Oxidized or Acetylated Low Density Lipoproteins are Rapidly Cleared by the Liver in Mice with Disruption of the Scavenger Receptor Class A Type I/II Gene

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Abstract

Oxidized low density lipoprotein (LDL) and acetyl LDL are recognized by the scavenger receptor class A type I/II (SR-AI/II) on macrophages and liver endothelial cells. Several investigators have suggested that there are additional receptors specific for oxidized LDL, but characterization of these alternate receptors for oxidized LDL and evaluation of their quantitative importance in uptake of oxidized LDL has been difficult because of overlapping ligand specificity with SR-AI/II. The purpose of this study was to determine the importance of SR-AI/II in the removal of modified LDL from the bloodstream in vivo. The clearance rate of oxidized LDL from plasma in normal mice was very rapid, and $>$ 90% of injected dose was removed from the blood within 5 min. Clearance rates of oxidized LDL were equally high in SR-AI/II knockout mice, indicating that this receptor is not required for removal of oxidized LDL from plasma. Surprisingly, there was no difference in the clearance rate of acetyl LDL in wild-type and SR-AI/II knockout animals. The plasma clearance of radioiodinated acetyl LDL was almost fully blocked by a 50-fold excess of unlabeled acetyl LDL, but the latter only inhibited oxidized LDL clearance by $\sim$ 5%. Both modified LDLs were cleared mostly by the liver, and there was no difference in the tissue distribution of modified LDL in control and knockout mice. Studies in isolated nonparenchymal liver cells showed that Kupffer cells accounted for most of the uptake of oxidized LDL. Extensively oxidized LDL and LDL modified by exposure to fatty acid peroxidation products were efficient competitors for the uptake of labeled oxidized LDL by SR-AI/II–deficient Kupffer cells, while acetyl LDL and malondialdehyde-modified LDL were relatively poor competitors. (*J. Clin. Invest.* 1997. 100:244–252.) Key words: scavenger receptor • oxidized low density lipoprotein • atherosclerosis • macrophages • Kupffer cells

Introduction

Oxidized or chemically modified low density lipoproteins (LDL) can promote cholesterol accumulation in cultured macrophages, and several lines of evidence indicate that oxidized LDL plays a role in the pathogenesis of atherosclerosis. Previous studies have shown that chemically modified (acetylated or acetoacetylated) LDL is removed rapidly from the circulation by nonparenchymal liver cells (1, 2). Fractionation of rat nonparenchymal liver cells after intravenous injection of radiolabeled acetyl LDL showed that most of the modified LDL was associated with endothelial cells, and a high-affinity uptake pathway for acetyl LDL was demonstrated in these cells in vitro (3). Pitas et al. (4) used fluorescence microscopy with indocarbocyanine-labeled acetyl LDL as well as autoradiography with radiolabeled acetyl LDL to confirm the preferential uptake of acetyl LDL by liver sinusoidal endothelial cells in rats, guinea pigs, and dogs. This uptake was thought to be mediated by the class A scavenger receptor type I/II (SR-AI/II),[1] which is a well-characterized trimeric integral membrane glycoprotein that binds a variety of negatively charged ligands, including acetylated or oxidized LDL, dextran sulfate, and polyinosinic acid (5–14). Studies with intravenously injected oxidized LDL revealed a similar, rapid uptake of oxidized LDL from the circulation by the liver (15, 16). It was reported initially that oxidized LDL was also taken up by liver endothelial cells (15), but subsequent studies showed that this extensively oxidized LDL was actually taken up by Kupffer cells (17). Studies of isolated human Kupffer cells demonstrated nonreciprocal cross-competition between acetyl LDL and oxidized LDL for degradation, and were interpreted as evidence for a specific receptor for oxidized LDL on these cells (18). It has been shown, however, that nonreciprocal cross-competition between modified lipoproteins can be explained sometimes by different binding sites on the same receptor, or by variation in the extent of oxidation or aggregation of oxidized LDL (10, 19).

Previous studies have identified several macrophage or Kupffer cell membrane proteins that can bind oxidized LDL, including the FcγRII-B2 receptor (20), human monocyte antigen CD36 (21), a CD36 homologue termed scavenger receptor BI (SR-BI) (22), and a 94–97-kD protein that has been identified recently as macrosialin, a murine lysosomal membrane protein homologous to human CD68 (23–25). The role that these candidate receptors play in oxidized LDL uptake remains unclear. Because of overlapping ligand specificities with

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1. *Abbreviations used in this paper:* SR-AI/II, class A scavenger receptor type I/II; SR-BI, scavenger receptor BI.

the SR-AI/II, it is often difficult to assess the relative contribution of such additional receptors to the uptake of modified lipoproteins. We found recently that macrophages from SR-AI/II knockout mice expressed a high-affinity receptor for oxidized LDL that accounted for ~ 70% of the oxidized LDL uptake in control animals (26). Uptake of acetyl LDL in SR-AI/II–deficient macrophages was reduced by > 80%, confirming the importance of this receptor in the uptake of acetyl LDL by macrophages. In the present studies, we determined the clearance rates and sites of uptake of intravenously injected acetyl LDL and oxidized LDL in mice in which the SR-AI/II gene had been inactivated, and compared the findings to those in wild-type mice. The clearance of oxidized LDL from the circulation was very rapid, and apparently was not altered by inactivation of the SR-AI/II. Surprisingly, we also found no difference in the plasma clearance of acetyl LDL. Analysis of the cell association and degradation of oxidized LDL and acetyl LDL by Kupffer cells and endothelial liver cells from SR-AI/II–deficient mice in comparison with those from wild-type mice showed a decrease in the rate of acetyl LDL uptake by endothelial cells from knockout animals. It appeared, however, that the uptake of acetyl LDL by alternate pathways in vivo was sufficient to compensate fully for the deficiency of SR-AI/II.

Methods

Carrier-free $Na^{125}I$ was purchased from Mandel Scientific (Guelph, Ontario). DME, bovine serum fibronectin, Hanks' balanced salt solution, and gentamicin were from Canadian Life Technologies (Burlington, Ontario). Defined fetal bovine serum was supplied by Professional Diagnostic Inc. (Edmonton, Alberta). Formaldehyde and paraformaldehyde were from J.B. EM Services (Dorval, Quebec). Collagenase (CLS 1) was obtained from Worthington Biochemical Corp. (Freehold, NJ). Endothelial cell growth supplement was from Collaborative Biomedical Products (Bedford, MA). Accudenz was purchased from Accurate Chemical and Science Corp. (Westbury, NY). Other chemicals were purchased from Fisher Scientific (Vancouver, British Columbia) or VWR Canlab (Edmonton, Alberta).

Animals. Inactivation of the scavenger receptor gene was performed in the laboratory of Dr. T. Kodama (University of Tokyo), using homologous recombination of a targeted construct spanning exon 4 of the type I/type II *MSR* gene in A3-1 embryonic stem cells. The description of the construct and the phenotypic expression in homozygous knockout mice have been reported elsewhere (27). There is a single copy of the *SR-AI/II* gene (14), and no SR-AI/II protein is detectable by immunostaining of the liver in homozygous knockout animals (27). SR-AI/II genotypes were verified by Southern blot analysis of tail DNA in all breeding animals, as well as in randomly selected experimental animals.

Lipoprotein isolation and labeling. LDL (d = 1.019–1.063) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma obtained from healthy normolipidemic volunteers (28). Radioiodination was performed using a modification of the iodine monochloride method of MacFarlane (29), using conditions that gave specific radioactivities of 100–150 cpm/ng. Iodination was performed before oxidation or acetylation of LDL.

Lipoprotein modification. The concentration of EDTA in LDL preparations was reduced before oxidation by dialysis against Dulbecco's PBS containing 10 μM EDTA. Standard conditions for LDL oxidation were: 200 μg/ml LDL in Dulbecco's PBS containing 5 μM $CuSO_4$ incubated at 37°C for 20 h (30). This oxidation typically resulted in a fourfold increase in electrophoretic mobility relative to native LDL. Very extensive oxidation was achieved by prolonging the incubation to 30 h, while mildly oxidized LDL was obtained by incubating under similar conditions for 4 h. Oxidation was arrested by addition of 100 μM EDTA and 40 μM butylated hydroxytoluene, and the oxidized LDL was concentrated to 1–2 mg/ml using CF-100 ultrafiltration cones (Amicon Corp., Oakville, Ontario). Acetylation, malondialdehyde modification, or modification of LDL by fatty acid oxidation products was performed as described previously (31). Acetylation or malondialdehyde modification resulted in derivatization of > 75% of free amino groups as evaluated using the trinitrobenzenesulfonic acid assay (30).

In vivo plasma clearance of LDL. Wild-type or SR-AI/II–deficient mice were anesthetized with 2% halothane. A 0.61-mm outer diameter polyethylene catheter (PE 10; VWR Canlab) was introduced into the superior vena cava via the internal jugular vein using standard microsurgical techniques and a Zeiss operating microscope (Carl Zeiss Canada Ltd., Don Mills, Ontario). Unless indicated otherwise in the figure legend, 20 μg protein (representing between 0.5 and 1 μCi) of each of two different modified LDLs, one labeled with ^{125}I and the other with ^{131}I, was pipetted into a 1-ml plastic syringe fitted with a 30-gauge needle, and mixed with 200 μl of 150 mM NaCl. The syringe was counted before and after the injection of labeled LDL to allow precise determination of the amount of radioactivity administered. After injection of LDL, the catheter was flushed three times with saline. Control studies showed negligible binding to or desorption of label from the catheter after these rinses. Seven or eight sequential 40–50 μl blood samples were withdrawn over ~ 20 min, and an aliquot of exactly 25 μl of each was dispensed into a calibrated capillary tube. When > 98% of the radioactivity from the first injection of modified LDLs had been cleared, a second injection was given of a different type or quantity of modified LDL together with native LDL, blood samples were obtained as above, and whole blood radioactivity was measured (1282 gamma counter operated in dual-isotope mode; LKB, Uppsala, Sweden). In many studies, > 60% of the radioactivity from modified LDL was cleared by the time the first time point was obtained (typically 2 min after injection), and hence it was not possible to estimate accurately the radioactivity at time 0 for modified LDLs by extrapolation of the respective plasma decay curves. The amount of each LDL injected, however, was known, and the volume of distribution was obtained from extrapolation of the decay curve of native LDL, which permitted calculation of the initial concentration of radioactivity for each modified LDL.

Isolation and culture of nonparenchymal liver cells. Mice were anesthetized by inhalation of 2% halothane. The portal vein was cannulated with a 0.61-mm diameter polyethylene catheter, and the liver was perfused at a flow of 2 ml/min with Hanks' buffer for 10 min, and then with Hanks' buffer containing 0.05% collagenase and 1 mM $CaCl_2$ for 20 min at 37°C. The liver was minced, suspended in ice-cold DME medium, and filtered through a 90-μm stainless steel mesh. Hepatocytes were removed from the filtrate by three sequential centrifugations at 50 *g* for 15 s. The supernatant was then centrifuged at 400 *g* for 10 min, and the pellet (containing mainly nonparenchymal cells) was resuspended in 4 ml of PBS, mixed with 5.6 ml of 30% Accudenz, and overlaid with 0.8 ml of PBS in a 15-ml centrifuge tube. After centrifugation for 15 min at 1,400 *g*, the cells at the interphase were collected, washed twice with PBS, and cultured for 30 min at 37°C in DME medium containing 10% FBS. Each dish was then washed several times with DME medium to remove loosely adherent sinusoidal endothelial cells. The endothelial cells were resuspended in medium 199 containing 20% FBS and 50 μg/ml endothelial cell growth supplement, and were cultured at 37°C on fibronectin-coated dishes. Confluent first-passage monolayers were used in experiments. Purity of endothelial cell preparations was verified by electron microscopy. The adherent Kupffer cells were cultured in DME medium with 10% FBS for 16 h at 37°C, washed twice with DME medium, and used for experiments. Purity of the Kupffer cell preparations was monitored by electron microscopy, and by staining freshly prepared cells for peroxidase activity. Cells grown on coverslips were fixed with 2% glutaraldehyde for 5 min, and were then incubated with 3,3-diaminobenzidine tetrahydrochloride for 2 h at 37°C.

Assays of LDL uptake and degradation. Kupffer cells or liver endothelial cells were incubated in serum-free medium containing ^{125}I-labeled lipoprotein with or without varying concentrations of unlabeled lipoprotein. After 5 h of incubation at 37°C, media were removed and assayed for trichloroacetic acid–soluble noniodide degradation products (32). Cells were then washed three times with Dulbecco's PBS, dissolved in 0.1 N NaOH, scraped from the plates, and assayed for radioactivity and protein content.

Analytic procedures. Protein determination was done by the method of Lowry (33) in the presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard. Lipoprotein electrophoresis was done (Corning apparatus and Universal agarose film; Corning Medical and Scientific, Medfield, MA) in 50 mM barbital buffer (pH 8.6). Bovine albumin was added to dilute lipoprotein samples to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with fat red.

Results

We found recently that acetyl LDL uptake is reduced by at least 80% in peritoneal macrophages from SR-AI/II–deficient mice, whereas the uptake of oxidized LDL was reduced by only 30% (26). This result was interpreted as evidence that acetyl LDL is taken up via SR-AI/II in these cells, but that oxidized LDL uptake is internalized mostly through alternate pathway(s). If these findings in cultured macrophages are representative of overall uptake mechanisms in vivo, one would expect that there would be little change in the clearance rate of oxidized LDL from plasma in SR-AI/II–deficient (knockout) animals, but that the clearance of acetyl LDL in these animals would be slow. To test this hypothesis, the clearance rates of radiolabeled oxidized LDL and acetyl LDL in SR-AI/II knockout mice were compared with those in wild-type mice. As shown in Fig. 1, there was no significant difference in the clearance of oxidized LDL in wild-type and knockout animals. Surprisingly, there was also no difference in the clearance rate of acetyl LDL or malondialdehyde-modified LDL. This result indicates that there are alternate pathway(s) for the uptake of modified LDL that can compensate fully for the deficiency of SR-AI/II. In animals injected with acetyl or malondialdehyde-modified LDL, blood radioactivity fell rapidly by ~ 6 min to

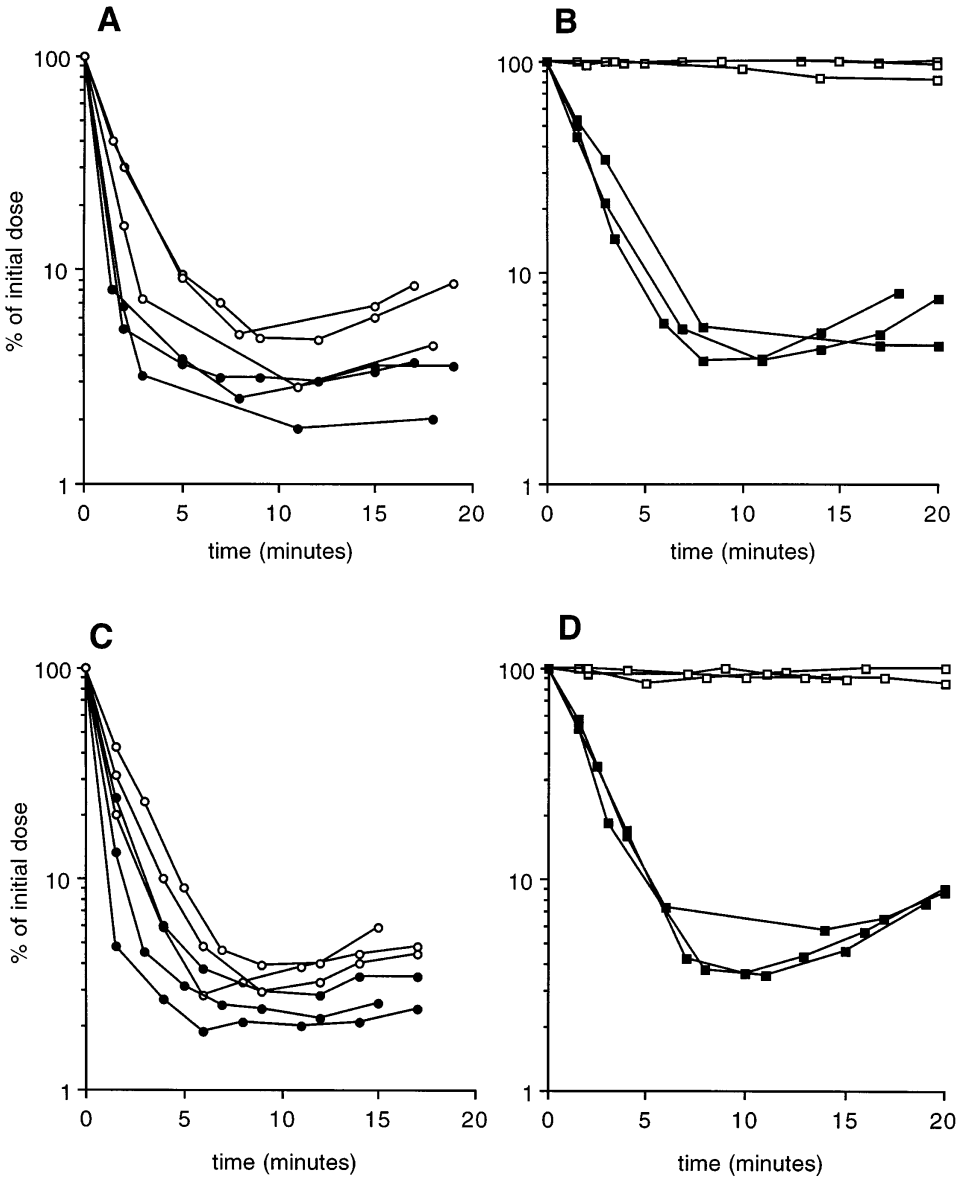

Figure 1. Clearance of intravenously injected oxidized LDL (*solid circles*), acetyl LDL (*open circles*), native LDL (*open squares*), and malondialdehyde-modified LDL (*solid squares*) in wild-type mice (*A* and *B*) and SR-AI/II–deficient mice (*C* and *D*). Mice were anesthetized with halothane, a jugular cannula was inserted, and 1–2 million cpm of each of two differently labeled LDLs were injected. Serial 20-μl blood samples were then withdrawn, and radioactivity was measured. Electrophoretic mobility of oxidized LDL in this experiment was 4.4-fold that of native LDL, while that of both acetyl LDL and malondialdehyde-modified LDL was 4.6-fold that of native LDL.

$\sim$ 5% of the initial blood radioactivity, and then slowly increased to 10%. This secondary increase probably reflects release of iodinated LDL degradation products from the liver into the bloodstream, as most of the radioactivity at later time points was not precipitable by 10% trichloroacetic acid. With oxidized LDL, there was only a minimal secondary increase in blood radioactivity. This increase can be explained by the fact that oxidized LDL has been shown to be resistant to lysosomal degradation in cultured macrophages (34), and it is possible that its degradation in vivo would also be slower than that of acetyl LDL or malondialdehyde LDL.

To determine the extent of LDL oxidation that was required for rapid clearance in scavenger receptor knockout animals, we compared the clearance rate of LDL samples that had been oxidized to different degrees by varying the time of exposure to copper. Results with moderately oxidized LDL (with electrophoretic mobility 3.5 times that of native LDL) showed very rapid clearance in both wild-type and knockout animals, essentially identical to findings with extensively oxidized LDL shown in Fig. 1. The clearance of mildly oxidized LDL (with electrophoretic mobility 1.6 times that of native LDL) was much slower than that of the more heavily oxidized LDL preparation, but was still more than threefold faster than that of native LDL (Fig. 2). Again, there was no difference in SR-AI/II knockout animals compared to wild-type controls. As less than half of the injected radioactivity remained in the circulation by the time the first time point was obtained (and often $< 10\%$ by the second time point), however, it is possible that a difference of as much as 25% in the clearance rate of acetyl LDL or oxidized LDL might not have been detectable.

One interesting observation was that the apparent volume of distribution (injected dose divided by blood radioactivity extrapolated to time 0) of mildly oxidized LDL was consistently smaller than that of native LDL when the two tracers were injected simultaneously into the same animal. In control mice, the apparent volume of distribution of mildly oxidized LDL was 45±5 (SD) ml/kg body wt, and that of native LDL was 56±7 ml/kg. In knockout mice, the volume of distribution of mildly oxidized LDL was 46±3 ml/kg, and that of native LDL was 63±3 ml/kg. The differences in volume of distribution between native and mildly oxidized LDL are significant at $P < 0.05$ by two-tailed paired t test. We have shown previously

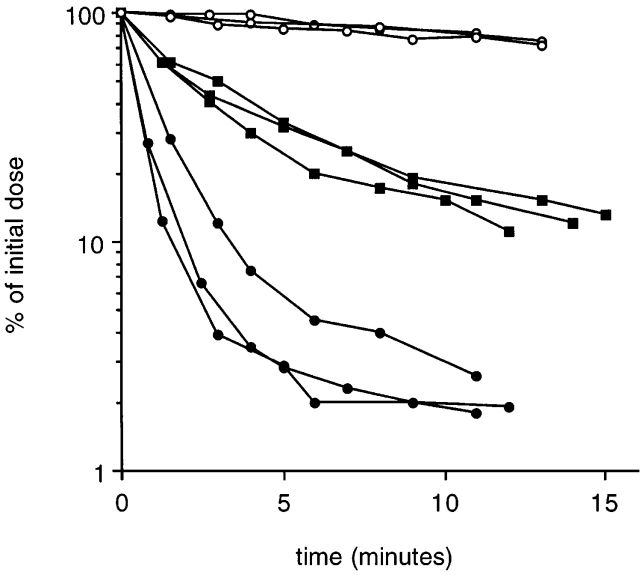

Figure 3. Capacity of the uptake pathway for oxidized LDL. ^{125}I-LDL (200 μg/ml) was exposed to 5 μM copper sulfate at 37°C for 24 h, which resulted in a 4.4-fold increase in electrophoretic mobility relative to native LDL. 50 μg (●), 200 μg (■), or 750 μg (○) of oxidized ^{125}I-LDL was then injected into SR-AI/II–deficient mice, and radioactivity in serial blood samples was then determined.

that even mild oxidation of LDL impairs its ability to bind to LDL receptors (30). This fact suggests that the larger apparent volume of distribution of native LDL might be explained by the preferential binding of native LDL to LDL receptors (or perhaps other lipoprotein binding sites) on vascular endothelium.

As it is unlikely that oxidized LDL is present in the circulation normally, the pool size of modified LDL is essentially the amount injected. As we typically injected only 20 μg of labeled LDL, rapid clearance from plasma might simply be due to a small number of high-affinity binding sites, and would not necessarily be evidence of an efficient, high-capacity degradative pathway. To gain an approximation of the capacity of the uptake process for oxidized LDL, we injected larger quantities

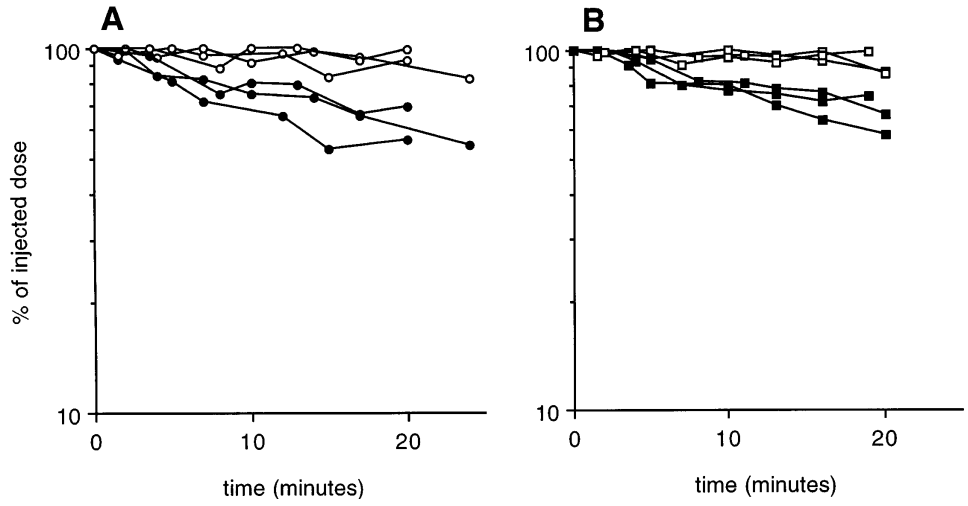

Figure 2. Mild oxidation of LDL increases its rate of clearance from plasma. ^{125}I-LDL (200 μg/ml) was exposed to 5 μM copper sulfate at 37°C for 4 h, which resulted in a 1.8-fold increase in electrophoretic mobility relative to native LDL. Oxidized ^{125}I-LDL (*closed symbols*) was then injected into wild-type (*A*, *circles*) or SR-AI/II–deficient mice (*B*, *squares*) together with ^{131}I-native LDL (*open symbols*). Radioactivity in serial blood samples was then determined. Note the expanded scale of the ordinate in this figure.

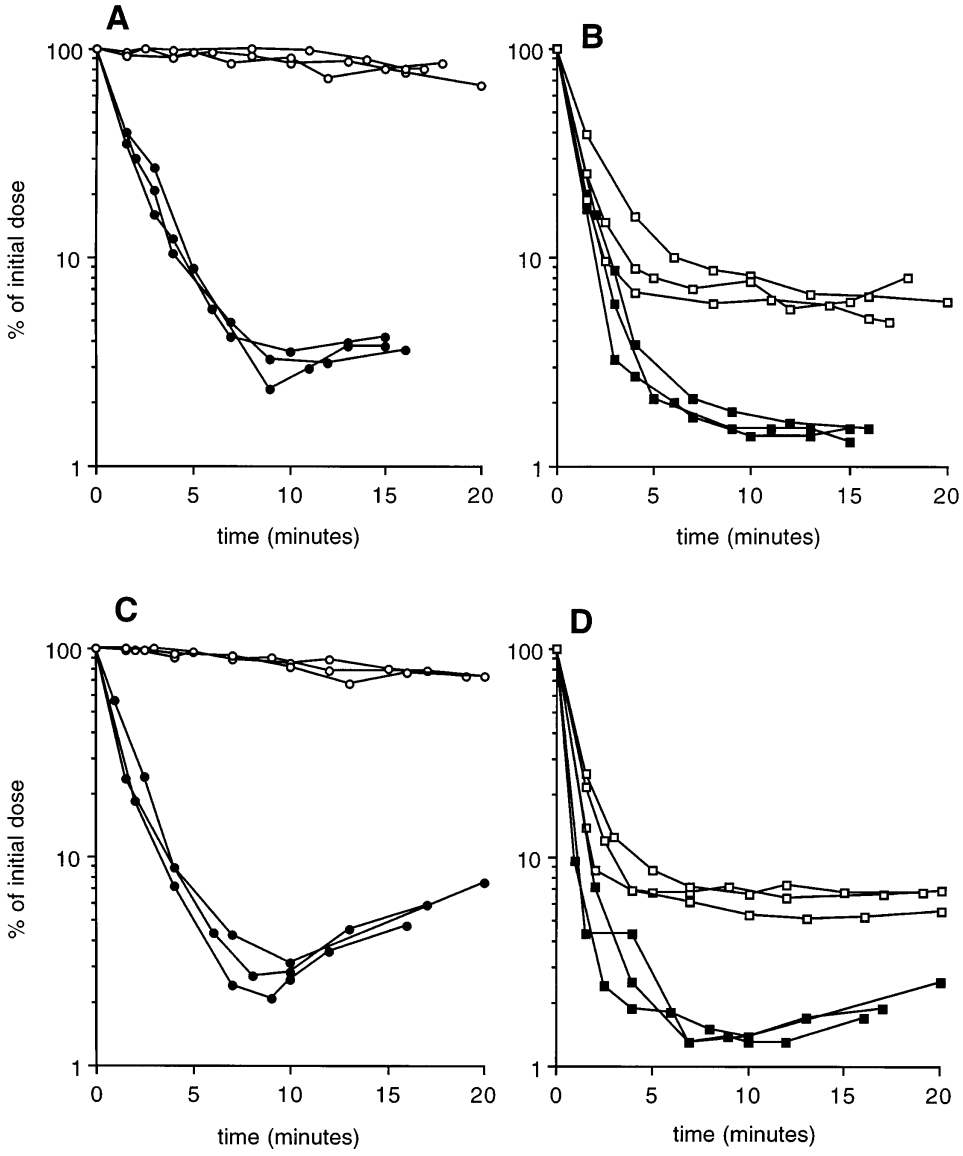

Figure 4. Effect of excess unlabeled acetyl LDL on the clearance of oxidized LDL or acetyl LDL. 20 μg each of ^{131}I-acetyl LDL (*circles*) or ^{125}I-oxidized LDL (*squares*) were injected into wild-type mice (*A* and *B*) or scavenger receptor knockout mice (*C* and *D*) with (*open symbols*) or without (*closed symbols*) 1 mg unlabeled acetyl LDL. Radioactivity was then measured in serial blood samples.

(up to 750 μg) of oxidized LDL. Results shown in Fig. 3 indicate that there was apparent saturation of the uptake pathway if $>$ 200 μg of oxidized LDL was injected. The capacity of this pathway was $\sim$ 15 μg of oxidized LDL cleared per minute. This value represents a fairly high capacity, equivalent to the removal of the entire circulating pool of native LDL in these animals within 10 min. We also tested the effect of a 50-fold excess of unlabeled acetyl LDL on the clearance of labeled modified LDL. Results shown in Fig. 4 indicate that in both wild-type and SR-AI/II–deficient mice, excess unlabeled acetyl LDL almost completely blocked the clearance of labeled acetyl LDL. The same amount of unlabeled acetyl LDL, however, had only a small effect on the clearance of labeled oxidized LDL. Results were identical in control and knockout mice, indicating that the clearance cannot be attributed to SR-AI/II. There are two possible interpretations of these competition results in SR-AI/II–deficient animals: first, the acetyl LDL and oxidized LDL interact with different receptors, or second, that they interact with the same receptor, but that the binding site for acetyl LDL on this receptor is different from the binding site for oxidized LDL.

Previous studies in rats and guinea pigs revealed that the liver is the major site responsible for the clearance of modified LDL (3, 15, 16). To determine if SR-AI/II was responsible for the hepatic uptake of modified LDL from plasma, we compared the organ-specific uptake of oxidized LDL and acetyl LDL in control and SR-AI/II knockout mice. As shown in Fig. 5, in both control and knockout animals, the uptake of oxidized LDL and acetyl LDL was mostly in the liver. The uptake of oxidized LDL in the lungs was greater than that of acetyl LDL in both knockout animals and controls, and amounted to $\sim$ 10% of the total uptake of oxidized LDL. The most likely explanation for this result is the trapping of aggregates of oxidized LDL in the pulmonary capillaries. With the exception of this difference in pulmonary uptake (and the consequent reduction in the percentage of radioactivity recovered in the liver), the tissue sites of uptake of oxidized and acetyl LDL in knockout animals were almost identical. In control animals,

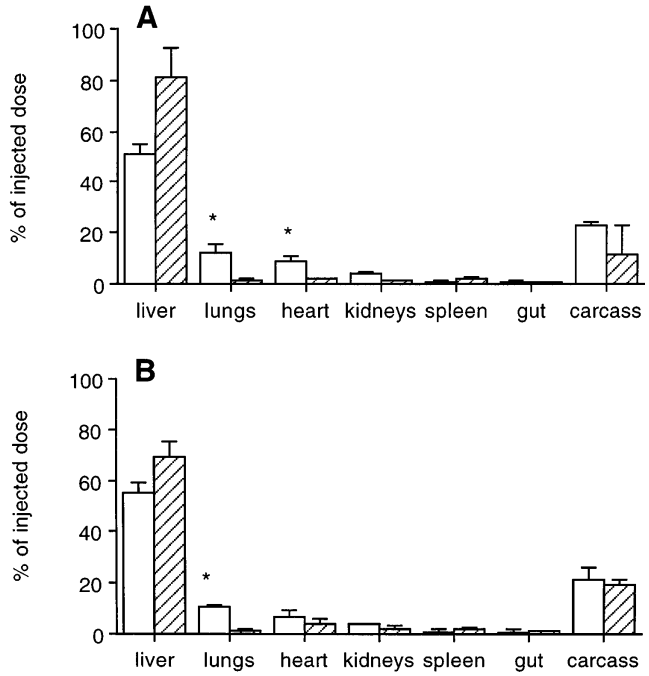

Figure 5. Organ and tissue sites of uptake of modified LDL. Radioiodinated acetyl LDL (*hatched bars*) or oxidized LDL (*open bars*) was injected into anesthetized wild-type mice (*A*) or scavenger receptor knockout mice (*B*). 5 min later, when 95% of the radioactivity had been cleared from the bloodstream, the animals were killed, and individual organs and tissues were analyzed for radioactivity. Values shown are the means±SD of three animals in each group. Comparisons between knockout and control animals, and oxidized LDL and acetyl LDL were evaluated with the *t* test and Bonferroni correction for multiple comparisons. The only significant differences were that uptake of oxidized LDL was greater than that of acetyl LDL in the lungs on both groups of animals, and in the heart in control animals.

there was also an increase in the proportion of oxidized LDL recovered in the heart. It is not certain if this is also attributable to aggregation of oxidized LDL, or to some other factor. The finding that there was no major difference in the organ and tissue distribution of radioactivity from each modified LDL suggests that SR-AI/II and the alternate oxidized LDL receptor(s) appear to be expressed in parallel. The lack of preferential uptake in the spleen of knockout animals indicates that the uptake cannot be because of MARCO (a recently described class A–like trimeric scavenger receptor), as this receptor is expressed only on macrophages in the spleen and lymph nodes (35).

It has been suggested previously that although acetyl LDL and oxidized LDL are both cleared by the liver, the cell types involved are different, with acetyl LDL being cleared mostly by sinusoidal endothelial cells (perhaps reflecting high expression of SR-AI/II on these cells), and oxidized LDL by Kupffer cells (15, 17). The implication of this finding is that alternate oxidized LDL receptors are preferentially expressed on Kupffer cells. If such alternate receptors are responsible for the uptake of both oxidized LDL and acetyl LDL in the knockout animals, then one would expect to see low uptake of acetyl LDL in liver endothelial cells reflecting the absence of scavenger receptors, but relatively active uptake of acetyl LDL by Kupffer cells. To test this hypothesis, we isolated liver endothelial cells and Kupffer cells from knockout and control mice, and compared the uptake of acetyl LDL and oxidized LDL by these cells. Purity of the nonparenchymal cell preparations was assessed by staining for peroxidase activity, and by electron microscopy. Approximately 80% of cells in Kupffer cell preparations were peroxidase-positive, whereas < 10% of cells in endothelial cell preparations were peroxidase-positive. By electron microscopy, 70–75% of cells in Kupffer cell preparations had morphologic features of Kupffer cells, 20–25% were endothelial cells, and 5% were lymphocytes or unidentified cells. More than 96% of cells in endothelial cell preparations were found to be endothelial cells by electron microscopy. This high purity reflects the selective proliferation of endothelial cells in culture. The rate of uptake of oxidized LDL by Kupffer cells from wild-type animals was high (25–30 μg/mg/5 h), and was similar to that of acetyl LDL in these cells (Fig. 6). With oxidized LDL, there was a high ratio of cell-associated radioactivity relative to the amount of LDL degraded, while the opposite was found with acetyl LDL. Similar results have been noted previously in peritoneal macrophages, and have been shown to reflect impaired lysosomal degradation of internalized oxidized LDL (34, 36). Uptake of acetyl LDL by liver endothelial cells from wild-type animals was rapid (around 30 μg/mg/5 h), but the rate of saturable uptake of oxidized LDL by these cells was only one-third of that of acetyl LDL. Cell association and degradation of oxidized LDL was reduced by 30% in both Kupffer cells and endothelial cells from knockout mice. This result is very similar to the findings with resident peritoneal macrophages from these animals (26). In contrast, the uptake of acetyl LDL was ~ 80% lower in endothelial cells from knockout mice than in controls, while the uptake of acetyl LDL in Kupffer cells was only reduced by 30% (Fig. 6). These results with isolated nonparenchymal liver cells are consistent with previous reports that the SR-AI/II is expressed preferentially on liver endothelial cells, and that a different receptor(s) expressed mainly on Kupffer cells accounts for most of the uptake of oxidized LDL. The finding that Kupffer cells from knockout animals showed a relatively high rate of saturable uptake of acetyl LDL suggests that the alternate receptor(s) can recognize acetyl LDL.

To determine if acetyl LDL interacts with the same binding site as oxidized LDL, competition studies were performed with Kupffer cells from knockout animals. As shown in Fig. 7, acetyl LDL was considerably less effective as a competitor for the uptake of labeled oxidized LDL than unlabeled oxidized LDL or LDL modified with fatty acid peroxidation products. Malondialdehyde-modified LDL was also a relatively weak competitor. The finding that acetyl LDL fails to compete for oxidized LDL uptake in knockout Kupffer cells, even though the rate of uptake of acetyl LDL by cells is similar to that of oxidized LDL, is evidence that acetyl LDL and oxidized LDL interact with different binding sites.

Discussion

This study demonstrates that inactivation of the *SR-AI/II* gene in mice does not affect the clearance rate of radioiodinated oxidized LDL or acetyl LDL from the circulation, nor does it alter the organ and tissue distribution of radioactivity after modified LDL is cleared from plasma. A 50-fold excess of

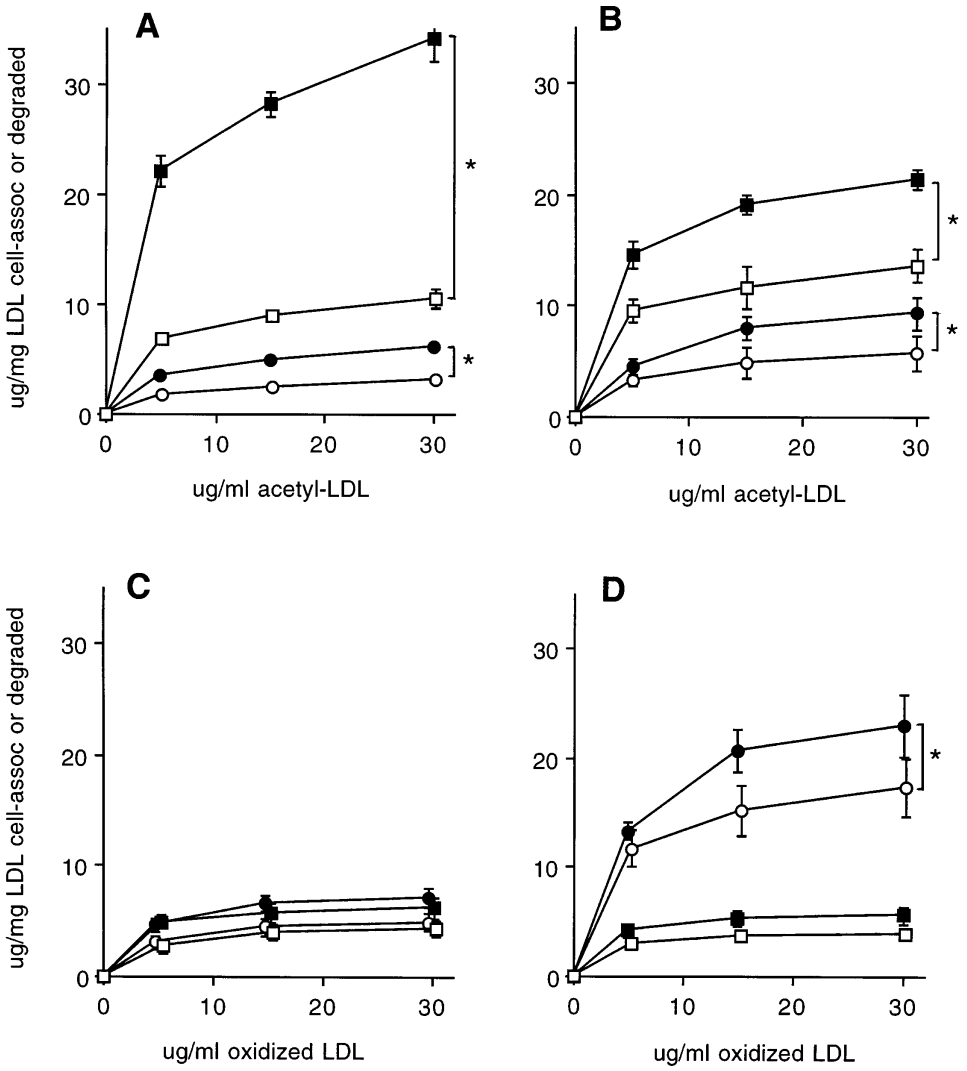

Figure 6. Cell association (*circles*) and degradation (*squares*) of acetyl ^{125}I-LDL (*A* and *B*) or oxidized ^{125}I-LDL (*C* and *D*) by cultured liver endothelial cells (*A* and *C*) or Kupffer cells (*B* and *D*) from wild-type mice (*closed symbols*) or SR-AI/II–deficient mice (*open symbols*). Cells were isolated by collagenase perfusion of the liver, followed by density gradient centrifugation and differential adherence as detailed in Methods, and were then incubated for 5 h at 37°C with the indicated concentration of modified LDL. The amount of cell-associated and degraded LDL was then measured. Error bars indicate standard deviation of triplicate dishes from one of three similar experiments. Significance of differences between controls and SR-AI/II–deficient animals was analyzed by one-way repeated measures ANOVA. *$P < 0.05$.

unlabeled acetyl LDL, which would have yielded plasma concentrations of ~ 500 μg/ml, completely blocked the uptake of acetyl LDL, but only prevented the uptake of ~ 5% of the injected mass of oxidized LDL. Even this 5% of oxidized LDL clearance could not be attributed to SR-AI/II, as the results were identical in knockout mice and controls. One possible explanation for the failure of acetyl LDL to block the clearance of oxidized LDL in SR-AI/II–deficient animals might be that the uptake of oxidized LDL and acetyl LDL is mediated by an alternate oxidized LDL receptor with a single binding site that has a higher affinity for oxidized LDL than does acetyl LDL. We have shown previously that the apparent affinities of oxidized LDL and of acetyl LDL for uptake in normal murine macrophages depend on the degree of LDL modification, and that in some cases nonreciprocal cross-competition can be explained simply on the basis of ligand heterogeneity (19). This conclusion, however, is unlikely in the present case, as the affinity of oxidized LDL would have to be several orders of magnitude higher than that of acetyl LDL, and no such difference was noted when the same oxidized LDL and acetyl LDL preparations were compared in cell culture studies with isolated nonparenchymal liver cells from SR-AI/II–deficient animals. Hence, it is more likely that oxidized LDL and acetyl LDL interact with different binding sites on the same receptor, or with entirely different receptors.

Van Berkel et al. (17) found that in normal rats, oxidized LDL was preferentially cleared from the circulation by Kupffer cells in the liver, whereas acetyl LDL was taken up by sinusoidal endothelial cells. This finding represented the first demonstration of different uptake pathways for oxidized LDL and acetyl LDL in vivo, and these investigators concluded that the uptake of acetyl LDL by sinusoidal endothelial cells was because of SR-AI/II, but that a second receptor on Kupffer cells accounted for most of the uptake of oxidized LDL. Our observations in SR-AI/II–deficient mice are in full agreement with their findings, and extend them by proving that the preferential uptake of oxidized LDL by Kupffer cells cannot be explained simply by phagocytic uptake of aggregated oxidized LDL. Evidence for the selective expression of a receptor for oxidized LDL has also been reported on Kupffer cells isolated from human liver (18), and from rabbit liver (37). In all three species, it was found that acetyl LDL competed for no more than one-half of the degradation of oxidized LDL. Our results support the inference that the failure of acetyl LDL to com-

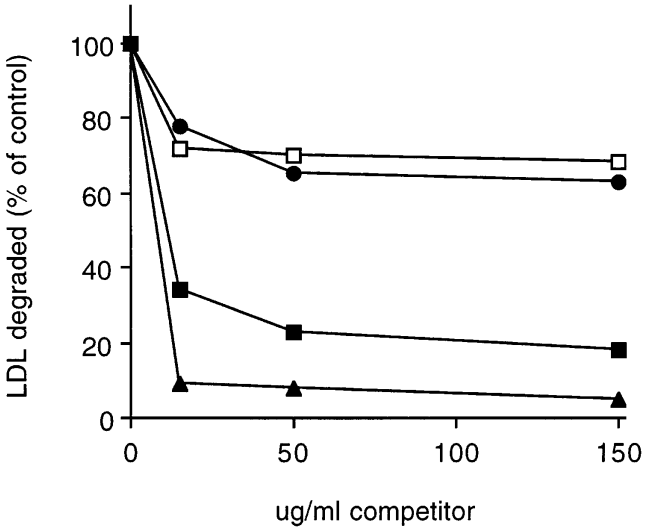

Figure 7. Comparison of the ability of unlabeled lipoproteins to compete for the degradation of oxidized ^{125}I-LDL by Kupffer cells from scavenger receptor knockout mice. Kupffer cells were incubated for 5 h at 37°C with 5 μg/ml of oxidized ^{125}I-LDL and the indicated amounts of unlabeled acetyl LDL (□), MDA LDL (●), oxidized LDL (■), or LDL modified with arachidonic acid peroxidation products (▲). Apolipoprotein degradation is expressed as the percentage of the radioactivity obtained in the absence of competitor. Values are means of duplicates from one of three similar experiments.

pete for oxidized LDL degradation reflects alternate oxidized LDL receptor(s). Furthermore, as acetyl LDL was found to compete partially for oxidized LDL uptake in SR-AI/II–deficient mice, it is possible that the proportion of oxidized LDL uptake that is mediated by alternate receptors on Kupffer cells in rats, rabbits, and humans is in fact greater than the 50–60% that would be predicted simply on the basis of the acetyl LDL–resistant component of oxidized LDL degradation.

The characterization of alternate receptors for oxidized LDL on Kupffer cells remains incomplete. A number of membrane proteins have been proposed as candidate oxidized LDL receptors, including the FcγIIB receptor (20), CD36 (21), SR-BI (22), and macrosialin, or its human homologue, CD68 (25). Ligand blot analysis of rat Kupffer cell membrane proteins showed that oxidized LDL bound to a 95-kD protein (24). This apparent molecular mass is similar to that of CD36 and SR-BI, but is larger than that of the FcγRII-B2 receptor. Binding to this blotted Kupffer cell membrane protein was inhibited $>$ 80% by mildly oxidized LDL (electrophoretic mobility 30% greater than native LDL), which would be compatible with the reported ligand specificity of SR-BI or CD36. The potential role of CD36 as an oxidized LDL receptor has been highlighted by the demonstration that macrophages from CD36-deficient human subjects degrade oxidized LDL at only half the rate of normal macrophages (38). CD68/macrosialin is another candidate receptor for oxidized LDL that might account for the uptake of modified LDL by Kupffer cells in the liver of SR-AI/II knockout mice. Its size (94–97 kD) is the same as the oxidized LDL-binding protein identified on ligand blots of Kupffer cell membranes, and its ligand specificity appears similar to that of the uptake pathway for oxidized LDL in SR-AI–deficient macrophages (26). It is evident that more work is required to identify definitively the receptor(s) responsible for oxidized LDL uptake in vivo, and to clarify its physiologic and pathogenic role. The paradigm illustrated by the present study, i.e., molecular characterization of a candidate receptor and then generation of a knockout to define the role of this receptor in vivo, would appear to be a useful one for this purpose.

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