Cellular uptake of lipoprotein[a] by mouse embryonic fibroblasts via the LDL receptor and the LDL receptor-related protein.

Tjark Reblin, Andreas Niemeier, Nicolette Meyer, Thomas E. Willnow,* Florian Kronenberg,† Hans Dieplinger,† Heiner Greten, and Ulrike Beisiegel

Medical Clinic, University Hospital Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany; Max-Delbrück-Center for Molecular Medicine,* Berlin-Buch, Germany; and Institute for Medical Biology and Human Genetics,† University of Innsbruck, Innsbruck, Austria

Abstract The sites and precise mechanisms of the catabolism of the atherogenic lipoprotein[a] (Lp[a]) are unknown. It has been proposed that the low density lipoprotein receptor (LDL-R) and the low density lipoprotein receptor-related protein (LRP) are involved in the catabolism of Lp[a]. To address the question whether and to what extent the LDL-R and/or LRP are involved in the catabolism of Lp[a], we studied the cellular uptake of Lp[a] via those two receptors using mouse embryonic fibroblast (MEF) cell lines lacking either the LDL-R, the LRP, or both receptors due to disruption of the respective mouse genes. ^{125}I-labeled LDL and ^{125}I-labeled Lp[a] uptake by wild-type fibroblasts (MEF1) was compared with that by fibroblasts homozygous for the disrupted LRP allele (MEF2), fibroblasts with two defective alleles for the LDL-R (MEF3), and fibroblasts homozygous for defects both in the LDL-R and LRP gene (MEF4). Compared with MEF1, ^{125}I-labeled LDL uptake by MEF2 was 77%, by MEF3 30%, and by MEF4 24% of that by MEF1. However, no significant differences in the specific ^{125}I-labeled Lp[a] uptake by the four mouse embryonic cell lines was observed. In comparison with MEF1, the ^{125}I-labeled Lp[a] uptake by MEF2 was 98%, by MEF3 111%, and 73% by MEF4. Approximately 50% of the total cellular uptake of ^{125}I-labeled Lp[a] was nonspecific. In conclusion, our results suggest that Lp[a] is a poor ligand for the LDL receptor and the LRP. The data of the displacement studies, however, indicated that the nonspecific uptake of Lp[a] constitutes a major route for the cellular Lp[a] catabolism in this study.—**Reblin, T., A. Niemeier, N. Meyer, T.E. Willnow, F. Kronenberg, H. Dieplinger, H. Greten, and U. Beisiegel.** Cellular uptake of lipoprotein[a] by mouse embryonic fibroblasts via the LDL receptor and the LDL receptor-related protein. *J. Lipid Res.* 1997. **38:** 2103–2110.

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The metabolism of lipoprotein[a] (Lp[a]) is independent of other lipoproteins (1). The major source of circulating plasma Lp[a] is the human liver (2, 3). Lp[a] serum concentrations are determined by the Lp[a] production rate (4, 5) and correlate directly with hepatic mRNA abundance (6). Studies in primary cultures of baboon hepatocytes showed that the majority of apo[a] is secreted by liver cells into the medium in its free form (7, 8). Wilkinson, Munro and Higgins (9), showed that apo[a] in the human liver is not associated with apoB, suggesting that the association of apolipoprotein [a] (apo[a]) and apolipoprotein B-100 (apoB) occurs extracellularly after secretion (8).

Conflicting results exist about the catabolism of Lp[a]. Several studies showed that Lp[a] binds to the low density lipoprotein receptor (LDL-R) (10–14). Lp[a] levels are elevated in FH patients (15–18) and overexpression of LDL-R in transgenic mice leads to accelerated catabolism of Lp[a] (19). Others found no or neglectable clearance of Lp[a] via the LDL-R (20–23) and unchanged Lp[a] levels in FH patients (24). These results are in agreement with the observation that lipid-lowering drugs, which lower LDL due to inhibition of HMG-CoA reductase and up-regulation of the LDL-R have no effect on Lp[a] serum levels (25–27). Even if some cellular Lp[a] uptake occurs via the LDL-R, this route does not seem to be the major determinant of Lp[a] catabolism (28). According to Gries et al. (29) potential reasons for the poor binding of Lp[a] to the LDL-R include *a*) covering of domains near the bind-

Abbreviations; apo[a], apolipoprotein[a]; apoB, apolipoprotein B-100; apoE, apolipoprotein E; DMEM, Dulbecco's modified Eagle's medium; gp330/megalin, Heymann Nephritis Autoantigen; LDL, low density lipoprotein; LDL-R, LDL receptor; Lp[a], lipoprotein[a]; LpL, lipoprotein lipase; LRP, LDL receptor-related protein; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; RAP, receptor-associated protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electro-phoresis.

[1]To whom correspondence should be addressed.

ing region of apoB by apo[a], *b*) steric hindrance in the interaction of Lp[a] with the LDL-R caused by apo[a], *or c) conformational changes in the binding region of* apoB by apo[a]. The interaction between of apo[a] and apoB may differ among the various apo[a] isoforms. This potential variability is usually not taken into account but might contribute to the conflicting results of the different studies. Alternatively, Lp[a] might be taken up via the low density lipoprotein receptor-related protein (LRP) (30) or, associated with LDL, in a "hitchhiking" process (31). This catabolic pathway is supported by recent data of Tam, Zhang, and Koschinsky (32) showing that recombinant apo[a], complexed with LDL is taken up by human fibroblasts and HepG2 cells via the LDL-R. Several other mechanisms which could play a role in the catabolism of Lp[a], have been proposed, some of which are independent of specific cell surface receptors. Williams et al. (33) showed an increased Lp[a] cell association and degradation by lipoprotein lipase (LpL) which promotes binding of Lp[a] to cell surface proteoglycans possibly by virtue of its structural, non-enzymatic properties. Several studies have demonstrated that Lp[a], due to the homology, of apo[a] with plasminogen (34), binds to plasminogen receptors on monocytoid cells, endothelial cells, human fibroblasts, and HepG2 cells (32, 35, 36).

Previous studies on the binding of Lp[a] to cell surface receptors were performed with human or monkey fibroblasts (10–12, 14, 19–21, 30), human macrophages (13, 22, 37, 38), primary human hepatocytes (38), or hepatoma cell lines (31, 32). All these cells express a multitude of receptors involved in the binding of lipoproteins. To specifically address the question whether and to what extent the LDL-R and/or LRP are involved in the cellular uptake of Lp[a], the present study was designed to investigate the uptake of Lp[a] via those two receptors into mouse embryonic fibroblast (MEF) lines genetically deficient of either the LDL-R, the LRP, or both receptors due to targeted gene disruption (39, 40; J. Hilpert, T. E. Willnow, S. Jonat, J. Herz, and U. Beisiegel, unpublished results).

MATERIAL AND METHODS

Animal breeding

Mouse embryonic fibroblasts (MEF) were all derived from C57Bl/6 × 129 strain of mice. Wild-type fibroblasts (MEF1), fibroblasts homozygous for the disrupted LRP-allele (MEF2), fibroblasts homozygous for the disrupted LDL-R allele (MEF3), and fibroblasts carrying two defective alleles for both LDL-R and LRP were prepared as described before (39, 40).

Isolation of primary fibroblasts

Isolation of the different fibroblasts was performed as described (41). Mouse embryos were taken out of the uterus by cesarean section. After dissection and incubation in 0.05% (v/v) trypsin solution, the softened tissue was disrupted by repeated pipetting. After separation of cell debris, the supernatant was plated on Costar 6-well plates. Fibroblasts were then expanded and transfected with 0.5 μg per ml of a plasmid containing the large T-antigen of the Simian Virus-40 using Transfectam (Promega, Madison, WI).

Lipid and protein quantifications

Triglycerides and cholesterol were determined enzymatically with colorimetric assays from Boehringer Mannheim. Lp[a] was measured by ELISA. LDL was determined as described (42). Protein concentration of cell protein and lipoproteins was determined according to Lowry et al. (43).

Lipoprotein isolation

Lp[a] and LDL were obtained from 480 ml fresh EDTA-plasma of a healthy individual undergoing plasmapheresis. The Lp[a] serum level was 60 mg/dl. The apo[a] isoform was determined as homozygous 20/20, implicating an apo[a] isoform consisting of 20 kringle 4 domains (44). The molecular weight of this isoform is approximately 560 kD. The apo[a] isoform 20/20 belongs to the low molecular weight isoform goups which are present in approximately 15% of individuals (44). PMSF, EDTA, BHT, merthiolate, and N3 were added to the fresh plasma to final concentrations of 37 mg/l, 0.5 mM, 0.1 mM, 0.1 mg/l, and 0.2 mg/l, repectively. Plasma was then adjusted to a density of 1.063 g/ml with KBr followed by centrifugation for 48 h at 49,000 rpm and 10°C using a 50.2 Ti Beckman rotor in a L8 Beckman ultracentrifuge. The floating material, containing VLDL and LDL, was dialyzed against PBS and EDTA. Subsequently, VLDL and LDL were separated by ultracentifugation at 49.000 rpm for 24 h at 10°C in the 50.2 Ti Beckman rotor. The orange band in the intermediate fraction after the first run containing Lp[a] was recovered by tube-slicing, adjusted to a density of 1.1 g/ml with KBr, and centrifuged for 24 h at 49.000 rpm and 10°C. The top fraction containing Lp[a] was then recovered and underlayered in a stepwise gradient consisting of 2 ml H_2O and KBr solutions (3 ml d 1.019 g/ml, 3 ml d 1.063 g/ml). The gradient was centrifuged for 24 h at 40,000 rpm and 10°C using a SW 41 Beckman rotor. The Lp[a]-containing band as analyzed by ELISA, was recovered and the protein concentration was determined according to Lowry et al. (43). The LDL and Lp[a] preparations were checked for purity

and integrity by sodium dodecyl sulfate (SDS) gel electrophoresis on 5% acylamide gels as described below and shown in Fig. 1. Lp[a] was not contaminated with LDL and purified LDL did not contain Lp[a] contamination.

Iodination

LDL and Lp[a] were radiolabeled with ^{125}Iodine by the ICI method (45). The specific activity of the different Lp[a] preparations varied between 35.1 cpm/ng protein and 303 cpm/ng protein. The specific activity of the different LDL preparations varied between 88.2 cpm/ng protein and 126 cpm/ng protein. The integrity and the purity of the radiolabeled LDL and Lp[a] was determined by 5% SDS gel electrophoresis as shown in Fig. 1.

Binding and uptake experiments

The cellular uptake experiments of Lp[a] and LDL were essentially performed as described (46, 47). Cells were grown in 24-well plates with 40,000 cells per well resulting in subconfluent monolayers after 48 h of incubation. Studies were performed with 2.5, 10.0, 15.0, and 20.0 pmol of ^{125}I-labeled Lp[a] or 10.0 pmol and 10.0, 20.0, 30.0 nmol of ^{125}I-labeled LDL in 1 ml of Dulbecco's modified Eagle's medium (DMEM, without glutamine; Gibco) containing 5% BSA (fraction V; Sigma), and 0.02 M HEPES (pH 7.4; Gibco). The cells were incubated for 90 min at 37°C. To determine the bound fraction, cells were washed with phosphate-buffered saline (PBS, pH 7.4), PBS with 2 mg/ml BSA, and finally PBS without BSA. Then the bound particles were released by 770 IU/ml of heparin in PBS. Subsequently, cells were solubilized in 0.1 M NaOH for determination of cell-associated radioactivity and cell protein content. Nonspecific uptake was determined in the presence of a 50-fold molar excess of the unlabeled ligand at all different concentrations for both LDL and Lp[a]. Specific uptake was calculated by subtracting nonspecific uptake values from the cell-associated radioactivity after the heparin wash.

SDS-PAGE and immunoblotting

Lp[a] was reduced as described (48). The reduced samples (20 µg protein/lane or 80,000 cpm/lane for ^{125}I-labeled Lp[a]) were applied to 5% polyacrylamide gel electrophoresis (SDS-PAGE) according to Neville (49). Immunoblotting was carried out according to Beisiegel (50) using the monoclonal anti-apo[a]-antibody 8D3 which has been described elsewhere (51).

Statistical procedures

Mean values and standard deviations were calculated using conventional statistical procedures. Comparison of non-paired data was performed using Mann-Whitney's U-test.

RESULTS

Ligand characterization

LDL preparations were free of relevant amounts of Lp[a] or other lipoproteins as shown in **Fig. 1.** The Lp[a] preparations, displaying the apo[a] isoform 20/20, were also checked for purity and integrity by sodium dodecyl sulfate (SDS) gel electrophoresis on 5% polyacrylamide gels. No other apolipoproteins were detected in the Lp[a] preparations as shown in Fig. 1.

Uptake of ^{125}I-labeled LDL by mouse embryonic fibroblasts

To confirm that the LDL-R and LRP were functionally intact in our cell system and thus able to clear apoB-containing lipoproteins, the ^{125}I-labeled LDL uptake by wild-type fibroblasts (MEF1) expressing both receptors was compared with fibroblasts homozygous for the disrupted LRP allele (MEF2), fibroblasts with two defective alleles for the LDL-R (MEF3), and fibroblasts homozygous for defects both in the LDL-R and LRP (MEF4). The cells were incubated with ^{125}I-labeled LDL (10.0 pmol/ml and 10.0, 20.0, 30.0 nmol/ml). Nonspecific uptake was determined in the presence of a 50-fold molar excess of unlabeled LDL at all different concentrations. The specific uptake was calculated by subtracting nonspecific uptake values from the cell-associated radioactivity after the heparin wash. One representative uptake experiment for ^{125}I-labeled LDL at 10 pmol/ml is shown in **Fig. 2.** As expected, uptake was highest in MEF-1 and MEF2 cells, both expressing LDL-R, whereas much lower values were found for MEF3 and MEF4.

Uptake of ^{125}I-labeled Lp[a] by mouse embryonic fibroblasts

Possible interactions of ^{125}I-labeled Lp[a] with the LDL-R and/or the LRP were studied using the different mouse embryonic fibroblast cell lines. The cells were incubated with increasing concentrations of ^{125}I-labeled Lp[a] (2,5 pmol/ml–20 pmol/ml) for 90 min at 37°C. Saturation of the specific ^{125}I-labeled Lp[a] uptake was reached at 15 pmol ^{125}I-labeled Lp[a]/ml for MEF1–3. Therefore the focus of this study was on 10 pmol/ml. No saturation could be reached for the specific uptake of ^{125}I-labeled Lp[a] by MEF4 using concentrations of the radiolabeled ligand within the range of 2,5 and 20 pmol/ml (**Fig. 3**).

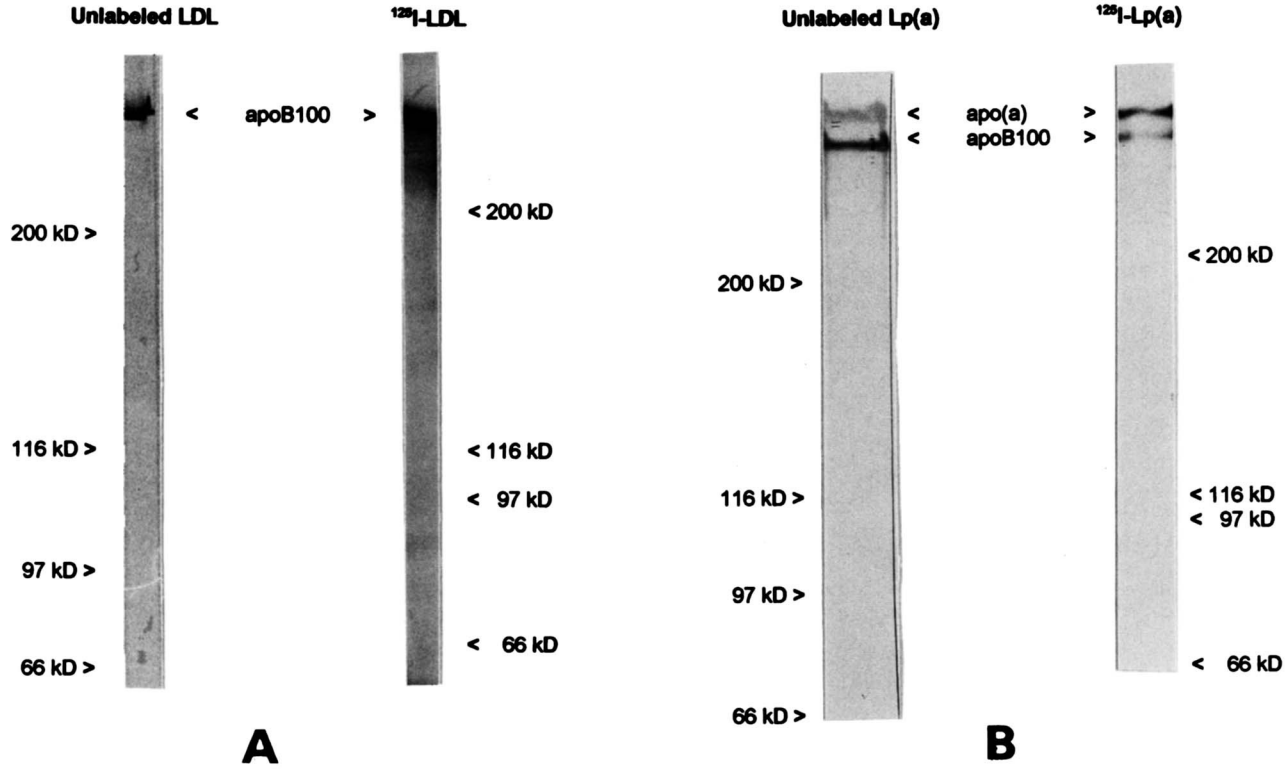

Fig. 1. 5% SDS-PAGE of unlabeled LDL and ^{125}I-labeled LDL and unlabeled Lp[a] and ^{125}I-labeled Lp[a]. A. For the unlabeled LDL sample (left), 20 μg protein was loaded per lane. For the ^{125}I-labeled LDL, 80,000 cpm was applied per lane. B: For the right side of the figure, Lp[a] containing the apo[a] isoform 20/20 was reduced using mercaptoethanol. Twenty μg protein and 80,000 cpm were loaded per lane. For the unlabeled ligands, after transfer of the proteins to nitrocellulose, proteins were stained with Ponceau. For the radiolabeled ligands, the dried gels were exposed to a Dupont Cronex 10s film for 12h at −80°C.

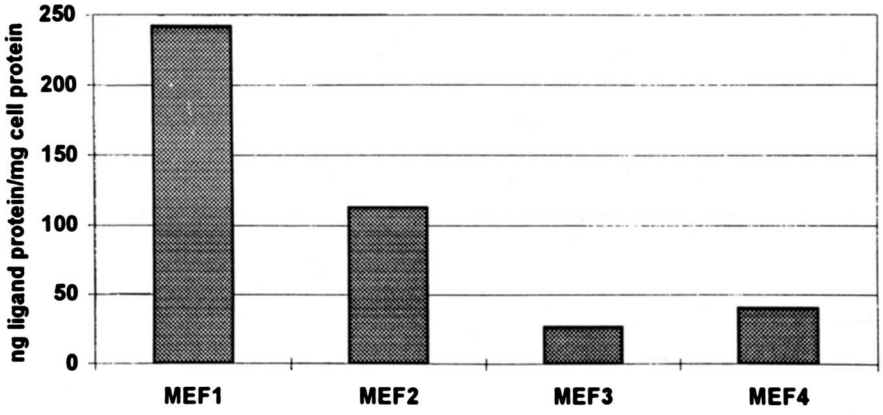

Fig. 2. Uptake of ^{125}I-labeled LDL by MEF1–4 at 10 pmol ligand/ml. One representative experiment (out of 11) studying the cellular uptake of ^{125}I-labeled LDL at 10 pmol/ml is shown. Cells were grown in 24-well plates with 40,000 cells per well resulting in subconfluent monolayers after 48 h of incubation. The cells were incubated for 90 min at 3°C. Cell surface bound particles were released by 770 lU/ml of heparin in PBS. Subsequently, cells were solubilized in 0.1 M NaOH for determination of cell-associated radioactivity and cell protein content. Nonspecific uptake was determined in the presence of a 50-fold molar excess of unlabeled LDL. Specific uptake is expressed as ng ligand protein/mg cell protein.

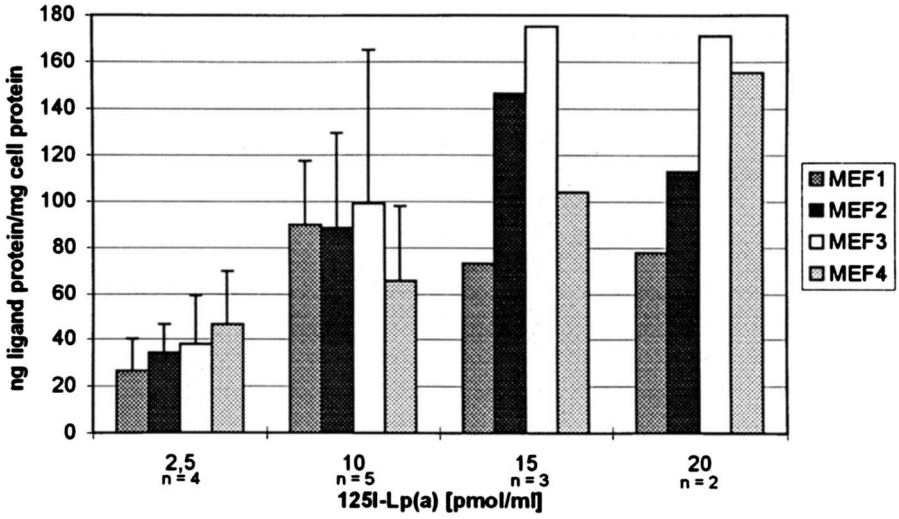

Fig. 3. Saturation of specific uptake of ^{125}I-labeled Lp[a] by MEF1–4. Specific uptake of ^{125}I-labeled Lp[a] was measured at 2.5 pmol/ml (n = 4), 10 pmol/ml (n = 5), 15 pmol/ml (n = 3), and at 20 pmol/ml (n = 2). Experiments were performed as described in the legend to Fig. 2. Values for specific uptake are given as ng ligand protein/mg cell protein. Nonspecific uptake was determined in the presence of a 50-fold molar excess of unlabeled Lp[a]. Due to the small number of studies at 15 and 20 pmol/ml, no standard deviations are shown for these concentrations.

No statistically significant differences among the specific ^{125}I-labeled Lp[a] uptake by the four mouse embryonic cell lines was observed. At 10 pmol/ml the uptake of ^{125}I-labeled Lp[a] by MEF1 reached 90 ± 28 ng ligand protein/mg cell protein, by MEF2 88 ± 41, by MEF3 100 ± 66, and by MEF4 66 ± 32. Although the uptake by MEF4 was slightly lower than the uptake by the other fibroblast cell lines, this difference was not statistically significant. No significant differences between MEF4 and the other cell lines were observed at 2.5, 15.0, and 20.0 pmol/ml.

Uptake of LDL and Lp[a] by MEF2–4 relative to MEF1

Taking together the results of all 11 ^{125}I-labeled LDL uptake assay we found a significantly lower uptake by MEF2–4 as compared with ^{125}I-labeled LDL uptake by MEF1. Uptake by MEF2 was 77% ($P = 0.011$), by MEF3 30% ($P < 0.0001$), and by MEF4 24% ($P < 0.0001$) of that by MEF1. To further explore possible differences in the cellular uptake of ^{125}I-labeled Lp[a] mediated by either the LDL-R or LRP, the uptake by MEF2–4 at 10 pmol/ml was also compared relative to MEF1, which express both the LDL-R and LRP. ^{125}I-labeled Lp[a] uptake by MEF2–4 was equal to or lower than that by MEF1 (MEF2: 98%, MEF3: 111%, MEF4: 73%) but no significant differences were observed between the cell lines.

As the LDL-R was proposed to clear Lp[a] from the circulation we compared the uptake of ^{125}I-labeled LDL and ^{125}I-labeled Lp[a] between MEF1 and MEF3 which lack the LDL-R but express LRP. As expected ^{125}I-labeled LDL uptake by MEF3 was significantly lower relative to MEF1 (30% ± 20% of uptake by MEF1). No significant difference between the two cell lines was observed for ^{125}I-labeled Lp[a]. The uptake of ^{125}I-labeled Lp[a] by MEF3 was only slightly higher relative to MEF1 (111% ± 39% of uptake by MEF1).

Specific LDL and Lp[a] uptake of total cellular uptake

We compared the fraction of specific ^{125}I-labeled LDL and ^{125}I-labeled Lp[a] uptake as percentage of total uptake among the different fibroblast lines. Marked differences between LDL and Lp[a] were observed in the percentage of specific uptake by the different cell lines in the presence of a 50-fold molar excess of the unlabeled ligand (**Table 1**). Whereas specific ^{125}I-labeled LDL up-

TABLE 1. Specific cellular uptake of ^{125}I-labeled LDL and ^{125}I-labeled Lp[a]

	MEF 1	MEF 2	MEF 3	MEF 4
^{125}I-LDL	71 ± 11	62 ± 10	37 ± 13	41 ± 14
^{125}I-Lp[a]	46 ± 19	50 ± 15	50 ± 28	49 ± 18

Experiments were performed as described in the legend to Fig. 2. The results of specific uptake are expressed as % of total uptake. The data represent the mean values of 11 independent experiments for ^{125}I-labeled LDL (uptake at 10.0 pmol/ml, 10.0, 20.0, and 30.0 nmol/ml) and for 14 independent experiments for ^{125}I-labeled Lp[a] (uptake at 2.5, 10.0, 15.0, and 20.0 pmol/ml).

take was highest by the MEF1 (71%) and MEF2 (62%) cells, both expressing the LDL-R, the percentage of specific uptake by MEF3 (37%) and MEF4 (41%) was much lower. With respect to ^{125}I-labeled Lp[a], no significant differences in the specific cellular uptake were observed among the four different mouse embryonic fibroblast cell lines. At 10 pmol ^{125}I-labeled Lp[a]/ml, the total uptake of ^{125}I-labeled Lp[a] to the mouse fibroblasts was inhibited to a residual value of 54% for MEF1, 55% for MEF2, 48% for MEF3, and 57% for MEF4. At 2, 5, 15, and 20 pmol/ml, inhibition was also approximately 50% for all cell lines.

DISCUSSION

The current study was initiated to investigate the potential role of the LDL receptor and the LDL receptor-related protein in the cellular uptake of Lp[a]. To address the question to what extent the LDL-R and/or the LRP are involved in the cellular uptake of Lp[a], mouse embryonic fibroblast (MEF) cell lines lacking either the LRP (MEF2), the LDL-R (MEF3), or both receptors (MEF4) due to targeted gene disruption (39, 40) were compared with wild-type fibroblasts expressing both receptors (MEF1). Although Lp[a] undergoes temperature-induced conformational changes that allow better recognition of Lp[a] by the LDL-R at 4°C (22), the uptake was studied at the physiologic temperature of 37°C. As demonstrated by SDS-PAGE, LDL-R and LRP were structurally intact. The uptake experiments with ^{125}I-labeled LDL showed that the LDL-R was also functionally intact.

Taken together, the results of the cellular uptake assays are in good agreement with several previous reports that have come to the conclusion that Lp[a] is catabolized via the LDL-R only to a minor extent. Some groups demonstrated that Lp[a] enters fibroblasts either independently of the LDL-R (10) or that the LDL-R plays only a minor role in the clearance of Lp[a] from the plasma (28, 38). Compared with LDL, the maximal LDL-R-mediated uptake of Lp[a] by fibroblasts was shown to be as low as 20–30% (28, 38). In a recent study, the LDL-R-mediated cellular binding of Lp[a] to human hepatocytes, macrophages, and fibroblasts did not account for more than 10%, 29%, and 29%, respectively of the values obtained with LDL. The LDL-R-mediated degradation of Lp[a] in human hepatocytes, macrophages, and fibroblasts was similar: 17%, 22% and 26% of LDL degradation, respectively (38). In general, diverging results about the interaction of Lp[a] with the LDL-R may be due to differences in apo[a] isoform size. Differences in the apo[a] protein could explain variations in the covering of domains near the binding region of apoB by apo[a], thus causing steric hindrance in the interaction of Lp[a] with the LDL-R (29). Other factors, such as LpL, have been shown to play a role in variations of the affinity of Lp[a] for the LDL-R (33). LpL possibly mediates conformational changes in the Lp[a] molecule, thereby exposing previously hidden receptor-binding domains on the apo[a]–apoB complex (33). LDL could facilitate cellular Lp[a] uptake as proposed by Kostner (31) in a "hitchhiking" process. This hypothesis is supported by the fact that no uptake of recombinant apo[a] via the LDL-R occurred in the absence of LDL (32). Our present in vitro study indicates that Lp[a] is no or a very poor ligand for the LDL-R, a finding mutually supportive with in vivo turnover studies in FH patients showing that the LDL-R is not required for the normal catabolism of Lp[a] in humans (23).

To date, only few data exist about the possible interaction of Lp[a] with the LRP. In our study we found only a minor cellular uptake of Lp[a] mediated by LRP. A potential interaction between LRP and Lp[a] may also depend on the apo[a]-isoform size, as has been proposed for the interaction of Lp[a] with the LDL-R. This aspect could explain the apparent contradiction between our study and data showing the cellular internalization of Lp[a] with a high molecular weight apo[a] via the LRP (30). A further factor which could possibly influence the uptake of Lp[a] via the LRP is apolipoprotein E (apoE). The interaction between some species of Lp[a] and LRP could, in part, be mediated by apoE which is present in approximately 20% of Lp[a] particles (52). Except for the one above-mentioned study (32) no other publication that addressed the interaction of Lp[a] and LRP found a significant uptake of Lp[a] or apo[a] by LRP.

Other possible receptors responsible for the specific uptake of Lp[a] are the plasminogen receptor (32, 35) and the scavenger receptor (22, 37, 53, 54). However, the specific fraction of total Lp[a] uptake by mouse embryonic fibroblast cell lines was only 50% in our study, indicating that the unspecific cellular uptake of Lp[a] plays a major role in the clearance of this lipoprotein under in vitro conditions. These findings support studies by Snyder and coworkers (38) who reported a fraction of nonspecific degradation of ^{125}I-labeled Lp[a] of 76% in primary human hepatocytes, 58% in human macrophages, and 33% in human fibroblasts.

In conclusion, our results demonstrate that Lp[a] is no or only a poor ligand for the LDL receptor and the LRP. This has been shown by comparing the uptake of ^{125}I-labeled Lp[a] by wild-type mouse embryonic fibroblasts with three different mouse embryonic fibroblast cell lines lacking either the LDL-R, the LRP, or both receptors due to targeted gene disruption of the respective mouse genes. Whether other cell surface receptors

play a more important role in the cellular catabolism of Lp[a], remains a subject of further investigations.

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