Differentiation of binding sites on reconstituted hepatic scavenger receptors using oxidized low-density lipoprotein

Elke OTTNAD,* David P. VIA,† Jochen FRÜBIS,* Hansjörg SINN,‡ Eckhard FRIEDRICH,‡ Reinhard ZIEGLER* and Hans Alois DRESEL*§

*Department of Medicine, University of Heidelberg, Bergheimer Str. 58 D-69 Heidelberg, Federal Republic of Germany, †Baylor College of Medicine, Department of Medicine, 6565 Fannin A-601, Houston, TX 77030, U.S.A., and ‡German Cancer Research Center, Department for Radiobiology and Pathophysiology, Im Neuenheimer Feld 280, D-69 Heidelberg, Federal Republic of Germany

Reduced hepatic membrane receptors for acetylated low-density lipoprotein (acetyl-LDL) and maleylated BSA (Mal-BSA) with apparent molecular masses of 35 kDa, 85 kDa and 15 kDa have been extracted from rat liver and separated by affinity chromatography as described by us previously [Ottnad, Via, Sinn, Freidrich, Ziegler & Dresel (1990) Biochem. J. **265**, 689–698]. Binding of these three reduced scavenger receptors to oxidatively modified LDL has been now examined. Competition studies with receptor–phosphatidylcholine complexes and ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA as ligands were conducted. Mal-BSA, acetyl-LDL and fully oxidized LDL were used as competitors, and differentiated in the three receptors three types of binding site: a class I binding site for acetyl-LDL, Mal-BSA and fully oxidized LDL; a class II binding site recognizing only ^{131}I-Mal-BSA and class III binding sites recognizing ^{131}I-Mal-BSA and fully oxidized LDL. The results of competition studies with mildly oxidized LDL and polyadenylic acid demonstrated that the binding sites might be even more heterogeneous. Thus there is evidence that the reconstituted receptors either have several binding sites for each of the various ligands or are functionally different, despite the fact that they do not differ in their apparent molecular masses.

INTRODUCTION

Rat hepatic sinusoidal cells and endothelial cells recognize acetylated low-density lipoprotein (acetyl-LDL) via specific receptors (Nagelkerke *et al.*, 1983; Pitas *et al.*, 1985). Acetyl-LDL receptors detected by Goldstein *et al.* (1979) on cultured macrophages were termed 'scavenger receptors' (Brown & Goldstein, 1983). Physiological ligands of the scavenger receptors have not yet been identified. Steinberg and coworkers discovered that oxidized LDL is a competitor for binding of acetyl-LDL to the macrophage scavenger receptors (Sparrow *et al.*, 1989). Since oxidized LDL can be detected in the atherosclerotic lesion, a role for macrophage scavenger receptors in atherogenesis has been suggested. It has been hypothesized that uptake of oxidized LDL by arterial wall macrophages might cause the conversion of these cells to cholesterol-ester-rich foam cells (Palinski *et al.*, 1989; Ylä-Herttuala *et al.*, 1989; Steinberg *et al.*, 1989).

A protective role for scavenger receptors in the liver has also been suggested. Hepatic scavenger receptors might act as a filter to remove modified LDL from the bloodstream (Nagelkerke *et al.*, 1983; van Berkel *et al.*, 1991). Unreduced high-molecular-mass acetyl-LDL receptors (~ 250 kDa) and three reduced binding proteins (~ 85 kDa, 35 kDa and 12–15 kDa) have been solubilized and purified from rat liver by us previously (Dresel *et al.*, 1987; Ottnad *et al.*, 1990). A similar unreduced high-molecular mass acetyl-LDL receptor was isolated by us from a macrophagocytic cell line (Via *et al.*, 1985) and by others from bovine lung macrophages (Kodama *et al.*, 1988). Two closely related cDNAs of a putative 77 kDa monomer of the bovine scavenger receptors were cloned recently from a lung library (Kodama *et al.*, 1990; Rohrer *et al.*, 1990).

Our previous studies have included saturation binding analyses on purified hepatic scavenger receptors with acetyl-LDL and maleylated BSA (Mal-BSA) (Ottnad *et al.*, 1990). Mal-BSA was described by Brown *et al.* (1980) as an inhibitor of acetyl-LDL binding to the scavenger receptors, and was used by us as a second ligand. A 4:1 binding ratio for Mal-BSA:acetyl-LDL at the 85 kDa and the 15 kDa binding proteins, and a 2:1 binding ratio at the 35 kDa binding protein, were observed. By cross-competition studies with acetyl-LDL and Mal-BSA, at least two classes of binding sites for Mal-BSA could be separated. In the present paper we analyse the interaction of oxidized LDL with the three reduced 85 kDa, 35 kDa and 12–15 kDa acetyl-LDL/Mal-BSA-binding proteins from rat liver. We demonstrate competition of acetyl-LDL binding and Mal-BSA binding by native LDL, mildly oxidized LDL and fully oxidized LDL in a reconstituted system.

MATERIALS AND METHODS

Materials and chemicals were obtained from sources described previously (Ottnad *et al.*, 1990). With the exception of Trasylol, which was obtained from Bayer (Leverkusen, Germany), the proteinase inhibitors were purchased from Boehringer Mannheim G.m.b.H. (Mannheim, Germany).

Preparation and characterization of lipoprotein and radioligands

Human LDL was prepared from the plasma of four male healthy blood donors (non-smokers) by sequential ultracentrifugation in NaBr solution in the presence of 0.01 % EDTA (Havel *et al.*, 1955). The isolated LDL was stored in the presence of EDTA under nitrogen for up to 10 days before use. LDL was dialysed before oxidation at 4 °C for 24 h against 4 × 4 litres

Abbreviations used: LDL, low-density lipoprotein; acetyl-LDL, acetylated LDL; E64, *N*-[*N*-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agmatin; Mal-BSA, maleylated BSA; OG, octyl glucoside (*n*-octyl β-D-glucopyranoside); PMSF, phenylmethanesulphonyl fluoride; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TNE buffer, 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/0.1 mM-EDTA.

§ To whom correspondence and reprint requests should be addressed at: Boehringer Mannheim G.m.b.H., Department for Atherosclerosis Research, FM 1A, Sandhofer Str. 116, D-6800 Mannheim 31, Federal Republic of Germany.

Table 1. ^{131}I-Acetyl-LDL and ^{131}I-Mal-BSA binding to scavenger receptor proteins from rat liver

The hepatic proteins were prepared and partially purified from rat liver by the procedure described in the Materials and methods section. Specific binding is expressed per mg of liposome-associated protein. Maximal specific binding and K_d were determined by filtration assay and Scatchard analysis of the binding data. Molecular masses were determined by SDS/polyacrylamide-slab-gel electrophoresis as described in the Materials and methods section.

		^{131}I-Acetyl-LDL binding		^{131}I-Mal-BSA binding	
Molecular mass (kDa)	Purification (-fold)	Specific binding (nmol/mg of protein)	K_d (nM)	Specific binding (nmol/mg of protein)	K_d (nM)
35	18000	2.34	5.0	4.5	4.8
85	> 440	0.85	6.5	3.7	30
12–15	> 440	0.30	2.3	1.2	5.6

of 50 mM-Tris/HCl (pH 8)/150 mM-NaCl (TN buffer) under nitrogen. The concentration of LDL was expressed in terms of protein content/ml of solution, and was determined according to Lowry *et al.* (1951). The integrity of the apolipoprotein B and the purity of the LDL was checked by SDS/polyacrylamide slab-gel electrophoresis (Laemmli, 1970). A 2 μg sample of protein was applied per lane. Apolipoprotein B moved as a single band. No lipoprotein(a) was detected by staining with the Bio-Rad silver staining kit.

In order to characterize the stability of the LDL preparation, LDL (125 μg/ml) was oxidized in a 2 ml cuvette with 10 μM-Cu(II) as pro-oxidant, as described previously (Huber *et al.*, 1990). Briefly, the formation of dienes was recorded at A_{234}, and the so-called 'lag phase' (defined as the interval between the intercept of the tangent of the slope of the curve with the time-scale axis), and the maximal rate of A_{234} increase (calculated from the slope of the absorbance curve during the propagation reaction) were estimated. The LDL preparation was analysed three times within 10 days. The lag phase of the probe was 77 ± 8 min (mean $\pm$ S.D.), and the mean maximal rate of the diene formation was 0.027 ± 0.002 A_{234} units/min. There was no evidence for a shortening of the lag phase over the 10 days of storage.

Acetylation of LDL was performed by treatment with acetic anhydride (Basu *et al.*, 1976). BSA was maleylated with maleic anhydride (Imber *et al.*, 1982). In our experiments 50 of 59 lysine residues were maleylated, as determined by the method of Fields (1972). Protein concentrations of the modified albumin were also determined according to Lowry *et al.* (1951).

Radioiodination of LDL and Mal-BSA was performed with Na^{131}I and *N*-bromosuccinimide as oxidant (1 μg of NBS/mCi of ^{131}I) and analysed by t.l.c. as described (Sinn *et al.*, 1988). The following specific radioactivities were obtained: ^{131}I-LDL, 200–300 c.p.m./fmol; ^{131}I-acetyl-LDL, 200–460 c.p.m./fmol; ^{131}I-Mal-BSA, 80–90 c.p.m./fmol; more than 97% of the label was bound to the protein.

Preparation and characterization of mildly and fully oxidized LDL

The oxidation of LDL (400 μg/ml) was carried out in TN buffer at 37 °C in the presence of 10 μM-$CuCl_2$ in plastic tubes. To stop the oxidative modification of LDL and to adjust for the binding assay conditions, the samples were chilled on ice at the indicated times. EDTA as a chelator for the redox metal was added to a final concentration of 20 mM; in addition, those probes used for the binding studies were adjusted with albumin to a final concentration of 1% to scavenge excessive aldehydes in the aqueous phase. The modification of LDL was estimated by the thiobarbituric acid (TBA) reaction for lipid peroxidation products, as described by Slater & Sawyer (1971) with minor modifications. Portions of LDL and oxidized LDL were precipitated with 30% trichloroacetic acid (final concn. 5%) and an equal volume of 1% TBA and then heated for 20 min at 98 °C. After centrifugation (10000 *g*, 10 min) of the precipitate, absorbance was recorded at 532 nm. The TBA-reactive substances (TBARS) were calculated from a standard curve obtained with malondialdehyde bis(dimethylacetal) and expressed as nmol of malondialdehyde/total LDL. The electrophoretic mobilities of native LDL and of the various oxidized LDL samples were analysed by 1% agarose gel electrophoresis using the commercially available Tris/Veronal buffer obtained from Biotec Fischer. A 10 μg sample of LDL protein was applied to the gel. Electrophoresis was performed at 200 V for 2.5 h. Results are expressed as the relative electrophoretic mobility of the lipoprotein, i.e. the ratio of the electrophoreic mobility of the oxidized LDL to the electrophoretic mobility of the native LDL.

Preparation of reduced binding proteins

Three rat liver membrane proteins with apparent molecular masses of 85 kDa, 35 kDa and 15 kDa and having binding activity for acetyl-LDL and Mal-BSA were solubilized and separated under reducing conditions by Mal-BSA–Sepharose affinity chromatography followed by gel-permeation chromatography, essentially as described previously (Ottnad *et al.*, 1990). Briefly, the 35 kDa and 15 kDa binding proteins were solubilized from membrane pellets in TNE buffer [50 mM-Tris/HCl (pH 8)/150 mM-NaCl/0.1 mM-EDTA] containing 0.2% β-mercaptoethanol, 40 mM-octyl glucoside (OG), 23 μg of phenylmethanesulphonyl fluoride (PMSF)/ml and 0.1% Trasylol. The 85 kDa binding protein was solubilized in TNE buffer containing 5 mM-GSSG and 5 mM-GSH (instead of β-mercaptoethanol) and 1% Triton X-100. The yield of the 35 kDa, 15 kDA and 85 kDa binding proteins was greatly influenced by the type of detergent and also by the reducing agent in the solubilization buffer. However, when some proteinase inhibitors were included in the TNE buffer (23 μg of PMSF/ml, 0.1% Trasylol, 25 μg of leupeptin/ml, 2.5 μg of cystatin/ml and 25 μg of E64/ml), no difference in binding protein yield or integrity was observed. To purify the 35 kDa and 15 kDa binding proteins, the solubilized membrane proteins were applied to a Mal-BSA–Sepharose 4B column equilibrated with 50 mM-Tris/HCl (pH 8)/0.1 mM-EDTA/40 mM-OG/0.2% β-mercaptoethanol and eluted in the same buffer containing 250 mM-NaCl and then 1 M-NaCl. Subsequently, separation and further purification could be obtained by gel-permeation chromatography in 125 mM-Tris/HCl (pH 6.8)/0.1% β-mercaptoethanol/0.1% SDS and characterized for their binding activities (Table 1). The solubilized 85 kDa binding protein was diluted to 0.5% Triton X-100, added to a

Mal-BSA–Sepharose 4B column equilibrated with 50 mM-Tris/HCl (pH 8)/50 mM-NaCl/0.1 mM-EDTA/5 mM-GSSG/5 mM-GSH/0.1 % Triton X-100, and eluted in the same buffer containing 250 mM-NaCl, but no GSSG, and characterized for its binding activities (Table 1).

Assay for competition of oxidized LDL with ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL binding

Competition with ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA binding to their reconstituted reduced hepatic binding proteins was determined by the filter assay of Schneider *et al.* (1980), modified as described (Via *et al.*, 1985). The purified 85 kDa binding protein was in 50 mM-Tris/HCl (pH 8)/250 mM-NaCl/0.1 mM-EDTA/5 mM-GSH/0.1 % Triton X-100. The 35 kDa protein and the 12–15 kDa proteins were in 125 mM-Tris/HCl (pH 6.8)/0.1 % β-mercaptoethanol/0.1 % SDS. Samples were diluted, complexed with egg phosphatidylcholine and precipitated by 33 % acetone as described previously (Via *et al.*, 1985). The precipitated liposome–protein complexes were suspended in 10 mM-Tris/HCl (pH 8) and incubated with ^{131}I-Mal-BSA, ^{131}I-LDL or ^{131}I-acetyl-LDL in 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/10 mM-EDTA/BSA (1 mg/ml), for 30 min at 20 °C in the absence or the presence of a second ligand. Unbound radioligands were separated from bound radioligands on cellulose acetate filters which were extensively washed with binding buffer. All competition experiments which resulted in a competition for radioligand binding of less than 80 % were repeated twice; however, the presented binding data are always duplicates from a single but representative experiment.

RESULTS

Assay conditions for ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA binding in the presence of oxidized LDL

LDL was incubated under oxidative conditions for 0–18 h. The formation of dienes and fluorescent lipid–protein conjugates was as shown previously (Huber *et al.*, 1990). However, since binding of oxidized LDL has been analysed by others (Sparrow *et al.*, 1989) using a ligand characterized by TBARS, we show in Fig. 1 the TBARS in the oxidized LDL samples we call mildly, moderately and fully oxidized LDL. In a typical LDL incubation experiment with Cu(II) as pro-oxidant, the TBARS were

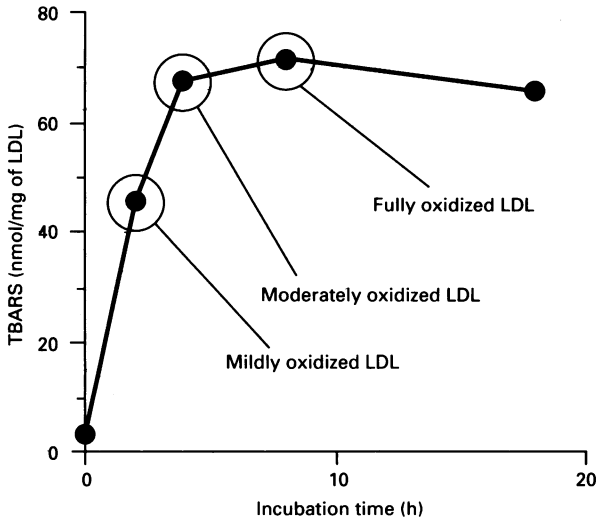

Fig. 1. Kinetics of TBARS formation during oxidation of LDL

Protein (125 μg) was incubated at 37 °C in 1 ml of 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/10 μM-$CuCl_2$. At the indicated times the samples were chilled on ice and precipitated by addition of 0.2 ml of 30 % trichloroacetic acid. The protein was pelleted, the supernatant was incubated with 1.2 ml of TBA at 98 °C for 20 min and then analysed at 532 nm. The TBARS are expressed as nmol/mg of LDL.

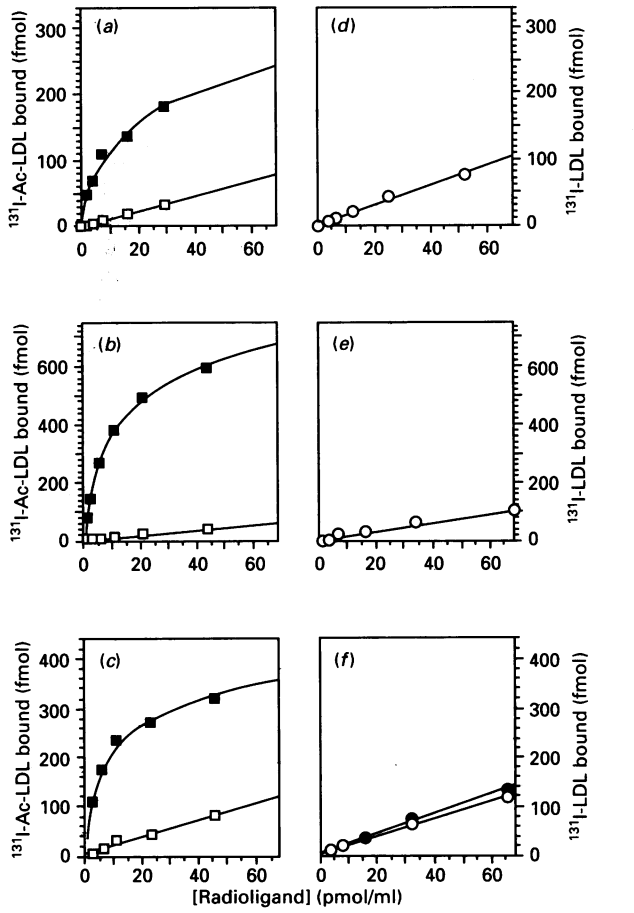

Fig. 2. Saturation analysis of binding to the 35 kDa, 12–15 kDa and 85 kDa binding proteins

Solubilized hepatic membrane proteins from rat liver were fractionated by Mal-BSA–Sepharose 4 B column affinity chromatography and h.p.l.c. gel chromatography. Portions (200 μl) of the fractions containing the 35 kDa, 12–15 kDa and 85 kDa proteins with binding activity for acetyl-LDL and Mal-BSA were reconstituted with egg phosphatidylcholine. Complexes were analysed for saturation binding with ^{131}I-radioligands in the absence and the presence of a second unlabelled ligand at room temperature for 30 min before filtration (to separate bound from free radioligand). (*a*), (*d*) Binding of ^{131}I-acetyl-LDL and ^{131}I-LDL to the fraction containing the 35 kDa protein (4.5 μg of protein/assay): ■, total binding of ^{131}I-acetyl-LDL in the absence of oxidized LDL; □, total binding in the presence of fully oxidized LDL (800 pmol/ml); ●, total binding of ^{131}I-LDL in the absence of Mal-BSA; ○, total binding in the presence of Mal-BSA (1480 pmol/ml). (*b*), (*e*) Binding of ^{131}I-acetyl-LDL and ^{131}I-LDL to the fraction containing the 12–15 kDa protein (1.5 μg of protein/assay): ■, total binding of ^{131}I-acetyl-LDL in the absence of oxidized LDL; □, total binding in the presence of fully oxidized LDL (800 pmol/ml); ●, total binding of ^{131}I-LDL in the absence of Mal-BSA; ○, total binding in the presence of Mal-BSA (1480 pmol/ml). (*c*), (*f*) Binding of ^{131}I-acetyl-LDL and ^{131}I-LDL to the fraction containing the 85 kDa protein (3.8 μg of protein/assay): ■, total binding of ^{131}I-acetyl-LDL in the absence of oxidized LDL; □, total binding in presence of fully oxidized LDL (800 pmol/ml); ●, total binding of ^{131}I-LDL in the absence of Mal-BSA; ○, total binding in the presence of Mal-BSA (1480 pmol/ml). The specific radioactivity of ^{131}I-acetyl-LDL was 293 c.p.m./fmol, and that of ^{131}I-LDL was 290 c.p.m./fmol. Values are means of duplicate determinations.

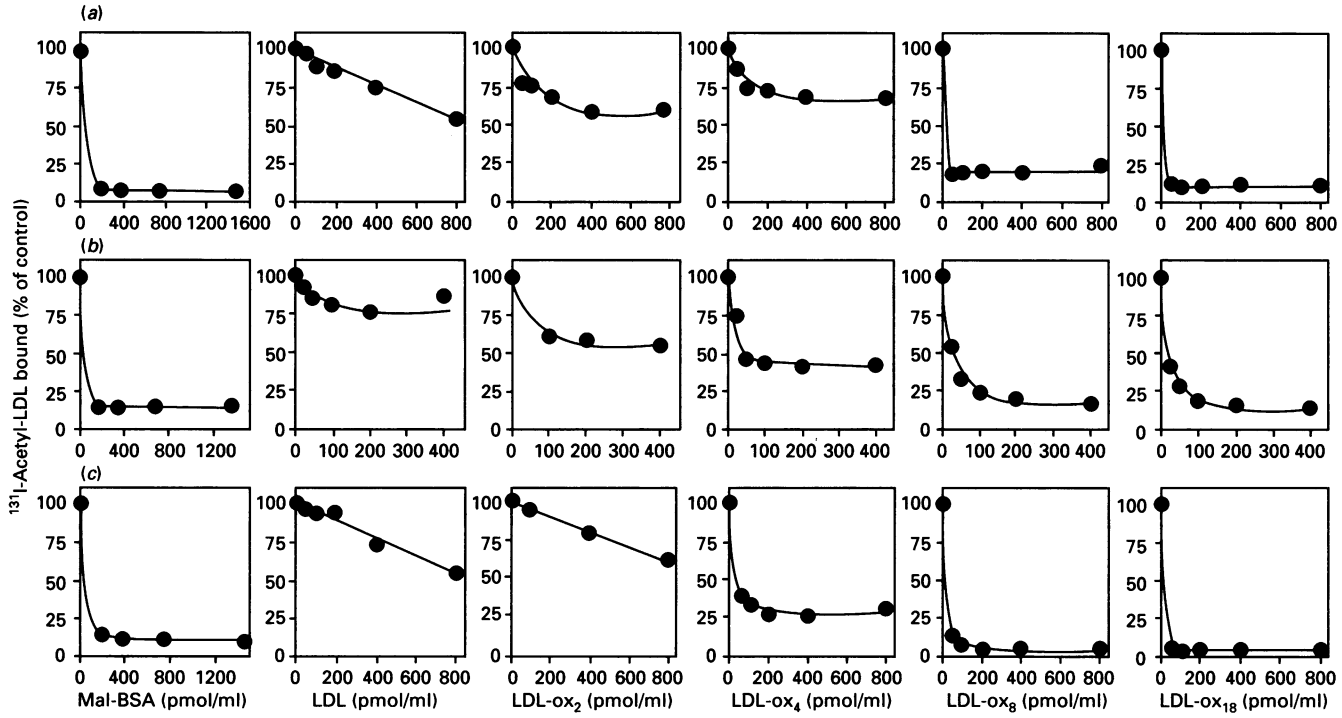

Fig. 3. Inhibition of ^{131}I-acetyl-LDL binding to the 35 kDa, 15 kDa and 85 kDa hepatic receptor proteins by various concentrations of unlabelled Mal-BSA, LDL and oxidized LDL

Liposome–protein complexes containing the 35 kDa and 85 kDa binding proteins were made as described in the Materials and methods section. The complexes were incubated with 20 pmol of ^{131}I-acetyl-LDL/ml (200–460 c.p.m./fmol) in the absence or the presence of increasing concentrations of the inhibitors. (*a*) Inhibition of total ^{131}I-acetyl-LDL binding to the 35 kDa protein (4.5 μg of protein/assay). The 100% value was 0.54 pmol of ^{131}I-acetyl-LDL bound/assay. (*b*) Inhibition of total ^{131}I-acetyl-LDL binding to 1.5 μg of the reconstituted 15 kDa binding protein. The 100% value was 2.287 pmol of ^{131}I-acetyl-LDL bound/assay. (*c*) Inhibition of total ^{131}I-acetyl-LDL binding to 3.8 μg of the reconstituted 85 kDa binding protein. The 100% value was 0.811 pmol of ^{131}I-acetyl-LDL bound/assay. Abbreviation: LDL-ox, oxidized LDL; the subscript indicates the oxidation period (h).

3.3 nmol/mg of LDL immediately after the addition of $CuCl_2$. This had increased to 45.9 nmol/mg after 2 h (mildly oxidized LDL). TBARS reached 67.2 nmol/mg at 4 h (moderately oxidized LDL), and reached a plateau: values after 8 h and 18 h were 71.5 nmol/mg and 65.5 nmol/mg respectively (fully oxidized LDL). The electrophoretic mobility relative to native LDL on agarose gel electrophoresis increased from 1.2 (mildly oxidized) and 1.35 (moderately oxidized) to 2.0 at the 18 h time point (fully oxidized LDL) (results not shown).

For analysis of the competitor activities of the partially modified LDLs, it was necessary to establish conditions with which to stop oxidative LDL modification and to prevent the continuation of LDL modification by lipid peroxidation products. The oxidized LDL samples were chilled on ice, and EDTA and albumin were added to the samples. These manipulations are necessary, since after this treatment formation of TBARS was prevented in the samples if the LDL was further incubated with liposomes or with receptor–liposome complexes in competition assays (results not shown). Also, preincubation of the reconsituted receptors with an ultrafiltrate from oxidized LDL supplemented with EDTA/albumin (obtained by centrifugation of oxidized LDL on a Centricon C30 membrane) at 37 °C for 30 min did not significantly decrease the binding capacity of the receptors. Thus the oxidized LDL samples treated with EDTA/albumin can be used for competition studies at room temperature.

Binding of ^{131}I-acetyl-LDL in the absence and the presence of oxidized LDL to the hepatic scavenger receptors

The reduced 85 kDa, 35 kDa and 12–15 kDa binding proteins were separated, reconstituted into liposomes and characterized as described (Table 1). Figs. 2(*a*)–2(*c*) show ^{131}I-acetyl-LDL saturation binding in the absence and the presence of LDL which was oxidized for 18 h. This LDL had a TBARS concentration of 65.5 nmol/mg of LDL. ^{131}I-Acetyl-LDL exhibited high-affinity saturation binding to all three reduced binding proteins. In the presence of an excess of the fully oxidized LDL, however, all of the high-affinity saturation binding of ^{131}I-acetyl-LDL was inhibited. The results are similar to those seen with an excess of unlabelled acetyl-LDL (Ottnad *et al.*, 1990). The residual ^{131}I-acetyl-LDL binding in the presence of oxidized LDL was unspecific binding: saturation binding analysis with ^{131}I-LDL in the absence and the presence of an excess of Mal-BSA (Figs. 2*d*–2*f*) resulted in unspecific binding similar to that seen in incubations of ^{131}I-acetyl-LDL in the presence of oxidized LDL for all three hepatic binding proteins. Thus the three proteins exhibit only high-affinity ^{131}I-acetyl-LDL binding, but not high-affinity binding of native LDL to any measurable extent. Competition of oxidized LDL for the reduced acetyl-LDL receptors is a specific inhibition for the modified LDL only, and not for native LDL.

Competition with ^{131}I-acetyl-LDL binding and ^{131}I-Mal-BSA binding to reduced hepatic binding proteins by LDL oxidized to various extents

When competition with ^{131}I-acetyl-LDL binding by the mildy and fully oxidized LDLs was compared with competition with Mal-BSA binding, clear differences in the competitor activities could be observed (Figs. 3 and 4). The inhibition studies with ^{131}I-acetyl-LDL (Fig. 3) were performed at saturating ligand concentrations for binding (20 pmol of ^{131}I-acetyl-LDL/ml) in

the presence of increasing concentrations of native LDL, Mal-BSA, acetyl-LDL and LDL oxidized for 2, 4, 8 and 18 h. An excess of Mal-BSA or of LDL oxidized for 8 or 18 h inhibited more than 80% of ^{131}I-acetyl-LDL binding to the three binding proteins at concentrations less than 100 pmol/ml. LDL oxidized for 4 h inhibited 50% of ^{131}I-acetyl-LDL binding to the 12–15 kDa protein and 75% of ^{131}I-acetyl-LDL binding to the 85 kDa protein at concentrations < 100 pmol/ml. However, native LDL did not compete significantly for ^{131}I-acetyl-LDL binding to the 35 kDa receptor at low concentrations. LDL oxidized for 2 h decreased ^{131}I-acetyl-LDL binding to some extent at very high concentrations (400–800 pmol/ml), but displaced less than 20% of the ^{131}I-acetyl-LDL binding at concentrations < 100 pmol/ml. Thus only LDL particles oxidized for more than 4 h are active competitors for ^{131}I-acetyl-LDL binding to the 15 kDa and 85 kDa protein. LDL oxidized for 4 h differentiates between the three reduced binding proteins by its competitive activity for ^{131}I-acetyl-LDL binding. It is a significant competitor for ^{131}I-acetyl-LDL binding to the 12–15 kDa and the 85 kDa proteins, but only a weak competitor at the 35 kDa binding protein.

Competition with ^{131}I-Mal-BSA binding by oxidized LDL, LDL and acetyl-LDL

The binding of iodinated Mal-BSA, the non-lipoprotein ligand for the scavenger receptor, was analysed (Fig. 4). These competition studies were performed at a saturating radioligand concentration of 58.8 pmol of ^{131}I-Mal-BSA/ml in the presence of increasing concentration of native LDL, acetyl-LDL and oxidized LDLs. Acetyl-LDL competed with ^{131}I-Mal-BSA for 20–25% of its binding to the 35 kDa binding protein, and with binding to the 15 kDa and 85 kDa binding proteins as well. Native LDL did not compete for ^{131}I-Mal-BSA binding to the 35 kDa or 85 kDa proteins. However, a decrease of up to 20% in ^{131}I-Mal-BSA binding of the 15 kDa protein in the presence of a very high excess of native LDL was observed in our filter assay. LDL oxidized for 2 h was a weak competitor, and decreased ^{131}I-Mal-BSA binding by not more than 20% even at high competitor concentrations. Fully oxidized LDL competed with about 25% of ^{131}I-Mal-BSA binding to the 35 kDa binding protein, and with about 50% of ^{131}I-Mal-BSA binding to the 85 kDa and 15 kDa receptors. Thus the 35 kDa binding protein on the one hand and the 15 kDa and 85 kDa proteins on the other differ in their binding stoichiometry for ^{131}I-Mal-BSA and oxidized LDL.

DISCUSSION

In this study we have characterized three detergent-solubilized and partially purified acetyl-LDL binding proteins from rat liver in a reconstituted system. This system was used by others to analyse the solubilized LDL receptors (Schneider *et al.*, 1980) and by our group to analyse solubilized scavenger receptors (Via *et al.*, 1985; Dresel *et al.*, 1987; Ottnad *et al.*, 1990). This system allowed us to compare the binding selectivity, binding stoichiometry and affinity constants for the binding of various ligands to solubilized scavenger receptors and to receptors on intact cells, and to study the relationship with liver uptake in the

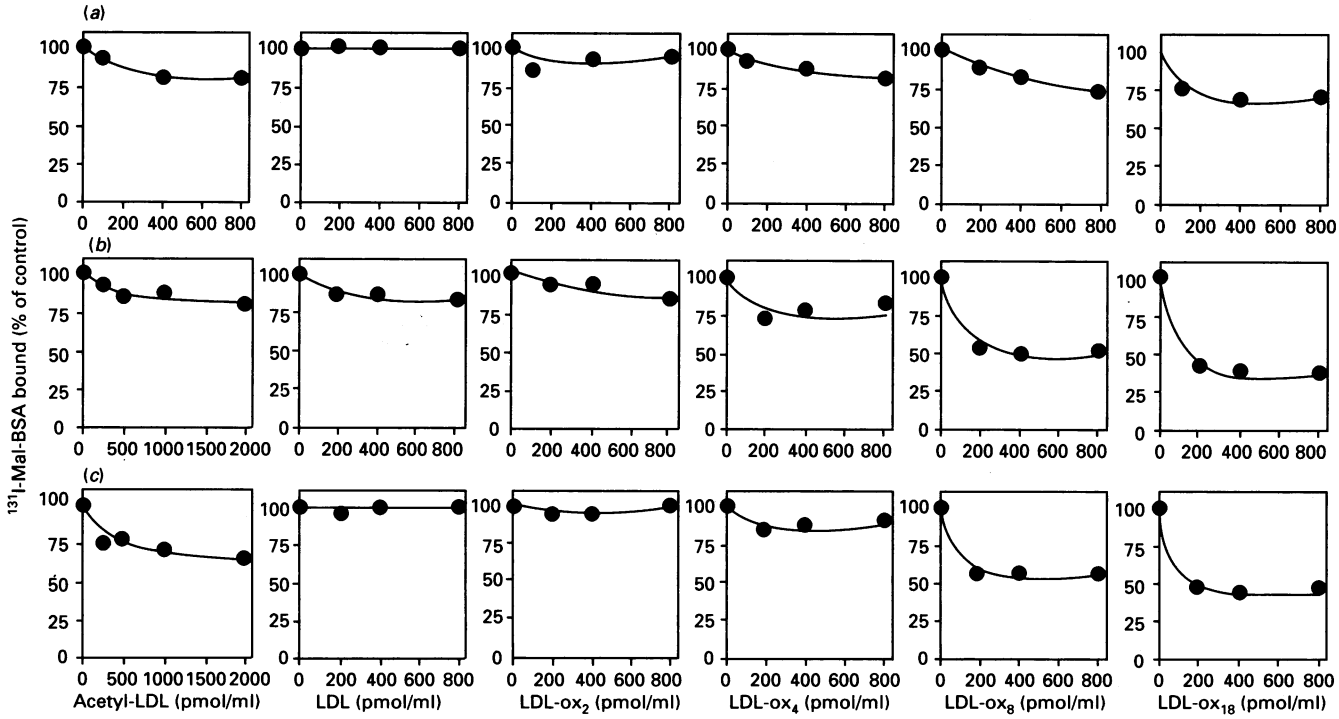

Fig. 4. Inhibition of ^{131}I-Mal-BSA binding to the 35 kDa, 15 kDa and 85 kDa hepatic receptor proteins by various concentrations of unlabelled acetyl-LDL, LDL and oxidized LDL

Liposome–protein complexes containing the 35 kDa, 15 kDa and 85 kDa binding proteins were made as described in the Materials and methods section. The complexes were incubated with 58.8 pmol of ^{131}I-Mal-BSA/ml (80–90 c.p.m./fmol) in the absence or the presence of increasing concentrations of the inhibitors. (*a*) Inhibition of total ^{131}I-Mal-BSA binding containing the 35 kDa protein (4.5 μg of protein/assay). The 100% value was 1.1 pmol of ^{131}I-Mal-BSA bound/assay. (*b*) Inhibition of the total ^{131}I-Mal-BSA binding to 1.5 μg of the reconstituted 15 kDa binding protein. The 100% value was 2.41 pmol of ^{131}I-Mal-BSA bound/assay. (*c*) Inhibition of total ^{131}I-Mal-BSA binding to 3.8 μg of the reconstituted 85 kDa protein. The 100% value was 3.18 pmol of ^{131}I-Mal-BSA bound/assay. Abbreviation: LDL-ox, oxidized LDL; the subscript indicates the oxidation period (h).

whole animal as well (Dresel *et al.*, 1987; Ottnad *et al.*, 1990). The results obtained with this reconstituted system were always in good agreement with the data from macrophage studies reported by Brown *et al.* (1980), and also reflected the observed competition of polyanionic compounds and Mal-BSA for acetyl-LDL uptake in the liver *in vivo*. We decided, therefore, to use the reconstituted system for competition analyses with various oxidized LDLs and highly purified binding proteins extracted from rat liver. However, the data have to be interpreted with caution, since in the reconstituted system the possibility exists that some binding proteins are present in an alternative orientation and that artefactual binding might occur. Since the purified receptor proteins were rather limited, we could not control the orientation of the binding proteins in the liposomes. The data and interpretations can only be relevant if they are in agreement with results from cellular studies and studies *in vivo*.

Our results demonstrate that inhibition of ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA binding to the three different receptor proteins by native LDL, mildly oxidized LDL, moderately oxidized LDL and fully oxidized LDL shows certain similarities and various differences. The differences in the degree of competition with the various oxidized LDLs suggest that several classes of ligand binding sites exist on the receptor proteins.

LDL oxidized for 8 h or 18 h (fully oxidized LDL) inhibited all ^{131}I-acetyl-LDL high-affinity binding to the 85 kDa, 35 kDa and 12–15 kDa receptors. The fully oxidized LDLs therefore seem to compete with ^{131}I-acetyl-LDL binding in a manner similar to Mal-BSA (Ottnad *et al.*, 1990). However, a comparison of the inhibitory potencies of acetyl-LDL, mildly oxidized LDL and Mal-BSA for ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA binding at the receptors indicate a more complex interaction of the ligands with the receptors. Acetyl-LDL inhibits only 25% of the ^{131}I-Mal-BSA binding to the three receptors. Fully oxidized LDL inhibits, like acetyl-LDL, ~25% of the ^{131}I-Mal-BSA binding to the 35 kDa receptor. However, unlike acetyl-LDL, it inhibits 50% of the ^{131}I-Mal-BSA binding to the 85 kDa and 12–15 kDa binding proteins. These findings suggest the existence of several classes of binding sites on the three hepatic proteins. On the reconstituted 35 kDa receptor they appear to fall into three categories: a class I binding site recognizing acetyl-LDL, Mal-BSA and fully oxidized LDL, a class II binding site recognizing only Mal-BSA, and a class III site which recognizes Mal-BSA and oxidized LDL. Based on its binding stoichiometry, the 35 kDa protein would then contain a single class I binding site and three class II binding sites. It appears that the 85 kDa protein and the 12–15 kDa protein contain similar class I and class II binding sites, since binding of ^{131}I-Mal-BSA to these proteins is also inhibited only partially by acetyl-LDL and fully oxidized LDL. Evidence for the third class of binding sites arises from the observation that excess acetyl-LDL diminished ^{131}I-Mal-BSA binding by only 25%, and not by 50% as did fully oxidized LDL. Apparently this class III binding site interacts only with Mal-BSA and fully oxidized LDL, but not with acetyl-LDL. Competition for binding of ^{131}I-Mal-BSA to the class III binding sites of the 85 kDa and 12–15 kDa receptors by fully oxidized LDL resembles the competition obtained with polyadenylic acid in our previous studies (Ottnad *et al.*, 1990). However, on the 35 kDa receptor, polyadenylic acid competed with 75% of the ^{131}I-Mal-BSA binding (Ottnad *et al.*, 1990), greater than the 25% obtained with fully oxidized LDL in this study. Therefore differences in ligand binding between the class III binding sites of the 85 kDa and 12–15 kDa receptors on the one hand and the 35 kDa receptors on the other hand can be seen when various competitors are compared.

The assumption of the existence of three classes of binding sites on the receptors still does not sufficiently explain all of the data on competition with ^{131}I-acetyl-LDL binding since, as with the class III binding sites, the class I and class II binding sites can be further differentiated. Mildly oxidized LDL competes for 25%, 50% and 75% of ^{131}I-acetyl-LDL binding to the 35 kDa, 85 kDa and 12–15 kDa receptors respectively. Thus at the class I binding sites for ^{131}I-acetyl-LDL, mildly oxidized LDLs are partial inhibitors for ^{131}I-acetyl-LDL binding to the three receptors with different potencies. Also, class II binding sites classified above as binding only ^{131}I-Mal-BSA can be further differentiated when oxidized LDL and polyadenylic acid are compared for their inhibitory potency at the three receptor species. Our previous data using polyanionic competitors demonstrated total competition with ^{131}I-Mal-BSA binding at these sites by poly(vinyl sulphate) and polyinosinic acid. Neither mildly nor fully oxidized LDL further differentiates the class II binding sites on the three receptors, but polyadenylic acid competes with 75% of ^{131}I-Mal-BSA binding to the 35 kDa receptors, 50% more of the ^{131}I-Mal-BSA binding than is displaced by fully oxidized LDL (Ottnad *et al.*, 1990).

More recently, van Berkel *et al.* (1991) compared the uptake of hepatic acetyl-LDL and oxidized LDL in both endothelial and Kupffer cells *in vivo* and *in vitro*. They found that the classical scavenger receptors on the endothelial cells recognized acetyl-LDL and oxidized LDL. van Berkel *et al.* (1991) noted a 6,8-fold higher uptake of oxidized LDL than of acetyl-LDL, and an only partial competition of acetyl-LDL for binding and uptake of oxidized LDL by Kupffer cells. These researchers postulated in addition to a relatively smaller number of acetyl-LDL receptors, additional receptors for oxidized LDL that are highly concentrated on Kupffer cells. When we take the data of van Berkel's group into consideration and assume that the binding proteins characterized in the reconstituted system are truly active receptors as are expressed at the cellular surface of the Kupffer cells, we can speculate that the class III binding sites on these binding proteins might be the oxidized LDL receptors demonstrated by van Berkel *et al.* in their *in vivo* and *in vitro* studies with cultured cells. To analyse this possibility in a greater detail, quantification and competition analyses of the Kupffer cell receptors and endothelial cell receptors by ligand blotting and perhaps better defined reconstituted systems would be necessary.

Evidence to support the existence of multiple receptors for modified LDLs has been reported also by Arai *et al.* (1989) and Sparrow *et al.* (1989). The existence of at least three receptors has been suggested on cultured peritoneal macrophages, one specific receptor for ^{125}I-acetyl-LDL, another receptor for ^{125}I-(oxidized LDL), and a common receptor for both radioligands. In those studies, however, receptors were tested in intact cells and were not physically separated before ligand binding. Furthermore, ligand binding was not differentiated by using LDL particles oxidized to varying degrees.

Taken together, the results of our previous work (Ottnad *et al.*, 1990) and the results of this series of competition experiments with mildly and fully oxidized LDL as competitors suggest that the reduced hepatic scavenger receptors bind acetyl-LDL and Mal-BSA, and recognize mildy and fully oxidized forms of LDL. These hepatic binding proteins, which were highly enriched by affinity chromatography, separated by h.p.l.c. and visualized by ligand blotting as single bands on nitrocellulose strips after further separation by SDS/PAGE, appear to be a homogeneous population (Ottnad *et al.*, 1990). If the fractions are truly homogeneous, the binding proteins must contain several binding sites differing in ligand recognition. However, the fractions could also contain proteins with similar molecular masses which vary functionally but are not separated on SDS/polyacrylamide gels. If this possibility is true, the binding proteins for ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA could be a heterogeneous family of scavenger

receptors with similar molecular masses but functional differences. If the reconstituted system is not showing artefactual binding, then our experiments indicate an even greater diversity in ligand binding specificity for the hepatic scavenger receptor system as shown by Arai *et al.* (1989) and Sparrow *et al.* (1989) in their studies with mouse peritoneal macrophages.

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