Recognition Sites on Rat Liver Cells for Oxidatively Modified \(\beta\)-Very Low Density Lipoproteins

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The in vivo fate of \(\beta\)-very low density lipoproteins (\(\beta\)-VLDLs) was investigated after \(\text{Cu}^{2+}\)-mediated oxidative modification (Ox-\(\beta\)-VLDL). Ox-\(\beta\)-VLDL may be physiologically relevant under conditions of defective VLDL removal by the liver (type III hyperlipoproteinemia) or overloading of the remnant receptor (high cholesterol feeding). On oxidation of \(\beta\)-VLDL, the kinetics of its removal from the blood and uptake by the liver are unchanged. However, in contrast to \(\beta\)-VLDL, which is recognized by the remnant receptor of parenchymal cells, liver uptake of Ox-\(\beta\)-VLDL is mediated mainly by Kupffer cells (65% of liver-associated radioactivity). In vitro competition studies show that the cell association and degradation of iodine-125-labeled Ox-\(\beta\)-VLDL by both liver endothelial and Kupffer cells are only marginally competed for by acetylated LDL (10–20%), while an efficient blockade is noted with Ox-\(\beta\)-VLDL, oxidized low density lipoproteins, or polyniosinic acid (80–90%). The capacity of Kupffer cells to associate with and degrade \(^{125}\text{I}-\text{Ox}-\beta\)-VLDL appears to be twofold higher than for endothelial cells. It is concluded that on oxidation of \(\beta\)-VLDL, the recognition system responsible for the uptake of \(\beta\)-VLDL from the blood circulation is shifted from the remnant receptor to a specific oxidized-lipoprotein receptor. The efficiency of the scavenger activity on Kupffer cells will then form the protection system against the prolonged circulation of these atherogenic lipoproteins in the blood. (Arteriosclerosis and Thrombosis 1992;12:41–49)

An accelerated rate of the vascular deposition of lipids is observed in patients suffering from type II or type III hyperlipoproteinemia.\(^1,2\) While type II hyperlipoproteinemia is associated with an increased low density lipoprotein (LDL) cholesterol level, in type III patients the majority of cholesterol is present in very low density lipoproteins with a \(\beta\)-mobility on agarose gels (so-called \(\beta\)-VLDLs). \(\beta\)-VLDL, in humans, is a cholesteryl ester–rich lipoprotein, with the characteristic phenotype of apolipoprotein (apo) E2/E2.\(^3\)

The ineffectiveness of the apo E2/E2 phenotype to mediate binding to the LDL receptor and/or the remnant receptor will lead to an increased circulation time and accumulation of \(\beta\)-VLDL in the blood compartment. However, it is remarkable that cardiovascular disease is not found in all persons with the apo E2/E2 phenotype.\(^3,4\) It is suggested that an increased risk for peripheral vascular disease is found when the apo E2/E2 phenotype becomes associated with secondary factors such as smoking, a lipid-rich diet, or diabetes.\(^5,6\) It seems possible that, as with LDL, the atherogeneity of \(\beta\)-VLDL may be influenced by its susceptibility for oxidation during its prolonged blood circulation time. Such a hypothesis is supported by experiments performed in cholesterol-fed rabbits, which accumulate \(\beta\)-VLDL in their plasma. The rabbits appear to develop atherosclerosis,\(^7,8\) the severity of which is decreased by the administration of probucol.\(^9,10\) Besides a cholesterol-lowering effect, probucol can also function as an antioxidant.\(^11\) For Watanabe heritable hyperlipidemic rabbits (LDL-receptor deficient), it has been reported that probucol can prevent the progression of atherosclerotic lesions due to its antioxidant effect.\(^12,13\) These considerations led us to investigate the effect of oxidation of \(\beta\)-VLDL on its in vivo fate.

Different receptor systems responsible for the recognition of modified lipoproteins have recently been reported to be present on mouse peritoneal macrophages.\(^14-16\) Besides the acetylated (Ac) LDL receptor, which recognizes both Ac-LDL and oxidized (Ox) LDL, an additional specific receptor has been found that recognizes oxidatively modified LDL but...
not Ac-LDL, the so-called “Ox-LDL” receptor. Recent evidence indicates that the liver is responsible for the rapid elimination of modified lipoproteins from the blood circulation by various scavenger receptors. In the rat liver, the Ox-LDL receptor is concentrated on Kupffer cells and is mainly responsible for the removal of Ox-LDL particles from the blood compartment, while the Ac-LDL receptor, which also interacts with Ox-LDL, is highly concentrated on endothelial liver cells. In the present investigation, we determined to what extent oxidative modification of β-VLDL affected its in vivo fate in rats. Furthermore, the cellular fate and the recognition system responsible for the removal of Ox-β-VLDL from the blood were determined.

**Materials**

Collagenase (types I and IV), bovine serum albumin (BSA; fraction V), and polyinosinic acid (poly I; 5’)-polycytidylic acid (poly C) were obtained from Sigma Chemical Co., St. Louis, Mo.; pronase (B grade), from Calbiochem Behring Corp., La Jolla, Calif.; Dulbecco’s modified Eagle’s medium (DMEM), from Gibco Laboratories, Grand Island, N.Y.; and iodine-125 (carrier free), from New England Nuclear Chemicals, Dreieich, FRG.

**Animals and Diet**

Throughout the study, we used male Wistar rats weighing 200–250 g. Cholesterol-fed rats were maintained on the diet for 14 days. The cholesterol-enriched chow included 2% cholesterol, 5% olive oil, and 0.5% cholic acid (Hope Farms, Woerden, The Netherlands). Before blood was collected from the abdominal aorta, rats were fasted for 1 day.

**Lipoproteins**

Isolation and storage of lipoproteins were done in the presence of 1 mM EDTA at 4°C. β-VLDL, the d<1.006 g/ml fraction, was isolated from the plasma of cholesterol-fed rats according to Redgrave et al.20 Rat β-VLDL showed a so-called β-mobility on agarose gels (Rf=0.38±0.02; rabbit β-VLDL showed an Rf value of 0.37±0.01). LDL (1.024<d<1.055 g/ml) was isolated from human plasma by two repetitive isoelectric focussing. LDL was enriched by exposure of 200 μg apoprotein/ml to 10 μM free Cu²⁺ at 37°C for 20 hours, after which the oxidation was stopped by the addition of EDTA (to a final concentration of 1 mM).

On 0.75% agarose gel electrophoresis, the oxidized lipoproteins showed an increased electrophoretic mobility. The relative electrophoretic mobility (compared with native lipoprotein) of Ox-β-VLDL was 1.39±0.05 (Rf value, 0.53±0.02; n=8, mean±SEM) and for Ox-LDL, 2.58±0.03 (Rf value, 0.54±0.01; n=6, mean±SEM). As reported earlier, the relative electrophoretic mobility of Ox-LDL is 1.37±0.05 (Rf value, 0.53±0.02; n=8, mean±SEM).

**Methods**

**Oxidation of Lipoproteins**

Before oxidation of β-VLDL or human LDL, lipoproteins were dialyzed extensively at 4°C against phosphate-buffered saline (PBS) containing 10 μM EDTA. Oxidation of β-VLDL or LDL was performed by exposure of 200 μg apoprotein/ml to 10 μM free Cu²⁺ at 37°C for 20 hours, after which the oxidation was stopped by the addition of EDTA (to a final concentration of 1 mM). On 0.75% agarose gel electrophoresis, the oxidized lipoproteins showed an increased electrophoretic mobility. The relative electrophoretic mobility (compared with native lipoprotein) of Ox-β-VLDL was 1.39±0.05 (Rf value, 0.53±0.02; n=8, mean±SEM) and for Ox-LDL, 2.58±0.03 (Rf value, 0.54±0.01; n=6, mean±SEM). As reported earlier, the relative electrophoretic mobility of Ox-LDL is 1.37±0.05 (Rf value, 0.53±0.02; n=8, mean±SEM).

**FIGURE 1. Gel electrophoretic characterization of native and oxidatively modified rat β-very low density lipoprotein (VLDL). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (left): The gel was stained with 0.2% Coomassie blue and dried. Various protein bands were identified. Lane 1, oxidized (Ox) β-VLDL; lane 2, β-VLDL. Agarose gel electrophoresis (right): Iodine-125-labeled β-VLDL (lane 3) and 125I–Ox–β-VLDL (lane 4) (50,000 cpm each) were subjected to agarose electrophoresis. After 2 hours of electrophoresis, the agarose plate was dried by hot air and subjected to autoradiography for 24 hours. See “Methods” for characterization of both β-VLDL and its oxidized form. Molecular weight markers at left of lane 1; apoproteins B-100, B-48, A IV, E, A I, A II, Cs at right of lane 2.**
The electrophoretic mobility for Ac-LDL was 2.50±0.05 (R, value, 0.52±0.01; n=6, mean±SEM).

Thiobarbituric-acid reactivity of Ox-β-VLDL was 22.5±17.5 (n=7, mean±SEM) compared with 1.1±0.1 nmol malondialdehyde/mg protein with native β-VLDL. The chemical composition of β-VLDL did not change significantly after oxidation (11±1% free cholesterol, 48±15% cholesteryl ester, 14±8% triglyceride, 16±6% phospholipids, and 11±1% protein; n=3, mean±SD). After oxidation of β-VLDL, the size of the particles was measured with laser-light scattering (submicron particle analyzer, Malvern Instruments Inc.). The diameter of the Ox-β-VLDL particles (66±10 nm; n=3) was not changed significantly compared with native β-VLDL (55±13 nm; n=3, mean±SD).

In Vivo Serum Clearance and Liver Association

Rats were anesthetized by intraperitoneal injection of 80 mg/kg body wt Nembutal (Abbott Laboratories, North Chicago, Ill.). The abdomen was opened, and radiolabeled lipoproteins were injected into the vena penis. The body temperature was maintained at 36.5–37°C. At the indicated times, 0.2 ml blood was taken from the inferior vena cava, and the radioactivity was counted. Liver lobules were tied off and excised at the indicated times. After weighing the lobule and counting the radioactivity, we calculated the total liver uptake. Liver lobules were not perfused, and values were corrected for the amount of radiiodinated lipoproteins entrapped in the blood present in the lobule. Values are expressed as percentages of injected dose. Bars represent SEM for three to six animals.
of radioiodinated lipoproteins entrapped in the blood present in the lobule. Poly I or PBS tested for its effect on the lipoprotein uptake by the liver was injected into rats as a bolus 1 minute before the radiiodinated compound.

**Rat Liver Cells**

Rats were anesthetized by intraperitoneal injection of 80 mg/kg Nembutal (Abbott). To determine the contribution of the various liver cells to the total liver uptake of β-VLDL and Ox-β-VLDL, the radiolabeled lipoproteins were injected into the vena penis. Routinely, 25 μg apoprotein per rat was injected. In some experiments, 3-5 μg apoprotein per rat was injected, and no difference in the percent uptake was found. After a 10-minute circulation, liver parenchymal, endothelial, and Kupffer cells were separated with a collagenase/pronase method at 8°C according to Nagelkerke et al.¹⁷ and Van Berkel et al.²⁹

For in vitro studies, endothelial and Kupffer cells were isolated with collagenase (0.05% type I) at 37°C. Endothelial and Kupffer cell preparations were controlled for purity by peroxidase staining and were found to contain 95% of the indicated cell type. Parenchymal cells were isolated by perfusion with collagenase (0.05% type IV) according to the method of Seglen.³⁰

The viability of the cells used for in vitro experiments was ≥90% as tested by trypan blue exclusion. Incubations of freshly isolated liver cells with the indicated amounts of lipoproteins were performed in 0.5 ml DMEM containing 2% (wt/vol) BSA at 37°C.
for the indicated times. The cell-associated radioactivity and degradation of the lipoproteins were determined as described elsewhere. The radioactivity in cell-free control incubations was routinely determined and subtracted from that obtained in the presence of cells.

Results

Identification of β-VLDL on Oxidation

In Figure 1, the electrophoretic mobility and apoprotein pattern of both β-VLDL and Ox-β-VLDL are compared. Exposure of β-VLDL to Cu\(^{2+}\) ions led to an enhanced electrophoretic mobility and fragmentation of the apoproteins. It appears that the electrophoretic mobility of oxidized β-VLDL is comparable to that of Ox-LDL, while the fragmentation of apoproteins has been noted earlier with rabbit Ox-β-VLDL. In addition to apo B, other apolipoproteins were also modified by the oxidative procedure.

Serum Decay and Liver Association

The disappearance of native β-VLDL and Ox-β-VLDL from the blood was equally rapid, and we observed that the blood decay and liver association of Ox-β-VLDL were kinetically indistinguishable from those of native β-VLDL (Figure 2). With both ligands, 50–60% of the injected dose became associated with the liver within 15 minutes. For rabbit Ox-β-VLDL, the liver-associated radioactivity reached 66% of the injected dose in a similar time frame (not shown).

At the indicated times, analysis of the radioactivity associated with the liver indicated that the distribution of radioactive label reflected that of the administered particle, indicating that the whole (oxidized) β-VLDL particles became associated with the liver. Preinjection of 5 mg poly I (1 minute before injection of radiolabeled compounds) did not have any effect on the clearance or liver association of native β-VLDL, while the clearance and liver association of Ox-β-VLDL were greatly retarded.

Cellular Distribution

We have shown previously that within the liver, β-VLDL is mainly taken up by parenchymal cells. In agreement with these data, 94±4% of the liver-associated radioactivity from β-VLDL appeared to be associated with parenchymal cells (Figure 3). For Ox-β-VLDL, however, the endothelial and Kupffer cell uptake was sixfold and 21-fold higher, respectively, as with native β-VLDL, leading to the Kupffer cells as the main liver site for uptake (65±2% of the total liver uptake). So, it appears that despite the identical blood decay and kinetics of liver association for β-VLDL and Ox-β-VLDL, their intrahepatic fates are markedly different.

When the uptake of Ox-β-VLDL is expressed per milligram cell protein (Figure 4), it is clear that the specific uptake of Ox-β-VLDL by Kupffer cells is fourfold and 116-fold higher than that of endothelial and parenchymal cells, respectively. For all three cell types, preinjection of poly I appeared to inhibit the uptake of Ox-β-VLDL.

Capacity of Cell Association and Degradation for Oxidized β-VLDL

With freshly isolated cells, the cell association and degradation of Ox-β-VLDL were characterized. The time course of cell association of Ox-β-VLDL with Kupffer and endothelial cells showed an initially rapid phase, whereas at later time points, an apparent equilibrium was reached (Figure 5). In contrast, the time course of the trichloroacetic acid-soluble radioactivity appearing in the aqueous phase showed a clear lag phase of 10 minutes. The degradation of Ox-β-VLDL by Kupffer and endothelial cells was clearly inhibited in the presence of chloroquine (100
FIGURE 7. Line plots showing comparison of the ability of unlabeled lipoproteins or polynosinic acid (poly I) to compete with the cell association (ass.) or degradation (degr.) of iodine-125-labeled oxidized β-very low density lipoprotein (Ox-β-VLDL) by parenchymal, endothelial, and Kupffer cells. Parenchymal, endothelial, or Kupffer cells were incubated with 5 μg/ml 125I-Ox-β-VLDL and the indicated amounts of unlabeled Ox-β-VLDL (■), oxidized low density lipoprotein (Ox-LDL) (○), acetylated low density lipoprotein (Ac-LDL) (●), or poly I (▲) at 37°C for 2 hours, and the cell association (upper panels) or degradation (lower panels) was determined. Values are expressed as percentage of radioactivity obtained in the absence of competitor. One hundred percent values for association and degradation of 125I-Ox-β-VLDL by Kupffer cells were 1,978±270 and 980±194 ng apoprotein/mg cell protein, respectively, and for endothelial cells, 1,360±242 and 562±86 ng apoprotein/mg cell protein, respectively (n=9; mean±SEM). In the absence of competitors, 198±28 ng apoprotein/mg cell protein became cell associated and 18±8 ng/ml cell protein was degraded by parenchymal cells (n=3).

In the presence of 10 mM NH₄Cl, a similar inhibition was found (not shown). The inhibition of Ox-β-VLDL degradation did not lead to accumulation of cell-associated Ox-β-VLDL, probably because chloroquine, in addition to raising the lysosomal pH, also inhibits the fusion of endocytic vesicles or multivesicular bodies with lysosomes. The capacity of Kupffer and endothelial cells to interact with Ox-β-VLDL is indicated in Figure 6. With increasing concentrations of ligand, the cell association and degradation values reached indicate the relatively high capacity of endothelial and Kupffer cells to interact with Ox-β-VLDL compared with parenchymal cells. The capacity of Kupffer cells to interact with and to degrade Ox-β-VLDL appears to be twofold and 30-fold higher than for endothelial and parenchymal cells, respectively.

Nature of Recognition Site

Competition experiments were performed to obtain information about the specificity of the recognition site for Ox-β-VLDL on the various liver cell types (Figures 7 and 8). Recently, we have established that various scavenger receptors may mediate the in vivo clearance of modified lipoproteins. In addition to the scavenger (Ac-LDL) receptor, which interacts efficiently with both Ac-LDL and Ox-LDL and which is concentrated on endothelial cells, an additional specific Ox-LDL receptor appears to be highly concentrated on Kupffer cells. To discriminate between the various receptor systems, the radiolabeled Ox-β-VLDL was competed for with poly I, Ac-LDL, Ox-LDL, and Ox-β-VLDL itself. Furthermore, we analyzed to what extent β-VLDL was able to displace Ox-β-VLDL. The association and degradation of 125I-Ox-β-VLDL by both endothelial and Kupffer cells were inhibited 70–80% by Ox-β-VLDL itself, Ox-LDL, or poly I. With endothelial cells, Ox-LDL appeared to be slightly more efficient at lower apo-protein concentrations than was Ox-β-VLDL itself. The association and degradation of 125I-Ox-β-VLDL were inhibited by Ac-LDL only 30% and 20% with
endothelial and Kupffer cells, respectively. The association of Ox-β-VLDL with parenchymal cells also appeared to show a specific component, whereby both Ox-β-VLDL and Ox-LDL efficiently competed for the association and degradation of 125I-Ox-β-VLDL by parenchymal cells. Ac-LDL and poly I inhibited the cell association and degradation only 10–20%. The degradation of 125I-Ox-β-VLDL by Kupffer and endothelial cells was slightly competed for by native β-VLDL, as reflected in an inhibition of the degradation of about 20% (Figure 8).

Discussion

The aim of the present study was to investigate to what extent oxidation of β-VLDL would change its in vivo behavior and fate. Although the liver is highly active in the elimination of β-VLDL from the blood circulation,32 cholesterol feeding of rats and rabbits still leads to the accumulation of β-VLDL in the plasma.36 Under these conditions, the high capacity of the liver to remove these atherogenic lipoproteins is apparently not sufficient. The prolonged circulation of β-VLDL may allow oxidative modification of these particles by a mechanism similar to that with LDL.19 In type III hyperlipoproteinemia, the E2/E2 phenotype is thought to lead to an ineffective removal of β-VLDL from the plasma, also resulting in the prolonged exposure of the particles to the blood compartment. After its intravenous injection into rats, native rat or rabbit β-VLDL is fully recognized by the remnant receptor, which is concentrated on rat liver parenchymal cells.32 The present data indicate that on oxidation of β-VLDL, its kinetics of removal from the blood and uptake by the liver remain identical. However, its intrahepatic cellular fate is markedly changed, and instead of parenchymal liver cells, the nonparenchymal cell types are responsible for uptake from the blood compartment. Recognition of Ox-β-VLDL is changed to scavenger receptors present on the Kupffer, endothelial, and parenchymal cells. From the total liver-associated radioactivity, 67% is recovered with Kupffer cells, whereas this percentage for endothelial and parenchymal cells was 16% and 17%, respectively. The in vivo involvement of scavenger receptors is indicated by the blockade of cell uptake by preinjection of poly I. It is apparent that the remnant receptor on parenchymal cells is unable to interact with Ox-β-VLDL particles even under conditions that prolonged circulation of Ox-β-VLDL is induced by the presence of poly I. The observed fragmentation of apo E explains this behavior. This apoprotein is considered to be the main epitope for recognition by the parenchymal remnant receptor.

Recently we showed that in addition to the Ac-LDL receptor, which is concentrated on endothelial liver cells,17 Kupffer cells do contain a scavenger receptor that specifically interacts with Ox-LDL (the so-called Ox-LDL receptor19). The present in vitro competition studies show that the specific recognition site for Ox-β-VLDL on parenchymal, endothelial, and Kupffer cells is different from the Ac-LDL receptor. The Ox-β-VLDL recognition on both endothelial and Kupffer cells is only partially competed for by Ac-LDL, while an efficient blockade is noted with poly I or Ox-LDL. Although a specific “oxidized lipoprotein recognition site” is also noted on liver parenchymal cells, the expression of scavenger activity per milligram of cell protein is low compared with that on Kupffer and endothelial cells. The capacity of endothelial and Kupffer cells to degrade Ox-β-VLDL appears to be 30- and 55-fold higher, respectively, than by parenchymal cells.

As with Ox-LDL19 both in vivo and in vitro, the association of Ox-β-VLDL with Kupffer cells is higher than with endothelial cells. It is remarkable to note the high degradation capacity coupled to recognition of oxidized lipoproteins by the oxidized lipoprotein receptor. The relation of this receptor with the recently cloned scavenger receptor cDNA is unclear.37,38
The present data indicate that the specific oxidized lipoprotein recognition site on Kupffer cells is not exclusive for the oxidative form of LDL but apparently recognize an epitope generated on several lipoproteins after oxidative modification.

The atherogeneity of \( \beta \)-VLDL is presumably to be augmented by oxidation.\(^{16}\) As with Ox-LDL, oxidation of \( \beta \)-VLDL mediated by both bovine aortic smooth muscle cells and \( \text{Cu}^{2+} \) ions led to an enhanced degradation by mouse peritoneal macrophages and an enhanced cholesterol esterification in the cells compared with native \( \beta \)-VLDL.\(^{15,19}\) The uptake of Ox-\( \beta \)-VLDL by macrophages was also found to be mediated by the specific oxidized recognition site.\(^{15}\) The involvement of the oxidation of \( \beta \)-VLDL in inducing atherosclerosis is also indicated by the preventive effect of probucol in the cholesterol-fed rabbits. It is concluded that the active high-affinity oxidized lipoprotein receptor on Kupffer cells will prevent the uptake of these atherogenic particles by cells in the arterial wall if Ox-\( \beta \)-VLDL is formed in plasma or if it enters the blood circulation. Therefore, the liver will form an efficient protection mechanism against these atherogenic lipoproteins.

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