Endogenously produced lipoprotein lipase enhances the binding and cell association of native, mildly oxidized and moderately oxidized low-density lipoprotein in mouse peritoneal macrophages

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It has been well established that purified lipoprotein lipase (LPL) can facilitate the cellular uptake of various native and modified lipoproteins when added exogenously to macrophages. Because activated macrophages express LPL endogenously, it was the aim of this study to investigate the effect of macrophage-produced LPL on the uptake of native low-density lipoprotein (LDL) and LDL that has been modified to various degrees by Cu^{2+}-mediated oxidation. Cell binding and uptake of Eu^{3+}-labelled native and oxidized LDL was determined in mouse peritoneal macrophages (MPM) from normal mice and induced mutant mice that lack LPL expression in MPM. We found that LPL expressed by MPM was able to increase cell binding and association of native LDL (by 121 % and 101 % respectively), mildly oxidized LDL (by 47 % and 43 %) and moderately oxidized LDL (by 30 % and 22 %). With increased levels of lipoprotein oxidation, the relative proportion of LPL-mediated LDL uptake decreased. This decrease was not due to weakened binding of LPL to oxidized LDL. The drastically increased uptake of highly oxidized LDL in MPM by scavenger-receptor-mediated pathways might dominate the simultaneous exogenous or endogenous LPL-mediated uptake of this lipoprotein. Competition experiments with positively charged poly(amino acids) furthermore suggested that histidine, arginine and lysine residues in LPL are important for the interaction between LDL and LPL. Our results imply that physiological levels of LPL produced by macrophages facilitate the uptake of native LDL as well as mildly and moderately oxidized LDL. This process might, in the micro-environment of arteries, contribute to the accumulation of macrophage lipids and the formation of foam cells.

Key words: europium, polyamino acids, time-resolved fluorimetric assay.

INTRODUCTION

Lipoprotein lipase (LPL) is the major enzyme responsible for the hydrolysis of triacylglycerols present in circulating chylomicrons and very-low-density lipoproteins. On hydrolysis, fatty acids are liberated for subsequent use in adipose tissue and muscle [1,2]. The mature protein is secreted from parenchymal cells in tissues such as adipose, skeletal and heart muscle, mammary gland and brain, and transported to capillaries, where LPL is bound to glycosaminoglycan components of the capillary endothelium [3]. It was first suggested by Zilversmit [4] that LPL might be important in atherogenesis in that the hydrolysis of triacylglycerol-rich lipoproteins by LPL at the endothelial lining of arteries would lead to the formation of atherogenic remnants, which are then taken up by the cells in the artery wall, resulting in lipid deposition.

Aside from its enzymic function, LPL mediates several other biological processes, which might be relevant to atherogenesis. (1) LPL can act as a bridge linking apolipoprotein B (apoB) of low-density lipoprotein (LDL) with heparan sulphate proteoglycans [5] and with a fragment of apoB [6] on endothelial cells. (2) Products resulting from chylomicron lipolysis by LPL were shown to increase the permeability of the endothelial layer, thus enhancing the influx of atherogenic LDL into the arterial wall [7]. (3) In the subendothelial intima of the artery, LPL can mediate the binding of LDL to intimal proteoglycans [8], to cell-surface heparan sulphate proteoglycans and to the LDL-receptor-related protein ('LRP') [9–11]. In this way LPL helps to entrap LDL in the intima and enhances the uptake of LDL by macrophages and, presumably, smooth-muscle cells. It was shown that the oxidation of LDL strikingly changes the structural and functional properties of this lipoprotein [12–14]. Binding followed by the uptake of oxidized LDL in macrophages in an unregulated fashion would lead to the formation of foam cells, the hallmark of early atherosclerotic lesions [15]. The oxidation of LDL increased its affinity for extracellular matrix proteins [16,17]; exogenously added LPL from bovine milk further enhanced the entrapment of oxidized LDL by bridging it with the matrix [18]. It has been shown that the binding and uptake of moderately oxidized LDL by J774 macrophages can also be stimulated by exogenously added LPL from bovine milk [19].

In all the experiments mentioned above, exogenously added LPL purified from milk was used. Because it is improbable that such high experimental concentrations of LPL exist *in vivo*, the question arose whether a variation in LPL concentration within the physiological range would influence the uptake of native and

Abbreviations used: apoB, apolipoprotein B; DMEM, Dulbecco's modified Eagle's medium; HNE, 4-hydroxynonenal; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MCK, muscle-specific creatine kinase; MDA, malondialdehyde; MPM, mouse peritoneal macrophages; REM, relative electrophoretic mobility.

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oxidized LDL by macrophages. Because LPL can bind native and oxidized LDL [18], a complex might be formed when exogenous LPL is mixed with LDL or oxidized LDL before LPL interacts with the cells, as in the above-mentioned study [19]. LPL in this complex might function in a different way from LPL produced by macrophages and translocated to the cell surface. In the present study we compared the binding and uptake of native and oxidized LDL in mouse peritoneal macrophages (MPM) from genetically engineered mice that lacked LPL expression in MPM, with MPM from littermate mice expressing normal LPL. Additionally, to avoid the lipid peroxidation of LDL by labelling with radioactive iodine [20], which might change the metabolic fate of the labelled LDL in macrophages [21], LDL was gently labelled with europium (Eu^{3+}) ions to ensure that no lipid peroxidation occurred during the labelling procedures [22].

EXPERIMENTAL

Lipoprotein preparation

LDL was isolated from plasma of normolipidaemic, fasting (12–14 h) young male human donors with serum lipoprotein(a) levels lower than 1 mg/100 ml. EDTA (1 mg/ml; Merck) and the protease inactivators aprotinin (100 i.u./ml; Bayer) and Pefabloc (50 μM; Merck) and the antioxidant butylated hydroxytoluene ('BHT') (20 μM; Merck) were present during LDL preparation by differential ultracentrifugation at a density range between 1.020 and 1.050 g/ml; NaBr was used to adjust the density. The protein concentration of LDL was measured by the method of Lowry et al. [23], with BSA as standard. LDL concentrations are expressed in terms of the protein content. LDL was sterile-filtered and stored at 4 °C. Lipoprotein-deficient serum was prepared by ultracentrifugation of the serum at $d =$ 1.235 and removal of the lipoprotein-containing supernatant followed by extensive dialysis against PBS.

Labelling of LDL with Eu^{3+}

Labelling of LDL with Eu^{3+} was performed as described previously [16]. In brief, 2 mg of LDL in 50 mM $NaHCO_3$, pH 8.4, containing 20 μM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Hofmann LaRoche), was incubated with 0.2 mg Eu^{3+} chelate of N^1-(*p*-isothiocyanatobenzyl)-diethylene triamine-N^1,N^2,N^3,N^3-tetra-acetic acid (DELFIA Eu-labelling kit; Wallac Oy) at 25 °C in the dark for 12 h. Sephadex G-25 chromatography (Pharmacia Biotech) was used for the separation of labelled protein from unreacted chelate in 50 mM Tris/HCl, pH 7.8, containing 0.05 % NaN_3 and 20 μM Trolox. The labelling yield of Eu^{3+}-LDL was between 4 and 22 Eu^{3+} ions per protein molecule, or between 7 and 40 nmol of Eu^{3+} per mg of protein.

Cu^{2+}-mediated oxidation of LDL and Eu^{3+}-LDL

Before oxidation, LDL and Eu^{3+}-LDL were dialysed against 10 mM PBS, pH 7.4, carefully degassed and then saturated with N_2. Cu^{2+}-mediated oxidation of LDL (500 μg/ml) was performed at 37 °C with 30 μM $CuCl_2$. At intervals between 0 and 24 h the reaction was terminated by the addition of a stop solution to achieve a final EDTA concentration of 2.7 mM. The samples were saturated with N_2 and stored at 4 °C. The degree of LDL modification was estimated as the relative electrophoretic mobility (REM) in comparison with the respective labelled and unlabelled native LDL on 1 % (w/v) agarose gels at pH 8.05 with the Lipidophor system (Immuno AG). In our experiments, LDL and Eu^{3+}-LDL that had been oxidized for 1 h (REM approx. 1.3) and 4 h (REM approx. 2.6) were designated mildly or moderately oxidized LDL; those oxidized for 8 h (REM approx. 2.9) and 24 h (REM approx. 3.5) were designated strongly and heavily oxidized LDL respectively. In some samples lipidhydroperoxides were estimated by a spectrophotometric assay with cholesterol oxidase/iodide colour reagent (Merck) at 365 nm, as developed in this laboratory [24].

Generation of transgenic mice

The detailed description of the transgenic mice is described elsewhere [25]. In brief, by cross-breeding of heterozygous LPL knock-out mice [26] with transgenic mice expressing LPL under the control of a muscle-specific creatine kinase (MCK), animals were obtained that express human LPL in skeletal and cardiac muscle on either the null (L0-MCK) or normal (L2-MCK) LPL background. Whereas L2-MCK mice show normal LPL expression in macrophages, L0-MCK mice show no LPL expression in macrophages. All animals were fed with a standard laboratory chow diet.

Cell cultures

Resident MPM from MCK mice and their littermate controls were elicited by the intraperitoneal injection of 2 ml of 3 % (w/v) thioglycollate medium (Gibco BRL) 3 days before the mice were killed. Primary cultures were prepared at a density of 1.5×10^5 per well in 96-well plates (Costar, Vienna, Austria), in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10 % (v/v) foetal calf serum (Gibco BRL), 100 i.u./ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified incubator with air/CO_2 (19:1); 3 h after the plating non-adherent cells were washed out with 10 mM PBS, pH 7.4. The cells were cultured in the above medium overnight before use. Cell viability was greater than 98 % as assessed by Trypan Blue exclusion.

Purification of LPL from bovine milk

Bovine milk LPL was purified from fresh unpasteurized milk by heparin–Sepharose (Pharmacia) chromatography as described by Saxena et al. [27], followed by affinity chromatography on a HiTrap Heparin column (Pharmacia) eluted with 10 mM phosphate buffer, pH 6.8, containing 0.75–2.00 M NaCl. Purified LPL showed a major protein band of approx. 55 kDa and a minor band of approx. 40 kDa in some fractions when analysed by SDS/PAGE and stained with Coomassie Blue. The band at approx. 55 kDa was also observed in Western blot analysis with an anti-LPL monoclonal antibody. Purified LPL was stored at −70 °C. The LPL that we used in each experiment contained mostly the approx. 55 kDa protein and only a small amount of approx. 40 kDa protein. The LPL activity was 650 ± 145 nmol of non-esterified fatty acid/h per μg of LPL protein.

LPL activity measurement

LPL activity was measured as described previously [28]. Before each experiment, 0.1 i.u./ml heparin was added to the medium and allowed to incubate with the peritoneal macrophages from both L0-MCK and L2-MCK mice for 2 h at 37 °C to release the LPL from the cells into the medium. LPL activity was calculated as the amount of non-esterified fatty acid released/h, after subtraction of the background.

Table 1 Binding of Eu^{3+}-labelled native and oxidized LDL to MPM from L0-MCK and L2-MCK mice in the absence or presence of exogenous bovine milk LPL and heparinase

MPM from L0-MCK and L2-MCK mice were incubated with 10 μg/ml Eu^{3+}-labelled native and oxidized LDL (oxLDL) in the absence or presence of 10 μg/ml bovine milk LPL for 4 h at 4 °C, with or without preincubation for 2 h at 37 °C with 2.5 units/ml heparinase (H-ase). Values were corrected for non-specific binding and are means ± S.D. for three separate experiments. Percentage values in parentheses in the L0-MCK + LPL and L2-MCK + LPL columns are the percentage increases in binding compared with L0-MCK and L2-MCK respectively; those in the L2-MCK column are the percentage increases in binding compared with L0-MCK; and those in the L0-MCK + H-ase + LPL and L2-MCK + H-ase + LPL columns are the percentage decreases in LPL-mediated increased binding in L0-MCK and L2-MCK MPM respectively after incubation with heparinase (H-ase). *$P < 0.01$ compared with L0-MCK, †$P < 0.01$ compared with L2-MCK, calculated with Student's two-tailed t test.

	Lipoprotein binding (ng of labelled lipoprotein/mg of cell protein)					
Lipoprotein	L0-MCK	L0-MCK + LPL	L0-MCK + H-ase + LPL	L2-MCK	L2-MCK + LPL	L2-MCK + H-ase + LPL
LDL	0.52 ± 0.09	6.16 ± 0.43* (1084.6%)	1.28 ± 0.12 (86.5%)	1.15 ± 0.07* (121.2%)	5.92 ± 0.38† (414.8%)	1.64 ± 0.05 (89.7%)
oxLDL, 1 h	1.35 ± 0.15	14.20 ± 0.80* (951.9%)	2.21 ± 0.55 (93.3%)	1.99 ± 0.13* (47.4%)	15.01 ± 1.46† (654.3%)	3.09 ± 0.32 (91.5%)
oxLDL, 4 h	7.12 ± 0.51	13.07 ± 1.36* (83.6%)	9.10 ± 0.85 (66.7%)	9.22 ± 0.15* (29.5%)	13.53 ± 0.90† (46.7%)	10.24 ± 0.12 (76.3%)
oxLDL, 8 h	58.26 ± 0.92	81.20 ± 2.62* (39.3%)	63.26 ± 4.35 (78.3%)	65.23 ± 2.41* (12.0%)	80.33 ± 4.81† (23.1%)	70.89 ± 2.64 (62.5%)
oxLDL, 24 h	572.33 ± 40.25	539.14 ± 65.33 (−6.0%)	568.12 ± 39.81 (–)	592.30 ± 17.55 (3.5%)	582.34 ± 11.79 (−1.7%)	633.90 ± 5.65 (–)

Cell binding and association studies with time-resolved fluorimetric assay

Time-resolved fluorimetric assay, a non-radioactive assay that is highly sensitive and specific (developed recently in this laboratory), was used to measure the cell binding and association of lipoproteins to macrophages [22]. Cell binding and association studies were performed in 96-well plates in DMEM containing 10% (v/v) lipoprotein-deficient serum and 25 mM Hepes, pH 7.4, by incubating the cells with the medium containing Eu^{3+}-labelled native or oxidized LDL for 4 h at 4 °C (binding: the lipoproteins bind to the surface of the cells) or 37 °C (cell association: the lipoproteins are both on the surface of and inside the cells) respectively, in the absence or presence of exogenous LPL. In some experiments, cells were preincubated with medium containing 2.5 units/ml heparinase I (EC 4.2.2.7; Sigma) for 2 h at 37 °C and washed with medium; Eu^{3+}-labelled lipoproteins and LPL were then added as above. Specific cellular binding and association were calculated by subtracting the amounts of labelled native and oxidized LDL, which were bound or cell-associated in the presence of a 40-fold excess of unlabelled native or oxidized LDL with the same REM as the labelled ones (non-specific), from those in the absence of unlabelled native or oxidized LDL. Cells were washed three times with 10 mM PBS containing 0.1% BSA and twice with PBS without BSA after incubation. Triton X-100 (0.05%, v/v) was then added to each well to dissolve the cells for 10 min, with shaking at room temperature. Time-resolved fluorescence in the cell lysate was measured in duplicates in enhancement solution with a 1234 DELFIA research fluorimeter (Wallac Oy). The cell protein content was estimated by the method of Lowry et al. [23], with BSA as standard [containing 0.05% (v/v) Triton X-100]. The non-specific cellular binding or association was less than 15% of the total cellular binding or association.

Modification of poly(amino acids) with malondialdehyde (MDA) and 4-hydroxynonenal (HNE)

Modification of poly(L-amino acids) (all from Sigma), including poly(L-lysine) (molecular mass 7500 Da), poly(L-arginine) (12100 Da), poly(L-histidine) (11100 Da), by the lipid peroxidation products HNE and MDA was performed as described previously [29]. HNE was synthesized as described [30]. MDA was obtained by the acid hydrolysis of 1,1,3,3-tetramethoxypropane. Poly(L-amino acids) in 0.1 M PBS were incubated with 20 mM MDA or 3 mM HNE in the dark at 37 °C for 5 h. Free aldehydes were removed by extensive dialysis against PBS.

Binding of Eu^{3+}-labelled native and oxidized LDL to LPL

Bovine milk LPL (1.5 μg) in 100 μl PBS was coated on to each well of the microtitration plates (Nunc) at 4 °C for 18 h. After three washes with PBS, each well was blocked with 200 μl of PBS containing 3% (w/v) BSA for 1 h at room temperature. The plates were washed three times with PBS; DMEM containing 1% (w/v) BSA and Eu^{3+}-labelled native and oxidized LDL, in the absence or presence of competitors (polyinosinic acid, fucoidan, heparan sulphate and heparin), was then added to each well. When poly(L-amino acids) and MDA- or HNE-modified poly(L-amino acids) were used as competitors, 10 mM Tris/HCl containing 50 mM NaCl, 2 mM $CaCl_2$ and 1% (w/v) BSA was used instead of DMEM, because DMEM itself already contains large quantities of various amino acids. After four washes with PBS, the fluorescence of bound Eu^{3+} was measured in the presence of enhancement solution (200 μl per well). To measure the non-specific binding, BSA instead of bovine milk LPL was coated on the plates and the binding was measured in the same way. The non-specific binding of Eu^{3+}-labelled lipoproteins to BSA was less than 5%.

RESULTS

Effect of endogenous and exogenous LPL on the cell binding and association of Eu^{3+}-labelled native and oxidized LDL to MPM

The generation of mice expressing human LPL exclusively in muscle but not in other tissues was reported elsewhere [25]. To ensure that there was no detectable LPL activity in MPM from L0-MCK mice but LPL activity was normal in those from L2-MCK mice, LPL activity in the medium was measured before each experiment. In a typical experiment, LPL activity in the conditioned medium of the MPM from the L2-MCK mouse was 2.1 ± 0.2 nmol of non-esterified fatty acid/h per μg of cell protein ($n = 3$) but was not detectable in the conditioned medium of the MPM from the L0-MCK mice.

When the binding of Eu^{3+}-labelled native and oxidized LDL to MPM from L2-MCK and L0-MCK mice was compared, it was found that the binding of native LDL and that of LDL oxidized

Table 2 Cell association of Eu^{3+}-labelled native and oxidized LDL to MPM from L0-MCK and L2-MCK mice in the absence or presence of exogenous bovine milk LPL and heparinase

MPM from L0-MCK and L2-MCK mice were incubated with 10 μg/ml Eu^{3+}-labelled native and oxidized LDL (oxLDL) in the absence or presence of 10 μg/ml bovine milk LPL for 4 h at 4 °C, with or without preincubation for 2 h at 37 °C with 2.5 units/ml heparinase (H-ase). Values were corrected for non-specific cell association and are means ± S.D. for three separate experiments. Percentage values in parentheses in the L0-MCK + LPL and L2-MCK + LPL columns are the percentage increases in cell association compared with L0-MCK and L2-MCK respectively; those in the L2-MCK column are the percentage increases in cell association compared with L0-MCK; and those in the L0-MCK + H-ase + LPL and L2-MCK + H-ase + LPL columns are the percentage decreases in LPL-mediated increased cell association in L0-MCK and L2-MCK MPM respectively after incubation with heparinase (H-ase). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with L0-MCK; †$P < 0.01$ compared with L2-MCK, calculated with Student's two-tailed t test.

		Lipoprotein cell association (ng of labelled lipoprotein/mg of cell protein)				
Lipoprotein	L0-MCK	L0-MCK + LPL	L0-MCK + H-ase + LPL	L2-MCK	L2-MCK + LPL	L2-MCK + H-ase + LPL
LDL	20.56 ± 1.32	152.36 ± 9.42** (641.1%)	44.32 ± 4.99 (81.9%)	41.32 ± 1.78** (101.0%)	153.08 ± 11.24† (270.5%)	59.54 ± 3.90 (84.0%)
oxLDL, 1 h	19.20 ± 1.69	125.49 ± 10.19** (553.6%)	29.79 ± 3.45 (90.0%)	27.47 ± 1.10** (43.1%)	124.62 ± 7.70† (353.7%)	42.02 ± 1.00 (85.0%)
oxLDL, 4 h	29.30 ± 2.51	46.22 ± 3.10** (57.7%)	36.72 ± 1.72 (56.1%)	35.59 ± 1.21* (21.5%)	47.71 ± 4.53† (34.1%)	39.05 ± 3.62 (71.5%)
oxLDL, 8 h	133.93 ± 12.42	160.09 ± 4.55* (19.5%)	145.06 ± 8.85 (57.5%)	159.64 ± 2.58* (19.2%)	169.90 ± 8.43 (6.4%)	163.34 ± 9.56 (63.9%)
oxLDL, 24 h	1268.59 ± 52.60	1252.50 ± 41.92 (−1.3%)	1289.26 ± 59.03 (–)	1299.80 ± 56.31 (2.4%)	1243.28 ± 101.92 (−4.3%)	1208.04 ± 74.50 (–)

for 1, 4 and 8 h to MPM from L2-MCK mice was significantly higher than that of LDL from L0-MCK mice (Table 1). Generally, the relative increase in the binding of Eu^{3+}-labelled LDL to MPM from the L2-MCK mouse compared with that from the L0-MCK mouse decreased with the degree of oxidative modification. However, the absolute increase in the binding of Eu^{3+}-labelled LDL to MPM from the L2-MCK mouse compared with that from the L0-MCK mouse increased with the degree of oxidative modification. Similar results were obtained in cell association experiments (Table 2). This indicated that endogenous LPL produced by macrophages could enhance the cell binding and association of Eu^{3+}-labelled native, mildly oxidized and moderately oxidized LDL to macrophages. However, in comparison with the high-capacity uptake of heavily oxidized LDL through scavenger receptors, endogenous LPL-mediated LDL uptake was of insignificant relevance in quantitative terms.

Next we investigated whether exogenously added LPL was able to increase the cell binding and association of native and oxidized LDL to both LPL-deficient and control MPM. As shown in Table 1, exogenously added bovine LPL significantly increased the binding of Eu^{3+}-labelled native LDL and LDL oxidized for 1, 4 and 8 h to both LPL-deficient and control macrophages. LPL increased to a relatively greater extent the binding of Eu^{3+}-labelled native and less modified LDL to both LPL-deficient and control macrophages, as shown by the percentage increase. Similar results were obtained in cell association studies.

When L0-MCK and L2-MCK MPM were preincubated with heparinase, the exogenous LPL-mediated enhanced binding and cell association of native, mildly and moderately oxidized LDL to both types of cells was decreased by 56–93 % (Tables 1 and 2). To investigate whether pretreatment with heparinase could also have some influence on endogenous LPL, the binding and cell association of native and oxidized LDL to LPL-deficient and control MPM, with or without pretreatment with heparinase, were measured (Tables 3 and 4). After pretreatment of the L0-MCK MPM with heparinase, the binding and cell association of native and oxidized LDL to these cells did not change significantly. For L2-MCK MPM, there was a decrease in the binding and cell association after the pretreatment with heparinase, although the decrease was statistically significant only for native LDL. The difference in the binding of native LDL and mildly and moderately oxidized LDL to L0-MCK MPM compared with L2-MCK MPM still persisted even after the latter cells had been pretreated with heparinase.

Table 3 Binding of Eu^{3+}-labelled native and oxidized LDL to MPM from L0-MCK and L2-MCK mice in the absence or presence of heparinase

MPM from L0-MCK and L2-MCK mice were preincubated with 2.5 units/ml heparinase (H-ase) for 2 h at 37 °C before incubation with 10 μg/ml Eu^{3+}-labelled native and oxidized LDL (oxLDL) for 4 h at 4 °C. Values were corrected for non-specific binding and are means ± S.D. for three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with L0-MCK, †$P < 0.05$ compared with L2-MCK, calculated with Student's two-tailed t test.

	Lipoprotein binding (ng of labelled lipoprotein/mg of cell protein)			
Lipoprotein	L0-MCK	L0-MCK + H-ase	L2-MCK	L2-MCK + H-ase
LDL	0.61 ± 0.09	0.63 ± 0.19	1.66 ± 0.12**	1.36 ± 0.12**†
oxLDL, 1 h	1.87 ± 0.17	1.86 ± 0.14	2.42 ± 0.31*	2.18 ± 0.23
oxLDL, 4 h	9.67 ± 0.50	9.94 ± 0.60	13.01 ± 0.65**	11.75 ± 0.51**
oxLDL, 8 h	72.06 ± 0.75	72.17 ± 5.03	84.40 ± 6.38**	77.95 ± 7.47
oxLDL, 24 h	634.75 ± 49.15	639.52 ± 29.42	654.33 ± 134.01	638.85 ± 17.04

Binding of Eu^{3+}-labelled native and oxidized LDL to bovine LPL

It has been reported that the N-terminal part of apolipoprotein B, especially the positively charged basic amino acid clusters, are responsible for the binding of apolipoprotein B to LPL [31]. Because the basic amino acids such as lysine, arginine and histidine are the targets of modification by lipid-peroxidation-derived aldehydes such as MDA and HNE during LDL oxidation [12,13,32], we wished to investigate whether the ability of LDL to bind LPL decreased after the oxidative modification of this lipoprotein. Microtitration plates were coated with LPL from bovine milk and the direct binding of Eu^{3+}-labelled native and oxidized LDL to LPL was estimated. As shown in Figure 1, similar amounts of native and mildly oxidized LDL bound to LPL. The binding increased with the degree of oxidative modification of LDL. To investigate whether negative charge had a role in the binding of LPL to native and oxidized LDL, we measured the binding of Eu^{3+}-labelled native LDL and LDL oxidized for 24 h to LPL, in the absence or presence of heparan sulphate, heparin, polyinosinic acid and fucoidin. The result

Table 4 Cell association of Eu^{3+}-labelled native and oxidized LDL to MPM from L0-MCK and L2-MCK mice in the absence or presence of heparinase

MPM from L0-MCK and L2-MCK mice were preincubated with 2.5 units/ml heparinase for 2 h at 37 °C before incubation with 10 μg/ml Eu^{3+}-labelled native and oxidized LDL for 4 h at 37 °C. Values were corrected for non-specific cell association and are means ± S.D. for three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with L0-MCK, †$P < 0.05$ compared with L2-MCK, calculated with Student's two-tailed t test.

	Lipoprotein cell association (ng of labelled lipoprotein/mg of cell protein)			
Lipoprotein	L0-MCK	L0-MCK + H-ase	L2-MCK	L2-MCK + H-ase
LDL	25.04 ± 2.14	24.66 ± 4.29	42.31 ± 1.49**	34.53 ± 3.67*†
oxLDL, 1 h	22.17 ± 2.62	21.98 ± 4.46	32.22 ± 4.01*	29.36 ± 3.10*
oxLDL, 4 h	26.94 ± 3.54	26.82 ± 2.79	35.38 ± 1.62**	32.84 ± 2.58**
oxLDL, 8 h	173.91 ± 13.51	167.33 ± 14.44	196.33 ± 5.14**	187.07 ± 29.50
oxLDL, 24 h	1404.43 ± 54.94	1372.42 ± 778.54	1339.82 ± 63.91	1333.07 ± 79.70

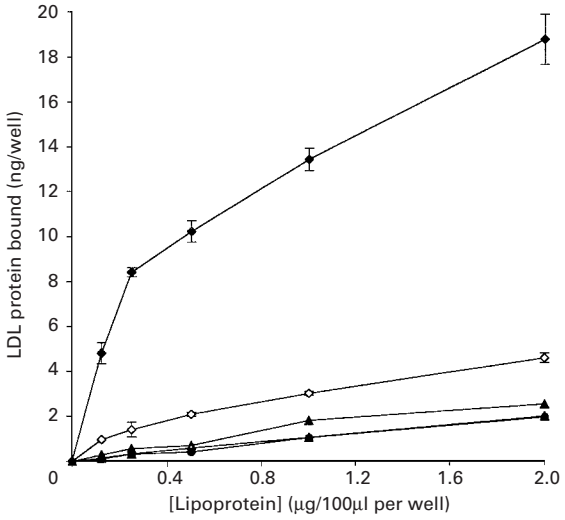

Figure 1 Binding of Eu^{3+}-labelled native and oxidized LDL to bovine milk LPL

Microtitration plates were coated with 15 μg/ml LPL for 18 h at 4 °C. The binding to LPL of increasing concentrations of Eu^{3+}-labelled native LDL (●, REM = 1) and differently oxidized LDL (△, 1 h, REM = 1.3; ▲, 4 h, REM = 2.6; ◇, 8 h, REM = 3.0; ◆, 24 h, REM = 3.6) was measured. Values have been corrected for non-specific binding by using BSA instead of LPL, and are expressed as means ± S.D. ($n = 3$) representative of four experiments. The non-specific binding was less than 2%.

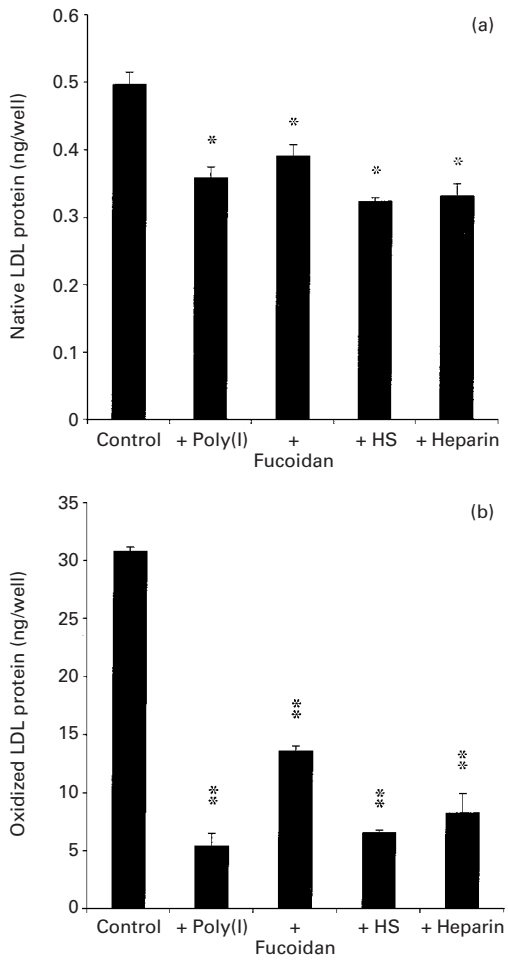

Figure 2 Effect of negative charge on the binding of Eu^{3+}-labelled native and oxidized LDL to bovine milk LPL

Microtitration plates were coated with 15 μg/ml LPL for 18 h at 4 °C. The binding to LPL of Eu^{3+}-labelled native LDL (**a**) and LDL oxidized for 24 h (**b**), in the absence (control) and presence of poly(inosinic acid) [poly(I), 100 μg/ml], fucoidan (20 μg/ml), heparan sulphate (HS, 15 μg/ml) and heparin (15 μg/ml), was measured. Values were corrected for non-specific binding by using BSA instead of LPL and are expressed as means ± S.D. for three separate experiments. Note that different scales are used for native and oxidized LDL. The non-specific binding was less than 3%. $^{*}P < 0.05$, $^{**}P < 0.001$ compared with the control, calculated with Student's two-tailed t test.

(Figure 2) shows that all these negatively charged molecules potently competed for the binding to LPL of Eu^{3+}-labelled LDL oxidized for 24 h, but were much less effective in competing for the binding to LPL of Eu^{3+}-labelled native LDL.

Effect of poly(amino acids) and modified poly(amino acids) on the binding of Eu^{3+}-labelled native and oxidized LDL to bovine milk LPL

To explore further the mechanism of the LPL-mediated binding of native and oxidized LDL to macrophages, we tested the effect of polylysine, polyarginine, polyhistidine and polyproline on the binding of native and oxidized LDL to LPL. Microtitration plates were coated with LPL and the binding of Eu^{3+}-labelled native LDL and LDL oxidized for 24 h to the LPL in the absence or presence of these poly(amino acids) or their MDA- and HNE-modified derivatives was estimated. At a concentration of 50 μg/ml, positively charged polylysine, polyarginine and polyhistidine competed for 47%, 32% and 93% respectively of the binding of Eu^{3+}-labelled native LDL to LPL, whereas for Eu^{3+}-labelled oxidized LDL, the degrees of competition were 62%, 26% and 90% respectively. In comparison, polyproline showed no effect on the binding to LPL of both Eu^{3+}-labelled native and oxidized LDL (Figure 3). Modification of polylysine with lipid peroxidation products MDA or HNE could decrease or even abolish its ability to compete for the binding to LPL of Eu^{3+}-labelled native and oxidized LDL, whereas modification of polyarginine or polyhistidine by MDA or HNE did not decrease their competition effect (results not shown).

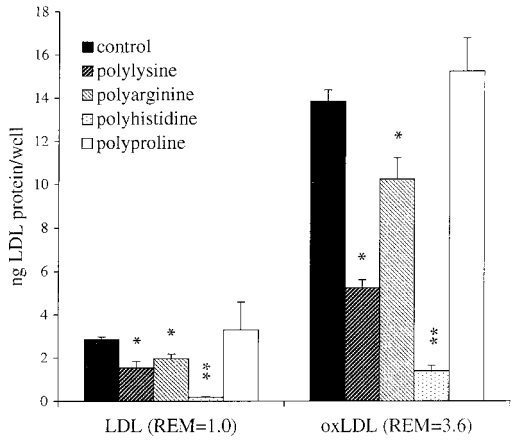

Figure 3 Effect of poly(amino acids) on the binding of Eu^{3+}-labelled native and oxidized LDL to bovine milk LPL

Microtitration plates were coated with 15 μg/ml LPL for 18 h at 4 °C. The binding to LPL of Eu^{3+}-labelled native LDL and LDL oxidized for 24 h, in the absence (control) and presence of 50 μg/ml poly(amino acids), was measured. Values were corrected for non-specific binding using BSA instead of LPL and are expressed as means ± S.D. for three separate experiments. The non-specific binding was less than 5%. $^{*}P < 0.01$, $^{**}P < 0.001$ compared with the control, calculated with Student's two-tailed *t* test.

DISCUSSION

Apart from its enzymic function as a lipase, exogenous LPL has been shown to mediate the uptake of very-low-density lipoprotein and LDL by human fibroblasts, HepG2 cells and THP-1 monocytes and macrophages [10,11,33,34]. The physiological significance of these observations is supported by the findings that (1) cultured macrophages [35–37] and smooth-muscle cells [38] are able to produce LPL, (2) LPL activity has been detected in the artery wall [39,40] and (3) LPL protein and mRNA have been found to be associated with macrophages, and to a smaller extent with smooth-muscle cells, in atherosclerotic lesions [41,42]. LDL is extremely susceptible to oxidative damage; some enzyme systems such as 15-lipoxygenase and myeloperoxidase and all major cell types in the artery wall might contribute to the oxidation of LDL (reviewed in [43]). In fact, the presence of oxidatively modified LDL has been shown in human arteries [44,45]. It was therefore of considerable interest to study whether LPL produced by macrophages was able to increase the uptake of oxidized LDL by these cells, the major precursor of foam cells in atherosclerotic lesions.

It has been shown that exogenously added LPL from bovine milk can stimulate the binding and uptake of moderately oxidized LDL by J774 macrophages [19]. The results from our experiments with MPM and exogenous LPL from bovine milk are in agreement with these findings. It should be pointed out that, when exogenous LPL was mixed with lipoproteins and added to the cells, LPL formed a complex with the lipoproteins before acting as a bridge. LPL in this complex might function in a different way from LPL produced by macrophages and situated on the surface of these cells. Additionally, we wished to know whether a physiological level of LPL produced by macrophages could also enhance the binding of native and oxidized LDL to macrophages. We found that both LPL produced by macrophages and LPL added exogenously could significantly enhance the cell binding and association of native, mildly oxidized or moderately oxidized LDL to macrophages. Pretreatment of the cells with heparinase decreased by 56–93 % the enhanced binding and cell association of native and oxidized LDL by exogenous LPL, whereas it was less efficient in inhibiting the enhanced binding and cell association by endogenously produced LPL. This suggests that there might be some common, but not identical, pathways for exogenous and endogenous LPL with regard to their ability to enhance the binding and cell association of native and oxidized LDL to MPM.

It was found that a fragment of bovine LPL, presumably generated by proteolytic degradation during the isolation of LPL from bovine milk, was mostly responsible for tthe enhanced uptake of lipoproteins by the cells [46]. We were able to show that LPL produced by macrophages, thus avoiding any modification due to the isolation processes, also stimulates the uptake of native, minimally and moderately oxidized LDL by macrophages. We therefore conclude that macrophages are able to produce LPL in amounts sufficient to enhance the binding and uptake of native, mildly oxidized or moderately oxidized LDL by them. This implies that LPL synthesized locally by macrophages in atherosclerotic lesions has a role in the metabolism of native and oxidized LDL.

LDL was reported to become aggregated during oxidation, depending on the degree of oxidative modification [47]. Because we used the same Eu^{3+}-labelled native or oxidized LDL samples in one experiment, the contribution of aggregation to increased binding and cell association of oxidized LDL in the macrophages should have been similar in the presence and in the absence of endogenous or exogenous LPL. The cell binding and association of native, mildly oxidized or moderately oxidized LDL to macrophages, even in the presence of LPL, are still much less than those of heavily oxidized LDL, the latter being mediated by scavenger receptors. However, this does not obviate or weaken the significance of the bridging effect of LPL, because LDL in atherosclerotic lesions was found to be mildly or moderately oxidized [44], and such a strong degree of oxidation as that of LDL oxidized by copper for 24 h seems unlikely to occur *in vivo*.

Various structurally unrelated negatively charged substances competed for the binding of oxidized LDL to LPL (Figure 2), suggesting that negative charges in oxidized LDL or LPL, or both, were involved in the binding of oxidized LDL to LPL. Although more of the heavily oxidized LDL bound to LPL than less-oxidized LDL or native LDL, LPL did not increase the binding of the former to MPM to a significant degree. This is because heavily oxidized LDL bound with high affinity and in large amounts to the abundant scavenger receptors on MPM [21] and the LPL-mediated binding of heavily oxidized LDL to MPM was probably overwhelmed by this predominant binding to scavenger receptors. For native LDL, which does not bind to scavenger receptors, or minimally and moderately oxidized LDLs, which bind to scavenger receptors to a much lower degree than heavily oxidized LDL ([48], and X. Wang, J. Greilberger and G. Jürgens, unpublished work), LPLs still had a significant role in increasing their binding to MPM.

It was previously found that positively charged lysine and arginine residues in LDL were important for its binding to LPL [31]. Our results are in agreement with this finding, because positively charged polylysine and polyarginine were able to compete for the binding of LDL to LPL. In addition, we found that polyhistidine, another basic amino acid, was an even more potent competitor, suggesting that histidine is also important in the interaction between LPL and LDL. Our results demonstrate further that the interaction between oxidized LDL and LPL also involves the basic amino acids lysine, arginine and histidine, because the polymers of these amino acids were able to compete for the binding of oxidized LDL to LPL.

The finding that polyarginine and polylysine could compete for the binding of LDL to LPL has previously been explained as an involvement of arginine and lysine residues of LDL in the interaction between LDL and LPL [31]. However, the possibility that arginine and lysine residues on LPL are also involved in this interaction cannot be excluded. We found that the basic amino acids lysine, arginine and histidine could compete for the binding of native and oxidized LDL to LPL, which meant that these basic amino acids, either in LPL or in native and oxidized LDL, were important in the interaction between LPL and native or oxidized LDL. Because more LDL binds to LPL as the degree of oxidative modification of this lipoprotein increases, whereas basic amino acids such as lysine, arginine and histidine in LDL are modified and lose their positive charges during LDL oxidation [12,13,32], our findings suggest that basic amino residues in LPL, in addition to those in LDL, are involved in its binding to native and oxidized LDL.

We thank Alexandra Tieber, Gerd Kager and Gerhard Ledinski for technical assistance. This work was supported by the Austrian Science Fund, special research center 'Biomembranes', projects F00710 (G.J.), F00713 and P11696 (R. Zechner) and by the Jubiläumsfonds der Österreichischen Nationalbank, project 6941 (G.J.).

REFERENCES

1 Havel, R. J., Kane, J. P. and Kashyap, M. L. (1973) J. Clin. Invest. **52**, 32–38
2 Nilsson-Ehle, P. A., Garfinkel, A. S. and Schotz, M. C. (1980) Annu. Rev. Biochem. **49**, 667–693
3 Olivecrona, T. and Bengtsson-Olivecrona, G. (1990) Curr. Opin. Lipidol. **1**, 116–122
4 Zilversmit, D. B. (1973) Circ. Res. **33**, 633–638
5 Saxena, U., Klein, M. G. and Goldberg, I. J. (1991) J. Biol. Chem. **266**, 17516–17521
6 Sivaram, P., Choi, S. Y., Curtiss, L. K. and Goldberg, I. J. (1994) J. Biol. Chem. **269**, 9409–9412
7 Rutledge, J. C., Woo, M. M., Rezai, A. A., Durtiss, L. K. and Goldberg, I. J. (1997) Circ. Res. **80**, 819–828
8 Saxena, U., Klein, M. G., Vanni, T. M. and Goldberg, I. J. (1992) J. Clin. Invest. **89**, 373–380
9 Beisiegel, U., Weber, W. and Bengtsson-Olivecrona, G. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8342–8346
10 Rumsey, S. C., Obunike, J. C., Arad, Y., Deckelbaum, R. J. and Goldberg, I. J. (1992) J. Clin. Invest. **90**, 1504–1512
11 Mulder, M., Lombardi, P., Jansen, H., van Berkel, T. J. C., Frant, R. R. and Havekes, L. M. (1992) Biochem. Biophys. Res. Commun. **185**, 582–587
12 Jürgens, G., Hoff, H. F., Chisolm, G. M. and Esterbauer, H. (1987) Chem. Phys. Lipids **45**, 315–336
13 Steinbrecher, U. P. (1987) J. Biol. Chem. **262**, 3603–3608
14 Esterbauer, H., Gebicki, J., Puhl, H. and Jürgens, G. (1992) Free Radicals Biol. Med. **13**, 341–390
15 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) New Engl. J. Med. **320**, 915–924
16 Greilberger, J., Schmut, O. and Jürgens, G. (1997) Arterioscler. Thromb. Vasc. Biol. **17**, 2721–2728
17 Kaplan, M. and Aviram, M. (1997) Biochem. Biophys. Res. Commun. **237**, 271–276
18 Auerbach, B. J., Bisgaier, C. L., Wölle, J. and Saxena, U. (1996) J. Biol. Chem. **271**, 1329–1335
19 Hendriks, W. L., van der Boom, H., van Vark, L. C. and Havekes, L. M. (1996) Biochem. J. **314**, 563–568
20 Khouw, A. S., Parthasarathy, S. and Witztum, J. L. (1993) J. Lipid Res. **34**, 1483–1496
21 Chisolm, G. M. and Penn, M. S. (1996) in Atherosclerosis and Coronary Artery Disease (Fuster, V., Ross, R. and Topol, E. J., eds.), pp. 129–149, Lippincott-Raven Publishers, Philadelphia
22 Wang, X., Greilberger, J. and Jürgens, G. (1999) Anal. Biochem. **267**, 271–278
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
24 El-Saadani, M., Esterbauer, H., El-Sayed, M., Goher, M., Nassar, A. Y. and Jürgens, G. (1989) J. Lipid Res. **30**, 627–629
25 Levak-Frank, S., Weinstock, P. H., Hayek, T., Verdery, R., Hofmann, W., Ramakrishnan, R., Sattler, W., Breslow, J. L. and Zechner, R. (1997) J. Biol. Chem. **272**, 17182–17190
26 Weinstock, P. H., Bisgaier, C. L., Aalto-Setälä, K., Radner, H., Ramakrishnan, R., Levak-Frank, S., Essenburg, A. D., Zechner, R. and Breslow, J. L. (1995) J. Clin. Invest. **96**, 2555–2568
27 Saxena, U., Witte, L. D. and Goldberg, I. J. (1989) J. Biol. Chem. **264**, 4349–4355
28 Zechner, R. (1990) Biochim. Biophys. Acta **1044**, 20–25
29 Chen, Q., Esterbauer, H. and Jürgens, G. (1992) Biochem. J. **288**, 249–254
30 Jürgens, G., Lang, J. and Esterbauer, H. (1986) Biochim. Biophys. Acta **875**, 103–114
31 Saxena, U., Auerbach, B. J., Ferguson, E., Wölle, J., Marcel, A. L., Weisgraber, K. H., Hegele, R. A. and Bisgaier, C. L. (1995) Arterioscler. Thromb. Vasc. Biol. **15**, 1240–1247
32 Esterbauer, H., Jürgens, G., Quehenberger, O. and Koller, E. (1987) J. Lipid Res. **28**, 495–509
33 Mulder, M., Lombardi, P., Jansen, H., van Berkel, T. J. C., Frants, R. R. and Havekes, L. M. (1993) J. Biol. Chem. **268**, 9369–9375
34 Obunike, J. C., Edwards, I. J., Rumsey, S. C., Surtiss, L. K., Wagner, W. D., Deckelbaum, R. J. and Goldberg, I. J. (1994) J. Biol. Chem. **269**, 13129–13135
35 Khoo, J. C., Mahoney, E. M. and Witztum, J. L. (1981) J. Biol. Chem. **256**, 7105–7108
36 Chait, A., Iverius, P. H. and Brunzell, J. D. (1982) J. Clin. Invest. **69**, 490–493
37 Mahoney, E. M., Khoo, J. C. and Steinberg, D. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 1639–1642
38 Vance, J. E., Khoo, J. C. and Steinberg, D. (1982) Arteriosclerosis **2**, 390–395
39 DiCorleto, P. E. and Zilversmit, D. B. (1975) Proc. Soc. Exp. Biol. Med. **148**, 1101–1105
40 Henson, L. C. and Schotz, M. C. (1975) Biochim. Biophys. Acta **409**, 360–366
41 Ylä-Herttuala, S., Lipton, B. A., Rosenfeld, M. E., Goldberg, I. J., Steinberg, D. and Witztum, J. L. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10143–10147
42 O'Brien, K. D., Gordon, D., Deeb, S., Ferguson, M. and Chait, A. (1992) J. Clin. Invest. **89**, 1544–1550
43 Steinberg, D. (1997) J. Biol. Chem. **272**, 20963–20966
44 Ylä-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Sigal, E., Särkioja, T., Witztum, J. L. and Steinberg, D. (1991) J. Clin. Invest. **87**, 1146–1152
45 Hammer, A., Kager, G., Dohr, G., Rabl, H., Ghassempur, I. and Jürgens, G. (1995) Arterioscler. Thromb. Vasc. Biol. **15**, 704–713
46 Hendriks, W. L., van Vark, L. C., Schoonderwoerd, K., Jansen, H. and Havekes, L. M. (1998) Biochem. J. **330**, 765–776
47 Aviram, M. (1996) Eur. J. Clin. Chem. Clin. Biochem. **34**, 599–608
48 Lougheed, M. and Steinbrecher, U. P. (1996) J. Biol. Chem. **271**, 11798–11805

Received 7 April 1999/2 July 1999; accepted 6 August 1999