INTERACTION OF LOW DENSITY LIPOPROTEINS WITH RAT LIVER CELLS

INTERAKTIE VAN LAGE DICHTHEIDS LIPOPROTEINEN MET LEVERCELLEN VAN DE RAT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. M.W. VAN HOF
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 19 JUNI 1985 TE 15.45 UUR

DOOR

LEENDERT HARKES

GEBOREN TE ZOETERWOUDE

1985

OFFSETDRUKKERIJ KANTERS B.V.,
ALBLASSERDAM

Begeleidingscommissie

Promotor: Prof. Dr. W.C. Hülsmann
Overige leden: Prof. Dr. J.C. Birkenhäger
Prof. Dr. M.B. Katan
Prof. Dr. H.R. Scholte

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Aan mijn ouders

Myra

CONTENTS

APPENDIX PAPERS

I Quantitative role of parenchymal and non-parenchymal liver cells in the uptake of [^{14}C]sucrose-labelled low density lipoprotein *in vivo*. Harkes, L. and Van Berkel, Th.J.C. Biochem. J. 224 (1984) 21-27.

II *In vivo* characteristics of a specific recognition site for LDL on non-parenchymal rat liver cells which differs from the 17α-ethinyl estradiol-induced LDL receptor on parenchymal liver cells. Harkes, L. and Van Berkel, Th.J.C. Biochim. Biophys. Acta 794 (1984) 340-347.

III Cellular localization of the receptor-dependent and receptor-independent uptake of human LDL in the liver of normal and 17α-ethinyl estradiol-treated rats. Harkes, L. and Van Berkel, Th.J.C. FEBS Lett. 154 (1983) 75-80.

IV A saturable, high-affinity binding site for human low density lipoprotein on freshly isolated rat hepatocytes. Harkes, L. and Van Berkel, Th.J.C. Biochim. Biophys. Acta 712 (1982) 677-683.

V Processing of acetylated human low-density lipoprotein by parenchymal and non-parenchymal liver cells. Involvement of calmodulin? Van Berkel, Th.J.C., Nagelkerke, J.F., Harkes, L. and Kruijt, J.K. Biochem. J. 208 (1982) 493-503.

VI *In vivo* and *in vitro* interaction of lipoprotein(a) with the apolipoprotein B,E and acetyl-LDL receptor on parenchymal and non-parenchymal rat liver cells. Harkes, L., Jürgens, G., Holasek, A., Nagelkerke, J.F. and Van Berkel, Th.J.C. Submitted for publication.

VOORWOORD

Het in dit proefschrift beschreven onderzoek werd uitgevoerd op de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam. Graag wil ik alle medewerk(st)ers van Biochemie I en iedereen die aan de totstandkoming van dit proefschrift heeft bijgedragen bedanken. Met name noem ik: Theo van Berkel, die als projectleider uitstekend voldeed; Cecile Hanson die de verschillende artikelen nauwgezet typte; Wim Hülsmann, die als promotor het proefschrift kritisch en snel beoordeelde; Kar Kruijt, die in tijden van zeer grote drukte, vakbekwaam de nodige technische assistentie verleende en Martha Wieriks, die zo virtuoos de tekstverwerker bespeelde. Ook de vruchtbare samenwerking met Günther Jürgens van de Karl-Franzens Universiteit te Graz dient zeker niet onvermeld te blijven.

De medewerkers van het Audio Visuele Centrum wil ik bedanken voor hun grafische en fotografische bijdragen en de leden van de begeleidingscommissie voor het snelle beoordelen van het proefschrift. Tenslotte dank ik mijn eerste Rotterdamse huisgenoten, Evelien en Helmer, voor hun morele steun tijdens het promotieonderzoek.

ABBREVIATIONS

ACAT	acyl-CoA: cholesterol acyltransferase
acetyl-LDL	acetylated low density lipoprotein
apo	apo(lipo)protein
β-VLDL	β-migrating very low density lipoprotein
CHD-LDL	1,2-cyclohexanedione treated low density lipoprotein
EGTA	ethyleneglycol-2-(2-aminoethyl)-tetracetic acid
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HDL_1	α_2-migrating high density lipoprotein
HDL_c	cholesterol-induced α_2-migrating high density lipoprotein
HMG-CoA	hydroxymethylglutaryl-coenzym A
IDL	intermediate density lipoprotein
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
Lp(a)	lipoprotein(a)
LPL	lipoprotein lipase
Me-LDL	reductive-methylated low density lipoprotein
VLDL	very low density lipoprotein
WHHL	Watanabe heritable hyperlipidemic

SAMENVATTING

Uitgebreid bevolkingsonderzoek naar het verband tussen cholesterolniveau's in het bloed en het ontstaan van hart- en vaatziekten enerzijds en de ontdekking van de "low density lipoprotein (LDL) receptor" anderzijds hebben geleid tot de overtuiging dat er een oorzakelijk verband bestaat tussen een hoog LDL cholesterol gehalte in het bloed en het ontstaan van atherosclerose. Uit onderzoek met patiënten die geen functionerende LDL receptor bezitten is naar voren gekomen dat de LDL receptor een sleutelrol speelt in de afbraak van LDL en in het handhaven van normale cholesterol niveau's. Men probeert op allerlei manieren de verhoogde LDL concentraties in het bloed van hypercholesterolemische patiënten te verlagen. Veel behandelingen zijn er op gericht de afbraak van LDL te stimuleren. Daar de lever het enige orgaan is dat in belangrijke mate cholesterol uit het lichaam verwijdert, wordt vooral een stimulatie van de opname van LDL door de lever nagestreefd.

Het in dit proefschrift beschreven onderzoek handelt over de rol van de lever en de verschillende leverceltypen in de afbraak van "low density" lipoproteïnen, waarbij de rat als proefdier is gebruikt. De kwantitatieve rol van de lever in de afbraak van LDL werd in vivo bepaald. Daar we de rol van de LDL receptor (ook wel apolipoproteïne-B,E receptor genoemd) wilden ophelderen, werd humaan LDL gebruikt omdat dit uitsluitend apolipoproteïne-B (apo-B) bevat. Hiermee wordt voorkomen dat er een interactie met de ook in de lever aanwezige apo-E receptor optreedt. Na intraveneuze inspuiting van met [^{14}C]sucrose gemerkt LDL werd de tijdsafhankelijke opname van radioactiviteit door de lever bepaald. Na opname en afbraak van het LDL blijft het [^{14}C]sucrose in de cel aanwezig en vormt daarmee een maat voor de kwantitatieve opname van LDL. Gevonden werd dat 70-80% van het LDL dat uit het serum verdwijnt, door de lever wordt opgenomen. De herkenning van LDL door de LDL receptor kan worden verhinderd door de lysine residuen van het LDL apoproteïne te methyleren. De opname van het gemethyleerde LDL (Me-LDL) door de lever is ongeveer 65% lager dan de opname van het natieve LDL. Geconcludeerd kan worden dat 65% van de LDL opname door de lever via de receptor verloopt.

De bijdrage van de parenchymale en niet-parenchymale cellen aan de opname van LDL in vivo is bepaald door de verschillende leverceltypen op verschillende tijdstippen na [^{14}C]sucrose LDL injectie te isoleren. De

niet-parenchymale cellen blijken voor tenminste 70% van de totale leveropname verantwoordelijk te zijn. Proeven met gemethyleerd LDL geven aan dat 79% van de niet-parenchymale celopname via de receptor verloopt. Wanneer de niet-parenchymale celfractie met behulp van centrifugale elutriatie verder wordt gescheiden in endotheel en Kupffercellen dan blijkt dat alleen de Kupffercellen verantwoordelijk zijn voor de receptor afhankelijke opname van LDL. Met de parenchymale cellen werd evenals met de endotheelcellen geen receptor afhankelijke opname van LDL vastgesteld.

De eigenschappen van de herkenningsplaatsen voor LDL zijn bepaald door gejodeerd LDL in te spuiten in ratten en vervolgens de levercellen na een relatief korte circulatietijd van LDL (30 min) te isoleren. Om te voorkomen dat er tijdens de leverperfusie en celisolatie een herverdeling van het geassocieerde radioactieve jodium plaats vindt, werden de leverperfusie en celisolatie uitgevoerd bij lage temperatuur ($8^{o}C$).

De arginine en lysine residuen van het apoproteïne van LDL zijn van essentieel belang zijn voor de herkenning door de LDL receptor van fibroblasten. Deze herkenning kan worden verhinderd door de arginine residuen te modificeren met cyclohexaandion of de lysine residuen te modificeren door middel van reductieve methylering. De associatie van deze gemodificeerde LDL deeltjes met de lever werd nu vergeleken met de associatie van het natieve deeltje. Gevonden werd dat de interaktie van LDL met de niet-parenchymale cellen wordt geremd door methylering maar niet door cyclohexaandion behandeling. Dit wijst erop dat de specifieke herkenningsplaats voor LDL op niet-parenchymale levercellen unieke LDL herkenningseigenschappen bezit. De regulatie van de LDL receptoren op parenchymale en niet-parenchymale cellen werd onderzocht door de ratten te behandelen met ethinyl estradiol of ethyl oleaat. Ethinyl estradiol behandeling van de ratten verhoogt (17-voudig) specifiek de associatie van LDL met de parenchymale cellen, terwijl het geen effect heeft op de associatie van LDL met niet-parenchymale cellen. De verhoogde interaktie van LDL met de parenchymale cellen wordt door zowel methylering als cyclohexaandion behandeling van LDL geremd, waaruit blijkt dat zowel arginine als lysine residuen nodig zijn voor de herkenning van LDL door de estradiol-geïnduceerde LDL receptor. De herkenning van LDL door de niet-parenchymale cellen was ook in deze experimenten alleen afhankelijk van de lysine residuen. De specifieke herkenningsplaats voor LDL op niet-parenchymale cellen verdwijnt na voorbehandeling van de ratten met ethyl oleaat. De herkenning van LDL door

parenchymale levercellen wordt daarentegen door deze behandeling niet beïnvloed. De specifieke beïnvloeding van de LDL herkenning door parenchymale cellen tengevolge van estradiol behandeling en door niet-parenchymale cellen tengevolge van ethyl oleaat behandeling wijzen erop dat er een onafhankelijke regulatie van de LDL receptoren op de verschillende leverceltypen bestaat.

De cellulaire verwerking van LDL werd onderzocht door levercellen die in vivo LDL hebben opgenomen te isoleren, waarna deze in vitro bij 37°C werden geïncubeerd. LDL blijkt voornamelijk afgebroken te worden door de niet-parenchymale cellen. Deze afbraak wordt gedeeltelijk geremd door chloroquine en ammonia, zodat aangenomen kan worden dat de lysosomen een rol spelen bij de afbraak. Na de oestrogeen behandeling van de ratten kon ook met de geïsoleerde parenchymale cellen een lysosomaal afbraakpad voor LDL worden vastgesteld.

Hoewel er een relatie is tussen het LDL niveau en atherosclerose is het niet mogelijk om schuimcellen, zoals aangetroffen in de atherosclerotische plaque, te verkrijgen door natief LDL met macrofagen te incuberen. Dit is wel mogelijk met biologisch of chemisch gemodificeerd LDL (bijvoorbeeld met azijnzuuranhydride) en er is dan ook gesuggereerd dat vooral de gemodificeerde vormen van LDL atherogeen zijn. Wanneer chemisch gemodificeerd LDL (acetyl-LDL) intraveneus wordt ingespoten in ratten, leidt dit tot een snelle opname door de lever en vooral de niet-parenchymale cellen blijken hiervoor verantwoordelijk. De opname van acetyl-LDL verloopt met behulp van de zogenaamde "scavenger" of "acetyl-LDL receptor" die vooral in de endotheelcelfractie van de lever verrijkt wordt aangetroffen. Of deze atherogene LDL deeltjes ook in vivo kunnen voorkomen is onduidelijk. Een aanwijzing voor het bestaan van een atherogene subklasse is afkomstig uit epidemiologisch onderzoek. Hierbij is aangetoond dat er een deeltje bestaat, "lipoprotein(a)" (Lp(a)) genaamd (een lipoproteïne met zowel apolipoproteïne-B als apolipoproteïne(a)) dat een risikofaktor voor atherosclerose vormt. Opnamestudies met Lp(a) in vivo en verdringingsproeven in vitro laten zien dat Lp(a) een interaktie kan aangaan met de acetyl-LDL receptor van leverendotheelcellen. Deze eigenschap van Lp(a) zou mogelijk een verklaring kunnen vormen voor het atherogene karakter van Lp(a).

De gevonden, kwantitatief belangrijke, receptor afhankelijke opname van acetyl-LDL en Lp(a) door de leverendotheelcellen, en van LDL door de

Kupffercellen leidt tot de conclusie dat een goed functioneren van de niet-parenchymale celtypen binnen de lever van groot belang kan zijn voor de bescherming van het lichaam tegen atherosclerose.

SUMMARY

Extensive epidemiological studies on the relation between plasma cholesterol levels and atherosclerosis and the discovery of the low density lipoprotein (LDL) receptor have led to evidence for a causal relation between a high LDL cholesterol level in the blood and coronary heart diseases. The key role of the LDL receptor in LDL catabolism and cholesterol homeostasis has become clear from studies with patients which lack a functional LDL receptor. Many attempts have been performed to decrease the elevated LDL levels from hypercholesterolemic patients. In this respect the attention is focused on treatments which stimulate LDL catabolism. An important beneficial role of the liver is expected because the liver is the only organ which can remove cholesterol irreversible from the circulation.

This thesis deals with the role of the liver and the various types of liver cells in the catabolism of low density lipoproteins, whereby the rat has been taken as experimental animal. The quantitative role of the liver in LDL catabolism was determined in vivo. As we wanted to clarify the role of the LDL receptor (also called apolipoprotein-B,E receptor) without interference with the apolipoprotein-E receptor, the solely apolipoprotein-B (apo-B) containing human LDL was used. The time dependent uptake of LDL by the liver was determined after intravenous injection of [^{14}C]sucrose-labelled LDL into rats. After uptake and degradation of LDL, [^{14}C]-sucrose remains entrapped in the lysosomes and so forms a cumulative measure for the uptake of LDL. It is found that from the LDL which is removed from serum, 70-80% is present in liver. Reductive methylation of the lysine residues of the LDL apoprotein blocks the interaction with the LDL receptor. The uptake of reductive methylated LDL (Me-LDL) with the liver is about 35% of that of native LDL, indicating that 65% of the liver uptake is receptor-dependent.

The quantitative contribution of the parenchymal and non-parenchymal liver cells to the in vivo uptake of LDL by the liver has been assessed by separation of the various liver cells at different times after injection of [^{14}C]sucrose-labelled LDL. The non-parenchymal cells are responsible for at least 70% of the total liver uptake. Experiments with methylated LDL indicate that 79% of this uptake is receptor-dependent. Separation of the non-parenchymal cell fraction into endothelial and Kupffer cells by

centrifugal elutriation makes it clear that the receptor-dependent uptake has been located solely on the Kupffer cells. With endothelial and parenchymal cells no receptor-dependent liver uptake of LDL could be observed.

The characteristics of the recognition sites for LDL has been determined by injection of iodine-labelled LDL and isolation of the cells at a relatively short time after injection (30 min). To minimalize loss or redistribution of [^{125}I]label, a low temperature (8^{o}C) liver perfusion and cell isolation procedure was applied.

As the arginine and lysine residues of the apolipoprotein are essential for the recognition of LDL by the LDL receptor on fibroblasts, either the arginine residues in LDL were modified by cyclohexadione treatment or the lysine residues were modified by reductive methylation. The association of native LDL was compared with that of the modified forms and the data show that the non-parenchymal cell-association of LDL is inhibited upon methylation but not upon cyclohexadione treatment of LDL. This indicates that non-parenchymal liver cells do possess a unique specific recognition site for LDL. In order to investigate the relation between the LDL receptors on parenchymal and non-parenchymal cells, rats were pretreated with ethinyl estradiol or ethyl oleate. It is found that ethinyl estradiol treatment of rats specifically increases the association of LDL to parenchymal cells (17-fold) and have no effect at all on the association to non-parenchymal cells. The interaction of LDL with the estrogen-induced recognition site on parenchymal cells is blocked by methylation or cyclohexadione treatment of LDL so indicating that the recognition of LDL by the induced recognition site is dependent on both the lysine and arginine residues. In the same experiments the recognition of LDL by non-parenchymal cells was only dependent on lysine residues. The specific recognition of LDL by the non-parenchymal cells disappeared upon ethyl oleate treatment of the rats, while the parenchymal cell recognition of LDL was not influenced under these conditions. The specific modulation of the LDL recognition by parenchymal cells upon estrogen treatment and by non-parenchymal cells upon ethyl oleate treatment indicate an independent regulation of LDL receptors in the various liver cell types.

The cellular processing of LDL was investigated by incubating the isolated cells <u>in vitro</u> while the cells were preloaded with LDL <u>in vivo</u>. It appears that in control rats mainly the non-parenchymal cells degrade LDL. The degradation of LDL by non-parenchymal cells was inhibited by

lysosomotropic agents suggesting that degradation at least partly occurs in the lysosomal compartment. In estrogen-treated animals a lysosomal degradation pathway was evident for both cell types.

Although LDL itself can be considered as atherogenic, in vitro formation of foam cells cannot be induced by incubating macrophages with native LDL. In contrast biologically or chemically modified LDL (for instance with acetic anhydride) can convert macrophages to cells with a foam cell like appearance and therefore it has been suggested that especially such modified forms of LDL are atherogenic. When chemically modified LDL (acetyl-LDL) is injected into rats, the liver and specifically non-parenchymal liver cells do clear these particles very fast from the circulation. The uptake of acetyl-LDL is mediated by the so-called scavenger or acetyl-LDL receptor which is highly enriched on liver endothelial cells. The in vivo occurrence of such atherogenic LDL particles is unclear. However, epidemiological studies have indicated that a certain lipoprotein subclass (called Lp(a), a lipoprotein which contains both apolipoprotein-B and apolipoprotein(a)) forms a risk factor for atherosclerosis. In vivo injection of Lp(a) into rats and in vitro competition studies indicated that Lp(a) can interact with this acetyl-LDL receptor in the liver. This property of Lp(a) might be related to the action of Lp(a) as an atherogenic lipoprotein.

The described quantitatively important, receptor-dependent uptake of acetyl-LDL and Lp(a) by the liver endothelial cells and LDL by the Kupffer cells lead to the conclusion that a proper functioning of the non-parenchymal celltypes inside the liver can play a crucial role in the protection of the body against atherosclerosis.

1. INTRODUCTION

1.1. LOW DENSITY LIPOPROTEINS AND ATHEROSCLEROSIS

Plasma neutral lipid transport is exerted by at least four discrete classes of lipoproteins: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The lipoproteins are composed of lipids and specific protein components, called apo(lipo)proteins (table 1). The lipoprotein molecule consists of two distinct domains, a lipid core of triglycerides and cholesterolesters with an outer shell of apoproteins, phospholipids and free cholesterol.

Table 1.

Composition of human lipoproteins*

Lipoprotein class	Triglycerides (% wt)	Phospholipids (% wt)	Free Cholesterol (% wt)	Esterified cholesterol (% wt)	Proteins (% wt)	Major apoproteins
Chylomicrons	80-95	3-6	1-3	2- 4	1- 2	A-I, A-IV, B, CI, CIII, E
VLDL	45-65	15-20	4-8	16-22	6-10	B, E, CI,CII, CIII
LDL	4- 8	18-24	6-8	45-50	18-22	B
HDL	2- 7	26-32	3-5	15-20	45-55	A-I, A-II, E

*From ref. 1.

During the last decade it has become clear that the plasma lipoproteins must be regarded as interrelated parts of one or more metabolic cycles. The pathways of lipoprotein formation, interconversion and catabolism in the body are complex, as summarized in some recent reviews (2-5). The major metabolic cycles are illustrated in fig. 1. Chylomicrons are produced in the intestine from dietary fat and contain mainly triglycer-

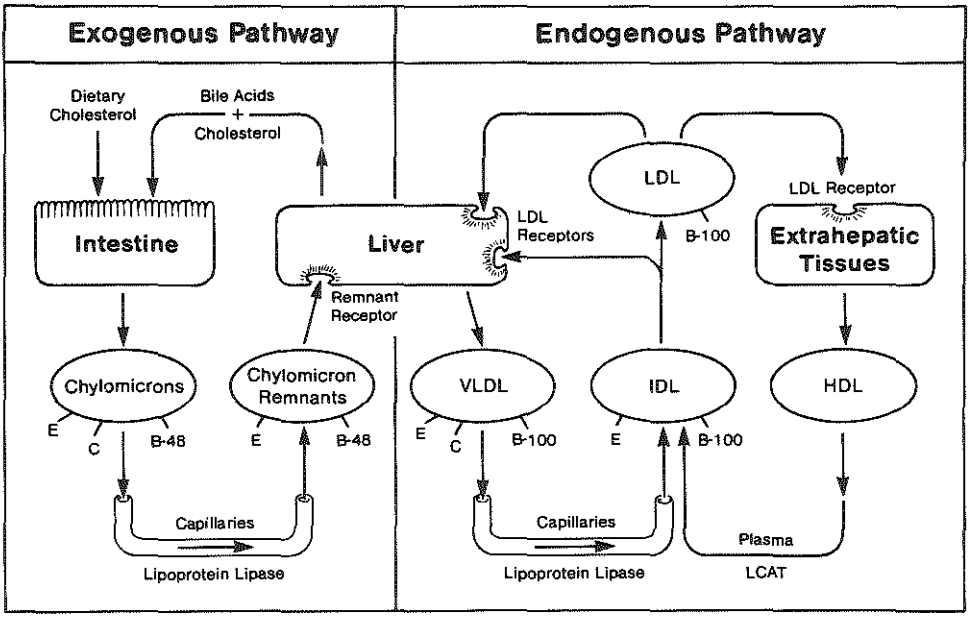

Fig. 1. Separate pathways for receptor-mediated metabolism of lipoproteins carrying endogenous and exogenous cholesterol. Abbreviations are as follows: LPL, lipoprotein lipase; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT, lecithin-cholesterol acyltransferase (scheme from ref. 6.).

ides. Chylomicrons are secreted in the lymph, enter the blood circulation through the thoracic duct and pass into the peripheral circulation. Here lipoprotein lipase (LPL) located on the surface of vascular endothelium, hydrolyses most triglycerides. After lipolysis, the remaining chylomicron remnants are rapidly cleared by the liver. VLDL is produced by the liver and just like the chylomicrons, the triglycerides are hydrolyzed by lipoprotein lipase and smaller VLDL-remnants are formed (also called intermediate density lipoproteins (IDL)). In normal humans about half of the VLDL remnants are directly removed by the liver. The remainder is converted to LDL, the major cholesterol carrier in human plasma. This particle is cleared from plasma by liver and extra hepatic cells. HDL serves as acceptor of the excess surface materials from the triglyceride depleted chylomicron and VLDL particles. The excess free cholesterol is esterified in the lecithine-cholesterol acyltransferase (LCAT) reaction and is transferred back to lower density lipoproteins through the action of a plasma cholesterylester exchange/transfer protein (3, 5). Furthermore it has been postulated that HDL is directly involved in the reverse transport of

cholesterol from various tissues to the liver (4).

As early as in 1913 Anitschkow (7) demonstrated that a high blood level of cholesterol in rabbits can produce atherosclerosis. Animal studies have shown that when plasma cholesterol levels are raised by cholesterol feeding this results in atherosclerosis (8, 9). Two thirds of the cholesterol in human plasma is present in LDL. Epidemiologic data support a relation between elevated LDL levels and the incidence of coronary heart disease in the human population (10, 11). The role of high LDL levels in the genesis of atherosclerosis is established by the human genetic disorder familial hypercholesterolemia (FH). It is inherited as an autosomal dominant disease. The homozygous form in which two abnormal genes are inherited is rare; about one per million people. Plasma cholesterol can reach 1000 mg/dl as a consequence of a six to eight times higher LDL level. Coronary atherosclerosis develops before the age of 20 years. Heterozygotes for this disorder are relative common, about one in 200-500 people. The heterozygote individuals have LDL levels that are two to three fold above normal. Severe atherosclerosis often becomes manifest in the third to fifth decade.

The molecular mechanism by which LDL or other lipoproteins are atherogenic is not elucidated. However several possible mechanisms are proposed: 1. Elevated LDL levels may damage vascular endothelial cells (12, 13) whereafter the smooth muscle cells will be directly exposed to LDL and other blood constituents. Platelet aggregation causes release of a growth factor which can stimulate smooth muscle cells to proliferate and secrete connective tissue matrix elements. Together with lipid infiltration, an atherosclerotic plaque might be generated. 2. Foam cells have been recognized as a characteristic feature of the atheroma. These cells are supposed to be derived from circulating monocytes (14, 15). Monocyte derived macrophages cannot readily catabolize native LDL but a chemically modified form (acetyl-LDL) is rapidly internalized with a concurrent change of the cells to a form which resembles the foam cells (16). Recently it has been demonstrated that LDL can be modified biologically by cultured endothelial cells, whereafter the modified LDL shows uptake characteristics by monocytes, similar to chemically modified LDL (17). Also uptake of β-VLDL (beta migrating VLDL) (a form of VLDL which accumulates in plasma of cholesterol-fed animals (18, 19) and patients with familial dysbetalipoproteinemia (Type III hyperlipoproteinemia) (20)) by macrophages leads to

accumulation of cholesterol (21, 22). These findings suggest that besides the major lipoproteins certain subclasses of lipoproteins can exist (modified LDL, β-VLDL) which can be considered as specifically atherogenic.

Firm evidence exists that lowering of plasma LDL will reduce the risk for coronary heart diseases in hypercholesterolemic patients (23, 24).

1.2. CATABOLISM OF LOW DENSITY LIPOPROTEINS

1.2.1. The LDL receptor

Comparison of the interaction of LDL with cultured fibroblasts of FH patients and healthy persons have led to the discovery of a specific binding site for LDL, the LDL receptor (2). This important observation, established by the studies of Goldstein and Brown, has advanced our understanding of lipoprotein metabolism and cholesterol homeostasis. Receptor mediated endocytosis of LDL is initiated by binding of LDL to a specific protein (fig. 2.), located in regions of the plasma membrane called "coated pits". The binding process of LDL is followed by invagination of the LDL containing pits into the cell, whereafter the formed vesicles migrate towards the lysosomes. After fusion with the lysosomes the protein component of LDL is degraded by proteases to amino acids while the choles-

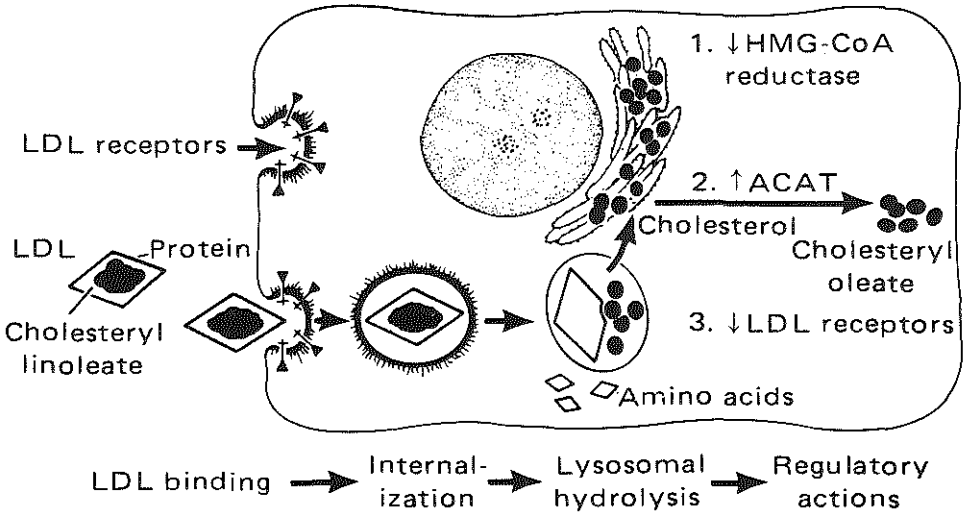

Fig. 2. The intracellular LDL pathway in cultured mammalian cells. HMG-CoA reductase denotes 3-hydroxy 3-methylglutaryl coenzyme A reductase; ACAT denotes acyl-CoA-cholesterol acyltransferase; vertical arrows suggest regulatory effects (scheme from ref. 25).

terylesters are hydrolysed by an acid lipase to fatty acids and cholesterol. When the need for cholesterol in the cells is met, the free cholesterol mediates feedback regulatory actions, whereby the further synthesis of LDL receptors is inhibited and the intracellular synthesis of cholesterol is blocked by the suppression of hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase). Furthermore the esterification of free cholesterol for storage is stimulated by an increase in the activity of acyl-CoA: cholesterol acyltransferase (ACAT) (for review see refs. 2, 3).

The number of LDL receptors on cells is an important determinant in the regulation of the LDL level in blood as clearly demonstrated by the increased LDL levels found in FH patients. In healthy persons two thirds of the LDL particles are metabolized by the specific receptor of liver cells and other body cells (26). In FH heterozygotes which possess half of the normal number of functional LDL receptors, LDL is removed from plasma at two thirds the normal rate, while in the homozygous form of FH no functional LDL receptors are present, resulting in an LDL removal from plasma at only one third the normal rate (26).

Elucidation of the role of LDL receptors for the turnover of LDL was also facilitated by the availability of a strain of rabbits with a genetic defect apparently identical to the LDL receptor defect in FH patients (27). This rabbit strain with spontaneous hypercholesterolemia and atherosclerosis was first described by Watanabe and therefore called Watanabe heritable hyperlipidemic (WHHL) rabbits (28). It has been demonstrated that these rabbits lack the LDL receptor also in the liver (29, 30). As a consequence changes in hepatic cholesterol metabolism occur; the receptor-independent uptake of LDL is <u>not</u> accompanied by a decrease in HMG-CoA reductase activity (30).

The LDL receptor has been purified to apparent homogeneity from membranes of the bovine adrenal cortex (31). In its functional form the receptor is a glycoprotein with an apparent moleculair weight of 160,000. Initially a precurser form is synthesized with an apparent molecular weight of 120,000. After synthesis, the 120,000 dalton precursor is converted to a 160,000 dalton mature form, which is inserted into the plasma membrane (32). The mature receptor on the cell surface recognizes the apoprotein-B component of LDL with high affinity. The receptor also interacts with apoprotein-E (33), hence the LDL receptor is also called the apo-B,E receptor. Apo-E is found in various lipoprotein subclasses namely

IDL, chylomicron remnants and in a subfraction of HDL called HDL_1 or HDL_c.

The importance of the apoproteins B or E for the interaction of lipoproteins with the apo-B,E receptor is established by determination of the effect of apoprotein modification on the receptor binding. Modification of a limited number of arginine residues of apo-B or E by 1,2-cyclohexanedione (34) or of lysine residues by acetoacetylation, reductive methylation or carbamylation (35) totally prevents LDL or HDL_c binding to the LDL receptor of fibroblasts. It must be mentioned that the 1,2-cyclohexanedione modification is completely reversible and it was shown that the binding properties of the LDL were reintroduced again after reversal of the modification (34).

1.2.2. The remnant receptor

In addition to the LDL receptor the liver contains a second lipoprotein receptor which recognizes apo-E. The presence of such a receptor was already suggested by the apparant normal clearance of of chylomicron-remnants in FH patients while also the apo-E levels in such patients were virtually normal. These observations suggested that a genetic distinct remnant (apo-E) receptor might exist for the uptake of apo-E containing lipoproteins (36). Binding studies with cell membranes established the existence of liver receptors that bind apo-E-HDL_c (HDL_c with only apo-E as apoprotein constituent), chylomicron remnants and HDL with apo-E but not apo-E free HDL or LDL (36-38). Further evidence for a distinct lipoprotein receptor, different from the apo-B,E receptor, has been derived from lipoprotein binding studies with intact liver cells (39). Studies with the WHHL rabbit finally confirmed the distinct genetic origin (40) of the apo-B,E and remnant receptor.

1.2.3. Lipoprotein receptors on macrophages

On macrophages two different lipoprotein receptors are suggested to play a role in the conversion of these cells to foam cells. By these two receptors, the macrophages are able to internalize a lot of cholesterol which can accumulate in the cytoplasm as cholesterylester droplets (21, 22, 41, 42). These droplets give the cytoplasm a foamy appearance in the electron microscope.

The two independent receptors are:

1. The acetyl-LDL receptor which interacts with acetylated LDL (16) malondialdehyde treated LDL (43) and biologically modified LDL (17) but not with unmodified LDL or β-VLDL.
2. A specific receptor for β-VLDL which does not interact with native LDL or acetyl-LDL (21, 22)

Ad. 1) Specific chemical modifications that abolish positive lysine residues and increase LDL's net negative charge can convert the lipoprotein into a ligand for the acetyl-LDL receptor. In addition, the ability to bind to the classic LDL receptor is inhibited (44). Binding to the acetyl-LDL receptor leads to a rapid internalization and degradation of the lipoprotein. However, cellular cholesterol accumulation does not lead to down regulation of the acetyl-LDL receptor so that the excessive cholesterylester accumulation can be induced (16). **Ad. 2)** β-VLDL is supposed to be generated from remnant particles when these particles are not adequately catabolized. This may be due to an overloading of the hepatic clearance mechanism, which happens in cholesterol-fed animals (18. 19), or to an abnormal recognition mark (apo-E) as observed in patients with familial dyslipoproteinemia (Type III hyperlipoproteinemia) (45). Upon in vitro incubation of macrophages with β-VLDL, cholesterylester accumulation is observed similarly as in in macrophages of cholesterol-fed animals or type III patients (4).

1.3. SCOPE OF THE THESIS

The quantitative role of the liver in LDL uptake was unclear at the start of the present investigations. Currently the liver was considered as a homogeneous tissue and a possible role of the non-parenchymal liver cells in lipoprotein catabolism was ignored. Although only 7.5% of liver protein is attributed to non-parenchymal cells, as many as 26.5% of the liver plasma membranes and 43% of the liver lysosomes are located in non-parenchymal cells (46). The non-parenchymal cells themselves also form a heterogeneous population of Kupffer cells, endothelial cells, fat storing cells and pit cells (46, 47). The Kupffer and the endothelial cells are in direct contact with the circulating blood, including lipoproteins. Initial studies from our laboratory (48) had indicated that after LDL injection in rats, non-parenchymal liver cells accumulate LDL 12 times as much LDL per

mg cell protein as parenchymal cells. The recovery of the total liver associated radioactivity in the isolated cells was however not quantitative and the relative importance of receptor-dependent and receptor-independent uptake was not indicated. Furthermore it was not possible to measure the accumulation of LDL at longer circulation times, as the radioactive degradation products of the iodinated lipoproteins escape rapidly from the cells. To elucidate the role of the apo-B,E receptor in the liver and the various liver cell types in LDL catabolism, without interference with the apo-E (remnant)receptor (38, 39, 49), we have studied the fate of an LDL particle which contains only apo-B. For reasons that rat LDL cannot be obtained in sufficient quantities in an apo-E free form, we used human LDL. In order to characterize the recognition sites on the various liver cell types, both in vivo and in vitro studies are performed. To discriminate between receptor-dependent and independent interaction, LDL was chemically modified. To indicate the independent regulation of the apo-B,E receptor activity on the various liver cell types, the rats were treated with various effectors. The degradation of LDL was taken into account, by labelling LDL with a radioactive marker ([^{14}C]sucrose) which accumulates in the lysosomes when the apoprotein is hydrolysed. By following the time-dependent cell-association of [^{14}C]sucrose it can be determined which cell types form an active catabolic site for LDL.

As mentioned before initial studies suggested that the non-parenchymal liver cells could play a quantitative important role in LDL catabolism. In addition also an important role of these cell types can be expected in acetyl-LDL or β-VLDL degradation. The non-parenchymal cells represent the greatest population of macrophages in the body (50), and some types of macrophages express active receptors for acetyl-LDL and β-VLDL (section 1.2.3.). In this study we used acetyl-LDL to test the possibility that the liver could play an important role in the uptake of this potentially atherogenic lipoprotein. Furthermore, the interaction of liver cells with another potentially atherogenic lipoprotein, lipoprotein(a) (Lp(a)), was investigated. This minor lipoprotein can be demonstrated in the blood of most people and its level is positively correlated with the occurence of coronary heart disease (51-54). It resembles LDL in lipid composition and apo-B content, however it possesses in addition a unique apolipoprotein(a) (55-57).

In summary: The thesis describes the role of the apo-B,E receptor, in the liver and the various liver cells, in the catabolism of low density lipoprotein. Furthermore the effectivity of liver in the uptake of potentially atherogenic lipoproteins like acetyl-LDL and lipoprotein(a) has been determined in order to establish to what extent the liver can be considered as a protection system for these lipoproteins. The specificity of the receptors, involved in the in vivo uptake of these lipoproteins, has been verified in vitro by studying the binding characteristics of the isolated liver cell types for LDL, acetyl-LDL and Lp(a).

2. EXPERIMENTAL WORK

2.1. QUANTITATIVE ROLE OF THE LIVER IN LDL CATABOLISM

Until recently no quantitative method was available to assess in vivo the contribution of the liver to LDL catabolism. The initial cell-association rate of radioiodinated LDL is not necessarily correlated with overall LDL uptake and catabolism. At longer circulation times of iodinated lipoproteins the degradation products (iodinated tyrosine or free iodine) will escape from the cell, and therefore determination of the radioactivity in the steady-state condition underestimates the total contribution of cells which avidly degrade LDL. Recently a technique became available which circumvents these problems (58). This technique is based on a method used to measure fluid endocytosis in cultured cells (59, 60). Radiolabelled sucrose is internalized by cells, once in the lysosomes it remains entrapped because it does not readily cross the lysosomal membrane while there is little sucrase activity in lysosomes (60, 61). According to this approach we coupled [^{14}C]sucrose covalently to the LDL apoprotein (58). After injection into rats the radioactive sucrose inside the cells then forms a cumulative measure for the uptake of LDL (58, 62, 63).

A discrimination between receptor-dependent and receptor-independent uptake can be made by modification of lysine residues of the LDL apolipoprotein by methylation, because the residues are involved in the recognition of the particle by the receptor. It has been reported that such a modification of the lysine residues prevents the association of LDL to the classical apo-B,E receptor (35). By comparing the uptake of native LDL and methylated LDL the difference in uptake will represent the quantitative contribution of this receptor.

After in vivo injection of [^{14}C]sucrose-labelled LDL or [^{14}C]sucrose-labelled reductive methylated LDL ([^{14}C]sucrose-labelled Me-LDL) the time dependent decay from serum and accumulation of [^{14}C]sucrose by the liver is determined (paper I). At the indicated time-intervals the liver is perfused with a cold (8^{o}C) Hanks' buffer, whereafter a lobule is tied off for determination of the uptake in whole liver. Table 2 shows the percentual uptake of LDL by the liver at various times after intravenous injection. At 24 hours after LDL injection 47% of the LDL which has been disappeared fom serum, can be found in liver. This value is comparable

Table 2.
Relative importance of the liver in accumulating screened [^{14}C]sucrose-labelled LDL at different times after injection.

Time after injection (h)	LDL cleared from serum (%)	LDL accumulated in liver (%)	Relative importance of the liver for the LDL decay (%)
2	25.1	20.1	80.1
4.5	48.7(30.8*)	33,7(12.1*)	69.3(39.4*)
12	70.4	34.7	49.3
24	86.7	40.5	46.8

*Value for [^{14}C]sucrose-labelled Me-LDL

with that reported by others (63, 64). For pig (65) and rabbit (66) the contribution of the liver to total LDL catabolism at 24 h after injection was also about 50%. However when the uptake of [^{14}C]sucrose LDL in liver is determined at respectively 2 and 4.5 h after injection, we observed that from the LDL which is removed from serum, 80% respectively 70% is present in liver. It appears that the linear uptake phase of [^{14}C]sucrose LDL during the first 4.5 h after injection is followed by a steady state level of [^{14}C]sucrose at prolonged circulation of LDL, probably indicating that the continuing uptake of [^{14}C]sucrose- labelled LDL is accompanied by a release of label from the cells (paper I, ref. 67). Release of [^{14}C]-sucrose from the total rat liver has been measured by Pittman et al. (63) and was reported to account for a loss of 10% of the total label per day from this organ to the bile. In addition release can be due to retro-endocytosis of LDL (68), a process by which LDL after uptake escapes degradation by re-excretion from the cells. A third possiblity is that a low sucrase activity in the lysosomes (61) will lead to hydrolysis of [^{14}C]sucrose to metabolizable products. Furthermore uptake of [^{14}C]-sucrose LDL may occur in cell types which show an active secretion of lysosomal constituents (69).

Although these aforementioned processes may influence the quantitative approach to assess the liver contribution in LDL catabolism at prolonged circulation time, it is clear that, as compared to iodine-labelled LDL, the liver accumulation of [^{14}C]sucrose-labelled LDL is substantially higher. With iodine-labelled LDL at any time after injection never more than 4% of the injected dose is recovered in liver. It can be argued that at circulation times of LDL up to 4.5 hours a quantitative determination of the liver contribution is possible, a time point at which about half of the LDL is cleared from the blood.

The quantitative importance of the LDL receptor for the uptake of LDL by the liver was determined at two time points after injection (30 min and 4.5 h). For both time points the association of methylated LDL to total liver is about 35% of that of unmodified LDL, indicating that 65% of the liver uptake is receptor-dependent.

2.2. INTERACTION OF LDL WITH PARENCHYMAL AND NON-PARENCHYMAL LIVER CELLS

In liver two different receptors for native lipoproteins are identified, the apo-B,E receptor and the apo-E receptor (for reviews see refs. 70, 71). In rabbits it was demonstrated that the apo-B,E receptor in liver can be regulated by the liver's demand for cholesterol. Receptor suppression occurs when a high cholesterol diet is consumed (37, 72). Conversely the amount of LDL receptors increase when hepatic cholesterol synthesis is blocked by the drugs compactin or mevinolin (73, 74), or when bile acid binding resins are given (75, 76). However, on isolated liver membranes of untreated rats it was impossible to demonstrate a high affinity receptor for LDL and only after treatment of the rats with 17α-ethinyl estradiol a specific binding site for human LDL was induced (77, 78). This binding site had similar properties as the apo-B,E receptor from fibroblasts (79).

It must be mentioned that these binding studies were performed at 0°C, a condition which not necessarily reflects the cell-association characteristics of LDL in vivo. We decided to characterize the properties of cell-association of LDL in vivo by intravenous injection of iodinated LDL preparations into the rats. Subsequently after a short circulation time (30 min) the liver cells are isolated by a procedure which prevents release of label from the cells and the amount of cell-associated radioactivity is determined. To assess the role of the various liver cells in the

processing of LDL, [^{14}C]sucrose-labelled LDL was used and the uptake of radioactivity was determined at a prolonged circulation time (4.5h).

The specificity of the interaction of human LDL with the various liver cell types and the saturation kinetics of cell-association were determined *in vitro* with freshly isolated cells. In addition, studies were performed in which the LDL was allowed to interact with the liver cells *in vivo*, whereafter *in vitro* the processing of the particle was followed.

2.2.1. **In vivo** association of LDL to liver cells.

Chemical modification of the arginine or lysine residues of apolipoprotein- B in human LDL with cyclohexanedione treatment or reductive methylation respectively, prevents LDL association to the apo-B,E receptor from fibroblasts (34, 35). We used cyclohexanedione-treated LDL (CHD-LDL) and reductive methylated LDL (Me-LDL) to investigate the nature and specificity of the recognition site for LDL on both parenchymal and non-parenchymal liver cells *in vivo*.

After intravenous injection of the radioiodinated lipoproteins into rats, the various liver cell types were isolated at 30 min after injection by a low temperature cell isolation technique, based on (80) and extensively described in paper I and (81). This low temperature technique was used in order to prevent degradation or redistribution of the lipoproteins during cell isolation. Pure parenchymal and pure non-parenchymal cells are obtained as checked microscopically and by determining the absence or specific presence of M_2-type pyruvate kinase in cell preparations (82). The validity of this newly developed technique, is further discussed at the end of this chapter.

It is found that reductive methylation of LDL inhibits the association of LDL to both parenchymal (66%) and non-parenchymal cells (44%) (Table IV in paper II), indicating that lysine residues are important for LDL recognition by both cell types. In contrast, cyclohexanedione treatment of LDL did not inhibit the cell association of LDL to non-parenchymal cells. These data indicate that apparently lysine residues on apo-B are important for the recognition of LDL by the non-parenchymal cells whereas arginine residues are not involved.

For reason that in rats 17α-ethinyl estradiol treatment leads to induction of a LDL receptor with characteristics similar to the fibroblast receptor (77-79), we decided to compare the properties of this receptor

with those of untreated rats. 17α-ethinyl estradiol treatment selectively increases the cell association of LDL to parenchymal cells (17-fold) leaving the non-parenchymal cell asssociation uninfluenced (paper III). The increased cell association of LDL to parenchymal cells is almost completely blocked by cyclohexanedione treatment of LDL (for 82%) or by reductive methylation of LDL (for 97%) (paper II). These data indicate that the arginine and lysine residues of LDL are essential for the recognition of LDL by the estrogen-induced LDL receptor on parenchymal cells, whereas in estrogen-treated rats for the recognition of LDL by the non-parenchymal cells still only lysine residues are essential. Furthermore these data indicate that an LDL receptor can be found in rats, with properties comparable to the LDL receptors on human fibroblasts, provided that the rats are pretreated with ethinyl estradiol. The difference in the interaction of Me-LDL and CHD-LDL with non-parenchymal cells might explain the slower decay of Me-LDL as compared to CHD-LDL as reported several times (75, 83-85).

The relative importance of the parenchymal and non-parenchymal liver cells for the receptor-dependent cell-association to total liver can be calculated on the basis of the cell-associated radioactivity and taking into account the composition of the liver. In estrogen-treated rats, parenchymal cells form the major tissue site for receptor-dependent cell-association of human LDL (92%). In contrast, in untreated rats the non-parenchymal cells are quantitatively more important and contribute for 57% to the total receptor-dependent cell-association of the liver.

Autoradiographic studies (86) also indicate an increased LDL uptake by parenchymal liver cells upon estrogen treatment of rats. Under these circumstances the total LDL uptake by non-parenchymal cells was only 10% of the total liver uptake, data comparable to ours. In the untreated liver the quantitatively important role for non-parenchymal liver cells could not be demonstrated in this system. The relative insensitivity of the autoradiographic method force the use of large doses of labelled LDL leading to serum LDL levels 10 times above the physiological range. Under these circumstances the cell-association may be largely unspecific. With our method, trace amounts of labelled LDL are used, whereafter both the receptor-dependent and independent cell-association of LDL to the various liver cells can be easily quantified. Similar results have been obtained when we injected 10 times more LDL (still in the physiologically range)

(see paper III).

Packard et al (83) and Slater et al (87) suggested that the reticulo endothelial cells could play a quantitatively important role in the receptor-independent catabolism of LDL (They defined the catabolism of CHD-LDL as receptor-independent). By blockade of the reticulo-endothelial system by ethyl oleate (88, 89), the LDL cholesterol level in rabbits increases by 33% (87). We determined the effect of ethyl oleate on the cell-association of LDL to both parenchymal and non-parenchymal cells.

The association of native LDL and CHD-LDL with non-parenchymal cells appears to be selectively decreased by ethyl oleate treatment and a cell-association level is measured comparable to that of Me-LDL. No effect of ethyl oleate treatment was observed on the association to parenchymal cells. These findings indicate that the LDL recognition sites on parenchymal and non-parenchymal cells differ in respect to both their regulatory respons and recognition properties.

The specific effect of estrogen on the cell-association of LDL to parenchymal cells and ethyl oleate on the cell-association to non-parenchymal cells form further evidence for the validity of the cold perfusion and cell isolation method. The validity of the applied low temperature method can now be justified on the following grounds: at 8°C, collagenase (0.05%) reduced minimally the amount of membrane associated lipoprotein to total liver in contrast to the situation at 37°C (see table I in paper II), while processing of lipoproteins hardly occurs at 8°C (69, 90). Consequently the recovery of the radioactivity in the isolated cells increases as compared to the method performed at 37°C and is now 100% (see table 1 in paper III). Specific modulation of the LDL association to the various liver cell types by 17α-ethinyl estradiol (parenchymal cells) or ethyl oleate treatment (non-parenchymal cells) can be demonstrated, forming circumstantial evidence for the absence of cross-contamination in the cell-preparations.

2.2.2. Catabolism of LDL by liver cells.

The cell association of lipoproteins is not necessarily coupled to cellular uptake and degradation of the apolipoproteins or may be coupled to it with varying efficiency. For instance during the vascular catabolism of chylomicrons, the apoproteins are not taken up by the endothelial cells while the lipid core is readily metabolized (91). To investigate to what

extent the initial cell-association of LDL is coupled to cellular uptake of the apolipoprotein we determined the processing of the *in vivo* internalized LDL. Thirty minutes after intravenous injection of the [^{125}I]-labelled LDL, the extracellulair associated LDL was removed by a short collagenase perfusion (37°C) of the liver (paper II). Subsequently the parenchymal and non-parenchymal cells were isolated, incubated in a Ham F-10 medium (37°C) and the release of LDL and the degradation products of LDL into the medium were measured. It appears that in control rats, mainly the non-parenchymal cells degrade LDL, and per mg of cell protein at least a 30-fold greater amount of degradation products of LDL are released into the medium than with parenchymal cells. This degradation is inhibited for about 50% by chloroquine, suggesting the involvement of the lysosomes. No effect of estrogen treatment is noticed on the release of intact or degraded LDL from non-parenchymal cells. In contrast, after estrogen treatment the parenchymal cells degrade LDL at a 15-fold increased rate, which degradation is for 30% inhibited by chloroquine, suggesting that at least partly it occurs in the lysosomes.

The important role of non-parenchymal liver cells in the degradation of LDL is further established *in vivo* by studies on the time dependent uptake of [^{14}C]sucrose-labelled LDL by the various liver cells (paper I).

As mentioned before [^{14}C]sucrose LDL can be used *in vivo*; at least up to 4.5 h after injection, as a cumulative measure for cell-association and uptake of LDL. When the time-dependent cell-association of [^{14}C]sucrose-labelled LDL is determined with parenchymal and non-parenchymal cells, it is clear that with both cell types the binding is about similarly coupled to uptake (Fig. 3, paper I). A calculation on the relative importance of the non-parenchymal cells for the uptake of LDL by total liver, indicates that these cells are for at least 70% responsible for the uptake. A comparison of the cellular uptake of [^{14}C]sucrose-labelled LDL and [^{14}C]-sucrose-labelled Me-LDL after 4.5 h circulation indicates that 79% of the uptake of LDL by non-parenchymal cells is receptor dependent. Within the non-parenchymal cells the Kupffer cell is the single cell type responsible for this receptor-dependent uptake. For parenchymal cells similarly as for endothelial cells no receptor-dependent uptake of LDL could be demonstrated.

Although the data on the receptor-dependent uptake of LDL by total liver are in agreement with the data of Pittman and coworkers (63, 64),

the distribution of the label between the various liver cell types is completely at variance. Pittman et al. (63, 64) suggest that the hepatocytes are quantitatively the most important cell type for LDL degradation. Unfortunately the authors do not report LDL uptake values for the non-parenchymal cells. Moreover no data are presented on the purity of their cell preparations. Our data on the relative importance of the non-parenchymal cells for LDL uptake are supported by the findings that in rabbits after blockade of the reticulo-endothelial system a rapid 33% increase in LDL cholesterol occurs (87).

In conclusion our in vivo and in vivo-in vitro data on the catabolism of LDL indicate that non-parenchymal liver cells (mainly Kupffer cells) not only bind, but also actively catabolize the apolipoprotein of LDL (about 70% of the total liver uptake of LDL). Furthermore we established that in untreated rats only the catabolism of LDL by Kupffer cells can be defined as receptor-dependent. The recognition site for LDL on non-parenchymal cells shows a unique recognition property, in that the arginine residues on LDL are not important for recognition, in contrast to the lysine residues. In this respect this recognition site differs from the classical LDL or apo-B,E receptor which can also be expressed in rats, specifically on hepatic parenchymal cells after estrogen treatment.

2.2.3. In vitro determination of binding sites for LDL on liver cells.

In order to determine the specificity and affinity of the LDL binding sites on both parenchymal and non-parenchymal cells we performed in vitro binding studies with freshly isolated parenchymal and non-parenchymal liver cells. These cell types were dissociated according to Seglen (92). The collagenase perfusion of the liver at 37°C was followed by differential centrifugation of the crude cell suspension at 8°C in order to separate parenchymal and non-parenchymal cells (48). There is a striking difference between human LDL and rat LDL in the interaction with both parenchymal or non-parenchymal rat liver cells. Cell association of rat LDL to both cell types reaches at least a 6 times higher level than with human LDL (paper IV, V and ref. 93). The difference in cell-association between rat and human LDL is probably due to a difference in apo-E content as it is shown that apo-E is an important determinant in the hepatic uptake of plasma lipoproteins (94) and can mediate an interaction with the so-called apo-E receptor (38, 39, 93, 95). In addition,apo-E containing

particles can interact with much greater affinity with the apo-B,E receptor than solely apo-B containing particles (96). The concentration dependency of the cell-association of human LDL with both parenchymal and non-parenchymal cells, clearly shows that high affinity binding sites for the only apo-B containing human LDL are present on these cells. The high affinity binding site on rat hepatocytes possesses a Kd of 2.6 x 10^{-8}M, a value comparable to that found with the receptor of liver membranes isolated from the estradiol-treated rat (79). However, the high-affinity binding of human LDL on rat hepatocytes is not very efficiently coupled to uptake and subsequent degradation of LDL because after one hour of incubation at 37°C still less than 30% of the cell-associated LDL is internalized and no evidence for any subsequent high affinity degradation is obtained (paper IV), In contrast, the cell-assocation of LDL with non-parenchymal cells, reaches a 6 times higher level as with parenchymal cells (expressed per mg cell protein), and is followed by degradation of the apolipoprotein (paper V). The high-affinity degradation of LDL by non-parenchymal cells is largely inhibited by 100 μM chloroquine or 10 mM NH_4Cl, indicating the involvement of the lysosomes in this process.

The properties of the human LDL binding site on rat hepatocytes are very similar to those of the human fibroblast receptor (2) in that the interaction of LDL with the cells is dependent on the extracellular Ca^{2+} concentration and that lipoproteins with either apo-B or apo-E compete with the association of the radiolabelled LDL. These in vitro studies give evidence that the binding site for human LDL as observed in vivo (paper I and II) is indeed a high-affinity binding site which shows an interaction both with apo-E and apo-B containing lipoproteins and can thus be defined as an apo-B,E receptor.

2.3. ROLE OF LIVER AND LIVER CELLS IN THE CATABOLISM OF ACETYLATED LDL AND LIPOPROTEIN(A).

The atheromatous lesions in patients with (familial) hypercholesterolemia are rich in cholesterol containing foam cells. Most of these cells are supposed to be derived from circulating monocytes (14). Since patients with homozygous FH lack the LDL receptor, the question arises how these

foam cells do obtain these cholesterol levels. When monocytes or macrophages are incubated with normal LDL, this lipoprotein is taken up very slowly and by in vitro incubations no cholesterol accumulation could be induced (16, 41). However, when LDL is modified by acetylation, malondialdehyde treatment or prolonged incubation with umbilical vein endothelial cells the modified lipoprotein is readily internalized and produces a cholesterol deposition comparable to that seen under pathological conditions (16, 41). Although acetylation of LDL destroys the ability of the lipoprotein to interact with the LDL receptor on fibroblasts (44), in macrophages this modified lipoprotein is recognized by the acetyl-LDL receptor which, due to its absent feedback regulation (16), can mediate excessive cholesterol deposition (41).

Untill now, no direct evidence is available for the presence of these potentially atherogenic lipoproteins in the circulation. However, Lp(a) a lipoprotein which can be demonstrated in small quantities in the blood of most people is positively correlated with the occurence of coronary heart disease (51-54), and no mechanism is known to indicate the reason why Lp(a) is a risk factor. A number of in vitro studies have shown that Lp(a), which contains both apo-B and apolipoprotein(a), binds to the apo-B,E receptors on fibroblasts (97-99), but also a contrasting view is reported (100). Untill now it is not clear to what extent, binding of Lp(a) to the apo B,E-receptor determines the in vivo turnover of this interesting lipoprotein, or that also other binding sites are involved.

The potential role of the liver as protection system against circulating atherogenic lipoproteins, made it interesting to investigate the uptake of Lp(a) and acetyl-LDL by the liver and the various liver cells.

2.3.1. Acetylated LDL.

Three minutes after intravenous injection of [^{125}I]-labelled acetyl-LDL into rats, already 94% of the [^{125}I]-label is removed from serum while at 10 min this value is 98% (paper V). The bulk of the radiolabelled acetyl-LDL 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 cell protein than the parenchymal cells. The separation of the cells was however effected by a liver perfusion at 37^{o}C with collagenase. Because at 37^{o}C the degradation or release from the cell-

membrane of the lipoproteins is not blocked, the recovery of the total liver-associated label in the subsequently isolated cells is low. Subsequent experiments performed in our laboratory (81) however confirm the already suggested important role of the non-parenchymal cells in acetyl-LDL uptake and it was reported that especially the liver endothelial cells actively metabolize this modified LDL.

In vitro experiments with isolated cells show that the degradation of acetyl-LDL by non-parenchymal cells is 50-fold higher per mg cell protein than by parenchymal cells. When the degradation of LDL or acetyl-LDL by non-parenchymal cells is compared, it appears that non-parenchymal cells degrade acetyl-LDL at a 50 times higher rate than native LDL (see also ref. 101). The very active degradation of acetyl-LDL by non-parenchymal cells occurs in the lysosomes because it is blocked by either chloroquine (50 μM) or NH_4Cl (10 mM). Competition experiments show that an excess of unlabelled human LDL, rat LDL or rat HDL does not compete for the [^{125}I]-labelled acetyl LDL binding in contrast to an excess of unlabelled acetyl-LDL, indicating that the acetyl-LDL binding site on non-parenchymal cells is specific for the modified lipoprotein. Recent studies (81, 102) have shown that within the non-parenchymal cell preparation also in vitro the endothelial liver cell is the main cell type that interacts with acetyl-LDL.

2.3.2. Lipoprotein(a)

Lp(a) is a rather labile lipoprotein and the properties of an Lp(a) preparation are easily influenced by the isolation method employed (103). We tested the properties of Lp(a) which was isolated by two different procedures (103, 104, paper VI). Lp(a) was isolated from pooled human sera; obtained from about 5-7 persons who were screened in advance, and appeared to be highly Lp(a) positive. The following experiments were performed in order to determine to what extent Lp(a) could interact in vivo with the apo-B,E receptor and/or acetyl LDL receptor. For a determination of the possible interaction of Lp(a) with the apo-B,E receptor the number of receptors was selectively increased in parenchymal liver cells by estrogen treatment of the rat. As mentioned before the interaction of LDL with apo-B,E receptor can be blocked by reductive methylation or cyclohexanedione treatment of the lipoprotein (34, 35). Cyclohexanedione treatment of Lp(a) results in a complete loss of specific binding to human

fibroblasts (99). This indicates that the apolipoprotein-B part of Lp(a) is responsible for the in vitro binding to the apo-B,E receptor. We used reductive methylation of Lp(a) to determine the role of lysine residues in the interaction of Lp(a) with the different cell types.

The different Lp(a) preparations were injected into rats and after 30 min circulation, variable amounts of Lp(a) were found to be associated to total liver (paper VI). To some extent the employed Lp(a) isolation method may be responsible for such a variation. However, independent of any isolation procedure it is clearly shown that Lp(a) is taken up to a higher extent than LDL by the liver endothelial cells. Because especially acetyl-LDL is rapidly taken up by liver endothelial cells (81), the observed change might be related to an acetyl-LDL like character of the particle. To investigate this possibility more clearly , in vitro competition studies between radiolabelled Lp(a) and unlabelled LDL or acetyl-LDL were performed (paper VI). For these studies an Lp(a) preparation was used with the highest uptake in endothelial cells. The data clearly indicate that the cell-association of Lp(a) to non-parenchymal cells can be inhibited even more efficiently by acetyl-LDL than by Lp(a) itself. These data at least allow the conclusion that Lp(a) can show a character which leads to recognition by the acetyl-LDL receptor. This behaviour appears to be influenced by the lysine residues of Lp(a) because methylation of Lp(a) largely blocks the high uptake of Lp(a) by the non-parenchymal liver cells. Upon estrogen treatment of the rat, the cell-association of Lp(a) to parenchymal cells is increased (paper VI), suggesting that Lp(a) is also recognized by the apo-B,E receptor, although much less efficiently than LDL. This conclusion is also derived from studies with fibroblasts (97-99).

The aforementioned data suggest that for Lp(a) a situation may exist, which can be compared with the malondialdehyde modification of LDL. Malondialdehyde modification of a few lysine residues of LDL strongly inhibits the interaction with the apo-B,E receptor. After modification of an increased number of lysine residues of LDL, an acetyl-LDL like character could be demonstrated (105). A comparable process has been demonstrated with the carbamylation of LDL (106). An analogous process might explain the present results. As compared to LDL, Lp(a) might be more susceptible to subtile changes in the environment of the lysine residues of the apolipoprotein leading to a more readily conversion of Lp(a) to an atherogenic

form which can be recognized by the acetyl-LDL receptor. Also Gianturco et al. (107) recently showed that unfiltered or aggregated Lp(a) can cause lipid accumulation in macrophages in contrast to filtered Lp(a). A ready induction of an acetyl-LDL character in Lp(a) in vivo might explain the positive correlation between the Lp(a) level in serum and coronary heart diseases.

3. CONCLUDING REMARKS

The most marked conclusion is the establishment of the important role of non-parenchymal cells in the catabolism of the low density lipoproteins by the rat liver. Because the liver is responsible for 70-80% of the removal of LDL from blood this conclusion can be extended to total LDL turnover. The relatively important role of the non-parenchymal liver cells in LDL uptake might be due to the low interaction of rat hepatocytes with human LDL, both in vivo and in vitro. This is in contrast to results obtained with hepatocytes from pig (108) and rabbit (30, 109). The role of non-parenchymal cells from pig, rabbit or human liver in LDL catabolism is unknown at the moment.

Beside the important role of the non-parenchymal cells in LDL catabolism, we also illustrate an important role of these cells in the uptake and degradation of the potentially atherogenic lipoproteins acetyl-LDL and lipoprotein(a). Just like acetyl-LDL and biologically modified LDL (110), Lp(a) is taken up by the endothelial liver cells to a higher extent than LDL, probably due to its interaction with the acetyl-LDL receptor. The receptor-dependent uptake of acetyl-LDL and Lp(a) by the liver endothelial cells and LDL by the Kupffer cells can have important consequences for the cholesterol metabolism in liver, because specifically receptor-dependent uptake regulates cholesterol synthesis and esterification (30, 108, 111).

The low temperature perfusion and cell isolation techniques have greatly improved the recoveries of the total liver-associated radioactivity in the isolated liver cell types. The applied method may be an important aid in future experiments on the role of parenchymal and non-parenchymal cells in lipoprotein catabolism, in which the consequence of the receptor-dependent and independent uptake of lipoproteins for cholesterol metabolism in the various liver cell types can be studied.

4. REFERENCES

1. **Breslow, J.L.** (1984) Circulation 69, 1190-1194
2. **Goldstein, J.L. and Brown, M.S.** (1977) Ann. Rev. Biochem. 46, 897-930
3. **Brown, M.S., Kovanen, P.T. and Goldstein, J.L.** (1981) Science 212, 628-635
4. **Mahley, R.W. and Innerarity, T.L.** (1983) Biochim. Biophys. Acta 737, 197-222
5. **Eisenberg, S.** (1983) Klin. Wochenschr. 61, 119-132.
6. **Goldstein, J.L., Kita, T. and Brown, M.S.** (1983) N. Engl. J. Med. 309, 288-296.
7. **Anitschkow, N. and Chalatow, S.** (1913) Centralbl. Allgem. Pathol. Pathol. Anat. 24, 1-9
8. **Katz, L.N. and Stamler, J.** (1953) Experimental Arteriosclerosis, Springfield Illinois, Charles C. Thomas.
9. **Wissler, R.W.** (1974) in: The Myocardium: Failure and Infarction, ed. Braunwald, E., New York, H.P. Publishing Co., Inc., 155-166
10. **Gordon, T., Castelli, W.P, Hjortland, M.C., Kannel, W.B. and Dauber, T.R.** (1977) JAMA 238, 497-499
11. **Wilson, P.W., Garrison, R.J., Castelli, W.P., Feinleib, M., McNamara, P.M. and Kannel, W.B.** (1980) Am. J. Cardiol. 46, 649-654
12. **Henriksen, T., Evensen, S.A. and Carlander, B.** (1979) Scand. J. Clin. Lab. Invest. 39, 361-368
13. **Ross, R. and Harker, L.** (1976) Science 193, 1094-1100
14. **Fowler, S., Shio, H. and Haley, W.J.** (1979) Lab. Invest. 41, 372-378
15. **Schaffner, T., Taylor, K., Bantucci, E.J., Fischer-Dzoga, K., Beenson, J.H., Glagov, S. and Wissler, R.** (1980) Am. J. Pathol. 100, 57-80
16. **Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S.** (1979) Proc. Natl. Acad. Sci. USA 76, 333-337
17. **Henrikson, T, Mahoney, E.M. and Steinberg, D.** (1981) Proc. Natl. Acad. Sci. USA 78, 6499-6503
18. **Shore, V.G., Shore, B. and Hart, R.G.** (1974) Biochemistry 13, 1579-1585
19. **Mahley, R.W.** (1979) Atherosclerosis Rev. 5, 1-34
20. **Fredrickson, D.S., Levy, R.I. and Lindgren, F.T.** (1968) J. Clin. Invest. 47, 2446-2457

21. **Goldstein, J.L, Ho, Y.K., Brown, M.S., Innerarity, T.L. and Mahley, R.W.** (1980) J. Biol. Chem. 255, 1839-1848

22. **Mahley, R.W., Innerarity, T.L., Brown, M.S., Ho, Y.K. and Goldstein, J.L.** (1980) J. Lipid. Res. 21, 970-980

23. The Lipid Research Clinics Coronary Primary Prevention Trial Results. I. (1984) JAMA 251, 351-364

24. The Lipid Research Clinics Coronary Primary Prevention Trial Results. II. (1984) JAMA 251, 365-374

25. **Brown, M.S. and Goldstein, J.L.** (1979) Proc. Natl. Acad. Sci. USA 76, 3330-3337

26. **Goldstein, J.L. and Brown, M.S.** (1983) in: The Metabolic Basis of Inherited Disease, eds. Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S, Goldstein, J.L., Brown, M.S., 5th ed. New York, McGraw-Hill, 672-712

27. **Tanzawa, K., Shimada, Y., Kuroda, M., Tsujita, Y., Arai, M. and Watanabe, Y.** (1980) FEBS Lett. 118, 81-84

28. **Watanabe, Y.** (1980) Atherosclerosis 36, 261-268

29. **Kita, T., Brown, M.S., Watanabe, Y. and Goldstein, J.L.** (1981) Proc. Natl. Acad. Sci. USA 78, 2268-2272

30. **Attie, A.D., Pittman, R.C., Watanabe, Y. and Steinberg, D.** (1981) J. Biol. Chem. 256, 9789-9792

31. **Schneider, W.J., Beisiegel, U., Goldstein, J.L. and Brown, M.S.** (1982) J. Biol. Chem. 257, 2664-2673

32. **Tolleshaug, H., Goldstein, J.L., Schneider, W.J. and Brown, M.S.** (1982) Cell 30, 715-724

33. **Innerarity, T.L. and Mahley, R.W.** (1978) Biochemistry 17, 1440-1447

34. **Mahley, R.W., Innerarity, T.L., Pitas, R.E., Weisgraber, K.H., Brown, J.H. and Gross, E.** (1977) J. Biol. Chem. 252, 7279-7287

35. **Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W.** (1978) J. Biol. Chem. 253, 9053-9062

36. **Mahley, R.W., Hui, D.Y., Innerarity, T.L. and Weisgraber, K.H.** (1981) J. Clin. Invest. 68, 1197-1206

37. **Hui, D.Y., Innerarity, T.L. and Mahley, R.W.** (1981) J. Biol. Chem. 256, 5646-5655

38. **Cooper, A.D., Erickson, S.K., Nutik, R. and Shrewsbury, M.A.** (1982) J. Lipid Res. 23, 42-52

39. **Van Berkel, Th.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A.** (1981) Biochim. Biophys. Acta 665, 22-33

40. **Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornick, C.A. and Havel, R.J.** (1982) Proc. Natl. Acad. Sci. USA 79, 3623-3627

41. **Brown, M.S., Goldstein, J.L., Krieger, M., Ho, Y.K. and Anderson R.G.W.** (1979) J. Cell Biol. 82, 597-613

42. **Brown, M.S., Ho, Y.K. and Goldstein, J.L.** (1980) J. Biol. Chem. 255, 9344-9352

43. **Fogelman, A.M., Shechter, I., Seager, J., Hokom, M., Child, J.S. and Edwards, P.A.** (1980) Proc. Natl. Acad. Sci. 77, 2214-2218

44. **Basu, S.K., Golstein, J.L., Anderson, R.G.W. and Brown, M.S.** (1976) Proc. Natl. Acad. Sci. USA 73, 3178-3182

45. **Utermann, G., Jaeschke, M. and Menzel, J.** (1975) FEBS Lett 56, 352-355

46. **Blouin, A., Bolender, R.P. and Weibel, E.R.** (1977) J. Cell Biol. 72, 441-455

47. **Wisse, E., Van 't Noordende, J.M., Van der Meulen, J. and Daems, W.Th.** (1976) Cell Tiss. Res. 173, 423-435

48. **Van Berkel, Th.J.C. and Van Tol, A.** (1978) Biochim. Biophys. Acta 530, 299-304

49. **Sherrill, B.C., Innerarity, T.L. and Mahley, R.W.** (1980) J. Biol. Chem. 255, 1804-1807

50. **Saba, T.M.** (1970) Arch. Inter. Med. 126, 1031-1052

51. **Berg, K.** (1963) Acta Pathol. Microbiol. Scand. 59, 369-382

52. **Berg, K., Dahlen, G. and Frick, M.H.** (1974) Clin. Genet. 6, 230-235

53. **Dahlen, G., Berg, K., Ramberg, U. and Tamm, A.** (1974) Acta Med. Scand. 196, 327-331

54. **Albers, J.J., Adolphson, J.L. and Hazzard, W.R.** (1977) J. Clin. Invest. 18, 331-338

55. **Simons, K., Ehnholm, C., Renkonen, O. and Bloth, B.** (1970) Acta Pathol. Microbiol. Scand. sect. B. 78, 459-466

56. **Ehnholm, C, Garoff, H., Renkonen, O. and Simons, K.** (1972) Biochemistry 11, 3229-3232

57. **Jürgens, G. and Kostner, G.M.** (1975) Immunogenetics 1, 560-574

58. **Pittman, R.C., Green, S.R., Attie, A.D. and Steinberg, D.** (1979) J. Biol. Chem. 254, 6876-6879

59. **Wagner, R. Rosenberg, M. and Estensen, R.** (1971) J. Cell Biol. 50, 804-817

60. **Cohn, Z.A. and Ehrenreich, B.A.** (1969) J. Exp. Med. 129, 201-225

61. **Horvat, A.** (1973) Biochim. Biophys. Acta 297, 413-422

62. **Tolleshaug, H. and Berg, T.** (1981) Exp. Cell Res. 134, 207-217

63. **Pittman, R.C., Attie, A.D., Carew, T.E. and Steinberg, D.** (1982) Biochim. Biophys. Acta, 710, 7-14

64. **Carew, T.E., Pittman, R.C. and Steinberg, D.** (1982) J. Biol. Chem. 257, 8001-8008

65. **Pittman, R.C., Attie, A.D., Carew, T.E. and Steinberg, D.** (1979) Proc. Natl. Acad. Sci. USA 76, 5345-5349

66. **Pittman, R.C., Carew, T.E., Attie, A.D., Witzum, J.L., Watanabe, Y. and Steinberg, D.** (1982) J. Biol. Chem. 257, 7994-8000

67. **Bhattacharya, S,. Balasubramanian, S. and Simons, L.A.** (1984) Biochem. J. 220, 330-336

68. **Aulinskas, T.H., Van der Westhuyzen, D.R., Bierman, E.L., Gevers, W. and Coetzee, G.A.** (1981) Biochim. Biophys. Acta 664, 255-265

69. **Silverstein, S.C., Steinman, R.M. and Cohn, Z.A.** (1977) Ann. Rev. Biochem. 46, 669-722

70. **Attie, A.D., Pittman, R.C. and Steinberg, D.** (1982) Hepatology 2, 269-281

71. **Brown, M.S. and Goldstein, J.L.** (1983) J. Clin. Invest. 72, 743-747

72. **Kovanen, P.T., Brown, M.S., Basu, S.K., Bilheimer, D.W. and Goldstein, J.L.** (1981) Proc. Natl. Acad. Sci. USA 78, 1396-1400

73. **Mabuchi, H., Haba, T., Tatami, R., Miyamoto, S., Sakai, Y., Wakasugi, T., Watanabe, A., Koizumi, J. and Takeda, R.** (1981) N. Engl. J. Med. 305, 478-482

74. **Bilheimer, D.W., Grundy, S.M., Brown, M.S. and Goldstein, J.L.** (1983) Proc. Natl. Acad. Sci. USA 80, 4124-4128

75. **Shepherd, J., Packard, C.J., Bicker, S., Lawrie, T.D.V. and Morgan, H.G.** (1980) N. Engl. J. Med. 302, 1219-1222

76. **Kovanen, P.T., Bilheimer, D.W., Goldstein, J.L., Jaramillo, J.J. and Brown, M.S.** (1981) Proc. Natl. Acad. Sci. USA 78, 1194-1198

77. **Kovanen, P.T., Brown, M.S. and Goldstein, J.L.** (1979) J. Biol. Chem. 254, 11367-11373

78. **Chao, Y.-s., Windler, E.E., Chen, G.C. and Havel, R.J.** (1979) J. Biol. Chem. 254, 11360-11366

79. **Windler, E.E.T., Kovanen, P.T., Chao, Y.-s., Brown, M.S., Havel, R.J. and Goldstein, J.L.** (1980) J. Biol. Chem. 255, 10464-10471

80. **Praaning-Van Dalen, D.P. and Knook, D.L.** (1982) FEBS Lett. 141, 229-232

81. **Nagelkerke, J.F., Barto, K.P. and Van Berkel, Th.J.C.** (1983) J. Biol. Chem. 258, 12221-12227

82. **Van Berkel, Th.J.C., Kruijt, J.K., Slee, R.G. and Koster, J.F.** (1977) Arch. Biochem. Biophys. 197, 1-7

83. **Packard, C.J., Slater, H.R., and Shepherd, J.** (1982) Biochim. Biophys. Acta 712, 412-419

84. **Slater, H.R., Packard, C.J. and Shepherd, J.** (1982) J. Lipid. Res. 23, 92-96

85. **Steinbrecher, U.P., Witzum, J.L., Kesaniemi, Y.A. and Elam, R.L.** (1983) J. Clin. Invest. 71, 960-964

86. **Chao, Y.-s., Jones, A.L., Hradek, G.T., Windler, E.E.T. and Havel, R.J.** (1981) Proc. Natl. Acad. Sci. USA 78, 597-601

87. **Slater, H.R., Packard, C.J. and Shepherd, J.** (1982) J. Biol. Chem. 257, 307-310

88. **Stuart, A.E., Biozzi, G., Stiffel, C., Halpern, B.N. and Mouton, D.** (1960) Br. J. Exp. Pathol. 41, 599-604

89. **Conning, D.M. and Heppleston, A.G.** (1966) Br. J. Exp. Pathol. 47, 388-400

90. **Steinman, R.M., Silver, J.M. and Cohn, Z.A.** (1974) J. Cell Biol. 63, 949-969

91. **Fielding, C.J., Vlodavsky, I., Fielding, P.E. and Gospodarowicz, D** (1979) J. Biol. Chem. 254, 8861-8868

92. **Seglen, P.O.** (1973) Exp. Cell Res. 82, 391-398

93. **Van Berkel, Th.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A.** (1980) Biochem. Biophys. Res. Commun. 92, 1002-1008

94. **Shellburne, F., Hanks, J., Meyers, W. and Quarfordt, S.** (1980) J. Clin. Invest. 65, 652-658

95. **Mahley, R.W.** (1978) in: Disturbances in Lipid and Lipoprotein Metabolism, eds. Dietschy, J.M., Gotto Jr., A.M. and Ontko, J.A., American Physiological Society, Bethesda Maryland, 181-197

96. **Pitas, R.E., Innerarity, T.L., Arnold, K.S. and Mahley, R.W.** (1979) Proc. Natl. Acad. Sci. USA 76, 2311-2315

97. **Havekes, L., Vermeer, B.J., Brugman, T. and Emeis, J.** (1981) FEBS Lett. 132, 169-173

98. **Floren, C.H., Albers, J.J. and Bierman, E.L.** (1981) Biochem. Biophys. Res. Commun. 102, 636-639

99. **Krempler, F., Kostner, G.M., Roscher, A., Haslauer, F., Bolzano, K. and Sandhofer, F.** (1983) J. Clin. Invest. 71, 1431-1441

100. **Maartman-Moe, K. and Berg, K.** (1981) Clin. Genet. 20, 352-362

101. **Van Berkel, Th.J.C., Nagelkerke, J.F. and Kruijt, J.K.** (1981) FEBS Lett. 132, 61-66

102. **Blomhoff, R., Drevon, C.A., Eskild, W., Helgerud, P., Norum, K.R. and Berg, T.** (1984) J. Biol. Chem. 259, 8898-8903

103. **Gaubatz, J.W., Heideman, C., Gotto Jr., A.M., Morrisett, J.D. and Dahlen, G.H.** (1983) J. Biol. Chem. 258, 4582-4589

104. **Eigner, D., Schurz, J. Jürgens, G. and Holasek, A.** (1979) FEBS Lett. 106, 165-168

105. **Haberland, M.E., Fogelman, A.M. and Edwards, P.A.** (1982) Proc. Natl. Acad. Sci. USA 79, 1712-1716

106. **Gonen, B., Cole, T. and Hahm, K.S.** (1983) Biochim. Biophys. Acta 754, 201-207

107. **Gianturco, S.H., Bradley, W.A., Dahlen, G.H., Gaubatz, J.W., Gotto Jr., A.M., Morrisett, J.D. and Peavy, D.L.** (1983) Arteriosclerosis 3, 500a, abstr.

108. **Pangburn, S.H., Newton, R.S., Chang, C.-M., Weinstein, D.B. and Steinberg, D.** (1981) J. Biol. Chem. 256 3340-3347

109. **Soltys, P.A. and Portman, O.W.** (1979) Biochim. Biophys. Acta 574, 505-520

110. **Nagelkerke, J.F., Havekes, L., Van Hinsbergh, V.W.M. and Van Berkel, Th.J.C.** (1984) Arteriosclerosis 4, 256-264

111. **Attie, D., Pittman, R.C. and Steinberg, D.** (1980) Proc. Natl. Acad. Sci. USA 77, 5923-5927

APPENDIX PAPER I

Biochem. J. (1984) **224**, 21–27
Printed in Great Britain

Quantitative role of parenchymal and non-parenchymal liver cells in the uptake of [^{14}C]sucrose-labelled low-density lipoprotein *in vivo*

Leen HARKES and Theo J. C. VAN BERKEL
Department of Biochemistry I, Faculty of Medicine, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

(*Received 2 April 1984/Accepted 26 July 1984*)

1. In order to assess the relative importance of the receptor for low-density lipoprotein (LDL) (apo-B,E receptor) in the various liver cell types for the catabolism of lipoproteins *in vivo*, human LDL was labelled with [^{14}C]sucrose. Up to 4.5h after intravenous injection, [^{14}C]sucrose becomes associated with liver almost linearly with time. During this time the liver is responsible for 70–80% of the removal of LDL from blood. A comparison of the uptake of [^{14}C]sucrose-labelled LDL and reductive-methylated [^{14}C]sucrose-labelled LDL ([^{14}C]sucrose-labelled Me-LDL) by the liver shows that methylation leads to a 65% decrease of the LDL uptake. This indicated that 65% of the LDL uptake by liver is mediated by a specific apo-B,E receptor.
2. Parenchymal and non-parenchymal liver cells were isolated at various times after intravenous injection of [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL. Non-parenchymal liver cells accumulate at least 60 times as much [^{14}C]sucrose-labelled LDL than do parenchymal cells when expressed per mg of cell protein. This factor is independent of the time after injection of LDL. Taking into account the relative protein contribution of the various liver cell types to the total liver, it can be calculated that non-parenchymal cells are responsible for 71% of the total liver uptake of [^{14}C]sucrose-labelled LDL. A comparison of the cellular uptake of [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL after 4.5h circulation indicates that 79% of the uptake of LDL by non-parenchymal cells is receptor-dependent. With parenchymal cells no significant difference in uptake between [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL was found. A further separation of the non-parenchymal cells into Kupffer and endothelial cells by centrifugal elutriation shows that within the non-parenchymal-cell preparation solely the Kupffer cells are responsible for the receptor-dependent uptake of LDL. It is concluded that in rats the Kupffer cell is the main cell type responsible for the receptor-dependent catabolism of lipoproteins containing only apolipoprotein B.

The liver plays a key role in lipoprotein metabolism because it is the only organ that can eliminate cholesterol from the body (Langer *et al.*, 1970; Lindstedt, 1970). Studies on the contribution of the various tissues to LDL catabolism indicate that the liver is responsible for about 50% of the LDL turnover in rat (Pittman *et al.*, 1982) and in the pig (Pittman *et al.*, 1979*a*). In those studies the apolipoprotein B in LDL was labelled with [^{14}C]sucrose, and it was suggested that upon apolipoprotein B degradation the [^{14}C]sucrose remains trapped intracellularly and forms a cumulative measure for the uptake of LDL (Pittman *et al.*, 1979*b*; Tolleshaug & Berg, 1981; Pittman *et al.*, 1982). By comparing the uptake of native [^{14}C]-sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL it is possible to assess the involvement of specific LDL receptors in the cellular uptake, because methylation of LDL blocks recognition by these receptors (Weisgraber *et al.*, 1978). By application of this method, Carew *et al.* (1982)

Abbreviations used: LDL, low-density lipoprotein; Me-LDL, reductive-methylated LDL.

found that in rats about two-thirds of the hepatic uptake of human LDL can be attributed to specific LDL receptors.

More recently we have compared the initial rates of cell association of iodine-labelled human LDL to the various liver cell types (Harkes & Van Berkel, 1984). A comparison of the cell association *in vivo* of LDL, methylated LDL and cyclohexanedione-treated LDL determined 30min after injection indicated that non-parenchymal liver cells do contain an LDL-recognition site. However, LDL recognition is blocked by methylation but not by cyclohexanedione treatment of LDL. This unique property is in contrast with the recognition characteristics of the oestrogen-induced LDL receptor on parenchymal cells, where recognition of LDL is blocked by both modifications (Harkes & Van Berkel, 1984), as with the classical LDL receptor on fibroblasts (Mahley *et al.*, 1977; Weisgraber *et al.*, 1978). The present work was performed in order to assess the quantitative importance of this unique LDL-recognition site on non-parenchymal liver cells for the catabolism of LDL *in vivo*. For this purpose the time-dependent accumulation of [^{14}C]sucrose-labelled human LDL and [^{14}C]sucrose-labelled Me-LDL by the various liver cell types was determined.

Experimental

Materials

Collagenase (type I) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Pronase (B grade) from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.), metrizamide from Nyegaard A/S (Oslo, Norway) and [U-^{14}C]sucrose from Amersham International (Amersham, Bucks., U.K.).

Lipoproteins

Human LDL ($1.024 < d < 1.055$) was isolated by the method of Redgrave *et al.* (1975) as previously described (Harkes & Van Berkel, 1983). The isolated LDL was subjected to a second identical centrifugation. Apolipoprotein E content of this LDL fraction was less than 0.03% of total apolipoprotein (Harkes & Van Berkel, 1982), as tested in a radial immunodiffusion system (Mancini *et al.*, 1965). Labelling of LDL with [U-^{14}C]sucrose (specific radioactivity 552Ci/mol) was performed exactly as described by Pittman *et al.* (1979*b*). A 0.2μmol portion of [U-^{14}C]sucrose was activated with cyanuric chloride, whereafter 7mg of LDL protein in 0.5ml of 0.15M-NaCl/20mM-sodium phosphate/1mM-EDTA buffer, pH7.2, was added. After 2h at room temperature the [^{14}C]sucrose-labelled LDL was separated from free [^{14}C]sucrose by dialysis twice against 0.15M-NaCl/20mM-sodium phosphate/1mM-EDTA buffer, pH6.8, then against the same buffer at pH7.0 and finally against 0.15M-NaCl/0.3mM-EDTA, pH7.0. The extent of labelling was 0.014μmol of [^{14}C]-sucrose/mg of LDL apoprotein. Reductive methylation of [^{14}C]sucrose-labelled LDL was carried out as described by Weisgraber *et al.* (1978). The extent of methylation of the lysine residues as determined by the trinitrobenzenesulphonic acid method was greater than 80%.

The [^{14}C]sucrose-labelled LDL and [^{14}C]-sucrose-labelled Me-LDL were screened by injecting 2mg of protein of either preparation intravenously into rats. After 2.5h of circulation total serum was collected and directly used, without prior isolation of the [^{14}C]sucrose-labelled LDL or [^{14}C]sucrose-labelled Me-LDL.

Methods

Male Wistar rats (12 weeks old) were used throughout the study. Rats were anaesthetized by intraperitoneal injection of 20mg of Nembutal. The abdomen was opened, and screened [^{14}C]-sucrose-labelled LDL was injected as a 0.5ml serum sample into the inferior vena cava at the level of the renal veins. In experiments with circulation times of LDL of more than 30min, the sample was injected in a tail vein under diethyl ether anaesthesia.

At the indicated circulation time, the vena porta was cannulated and the liver perfused with oxygenated Hanks' medium {8.0g of NaCl, 0.4g of KCl, 0.2g of $MgSO_4,7H_2O$, 0.06g of $Na_2HPO_4,2H_2O$, 0.06g of KH_2PO_4, 1.0g of glucose and 4.77g of Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] per litre, pH7.4} at 8°C. After 8min perfusion (flow rate 14ml/min) a lobule was tied off for determination of the total liver uptake. In order to separate the various cell types, the liver was further subjected to a low-temperature (8°C) perfusion with 0.05% collagenase (Harkes & Van Berkel, 1983; Nagelkerke *et al.*, 1983). After 20min of perfusion with collagenase, the liver was minced and the crude cell suspension was filtered (90μm mesh) from debris. The filtrate (containing parenchymal and non-parenchymal cells) was subjected to differential centrifugation exactly as described previously (Van Berkel & Van Tol, 1978). The parenchymal cells were completely free from non-parenchymal cells, as judged by microscopy and the absence of M_2-type pyruvate kinase (Van Berkel *et al.*, 1977) from this preparation. The non-parenchymal cells were collected from the first two supernatants of the parenchymal-cell centrifugations. In order to increase the recovery of non-parenchymal cells from the liver, the residue on the 90μm-mesh filter was incubated for 20min at 8°C with 0.25% Pronase (which

destroys parenchymal cells), and the non-parenchymal cells were collected and washed (twice) by centrifugation at 400*g* for 5min. The non-parenchymal-cell fractions (both from the supernatants from the parenchymal-cell isolation and the Pronase-treated filter residue) were combined. The cells were suspended in 5ml of Hanks' medium, mixed with 7.2ml of 30% (w/v) metrizamide and divided over two Sorvall tubes. Then 1ml of Hanks' medium was layered on top of the mixture, and the tubes were spun at 1500*g* for 15min. The cells that floated into the top phase were aspirated and subjected to a 30s 50*g* centrifugation to remove any remaining parenchymal cells. The non-parenchymal-cell preparation was collected and washed by two 400*g* centrifugations. The non-parenchymal-cell preparation was completely free from parenchymal cells or parenchymal-cell-derived particles, as judged by phase-contrast microscopy and the exclusive presence of M_2-type pyruvate kinase in this preparation (Van Berkel *et al.*, 1977). The purity of the parenchymal-cell and non-parenchymal preparations is also indicated by the specific modulation of LDL association to the various cell types by oestrogen (parenchymal cells) or ethyl oleate treatment (non-parenchymal cells) (Harkes & Van Berkel, 1984). By peroxidase staining with diaminobenzidine (Fahimi, 1970), about 30% of the isolated non-parenchymal cells were peroxidase-positive, indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells. This relative proportion is similar to that *in vivo* (Knook & Sleyster, 1980). Because a Kupffer cell contains twice as much protein as an endothelial cell (Knook & Sleyster, 1980), the non-parenchymal-cell preparation contains, calculated on a protein basis, 50% Kupffer cells and 50% endothelial cells.

In some experiments endothelial and Kupffer cells were obtained by subjecting the liver to a direct 8°C Pronase perfusion, whereafter the cells were purified by centrifugal elutriation exactly as described previously (Nagelkerke *et al.*, 1983). The Kupffer-cell preparation contained 70–90% Kupffer cells, the remainder being endothelial cells; the endothelial-cell preparation contained more than 95% endothelial cells, with less than 5% white blood cells, as determined by the method of Nagelkerke *et al.* (1983).

The amount of ^{14}C in the liver samples (0.2–0.3g) and cell preparations (0.5–1.0ml) was determined after digestion in 1ml of Soluene-350 and bleaching with 0.2ml of 30% H_2O_2 in a Packard Tri-Carb liquid-scintillation spectrometer. The relative proportion of protein-linked and small-peptide-bound [^{14}C]sucrose was determined by heating the various samples for 15min at 95°C. After centrifugation (10000*g* for 10min), the radioactivities of the precipitate and supernatant were counted.

Liver wet weight was taken as 3.75% of total body weight (Van Berkel & Van Tol, 1978). Protein determination was determined by the method of Lowry *et al.* (1951).

Results

Initial studies indicated a quantitatively important role for the non-parenchymal liver cells in the uptake of [^{14}C]sucrose-labelled LDL. As the Kupffer cells especially are known for their effective uptake of denatured protein (Buys *et al.*, 1975), we decided to subject the LDL preparations to a screening procedure (2.5h) in order to remove any possible denatured protein. Determination of the decay in serum after injection of the screened [^{14}C]sucrose-labelled LDL into rats revealed biphasic kinetics (Fig. 1) similar to those for the unscreened preparation. During the rapid phase ($t_{\frac{1}{2}}$ about 5h) about 50% of the LDL is removed from serum. During the first 4.5h after injections of [^{14}C]sucrose-labelled LDL, the liver radioactivity increases almost linearly with time (Fig. 2). Thereafter a steady-state value is observed.

From the results shown in Figs. 1 and 2 we can calculate the relative importance of the liver in accumulating LDL at different times after injection (Table 1). This contribution is 80.1% after 2h and 69.3% after 4.5h. This indicates that the liver is by far the most important site for LDL uptake. After 4.5h the calculated relative importance of the liver in LDL uptake apparently decreases, probably as a consequence of release of [^{14}C]-sucrose from the liver (see the Discussion section). The uptake of [^{14}C]sucrose-labelled LDL in parenchymal and non-parenchymal liver cells at different times after injection of the labelled LDL is shown in Fig. 3. During the total time course after LDL injection (24h), the LDL uptake in non-parenchymal cells is at least 60 times higher than in parenchymal cells (expressed per mg of protein). Taking into account that parenchymal cells constitute 92.5% of the total liver protein (Blouin *et al.*, 1977), it can be calculated that the parenchymal cells are responsible for 29% of the LDL uptake by liver and the non-parenchymal cells for 71%. This proportion is fairly constant at the various times after LDL injection. On the basis of the radioactivity found in the parenchymal cells and in the non-parenchymal cells, it can be concluded that non-parenchymal cells are quantitatively the most important site for LDL uptake in liver.

The role of a specific recognition site for apolipoprotein B in the uptake of LDL by the various liver cell types was determined at two time

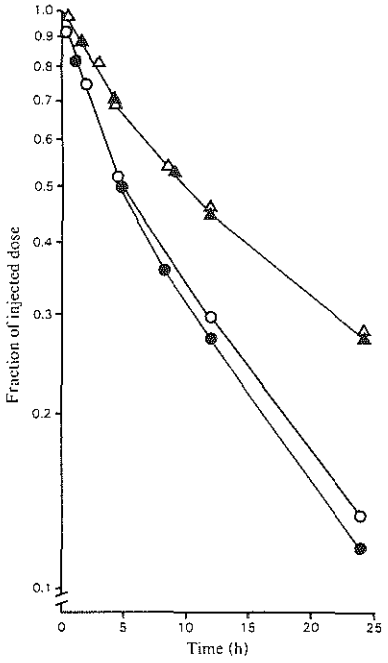

Fig. 1. *Decay in serum of* [^{14}C]*sucrose-labelled LDL and* [^{14}C]*sucrose-labelled Me-LDL in rats*

[^{14}C]Sucrose-labelled LDL (○, ●) or [^{14}C]sucrose-labelled Me-LDL (△, ▲) preparations were injected as a 0.5 ml serum sample for the screened lipoprotein, or as a 0.5 ml saline sample for the unscreened lipoprotein, into a tail vein, and the radioactivity was determined in 0.05 ml samples of serum. The results are expressed as fraction of the 3 min value. ○, △, Screened preparations; ●, ▲, unscreened preparations.

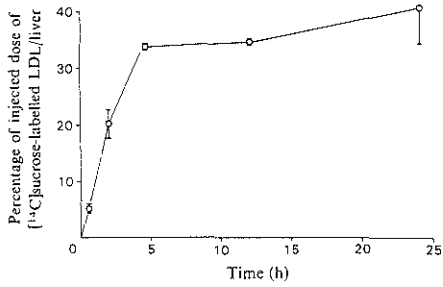

Fig. 2. *Association of* [^{14}C]*sucrose-labelled LDL with liver at different times after injection*

After injection of the screened [^{14}C]sucrose-labelled LDL, a perfusion of the liver with an 8°C Hanks' medium was started at the indicated times. Then 8 min later a liver sample was taken. Values are means ± S.E.M. for three experiments and expressed as percentages of the injected dose/liver.

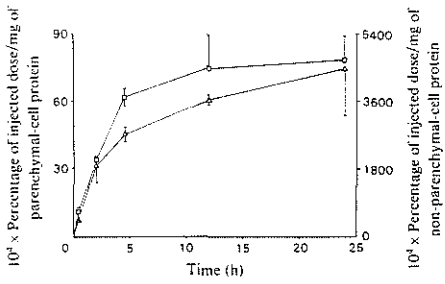

Fig. 3. *Cell association of* [^{14}C]*sucrose-labelled LDL with parenchymal cells and non-parenchymal cells at different times after intravenous injection*

[^{14}C]Sucrose-labelled LDL association with parenchymal (△) and non-parenchymal (□) liver cells was determined after a low-temperature (8°C) isolation and purification procedure started at different times after LDL injection. Results are expressed as 10^4 × percentages of the injected dose/mg of cell protein and are means ± S.E.M. for two or three experiments.

points after injection. For this purpose the cellular uptake of [^{14}C]sucrose-labelled Me-LDL was compared with that of the native particle (Fig. 4). The time intervals chosen represent the initial association (30 min) and the uptake at a time point when about 50% of the total LDL has disappeared from serum (4.5 h). It can be determined that at 30 min after injection the Me-LDL association with total liver is 35% of that of unmodified LDL. After a circulation time of 4.5 h the liver uptake of Me-LDL is 36% of that of LDL. For non-parenchymal cells especially the uptake of LDL (4.5 h value) is greatly diminished upon methylation (by 79%), indicating the essential role of lysine residues in the uptake of LDL by these cell types. The total non-parenchymal-cell preparation contains, on a protein basis, about 50% endothelial and 50% Kupffer cells (see the Experimental section). A purification of the non-parenchymal cells into Kupffer and endothelial cells shows that within the non-parenchymal cell population the Kupffer cells are responsible for this receptor-dependent uptake. The endothelial-cell uptake of LDL is unaffected by methylation of LDL. For parenchymal cells the initial recognition is inhibited by 33% by methyla-

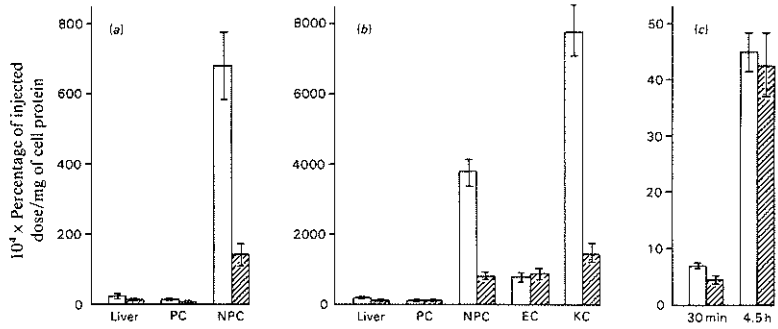

Fig. 4. *Distribution of* [^{14}C]*sucrose-labelled LDL or* [^{14}C]*sucrose-labelled Me-LDL between the various liver cell types at 30 min and at 4.5 h after injection*

The cell association of [^{14}C]sucrose-labelled LDL (□) or [^{14}C]sucrose-labelled Me-LDL (▨) with liver parenchymal (PC) and non-parenchymal cells (NPC) was determined 30 min (*a*) and 4.5 h (*b*) after intravenous injection of the screened lipoproteins. In panel (*b*), two additional experiments show the distribution of the labelled lipoproteins between endothelial (EC) and Kupffer cells (KC). In panel (*c*) the parenchymal-cell values obtained at 30 min and 4.5 h after injection are directly compared. Values are the means ± S.E.M. for two or three experiments and are expressed as 10^4 × percentages of the injected dose/mg of cell protein.

tion of LDL. However, binding to this specific recognition site is apparently not effectively coupled to uptake, as at the longer circulation time (4.5 h) the LDL accumulation in parenchymal cells (Fig. 4*c*) is inhibited only to a low extent by methylation.

The relative proportion of heat-soluble and heat-precipitable [^{14}C]sucrose label was determined in order to decide whether intracellular degradation of LDL occurs. With both parenchymal and non-parenchymal liver cells, isolated 30 min after LDL injection, 60% of the total radioactivity is precipitated by this treatment. When the cells are isolated 4.5 h after LDL injection, this percentage decreases to 35%.

Discussion

We previously reported that non-parenchymal liver cells do contain a specific recognition site for LDL; this recognition site differs from the classical LDL receptor from fibroblasts or the oestrogen-stimulated LDL receptor from parenchymal rat liver cells (Harkes & Van Berkel, 1984). Modification of arginine or lysine residues in LDL by respectively cyclohexanedione treatment or reductive methylation both blocks the interaction of LDL with the LDL receptor (apo-B,E receptor) on human fibroblasts or oestrogen-stimulated rat hepatocytes, whereas with non-parenchymal cells only methylation appears to inhibit the recognition. In the present study we evaluate the importance of this unique recognition site for LDL on (56%). However, when calculated in the linear non-parenchymal cells for LDL catabolism *in vivo*. LDL was labelled with [^{14}C]sucrose because it was reported (Pittman *et al.*, 1979*b*, 1982; Tolleshaug & Berg, 1981) that, on degradation of LDL, the [^{14}C]-sucrose-containing degradation products accumulate inside the cells. Furthermore, studies by Pittman *et al.* (1982) have shown that in the rat the decay rates of [^{14}C]sucrose-labelled and ^{125}I-labelled LDL are identical. The decay of [^{14}C]-sucrose-labelled LDL as observed in the present studies is identical with what was shown by Pittman *et al.* (1982) and Carew *et al.* (1982). In contrast with those previous studies, where the liver uptake of [^{14}C]sucrose-labelled LDL or [^{14}C]sucrose-labelled Me-LDL was only determined at 24 h after injection, we measured the accumulation of [^{14}C]sucrose in liver at different times after injection of [^{14}C]-sucrose-labelled LDL. The data indicate that up to 4.5 h after LDL injection the accumulation of [^{14}C]-sucrose-labelled LDL in liver is linear with time. This linear uptake phase is observed in both parenchymal and non-parenchymal cells. At longer circulation times, however, a near-equilibrium situation is reached, and apparently the uptake of [^{14}C]sucrose-labelled LDL is compensated by a release of label from the cells. Release of label from liver will result in an underestimation of the contribution of this organ to LDL catabolism at the longer time intervals, as indicated in Table 1. After 24 h the measured contribution of the rat liver to human LDL catabolism is 47%, a value comparable with that reported for [^{14}C]sucrose-labelled LDL by Carew *et al.* (1982) (44%) or Pittman *et al.* (1982)

Table 1. *Relative importance of the liver in accumulating screened [^{14}C]sucrose-labelled LDL at different times after injection*
The percentages are calculated from the data plotted in Figs. 1 and 2.

Time after injection (h)	LDL cleared from serum (%)	LDL accumulated in liver (%)	Relative importance of the liver for the LDL decay (%)
2	25.1	20.1	80.1
4.5	48.7 (30.8*)	33.7 (12.1*)	69.3 (39.4*)
12	70.4	34.7	49.3
24	86.7	40.5	46.8

* Value for [^{14}C]sucrose-labelled Me-LDL.

(56%). However, when calculated in the linear uptake phase, the liver contribution is much higher, and up to 80% of the LDL that is cleared from the circulation is found in the liver.

By using a low-temperature perfusion and cell-separation method, the liver can be subdivided into parenchymal and non-parenchymal cells, with a quantitative recovery of the label (Harkes & Van Berkel, 1983; Nagelkerke *et al.*, 1983). Furthermore the specific effects of oestrogen treatment on parenchymal cells or ethyl oleate on non-parenchymal cells indicate that no redistribution of label occurs during the cell-separation procedure (Harkes & Van Berkel, 1984). At all time intervals after injection, the amount of [^{14}C]sucrose label found associated with non-parenchymal liver cells appears to be 60 times (per mg of cell protein) that with parenchymal cells. The uptake of LDL by the non-parenchymal-cell preparation can primarily be ascribed to the Kupffer cells. It can be argued that the high uptake of LDL in Kupffer cells is the consequence of a denaturation of LDL during labelling or storage, as Kupffer cells are general consumers of modified proteins (Buys *et al.*, 1975). However, on several grounds we think that it is justified to exclude such a possibility. (1) The [^{14}C]-sucrose-labelled LDL was screened, and after this screening was directly injected without further isolation. Although the decay rate of [^{14}C]sucrose-labelled LDL was not influenced by screening, and identical decay curves and liver uptake values were measured, as reported previously (Carew *et al.*, 1982; Pittman *et al.*, 1982), we routinely still subjected the LDL preparation to a screening procedure. (2) The uptake of [^{14}C]sucrose-labelled LDL by non-parenchymal cells was greatly diminished (by 79%) by methylation of the lysine residues of LDL. As methylation does not lead to any charge changes in LDL, this shows that the lysine residues in LDL are mainly responsible for the LDL uptake by Kupffer cells. (3) It is not a small part of the LDL fraction that is initially rapidly taken by the non-parenchymal cells, because up to 4.5 h the uptake of LDL is almost linear with time (as with parenchymal cells), and at this time point half of the LDL is cleared from the circulation. Even at 24 h, when 87% of the LDL is cleared, the uptake in non-parenchymal cells appears to be 60 times that in parenchymal cells (expressed per mg of cell protein). This value is similar to that at the short circulation time (0.5 and 2 h). Taking into account the relative protein contribution of the various liver cell types to total liver, it can be calculated that non-parenchymal cells are responsible for 71% of the total liver uptake of LDL. A comparison of the cellular uptake of [^{14}C]sucrose-labelled LDL and [^{14}C]-sucrose-labelled Me-LDL after 4.5 h circulation indicates that 79% of the uptake of LDL by non-parenchymal cells is receptor-dependent. With parenchymal cells no significant difference in uptake between [^{14}C]sucrose-labelled LDL and [^{14}C]sucrose-labelled Me-LDL was found. A further separation of the non-parenchymal cells into Kupffer and endothelial cells by centrifugal elutriation shows that within the non-parenchymal cell preparation solely the Kupffer cells are responsible for the receptor-dependent uptake of LDL.

The importance of the non-parenchymal-cell fraction in LDL uptake is further sustained by the findings by Slater *et al.* (1982*a*,*b*) and Packard *et al.* (1982). They showed in rabbits that the reticulo-endothelial system in liver and spleen plays a quantitative important role in LDL catabolism. Blockade of the reticulo-endothelial system by ethyl oleate leads to a rapid 33% increase in LDL cholesterol (Slater *et al.*, 1982*a*). Our data, together with those obtained by Slater *et al.* (1982*a*,*b*) and Packard *et al.* (1982), indicate that the quantitatively important specific uptake of the apolipoprotein of LDL by the reticulo-endothelial system of the liver has also quantitatively important implications for serum cholesterol metabolism. Further studies on the regulatory aspect of this uptake are therefore required.

We thank Mr. Kar Kruijt for his technical assistance, Dr. Fred Nagelkerke for separating endothelial and Kupffer cells, Professor W. C. Hülsmann for critically reading the manuscript and Ms. Cecile Hanson and Ms. Martha Wieriks for typing. This research was supported by Grant No. 79.001 from the Netherlands Heart Foundation.

References

Blouin, A., Bolender, R. P. & Weibel, E. R. (1977) *J. Cell Biol.* **72**, 441–455

Buys, C. H. M. C., De Jonge, A. S., Bouma, J. M. & Gruber, M. (1975) *Biochim. Biophys. Acta* **392**, 95–100

Carew, T. E., Pittman, R. C. & Steinberg, D. (1982) *J. Biol. Chem.* **257**, 8001–8008

Fahimi, H. D. (1970) *J. Cell Biol.* **47**, 247–261

Harkes, L. & Van Berkel, Th. J. C. (1982) *Biochim. Biophys. Acta* **712**, 677–683

Harkes, L. & Van Berkel, Th. J. C. (1983) *FEBS Lett.* **154**, 75–80

Harkes, L. & Van Berkel, Th. J. C. (1984) *Biochim. Biophys. Acta* **794**, 340–347

Knook, D. L. & Sleyster, E. Ch. (1980) *Biochem. Biophys. Res. Commun.* **96**, 250–257

Langer, T., Strober, W. & Levy, R. I. (1970) in *Plasma Protein Metabolism* (Rothschild, M. A. & Waltman, T., eds.), pp. 483–503, Academic Press, New York

Lindstedt, S. (1970) *Proc. Int. Symp. Atherosclerosis 2nd* 262–271

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mahley, R. W., Innerarity, T. L., Pitas, R. E., Weisgraber, K. H., Brown, J. H. & Gross, E. (1977) *J. Biol. Chem.* **252**, 7279–7287

Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry* **2**, 235–254

Nagelkerke, J. F., Barto, K. P. & Van Berkel, Th. J. C. (1983) *J. Biol. Chem.* **258**, 12221–12227

Packard, C. J., Slater, R. H. & Shepherd, J. (1982) *Biochim. Biophys. Acta* **712**, 412–419

Pittman, R. C., Attie, A. D., Carew, T. E. & Steinberg, D. (1979*a*) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5345–5349

Pittman, R. C., Green, S. R., Attie, A. D. & Steinberg, D. (1979*b*) *J. Biol. Chem.* **254**, 6876–6879

Pittman, R. C., Attie, A. D., Carew, T. E. & Steinberg, D. (1982) *Biochim. Biophys. Acta* **710**, 7–14

Redgrave, T. G., Roberts, D. C. K. & West, C. E. (1975) *Anal. Biochem.* **65**, 42–49

Slater, H. R., Packard, C. J. & Shepherd, J. (1982*a*) *J. Biol. Chem.* **257**, 307–310

Slater, H. R., Packard, C. J. & Shepherd, J. (1982*b*) *J. Lipid Res.* **23**, 92–96

Tolleshaug, H. & Berg, T. (1981) *Exp. Cell Res.* **134**, 207–217

Van Berkel, Th. J. C. & Van Tol, A. (1978) *Biochim. Biophys. Acta* **530**, 299–304

Van Berkel, Th. J. C., Kruijt, J. K., Slee, R. G. & Koster, J. F. (1977) *Arch. Biochem. Biophys.* **179**, 1–7

Weisgraber, K. H., Innerarity, T. L. & Mahley, R. W. (1978) *J. Biol. Chem.* **253**, 9053–9062

APPENDIX PAPER II

Biochimica et Biophysica Acta, 794 (1984) 340–347
Elsevier

BBA 51668

IN VIVO CHARACTERISTICS OF A SPECIFIC RECOGNITION SITE FOR LDL ON NON-PARENCHYMAL RAT LIVER CELLS WHICH DIFFERS FROM THE 17α-ETHINYL ESTRADIOL-INDUCED LDL RECEPTOR ON PARENCHYMAL LIVER CELLS

LEEN HARKES and THEO J.C. VAN BERKEL

Department of Biochemistry I, Faculty of Medicine, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

(Received September 16th, 1983)
(Revised manuscript received February 16th, 1984)

Key words: LDL; Lipoprotein receptor; 17α-Ethinyl estradiol; (Rat liver)

Chemical modification of lysine or arginine residues of apolipoprotein B-100 in human low-density lipoprotein (LDL) with respectively reductive methylation (Me-LDL) or cyclohexanedione treatment (CHD-LDL) was applied to determine the role of these amino acids in LDL recognition by the various liver cell types. The cell association of native human LDL, Me-LDL and CHD-LDL to parenchymal and non-parenchymal cells was determined in vivo by isolating the various cell types 30 min after intravenous injection of the lipoproteins. In order to prevent degradation or release of cell-bound apolipoproteins during cell dissociation and purification, a low-temperature (8°C) liver perfusion and cell isolation procedure was performed. It was found that reductive methylation of LDL inhibits the association of LDL to both parenchymal and non-parenchymal cells, indicating that lysine residues are important for recognition of LDL by both these cell types. In contrast, cyclohexanedione treatment of LDL did not influence the cell association of LDL to non-parenchymal cells. 17α-Ethinyl estradiol treatment selectively increases the cell association of LDL by parenchymal cells (16-fold), leaving the non-parenchymal cell association uninfluenced. The increased cell-association of LDL to parenchymal cells is almost completely blocked by cyclohexanedione treatment of LDL (by 81%) or by methylation of LDL (by 97%). These data indicate that the arginine residues in LDL are not important for the recognition of LDL by non-parenchymal cells, whereas for the cell association of LDL to the estrogen-stimulated binding site on parenchymal cells both arginine and lysine residues are essential. The in vivo cell association of CHD-LDL or native LDL to non-parenchymal cells was lowered to the level of Me-LDL by ethyl oleate treatment of the rats, while no effect of ethyl oleate on parenchymal cells was noticed. These data suggest that the specific site for LDL on non-parenchymal cells, which need lysine residues on LDL for recognition, can be down-regulated by ethyl oleate treatment. The LDL, internalized by non-parenchymal cells, is effectively degraded. This degradation occurs at least partly in the lysosomes. It is suggested that the unique recognition site for LDL on non-parenchymal cells may be quantitatively important for serum LDL catabolism.

Introduction

The liver plays an important role in lipoprotein metabolism and is the only organ where cholesterol can be removed from the body and degraded to

Abbreviations: LDL, low-density lipoprotein; Me-LDL, reductively methylated LDL; CHD-LDL, cyclohexanedione-treated LDL.

bile acids [1,2]. Recent evidence indicates that various distinct types of high-affinity receptors are responsible for the uptake of apolipoprotein B,E-containing lipoproteins. The receptors are tentatively named
(1) the remnant (apolipoprotein E) receptor [3–6];
(2) the LDL (apolipoprotein B,E) receptor [3,7].
Mahley et al. [3] indicated that in dogs, swine and man the apolipoprotein B,E receptor is metabolically independent from the apolipoprotein E receptor. In rats the apolipoprotein B,E receptor in liver can be increased by 17α-ethinyl estradiol treatment [8,9], which increase is specific for parenchymal liver cells [10]. Human LDL, prepared free from apolipoprotein E [7,10] can be used to discriminate between the apolipoprotein B,E and apolipoprotein E receptor. Specific modification of the arginine or lysine residues of human LDL with respectively cyclohexanedione or reductive methylation prevents the association of LDL to the apolipoprotein B,E receptor [11,12]. This allows discrimination between the receptor-dependent and independent cell-association of LDL both in vitro and in vivo.

In the present study we have used CHD-LDL and Me-LDL to investigate the nature and specificity of the recognition site for LDL on both parenchymal and non-parenchymal cells in vivo. The possibility of an independent regulation of the parenchymal and non-parenchymal cell recognition sites for LDL was explored by 17α-ethinyl estradiol [8–10] or ethyl oleate treatment [13] of the rats.

Materials and Methods

Materials

17α-Ethinyl estradiol was obtained from Brocacef B.V., Maarssen, The Netherlands; collagenase (type I) and ethyl oleate from Sigma, St. Louis, U.S.A.; pronase B-grade from CalBiochem-Behring Corp., La Jolla, U.S.A.; Ham's F-10 medium from Gibco-Europe, Hoofddorp, The Netherlands; sodium [^{125}I]- and [^{131}I]iodine (carrier-free) was purchased from Amersham International, Amersham, U.K., and metrizamide from Myegaard & Co. A/S, Oslo, Norway.

Animals

12-week-old male Wistar rats were used throughout the study. 17α-Ethinyl estradiol in propylene glycol at a dose of 5 mg/kg body weight [8] was injected subcutaneously every 24 h during 3 days; control rats received equal volumes of the solvent. Ethyl oleate was injected 96 and 48 h [14] before lipoprotein injection as a 10% emulsion in 0.15 M NaCl with 0.7% Tween-20 as stabilizer [13] in a tail vein at a dose of 1 ml of lipid/kg body weight; control rats received an equal volume of 0.7% Tween-20 in 0.15 M NaCl.

Lipoproteins

Human LDL ($1.024 < d < 1.055$ g/ml) was isolated by two repetitive centrifugations according to Redgrave et al. [15] as previously described [10]. The human LDL preparation used in this study contains mostly apolipoprotein B (99.97%) and no degradation products were noticeable as checked by electrophoresis in SDS (sodium dodecyl sulfate) gels. With a high LDL concentration (5 mg apolipoprotein/ml) in a radial immunodiffusion system according to Mancini et al. [16], apolipoprotein E was noticeable at the detection limit and contributed maximally 0.02–0.03% of the total apolipoprotein.

Radioiodination of LDL was done according to a modification [17] of the ICl method described by McFarlane [18]. Reductive methylation of LDL was carried out as described in Ref. 12. The extent of methylation of lysine residues of Me-LDL as determined by the trinitrosulfonic acid method was greater than 80%. 1,2-Cyclohexanedione treatment of LDL was done according to Ref. 11, which results in a derivatization of at least 50% of the arginine residues. Radioiodinated human LDL, Me-LDL and CHD-LDL were always prepared from the same LDL preparation.

Methods

Lipoprotein preparations (about 40 μg) were injected in the inferior vena cava as described [10]. After the indicated circulation time the liver was perfused with an oxygenated Hanks buffer at 8°C. After 8 min perfusion a lobule was tied off for determination of the total liver uptake. To determine the uptake by the various cell types, the cell types were isolated by low-temperature procedures. After the 8 min perfusion at 8°C, the liver was subjected to a low-temperature (8°C) perfu-

sion with 0.05% collagenase [10,19]. After 20 min of perfusion with collagenase the liver was minced and the crude cell suspension filtered (90-μm mesh) from debris. The filtrate (containing parenchymal and non-parenchymal cells) was subjected to differential centrifugation exactly as described earlier [20]. The parenchymal cells were completely free from non-parenchymal cells as judged by microscopy and the absence of M_2-type pyruvate kinase [21] in this preparation. The non-parenchymal cells were collected from the first two supernatants of the parenchymal cell centrifugations. In order to increase the recovery of non-parenchymal cells from the liver the residue on the 90-μm mesh was incubated for 20 min at 8°C with 0.25% pronase (which destroys parenchymal cells), and the non-parenchymal cells were collected and washed (two times) by centrifugation at $400 \times g$ for 5 min. The non-parenchymal cell fractions (both from the supernatants from the parenchymal cell isolation and the pronase-treated filter residue) were combined. The cells were suspended in 5 ml Hank's buffer, mixed with 7.2 ml 30% metrizamide and divided over two Sorvall tubes. 1 ml Hank's buffer was layered on top of the mixture and the tubes are spun for 15 min at $1500 \times g$. The cells which floated into the top phase were aspirated and subjected to a 30 s $50 \times g$ centrifugation to remove any remaining parenchymal cells. The non-parenchymal cell preparation was collected and washed by two $400 \times g$ centrifugations. The non-parenchymal cell preparation was completely free from parenchymal cells or parenchymal cell-derived particles, as judged by phase-contrast microscopy and the exclusive presence of M_2-type pyruvate kinase in this preparation [21]. The purity of the parenchymal and non-parenchymal cell preparations is also indicated in the paper by the specific modulation of LDL association to the various cell types by estrogen (parenchymal cells) or ethyl oleate treatment (non-parenchymal cells). By peroxidase staining with diaminobenzidine [22] about 30% of the isolated non-parenchymal cells were peroxidase-positive, indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells. This relative proportion is similar to that found in vivo [23].

The degradation of the in vivo internalized lipoproteins was determined by isolating the various cell types by a short, warm (37°C) recirculating perfusion method based upon the method in Ref. 24 (perfusion flow: 40 ml/min). 10 min after intravenous injection of the labelled lipoproteins, perfusion of the liver was started at 37°C for 10 min with Hank's solution, 9 min with Hank's solution plus 0.05% collagenase and again 1 min with Hank's solution in order to remove the collagenase. As shown earlier [5,7] and also indicated in the paper, cell-bound LDL will be removed by collagenase. Hereafter the parenchymal and non-parenchymal cells were isolated and purified as described above, except that the pronase treatment of the filter residue was omitted because with the applied procedure at 37°C no residue is left. Subsequently the cells were incubated in vitro at 37°C in a Hams F-10 medium supplemented with 5% human lipoprotein-deficient serum during 2 h, and the cell-association and degradation was determined at the indicated times. Cell-association, trichloro-acetic acid-soluble degradation products and trichloroacetic acid-precipitable products were determined as described before [5].

Results

Cell isolation conditions

A quantitative recovery of the total liver-associated radioactivity with the isolated parenchymal and non-parenchymal cells is necessary in order to perform a reliable determination of the characteristics of the lipoprotein receptors on these cells. Because a perfusion with collagenase is obligatory for cell preparation, we investigated conditions in which collagenase should not influence the amount of liver-associated LDL, but is still able to separate cells. From Table I it can be seen that the conditions are fulfilled at a perfusion temperature of 8°C. As compared to the condition at 37°C + collagenase, the amount of liver-associated LDL or Me-LDL is only minimally reduced during the perfusion at 8°C. During the further purification steps, as shown before [10], no cell-associated radioactivity is lost and a quantitative recovery of the total liver-associated radioactivity in the subsequently isolated cells is obtained.

TABLE I

LIVER-ASSOCIATED LDL OR Me-LDL UNDER THREE DIFFERENT PERFUSION CONDITIONS

^{131}I-labelled LDL and ^{125}I-labelled Me-LDL were injected intravenously into rats. 10 min after injection a liver perfusion was started at the indicated temperature and after 8 min (time point 18) a liver lobule was tied off and the liver-associated radioactivity determined (100%). At time point 20 min, collagenase (0.05%) was added to the perfusion medium when indicated. After an additional 10 min of perfusion plus or minus collagenase at time point 30 min a second liver lobule was taken and the amount of radioactivity in the perfusion medium and liver determined. The perfusion flow was 40 ml/min (identical to that for the cell separation for the in vitro degradation studies; see Materials and Methods). Values are means of two experiments ± S.E. The perfusate radioactivity is the amount of radioactivity that accumulated in the perfusate from time point 18 to 30 min.

	Time after lipoprotein injection			
	18 min		30 min	
	LDL	Me-LDL	LDL	Me-LDL
Perfusion at 37°C minus collagenase				
Liver-associated	100	100	76 ± 3	77 ± 4
Perfusate: TCA-precipitable	–	–	14 ± 4	15 ± 5
TCA-soluble	–	–	10 ± 1	8 ± 1
Perfusion at 37°C plus collagenase				
Liver-associated	100	100	55 ± 2	54 ± 7
Perfusion at 8°C plus collagenase				
Liver-associated	100	100	93 ± 7	87 ± 4

In vivo recognition

Administration of 17α-ethinyl estradiol to rats leads to an induction of apolipoprotein B,E receptors in liver [8,9]. The properties of the induced receptor are comparable to those of the classical LDL receptor, as originally described in fibroblasts [25]. In a previous study [10] we showed that the increased interaction of human LDL with the liver occurs selectively in parenchymal cells. In the present study we used estrogen-treated rats to compare the in vivo characteristics of the estrogen-stimulated interaction site for LDL on parenchymal cells with the LDL interaction site on non-parenchymal cells. As shown in Table II, a 22-fold stimulation in the receptor-dependent cell association of LDL occurs (receptor-dependent as-

TABLE II

EFFECT OF 17α-ETHINYL ESTRADIOL UPON THE DISTRIBUTION OF NATIVE LDL, Me-LDL AND CHD-LDL BETWEEN PARENCHYMAL AND NON-PARENCHYMAL RAT LIVER CELLS IN VIVO

Native LDL, Me-LDL and CHD-LDL were labelled with ^{125}I or ^{131}I. Each rat was intravenously injected with both a ^{125}I- and a ^{131}I-labelled lipoprotein. 30 min after injection a liver perfusion was started with Hank's balanced salt solution at 8°C. After 8 min of perfusion a liver lobule was taken (whole liver) and subsequently the different cell types were isolated with a low-temperature procedure. The rats were treated with 17α-ethinyl estradiol; control rats received the solvent (propylene glycol). Values, expressed as % injected dose × 10^4/mg cell protein, are means ± S.E. with N in parentheses.

	LDL	Me-LDL	CHD-LDL
Whole liver			
Control	19.1 ± 5.5 (5)	5.8 ± 0.4 (3)	17.4 ± 3.1 (5)
Estradiol	107.2 ± 8.9 (6)	7.0 ± 0.6 (3)	30.1 ± 2.3 (6)
Parenchymal cells			
Control	7.3 ± 2.0 (5)	2.0 ± 0.3 (3)	4.3 ± 0.7 (5)
Estradiol	120.1 ± 5.4 (6)	4.2 ± 0.7 (3)	22.7 ± 1.4 (6)
Non-parenchymal cells			
Control	188.8 ± 65.6 (5)	55.0 ± 9.6 (3)	225.9 ± 46.7 (6)
Estradiol	163.8 ± 21.3 (6)	43.0 ± 3.8 (3)	193.1 ± 20.7 (6)

TABLE III

EFFECT OF ETHYL OLEATE UPON THE DISTRIBUTION OF NATIVE LDL, Me-LDL AND CHD-LDL BETWEEN PARENCHYMAL AND NON-PARENCHYMAL RAT LIVER CELLS IN VIVO

Native LDL, Me-LDL and CHD-LDL were labelled with ^{125}I or ^{131}I. Each rat was intravenously injected with both a ^{125}-I- and a ^{131}I-labelled lipoprotein. 30 min after injection a liver perfusion was started with Hank's balanced salt solution at 8°C. After 8 min of perfusion a liver lobule was taken (whole liver) and subsequently the different cell types were isolated with a low-temperature procedure. The rats were treated with ethyl oleate; control rats received the solvent (0.7% Tween-20 in 0.15 M NaCl). Values, expressed as % injected dose $\times 10^4$/mg cell protein, are means ± S.E. with N in parentheses.

	LDL	Me-LDL	CHD-LDL
Whole liver			
Control	18.8 ± 3.4 (5)	8.2 ± 0.6 (3)	22.6 ± 1.6 (6)
Ethyl oleate	12.7 ± 2.0 (5)	8.0 ± 1.5 (4)	10.5 ± 0.9 (5)
Parenchymal cells			
Control	8.2 ± 1.0 (5)	3.3 ± 0.3 (3)	7.3 ± 0.7 (5)
Ethyl oleate	8.0 ± 0.8 (5)	4.4 ± 0.4 (4)	6.7 ± 0.7 (5)
Non-parenchymal cells			
Control	164.5 ± 26.1 (5)	106.3 ± 19.8 (3)	317.4 ± 29.4 (5)
Ethyl oleate	67.2 ± 15.3 (5)	77.6 ± 35.9 (3)	94.7 ± 16.6 (5)

sociation is defined as the difference in native LDL and Me-LDL interaction). The estrogen-stimulated LDL interaction with parenchymal cells is not only blocked by methylation (97%) but also largely by cyclohexanedione treatment (81%). This indicates that for the estrogen-stimulated receptor both arginine and lysine residues in LDL are important for recognition. Furthermore, the data do indicate that during the circulation time chosen (30 min) the modification of LDL by cyclohexanedione is stable. In contrast with the estrogen-treated parenchymal cells, there is no effect of cyclohexanedione treatment of LDL on the interaction with non-parenchymal cells in estrogen-treated nor in control rats. As methylation of LDL leads to a strong inhibition of the interaction of LDL with non-parenchymal cells, indicating the importance of lysine residues for the recognition, it is clear that arginine residues in LDL do not participate in the interaction of LDL with these cell types.

As suggested by Slater et al. [13] and Packard et al. [26], the role of the reticuloendothelial system in the decay of LDL can be determined by blocking this system with ethyl oleate, whereafter the change in decay is quantified. We used an ethyl oleate treatment to determine its specific cellular effect on the liver association of LDL, CHD-LDL and Me-LDL (Table III). It appears that both treatment with Tween-20 (solvent for ethyl oleate and used here as control) and treatment with ethyl oleate specifically modify the LDL uptake by

TABLE IV

DISTRIBUTION OF NATIVE LDL, Me-LDL AND CHD-LDL BETWEEN PARENCHYMAL AND NON-PARENCHYMAL LIVER CELLS IN UNTREATED RATS 30 MIN AFTER INTRAVENOUS INJECTION

Native LDL, Me-LDL and CHD-LDL were labelled with ^{125}I or ^{131}I. Each rat was intravenously injected with both a ^{125}I- and a ^{131}I-labelled lipoprotein. 30 min after injection a liver perfusion was started with Hank's balanced salt solution at 8°C. After 8 min of perfusion a liver lobule was taken (whole liver) and subsequently the different cell types were isolated with a low-temperature procedure. Values, expressed as % injected dose $\times 10^4$/mg cell protein, are means ± S.E. with N in parentheses.

	LDL	Me-LDL	CHD-LDL
Whole liver	19.2 ± 4.0 (5)	7.3 ± 0.5 (4)	11.0 ± 1.1 (2)
Parenchymal cells	9.0 ± 2.4 (5)	3.1 ± 0.3 (4)	4.1 ± 0.1 (2)
Non-parenchymal cells	223.7 ± 21.5 (5)	125.9 ± 16.5 (3)	219.0 ± 6.9 (2)

non-parenchymal cells (cf. Table IV, which shows data with untreated rats). This enhanced cell association of CHD-LDL (as compared to native LDL, $P < 0.01$) by Tween-20 treatment is just like the cell association of native LDL, fully reduced to the level of Me-LDL by ethyl oleate treatment, indicating a loss of specific binding sites for LDL on non-parenchymal liver cells by ethyl oleate. No significant effect of Tween-20 or ethyl oleate on the interaction of LDL or Me-LDL with parenchymal liver cells is observed.

In vitro processing

Cell-association of lipoproteins is not necessarily coupled to cellular uptake and degradation of the apolipoproteins. Because cell isolation with collagenase might affect internalization steps we decided to follow the intracellular route of the lipoproteins in vitro after initial in vivo binding and internalization of the particle. The extracellular LDL was removed by a short collagenase perfusion at 37°C (Table I). Previously we applied such a collagenase incubation of cells to determine the amount of cell-bound versus internalized lipoprotein [5,7]. This allow us to follow the fate of LDL once it is internalized. As indicated by the '37°C plus collagenase' liver perfusion data (Table I), the amount of liver-associated radioactivity rapidly decreases and this condition is apparently due to release of not-internalized LDL (compared with data at '37°C minus collagenase'). After isolating the different cell types the fate of the internalized lipoproteins was followed by measuring the amount of trichloroacetic acid-soluble radioactivity released into the medium by parenchymal cells as well as non-parenchymal cells (Fig. 1). Furthermore the effect of chloroquine was determined. It appears that in control rats mainly non-parenchymal cells degrade LDL to trichloroacetic acid-soluble products and that this degradation is inhibited by about 50% by chloroquine. Also Me-LDL is degraded and also here chloroquine addition leads to an inhibition of the degradation, although the extent of inhibition is somewhat less (35%) than with LDL. No effect of estrogen-treatment is noticed on non-parenchymal cells, in contrast to parenchymal cells. After estrogen treatment also parenchymal cells degrade LDL at a significant rate and also with these parenchymal cells an inhibition (about 30%) of the degradation of LDL is observed in the presence of chloroquine.

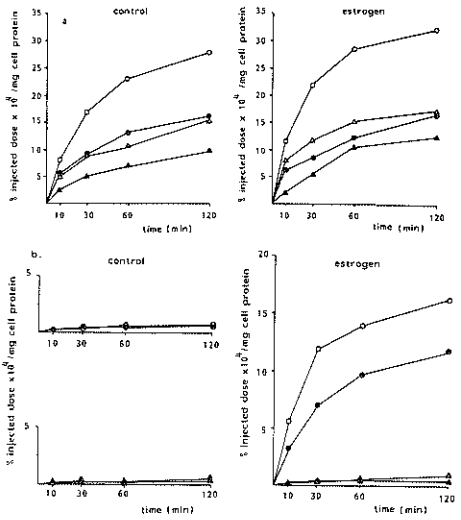

Fig. 1. Processing of native LDL (○, ●) and Me-LDL (△, ▲) by non-parenchymal (a) and parenchymal (b) liver cells from control and 17α-ethinyl estradiol-treated rats. Representative experiments are shown. The cells were isolated 10 min after intravenous injection of LDL or Me-LDL by the 37°C collagenase method (see Materials and Methods). The cells were incubated for the indicated times and the amount of trichloroacetic acid-soluble product was measured in the cell supernatant. Incubations with 100 μM chloroquine are indicated with closed symbols. The initial amount of cell-associated lipoprotein after isolation is expressed in % injected dose $\times 10^4$/mg cell protein: For control non-parenchymal cells; ○, 82.4; ●, 84.8; △, 65.6; ▲, 68.0. For estrogen-treated non-parenchymal cells; ○, 79.0; ●, 79.0; △, 63.3; ▲, 63.6. For control parenchymal cells; ○, 1.74; ●, 1.90; △, 0.86; ▲, 0.90. For estrogen-treated parenchymal cells; ○, 42.6; ●, 38.8; △, 2.12; ▲, 1.91.

Discussion

Chemical modification of the arginine or lysine residues by respectively cyclohexanedione treatment or reductive methylation of LDL inhibits the interaction of apolipoprotein B-100 with the apolipoprotein B,E receptor on cultured fibro-

lasts [11,12]. In vivo the relative importance of apolipoprotein B,E receptors for the catabolism of LDL has been determined by measuring the difference in decay between native and CHD-LDL or Me-LDL. In most studies the decay rate of Me-LDL is slightly slower than for CHD-LDL [14,26–28]. This difference is occasionally explained by the reversibility of the cyclohexanedione treatment [29]. However, during 30 min of circulation, as used in this study, no reconversion of CHD-LDL to LDL will occur [30], as is also illustrated by the inhibited association of CHD-LDL to the estrogen-stimulated receptor from the parenchymal cells. Packard et al. [26] suggested that the reticuloendothelial system is at least partly responsible for the difference in decay between CHD-LDL and Me-LDL, because especially liver and spleen show an increased uptake of CHD-LDL as compared to Me-LDL. Our results, obtained by isolating the various liver cells with a cold perfusion and isolation method, specify the cell types which are responsible for the difference in uptake between CHD-LDL and Me-LDL in vivo. The data clearly show that indeed mainly the non-parenchymal cells appear to be responsible for a higher CHD-LDL uptake as compared to Me-LDL. Specific modification of the lysine residues in native LDL does greatly diminish the uptake of LDL by the non-parenchymal cells. This suggests that lysine residues in LDL are of critical importance for recognition by non-parenchymal cells, implying a highly discriminating binding site for apolipoprotein B-100 on these cell types.

When the difference in cellular uptake between native LDL and Me-LDL is defined as receptor-dependent uptake [12] we can conclude that non-parenchymal liver cells do contain a receptor for LDL (presumably an apolipoprotein B,E receptor) whereby the apolipoprotein B-100 recognition involves lysine residues and not arginine residues. This binding site is markedly influenced by ethyl oleate treatment of the rats, and no receptor-dependent uptake of LDL by non-parenchymal cells is noticed after this treatment. In contrast, the receptor-independent uptake, represented by Me-LDL, is not influenced by ethyl oleate treatment. The interaction of LDL, Me LDL and CHD-LDL with parenchymal cells is not affected at all by ethyl oleate. From studies on the effect of ethyl oleate treatment with rabbits [13,26] it was concluded that the receptor-independent pathway was suppressed by ethyl oleate. Hereby, however, the decay of CHD-LDL is defined as receptor-independent catabolism, which is questioned now. Furthermore, our data indicate that the effect of ethyl oleate, when compared to rabbits treated with Tween-20, may be exaggerated, as Tween-20 treatment increases the uptake of CHD-LDL by non-parenchymal cells. Consequently the determination of the effect of ethyl oleate on the decay of CHD-LDL leads to an overestimation of the role of the reticuloendothelial cells in CHD-LDL clearance.

The processing of the LDL, once bound and internalized in vivo, by the various receptor sites was compared by applying a newly developed system. This system reveals information as closely as possible related to the in vivo situation. The recognition and internalization of LDL or Me-LDL occur in vivo, cell-bound lipoprotein is removed by the short 37°C collagenase treatment and for both parenchymal and non-parenchymal cells the intracellular pathway is monitored in vitro. It is not likely that the collagenase treatment of the cells will interfere with LDL metabolism once the particle is internalized. The data indicate that in control rats the degradation of LDL to trichloroacetic acid-soluble products is about 30-fold more active with non-parenchymal cells than with parenchymal cells (expressed per mg cell protein). With non-parenchymal cells a clearcut inhibition of LDL degradation by chloroquine is observed, suggesting at least the partial involvement of the lysosomes.

Me-LDL is also degraded by non-parenchymal cells; however, the involvement of the lysosomes in its degradation is somewhat less pronounced. Estrogen treatment does not influence the LDL and Me-LDL catabolism by non-parenchymal cells. It is clear that the increased uptake of LDL by the parenchymal cells upon estrogen treatment is coupled to a proportional increase in degradation. This degradation is not very effectively inhibited by chloroquine.

The aforementioned findings allow several conclusions. It appears that the non-parenchymal liver cells not only form a quantitatively important liver site for LDL interaction in vivo, but also are able

to degrade the cell-associated LDL efficiently. A similar conclusion is valid for LDL interaction with estrogen-stimulated parenchymal cells. The recognition site for LDL on non-parenchymal cells shows a unique recognition property in that the arginine residues on LDL are not important for recognition, in contrast to the lysine residues. In this respect this recognition site differs from the classical LDL or apolipoprotein B,E receptor which can also be expressed in rats on hepatic parenchymal cells after estrogen treatment.

Acknowledgements

The authors thank Kar Kruijt for his excellent technical assistance, Professor Dr. W.C. Hülsmann for critically reading and Cecile Hanson for typing the manuscript. This research was supported by a grant from the Netherlands Heart Foundation, grant No. 79.001/HA.

References

1 Langer, T., Strober, W. and Levy, R.I. (1970) in Plasma Protein Metabolism (Rothschild, M.A. and Waldman, T., eds.), pp. 483–503, Academic Press, New York
2 Lindstedt, S. (1970) in Proceedings of the Second International Symposium on Atherosclerosis (Jones, R.J., ed.), pp. 262–271, Springer-Verlag, New York
3 Mahley, R.W., Hui, D.Y., Innerarity, T.L. and Weisgraber, K.H. (1981) J. Clin. Invest. 68, 1197–1206
4 Hui, D.Y., Innerarity, T.L. and Mahley, R.W. (1982) J. Biol. Chem. 256, 5646–5655
5 Van Berkel, T.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A. (1981) Biochim. Biophys. Acta 665, 22–33
6 Van Berkel, T.J.C., Kruijt, J.K., Scheek, L.M. and Groot, P.H.E. (1983) Biochem. J. 216, 71–80
7 Harkes, L. and Van Berkel, T.J.C. (1982) Biochim. Biophys. Acta 712, 677–683
8 Chao, Y.-S., Windler, E.E.T., Chen, G.C. and Havel, R.J. (1979) J. Biol. Chem. 254, 11360–11366
9 Windler, E.E.T., Kovanen, P.T., Chao, Y.-S., Brown, M.S., Havel, R.J. and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464–10471
10 Harkes, L. and Van Berkel, T.J.C. (1983) FEBS Lett. 154, 75–80
11 Mahley, R.W., Innerarity, T.L., Pitas, R.E., Weisgraber, K.H., Brown, J.H. and Gross, E. (1977) J. Biol. Chem. 252, 7279–7287
12 Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1978) J. Biol. Chem. 253, 9053–9062
13 Slater, H.R., Packard, C.J. and Shepherd, J. (1982) J. Biol. Chem. 257, 307–310
14 Conning, D.M. and Heppleston, A.G. (1966) Br. J. Exp. Pathol. 47, 388–400
15 Redgrave, T.G., Roberts, D.C.K. and West, C.E. (1975) Anal. Biochem. 65, 42–49
16 Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235–254
17 Van Tol, A., Van 't Hooft, F.M. and Van Gent, T. (1978) Atherosclerosis 29, 449–457
18 McFarlane, A.S. (1958) Nature 182, 53
19 Nagelkeke, J.F., Barto, K.P. and Van Berkel, T.J.C. (1983) J. Biol. Chem. 258, 12221–12227
20 Van Berkel, T.J.C. and Van Tol, A. (1978) Biochim. Biophys. Acta 530, 299–304
21 Van Berkel, T.J.C. and Kruijt, J.K. (1977) Eur. J. Biochem. 73, 223–229
22 Fahimi, H.D. (1970) J. Cell Biol. 47, 247–261
23 Knook, D.L. and Sleyster, E.Ch. (1980) Biochem. Biophys. Res. Commun. 96, 250–257
24 Seglen, P.O. (1976) in Methods in Cell Biology (Prescott, D.M., ed.), Vol. XIII, pp. 28–93, Academic Press, New York
25 Brown, M.S. and Goldstein, J.L. (1974) Proc. Natl. Acad. Sci. USA 71, 788–792
26 Packard, C.J., Slater, H.R. and Shepherd, J. (1982) Biochim. Biophys. Acta 712, 412–419
27 Slater, H.R., Packard, C.J. and Shepherd, J. (1982) J. Lipid Res. 23, 92–96
28 Steinbrecher, U.P., Witzen, J.L., Kesaniemi, Y.A. and Elam, R.L. (1983) J. Clin. Invest. 71, 960–964
29 Mahley, R.W., Weisgraber, K.H., Melchior, G.W., Innerarity, T.L. and Holcombe, K.S. (1980) Proc. Natl. Acad. Sci. USA 77, 225–229
30 Shepherd, J., Bicker, S., Lorimer, A.R. and Packard, C.J. (1979) J. Lipid Res. 20, 999–1006

APPENDIX PAPER III

Volume 154, number 1 FEBS LETTERS April 1983

Cellular localization of the receptor-dependent and receptor-independent uptake of human LDL in the liver of normal and 17α-ethinyl estradiol-treated rats

Leen Harkes and Theo J.C. van Berkel

Department of Biochemistry I, Medical Faculty, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands

Received 28 December 1982

The cellular localization in the liver of the receptor-dependent and -independent uptake of human low density lipoprotein (LDL) in normal and 17α-ethinyl estradiol-treated rats was investigated by the simultaneous in vivo injection of human ^{131}I-LDL and human reductive methylated ^{125}I-LDL. The cells were subsequently isolated by a low temperature method. In untreated rats, after 30 min of in vivo circulation of human LDL, 57% of the receptor-dependent liver-association of human LDL occurs in non-parenchymal cells and 43% in parenchymal cells. Estradiol treatment of rats for 3 days selectively increases the receptor-dependent cell-association of human LDL with hepatocytes (17-fold), while the receptor-dependent cell-association with non-parenchymal cells is not affected.

Ethinyl estradiol *Low density lipoprotein* *Non-parenchymal liver cell* *Parenchymal liver cell* *Reductive methylated LDL*

1. INTRODUCTION

Administration of 17α-ethinyl estradiol in pharmacological amounts to rats produces a profound hypolipidemia [1]. The hepatic uptake and catabolism of both rat and human low density lipoprotein is stimulated many-fold in these rats [2]. This enhanced uptake is associated with an increased number of high affinity binding sites on membranes derived from the whole liver [3]. The binding site involved is described to be a functional lipoprotein receptor that recognizes lipoproteins, containing apoprotein B or E [4]. From autoradiographic studies it was concluded that estrogen-treatment enhances the normal mechanism by which LDL is taken up by the liver and that both in control and estrogen-treated rats the parenchymal cell is the predominant cell type for LDL interaction [5].

Abbreviations: LDL, low density lipoprotein; Me-LDL, reductive methylated LDL

Here, we have applied a low-temperature cell isolation method to determine the tissue site of the estradiol-stimulated lipoprotein receptor in the rat liver. A discrimination between receptor-dependent and -independent association was made by the simultaneous injection of native human ^{131}I-LDL and human reductive methylated ^{125}I-LDL (Me-^{125}I-LDL). As reductive methylation of at least 30% of the lysyl residues blocks receptor–lipoprotein interaction [6], the difference between the amount of cell-association of native and Me-LDL can be defined as receptor-mediated cell-association or uptake.

2. MATERIALS AND METHODS

17α-Ethinyl estradiol was obtained from Brocacef BV (Maarssen); collagenase (type I) from Sigma (St Louis MO); pronase B-grade from CalBiochem Behring Corp. (La Jolla CA); sodium [^{125}I]- and [^{131}I]iodide (carrier-free) was purchased from the Radiochemical Centre (Amersham).

Published by Elsevier Science Publishers
00145793/83/0000–0000/$3.00

12-Week-old male Wistar rats (250 g av. body wt) which had free access to water and food (standard laboratory chow), were used. Rats were injected subcutaneously with 17α-ethinyl estradiol dissolved in propylene glycol at 5 mg/kg body wt [2] every 24 h for 3 days. At this time the maximal decrease in plasma cholesterol concentration [3,7] and increase in LDL receptor-activity [3] is attained. Control rats received equal volumes of the solvent.

2.1. *Preparation of lipoproteins*

Human LDL ($1.024 < d < 1.055$ g/ml) was isolated as in [8], the isolated LDL was subjected to a second identical centrifugation to avoid any contamination with other lipoproteins. Apo E content of this LDL fraction was < 0.03% of the total apoprotein [9]. Radioiodination of LDL was done by a modification [10] of the ICl method in [11]. Reductive methylation of LDL was done as in [6]. About 80% of the lysyl residues from human LDL were methylated as determined by using the 2,4,6-trinitrobenzene sulfonic acid colorimetric assay [12]. Human ^{131}I-LDL and human Me-^{125}I-LDL were always prepared from the same LDL-preparation, specific radioactivity of both preparations varied from 100–500 cpm/ng apoprotein.

2.2. *Fate of LDL in rat serum and liver*

Rats were anesthesized by intraperitoneal injection of 20 mg nembutal. The abdomen was opened and about 40 μg human ^{131}I-LDL and 40 μg human Me-^{125}I-LDL in a measured volume (usually 500 μl) of 0.15 M NaCl, 0.3 mM EDTA (pH 7.0) was injected in the inferior vena cava at the level of the renal veins. After 3, 8, 15 and 25 min 0.2 ml blood was taken from the inferior vena cava at least 2 cm distal of the injection point. ^{125}I and ^{131}I were determined in the serum and the results expressed as percentage of the ^{125}I or ^{131}I in the sample taken 3 min after the injection. In some experiments 500 μl 12% trichloroacetic acid was added to 100 μl serum samples to determine the serum acid-soluble and acid-precipitable radioactivity.

After 30 min circulation of the radiolabeled lipoproteins the vena porta was cannulated and the liver was preperfused with an oxygenated Hanks buffer at 8°C. After 8 min perfusion a lobule was tied off for determination of the total liver uptake. Subsequently, the liver was subjected to a low temperature (8°C) perfusion with 0.25% pronase for the isolation of nonparenchymal cells or a low temperature (8°C) collagenase (0.05%) perfusion for the isolation of parenchymal cells based upon [13] and extensively described in [28]. The parenchymal cells were completely free from non-parenchymal cells as checked microscopically. The non-parenchymal cell preparation was completely free from parenchymal cells as checked microscopically and biochemically by the pyruvate kinase assay [14]. The purity of the cell preparation is furthermore illustrated by the described selective increase of radioactivity in parenchymal cell preparations after estrogen treatment.

2.3. *Other determinations*

Protein determination was done as in [15]. Liver wet weight for the studied rats is 3.75% of the body weight [16] and as the protein concentration in both parenchymal and non-parenchymal cells is identical (in mg/ml cell volume) [17], the relative protein contribution of parenchymal and non-parenchymal cells to total liver will be 92.5% and 7.5%, respectively [16,18]. These calculations based upon morphometric data are further sustained by our earlier studies on enzyme distribution between the different cell types [19].

3. RESULTS

The isolation of parenchymal and non-parenchymal cells was performed at a low temperature to prevent degradation of lipoproteins during the isolation procedure.

A comparison of the low temperature procedure with the method exerted at 37°C [20] indicates that the recovery of radioactivity in the isolated cells, as compared to total liver, is quantitative for the cells isolated at low temperature (table 1). The increase in recovery from 39 to 105% is caused by a 2-fold higher amount of radioactivity recovered in parenchymal cells and a 4-fold higher value in non-parenchymal cells leading to a doubling of the ratio of specific radioactivity of non-parenchymal over parenchymal cells.

In ethinyl estradiol-treated rats the disappearance rate of human LDL from the plasma is markedly increased as compared to untreated rats (fig.1) while the uptake of human LDL in the liver is 5-fold higher (table 2). The removal from serum

Table 1

Distribution of human ^{131}I-LDL between parenchymal and non-parenchymal liver cells, isolated at low (8°C) and high (37°C)[a] temperature 30 min after intravenous injection

	% × 10^4 of the injected dose/ mg cell protein[b]	
	8°C method	37°C method[a]
Whole rat liver	15.0 ± 1.2 (6)	14 ± 1
Parenchymal cells (PC)	5.8 ± 1.3 (3)	3 ± 0
Non-parenchymal cells (NPC)	138.3 ± 6.8 (3)	33 ± 6
Ratio NPC/PC	23.8	11
Recovery (%)[c]	105.3 ± 3.3 (3)	39 ± 4

[a] Values obtained from [20]

[b] Mean ± SE, n in parentheses

[c] The mean recovery of the radioactivity in the cells isolated by the 8°C method as compared to whole liver with inclusion of estradiol-treated rats and Me-LDL is 104.5 ± 3.6% (n = 10)

and uptake in liver of human Me-LDL is not influenced by estradiol treatment. For human LDL and human Me-LDL the radioactivity in plasma is for 98.3% and 99.4% trichloroacetic acid-precipitable, respectively. This percentage remains constant during the 30 min of circulation both for control and estradiol-treated rats.

Estradiol-treatment of rats leads to a 13-fold higher amount of human LDL associated with parenchymal cells, while no effect is seen on the association with non-parenchymal cells (table 3). For the human Me-LDL there is only a slight increment in the parenchymal cell-associated radioactivity. The difference in the amount of cell-association of native LDL and reductive methylated LDL can be considered to represent the receptor-mediated uptake [6]. This receptor-dependent uptake is clearly present in both parenchymal and non-parenchymal cells from untreated rats and increased 17-fold in parenchymal cells from estradiol-treated rats, while there is no significant effect of estradiol-treatment on the non-parenchymal cell uptake. The increased uptake of human LDL by parenchymal cells is not simply caused by the reduced mass of native rat LDL in ethinyl estradiol-treated rats, because the

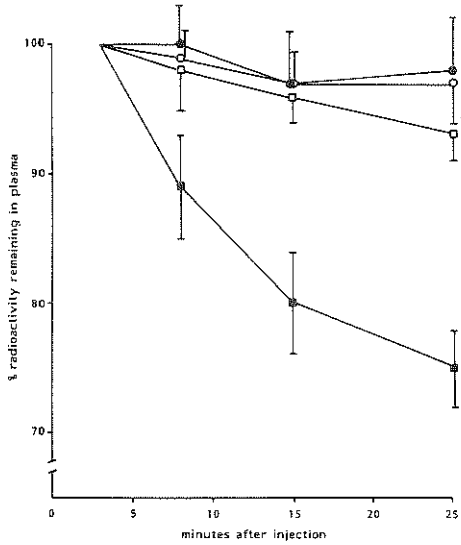

Fig. 1. Removal from blood plasma of human ^{131}I-LDL and reductive methylated human ^{125}I-LDL in control and 17α-ethinyl estradiol-treated rats. Human ^{131}I-LDL (□,■) and reductive methylated human ^{125}I-LDL (○,●) were injected intravenously in control (open symbols) and estradiol-treated rats (closed symbols). Radioactivity was determined in 50 μl samples of serum. Each point ± SE represents the mean value from 6 rats.

simultaneous injection of a 10-fold excess of human LDL did not change the relative uptake in control and estradiol-treated rats (table 4).

Taking into account the relative protein contribution of parenchymal and non-parenchymal

Table 2

Effect of estradiol treatment on the liver uptake of human ^{131}I-LDL and human Me-^{125}I-LDL 30 min after intravenous injection

	% of the injected dose[a]	
	Human LDL	Human Me-LDL
Untreated	2.8 ± 0.2 (6)	1.0 ± 0.1 (7)
Estradiol-treated	13.5 ± 1.2 (7)	1.0 ± 0.0 (8)

[a] Mean ± SE, n in parentheses

Table 3

Distribution of human ^{131}I-LDL and human Me-^{125}I-LDL between parenchymal and non-parenchymal cells in control and 17α-ethinyl estradiol-treated rats 30 min after intravenous injection

	% × 10^4 of the injected dose/mg cell protein[a]		
	Human LDL	Human Me-LDL	Receptor-dependent[b] cell association
Whole liver			
untreated	15.0 ± 1.2 (6)	5.8 ± 0.5 (6)	9.3 ± 1.0 (6)
estradiol-treated	82.2 ± 5.4 (6)	5.9 ± 0.2 (6)	75.4 ± 5.4 (6)
Parenchymal cells			
untreated	5.8 ± 1.3 (4)	1.5 ± 0.1 (3)	4.3 ± 1.2 (3)
estradiol-treated	78.0 ± 3.7 (4)	2.7 ± 0.1 (4)	75.0 ± 3.7 (4)
Non-parenchymal cells			
untreated	138.3 ± 6.8 (3)	67.4 ± 8.0 (3)	70.9 ± 8.7 (3)
estradiol-treated	126.7 ± 13.3 (3)	40.9 ± 6.8 (3)	85.9 ± 6.8 (3)

[a] Mean ± SE, n in parentheses

[b] Receptor-dependent cell association is the difference between human ^{131}I-LDL and human Me-^{125}I-LDL

cells to total liver it can be calculated that in the estrogen-treated rats, the parenchymal cells form the major tissue site for receptor-dependent liver uptake of human LDL (92%). In contrast, in untreated rats the non-parenchymal cells are quantitatively more important with 57% of the total receptor-dependent cell-association for human LDL.

Table 4

Effect of a 10-fold excess of unlabeled human-LDL on the uptake of human ^{131}I-LDL and human Me-^{125}I-LDL in parenchymal cells of untreated and 17α-ethinyl estradiol-treated rats, 30 min after intravenous injection[a]

	% × 10^4 of the injected dose /mg cell protein[b]	
	Human LDL	Human Me-LDL
Whole liver		
untreated	16.5 ± 0.8	8.9 ± 1.6
estradiol-treated	86.1 ± 15.2	13.5 ± 3.9
Parenchymal cells		
untreated	8.1 ± 0.2	3.9 ± 0.2
estradiol-treated	102.9 ± 1.4	8.0 ± 4.4

[a] Containing: (1st expt) 34 μg human ^{131}I-LDL, 19 μg human Me-^{125}I-LDL and 335 μg unlabeled human LDL; (2nd expt) 51 μg human ^{131}I-LDL, 19 μg human Me-^{125}I-LDL and 670 μg unlabeled human LDL

[b] Mean ± SE, n = 2

4. DISCUSSION

The cellular localization of the liver uptake of human LDL in vivo could be determined quantitatively by taking 2 precautions to prevent loss of lipoprotein degradation products from the cells.

(1) A circulation time of 30 min was chosen. In this time interval no increase of trichloroacetic acid-soluble products in serum occurs, nor is there any difference in the amount of the acid-soluble products between treated and control rats. A similar lag phase for human LDL degradation in rats is found in [2].

(2) A cell isolation procedure for parenchymal and non-parenchymal cells was performed in which no loss of degradation products occurs. This was achieved by maintaining a low temperature during the initial liver perfusion and subsequent isolation of the liver cells. The data indicate that this procedure leads to a quantitative recovery of the total liver-associated radioactivity in the subsequently isolated cells.

The simultaneous circulation of native and Me-LDL enables us to discriminate between receptor-dependent and receptor-independent uptake [6]. By applying a sucrose-label [27] in total liver 67.4% of the total LDL uptake was shown to be receptor-mediated. For whole rat liver we obtain a value of 61.3%. The present results indicate that in normal rats both in parenchymal and non-parenchymal cells a receptor-mediated uptake mechanism for human LDL is present. These data obtained in vivo, confirm data obtained in vitro, which showed the presence of a human LDL receptor in freshly isolated parenchymal cells [9] and non-parenchymal cells [21] from untreated rats. Estradiol treatment of the rats selectively increases the receptor-mediated uptake of human LDL in parenchymal cells, while the uptake in non-parenchymal cells is not affected. The parenchymal liver cells are therefore solely responsible for the increased liver-association of human LDL in estradiol-treated rats and form then the major liver site for receptor-dependent cell-association of human LDL with 92% of the total liver amount. This value agrees with autoradiographic data [5], which indicated that 5–15% of the grains of radiolabeled human LDL were seen over non-parenchymal cells after estrogen-treatment. In untreated rats, however, the non-parenchymal cells are quantitatively an important liver site for receptor-dependent cell-association of human LDL with 57% of the total liver uptake. The receptor-independent uptake of human LDL in rat liver is not influenced by estrogen-treatment and is mainly exerted by the non-parenchymal cells.

The properties of the estradiol-stimulated lipoprotein receptor of rat liver are extensively described in [2–4] and it appears that it reflects the LDL receptor characterized on extrahepatic cells (review [22]). However, in these studies membrane preparations from total liver or liver perfusions are used and it was not possible to decide if the estradiol-induced LDL binding sites and the enhanced LDL uptake occurs in parenchymal or non-parenchymal cells [3]. Our data indicate that selectively parenchymal cells show an increased uptake of human LDL as a result of estrogen-treatment and consequently the metabolism of LDL inside the liver is not only quantitatively but also in relation to cellular sites greatly changed.

With parenchymal liver cells isolated from untreated rats, we investigated the properties of a binding site for human LDL [9]. It was found that human LDL is bound with high affinity and the binding site recognizes both apo B as well as apo E containing lipoproteins and resembles the inducible apo B,E receptor [23]. These properties differ from the receptor which mediates the uptake of rat very low density lipoprotein (VLDL)-remnants [24] or rat chylomicron-remnants [25,26]. This remnant receptor, recognizing apo E, does not interact with human LDL. The ability to induce selectively the apo B,E receptor in parenchymal liver cells and not in non-parenchymal cells may form an important tool to determine the relative importance of the different cell types and receptors for liver lipoprotein metabolism.

ACKNOWLEDGEMENTS

We thank Kar Kruijt and Koen Barto for their excellent technical assistance and Cecile Hanson for typing the manuscript. This research was supported by a grant from the Netherlands Heart Foundation, grant No. 79.001/HA.

REFERENCES

[1] Davis, R.A. and Roheim, P.S. (1978) Atherosclerosis 30, 293–299.
[2] Chao, Y.-S., Windler, E.E.T., Chen, G.C. and Havel, R.J. (1979) J. Biol. Chem. 254, 11360–11366.
[3] Kovanen, P.T., Brown, M.S. and Goldstein, J.L. (1979) J. Biol. Chem. 254, 11367–11373.
[4] Windler, E.E.T., Kovanen, P.T., Chao, Y.-S., Brown, M.S., Havel, R.J. and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464–10471.
[5] Chao, Y.-S., Jones, A.L., Hradek, G.T., Windler, E.E.T. and Havel, R.J. (1981) Proc. Natl. Acad. Sci. USA 78, 597–601.
[6] Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1978) J. Biol. Chem. 253, 9053–9062.
[7] Arbeeny, C.M. and Eder, H.A. (1980) J. Biol. Chem. 255, 10547–10550.
[8] Redgrave, T.G., Roberts, D.C.K. and West, C.E. (1975) Anal. Biochem. 65, 42–49.
[9] Harkes, L. and Van Berkel, Th.J.C. (1982) Biochim. Biophys. Acta 712, 677–683.
[10] Van Tol, A., Van 't Hooft, F.M. and Van Gent, T. (1978) Atherosclerosis 29, 449–457.
[11] McFarlane, A.S. (1958) Nature 182, 53.

[12] Habeeb, A.F.S.A. (1966) Anal. Biochem. 14, 328–336.
[13] Praaning-Van Dalen, D.P. and Knook, D.L. (1982) FEBS Lett. 141, 229–232.
[14] Van Berkel, Th.J.C. and Kruijt, J.K. (1977) Eur. J. Biochem. 73, 223–229.
[15] Lowry, O.H., Rosebrough, R.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell Biol. 72, 441–455.
[17] Munthe-Kaas, A.C., Berg, T. and Sjeljelid, R. (1976) Exp. Cell Res. 99, 146–154.
[18] Groot, P.H.E., Van Berkel, Th.J.C. and Van Tol, A. (1981) Metabolism 30, 792–797.
[19] Van Berkel, Th.J.C., Kruijt, J.K. and Koster, J.F. (1975) Eur. J. Biochem. 58, 145–152.
[20] Van Tol, A. and Van Berkel, Th.J.C. (1980) Biochim. Biophys. Acta 619, 156–166.
[21] Van Berkel, Th.J.C., Nagelkerke, J.F., Harkes, L. and Kruijt, J.K. (1982) Biochem. J. 208, 493–503.
[22] Brown, M.S., Kovanen, P.T. and Goldstein, J.L. (1981) Science 12, 628–635.
[23] Hui, D.Y., Innerarity, T.L. and Mahley, R.W. (1981) J. Biol. Chem. 256, 5646–5655.
[24] Van Berkel, Th.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A. (1981) Biochim. Biophys. Acta 665, 22–33.
[25] Cooper, A.D., Ericksón, S.K., Nutik, R. and Shrewsbury, M.A. (1982) J. Lipid Res. 23, 42–52.
[26] Kita, T., Goldstein, J.L., Brown, M.S., Watanabe, Y., Hornich, C.A. and Havel, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 3623–3627.
[27] Carew, T.E., Pittman, R.C. and Steinberg, D. (1982) J. Biol. Chem. 257, 8001–8008.
[28] Nagelkerke, J.F., Barto, K.P. and Van Berkel, Th.J.C. (1982) in: Sinusoidal Liver Cells (Knook, D.L. and Wisse, E. eds) pp.319–326, Elsevier Biomedical, Amsterdam, New York.

APPENDIX PAPER IV

Biochimica et Biophysica Acta, 712 (1982) 677–683
Elsevier Biomedical Press

BBA 51200

A SATURABLE, HIGH-AFFINITY BINDING SITE FOR HUMAN LOW DENSITY LIPOPROTEIN ON FRESHLY ISOLATED RAT HEPATOCYTES

LEEN HARKES and THEO J.C. VAN BERKEL

Department of Biochemistry I, Faculty of Medicine, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

(Received April 28th, 1982)

Key words: Lipoprotein receptor; LDL; Lipid binding; (Rat hepatocyte)

Freshly isolated rat hepatocytes bind the solely apolipoprotein B-containing human low density lipprotein (LDL) with a high-affinity component. After 1 h of incubation less than 30% of the cell-associated human LDL is internalized and no evidence for any subsequent high-affinity degradation was obtained. Scatchard analysis of the binding data for human ^{125}I-labeled LDL indicates that the high-affinity receptor for human LDL on rat hepatocytes possesses a K_d of $2.6 \cdot 10^{-8}$ M, while the binding is dependent on the extracellular Ca^{2+} concentration. Competition experiments indicate that both the apolipoprotein B-containing lipoproteins (human LDL and rat LDL) as well as the apolipoprotein E-containing lipoproteins (human HDL and rat HDL) do compete for the same surface receptor. It is concluded that hepatocytes freshly isolated from untreated rats do contain, in addition to the earlier described rat lipoprotein receptor which does not interact with human apolipoprotein B-containing LDL, a high-affinity receptor which interacts both with solely apolipoprotein B-containing human LDL and apolipoprotein E-containing lipoproteins.

Introduction

Low density lipoprotein (LDL) is the major carrier of plasma cholesterol in man and has been implicated as an independent risk factor in atherogenesis. The liver plays an important role in serum lipoprotein catabolism [1] and is the only organ where cholesterol can be removed from the circulation and degraded to bile acids [2]. A high-affinity receptor for LDL with a K_d of around 10^{-9} M [3] has been found on the cell surface of a number of cell types of extrahepatic origin (for a review see Ref. 4). This receptor binds specifically apolipoprotein B-containing LDL and apoliprotein E-containing high density lipoprotein c (HDL c) [5], the latter with a much greater affinity [3]. The role of this high-affinity receptor in vivo is indicated by the much-elevated plasma LDL levels in patients [6,7] and rabbits [8,9] with genetically determined receptor deficiency. The importance of the liver in LDL catabolism is underlined by a rise in the plasma level of LDL of rabbits which lack the LDL recpetor in liver [9] and the reduction of the fractional catabolic rate of LDL apolipoprotein in rat proportional to the amount of liver removed [10].

The existence of hepatic high-affinity binding of LDL has been shown by studies with cultured rabbit hepatocytes [11] and freshly isolated rat hepatocytes [12]. However, the aforementioned preparations contain not only apolipoprotein B, but also apolipoprotein E [12,13], and it is suggested that apolipoprotein E is an important determinant in the hepatic uptake of plasma lipoproteins [14,15]. With human LDL, which contains no apolipoprotein E, no high-affinity binding to rat liver membranes isolated from untreated rats could

Abbreviations: LDL, low density lipoprotein (d 1.019–1.063 g/ml for human LDL, or 1.019–1.050 for rat LDL); HDL, high density lipoprotein (d 1.063–1.21 for human HDL, or 1.050–1.13 for rat HDL); EGTA, ethyleneglycol-2-(2-aminoethyl)-tetracetic acid.

be found [16]. So whether normal rat liver contains a receptor analogous to those found in extrahepatic tissues and which recognizes the solely apolipoprotein B-containing human LDL, is still questionable. Bachorik et al. [17,18] demonstrated high-affinity binding of homologous apolipoprotein E-deficient LDL to porcine hepatic membranes with binding characteristics which differ from those of the extrahepatic LDL receptor [4].

The present studies were undertaken to investigate the receptor binding of solely apolipoprotein B-containing human LDL, using hepatocytes freshly isolated from untreated rats. This system allows the measurement of both binding and subsequent handling of the lipoproteins. In addition, isolated hepatocytes offer a homogeneous population of the predominant cell type of liver, in contrast to membranes isolated from total liver, from which the non-parenchymal cells can contribute up to 25–30% [19]. In this paper we report evidence for high-affinity binding of human LDL to rat hepatocytes.

Materials and Methods

Isolation and labeling of serum lipoproteins

Human LDL and HDL were isolated exactly as described by Redgrave et al. [20]. Rat LDL and HDL were isolated as described in Ref. 21. The radioiodination of LDL, using a modification of the ICl method originally described by McFarlane [22], is described in Ref. 10.

The distribution of the apolipoproteins and the radioactivity in rat LDL (containing apolipoprotein E) is the same as described in Refs. 13 and 10. The distribution of the radioactivity in human LDL is: 88% in protein; 8% in phospholipids and 4% free, determined according to the method of Folch et al. [23]. The human LDL preparation used in this study contains mostly apolipoprotein B (99.92%). With a high LDL concentration (5 mg apolipoprotein/ml) in a radial immunodiffusion system according to the method of Mancini [33], apolipoprotein E was noticable at the detection limit and contributed maximally 0.06–0.1% of the total apolipoprotein. On some occasions, the isolated LDL was subjected to a second gradient centrifugation according to the method of Redgrave et al. [20] and in these preparations the relative amount of apolipoprotein E was approximately 2–3-fold lower.

Isolation of rat hepatocytes

Hepatocytes (parenchymal liver cells) were isolated from male Wistar rats of about 3 months old, according to the method of Seglen [24], with a few modifications. The whole perfusion procedure takes place in situ. First, the liver was pre-perfused during 10 min with a 37°C Carbogen (95% 0_2/5% CO_2) -saturated, Ca^{2+}-free Hank's buffer (8.0 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.06 g $Na_2HPO_4 \cdot 2H_2O$), 0.06 g KH_2PO_4, 1 g glucose, 4.77 g 2-(4-(2-hydroxyethyl)1-piperazinyl)ethanesulfonic acid and double-destilled H_2O per 1000 ml, pH 7.4) with 10 μM MgEGTA at a rate of 14 ml/min. Then, collagenase, final concentration 0.05%, and Ca^{2+}, final concentration 1 mM, was added to the buffer and after 20 min perfusion the liver was removed and gently dispersed in the collagenase buffer. The crude cell suspension was incubated for 10 min at 37°C under a constant Carbogen supply, whereafter the cell suspension was filtered through a 250 μm and a 90 μm nylon mesh.

Hepatocytes were isolated by differential centrifugation as described by Van Berkel and Van Tol [25]. After the second wash, the cell suspension was filtered through a 44 μm nylon mesh to obtain a cell suspension without cell aggregates. Cell washing was carried out with the preperfusion medium.

Lipoprotein association and degradation

Lipoproteinbinding, uptake and degradation was determined as described by Van Berkel et al. [13] with a few modifications. The incubations were carried out in plastic tubes under constant Carbogen supply. At the indicated time, 0.2-ml samples were withdrawn and the cells were obtained by centrifugation in an Eppendorf centrifuge for 2 min at 1700 rpm. After washing the cell pellets three times with 1 ml 50 mM Tris-HCl/0.15 M NaCl, pH 7.4 (twice with and once without 0.2% albumin), the pellets were counted in a LKB-Wallace ultrogamma counter. To 0.1 ml supernatant of the incubation sample, 0.5 ml 12% trichloroacetic acid was added to precipitate the protein present. The free I^- present in 0.5 ml

trichloroacetic acid-soluble phase was oxidized and the formed I_2 was extracted with 0.75 ml $CHCl_3$. 0.4 ml of the aqueous and 0.5 ml of the chloroform phase were counted for ^{125}I.

Protein determination was carried out according to the method of Lowry et al. [26].

Results

The cell association of rat LDL and human LDL is compared in Fig. 1. Although a rat LDL concentration 75% lower (2.7 μg apoprotein/ml) than that of human LDL (11.1 μg apoprotein/ml) was used, double the amount of cell-associated rat LDL is still found, compared to human LDL, after 60 min incubation at 37°C. After 60 min of incubation no further increase in cell-associated radioactivity occurred, so that equilibrium is apparently reached. Eight other experiments with different human LDL preparations, done under similar conditions, show that the association after 60 min incubation with LDL (10 μg apolipoprotein/ml) is rather constant (13.5 ± 1.7 ng apolipoprotein/mg cell protein (± S.E.)).

As reported earlier [13], hepatocytes show high-affinity degradation of rat LDL. However, no

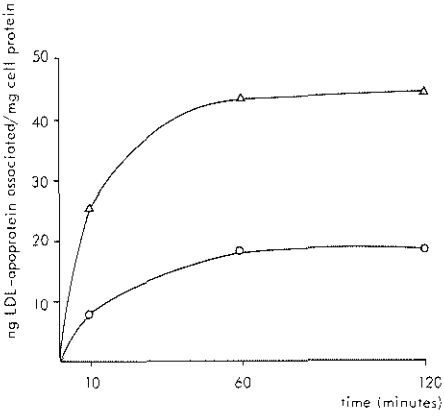

Fig. 1. Time course of the cell association of rat and human low density lipoproteins. Hepatocytes (0.8 mg cell protein/ml) were incubated with 2.7 μg rat ^{125}I-LDL apolipoprotein/ml (△) or 11.1 μg human ^{125}I-labeled LDL apoprotein/ml (○). Cell association is expressed as ng apolipoprotein/mg cell protein.

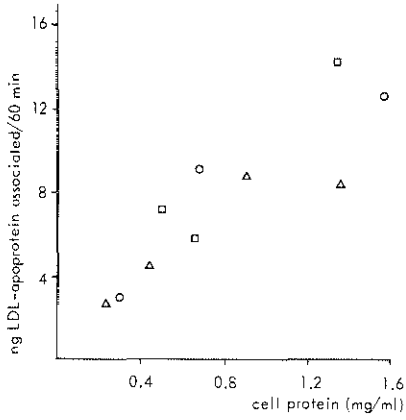

Fig. 2. The amount of cell-associated human ^{125}I-labeled LDL at increasing amounts of rat hepatocytes. Hepatocytes were incubated with 10.0 μg ^{125}I-labeled LDL apolipoprotein/ml. Incubations were carried out with three different cell preparations (△, ○, □).

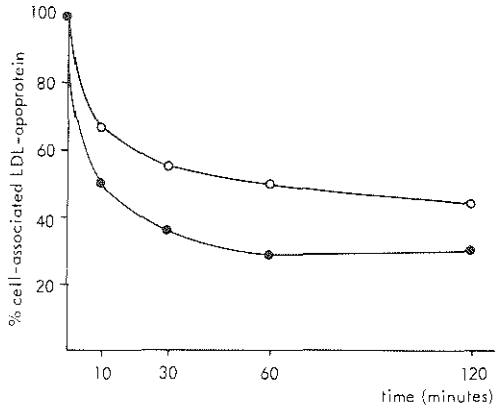

Fig. 3. The effect of incubation in a lipoprotein-free medium in the absence and presence of collagenase (0.05%) upon the amount of cell-associated human ^{125}I-labeled LDL. Cells were preincubated for 60 min with 10 μg ^{125}I-labeled LDL apolipoprotein/ml. The initial cell-associated ^{125}I-labeled LDL ($t = 0$) was determined in three 0.2 ml samples at the end of the preincubation. The rest of the cells were centrifuged (30 s, 50 × g), washed twice with medium A and subsequently incubated in medium A in the absence (0) or presence (●) of 0.05% collagenase.

evidence for the time-dependent appearance of trichloroacetic acid-soluble degradation products, originating from human LDL, under similar conditions was obtained, even when the time course of incubation was extended to 3 h.

Fig. 2 shows the amount of cell-associated radio-activity as a function of the amount of cell protein after a 60 min incubation with human ^{125}I-labeled LDL (10 μg apolipoprotein/ml). The obtained values are from three different cell preparations and it seems evident that the extent of association is linear with the amount of cell protein up to 1.6 mg protein/ml. Evidence that up to 1 h of incubation only binding, instead of binding plus uptake, is measured can be derived from Fig. 3. Cells which were incubated for 1 h with ^{125}I-labeled LDL were subsequently incubated in a lipoprotein-free medium. 56% of the associated radioactivity is the dissociated from the cells. When 0.05% collagenase is included in the medium then this percentage increases up to 70% from the total associated radioactivity. When the same experiment was repeated in the absence and presence of a 20-fold excess of unlabled LDL, it was found that the amount of releasable radioactivity was also 70% for the high-affinity component.

Fig. 4 shows the fate of the cell-associated human ^{125}I-labeled LDL. No evidence for a time-dependent degradation to water-soluble amino acids could be obtained, and only little deiodination occurs. It appears that most of the cell-associated radioactivity is released from the cells in particle form. Adding 0.05% collagenase to the incubation medium results in an initial increase to the water-soluble degradation products, which stops after 60 min. In the first 60 min more radioactivity is released from the cells but less in the trichloroacetic acid-precipitable form. Unlike in Fig. 3, the zero time here includes the two washings of the cells with Ham's F-10 medium with 5% lipoprotein-deficient serum (medium A) so that the initial cell-associated radioactivity was decreased as compared to Fig. 3 (see also the legends of Figs. 3 and 4).

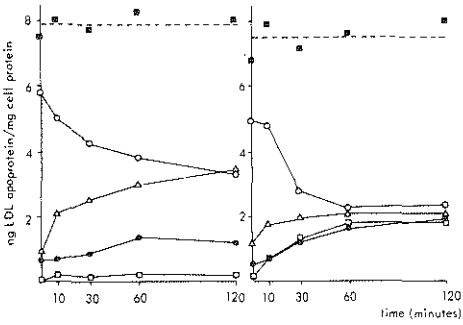

Fig. 4. Release and degradation of cell-associated ^{125}I-labeled LDL in medium A in the absence (left) or presence (right) of 0.05% collagenase. Isolated cells were preincubated with 10 μg ^{125}I-labeled LDL apolipoprotein for 60 min. Subsequently the cells were centrifuged (30 s, 50 × g) and washed twice with medium A. These cells were incubated in medium A in the absence or presence of 0.05% collagenase. Cell-associated (○), trichloroacetic acid precipitable (△), trichloroacetic acid-soluble (water-soluble (□) and $CHCl_3$-soluble (●)) radioactivity was determined. Calculated sum of the four different fractions.

The amount of cell association as a function of the extracellular LDL concentration is shown in Figs. 5A and B. The data obtained after 60 min of incubation, when equilibration is reached (see Fig. 1), can be analyzed in a Scatchard plot [27,28]. Two components can be detected, one that is saturable and possesses a high affinity for human LDL and non-saturable low-affinity one. The slope k_1 and the intercepts of the straight line (representing the saturable association) indicate an apparent K_d of 15.3 μg apolipoprotein/ml ($2.55 \cdot 10^{-8}$ M) and a maximal association of 32 ng apolipoprotein/mg cell protein (32 000 LDL particles/cell for the high-affinity binding (molecular weight apolipoprotein LDL $0.6 \cdot 10^6$ [29]).

It was investigated to what extent the low amount of apolipoprotein E in human LDL preparations (0.06–0.1%) could be responsible for this binding. Therefore LDL was subjected to a second gradient centrifugation according to the method of Redgrave et al. [20]. Although the amount of apolipoprotein E of this LDL preparation was 2–3-fold lower (tested by immunodiffusion according to the method of Mancini [33]), a similar time-dependent association (not shown) and K_d (13.7 μg/ml) was obtained. Table I indicates the competition of unlabeled rat and human lipoproteins with the association of human LDL to rat hepatocytes. It can be seen that both the solely apolipoprotein B-containing unlabeled human LDL and the apolipoprotein E-containing rat LDL compete with the association of the radioactively

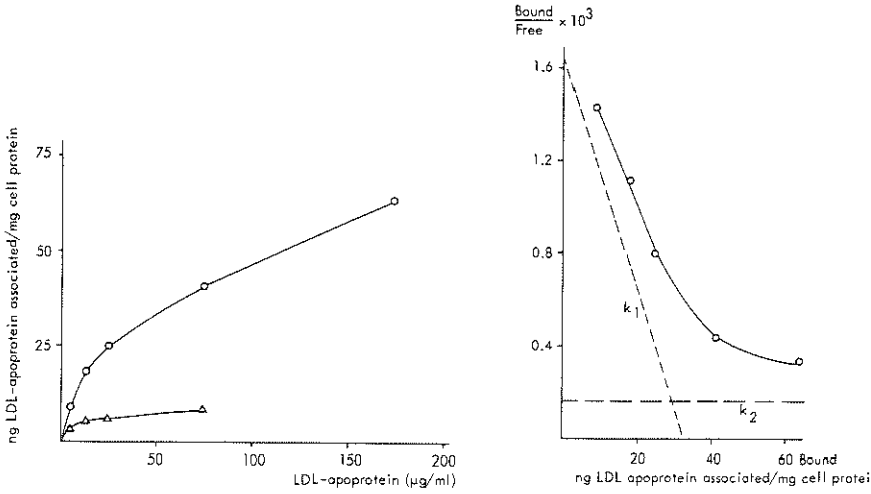

Fig. 5A. Relation between increasing amount of human ^{125}I-labeled LDL and the extent of cell association. Hepatocytes (0.8 mg cell protein/ml) were incubated with ^{125}I-labeled LDL (4.7–173 μg apolipoprotein/ml). Samples were taken after 10 (△) and 60 (○) min of incubation. B. Scatchard plot of the ^{125}I-labeled association after 60 min of incubation. k_2 represents the non-saturable, low-affinity component, k_1 represents the high-affinity component of the ^{125}I-labeled LDL association.

TABLE I

EFFECT OF THE PRESENCE OF MgEGTA AND COMPARISON OF THE ABILITY OF UNLABELED LIPOPROTEINS TO COMPETE WITH HUMAN ^{125}I-LABELED LDL FOR BINDING TO RAT HEPATOCYTES

Each incubation contained 0.8 mg cell protein/ml and human ^{125}I-labeled rat LDL at an apolipoprotein concentration of 10 μg/ml. The amount of cell-associated radioactivity without further additions, at 0.3 mM Ca^{2+}, is taken as 100%. Figures in parentheses the amount of different experiments in which the unlabeled lipoproteins were added. The concentrations of LDL and HDL were 190 μg/ml.

MgEGTA or unlabeled lipoprotein added	Amount of human ^{125}I-labeled LDL associated to cells ± S.E. after 1 h of incubation (%)
None	100
MgEGTA (0.3 mM)	55±5 (3)
Human LDL	48±5 (5)
Rat LDL	46±6 (5)
Human HDL	46±2 (2)
Rat HDL	42±5 (2)

labeled human LDL. Furthermore, apolipoprotein B-deficient, apolipoprotein E-containing, HDL also competes with the association of human LDL. The presence of 0.3 mM MgEGTA in the incubation medium decreases the binding of the human ^{125}I-labeled LDL by 45%.

Discussion

The data in this paper demonstrate that hepatocytes from untreated rat possess a binding site that recognizes human ^{125}I-labeled LDL. It is unlikely that the presence of 0.06–0.1% apolipoprotein E in our human LDL preparation is responsible for the observed association. Furthermore, a 2–3-fold decrease of the apolipoprotein E content does not change the association properties of the human LDL preparation. We used freshly isolated hepatocytes because we wanted to correlate in vivo uptake data [21] with the presence of specific receptors on the cells. Earlier in our

laboratory it was shown that the in vivo uptake of rat very low density lipoprotein remnants, LDL and HDL can be explained by the presence of a rat lipoprotein receptor which recognizes all these three rat lipoproteins [13]. The in vivo uptake of human LDL by rat hepatocytes is, however, considerably less than that of the rat lipoproteins [21]. Two explanations are possible to explain this low uptake: 1, The human LDL interacts with the rat lipoprotein receptor with a much lower affinity. 2, A different receptor for human LDL is present with a high affinity for LDL but much lower capacity than the rat lipoprotein receptor.

The present paper shows that the second explanation is valid. Because of the low amount of high-affinity binding of human LDL and the small amount of the cell-associated lipoprotein that is internalized (less than 30% after 1 h of incubation), we could not demonstrate any high-affinity degradation. The presence of deiodinase in hepatocytes [11], furthermore, complicates the sensitive detection of such specific degradation. However, if any high-affinity degradation of human LDL occurs, it can be concluded that it will be low.

The properties of the human LDL receptor on rat hepatocytes are very similar to those of the human fibroblast receptor, originally described by Brown and Goldstein [34], and to those of a number of other cell types of extrahepatic origin (for a review, see Ref. 4), but dissimilar to the recently described rat lipoprotein receptor [13,30], of which it was shown that the human LDL preparation did not compete with the binding of rat lipoproteins [13]. The human LDL receptor recognizes human apolipoprotein B, and shows also an interaction with apolipoprotein E-containing lipoproteins such as rat LDL, rat HDL and human HDL. Furthermore, it shows a high-affinity binding with a K_d of $2.6 \cdot 10^{-8}$ M for human LDL, which value is similar to the high-affinity binding site for human LDL in liver membranes from ethinyl estradiol-treated rats ($K_d = 3.8 \cdot 10^{-8}$ M) [16]. In untreated rats, Kovanen et al. [31] were unable to characterize such binding sites, probably because the concentration of the receptor is low and difficult to measure in a total liver membrane preparation. Using the freshly isolated hepatocytes, we were able to show that these cells have a maximal high-affinity binding of 32 ng human LDL apolipoprotein/mg cell protein (32000 LDL particles/cell). This is only 10–15% of what can be bound to the rat lipoprotein receptor [12,13]. Similar to the properties of the extrahepatic receptor and the receptor on liver membranes of ethinyl estradiol-treated rats, the association of human LDL demonstrated here depends on the Ca^{2+} concentration.

The physiologic significance of this receptor on rat hepatocytes is still unclear; however, recently Hui et al. [32] showed that under certain conditions, growth or drug treatment, expression of an apolipoprotein B, E receptor on canine liver membranes can be induced. It might be possible that the rat lipoprotein receptor, which probably recognizes apolipoprotein E, does not interact with human apolipoprotein B function as a constitutive receptor, while the receptor characterized in this paper does accommodate its level to the metabolic requirements. Further studies on the inducible expression of both receptors are clearly needed to correlate their presence with the cellular in vivo uptake of the various lipoproteins.

Acknowledgements

The authors thank Dr. Louis Havekes (Gaubius Institute, Leiden) for the apolipoprotein E determination. Cecile Hanson is thanked for secretarial help. The Netherlands Heart Foundation is acknowledged for financial support (grant 79.001/HA).

References

1 Langer, T., Strober, W. and Levy, R.I. (1970) in Plasma Protein Metabolism (Rothschild, M.A. and Waldman, T., eds.), pp. 483–503, Academic Press, New York
2 Lindstedt, S. (1970) in Proceedings of the Second International Symposium on Atherosclerosis (Jones, R.J., ed.), pp. 262–271, Springer Verlag, New York
3 Pitas, R.E., Innerarity, T.L., Arnold, K.S. and Mahley, R.W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2311–2315
4 Brown, M.S., Kovanen, P.T. and Goldstein, J.L. (1981) Science 12, 628–635
5 Mahley, R.W. and Innerarity, T.L. (1978) in Proceedings of the Sixth International Symposium on Drugs Affecting Lipid Metabolism (Kritchevsky, D., Paoletti, R. and Holmes, W.L., eds.), pp. 99–127, Plenum Press, New York
6 Goldstein, J.L. and Brown, M.S. (1974) J. Biol. Chem. 249, 5153–5162

7 Albers, J.J. and Bierman, E.L. (1976) Biochim. Biophys. Acta 424, 422–429
8 Tanzawa, K., Shimada Y., Kuroda, M., Tsujita, Y., Arai, M. and Watanabe, Y. (1980) FEBS Lett. 118, 81–84
9 Kita, T., Brown, M.S., Watanabe, Y. and Goldstein, J.L. (1981) Proc. Nat. Acad. Sci. U.S.A. 78, 2268–2272
10 Van Tol, A., Van 't Hooft, F.M. and Van Gent, T. (1978) Atherosclerosis 29, 449–457
11 Soltys, P.A. and Portman, O.W. (1979) Biochim. Biophys. Acta 574, 505–520
12 Van Berkel, T.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A. (1980) Biochem. Biophys. Res. Commun. 92, 1002–1008
13 Van Berkel, T.J.C., Kruijt, J.K., Van Gent, T. and Van Tol, A. (1981) Biochim. Biophys. Acta 665, 22–33
14 Shelburne, F., Hanks, J., Meyers, W. and Quarfordt, S. (1980) J. Clin. Invest. 65, 652–658
15 Mahley, R.W., Weisgraber, K.H., Melchior, G.W., Innerarity, T.L. and Holcombe, K.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 225–229
16 Windler, E.E.T., Kovanen, P.T., Chao, Y-S., Brown, M.S., Havel, R.J. and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464–10471
17 Bachorik, P.S., Livingston, J.L., Cooke, J. and Kwiterovich, P.O. (1976) Biochem. Biophys. Res. Commun. 69, 927–935
18 Bachorik, P.S., Kwiterovich, P.O. and Cooke, J.C. (1978) Biochemistry 17, 5287–5299
19 Blouin, A., Bolender, R.P. and Weibel, E.R. (1977) J. Cell Biol. 72, 441–455
20 Redgrave, T.G., Roberts, D.C.K. and West, C.E. (1975) Anal. Biochem. 65, 42–49
21 Van Tol, A. and Van Berkel, T.J.C. (1980) Biochim. Biophys. Acta 619, 156–166
22 McFarlane, A.S. (1958) Nature 182, 53
23 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
24 Seglen, P.O. (1973) Exp. Cell Res. 82, 391–398
25 Van Berkel, T.J.C. and Van Tol, A. (1978) Biochim. Biophys. Acta 530, 299–304
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
27 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
28 Rosenthal, H.E. (1967) Anal. Biochem. 20, 525–532
29 Innerarity, T.L. and Mahley, R.W. (1978) Biochemistry 17, 1440–1447
30 Ose, T., Berg, T., Norum, K.R. and Ose, L. (1980) Biochem. Biophys. Res. Commun. 97, 192–199
31 Kovanen, P.T., Brown, M.S. and Goldstein, J.L. (1979) J. Biol. Chem. 254, 11367–11373
32 Hui, D.Y., Innerarity, T.L. and Mahley, R.W. (1981) J. Biol. Chem. 256, 5646–5655
33 Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235–254
34 Brown, M.S. and Goldstein, J.L. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 788–792

APPENDIX PAPER V

Biochem. J. (1982) **208**, 493–503
Printed in Great Britain

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

Involvement of calmodulin?

Theo J. C. VAN BERKEL, Jan F. NAGELKERKE, Leen HARKES and Johan K. KRUIJT
Department of Biochemistry I, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

(*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 μM) 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 μM). 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 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

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).

0306-3283/82/110493-11$01.50/1

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 tranquillizer trifluoperazine, an inhibitor of calmodulin (Van Berkel *et al.*, 1981*b*). 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.*, 1981*b*). 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 μl) 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.*, 1981*a*).

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

^{125}I-Acetyl-LDL (15 μg) 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 NPC_1 preparation contained solely M_2-type pyruvate kinase. The non-parenchymal cell preparation obtained by differential centrifugation (termed 'NPC_2') 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 NPC_2 fraction were corrected for the contamination by parenchymal cells as described by Van Berkel & Van Tol (1978). Peroxidase staining indicates that the NPC_2 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 'NPC_2' (Van Berkel & Van Tol, 1978)]. No correction was made for the contamination of the NPC_2 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.* (1981*a*). Degradation of the lipoproteins was measured by the method of Bierman *et al.* (1974) as described previously (Van Berkel *et al.*, 1981*a*). 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, NH_4Cl, 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 ^{125}I 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 Ca^{2+}-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 ± S.E.M. The recovery values are expressed as percentages.

	10^4 × 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

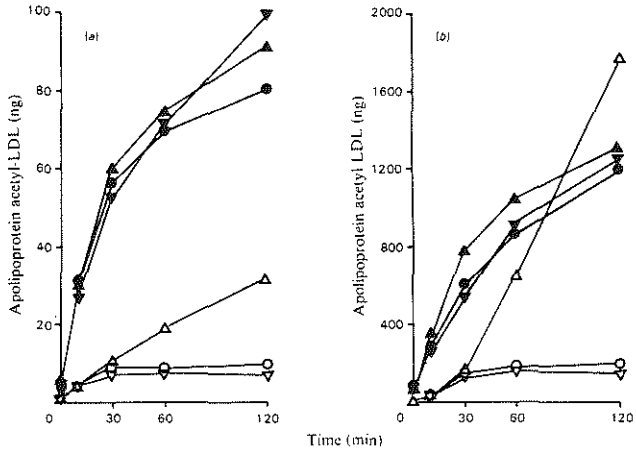

Fig. 1. *Effect of chloroquine and NH_4Cl on the time course of cell-association and degradation of acetyl-LDL by isolated parenchymal (a) and non-parenchymal (b) liver cells*

The cells were incubated with 10.1 μg of acetyl-LDL/ml in the absence (▲ and △) or presence of 100 μM-chloroquine (▼ and ▽) or 10 mM-NH_4Cl (● and ○). 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.*, 1981*a*). 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 μM) 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).

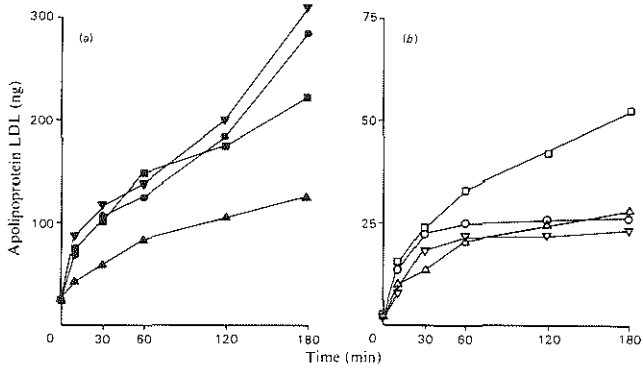

Fig. 2. *Effect of chloroquine, NH_4Cl and excess unlabelled LDL on the cell-association and degradation of native human LDL by non-parenchymal liver cells*

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.

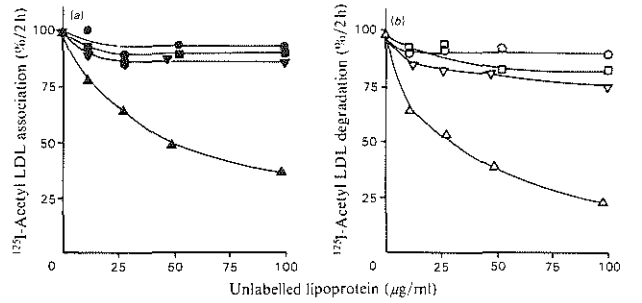

Fig. 3. *Comparison of the ability of unlabelled lipoproteins to compete with the cell association (a) and degradation (b) of ^{125}I-labelled acetylated human LDL by non-parenchymal liver cells*

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 μg 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 μM-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 μM, and with 50 μM-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. 5*a*).

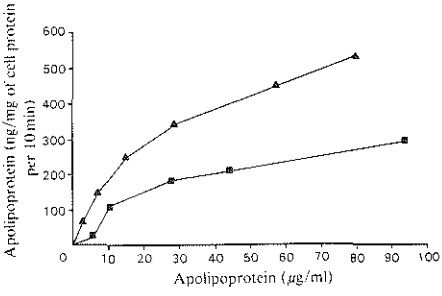

Fig. 4. *Relation of the concentration of acetyl-LDL or native LDL to the extent of cell-association with non-parenchymal liver cells*

The amount of labelled acetyl-LDL (▲) or native LDL (■) 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 μM-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 0.1–0.3 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 μg/ml) and the degradation was determined as trichloroacetic acid-soluble radioactivity in the water phase. The values are means (±S.E.M.) for three to four experiments. The 100% value represents 45 ± 6 ng of acetyl-LDL degradation/mg of cell protein.

Addition	Degradation (% of control)
None	100
Trifluoperazine (100 μM)	51 ± 5
Chloroquine (100 μM)	48 ± 4
NH_4Cl (10 mM)	53 ± 3
Unlabelled LDL (103 μg/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 μg/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.* (1981*a*). 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 ± 80 ng of acetyl-LDL/mg of cell protein ($n = 5$; means ± 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

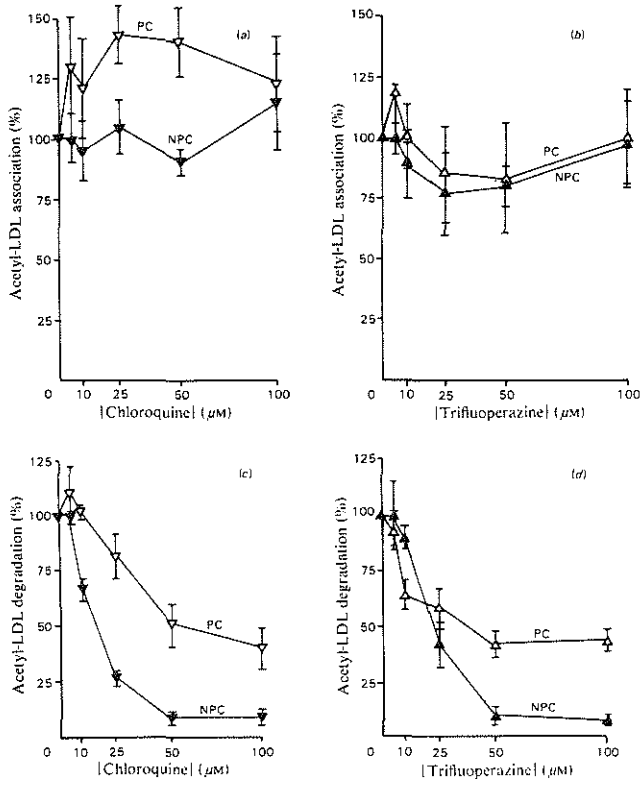

Fig. 5. *The effect of increasing chloroquine (a and c) or trifluoperazine (b and d) concentrations on the cell-association (a and b) and degradation (c and d) of acetyl-LDL by isolated parenchymal cells (PC) and non-parenchymal cells (NPC)* The cells were incubated for 2 h with 10.1 μg of acetyl-LDL/ml 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 ± 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 ± 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 radioactivity 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.

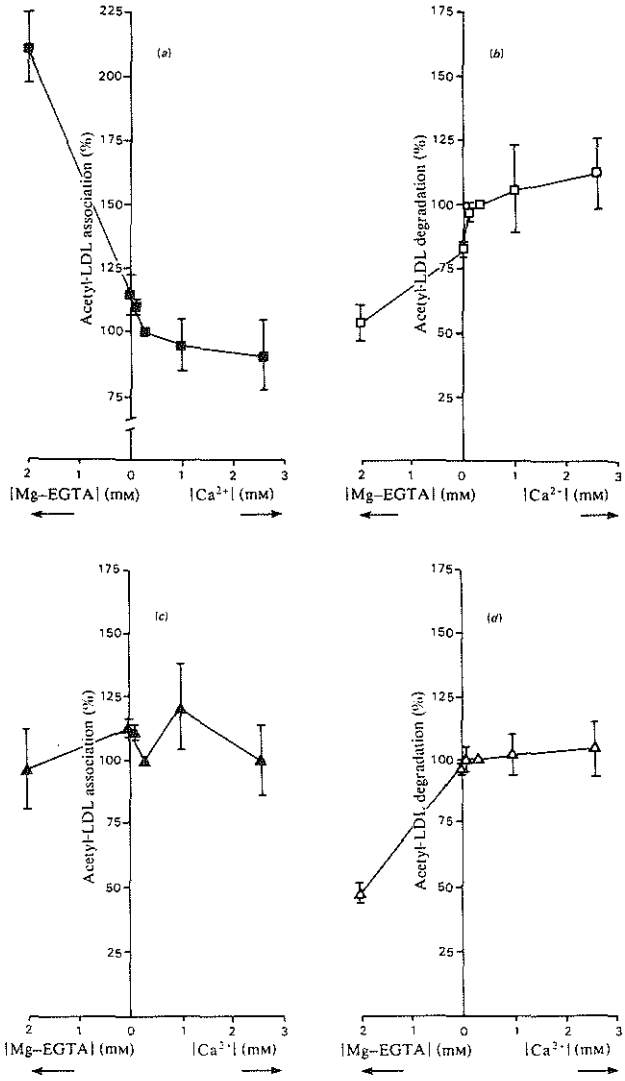

Fig. 6. *The effect of Mg–EGTA and Ca^{2+} on the cell-association (a and c) and degradation (b and d) of acetyl-LDL by parenchymal (a and b) and non-parenchymal (c and d) cells*

The cells were incubated for 2 h with 5.0 μg of acetyl-LDL/ml with the indicated |Mg–EGTA| or the different |Ca^{2+}|. 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-Ca^{2+} ± 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 ± S.E.M.).

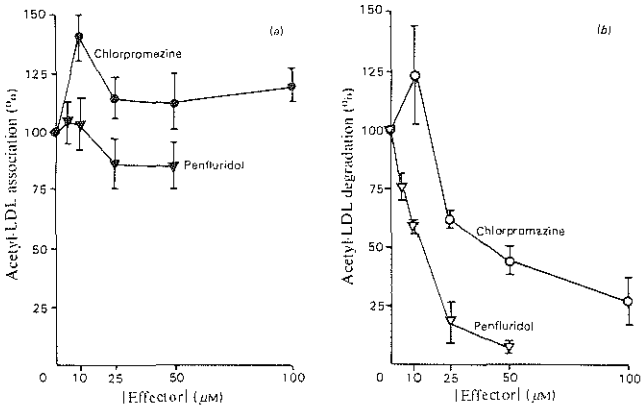

Fig. 7. *The effect of increasing penfluridol and chlorpromazine concentrations on the cell-association (a) and degradation (b) of acetyl-LDL by non-parenchymal liver cells*

The cells were incubated for 2 h with 10.1 μg 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 ± S.E.M. (indicated by the bars) The 100% value for the cell-association was 1642 ± 378 ng of acetyl-LDL/mg of cell protein and for the degradation 1947 ± 383 ng of acetyl-LDL/mg of cell protein ($n = 3$; means ± 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 μM 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 10–100 μg of unlabelled acetyl-LDL/ml was effective in showing competition, even though a 25–250-fold excess of extracellular protein was present (approx. 2500 μg 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.*, 1981*a*; 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 2h 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 12μM for penfluridol, 21μM for trifluoperazine and 35μM for chlorpromazine. The relative effectivity of these compounds to inhibit acetyl-LDL degradation corresponds to their effectivity as calmodulin inhibitors [half-maximal inhibition 2.5μM for penfluridol, 10μM for trifluoperazine and 42μM 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.*, 1981*b*). 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.

References

Basu, S. K., Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182

Bierman, E. L., Stein, O. & Stein, Y. (1974) *Circ. Res.* **35**, 136–150

Brown, M. S., Ho, Y. K. & Goldstein, J. L. (1980) *J. Biol. Chem.* **255**, 9344–9352

Chomette, G., De Gennes, J. L., Delcourt, A., Hammon, J. C. & Perie, G. (1971) *Ann. Anat. Pathol.* **16**, 233–250

Crisp, D. M. & Pogson, C. I. (1972) *Biochem. J.* **126**, 1009–1023

Fogelman, A. M., Shechter, I., Seager, J., Hokom, M., Child, J. S., & Edwards, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2214–2218

Fredrickson, D. S., Goldstein, J. L. & Brown, M. S. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wijngaarden, J. B. & Fredrickson, D. S., eds.), 4th edn., pp. 604–665, McGraw-Hill Book Co., New York

Goldstein, J. L. & Brown, M. S. (1978) *Johns Hopkins Med. J.* **143**, 8–16

Goldstein, J. L., Ho, Y. K., Basu, S. K. & Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337

Groot, P. H. E., Van Berkel, Th. J. C. & Van Tol, A. (1981) *Metab. Clin. Exp.* **30**, 792–797

Henriksen, T., Mahoney, E. M. & Steinberg, D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6499–6503

Ho, Y. K., Brown, M. S., Bilheimer, D. W. & Goldstein, J. L. (1976) *J. Clin. Invest.* **58**, 1465–1477
Knook, D. L. & Sleyster, E. Ch. (1976) *Exp. Cell Res.* **99**, 444–449
Kooistra, T., Duursma, A. M., Bouma, J. M. W. & Gruber, M. (1979) *Biochim. Biophys. Acta* **587**, 282–298
Langer, T., Strober, W. & Levy, R. I. (1972) *J. Clin. Invest.* **51**, 1528–1536
Levin, R. M. & Weiss, B. (1977) *Mol. Pharmacol.* **31**, 690–697
Levin, R. B. & Weiss, B. (1979) *J. Pharmacol. Exp. Ther.* **208**, 454–459
McFarlane, A. S. (1958) *Nature (London)* **182**, 53
Ose, T., Berg, T., Norum, K. R. & Ose, L. (1980) *Biochem. Biophys. Res. Commun.* **97**, 192–199
Redgrave, T. G., Roberts, D. C. K. & West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
Shechter, I., Fogelman, A. M., Haberland, M. E., Seager, J., Hokom, M. & Edwards, P. A. (1981) *J. Lipid Res.* **22**, 63–71
Tanaka, T. & Hidaka, H. (1980) *J. Biol. Chem.* **255**, 11078–11080
Van Berkel, Th. J. C. (1974) *Biochem. Biophys. Res. Commun.* **61**, 204–209
Van Berkel, Th. J. C. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 437–482, Academic Press Inc., London
Van Berkel, Th. J. C. & Van Tol, A. (1978) *Biochim. Biophys. Acta* **530**, 299–304
Van Berkel, Th. J. C., Kruijt, J. K., Slee, R. G. & Koster, J. F. (1977) *Arch. Biochem. Biophys.* **179**, 1–7
Van Berkel, Th. J. C., Kruijt, J. K., Van den Berg, G. B. & Koster, J. F. (1978) *Eur. J. Biochem.* **92**, 553–561
Van Berkel, Th. J. C., Kruijt, J. K., Van Gent, T. & Van Tol, A. (1981*a*) *Biochim. Biophys. Acta* **665**, 22–33
Van Berkel, Th. J. C., Nagelkerke, J. F. & Kruijt, J. K. (1981*b*) *FEBS Lett.* **132**, 61–66
Van Tol, A. & Van Berkel, Th. J. C. (1980) *Biochim. Biophys. Acta* **619**, 156–166
Van Tol, A., Van't Hooft, F. M. & Van Gent, T. (1978) *Atherosclerosis* **29**, 449–457
Wurster, N. B. & Zilversmit, D. B. (1971) *Atherosclerosis* **14**, 309–322

APPENDIX PAPER VI

In vivo and in vitro interaction of lipoprotein(a) with the apolipoprotein B,E and acetyl-LDL receptor on parenchymal and non-parenchymal rat liver cells.

Harkes, L,[@] Jürgens, G,[*] Holasek, A,[*] Nagelkerke, J.F.[@] and Van Berkel, Th.J.C.[@].

[@] Department of Biochemistry I
Faculty of Medicine, Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam, The Netherlands

[*] Institute of Medical Biochemistry
Karl-Franzens University Graz
Harrachgasse 21/III
A-8010 Graz, Austria

Submitted to Atherosclerosis

SUMMARY

The in vivo interaction of lipoprotein(a) (Lp(a)) with the liver and the various liver cell types was tested. Because the characteristics of Lp(a) can vary, dependent on the isolation method used, the Lp(a) was isolated in two different ways (defined $Lp(a)_I$ and $Lp(a)_{II}$). The serum decay of $Lp(a)_I$ was comparable with that of LDL, while $Lp(a)_{II}$ showed a faster decay. Upon estrogen-treatment of rats, serum decay and association of Lp(a) to parenchymal liver cells were only stimulated to a low extent, indicating that Lp(a) shows a less efficient interaction with the estrogen-induced apo B,E receptor than LDL. Both with $Lp(a)_I$ and $Lp(a)_{II}$, as compared to LDL, a higher cell-association to rat liver endothelial cells was found. In vitro competition experiments indicate that Lp(a) preparations with a high uptake by non-parenchymal cells achieved this by an interaction with the acetyl-LDL receptor. The interaction of Lp(a) with the acetyl-LDL receptor was blocked by methylation of Lp(a).

We conclude that lysine residues or their direct environment in Lp(a) are important for recognition by liver cells and we suggest that Lp(a)'s dual interaction with the apo B,E and acetyl-LDL receptor is caused by the high susceptibility of these residues towards environmental changes. This property of Lp(a) might be related to the action of Lp(a) as an atherogenic lipoprotein.

INTRODUCTION

Lipoprotein(a) (Lp(a)) can be demonstrated in the blood of most human people. Recently it has gained renewed interest because a number of studies have indicated a positive correlation between the serum level of Lp(a) and coronary vascular diseases (1-4).

After density gradient ultracentrifugation of human sera, Lp(a) is found in the density of 1.055-1.110 g/ml which borders the density range of low density lipoprotein (LDL) and coincides partly with that of high density lipoprotein (HDL). Lp(a) resembles LDL in lipid composition (5, 6) and also contains apo B as the major apoprotein (7). However Lp(a) can be distinguished from LDL by the presence of a unique Lp(a) apoprotein and by the high hexose, hexosamine and sialic acid content (7).

A number of in vitro studies have shown that Lp(a) can bind to the

apo B,E-receptor on fibroblasts (8-10), although it was also reported that Lp(a) does not interact with the apo B,E-receptor (11). Recently we described the intrahepatic cellular localization of lipoprotein receptors in rats. The presence of an apo B,E-receptor on parenchymal and Kupffer cells (12, 13) was demonstrated and the acetyl-LDL (scavenger) receptor appears to be very active on liver endothelial cells (14). Acetyl-LDL and other chemically modified LDL preparations can induce a cholesterol ester accumulation in macrophages in vitro (15-18) and it was suggested (15, 17) that the uptake of lipoproteins by the acetyl-LDL receptor is relevant for the formation of foam cells. In our view the acetyl-LDL receptor from liver endothelial cells protects against the formation of foam cells because the cells play a quantitative role in the removal of atherogenic lipoproteins from the blood (14). For reason of the above mentioned relation of Lp(a) with cardiovascular disease, the conflicting data on the interaction of Lp(a) with the apo B,E-receptor and the suggested protection of the liver cells against atherogenic particles, we determined in vivo the receptors involved in the interaction of Lp(a) with the liver.

In order to determine the interaction of Lp(a) with the apo B,E-receptor, the number of receptors was selectively increased in parenchymal liver cells (12) by estrogen-treatment of the rat (19). Involvement of the acetyl-LDL receptor was studied by determination of the uptake of Lp(a) in endothelial cells (14) and by performing in vitro competition studies.

Because the characteristics of the Lp(a) preparation can vary dependent on the isolation method used (20), the Lp(a) was isolated in two different ways (20, 21).

MATERIALS AND METHODS

Human LDL ($1.024 < d < 1.055$ g/ml) was isolated by two repetitive centrifugations according to Redgrave et al. (22) as previously described (12). The human LDL preparation used in this study contains almost only apolipoprotein B (99.97%) and no degradation products were detectable as checked by electrophoresis in SDS (sodium dodecyl sulfate) gels. With a high LDL concentration (5 mg apolipoprotein/ml) in a radial immunodiffusion system according to Mancini et al. (23), apolipoprotein E was noticeable at the detection limit and contributed maximally 0.02-0.03% of the total apolipoprotein.

Radioiodination of LDL was done according to a modification (24) of the ICl method described by McFarlane (25), using carrier-free [^{125}I] or [^{131}I] iodine. The distribution of radioactivity in human LDL is: 88% in protein; 8% in phospholipids and 4% free, as determined according to (26).

Human Lp(a) was isolated in two different ways. The first method was a combination of ultracentrifugation and gel chromatography (defined as $Lp(a)_I$) and the second method a combination of precipitation, ultracentrifugation and gelchromatography (defined as $Lp(a)_{II}$). Each Lp(a) batch was isolated from the pooled plasma of 5-7 highly positive donors. Method 1: Isolation of $Lp(a)_I$ and judging of the purity of the $Lp(a)_I$ fraction was done exactly as described by Gaubatz et al. (20). Method 2: Isolation of $Lp(a)_{II}$ and determination of the purity was done as described by Eigner et al. (21).

Radioiodination of the Lp(a) preparations was done according to a modification (27) of the ICl method (25). After iodination the Lp(a) preparation was dialyzed 3 times against 0.024 M NaBr, 0.01 M Tris-HCl, pH 8.0 and 2 times against 0.15 M NaCl, 0.3 M EDTA, pH 7.0. The distribution of radioactivity in Lp(a) is 87% in protein, 2% in phospholipids and 11% free as determined according to (26).

Reductive methylation of the lipoproteins was done according to (28). 0.5 ml lipoprotein (approximately 2.5 mg apolipoprotein/ ml) was mixed with 0.38 ml 0.3 M borate buffer, pH 9.0. On t=0 0.5 mg $NaBH_4$ and 0.5 µl formaldehyde were added, whereafter every 6 min (5 times) 0.5 µl formaldehyde was added. The extent of methylation of lysine residues was 80% as determined by the trinitrobenzenesulfonic acid method (29).

Animals

12 weeks old male Wistar rats were used throughout the study. 17α-ethinylestradiol in propyleneglycol at a dose of 5 mg/kg body weight (30) was injected subcutaneously every 24 hours during 3 days, control rats received equal volumes of the solvent.

In vivo uptake studies

Rats were anesthesized by intraperitoneal injection of 20 mg nembutal. The abdomen was opened and the radiolabelled lipoproteins were injected in the inferior vena cava at the level of the renal veins. After the indicated circulation time the liver was perfused with an oxygenated Hanks

buffer at 8°C. After 8 min perfusion a lobule was tied off for determination of the total liver uptake. To determine uptake by various cell types, the cell types were isolated by low temperature procedures. After the 8 min perfusion at 8°C, the liver was subjected to a low temperature (8°C) perfusion with 0.05% collagenase (12, 14). After 20 min of perfusion with collagenase the liver was minced and the crude cel suspension filtered (90 µm mesh). The filtrate (containing parenchymal and non-parenchymal cells) was subjected to differential centrifugation exactly as described earlier (31). The parenchymal cells were completely free from non-parenchymal cells as judged by microscopy and the absence of M_2-type pyruvate kinase (32) in this preparation. The non-parenchymal cells were collected from the first two supernatants of the parenchymal cell centrifugations. In order to increase the recovery of non-parenchymal cells from the liver the residue on the 90 µm mesh was incubated for 20 min at 8°C with 0.25% pronase (which destroys parenchymal cells) and the non-parenchymal were collected and washed (two times) by centrifugation at 400 g for 5 min. The non-parenchymal cell fractions (both from the supernatants from the parenchymal cell isolation and the pronase-treated filter residue) were combined. The cells were suspended in 5 ml Hanks buffer, mixed with 7.2 ml 30% metrizamide and divided over two Sorvall tubes. One ml Hanks buffer is layered on top of the mixture and the tubes are spun for 15 min at 1500 x g. The cells which floated into the top phase were aspirated and subjected to a 30 s 50 x g centrifugation to remove any left parenchymal cells. The non-parenchymal cell preparation was collected and washed by two 400 x g centrifugations. The non-parenchymal cell preparation was completely free from parenchymal cells or parenchymal cell derived particles, as judged by phase contrast microscopy and the exclusive presence of M_2-type pyruvate kinase in this preparation (32). The absence of any cross-contamination between the parenchymal and non-parenchymal cell preparation is also indicated by the selective effects of estrogen treatment on parenchymal cells and ethyloleate treatment on non-parenchymal cells as described earlier (13). By peroxidase staining with diaminobenzidine (33) about 30% of the isolated non-parenchymal cells were peroxidase-positive indicating that about 30% of these cells are Kupffer cells and about 70% endothelial cells. This relative proportion is similar as <u>in vivo</u> (34). For reason that a Kupffer cell contains twice as much protein as an endothelial cell (34), the non-parenchymal cell preparation contains 50% Kupffer cell

protein and 50% endothelial cell protein.

Alternatively a liver endothelial cell preparation and a Kupffer cell preparation were obtained by subjecting the liver directly to a 8°C pronase (0.25%) perfusion whereafter the cells were purified by centrifugal elutriation exactly as recently described (14). The Kupffer cell preparation contained 70-90% Kupffer cells, the remainder being endothelial cells; the endothelial cell preparation contained more than 95% endothelial cells with < 5% white blood cells (14). Radioactivity in the final cell preparations was counted in an LKB γ-counter. With the present cell isolation techniques it is possible to obtain a quantitative recovery of the total liver associated radioactivity in the subsequent isolated cells (12, 14).

In vitro processing of lipoproteins after in vivo uptake

The degradation of the in vivo internalized lipoproteins was determined by isolating the various cell types by a short, warm (37°C) recirculating perfusion method based upon (35) (perfusion flow: 40 ml/min). 10 min after intravenous injection of the labelled lipoproteins, perfusion of the liver was started at 37°C for 10 min with Hanks solution, 9 min with Hanks solution plus 0.05% collagenase and again 1 min with Hanks solution in order to remove the collagenase. As shown earlier (13, 36, 37), cell-bound LDL will be removed by collagenase at this temperature (37°C). Hereafter the parenchymal and non-parenchymal cells were isolated and purified as described above, except that the pronase treatment of the liver debris was omitted because with the applied procedure at 37°C no residue is left. Subsequently the cells were incubated in vitro at 37°C in a Hams F-10 medium supplemented with 2% bovine serum albumin during 2 h and the cell-association and degradation was determined at the indicated times (36).

In vitro binding and degradation studies

Parenchymal and non-parenchymal cells were isolated as described previously (31). Parenchymal and non-parenchymal cell preparations were pure as above mentioned. Freshly isolated parenchymal and non-parenchymal cells were incubated with the indicated amount of radiolabelled lipoproteins in Ham F-10 medium containing 2% bovine serum albumin. At the indicated time, samples were withdrawn and the cell-associated radioactivity

was determined as described by Van Berkel et al. (36). The content of trichloroacetic acid-soluble, noniodine radioactivity in the medium was used to calculate the amount of lipoprotein degraded (36).

Protein determination were done according to Lowry (38), with bovine serum albumin as a standard.

Materials

17α-ethinyl estradiol was obtained from Brocacef BV (Maarssen, The Netherlands; collagenase (type I) from Sigma, St. Louis, U.S.A.; pronase B-grade from CalBiochem. Behring Corp., La Jolla, U.S.A.; metrizamide was purchased from Nyegaard & Co. A/S, Oslo, Norway; Ham F-10 medium from Gibco-Europe, Hoofddorp, The Netherlands and [^{125}I] and [^{131}I] iodine (carrier-free) from Amersham International, Amersham, U.K.

RESULTS

As described in "Methods", 2 different methods for the isolation of Lp(a) were used. Lp(a) isolated according to method 1 or 2 is defined $Lp(a)_I$ or $Lp(a)_{II}$ respectively. The radiolabelled lipoproteins were injected into rats and after 30 min parenchymal and non-parenchymal cells were isolated and purified. During the 30 min circulation the serum decay of the lipoproteins was determined (Fig. 1). The serum decay of native LDL and $Lp(a)_I$ proceeds at the same rate but the serum decay of $Lp(a)_{II}$ is

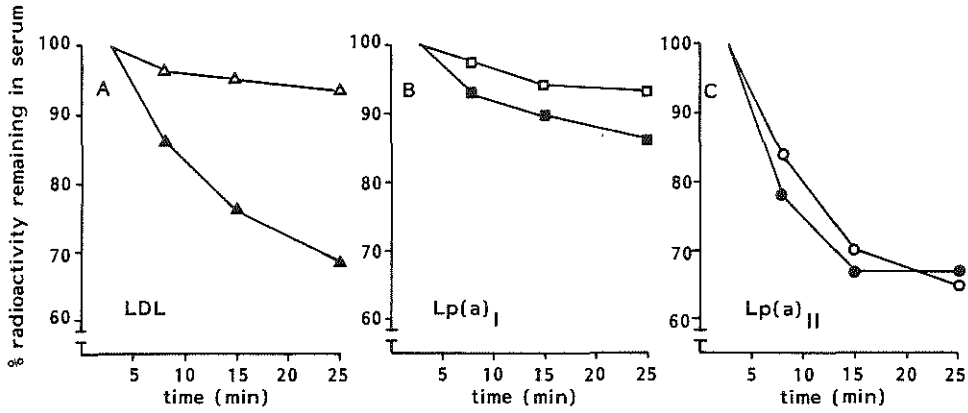

Fig. 1. Serum decay of LDL (A), $Lp(a)_I$ (B) or $Lp(a)_{II}$ (C) in estrogen treated (closed symbols) and control rats (open symbols). 3, 8, 15 and 25 minutes after injection of the ^{125}I-labelled lipoproteins, blood samples were drawn and the radioactivity is serum determined. The amount of radioactivity at 3 minutes after injection is taken as 100% value.

much faster, and biphasic. In the first 25 min 30% of the injected $Lp(a)_{II}$ is cleared but this sums up to only 50% in the next 2 hours (results not shown). In order to determine the involvement of the parenchymal apo B,E receptor in the clearance of Lp(a), rats were treated with 17α-ethinylestradiol. This treatment accelerated the clearance rate for native LDL, but had a small effect upon the decay of $Lp(a)_I$ and no effect upon that of $Lp(a)_{II}$.

Fig. 2A shows the in vivo uptake by the liver of Lp(a) as compared to

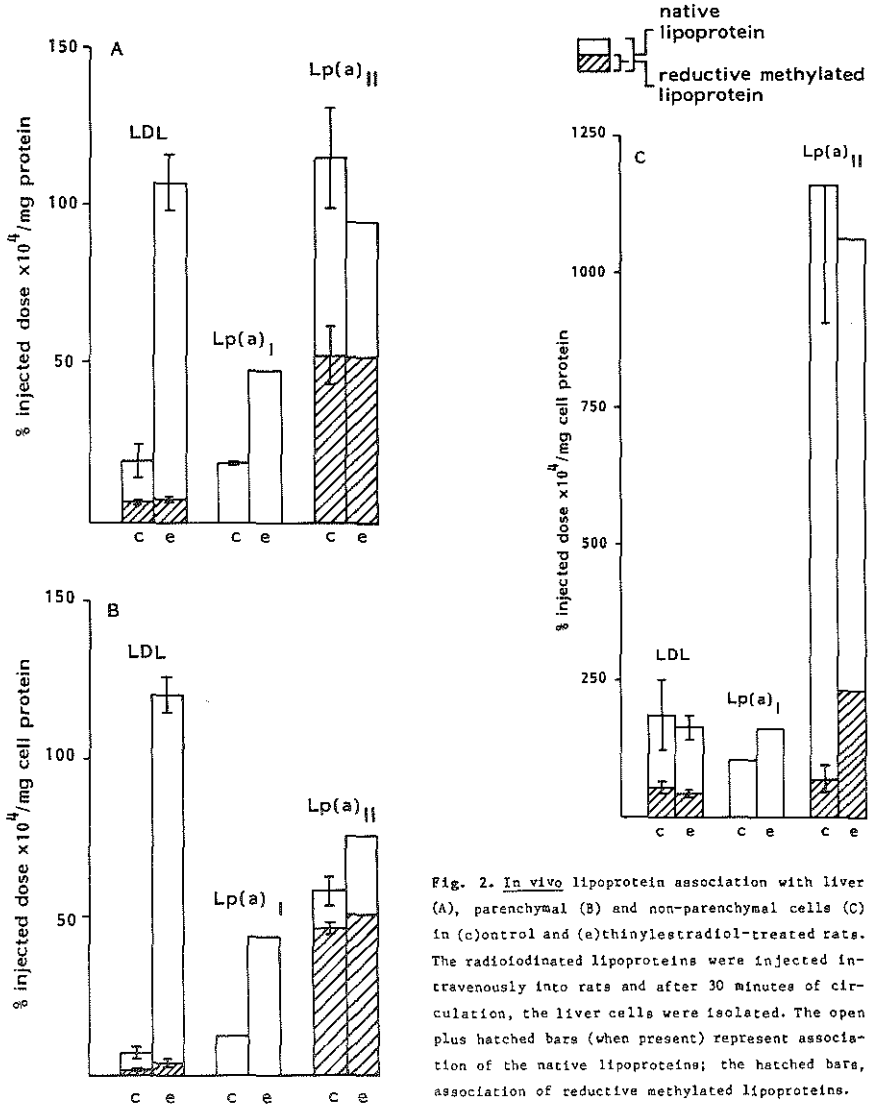

Fig. 2. In vivo lipoprotein association with liver (A), parenchymal (B) and non-parenchymal cells (C) in (c)ontrol and (e)thinylestradiol-treated rats. The radioiodinated lipoproteins were injected intravenously into rats and after 30 minutes of circulation, the liver cells were isolated. The open plus hatched bars (when present) represent association of the native lipoproteins; the hatched bars, association of reductive methylated lipoproteins.

LDL. The open plus hatched bars (when present) represent uptake of the native lipoproteins in c(ontrol) and e(strogen) treated animals. The hatched bars alone represent uptake of the reductive methylated lipoproteins. It is evident that the liver uptake of LDL and $Lp(a)_I$ is enhanced by the estrogen-treatment while there is no effect upon the uptake of $Lp(a)_{II}$. Reductive methylation of the lipoproteins inhibits the liver uptake of LDL and also affects the uptake of $Lp(a)_{II}$. With two other batches of $Lp(a)_{II}$, uptake values between those plotted for $Lp(a)_I$ and $Lp(a)_{II}$ were found. Figure 2B displays the amount of lipoprotein associated *in vivo* with the parenchymal cells. Liver cells from control rats were more active in the uptake of Lp(a) preparations than in that of LDL. However, estradiol treatment led to a 17-fold increment of the parenchymal cell uptake of LDL and in this situation the uptake of LDL exceeds that of Lp(a). The stimulation of the Lp(a) uptake by estrogen treatment was always much less than that of LDL. Reductive methylation of LDL leads to a decreased uptake by parenchymal cells both in control or estradiol treated rats. Modification of $Lp(a)_{II}$ does however hardly influence the uptake of Lp(a) by parenchymal cells. In figure 2C the *in vivo* uptake of the lipoproteins by the non-parenchymal cells is shown. Uptake of $Lp(a)_I$ is similar to LDL while the uptake of $Lp(a)_{II}$ is 6-fold higher than that of LDL. With two other batches of $Lp(a)_{II}$ intermediate values between $Lp(a)_I$ and $Lp(a)_{II}$ were found. There is no effect of estradiol treatment upon lipoprotein uptake in these cells. Surprising is that reductive methylation of $Lp(a)_{II}$ strongly inhibits the association of Lp(a) with these cells.

A further subdivision of non-parenchymal cells in endothelial and Kupffer cell fractions shows that all the Lp(a) preparations have an increased *in vivo* cell-association with the endothelial cells as compared to LDL (Table 1). The uptake of $Lp(a)_I$ by Kupffer cells was lower than that of LDL whereas all the $Lp(a)_{II}$ preparations show a clearcut (2-4 fold) higher uptake by Kupffer cells. The consistent high uptake of Lp(a) by endothelial liver cells might be related to an "acetyl-like character" of these preparations. To investigate this specific question we performed *in vitro* competition studies with the $Lp(a)_{II}$ batch which showed the highest *in vivo* uptake in these cells (Fig. 3). It appears that in contrast to LDL, acetyl-LDL is a potent inhibitor of the cell-association and degradation of $Lp(a)_{II}$ even more effective than $Lp(a)_{II}$ itself. The relatively lower potency of an excess $Lp(a)_{II}$ to inhibit its own cell-associa-

Table 1.

In vivo uptake of LDL, $Lp(a)_I$, $Lp(a)_{II}$ and acetyl-LDL in liver endothelial and Kupffer cells at 30 minutes after injection.

	% injected dose $x10^4$/mg cell protein	
	Endothelial cells	Kupffer cells
LDL[§]	32 ± 2 (3)	232 ± 15 (3)
$Lp(a)_I$	74	78
$Lp(a)_{II}$[o]	135 - 2094	597 - 950
Acetyl-LDL[*]	4700 ± 500 (3)	630 ± 100 (3)

§ Nagelkerke et al. Unpublished observations.

o 3 different $Lp(a)_{II}$ batches were tested and the range is indicated.

* Obtained from ref. (14).

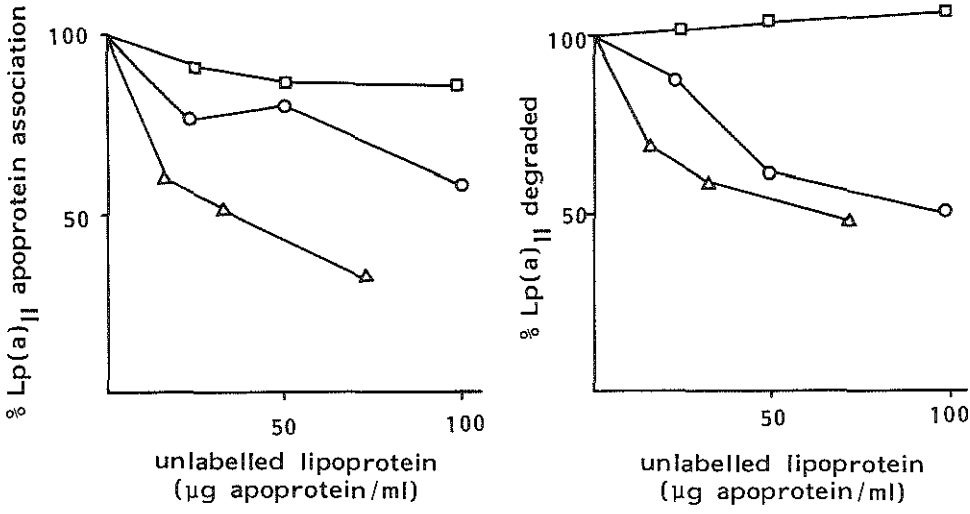

Fig. 3. Competition between ^{125}I-labelled $Lp(a)_{II}$ and unlabelled human LDL (□), acetyl-LDL (△) and $Lp(a)_{II}$ (○) for cell association and degradation by non-parenchymal liver cells. Non-parenchymal cells were incubated for 2h with 4.9 µg of ^{125}I-$Lp(a)_{II}$ and with the indicated amount of unlabelled lipoproteins. ^{125}I-labelled apolipoprotein association or degradation is expressed as the percentage of the amount obtained in the absence of unlabelled lipoprotein. The 100% value for the cell association is 779 ng $Lp(a)_{II}$/mg cell protein and for the degradation 302 ng of $Lp(a)_{II}$/mg cell protein.

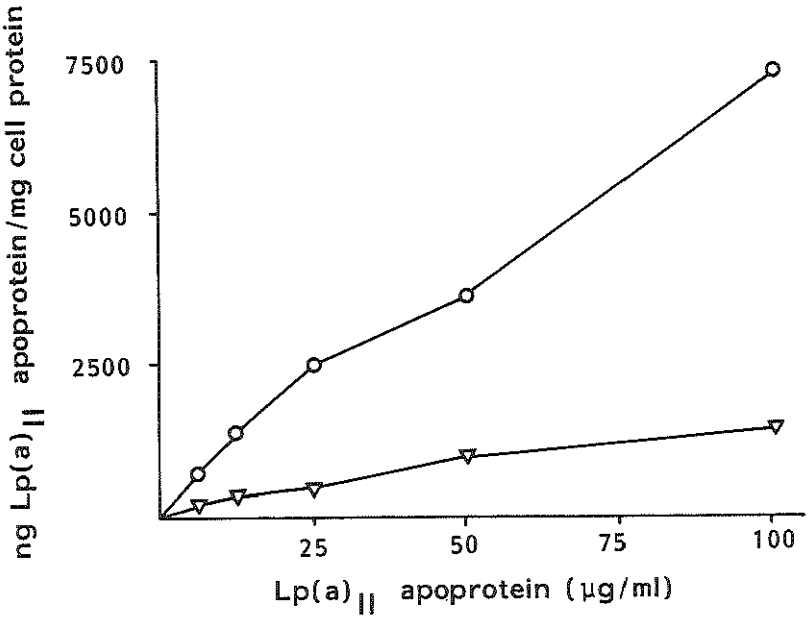

Fig. 4. Relation of the concentration of $Lp(a)_{II}$ to the extent of cell association (○) and degradation (▽) with non-parenchymal liver cells after 2 hours of incubation.

tion and degradation is due to the low affinity of $Lp(a)_{II}$ for the binding sites as shown in Fig. 4. Even at 100 µg $Lp(a)_{II}$/ml no clearcut saturation is observed.

In Fig. 5 the in vitro degradation of LDL and $Lp(a)_{II}$, which were taken up in vivo, is compared. The lipoproteins were injected into control and estrogen rats and after 10 min a perfusion of the liver at 37°C was started. Parenchymal and non-parenchymal cells were isolated and subsequently the isolated cells were incubated at 37°C and at different time intervals the amount of degraded lipoprotein was determined. In order to determine the role of the lysosomes in the degradation process, the cell incubations were done in the absence or presence of chloroquine. It is clear that in control rats more $Lp(a)_{II}$ than LDL is degraded by the parenchymal cells. Chloroquine substantially inhibits the degradation of Lp(a), suggesting that the lysosomes are involved. In estrogen-treated rats the initial amount of LDL associated to the parenchymal cells exceeds that of $Lp(a)_{II}$ (see also Fig. 2B). However, even under these conditions the amount of $Lp(a)_{II}$ degraded by these cells exceeds that of LDL. The association and degradation of Lp(a) by non-parenchymal cells is about 100

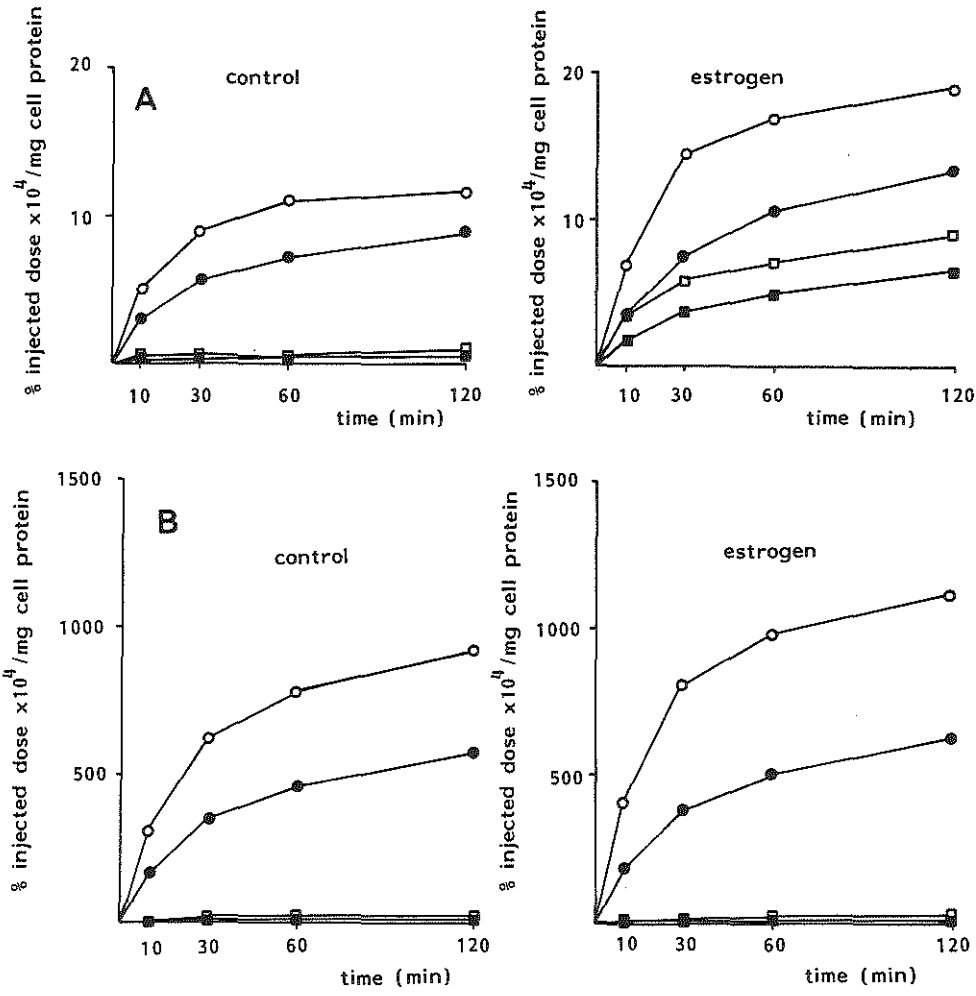

Fig. 5. In vitro degradation of LDL (□,■) or $Lp(a)_{II}$ (○,●) by parenchymal (A) and non-parenchymal liver cells (B) isolated after in vivo uptake of the lipoproteins by control and ethinylestradiol-treated rats. Liver cells were isolated 10 min. after intravenous injection of the lipoproteins by the 37° C collagenase method (see Materials and Methods). The cells were incubated in the absence (open symbols) or presence (closed symbols) of 100 μM chloroquine for the indicated times. The amount of trichloroacetic acid soluble radioactivity released in the medium is plotted. The cell associated radioactivity in these samples on t=0 is for A control □,1.4; ■,1.3; ○,16.1; ●,12.9; for A estrogen □,25.4; ■,26.7; ○,20.0; ●,19.4; for B control □,63.4; ■,58.8; ○,1458; ●,1307; and for B estrogen □,88.1; ■,92.0; ○,1577; ●,1621, in % injected dose x 10^4/mg cell protein.

fold higher (per mg cell protein) than with parenchymal cells. Irrespective of the estrogen-treatment Lp(a) is degraded by non-parenchymal cells to a higher extent than LDL while this degradation is for approximately 35% inhibited by chloroquine.

DISCUSSION

This study shows that Lp(a) can interact *in vivo* with the apo B,E receptor but also with a binding site for acetyl LDL. Most investigations on the binding properties of Lp(a) were done with fibroblasts (8-11). However, the results of these studies are not unequivocal. The present experiments also point to difficulties in assessing the properties of Lp(a), as variable results are obtained with various batches while the obligatory isolation of Lp(a) may also influence its properties. We applied two different isolated methods for Lp(a) and with both methods protease inhibitors are present. Although variations in cell-association were high, we think that the present results allow some conclusions which may be relevant to understand the atherogenic nature of this lipoprotein. 1) It is clear that Lp(a) can interact with the apo B,E receptor *in vivo*, although apparently less efficient than LDL. This conclusion is mainly based on the increase in cell-association to parenchymal cells upon estrogen-treatment which is lower for Lp(a) than for LDL . 2) The increased cell-association of Lp(a) with endothelial liver cells, as compared to LDL, is indicative for a property of Lp(a) similar to that of modified LDL (either acetylated (14) or endothelial-cell modified (39)). The *in vitro* competition experiments clearly indicate that isolated Lp(a) can possess properties enabling an interaction with the acetyl-LDL (scavenger) receptor. The different binding properties of the 4 Lp(a) batches, each obtained from 5-7 donors, might be explained by the inter- and intraindividual heterogeneity of Lp(a) (40). However, we want to mention that this acetyl-LDL-like property of Lp(a) might also be induced by the isolation procedure. Although this assumption may weaken the conclusion, it must be stressed that with LDL never such a "spontaneous acetyl-LDL character" has been observed.

Recent studies (41, 42) have shown that modification of a limited number of lysine-residues in LDL by malondialdehyde-treatment or carbamylation leads to a blockade of the interaction with the apo B,E receptor. When the extent of modification increases further the particle would bind neither to the LDL receptor nor to the acetyl-LDL receptor (41). Continuing modification results in an interaction of LDL with the acetyl-LDL receptor. Because we suspected that lysine-residues in Lp(a) might be important for recognition by receptors, these residues were methylated.

Surprisingly, we found that methylation of Lp(a) blocks greatly the interaction with non-parenchymal cells. This finding indicates that in Lp(a) the lysine-residues or their direct environment are involved in the recognition by the acetyl-LDL receptor.

The increased interaction of Lp(a) as compared to LDL with the liver endothelial cells probably mediated by the acetyl-LDL receptor, might be indicative for the action of Lp(a) as a risk factor in atherogenesis.If endothelial cells and/or monocytes/macrophages at other sites in the body show in vivo a similar behaviour, it can be speculated that Lp(a) can cause cholesterolester accumulation which can lead to foam cells. It must be mentioned that Gianturco et al. (43) also recently suggested that aggregated Lp(a) can cause lipid accumulation in macrophages in contrast to filtered Lp(a). Furthermore it has been reported that complexes of Lp(a) and sulfated polysaccharides can cause an increased cholesteryl ester deposition in mouse peritoneal macrophages (44). The well-known lability of Lp(a) might be related to its susceptibility to modification which, when exerted in vivo, can be responsible for foam cell formation and increased incidence of coronary heart disease.

ACKNOWLEDGEMENTS

The authors thank Kar Kruijt for his excellent technical assistance and Martha Wieriks for typing the manuscript. This research was partly supported by grants from the Netherlands Heart Foundation (grant nr. 84.012); the Österreichischer Funds zur Förderung des Wissenschaftlichen Forschung (nr. P5158) and the Jubiläumsfonds der Österreichischer National Bank.

REFERENCES

1. **Albers JJ, Adolphson JL and Hazzard WR.** Radioimmunoassay of human plasma Lp(a) lipoprotein. J Clin Invest 18 (1977) 331.
2. **Berg K.** A new serum type system in man - the Lp system. Acta Pathol Microbiol Scand 59 (1963) 369.
3. **Berg K, Dahlen G and Frick MH.** Lp(a) lipoprotein and pre-β_1-lipoproteins in patients with coronary heart disease. Clin Genet 6 (1974) 230.

4. **Dahlen G, Berg K, Ramberg U and Tamm A.** Lp(a) and pre-β_1- lipoprotein in young adults. Acta Med Scand 196 (1974) 327.

5. **Simons K, Ehnholm C Renkonen O and Bloth B.** Characterization of the Lp(a) lipoprotein in human plasma. Acta Pathol Microbiol Scand Sect B 78 (1970) 459.

6. **Jürgens G and Kostner GM.** Studies on the structure of the Lp(a) lipoprotein. Isolation and partial characterization of the specific antigen. Immunogenetics 1 (1975) 560.

7. **Ehnholm C, Garoff H, Renkonen O and Simons K.** Protein and carbohydrate composition of Lp(a) lipoproteins from human plasma. Biochemistry 11 (1972) 3229.

8. **Havekes L, Vermeer BJ, Brugman T and Emeis J.** Binding of Lp(a) to the low density lipoprotein receptor of human fibroblasts. FEBS Lett 132 (1981) 169.

9. **Floren GH, Albers JJ and Bierman EL.** Uptake of Lp(a) lipoprotein by cultured fibroblasts. Biochem Biophys Res Commun 102 (1981) 636.

10. **Krempler F, Kostner GM, Roscher A, Haslauer F, Bolzano K, and Sandhofer F.** Studies on the role of specific cell surface receptors in the removal of lipoprotein(a) in man. J Clin Invest 71 (1983) 1431

11. **Maartman-Moe K and Berg K.** Lp(a) lipoprotein enters cultured fibroblasts independently of the plasma membrane low density lipoprotein receptor. Clin Genet 20 (1981) 352.

12. **Harkes L and Van Berkel ThJC.** Cellular localization of the receptor-dependent and receptor-independent uptake of human LDL in the liver of normal and 17α-ethinyl estradiol-treated rats. FEBS Lett 154 (1983) 75.

13. **Harkes L and Van Berkel ThJC.** In vivo characteristics of a specific recognition site for LDL on non-parenchymal rat liver cells different from the 17α-ethinyl estradiol induced LDL receptor on parenchymal liver cells. Biochim Biophys Acta 794 (1984) 340.

14. **Nagelkerke JF, Barto KP and Van Berkel ThJC.** In vivo and in vitro uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer and parenchymal cells. J Biol Chem 258 (1983) 12221.

15. **Goldstein JL, Ho YK, Basu SK and Brown MS.** Binding sites on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 76 (1979) 333.

16. **Mahley RW Innerarity TL, Weisgraber KH and Oh SY.** Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. J Clin Invest 64 (1979) 743.

17. **Mahley RW.** Cellular and molecular biology of lipoprotein metabolism in atherosclerosis. Diabetes 30 suppl. 2 (1981) 60.

18. **Fogelman AM, Shechter I, Seager J, Hokom M, Child JS and Edwards PA.** Malondialdehyde alterations of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. Proc Natl Acad Sci USA 77 (1980) 2214.

19. **Kovanen PT, Brown MS and Goldstein JL.** Increased binding of low density lipoprotein to liver membranes from rats treated with 17α-ethinyl estradiol. J Biol Chem 254 (1979) 11367.

20. **Gaubatz JW, Heideman C, Gotto Jr AM, Morrisett JD and Dahlen GH.** Human plasma lipoprotein(a). Structural properties. J Biol Chem 258 (1983) 4582.

21. **Eigner D, Schurz J, Jürgens G and Holasek A.** Molecular parameters of Lp(a) lipoprotein by light scattering. FEBS Lett 106 (1979) 165.

22. **Redgrave TG, Roberts DCK and West CE.** Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem 65 (1975) 42.

23. **Mancini G, Carbonara AP and Heremans JF.** Immunochemical quantification of antigens by single radial immunodiffusion. Immunochemistry 2 (1965) 235.

24. **Van Tol A, Van Gent T, Van 't Hooft FM and Vlaspolder F.** High density lipoprotein catabolism before and after partial hepatectomy. Atherosclerosis 29 (1978) 439.

25. **McFarlane AS.** Efficient trace-labelling of proteins with iodine. Nature 182 (1958) 53.

26. **Folch J, Lees M and Sloane Stanley GH.** A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226 (1957) 497.

27. **Bilheimer DW, Eisenberg S and Levy RI.** The metabolism of low density lipoprotein proteins I. Preliminary in vitro and in vivo observations. Biochim Biophys Acta 260 (1972) 212.

28. **Weisgraber KH, Innerarity TL and Mahley RW.** Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J Biol Chem 253 (1978) 9053.

29. **Habeeb AFSA.** Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem 14 (1966) 328.

30. **Chao Y.-S, Windler EE, Chen GC and Havel RJ.** Hepatic catabolism of rat and human lipoproteins in rats, treated with 17α-ethinylestradiol. J Biol Chem 254 (1979) 11360.

31. **Van Berkel ThJC and Van Tol A.** In vivo uptake of human and rat low density and high density lipoprotein by parenchymal and non-parenchymal cells from rat liver. Biochim Biophys Acta 530 (1978) 299.

32. **Van Berkel ThJC, Kruijt JK, Slee RG and Koster JF.** Identity and activities of superoxide dismutase in parenchymal and non-parenchymal cells from rat liver. Arch Biochem Biophys 179 (1977) 1.

33. **Fahimi HD.** The fine structural localization of endogenous and exogenous peroxidase activity in Kupffer cells of rat liver. J Cell Biol 47 (1970) 247.

34. **Knook DL and Sleyster EC.** Isolated parenchymal, Kupffer and endothelial rat liver cells characterized by their lysosomal enzyme content. Biochem Biophys Res Commun 96 (1980) 250.

35. **Seglen PO.** Preparation of isolated rat liver cells. Methods Cell Biol 13 (1976) 29.

36. **Van Berkel ThJC, Kruijt JK, Van Gent T and Van Tol A.** Saturable high affinity binding, uptake and degradation of rat plasma lipoproteins by isolated parenchymal and non-parenchymal cells from rat liver. Biochim Biophys Acta 665 (1981) 22.

37. **Harkes L and Van Berkel ThJC.** A saturable high affinity binding site for human low density lipoprotein on freshly isolated rat hepatocytes. Biochim Biophys Acta 712 (1982) 677.

38. **Lowry OH, Rosebrough NJ, Farr AL and Randall RJ.** Protein measurement with the Folin phenol reagent. J Biol Chem 193 (1951) 265.

39. **Nagelkerke JF, Havekes L, Van Hinsbergh VWM and Van Berkel ThJC.** In vivo catabolism of biologically modified LDL. Arteriosclerosis 4(1984) 256.

40. **Fless GM, Rolih CA and Scanu AM.** Heterogeneity of human plasma lipoprotein(a). Isolation and characterization of the lipoprotein subspecies and their apoproteins. J Biol Chem 259 (1984) 1470.

41. **Haberland ME, Fogelman AM and Edwards PA.** Specificity of receptor-mediated recognition of malondialdehyde modified low density lipoproteins. Proc Natl Acad Sci USA 79 (1982) 1712.

42. **Gonen B, Cole T and Hahm KS.** The interaction of carbamylated low-density lipoprotein with cultured cells. Studies with human fibroblasts, rat peritoneal macrophages and human monocyte-derived macrophages. Biochim Biophys Acta 754 (1983) 201.

43. **Gianturco SH, Bradley WA, Dahlen GH, Gaubatz JW, Gotto Jr AM, Morrisett JD and Peavy DL.** Interaction of lipoprotein(a) with murine peritoneal macrphages and cultured human fibroblasts. Arteriosclerosis 3 (1983) 500a Abstr.

44. **Krempler F, Kostner GM, Roscher A, Bolzano K and Sandhofer F.** The interaction of human apo B-containing lipoproteins with mouse peritoneal macrophages: a comparison of Lp(a) with LDL. J Lipid Res, 25 (1984) 283.

CURRICULUM VITAE

Leen Harkes werd op 16 maart 1954 geboren te Zoeterwoude. Na het behalen van het diploma HBS-b aan de Christelijke Scholengemeenschap Westland-Zuid te Vlaardingen in 1972, begon hij in datzelfde jaar zijn studie aan de Landbouwhogeschool te Wageningen. Tijdens de praktijktijd deed hij onderzoek op de afdeling Veterinaire Biochemie van de Rijksuniversiteit te Utrecht. In januari 1980 slaagde hij voor het doctoraal examen met als hoofdvakken Humane Voeding en Dierfysiologie. Per februari 1980 trad hij in dienst bij de afdeling Biochemie I van de Erasmus Universiteit te Rotterdam, waar hij 3 jaar werkte aan een door de Nederlandse Hartstichting gesubsidieerd project (nr. 79.001). De resultaten van dit onderzoek zijn in dit proefschrift weergegeven. Thans participeert hij in het project "Cellulaire verwerking van atherogene lipoproteïnen: Pathologie versus bescherming" op dezelfde afdeling.