METABOLISM OF HDL_2 AND HDL_3 CHOLESTEROL BY MONOLAYERS OF RAT HEPATOCYTES

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1. Introduction

High density lipoproteins (HDL) are isolated from plasma in the density range between 1.063 and 1.210 mg/ml and constitute a heterogeneous lipoprotein family [1]. Subfractionation of HDL by ultracentrifugal procedure into HDL_2 (d = 1.063–1.125) and HDL_3 (d = 1.125–1.210 mg/ml) has indicated that the two subclasses may have different physiological roles [2]. Current interest in the HDL physiology has been stimulated by the observation that increased HDL_2 cholesterol levels or high HDL_2/HDL_3 ratios may be better correlated with a negative coronary risk than the whole HDL cholesterol levels, as generally maintained [3–5].

Studies on the regulation of the HDL_2 and HDL_3 levels in plasma have been numerous. Evidence has been indicated that HDL_3 may be the precursor of HDL_2, consequently to the transfer to HDL_3 of apoprotein subfractions and surface cholesterol esters, deriving from the breakdown of triglyceride-rich lipoproteins [6]. The HDL_2/HDL_3 ratio may be reduced following diets rich in polyunsaturated fatty acids [7] or, conversely, increased by drug treatments, such as with nicotinic acid and clofibrate [8].

The liver appears to be a major catabolic site for HDL [1]. Liver cells may also have high affinity uptake sites for the HDL apoproteins [9,10].

To identify differences in the catabolic pattern in liver of HDL_2 and HDL_3, an in vitro system of non-proliferating rat liver cells was used. This allows the maintenance of viable monolayer hepatocytes, in the complete absence of non-parenchymal cells, for $\leqslant 48$ h [11]. The effect of pharmacological concentrations of heparin in the system were also evaluated.

2. Materials and methods

Sprague-Dawley male rats (250–300 g) were used for lipoprotein collection and for the preparation of hepatocytes. Blood was held on ice in test tubes, containing EDTA (final conc. 0.015 M) from animals fasted for 12–14 h. Lipoprotein cholesterol labelling in vivo was achieved by the injection of 1 mCi [1,2-^{3}H]-cholesterol (spec. act. 58 Ci/mmol, The Radiochemical Center, Amersham) into the tail vein of rats 16 h before bleeding. The HDL_2 and HDL_3 subfractions were separated from the other lipoproteins by sequential ultracentrifugation [12], followed by extensive dialysis against 500 vol. saline EDTA (pH 7.4).

Free cholesterol of HDL_2 and HDL_3 was further labelled by incubating separated lipoproteins for 3–4 h at 37°C in silanized vials containing 25 μCi [4-^{14}C]-cholesterol (spec. act. 58.4 Ci/mmol, The Radiochemical Center, Amersham) [13]. The final specific activity of free cholesterol (^{3}H–^{14}C) ranged from 5940–7510 dpm/μg and 10 800–14 320 dpm/μg, respectively; that of esterified [^{3}H]cholesterol between 6000–6690 dpm/μg. Chemical analysis of the separated-lipoprotein classes was performed as in [14].

Rat liver cell monolayer cultures were prepared generally as in [15], with the exception that the CMRL 1969 formulation was included into the perfusion medium [16]. Parenchymal cells were seeded at $3–7 \times 10^4/cm^2$ density into 25 cm^2 Corning flasks. The culture medium was composed of 80% (v/v) CMRL 1969 and 20% (v/v) decomplemented rabbit serum. Porcine insulin (Sigma) was added at 0.5 μg/ml final conc. Cell incubation was performed in humidified air incubator at 37°C. The medium was changed 2 h after seeding and 24 h thereafter, at

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which time rabbit serum was substituted for an equal amount of lipoprotein deficient rabbit serum (LPDS) [17]. The labelled lipoproteins were added at the same time and a further incubation performed as described in the figure legends.

After incubation, the monolayers were washed 3 times with cold phosphate buffer and the cells scraped off with a rubber policeman. Lipids from the recovered cellular pellet were extracted according to the Folch procedure [18]. The free and esterified cholesterol bound radioactivity was determined by TLC separation of cellular lipids [19]. The silica gel zones corresponding to the sterols were scraped off, added to 5 ml Lipoluma (Lumac) and counted in a Packard liquid scintillation counter. Efficiency of lipid extraction and counting was determined by appropriate standards. Cellular protein contents of the lyophilized aqueous phase of the Folch extracts were measured following [20] using albumin as standard.

Lipoprotein free cholesterol entering the cells was determined as follows:

$$\mu g \text{ FC entered} = \frac{(\text{dpm } [^{14}C]FC + \text{dpm } [^{14}C] \text{ EC})}{\text{spec. act. } [^{14}C] \text{ FC}} \quad (1)$$

Free cholesterol entering the cells and ultimately esterified was:

$$\mu g \text{ FC esterified} = \text{dpm } [^{14}C]EC/\text{spec. act. } [^{14}C]FC \quad (2)$$

It is apparent from table 2 that the esterification rate of free cholesterol is very low. The flux of lipoprotein esters entering cells was therefore calculated from:

$$(\mu g \text{ EC entered})(\text{spec. act. } [^{3}H]EC) = \text{dpm } [^{14}C]FC - (\text{dpm } [^{14}C]FC)(\text{spec. act. } [^{3}H]FC)/\text{spec. act. } [^{14}C]FC + \text{dpm } [^{3}H]EC \quad (3)$$

The first addendum in (3) represents the mass of hydrolyzed cholesterol esters, whereas the second addendum is the mass of cholesterol esters interiorized by the hepatocytes, but not hydrolyzed.

In these formulae symbols are as follows: $[^{3}H]FC$ and $[^{3}H]EC$, tritium-labelled free and esterified cholesterol; $[^{14}C]FC$ and $[^{14}C]EC$, carbon-labelled free and esterified cholesterol.

Loss of cholesterol from cells was preliminarily evaluated by incubation with [1-^{14}C]acetate and determination of cholesterol-associated radioactivity in medium, in the presence of HDL_2 and HDL_3. The outflow was $\leqslant 18\%$ of cell-associated cholesterol radioactivity. No corrections were therefore introduced for cell cholesterol loss.

Heparin extracted from pig duodenum (170 U/mg) was from Calbiochem.

3. Results and discussion

In rats, in contrast to the human findings [2], only 20% of HDL cholesterol is associated with HDL_3 (table 1). The monolayer model offers the opportunity of following the fate of HDL_2 and HDL_3 associated cholesterol. The hepatic uptake of HDL_2 cholesterol esters, at similar lipoprotein concentrations, was 5-times higher than that of HDL_3 (table 2). Since esterified cholesterol does not readily exchange between membranes [21], these results may be considered as representative of a whole lipoprotein particle uptake process. The transfer of HDL free cholesterol from the subfractions to the hepatocytes was greater than that of cholesterol esters (table 2). Since for both subfractions, the mass of cholesterol esters is greater than that of free cholesterol, these data are consistent with a facilitated transport of free cholesterol, probably through a diffusive pathway, dissociated from the uptake of the lipoprotein particle.

Hypotheses suggesting that specific lipoprotein receptors may exist at the liver parenchymal cell surfaces have been raised [9,10]. From the data reported, some uptake specificity for different HDL subclasses, at least as relates to HDL_2 and HDL_3 cholesterol esters, is certainly indicated. The different apoprotein compositions of the two HDL subfractions may be regarded as important in hepatic uptake [1].

The concentration dependency of lipoprotein cholesterol transfer to cells was tested for HDL_2, where evidence for a more efficient cellular uptake was at hand. HDL_2 cholesterol ester internalization is not a linear function of pericellular HDL_2 levels, the efficiency of transport being decreased at high concentrations (fig.1). Saturation of transport, however, was not observed up to 51 μg/ml of medium HDL_2 cholesterol. The HDL_2 cholesterol ester hydrolysis was,

Table 1
Lipid and protein composition of rat plasma lipoproteins

	Esterified cholesterol	Free cholesterol	Triglycerides	Proteins
LDL d <1.063	9.7 ± 0.9	9.4 ± 0.5	92.1 ± 6.7	20.4 ± 2.0
HDL_2	42.2 ± 3.8	11.2 ± 1.3	6.7 ± 0.7	86.7 ± 5.7
HDL_3	8.3 ± 1.0	1.4 ± 0.6	0.6 ± 0.1	20.2 ± 1.3
Total	60.3 ± 5.0	20.3 ± 1.6	99.3 ± 7.0	126.8 ± 8.8

Each value (mg/dl plasma) is the mean ± SD from 6 independent determinations

Table 2
Internalization and cellular catabolism of free and esterified cholesterol from HDL_2 and HDL_3

	HDL_2: incubation time (min)				HDL_3: incubation time (min)			
	20	80	160	240	20	80	160	240
Internalized FC	116.2 ± 8.3	195.1 ± 11.4	298.8 ± 16.7	415.0 ± 17.8	74.7 ± 8.3	141.1 ± 14.2	224.1 ± 16.8	307.1 ± 21.4
Esterified FC	8.1 ± 1.7	8.1 ± 2.4	11.7 ± 3.1	12.0 ± 2.1	4.7 ± 3.0	5.3 ± 1.1	5.2 ± 0.6	8.7 ± 2.3
Internalized EC	20.7 ± 3.2	58.1 ± 5.8	166.0 ± 7.9	282.2 ± 13.3	8.3 ± 3.1	18.9 ± 3.0	29.2 ± 4.3	49.1 ± 5.1
Hydrolyzed EC	16.0 ± 2.2	50.4 ± 3.2	154.1 ± 16.3	260.0 ± 21.4	7.9 ± 3.6	18.0 ± 2.7	27.3 ± 4.7	46.2 ± 4.7
Non-hydrolyzed EC	4.2 ± 1.0	7.9 ± 0.6	11.9 ± 1.3	22.4 ± 0.7	0.4 + 0.1	0.8 + 0.2	2.1 + 0.7	3.0 + 1.0

The initial medium was changed after 24 h seeding for a medium containing 20% lipoprotein-deficient rabbit serum with either HDL_2 (8.4 μg cholesterol/ml) or HDL_3 (8.1 μg cholesterol/ml). Cells were incubated at 37°C in a humidified air incubator. At selected time intervals, cells were harvested and cellular lipid radioactivity determined as in section 2

Each value (ng cholesterol/mg cellular protein) is the mean ± SD from 3 independent determinations

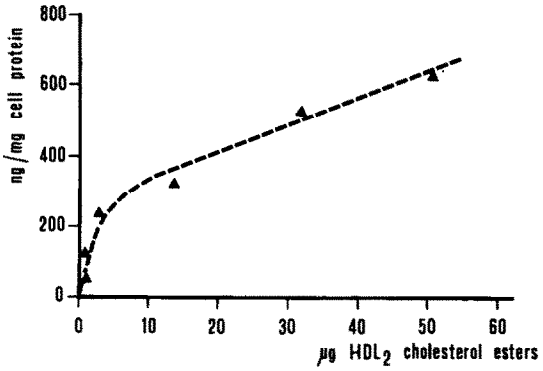

Fig.1. Cellular uptake of HDL_2 cholesterol esters: 24 h after seeding medium was changed for medium containing 20% lipoprotein-deficient rabbit serum and HDL_2 at different final cholesterol concentrations. Cells were incubated at 37°C for 4 h. Cellular lipid radioactivity was determined as in section 2. Each point is the mean from 2 independent determinations.

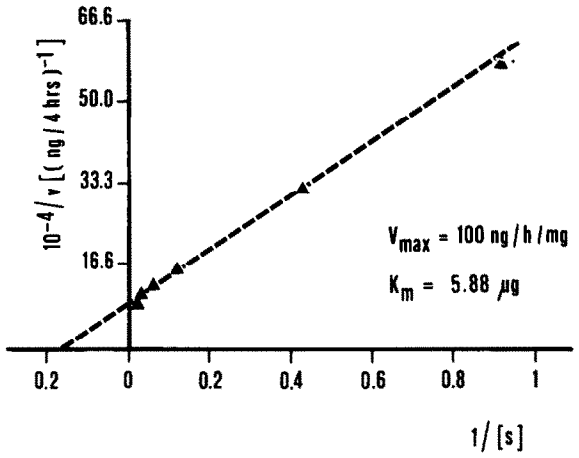

Fig.2. Lineweaver-Burk plot for HDL_2 cholesterol esters hydrolysis. Points were calculated from the results in fig.1, on knowledge of the mass of interiorized HDL_2 cholesterol esters undergoing hydrolysis (see section 2).

at any tested concentration, directly related to intracellular transport (table 2, fig.2). These findings clearly suggest that under the selected experimental conditions, liver parenchymal cells do not accumulate cholesterol esters. In the time interval of the study, moreover, free cholesterol entering the cells was only negligibly esterified (table 2). Similar findings have been reported in [22] for chylomicron remnant catabolism by liver parenchymal cells, and in [23] in human skin fibroblasts.

Hydrolytic activities for interiorized cholesterol esters were also calculated (fig.2). V_{max} was 100 μg . h^{-1} . mg cellular $protein^{-1}$ and K_m 5.88. These results are quite similar to those in [24] for the parenchymal cell hydrolysis of chylomicron remnant cholesterol esters. However, a lower figure for K_m was observed in [24]. Further experiments (unpublished) have shown that free and esterified cholesterol transfer for both HDL subfractions are temperature-dependent. At 4°C free and ester cholesterol internalization is reduced to 1/21 and 1/15, respectively.

The role of heparin in liver cell lipoprotein internalization, be it direct or mediated by cellular release of lipase activity [25], suggested testing the effect of the mucopolysaccharide in this system. Concentrations similar to those found in the plasma of treated patients [25] (1–10 U/ml) were used. The results (table 3) indicate a significant increase of cholesterol uptake, both free and ester, in parenchymal cells preincubated with heparin. This effect, although not significantly different between the two heparin concentrations, appears, on the other hand, related to the HDL_2 medium cholesterol, being evident only at >16 μg/ml.

Table 3
Effect of heparin on the cellular uptake of HDL_2 free and esterified cholesterol

HDL_2 cholesterol in medium (μg/ml)	Control		+ Heparin (1 U/ml)		+ Heparin (10 U/ml)	
	FC	EC	FC	EC	FC	EC
1.5	56.4 ± 4.2	69.7 ± 4.6	51.6 ± 4.7	73.7 ± 3.3	41.7 ± 3.9	62.8 ± 4.5
4.0	188.3 ± 7.8	126.7 ± 5.6	183.9 ± 4.8	102.0 ± 5.2	199.9 ± 8.7	117.5 ± 5.5
16.0	403.2 ± 10.9	187.2 ± 3.9	510.3 ± 8.8[a]	210.2 ± 7.8[a]	526.6 ± 13.4[a]	217.5 ± 9.8[a]
32.0	752.1 ± 18.7	322.2 ± 15.1	1000.6 ± 28.7[a]	418.4 ± 13.2[a]	941.9 ± 13.8[a]	387.1 ± 20.0[a]

[a] $p < 0.01$ *vs* control (Student's *t*-test for paired samples)

Each value (ng cholesterol/mg cellular protein) is the mean ± SD from 4 independent determinations

In conclusion, studies on the uptake of cholesterol associated with the two major HDL subfractions, showed that the cholesterol ester transport into cells is markedly higher for HDL_2, with an uptake pattern suggestive of a receptorial mechanism. Further studies are necessary to investigate whether HDL_2 inflow is associated with bile acid synthesis. The remarkable increase of HDL_2 cholesterol uptake induced by therapeutic concentrations of heparin, although with an unclear mechanism, is a finding of interest, in view of the proposed therapeutic effect of sulphated mucopolysaccharides in atherosclerotic conditions [26].

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