Short Communications

Cholesterol feeding to rats does not modulate the expression of binding sites for HDL on liver membranes

F. M. Maggi and A. L. Catapano*

Istitute of Pharmacology and Pharmacognosy, Via A. Del Sarto 21, I-20129 Milan (Italy), 4 November 1985

Summary. The binding of HDL, Apo-E-free, was studied in rats fed a cholesterol rich diet for 2, 4 and 7 days. Plasma cholesterol increased up to 16-fold (from 55 to 900 mg/dl); liver cholesterol was also raised, from 0.5 to 16 mg/g of tissue. The HDL binding to membrane preparations was not affected while the binding of β VLDL was reduced to about 50% of the controls. These data show, therefore, that liver binding sites for HDL are refractory to regulation by dietary cholesterol.

Key words. HDL; Apo-E; liver membranes; cholesterol feeding.

High density lipoproteins (HDL) are believed to play a key role in 'reverse cholesterol transport'[1]. This hypothesis has been validated at the cellular level by Oram et al. who have shown that HDL bind to specific sites present on human skin fibroblasts[2]. They have also shown that the HDL binding sites are up-regulated in cholesterol loaded cells. Furthermore, the binding of HDL to cells promotes cholesterol efflux[2].

Binding sites for DHL have been described in several organs[3–6] of rats as well as of other species[7,8]. We have recently found that a binding site for HDL is also present on human liver cell membranes[9].

The purpose of our work was to investigate whether the modulation of the cholesterol content of tissues, by cholesterol feeding of rats, results in an increased expression of binding sites for HDL on liver cell membranes.

Materials and methods. Male Sprague Dawley rats (200–225 g b.wt) were used throughout the study. They were fed a cholesterol rich diet (2% cholesterol, 1% cholic acid, Rieper Bolzano, Italy) for 2, 4 and 7 days. Two control groups were used; the first received the control diet ad libitum, the second the same amount of diet as the cholesterol fed rats, since the latter animals ate less of the diet than the controls. This was to avoid any possible effect of the level of food intake on plasma lipids as well as on the expression of lipoprotein receptors.

The animals were sacrificed at 09.00 h under ether anesthesia and blood was collected in the presence of sodium EDTA (1 mg/ml blood). Plasma cholesterol and triglycerides were determined by enzymatic procedures. Beta very low density lipoproteins (β VLDL) were obtained by ultracentrifugation at d. 1006 g/ml for 18 h at 12 °C in a 60 TI rotor at 40,000 rpm. HDL were isolated in the density range 1.050–1.21 g/ml for 48 h at 12 °C in a 60 TI rotor at 40,000 rpm. Lipoproteins were dialyzed against 0.15 M NaCl, pH 7.4, at 4 °C overnight and the HDL further fractionated by heparin-sepharose affinity chromatography as described[10]. The unretained fraction was free of Apo B or Apo E as demonstrated by SDS gel electrophoresis[11]. The plasma lipoprotein distribution was also determined by column chromatography of unfractionated serum on a biogel A 15 m column.

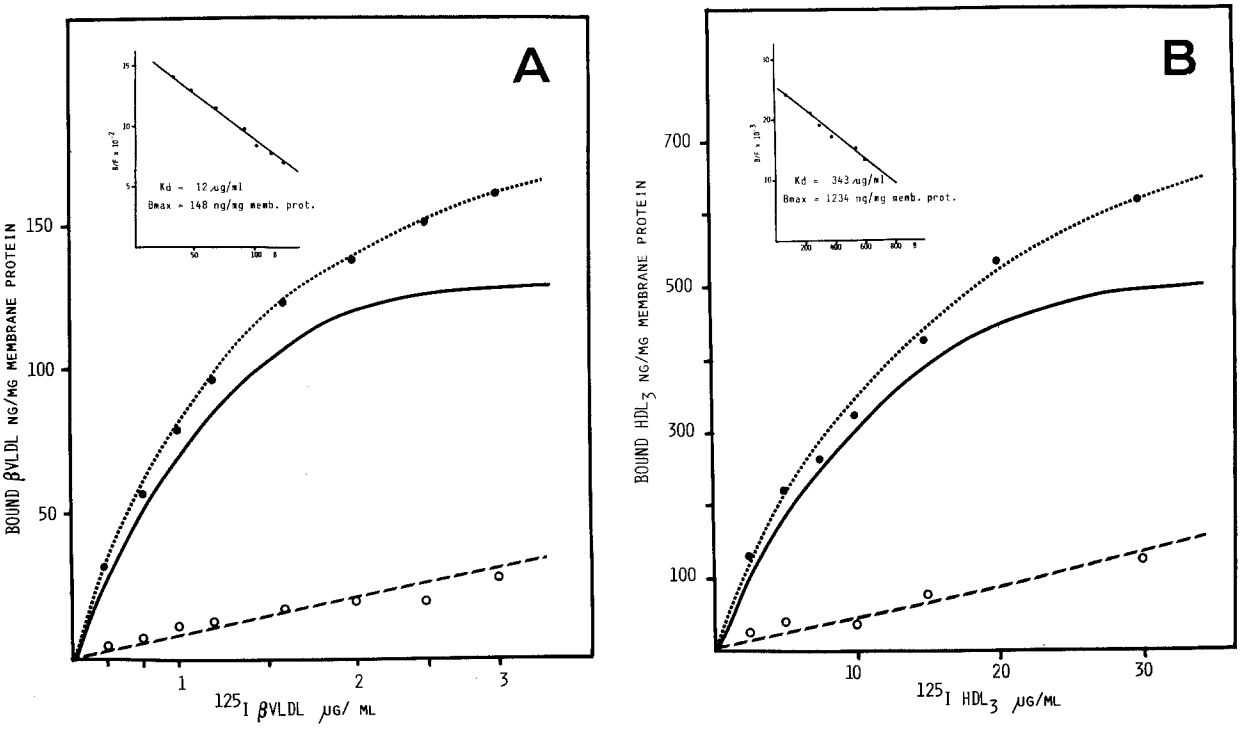

Figure 1. Saturation curve for the binding of ^{125}I β VLDL (left panel) and ^{125}I HDL (right panel). The data are means of duplicates that did not differ by more than 8%. The non specific binding was the binding in the presence of a 50-fold excess of unlabeled lipoprotein. (●) Total binding, (○) non specific binding, (–) specific binding.

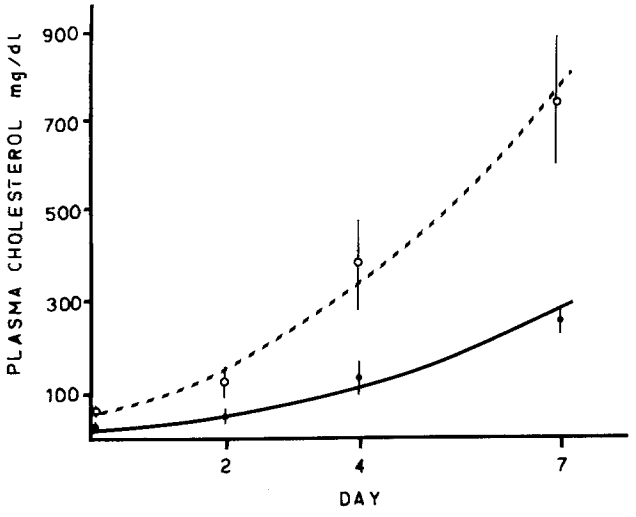

Figure 2. Plasma esterified (○) and free (●) cholesterol in cholesterol fed rats (x, n = 5). Bars are ± SD. All points differ significantly from control and from each other ($p < 0.01$).

Cholesterol was determined in each fraction by an enzymatic procedure. A VLDL+LDL and an HDL cholesterol peak were detectable by this method.

Lipoproteins were labeled with ^{125}I by the method of Mc Farlane as modified by Bilheimer et al.[12]. Specific activity ranged between 60 and 120 cpm/ng of protein for β VLDL and 140–200 cpm/ng of protein for HDL. Less than 1% of the radioactivity was non-precipitable with 10% tricloroacetic acid and less than 5% was lipid associated.

Membranes were prepared from liver homogenates as described by Kovanen et al.[9,13] and stored at −80 °C for up to 3 weeks with no apparent loss of binding activity. On the day of the experiment, the membranes were thawed and suspended in buffer B (50 mM NaCl, 1 mM $CaCl_2$ and 20 mM Tris HCl, pH 8) using a 22-gauge needle. The binding assay was performed in a final volume of 200 µl in 50 mM NaCl, 0.5 mM $CaCl_2$, 50 mM Tris HCl and 20 mg/ml bovine serum albumin pH 8 at a final membrane protein concentration of 1 mg/ml and 1 µg/ml of labeled β VLDL or 10 µg/ml of labeled HDL. Incubations were performed at 0 °C for 1 h; 75 µl of the incubation mixture were then applied at the top of a 150 µl cushion of fetal calf serum in a 42.2 Ti rotor. The membranes were pelletted by ultracentrifugation at 100,000 × g for 30 min, the supernatant carefully removed and the pellet washed with fetal calf serum and reprecipitated by ultracentrifugation. The bottoms of the tubes were then cut off using a mini drill; radioactivity was counted in a y spectrophotometer with less than 3% error[9,13]. The non specific binding was determined as the binding remaining in the presence of a 100-fold excess of unlabeled lipoproteins in the incubation medium. A typical saturation curve for β VLDL and HDL binding is shown in figure 1.

The cholesterol content of the liver was determined after lipid extraction of liver homogenates by the Folch method[14] using commercially available kits. Statistical analysis was performed by analysis of variance (Anova).

Results. Plasma cholesterol concentrations of the different experimental groups are reported in figure 2. No difference was observed in the plasma lipid concentration in the two groups fed control diet, even though at the end of the experimental period the b.wt of controls was 232 ± 10 versus 211 ± 26 g for pair-fed animals. Cholesterol fed rats had a b.wt of 217 ± 21. The differences between the three groups were not significant. Cholesterol feeding resulted in about a 16-fold increase of plasma total cholesterol as well as of the free cholesterol (fig. 2); the esterified/free cholesterol ratio was not dramatically affected. The distribution of cholesterol among the different lipoprotein classes was also modified, with a large increase of VLDL+LDL cholesterol as plasma cholesterol increased (table).

Upon cholesterol feeding, hepatic cholesterol increased about 6-fold. The free cholesterol increased 4 times from 1.7 to 6.5 mg/g fresh tissue while esterified cholesterol increased 30 times (from 0.3 to 6.2 mg/g fresh tissue). These data are presented in figure 3.

The results obtained in the experiments on the binding of β VLDL and Apo E-free HDL to membranes from livers of control and cholesterol fed animals are reported in figure 4. The binding of β VLDL to membranes decreased by about 50% at the second day of dietary treatment and remained low throughout the study ($p < 0.01$). The binding of HDL, however, was not affected significantly by the dietary treatment (fig. 4). Furthermore we have demonstrated that no change of apparent Kd could be detected suggesting that only the number of β VLDL binding sites was affected (data not shown).

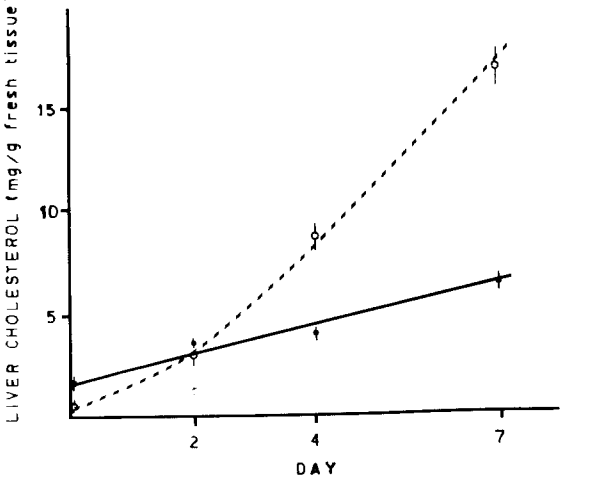

Figure 3. Hepatic esterified (○) and free (●) cholesterol in cholesterol fed rats (x, n = 5). Bars are ± SD. All points differ significantly from control and from each other ($p < 0.01$).

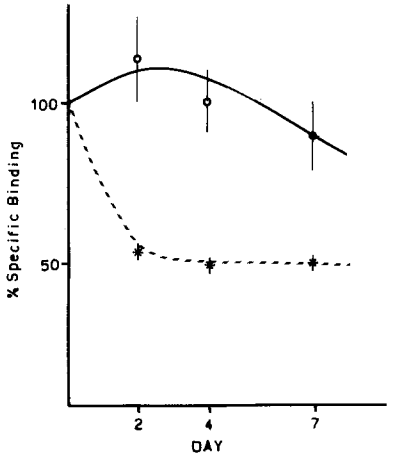

Figure 4. Specific binding of β VLDL (*) and Apo E free HDL (○) to liver membranes from control and cholesterol fed rats (x, n = 5). Bars are SD. Actual values for β VLDL binding were 104 ± 7.9 ng/mg protein 54.6 ± 3.7, 53.6 ± 3.7 and 52.8 ± 6.4 ng/mg protein at 0, 2, 4 and 7 days of cholesterol feeding ($p < 0.01$ vs controls) for HDL_3 the binding was 186 ± 12.8, 214 ± 19.8, 186 ± 18.7 and 165 ± 18.7 ng HDL_3/mg membrane protein (n.s. vs controls).

Cholesterol distribution among plasma lipoproteins in control and cholesterol fed rats

	Control	Control pair fed	Cholesterol fed		
			2	4	7 days
VLDL + LDL	24.6	25.8	81.9	86.8	97.8
HDL	73.4	74.2	18.1	50.7	2.2

Data are expressed as percent of total plasma cholesterol the values were 57.7 ± 5.5 mg/dl in controls, 55.7 ± 3.8 in controls pair fed, 168 ± 43, 389 ± 35 and 905 ± 147 in cholesterol fed animals at 2, 4 and 7 days (mean ± SD, N = 5). VLDL and LDL are calculated together since no apparent peak of LDL can be detected in control and in cholesterol fed rats; only a tail of β VLDL can be found in a region where human LDL are eluted from the column.

Discussion. The purpose of this work was to investigate whether cholesterol feeding resulted in a modulation of the HDL binding sites in the liver. Oram et al. reported that the loading of cultured fibroblasts with cholesterol results in an up-regulation of the binding sites for HDL_3[2]. Our data show that, while short term cholesterol feeding induces a dramatic increase of plasma cholesterol as well as of liver cholesterol in rats, this dietary manipulation does not affect HDL binding to liver membranes.

It has been reported that short term cholesterol feeding decreases the number of β VLDL and LDL receptors in the liver of rabbits and rats[15]. We therefore used β VLDL to show that under our experimental conditions cholesterol feeding results in a down-regulation of the LDL receptor also (fig. 4). The remaining binding of β VLDL can be explained either as binding to the E receptor[16] which is not modulated by cholesterol feeding or as binding that is not inhibited by EDTA[17].

The binding of HDL was not affected by cholesterol feeding while free and esterified cholesterol content of the liver increased. This finding does not support the hypothesis that in vivo loading of the liver with cholesterol results in up-regulation of HDL binding sites. A recent report by Hoeg et al. suggests, however, that in vitro the loading of hepatoma G-2 cells with cholesterol results in an up-regulation of the HDL binding sites[18]. These data disagree with our observation that cholesterol loading of Hepatoma G-2 cells in culture does not modify the HDL binding (Catapano et al., unpublished observations). Furthermore in enterocytes isolated from cholesterol fed rat the binding of HDL is not affected either[19]. We cannot exclude, however, that in vivo HDL binding sites on the liver are indeed up-regulated, but preparation of the membranes results in a loss of the sites induced by cholesterol feeding. This reasoning suggests that two different HDL binding sites may exist. Furthermore our data do not rule out the possibility that the binding of HDL may be up-regulated in peripheral tissues. A third possibility is that intra and extra cellular binding sites for HDL exist and cholesterol loading of the cell promotes their redistribution. Since the membrane preparations we used are a mixture of plasmatic and microsomal membranes the total binding would not be affected. This theory, however, is in disagreement with Oram et al. who found that sites induced by cholesterol loading of the cell are dependent upon protein synthesis[2].

In summary, our data suggest that in vivo in the rat the binding of Apo E-free HDL to liver membranes does not depend upon cholesterol loading of the cells. Whether this HDL binding site is relevant to the catabolism of HDL or to the 'release' of cholesterol by the HDL to the liver is unknown.

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Protein and lysozyme content of adult human nucleus pulposus

D.J. Sorce[1], C.A. McDevitt[2], R.A. Greenwald and S.A. Moak

Department of Pathology, State University New York at Stony Brook, Stony brook (New York 11794, USA), and Long Island Jewish Medical Center, New Hyde Park (New York 11042, USA), 22 July 1985

Summary. A radiologically normal human nucleus pulposus was extracted with 4 M guanidinium chloride and the non-collagenous proteins separated from the proteoglycans by dissociative density gradient centrifugation. Lysozyme was identified as a matrix constituent of the normal, mature human nucleus pulposus.
Key words. Lysozyme; nucleus pulposus; intervertebral disc; protein; proteoglycan.

Proteoglycans, type II and minor types of collagen, non-collagenous proteins and traces of elastin are the major macromolecules of the nucleus pulposus of the intervertebral disc[3]. Although the non-collagenous proteins can reportedly comprise up to 45% of the dry weight of the disc[4], their molecular forms and functions remain unknown. We here identify one of the non-collagenous proteins of the normal human disc as lysozyme.

Methods. A radiologically normal human spine (age: 32 years) was obtained at autopsy. The nuclei pulposi (L1/2 through L 4/5) were removed by dissection and extracted by gentle rotation