLDL from the circulation through an LPL-mediated mechanism.

LPL is highly expressed in macrophages (45,35). Obunike et al. (14) showed that the LDL-R-independent uptake of LDL via LPL is much more rapid in THP-1 macrophages than in fibroblasts. Our studies corroborate and extend these observations by demonstrating that LPL can drastically enhance the binding, uptake, and degradation of gLDL in macrophages. Additionally, using genetically modified macrophages, we were able to show that the amount of endogenously produced LPL from macrophages also affects the cellular uptake of gLDL. When mouse peritoneal macrophages do not express LPL, lipoprotein uptake is diminished. The LPL-mediated uptake of gLDL in macrophages might have important pathophysiological implications because the level of LPL activity in macrophages is directly correlated with the susceptibility of inbred mouse lines to develop atherosclerotic lesions (46). Direct evidence for such a proatherogenic role of LPL in macrophages was provided in experiments with transgenic mice demonstrating a reduced atherosclerosis susceptibility in animals that lack LPL in macrophages (47,48). In atherosclerotic lesions, LPL was found on the surface of macrophages and intimal smooth muscle cells and on extracellular matrix components (49). LPL bound to proteoglycans in the subendothelial matrix might cause...
the trapping of gLDL and prolong its residence time in the circulation similarly to the situation demonstrated for LDL (50,51). This process possibly permits more extensive oxidation termed glucoxidation (11) and could ultimately result in the formation of AGEs, such as pentosidine and vesperslysin (52,53). AGE-modified proteins and lipoproteins are avidly internalized by macrophages via specific AGE receptors and might contribute to lipid accumulation in macrophages, foam cell formation, and the development of atherosclerotic lesions (54).

The high affinity binding between LPL and lipoproteins is thought to involve multiple ionic and hydrophobic interactions (55) that allow the enzyme to interact not only with LDL but also chylomicrons, VLDL, and HDL (15). In this investigation, we show that LPL also binds to gLDL and that glycation does not affect the interaction of LPL with LDL. LPL binding to lipoproteins facilitated the cellular uptake and degradation of gLDL, gVLDL, and gHDL. LPL enzyme activity was not required for the enhancement of gLDL uptake. In contrast, binding of LPL to cell surface glycosaminoglycans was a prerequisite for LPL-mediated gLDL uptake because heparin treatment completely abolished this effect. Similar results were obtained when the LPL-mediated uptake of native LDL was studied (14,56).

Although LPL increased the binding of gLDL to cells dramatically, the amount of internalization and degradation did not reach the levels observed for nonglycated LDL. Therefore, we assume that a “slow, high-capacity” pathway, as originally described by Obunike et al. (14), for LPL-mediated uptake of LDL might be responsible for gLDL uptake. This hypothesis is in accordance with observations by Lopes-Virella et al. (57), who found that gLDL is internalized by an LDL-R–independent mechanism of high capacity in human macrophages.

In summary, we have shown that LPL can mediate the uptake of gLDL in different cell types even though the modified particle is not recognized by the LDL, LRP, or scavenger receptors. We speculate that LPL might play a role in the removal of gLDL from the circulation by LPL-containing cells. Additionally, the process of cellular binding of gLDL mediated by LPL might initiate the signal transduction pathway resulting in the cellular response of endothelial cells and macrophages after gLDL exposure.

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