Processing of acetylated human low-density lipoprotein by parenchymal and non-parenchymal liver cells

Involvement of calmodulin?

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(Received 28 June 1982/Accepted 11 August 1982)

1. Modified lipoproteins have been implicated to play a significant role in the pathogenesis of atherosclerosis. In view of this we studied the fate and mechanism of uptake *in vivo* of acetylated human low-density lipoprotein (acetyl-LDL). Injected intravenously into rats, acetyl-LDL is rapidly cleared from the blood. At 10 min after intravenous injection, 83% of the injected dose is recovered in liver. Separation of the liver into a parenchymal and non-parenchymal cell fraction indicates that the non-parenchymal cells contain a 30–50-fold higher amount of radioactivity per mg of cell protein than the parenchymal cells. 2. When incubated *in vitro*, freshly isolated non-parenchymal cells show a cell-association of acetyl-LDL that is 13-fold higher per mg of cell protein than with parenchymal cells, and the degradation of acetyl-LDL is 50-fold higher. The degradation of acetyl-LDL by both cell types is blocked by chloroquine (10–50 μ M) and NH_4Cl (10 mM), indicating that it occurs in the lysosomes. Competition experiments indicate the presence of a specific acetyl-LDL receptor and degradation pathway, which is different from that for native LDL. 3. Degradation of acetyl-LDL by non-parenchymal cells is completely blocked by trifluoperazine, penfluridol and chlorpromazine with a relative effectivity that corresponds to their effectivity as calmodulin inhibitors. The high-affinity degradation of human LDL is also blocked by trifluoperazine (100 μ M). The inhibition of the processing of acetyl-LDL occurs at a site after the binding-internalization process and before intralysosomal degradation. It is suggested that calmodulin, or a target with a similar sensitivity to calmodulin inhibitors, is involved in the transport of the endocytosed acetyl-LDL to or into the lysosomes. 4. It is concluded that the liver, and in particular non-parenchymal liver cells, are *in vivo* the major site for acetyl-LDL uptake. This efficient uptake and degradation mechanism for acetyl-LDL in the liver might form *in vivo* the major protection system against the potential pathogenic action of modified lipoproteins.

When the concentration of circulating lipoproteins rises to high levels in the plasma of man or experimental animals, lipid is deposited in macrophages throughout the body (Wurster & Zilversmit, 1971; Fredrickson *et al.*, 1978). This is especially evident in patients with familial hypercholesterolaemia (Goldstein & Brown, 1978). The cell types involved include the hepatic non-parenchymal cells

Abbreviations used: VLDL, very-low-density lipoprotein ($\rho < 1.006$ g/ml); LDL, low-density lipoprotein ($\rho = 1.019$ – 1.050 g/ml for rat LDL or 1.019 – 1.063 g/ml for human LDL); HDL, high-density lipoprotein ($\rho = 1.050$ – 1.13 g/ml for rat HDL).

and macrophages of the spleen, kidney, bone marrow, skin tendons and other organs (Chomette *et al.*, 1971; Goldstein & Brown, 1978).

The mechanism of the lipid deposition in the cells present in the atherosclerotic plaque has not yet been elucidated (Wurster & Zilversmit, 1971). When incubated with normal lipoproteins macrophages do not accumulate large amounts of cholesterol (Goldstein *et al.*, 1979; Brown *et al.*, 1980) and it has been proposed that modification of LDL, either by acetylation (Goldstein *et al.*, 1979) or by malonaldehyde treatment (Fogelman *et al.*, 1980; Shechter *et al.*, 1981), is necessary before the lipoprotein will

produce a deposition of a similar magnitude as seen under pathological conditions. When modified proteins are injected into animals they are cleared mainly by the liver non-parenchymal cells, which thus serve as the major scavenger site in the body (Kooistra *et al.*, 1979; Van Berkel, 1982). In view of the possible relevance of modified lipoproteins for the development of the atherosclerotic lesion it becomes of special interest to study the fate and uptake mechanism *in vivo* of these lipoproteins.

Recently we showed that the processing of acetyl-LDL by non-parenchymal liver cells is completely inhibited by the phenothiazine tranquilizer trifluoperazine, an inhibitor of calmodulin (Van Berkel *et al.*, 1981b). This suggested that calmodulin, or a still unknown target of trifluoperazine, is involved in the receptor-mediated endocytotic process (Levin & Weiss, 1977; Van Berkel *et al.*, 1981b). This idea is extended now by studying the effect of trifluoperazine on the processing of acetyl-LDL by parenchymal cells. Furthermore its effect on the handling of the unmodified LDL is studied. To specify the target for trifluoperazine in more detail, we determined the relative ability of two other potent inhibitors of calmodulin, penfluridol and chlorpromazine on the degradation of acetyl-LDL by non-parenchymal cells.

Experimental

Materials

Acetic anhydride was obtained from Merck, Darmstadt, Germany. [125 I]Iodide was purchased from NEN Chemicals, Dreieich, Germany and Ham's F-10 medium from Gibco (Europe) Paisley, Scotland, U.K. Penfluridol was a product of Janssen Pharmaceutica, Beerse, Belgium, and was dissolved in dimethyl sulphoxide. Trifluoperazine was a gift from Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A., and was dissolved in 20% propylene glycol. Another batch, in dihydrochloride form, was kindly donated by Rhône-Poulenc, Vitry, France. Pronase was obtained from Calbiochem, San Diego, CA, U.S.A. Collagenase (type I) and chloroquine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade.

Isolation, modification and labelling of LDL

Human LDL was isolated exactly as described by Redgrave *et al.* (1975). The density range was 1.019–1.063 g/ml. LDL was acetylated with repeated additions of acetic anhydride as described by Basu *et al.* (1976). LDL (1 ml; with a protein concentration between 1–10 mg/ml) in 0.15 M-NaCl/1 mM-EDTA/8 mM-phosphate buffer, pH 7.5, was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice/water bath.

Next, acetic anhydride was added in multiple small portions (2 μ l) over a period of 1 h. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min. The solution was then dialysed overnight at 6°C against buffer containing 0.15 M-NaCl, 1 mM-EDTA and 8 mM-phosphate buffer, pH 7.5. Both LDL and acetyl-LDL were iodinated at pH 10 by the ICl method (McFarlane, 1958) modified for lipoproteins by Langer *et al.* (1972). Free I $^-$ was removed by Sephadex G-50 filtration. The iodine/protein ratio was between 0.6 and 0.8 atoms/mol for both LDL and acetyl-LDL. Of the radioactivity in the preparation 3–4% was free, 3–5% was present in phospholipids and 92–94% was protein-bound. The only apoprotein present in LDL is apolipoprotein B, as determined on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Van Berkel *et al.*, 1981a).

Measurement of acetyl-LDL uptake by the rat liver cells *in vivo*

125 I-Acetyl-LDL (15 μ g) was injected at 09.00 h in chow-fed rats (250–350 g) under nembutal anaesthesia. After 3 min a blood sample was drawn from the retro-ocular sinus. At 10 or 30 min after injection, a liver perfusion with Ca $^{2+}$ -free Hank's medium was started. The liver was initially perfused at a flow rate of 4 ml/min. The flow rate was increased to 14 ml/min after cannulation of the caval vein. At 8 min after the start of the perfusion, a liver lobe was tied off to determine the total hepatic radioactivity. The remaining liver was perfused with 0.05% collagenase. Subsequently, parenchymal and non-parenchymal cells were isolated as described in detail previously (Van Berkel *et al.*, 1977; Van Berkel & Van Tol, 1978). The purity, composition and integrity of the different cell preparations were tested by phase-contrast light microscopy, by Trypan Blue exclusion, by peroxidase staining and by the relative distribution and specific activities of L- and M $_2$ -type pyruvate kinase. On the basis of light microscopy the final parenchymal-cell preparations were completely free from non-parenchymal cells and more than 95% of the isolated cells excluded Trypan Blue. The isolated cells were sensitive to glucagon in a similar way as a perfused liver system (Van Berkel *et al.*, 1978). The non-parenchymal cell preparation (termed 'NPC $_1$ '; Pronase method) excluded Trypan Blue almost completely and no parenchymal cells were present. By peroxidase staining with diaminobenzidine about 30% of the cells were peroxidase-positive, indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells (Knook & Sleyster, 1976). The purity of every individual cell preparation was also checked by determination of the distribution and activity of L-type and M $_2$ -type

pyruvate kinase, as described previously (Van Berkel *et al.*, 1977). In accordance with previous work (Crisp & Pogson, 1972; Van Berkel, 1974) the NPC₁ preparation contained solely M₂-type pyruvate kinase. The non-parenchymal cell preparation obtained by differential centrifugation (termed 'NPC₂') contained various amounts of parenchymal cell protein. This contamination by parenchymal cell protein can be corrected for, by measuring the pyruvate kinase activities of every individual cell preparation. The values for the uptake of acetyl-LDL *in vivo* in the NPC₂ fraction were corrected for the contamination by parenchymal cells as described by Van Berkel & Van Tol (1978). Peroxidase staining indicates that the NPC₂ preparation contains Kupffer cells and endothelial cells in a ratio of 1:7. Activity measurements of cathepsin D and peroxidase confirm the calculations mentioned above (Groot *et al.*, 1981).

Lipoprotein binding, uptake and degradation in vitro

Incubations of freshly isolated liver cells with the indicated amounts of lipoproteins and cells were performed in Ham's F-10 medium (modified), containing 5% (v/v) human lipoprotein-deficient serum (final protein concentration 2.5 mg/ml). The binding, uptake and degradation measurements *in vitro* were performed with the parenchymal- and non-parenchymal-cell preparations separated by differential centrifugation only ['PC' and 'NPC₂' (Van Berkel & Van Tol, 1978)]. No correction was made for the contamination of the NPC₂ preparation by parenchymal-cell protein. The incubations were carried out either in plastic tubes in a total volume of 1 ml or in 25 ml Erlenmeyer flasks (silicone-treated) stoppered with rubber caps with a total incubation volume and time as indicated in the legends to the Figures. At the indicated time 1 ml samples were withdrawn and the cell-associated radioactivity was determined as described by Van Berkel *et al.* (1981a). Degradation of the lipoproteins was measured by the method of Bierman *et al.* (1974) as described previously (Van Berkel *et al.*, 1981a). The degradation values represent radioactivity present in the acid-soluble water phase. In the corresponding blanks the lipoproteins were incubated in the absence of cells.

The viability of the cells during the long-term incubations was checked and remained higher than 85%. The viability was also checked after addition of chloroquine, NH₄Cl, trifluoperazine, penfluridol or chlorpromazine. It was found that at higher chloroquine or trifluoperazine concentrations (above 100 µM) the viability of the cells, especially at the longer incubation times, decreased significantly. With the concentrations reported in this paper no decrease in cell viability or cellular ATP level was

observed. When effectors were added, dissolved in dimethyl sulphoxide or propylene glycol, it was checked that a similar amount of the solvent did not influence the control values.

Results

Uptake of acetyl-LDL by parenchymal and non-parenchymal liver cells in vivo

In the rat, iodinated human LDL shows a monoexponential decay curve with a half-life of about 10 h (Van Tol *et al.*, 1978). On acetylation of human LDL the decay rate is strongly influenced and Table 1 shows that at 3 min after injection only 6% of the ¹²⁵I is still present in serum, and at 10 min this value is further decreased to 2%. The bulk of the radioactivity is recovered in the liver. Subsequent separation of the liver cells into a parenchymal and non-parenchymal cell fraction indicates that the non-parenchymal cells contain a more than 30-fold higher amount of radioactivity per mg of cell protein than the parenchymal cells (Table 2).

When the cells are isolated 30 min after injection of acetyl-LDL, a similar distribution is found. The data obtained 30 min after injection can be compared directly with the uptake of unmodified human LDL *in vivo* by the different cell types as reported previously (Van Tol & Van Berkel, 1980). Such a comparison indicates that on acetylation of LDL the radioactivity found in the parenchymal cells is increased three times, whereas the non-parenchymal cell radioactivity is increased 14 times.

Interaction of acetyl-LDL with parenchymal and non-parenchymal cells in vitro

Fig. 1 shows the time course of the cell-association and degradation of acetyl-LDL by freshly isolated parenchymal and non-parenchymal cells. The initial cell-association rate of acetyl-LDL is rapid and slows down after 30 min of incubation.

With non-parenchymal cells the time course of trichloroacetic acid-soluble radioactivity appearing

Table 1. *Distribution of radioactivity between liver and serum 3, 10 and 30 min after intravenous injection of acetylated human LDL*

Values are means of three different experiments ± S.E.M. The liver samples were obtained after 8 min pre-perfusion with Ca²⁺-free Hanks balanced salt solution.

Time after injection of acetyl-LDL (min)	Radioactivity distribution (% of injected dose)	
	Liver	Serum
3	—	6.0 ± 0.8
10	83.4 ± 1.7	2.2 ± 0.2
30	18.0 ± 1.2	8.4 ± 0.3

Table 2. Distribution of acetylated human LDL apoprotein between parenchymal and non-parenchymal liver cells 10 and 30 min after intravenous injection

Values are means of three to four different experiments $\pm$ S.E.M. The recovery values are expressed as percentages.

	$10^4 \times$ Distribution of acetylated LDL (% of injected dose/ mg of cell protein)	
	10 min	30 min
Whole rat liver	450 ± 19	89 ± 2
Parenchymal cells	20 ± 6	8 ± 2
Non-parenchymal cells (method 1)	639 ± 169	454 ± 126
Non-parenchymal cells (method 2)	453 ± 125	268 ± 71
Ratio of non-parenchymal cells (method 1) to parenchymal cells	32.9 ± 5.3	55.1 ± 8.1
Recovery of radioactivity in parenchymal and non-parenchymal cells (method 1) compared with whole rat liver	14.6 ± 3.3	46.9 ± 12.5

Fig. 1. Effect of chloroquine and NH_4Cl on the time course of cell-association and degradation of acetyl-LDL by isolated parenchymal (a) and non-parenchymal (b) liver cells

The cells were incubated with $10.1 \mu\text{g}$ of acetyl-LDL/ml in the absence ($\blacktriangle$ and $\triangle$) or presence of $100 \mu\text{M}$ -chloroquine ($\blacktriangledown$ and $\triangledown$) or 10 mM - NH_4Cl ($\bullet$ and $\circ$). The cell-association of acetyl-LDL is indicated with filled symbols, whereas for the degradation open symbols are used. The results are expressed as ng of apolipoprotein associated or degraded/mg of cell protein.

in the water phase shows a clear lag phase (10–30 min) before it reaches a constant rate (up to 3 h). With parenchymal cells, such a lag phase is not readily observed. Owing to the much lower degradation rate, a short-term aspecific degradation masks such a lag phase (Van Berkel *et al.*, 1981a). It can be seen that the degradation of acetyl-LDL by non-parenchymal cells is about 50 times higher than

by parenchymal cells, although the amount of cell-association is only 13-fold higher (per mg of cell protein). This very active degradation is totally blocked by both chloroquine ($100 \mu\text{M}$) or NH_4Cl (10 mM) as is the degradation by the parenchymal cells. (There is no increase in trichloroacetic acid-soluble radioactivity between 1 and 2 h of incubation.)

When native human LDL is incubated with parenchymal cells no high-affinity degradation is noticed (not shown). On incubation with non-parenchymal cells a high-affinity binding and degradation of human LDL is observed (Fig. 2). Both the amount of cell-associated radioactivity and the appearance of radioactivity in the trichloroacetic acid-soluble water phase is inhibited by about 50%

by an excess of unlabelled human LDL. This high-affinity degradation is inhibited nearly completely by NH_4Cl (10 mM) and chloroquine (100 μM).

The specificity of the acetyl-LDL processing by non-parenchymal cells was determined by investigating the effect of an excess of unlabelled acetyl-LDL, native human LDL, rat LDL and rat HDL on the cell-association process and degradation (Fig. 3).

Fig. 2. Effect of chloroquine, NH_4Cl and excess unlabelled LDL on the cell-association and degradation of native human LDL by non-parenchymal liver cells

The cells were incubated with 10.6 μg of LDL/ml in the absence (■ and □) or presence of 100 μM -chloroquine (▼ and ▽), 10 mM- NH_4Cl (● and ○) or 103 μg of unlabelled LDL/ml (▲ and △). At the indicated times samples were drawn and the amount of cell-associated radioactivity (a) as well as the radioactivity present in the acid soluble water phase were determined (b). Both cell-association and degradation are expressed as ng of apolipoprotein/mg of cell protein.

Fig. 3. Comparison of the ability of unlabelled lipoproteins to compete with the cell association (a) and degradation (b) of ^{125}I -labelled acetylated human LDL by non-parenchymal liver cells

Non-parenchymal cells were incubated for 2 h with 5.0 μg of ^{125}I -labelled acetyl-LDL/ml and with the indicated amounts of unlabelled human acetyl-LDL (△ and ▲), human native LDL (□ and ■), rat LDL (▼ and ▽) or rat HDL (○ and ●). ^{125}I -labelled apolipoprotein association or degradation is expressed as the percentage of the radioactivity obtained in the absence of unlabelled lipoprotein. The 100% value for the cell association is 632 ng of acetyl-LDL/mg of cell protein and for the degradation 876 ng of acetyl-LDL/mg of cell protein.

It is found that there is a competition between ^{125}I -acetyl-LDL and the unlabelled analogue both in the cell-association and degradation processes. These processes are not influenced by the presence of unlabelled native human LDL or rat LDL and HDL.

The dependency of the cell-association process on the concentration of both acetyl-LDL and unmodified LDL (Fig. 4) indicates that the non-parenchymal cells possess a high affinity for both the acetyl-LDL and native LDL with a half-maximal association rate at a concentration of about $20\text{ }\mu\text{g}$ of apolipoprotein/ml.

Effect of chloroquine, trifluoperazine and Ca^{2+} on the processing of acetyl-LDL

The relative abilities of chloroquine and trifluoperazine to influence the cell-association and degradation of acetyl-LDL by parenchymal and non-parenchymal cells are plotted in Figs. 5(a) and 5(b). Half-maximal inhibition of the degradation of acetyl-LDL occurs at 15 and $35\text{ }\mu\text{M}$ -chloroquine for non-parenchymal and parenchymal cells respectively.

The phenothiazine tranquillizer trifluoperazine appears to be a very active inhibitor of the degradation of acetyl-LDL both by parenchymal and non-parenchymal cells. A half-maximal effect is seen at about $20\text{ }\mu\text{M}$, and with $50\text{ }\mu\text{M}$ -trifluoperazine the maximal inhibitory effect is observed. The inhibitory effect of trifluoperazine on the degradation of acetyl-LDL is not caused by a blockade of the binding of acetyl-LDL to the cells, because the cell-association is only slightly influenced (Fig. 5a).

Fig. 4. Relation of the concentration of acetyl-LDL or native LDL to the extent of cell-association with non-parenchymal liver cells

The amount of labelled acetyl-LDL ($\blacktriangle$) or native LDL ($\blacksquare$) in the assay was varied as indicated and the amount of cell-associated radioactivity after 10 min of incubation was determined.

Also the high-affinity degradation of native human LDL is blocked by $100\text{ }\mu\text{M}$ -trifluoperazine (Table 3).

If calmodulin is involved in the processing of acetyl-LDL it is possible that an extracellular Ca^{2+} concentration change influences the degradation rate of acetyl-LDL. Fig. 6 shows that in the presence of Mg^{2+} -EGTA (2 mM) the degradation of acetyl-LDL is about half of that measured at 0.3 mM-Ca^{2+} . With parenchymal cells in the absence of Ca^{2+} or presence of Mg-EGTA a lower degradation rate is measured than that with $0.1\text{--}0.3\text{ mM-Ca}^{2+}$.

Table 3. Comparison of the effect of trifluoperazine, NH_4Cl and chloroquine on the high-affinity degradation of human LDL by non-parenchymal cells

The cells were incubated for 2 h with labelled human LDL ($9.9\text{ }\mu\text{g/ml}$) and the degradation was determined as trichloroacetic acid-soluble radioactivity in the water phase. The values are means ($\pm\text{S.E.M.}$) for three to four experiments. The 100% value represents $45 \pm 6\text{ ng}$ of acetyl-LDL degradation/mg of cell protein.

Addition	Degradation (% of control)
None	100
Trifluoperazine ($100\text{ }\mu\text{M}$)	51 ± 5
Chloroquine ($100\text{ }\mu\text{M}$)	48 ± 4
NH_4Cl (10 mM)	53 ± 3
Unlabelled LDL ($103\text{ }\mu\text{g/ml}$)	54 ± 5

Table 4. Relative contribution of binding to the total cell-associated radioactivity after 10, 60 and 120 min incubation of non-parenchymal cells with acetyl-LDL in the absence and presence of trifluoperazine or chloroquine

The cells were incubated for 10, 60 or 120 min with labelled acetyl-LDL ($10.1\text{ }\mu\text{g/ml}$). Subsequently the cells were centrifuged, washed and incubated in the absence and presence of collagenase (0.05%). The amount of cell-associated radioactivity was determined and the relative percentage released by collagenase was taken as cell-surface-bound and the values represent this percentage cell-surface binding. For further details see Van Berkel *et al.* (1981a). The values are means of at least two experiments. The 100% value for the incubation without further additions was for 10 min, 60 min and 120 min of incubation respectively 648 ± 112 , 1150 ± 121 and $1694 \pm 80\text{ ng}$ of acetyl-LDL/mg of cell protein ($n = 5$; means $\pm\text{S.E.M.}$). These values were not significantly different when trifluoperazine or chloroquine was present.

Addition	Cell-surface binding (%)		
	Incubation time (min) ... 10	60	120
None	50	32	17
Trifluoperazine	65	37	29
Chloroquine	60	33	19

Fig. 5. The effect of increasing chloroquine (a and c) or trifluoperazine (b and d) concentrations on the cell-association (a and b) and degradation (c and d) of acetyl-LDL by isolated parenchymal cells (PC) and non-parenchymal cells (NPC)

The cells were incubated for 2 h with $10.1 \mu\text{g}$ of acetyl-LDL/ml in the presence of the indicated amount of chloroquine or trifluoperazine. The results were obtained with three different acetyl-LDL and cell preparations and are given as mean percentages of the association or degradation in the absence of the effectors $\pm$ S.E.M. (indicated by the bars). The 100% value for cell-association with PC was 59 ± 7 and for NPC was 1211 ± 220 ng of acetyl-LDL/mg of cell protein. For the degradation the 100% value for PC was 44 ± 7 and for NPC 1462 ± 180 ng of acetyl-LDL/mg of cell protein ($n=3$; means $\pm$ S.E.M.)

To indicate more precisely the site of action of trifluoperazine we investigated to what extent the internalization of the acetyl-LDL particle is influenced by this compound.

Table 4 shows that internalization of acetyl-LDL still occurs in the presence of trifluoperazine. Furthermore we checked the possibility that trifluoperazine inhibits the excretion process of the degradation products of acetyl-LDL. The amount of cell-associated trichloroacetic acid-soluble radio-

activity was, however, not increased in the presence of trifluoperazine or chloroquine as compared with control incubations.

Effect of penfluridol and chlorpromazine

As well as trifluoperazine, other antipsychotic drugs acting as inhibitors of calmodulin are available (Levin & Weiss, 1979). Among these compounds penfluridol and chlorpromazine are most effective.

Fig. 6. The effect of Mg-EGTA and Ca²⁺ on the cell-association (a and c) and degradation (b and d) of acetyl-LDL by parenchymal (a and b) and non-parenchymal (c and d) cells

The cells were incubated for 2 h with 5.0 μ g of acetyl-LDL/ml with the indicated [Mg-EGTA] or the different [Ca²⁺]. For the cells incubated with Mg-EGTA the last two washings before incubation were also performed in the presence of 2 mM-Mg-EGTA. The results were obtained with four different acetyl-LDL and cell preparations and are given as mean percentages of the association or degradation at 0.3 mM-Ca²⁺ $\pm$ S.E.M. (indicated by the bars). The 100% values for the cell association were, for parenchymal cells 37.0 ± 6.3 and for non-parenchymal cells 955 ± 68 ng of acetyl-LDL/mg of cell protein. The 100% values for the degradation were, for parenchymal cells 22.5 ± 4.6 and for non-parenchymal cells 1216 ± 87 ng of acetyl-LDL/mg of cell protein ($n=4$; means $\pm$ S.E.M.).

Fig. 7. The effect of increasing penfluridol and chlorpromazine concentrations on the cell-association (a) and degradation (b) of acetyl-LDL by non-parenchymal liver cells

The cells were incubated for 2 h with $10.1 \mu\text{g}$ of acetyl-LDL/ml in the presence of the indicated amount of effector. The results were obtained with three different acetyl-LDL and cell-preparations and are given as mean percentages of the association or degradation in the absence of effectors $\pm$ S.E.M. (indicated by the bars) The 100% value for the cell-association was $1642 \pm 378 \text{ ng}$ of acetyl-LDL/mg of cell protein and for the degradation $1947 \pm 383 \text{ ng}$ of acetyl-LDL/mg of cell protein ($n = 3$; means $\pm$ S.E.M.).

Fig. 7 shows that, besides trifluoperazine, penfluridol and chlorpromazine are effective inhibitors of the degradation of acetyl-LDL by non-parenchymal cells, with half-maximal inhibitory concentrations of 12 and $35 \mu\text{M}$ respectively.

Discussion

The present results with ^{125}I -acetyl-LDL indicate that both parenchymal and non-parenchymal cells possess a site that recognizes acetyl-LDL. The competition experiments indicate that the acetyl-LDL recognition site is specific for acetyl-LDL, as no significant competition was observed with native human LDL or with the rat lipoproteins. Furthermore $10\text{--}100 \mu\text{g}$ of unlabelled acetyl-LDL/ml was effective in showing competition, even though a 25–250-fold excess of extracellular protein was present (approx. $2500 \mu\text{g}$ of protein/ml). According to the definition of Ho *et al.* (1976) these characteristics are indicative of the presence of a specific high-affinity receptor. The binding of acetyl-LDL to its receptor is effectively coupled to uptake, and after 10 min of incubation already half of the cell-associated radioactivity is internalized. Parenchymal cells also interact with acetyl-LDL, a binding that is similarly coupled to further intracellular processing. The amount of acetyl-LDL associated with non-parenchymal cells is, however, about 13-fold higher

per mg of cell protein than with parenchymal cells, with a degradation rate that is 50-fold higher. This indicates that, taking into account the relative protein contribution of non-parenchymal cells (7.5%) and parenchymal cells (92.5%) to total liver, the non-parenchymal liver cells are the major site for acetyl-LDL catabolism. That this is also the case *in vivo* can be concluded from the data on the uptake of acetyl-LDL by parenchymal and non-parenchymal cells *in vivo*.

At the moment it cannot be decided to what extent the different cell types present in the non-parenchymal cell preparations (endothelial or Kupffer cells) are responsible for the active interaction with acetyl-LDL. A further purification of the non-parenchymal cells by a procedure that does not affect the active endocytotic mechanism is therefore needed.

The present paper shows that once the acetyl-LDL is bound to its receptor on non-parenchymal cells, an efficient uptake and degradation process starts. This contrasts with previous data obtained with the native rat lipoproteins, of which the greater part (70–80%) remains extracellularly bound (Van Berkel *et al.*, 1981a; Ose *et al.*, 1980). The degradation of acetyl-LDL by freshly isolated non-parenchymal cells is completely blocked by low concentrations of chloroquine or NH_4Cl . These properties are consistent with a classical route for

receptor-mediated uptake, i.e. binding to a high-affinity receptor (specific for acetyl-LDL), uptake in endocytotic vesicles and degradation inside the lysosomes. A similar route can be described for the interaction of unmodified human LDL with non-parenchymal liver cells, although the amount of human LDL that is degraded relative to the amount that is cell-associated indicates a much less efficient intracellular processing. In the presence of chloroquine or NH_4Cl , the amount of acetyl-LDL associated with non-parenchymal cells at 2 h of incubation is similar to the amount obtained with the incubation in the absence of these agents. Because acetyl-LDL degradation hardly occurs, this indicates that the total amount of acetyl-LDL handled by the cells is considerably decreased. This might imply that chloroquine or NH_4Cl can also exert an effect on the receptor internalization or recycling. As shown in Table 4 the internalization of acetyl-LDL is not influenced by chloroquine, indicating that this additional action of chloroquine is exerted on the receptor recycling process.

In the present study we compared the relative ability of three of the most potent inhibitors of calmodulin (Levin & Weiss, 1979) on the processing of acetyl-LDL by non-parenchymal cells. The concentrations necessary for half-maximal inhibition of acetyl-LDL degradation were $12\mu\text{M}$ for penfluridol, $21\mu\text{M}$ for trifluoperazine and $35\mu\text{M}$ for chlorpromazine. The relative effectivity of these compounds to inhibit acetyl-LDL degradation corresponds to their effectivity as calmodulin inhibitors [half-maximal inhibition $2.5\mu\text{M}$ for penfluridol, $10\mu\text{M}$ for trifluoperazine and $42\mu\text{M}$ for chlorpromazine (Levin & Weiss, 1979)].

The site at which trifluoperazine interferes with the degradation of acetyl-LDL was investigated in more detail. Our data indicate that its complete inhibition of degradation cannot be explained by an effect on the initial binding or internalization process. The action of trifluoperazine is probably not at the level of the lysosome itself because the degradation of acetyl-LDL *in vitro* by cell homogenates at an acid pH is not inhibited by trifluoperazine (Van Berkel *et al.*, 1981b). Furthermore there is no accumulation of trichloroacetic acid-soluble radioactivity inside the cells, so that trifluoperazine does not exert its action on the secretion of the degradation products of acetyl-LDL. The action of trifluoperazine is then restricted either to the intracellular route from the internalization site of acetyl-LDL to the lysosomes or to the fusion process with the lysosomes. Although the relative effectivity of penfluridol, trifluoperazine and chlorpromazine on acetyl-LDL degradation can be considered as further evidence for the involvement of calmodulin, it must be stressed that these compounds do bind to calmodulin on a single site. Probably hydrophobic

regions in calmodulin are involved in this binding (Tanaka & Hidaka, 1980). Therefore it remains possible that the inhibition of acetyl-LDL degradation is either exerted at calmodulin or at a still unknown target with an active site similar to that of calmodulin.

As mentioned in the introduction section, the fate of the acetylated LDL *in vivo* was studied, in view of its possible relevance in the pathogenesis of atherosclerosis (Henriksen *et al.*, 1981). Both the uptake data *in vivo* and the data on the interaction of acetyl-LDL with the isolated liver cells *in vitro* indicate that the liver, and in particular the non-parenchymal liver cells, are the major site for acetyl-LDL uptake. Recently Henriksen *et al.* (1981) showed that native LDL can be converted by aortic endothelial cells into a form that is recognized by the macrophage receptor for acetyl-LDL. The presence of the highly active acetyl-LDL receptor in liver, as shown here, might form *in vivo* the major protection system against the potential pathogenic action of these modified lipoproteins.

We thank Mr. K. P. Barto for technical assistance and Miss A. C. Hanson for the preparation of the manuscript. Professor Dr. W. C. Hülsmann is thanked for critical reading of the manuscript. The Foundation for Fundamental Medical Research (FUNGO, grant 13-53-07) and the Dutch Heart Foundation (grant 79.001) are acknowledged for financial support.

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