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Interaction of low density lipoproteins with liver cells in rainbow trout

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Abbreviations: TC = tyramine cellobiose, LDL = low density lipoproteins, MeLDL = methylated low density lipoproteins, VLDL = very low density lipoproteins, HDL = high density lipoproteins, VTG = vitellogenin, EDTA = ethylenediamine tetraacetic acid, PBS = phospate buffered saline, SDS-PAGE = sodium dodecyl sulphatepolyacrylamide gel electrophoresis, DMPC = L-α-phosphatidylcholine-dimyristoyl.

Abstract

Liver is the main catabolic tissue for low density lipoprotein in rainbow trout (Gjøen and Berg 1992). We have investigated the interaction of LDL with isolated trout liver cells and liver membranes. ^{125}I-TC labelled trout LDL bound to isolated trout liver cells in a time dependent and saturable manner with an apparant K_d of 20.1 μg/ml, suggesting the existence of a specific binding site on the surface of these cells. The binding was Ca^{2+} dependent assessed by the 50% reduction obtained by 5 mM EDTA. Saturable binding to isolated trout liver membranes could also be demonstrated, but with lower affinity as compared to intact cells. Degradation of ^{125}I-TC-LDL in hepatocytes was also saturable as degradation could be inhibited about 60% by a 100 fold surplus of unlabelled LDL. The rate of degradation increased with temperature up to 20°C. Both cell association (binding + uptake) and degradation were reduced down to 20% of control in the presence of microtubular and lysosomal inhibitors. Hepatic catabolism of trout LDL therefore seems to depend on receptormediated endocytosis, followed by lysosomal degradation.

Introduction

Salmonid fishes live on a high lipid diet and may be well suited for studying the evolution of the LDL receptor pathway. These species develop atherosclerosis, although a direct correlation to dietary factors such as cholesterol is not clear (Farrell *et al.* 1986). This pathway has been extensively described in mammalian and partly in avian and amphibian species. In mammals, a 839 amino acid membrane glycoprotein that binds lipoproteins with apoB and/or apoE, whose synthesis is strictly regulated by the cholesterol status of the cell, has been characterized structurally and genetically by Goldstein *et al.* (1985). In lower vertebrates, Steyrer *et al.* (1990) have described two lipoprotein receptors from chicken that bind low density lipoproteins: one of 130 kDa in somatic cells and one 96 kDa receptor present in oocytes. The 96 kDa receptor binds VLDL, vitellogenin and mammalian apoE rich βVLDL while the 130 kDa receptor binds mainly LDL. Recently, a LDL receptor from *Xenopus laevis* was described; it resembles the human receptor in structure and mode of regulation (Metha *et al.* 1991a,b). Cells transfected with this receptor bind LDL and βVLDL but not vitelloge-

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nin. To date, only one report suggests specific LDL binding activity in piscine tissues (Fainaru *et al.* 1988). In this report, specific binding of carp and human LDL to carp liver membranes was demonstrated. Fishes possess virtually the same lipoprotein classes with similar apolipoprotein contents as mammals, except for the lack of apo E (Babin and Vernier 1989). Trout LDL normally contains two apolipoproteins of 240 kDa and 76 kDa respectively (Babin 1987). In a previous report we showed that iodinated trout LDL were mainly catabolized in liver (Gjøen and Berg 1992). In this report we have extended our studies, using primary cultures of trout hepatocytes to characterize further the interaction of LDL with its receptor on liver cells.

Materials and methods

Chemicals

Carrier free $Na^{125}I$ with a specific activity of 644 MBq per μg I, was obtained from the Institutt for Energiteknikk, Kjeller, Norway. Tyramine cellobiose was kindly donated by Dr. Helge Tolleshaug, Nycomed AS, Oslo. Collagenase Type I from C. histolytticum (EC 3.4.24.3) was bought from Sigma, U.S.A., and Nycodenz was obtained from Nycomed, Oslo, Norway. Ultroser serum replacement was obtained from IBF Biotechnics, France. Leibowitz L15 medium were from Flow Laboratories, Scotland.

Animals

Farmed rainbow trout (*Oncorhynchus mykiss*) weighing 400–600g were obtained from a local fish farm. They were kept in tanks containing running fresh water at a temperature of 12–14°C, and fed standard pellets for salmon, Ewos EST 93, Norway (40% protein, 49.5% fat and 10.5% carbohydrates).

Isolation of lipoproteins

Ten ml blood samples were pooled from three fishes anaesthetized with 0.03% chlorobutanol. The blood was withdrawn *via* the caudal vein and placed in tubes on ice. EDTA (0.01%) was present in the collection vessel and throughout centrifugation and dialysis to prevent oxidation and proteolysis. Plasma was obtained by centrifugation (3,000 × g, 20 min). Four lipoprotein fractions were obtained by sequential density ultracentrifugation in a TFT 70.38 rotor spun at 105,000 × g for 24h in a Centricon T-2060 ultracentrifuge. The four fractions; VLDL:$d < 1.015$; IDL:$1.015 < d < 1.040$; LDL:$1.040 < d < 1.085$; HDL:$1.085 < d < 1.210$ g/ml, were dialyzed against PBS-EDTA to remove NaBr, and stored at 4°C before characterization and labelling. All preparations were used within 3 weeks of isolation. Rabbit β-VLDL were isolated by standard procedures (overnight flotation at $d < 1.006$, 105,000 g) from plasma of animals fed a cholesterol rich diet for three weeks (2% w.w. cholesterol chow, Ewos Maintenance Food, Norway). Human LDL were isolated by the same procedures from fresh EDTA plasma at $1.019 < d < 1.063$ mg/ml.

Analysis of lipoproteins

The four fractions were analyzed by SDS-PAGE and for total protein by the protein assay from Bio-Rad GmBH, Germany (Bradford 1976) using bovine serum albumin as standard. Particle size was measured by a photon correlation spectroscopy method (Pecora, 1985). With this method trout LDL diameter was estimated to 22 nm (range 12–32 nm).

Labelling of lipoproteins with ^{125}I-tyramine-cellobiose

Trout LDL was labelled with ^{125}I-tyramine-cellobiose according to the method originally described by Pittman et al. (Pittman *et al.* 1983). We have used this labelling method to trace the sites of LDL catabolism *in vivo* (Gjøen and Berg 1992) and to follow its intracellular transport route. For reasons of consistency, LDL were labelled with the same residualizing adduct in this *in vitro* experi-

ments. A specific activity of 400–1000 cpm/ng LDL protein was obtained by this method. All radioactivity analyses were performed in a Packard Cobra Autogamma counter with 72% counting efficiency.

Isolation and cultivation of liver cells

Rainbow trout liver cells were isolated by a two step collagenase perfusion procedure essentially as described by Seglen (1976) and modified by Dannevig and Berg (1985). The parenchymal cells were suspended in buffer (NaCl 137 mM, KCl 5.4 mM, Na_2HPO_4 0.34 mM, KH_2PO_4 0.35 mM, $MgSO_4$ 0.81mM, HEPES 40 mM, $CaCl_2$ 2 mM, pH 7.5, 300 mOsm) containing 1% of BSA and enriched by two short (2 min) 50 g sedimentations. The second pellet was resuspended in 10 ml and mixed with 30 ml of 27% Nycodenz yelding a final density of 1.12. This suspension was overlaid carefully with 10 ml buffer and centrifuged at 1500 g for 15 minutes. The floating interphase was carefully pipetted off and the cells washed by one sedimentation in cold culture medium (Leibowitz L15 with 10 mM $NaHCO_3$, 100 U/ml pencillin, 100 μg/ml streptomycin and 2% Ultroser serum replacement). The cells were suspended to 1×10^6 cells/ml and seeded in 35 mm Costar dishes (2 ml/well). The cells were routinely incubated in a humid air atmosphere for 20h at 15°C before experimentation.

Binding, uptake and degradation of LDL in cultivated liver cells

The cells and medium were cooled on ice before addition of the indicated amounts of ^{125}I-TC labelled LDL. Nonspecific binding was estimated from samples containing 500 μg/ml of LDL. Samples were taken at intervals in the following manner: the cells were washed 4 times with ice cold buffer containing 150 mM NaCl, 50 mM Tris-HCl, 2 mg/ml of BSA, pH 7.4, and two times with buffer without BSA. The cells were solubilized in 1 ml of 0.1 M NaOH and assayed for radioactivity and protein by the Bio-Rad method using BSA as standard (Bradford 1976).

Analysis of degradation

The cells were grown at 15°C and ^{125}I-TC labelled LDL were added to the culture medium. Samples were taken essentially as described for binding except that the solubilised cells were precipitated with 1 ml of 20% TCA for analysis of degradation products.

Lipoprotein binding to trout liver membranes

Trout liver membrane fractions were prepared essentially as described by Kovanen *et al.* (1981). Briefly, trout livers were excised from female trout weighing 300–500g, homogenized in buffer A (150 mM NaCl, 10 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM PMSF, pH 7.5) with Dounce, and centrifuged at $500 \times g$ for 5 min. The resulting supernatant was centrifuged at $8,000 \times g$ for 15 min. The supernatant was then centrifuged at $100,000 \times g$ for 60 min and the pellet resuspended through a 22 G gauge and washed, then centrifuged again for 60 min at $100,000 \times g$. The final pellet was resuspended as above and analyzed for protein and 5′-nucleotidase (El-Aaser and Reid 1969). This enzyme was about 5 fold enriched in the final pellet.

The lipoprotein binding to these preparations was measured as described. Routinely, 100 μg liver membranes suspended in 150 μl buffer (25 mM NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$, 2 mg/ml BSA, pH 7.5) with the indicated amounts of ^{125}I-TC labelled LDL were incubated for 2h at room temperature. Nonspecific binding were estimated from samples containing 500 μg/ml LDL in addition to labelled lipoprotein. Bound and free ligand were separated by overlaying the incubation mixture with a 2:3 mixture of bis (3,5,5-trimethyl hexyl) phtalate:dibytylphtalate and centrifuged at 2,000 $\times$ g for 2 min. The pellet was cut off and assayed for radioactivity.

Results

Figure 1A and B demonstrate time and concentration dependent binding of ^{125}I-TC labelled LDL

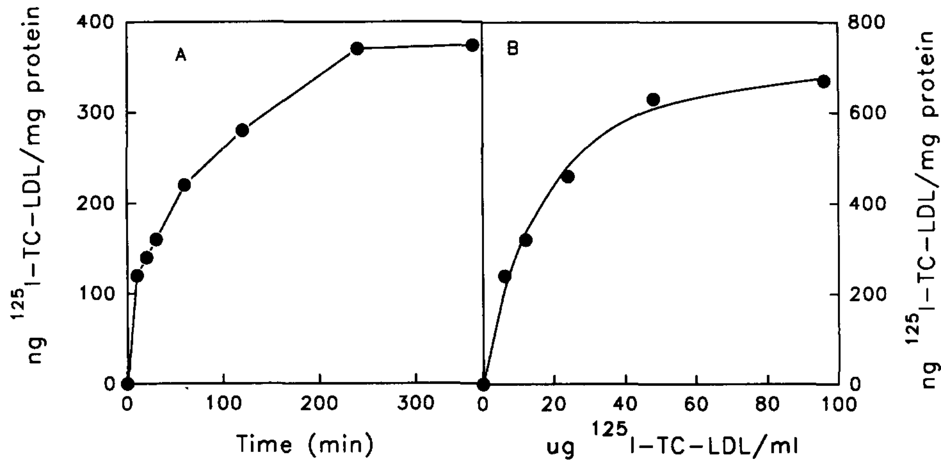

Fig. 1. Binding of ^{125}I-TC labelled LDL to cultured trout hepatocytes at 0°C. Cells were incubated with 5 μg/ml ^{125}I-TC labelled LDL and samples taken at intervals up to 6h (A) or after 4h in the presence of increasing concentrations of ^{125}I-TC-LDL (B). Nonspecific binding was corrected for by subtraction of binding in the presence of 500 μg/ml LDL. Data are mean of duplicate samples from one typical experiment.

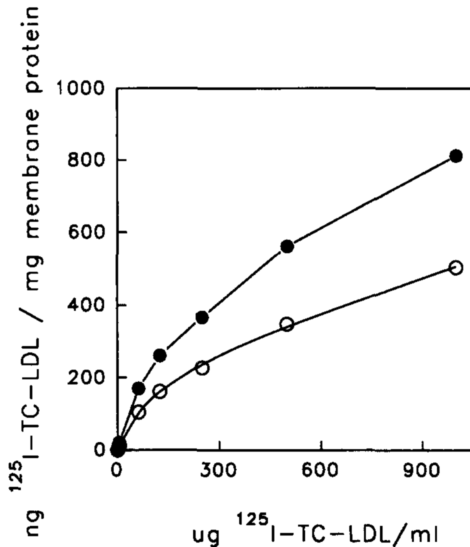

Fig. 2. Binding of ^{125}I-TC-LDL to liver membranes from rainbow trout. One hundred μg liver membranes were incubated for 4h with increasing amounts of labelled lipoprotein in the absence (closed circles) or presence of 500 μg/ml unlabelled LDL (open circles). Binding parameters were calculated by nonlinear curve-fitting.

to cultured trout hepatocytes. Maximum binding was reached after about 4h incubation at 0°C. When increasing amounts of ^{125}I-TC-LDL were incubated with cultured hepatocytes, a saturation of binding was observed. Binding parameters were calculated by nonlinear curve fitting using Enzfit computer program. The best fit was obtained using a 2 site model. Dissociation constant and maximal binding were calculated to 20.1 μg LDL/ml and 927.7 ng LDL/mg cell protein respectively. Fish cells may internalize plasma membrane even at low temperatures and this may affect binding data. This led us to investigate binding of ^{125}I-TC labelled LDL to isolated trout liver membranes. Figure 2 depicts a saturation curve for binding of ^{125}I-TC labelled LDL to trout liver membranes. This curve shows that binding of LDL to trout liver membranes was saturable, as was demonstrated for binding of LDL to cultured liver cells. When binding parameters were calculated for liver membranes K_d and B_{max} were found to be 112.2 μg LDL/ml and 236 ng LDL/mg membrane protein respectively. In Figure 3A, the displacement of ^{125}I-TC-LDL binding by trout LDL and HDL in addition to human LDL is shown. LDL from the two species were about equally effective while trout HDL posessed only about 50% competition efficiency compared to LDL. Apolipoprotein E has been shown to bind the mammalian LDL receptor with higher affinity than apoB. Although fish plasma contains no apolipoprotein E, we examined the binding of βVLDL from cholesterol fed rabbits. This apoE rich lipoprotein binds the mammalian LDL receptor with high affinity. Figure 3B shows that rabbit βVLDL bind to trout liver membranes

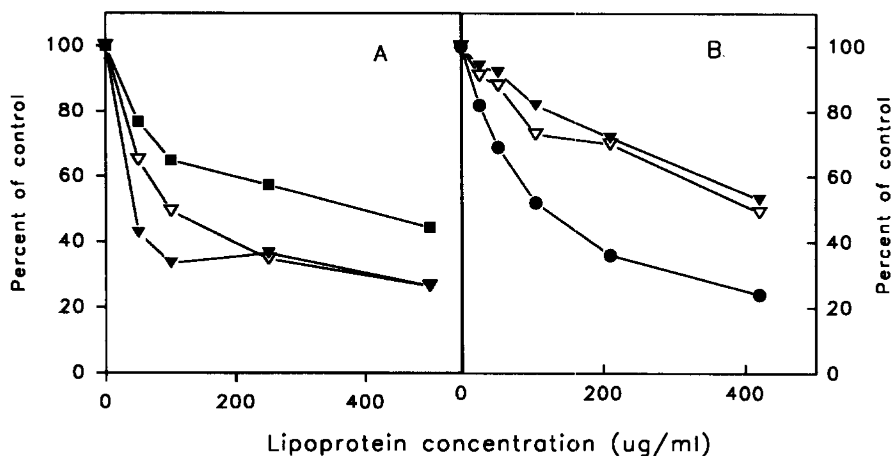

Fig. 3. Competition curves for binding of ^{125}I-TC labelled LDL 1251-TC-βVLDL (B) to trout liver membranes. Five μg/ml labelled lipoprotein were incubated for 4h with 100 μg liver membrane and increasing amounts unlabelled lipoproteins (closed triangle = trout LDL, open triangle = human LDL, closed circle = βVLDL, closed square = trout HDL). Data are mean of triplicate samples from one typical experiment.

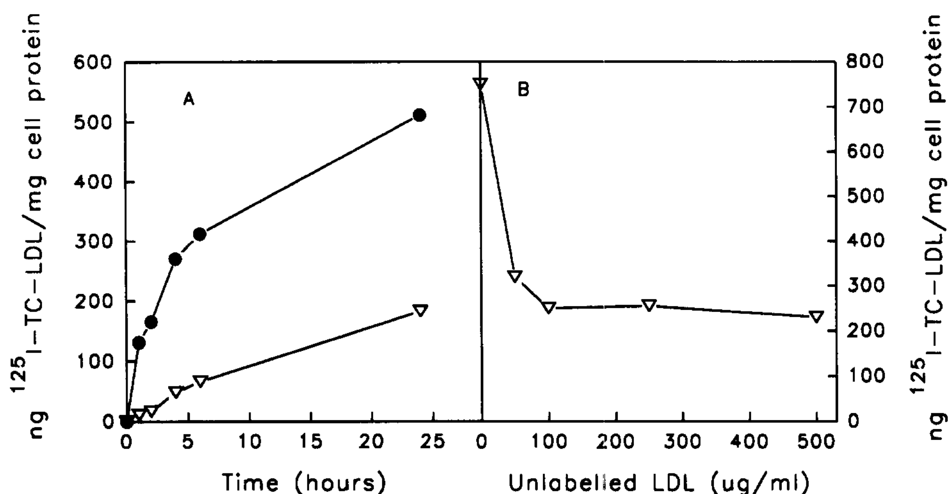

Fig. 4. Cell association (closed circles) and degradation (open triangles) of ^{125}I-TC labelled LDL in cultured trout hepatocytes at 15°C. Cells were incubated with 5 μg/ml ^{125}I-TC labelled LDL and samples taken at intervals (A) or after 5h in the presence of different concentrations unlabelled LDL (B, only degradation). Data are mean of duplicate samples from one typical experiment.

in a saturable manner and that both trout and human LDL were inferior to βVLDL in competing for this binding site. The effect of EDTA on LDL binding to hepatocytes was also tested. In the presence of 5 mM EDTA, binding was reduced about 50%, implicating a requirement for divalent cations for optimal binding to occur.

Figure 4A shows cell association (binding + uptake) and degradation of ^{125}I-TC labelled LDL in cultured trout hepatocytes as a function of time. After a rapid initial phase, the process seems to reach a steady state within 5 hours at this temperature (15°C). Acid soluble degradation products were observed after a lag phase of about 30–60 min. With 24h between 5 and 10% of added LDL/mg cell protein (5 μg/ml) were degraded. Degradation could be saturated upon incubation with increasing amounts of unlabelled LDL

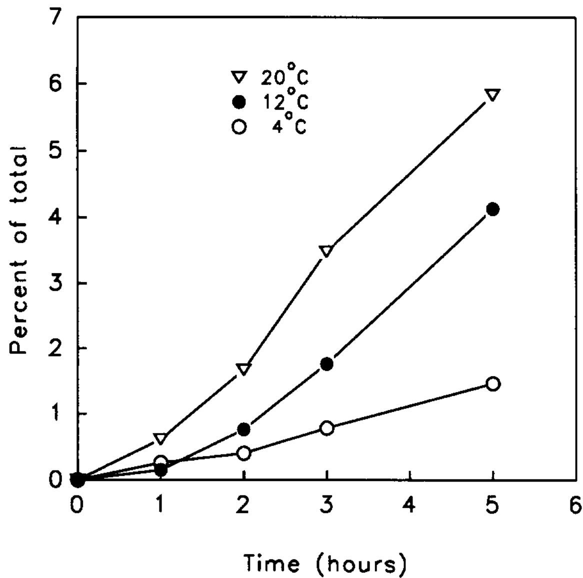

Fig. 5. Degradation of ^{125}I-TC labelled LDL in cultured trout hepatocytes at different temperatures. Cells were incubated with 5 μg/ml ^{125}I-TC labelled LDL and samples taken at intervals. Data are mean of duplicate samples from one typical experiment.

(Fig. 4B). When cultured hepatocytes were incubated at different temperatures it could be demonstrated that degradation was clearly temperature dependent, with an increase up to 20°C (Fig. 5). Degradation was about fourfold higher at 20°C compared to 4°C. At the highest temperature, the lag phase before appearance of degradation products, was also reduced.

Transport of endocytic organelles from the cell periphery to the perinuclear region rich in lysosomes, occurs along microtubules. We therefore tested the effect of inhibitors of microtubular (nocodazole, vinblastine, cholchicine) and lysosomal function (chloroquine, monensin, leupeptin) on the cellular processing of LDL in trout hepatocytes. Depolymerization of microtubuli (nocodazole, vinblastine, colchicine) reduced total cell association and degradation to about 30% of control. Vinblastine only reduced uptake to 73%. The lysosomotropic agents effectively suppressed LDL degradation up to 80%, but had less effect on cell association (Table 1).

Table 1. Effect of various inhibitors on cell association and degradation of ^{125}I-TC labelled LDL in trout hepatocytes. After overnight culture, 5 μg/ml ^{125}I-TC labelled LDL and inhibitor were added to the cells and incubated for 5h. The cells were washed and total and acid soluble activity analyzed. Data are expressed as percent of control and are mean of triplicate experiments. SD were within 10%.

Inhibitor	Cell associated	Degradation
None	100	100
Nocodazole 10 μM	36	29
Vinblastine 10 μM	73	44
Colchicine 100 μM	38	32
Monensin 100 μM	59	29
Leupeptin 2 mg/ml	69	44
Chloroquine 100 μM	33	20

Discussion

Although the liver takes up the majority of circulating LDL *via* receptor mediated endocytosis, reliable data on affinity and capacity of the hepatic LDL receptor in mammals, have been scarce. This is mainly due to the high degree of nonspecific binding of lipoproteins to liver cells (Pittman *et al.* 1982). An additional complication when studying this process in fish is that low temperature does not completely abolish the internalization of cell surface receptors in the fish cells. However, our findings are in agreement with published data on LDL-receptor properties in mammalian liver. In a study by Nenseter *et al.* (1989) with cultured rabbit hepatocytes, saturation of binding was reached around 10 μg/ml LDL, while total capacity was around 250 ng LDL/mg cell protein (values estimated directly from the figures). The corresponding calculated values in this report were 20.1 μg/ml and 927 ng LDL/mg cell protein, respectively (Fig. 1B). The trout hepatocytes therefore seem to display lower affinity, but higher total binding capacity than rabbit liver cells. In addition, the same study on rabbit hepatocytes demonstrated that removal of Ca^{2+} from the incubation medium (by EGTA) reduced binding to liver cells about 50%. Our results with trout hepatocytes show comparable Ca^{2+} dependency. About half of the hepatocyte binding capacity for trout LDL, was mediated via calcium dependent mechanisms, in

fish as in mammals. The nature of the LDL-receptor independent lipoprotein uptake is not clear, but the existence of a lipoprotein binding site different from the LDL receptor has been postulated (Pittman *et al.* 1982). When saturation experiments were performed with isolated liver membranes, both lower affinity and capacity were observed (112.2 μg LDL/ml and 236 ng LDL/mg membrane protein respectively) (Fig. 2). In rats, the corresponding binding parameters were estimated to 4,56 μg/ml and 454 ng/mg membrane protein respectively (Tripodi *et al.* 1991). This difference in affinity between assay with whole cells and membranes, probably reflects differences in pH, salts or degree of nonspecific binding in the two experimental systems. The affinity observed with whole cells are close to the value reported for interaction between human LDL and carp liver membranes (Fainaru *et al.* 1988), and in our opinion most reliable. A nonspecific lipid binding site seems to be present in trout liver, as we could demonstrate that high concentrations of trout HDL partially competed for LDL binding to liver membranes (Fig. 3A).

Hepatic lipoprotein binding in fish has previously been shown to exhibit species' cross-specificity. Carp liver membranes interact with human LDL and apoE-DMPC vesicles with an affinity comparable to homologous LDL (Fainaru *et al.* 1988). Our data demonstrate that this also holds true for trout liver membranes, although we observed lower affinity with our trout liver membranes. ApoE rich rabbit βVLDL was bound in a specific manner to isolated liver membranes, and was effectively displaced by unlabelled βVLDL. Human and trout LDL were about equally effective in displacing this lipoprotein, but inferior to βVLDL (Fig. 3B). However, βVLDL may bind to liver membranes *via* more than one receptor. When βVLDL is enriched with apoE, it also serves as a ligand for the α_2-macroglobulin receptor or LRP (LDL-receptor related protein) (Kowal *et al.* 1989). The role of LRP as a lipoprotein receptor is however still a matter of dispute, as it has been demonstrated that βVLDL and α2-macroglobolin do not compete for the same binding site (van Dijk *et al.* 1991). We have been able to demonstrate specific binding of activated α_2-macroglobulin to trout hepatocytes, indicating the presence of LRP on these cells (unpublished data).

Studies on the avian LDL receptors conducted by Schneider and coworkers demonstrate important differences in oviparous species, compared to mammalian lipoprotein receptors. In the laying hen, only the 95 kDa VLDL/VTG receptor expressed on oocytes, bind βVLDL, while the somatic 130 kDa LDL receptor show no affinity for apoE rich lipoproteins (Hayashi *et al.* 1989). The basis for these species differences in ligand specificity are not clear, but the avian oocyte receptor cross-react with antibodies against a mammalian (bovine) LDL receptor, whereas the somatic receptor does not. The oocyte receptor therefore corresponds better to the mammalian counterpart, both with respect to the ligand specificity and antigen epitopes. Deposition of yolk in salmonid ova is also mediated through vitellogenins secreted from the liver, and the receptors responsible for uptake have recently been identified (Stifani *et al.* 1990). This report states that salmon oocytes contain a 100 kDa protein that binds fish vitellogenin with an appearant K_d of 80 μg/ml. This receptor also displayed ligand cross-reactivity and immunological relationship with the chicken VTG receptor.

After binding to the cell surface receptors, lipoproteins are internalized and transported to the lysosomes for degradation. In mammalian hepatocytes, degradation products normally start to accumulate 10 min after initiation of endocytosis (Tolleshaug *et al.* 1977). In trout hepatocytes cultured at 15°C, this process took about 30 min. This is in accordance with previous studies showing that degradation of asialoglycoproteins in suspended hepatocytes commence at 30–60 min after start of endocytosis (Dannevig and Berg 1985). For aquatic poikiloterms, rapid changes in water temperature are an important source of physiological stress. Biological membranes are highly sensitive to temperature changes, and electrolyte permeability and membrane protein function are affected (Hazel and Williams 1990). We could demonstrated that degradation of LDL in trout hepatocytes was clearly temperature dependent (Fig. 4). The reduced degradation at lower temperatures probably reflects both

reduced transport rate from the cell surface to lysosomes and reduced enzymatic hydrolysis. The relatively high rate of degradation even at the lowest temperature, clearly demonstrates that binding data from fish cells should be judged with caution. The effects of temperature on endocytosis have been studied in cells both from homeotermic and poikilotermic animals (Opresko and Wiley 1987). In mammalian cells, internalization proceeds normally down to temperatures around 20°C, while transport to lysosomes are blocked due to cold-induced depolymerization of microtubuli (Dunn et al. 1980). Maximal VTG internalization in *Xenopus oocytes* occurs at 30°C, but is still significant at 4°C. The endocytic rate constant in *Xenophus* oocytes at 22°C are comparable to values obtained in mammalian cells cultured at 37°C, suggesting additional physical limitations on this process than temperature (Opresko and Wiley 1987).

The requirement of a functional endocytic-lysosomal apparatus for LDL degradation to ensue, was verified by use of inhibitors both against microtubuli and lysosomal function. Cholchicine was reported to be less effective against piscine microtubuli in an in vitro polymerization assay (Billger *et al.* 1991), although we found it efficient for inhibition of endocytosis in trout hepatocytes. Monensin reduced degradation both via its pH raising effect in organelles and its ability to block of transfer from early to late endocytic compartments (Berg *et al.* 1983).

In conclusion, our findings establish hepatocytes as an important site of lipoprotein catabolism in salmonid fishes. These cells take up the majority of circulating LDL *via* both specific and nonspecific mechanisms. The rate of degradation increased linearly with temperature, and could be disrupted by inhibitors of the endycytic pathway. In this respect, piscine lipoprotein catabolism do not deviate substantially from mammals, although the relative importance of the difference lipoproteins vary.

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