Regulation of Hepatic High Density Lipoprotein Binding Proteins after Administration of Simvastatin and Cholestyramine to Rats

Deirdre Mathai, Noel Fidge, Minoru Tozuka, and Alana Mitchell

We investigated the regulation of putative high density lipoprotein (HDL) receptors in rat liver after cholesterol feeding and the administration of cholesterol-lowering drugs to rats. The expression of two plasma membrane HDL binding proteins (HB, and HB2) were compared in control and treated livers by first separating membrane proteins on sodium dodecyl sulfate–polyacrylamide gels and quantitating HB, and HB2 levels with a specific ligand blot assay. Of the various treatments used, only simvastatin or simvastatin plus cholestyramine produced significant changes, with reductions of up to 40% and 60%, respectively, for HB, and HB2. The effect on the binding proteins was not associated with changes in serum cholesterol concentrations, which did not change significantly after either treatment, although a marked rise in liver cholesterol concentration after cholesterol was associated with a moderate increase in HB, expression. We show evidence for regulation of the levels of hepatic HDL binding proteins and provide another important criterion for the acceptance of HB, and HB2 as components of a functional HDL receptor. (Arteriosclerosis 10:1045–1050, November/December 1990)

We recently identified and purified from the plasma membranes of rat liver two proteins, HB, and HB2, which bind high density lipoprotein (HDL). The proteins, which preliminary structural studies suggest are different polypeptides, migrate on sodium dodecyl sulfate (SDS)–polyacrylamide gels with an apparent molecular weight of 120 and 100 kDa, respectively.1 On ligand blots, HB, and HB2 bound apolipoproteins A-I and A-II, as well as HDL, suggesting that these membrane proteins may function as specific cellular recognition sites for HDL. This suggestion is supported by previous reports from several laboratories on HDL binding and uptake by the liver in vitro2,3,4 or in vivo.5,6

Whether or not these binding proteins play a significant physiological role in HDL metabolism remains to be determined; if they do provide a mechanism for regulating HDL transport into or out of cells, it could be expected that their binding activity may respond to a variety of biochemical processes affected by HDL transport and metabolism. Such postulated changes in activity may result from altered rates of HDL synthesis or from modifications to their binding activity at the membrane surface. To determine whether the binding activities of HB, and HB2 are regulated, we treated rats with hypocholesterolemic drugs or diets known to affect lipoprotein concentrations, and we compared the activities of liver HDL binding proteins in these treated animals with those of control rats. To measure the activities, we extended the process of ligand blotting used initially for detecting the presence of HB, and HB2 during their isolation and purification1 to a quantitation system that depends on the radioassay of 125I-labeled HDL bound to HB, and HB2 after ligand blotting of purified plasma membrane proteins.

Materials

Animals

Male Sprague-Dawley rats, 6 to 7 weeks old with initial weights of 150 to 200 g, were housed in groups of three in cages with plastic floors. The rats were maintained in a temperature- and light-controlled room (20°C, 12 hours of light and 12 hours of dark) and given either a control diet of commercial rat pellets (G.R. 2+, Clark King & Co., Melbourne, Australia) or an experimental diet.

Diet or Drug Treatment

Cholestyramine (Questran) was obtained from Mead & Johnson, Australia, and simvastatin (MK-733) was donated by Merck, Sharp & Dohme. Cholesterol was purchased from Ajax Chemicals and animal fat “drippings,” from Wik Industries (Melbourne, Australia) or an experimental diet.

Method of Administration

Cholestyramine (4% wt/wt) and simvastatin (0.112% wt/wt) were added to ground rat pellets according to the method of Björkhem7 and Singer et al.8 Water was added to make a paste, which was then dried overnight in an oven at 56°C to form a dry biscuit. This was broken up into smaller cubes and fed ad libitum to the rats for 8 or 14 days before sacrifice. The control rats received the same biscuit but without drugs.
Rats on a cholesterol-enriched diet were given a biscuit made from ground rat pellets supplemented with 1% cholesterol (wt/wt), 5% drippings (wt/wt), and 0.3% taurocholic acid (wt/wt), in the same manner as described by Apostolopoulos et al.9 This was fed for 42 days before sacrifice.

At the end of the treatment period, the rats were fasted overnight and were killed by cervical dislocation. The procedures used were approved by the Alfred Hospital–Baker Medical Research Institute Animal Ethics Committee. Blood samples were taken from each rat by cardiac puncture. After in situ perfusion with 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (pH 7.4), the livers were excised, blotted, and weighed. About 0.5 g was set aside for a measurement of cholesterol content.

**Preparation of Plasma Membrane from Rat Liver**

Plasma membranes were prepared with minor modifications to the method described by Fleischer and Kerwin.10 The rat livers were perfused in situ with PMSF 150 mM NaCl/1 mM HEPES (pH 7.4); then they were removed and immersed in ice-cold 10 mM HEPES (pH 7.5)/0.25 M sucrose (homogenization buffer) and purified plasma membrane obtained as described previously.11

Examination of the plasma membranes by electron microscopy revealed typical vesicles with diameters of 200 to 500 nm and less than 2% contamination with mitochondria and other subcellular fractions. In addition, the purity of the plasma membranes was assessed by using the marker enzyme alkaline phosphatase,11 for which the specific activity was 10-fold higher in the plasma-membrane fraction compared with the whole homogenate.

**Isolation of Lipoproteins**

HDL3 (d=1.125 to 1.21 g/ml) and low density lipoprotein (LDL, d=1.019 to 1.055 g/ml) were isolated from human serum by ultracentrifugation as described previously.12 Lipoproteins were washed by recentrifugation at the appropriate densities until homogeneous by electrophoresis.

Lipoproteins and apoproteins were labeled with 125I by the iodine monochloride method13 as described previously. Isolation of lipoproteins from rat sera (d<1.21 g/ml) on native gradient polyacrylamide gels (PAA 4% to 30%, Pharmacia) and low density lipoprotein (LDL, d=1.019 to 1.055 g/ml) were isolated from human serum by ultracentrifugation as described above.

**Liver Cholesterol Determination**

Aliquots of liver (0.25 g) were digested with ethanol:30% KOH (1:1 vol/vol) at 80°C for 1 hour. Lipid was extracted three times with five volumes of petroleum spirit, and the combined extracts were washed twice with 2 M KOH and five times with H2O.

**Protein Determination**

Protein concentrations in isolated lipoprotein and plasma membrane preparations were measured by the method of Lowry et al.18 Bovine serum albumin was used as a standard in the concentration ranges of 10 μg/ml to 50 μg/ml.

**Serum Lipid Measurements**

Serum was obtained by centrifugation of blood at 2500 rpm (20 minutes, 4°C, Heraeus-Christ minifuge GL, Osterode am Harz, FRG). Serum total cholesterol and triglyceride concentrations were measured by automated enzymatic assays (Cobas Bio, Roche Diagnostics, Melbourne, Australia) with reagents supplied as a kit (Boehringer, Mannheim, FRG).

**Isolation of Lipoproteins from Rat Sera**

An aliquot of 500 μl of serum from each rat was adjusted to a density of 1.25 g/ml with KBr and was centrifuged at 100 000 rpm for 16 hours (Beckman rotor TL-100) to isolate the lipoprotein fraction (d<1.21 g/ml) from the remaining serum proteins. The recovery of cholesterol in the lipoprotein fraction was 75% to 80%.

**Lipoprotein Analysis by Size-Exclusion Chromatography**

The lipoprotein fraction (d<1.21 g/ml) was applied to an agarose-based, size-exclusion column (FPLC Superose 6-system, Pharmacia, Uppsala, Sweden) in a running buffer of 150 mM NaCl, 50 mM Tris (pH 7.4),14 and 0.6 ml fractions were collected at 30 ml/hour. The total cholesterol concentration of each fraction was measured as described above.

**Measurement of High Density Lipoprotein Particle Radius**

Particle size was estimated after electrophoresis of lipoprotein samples (d<1.21 g/ml) on native gradient polyacrylamide gels (PAA 4% to 30%, Pharmacia) as described by Blanche et al.16 Standard proteins of known radii (high molecular weight calibration kit, Pharmacia) were included to calibrate the gels in each run. After staining, gels were scanned by laser densitometry (LKB-Bromma Ultroscan XL).

**Assay for High Density Lipoprotein 3-binding Proteins of Rat Liver**

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Equal amounts of plasma membrane protein from control and treated rats were solubilized in 0.1% SDS in 0.05 M Tris HCl (pH 7), and 200 μg protein was loaded onto 10% polyacrylamide SDS-slab gels (15 cm x 11 cm x 1.5 mm) prepared according to the method of Weber and Osborne.19 Electrophoresis was performed at 30 mA for 6 hours in 25 mM Tris, 0.19 M glycine, and 0.1% SDS (pH 8.3).

**Ligand Blotting Assay**

This was carried out with a minor modification of the method previously described in detail.1 After transfer onto nitrocellulose sheets, the nonspecific binding sites on the membrane were blocked by incubation for 1 hour at room temperature in 3% casein, 150 mM NaCl, and 50 mM Tris-HCl (pH 8.0) (blocking buffer). This was followed by...
Table 1. Effect of Drug Treatment on Serum and Liver Lipid Values

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum cholesterol concentration (mM)</th>
<th>Serum triglyceride concentration (mM)</th>
<th>Liver cholesterol content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol*</td>
<td>12.0±2.2</td>
<td>1.25±0.18</td>
<td>71.0±12.0</td>
</tr>
<tr>
<td>Cholestyramine</td>
<td>1.72±0.09</td>
<td>0.16±0.02</td>
<td>2.67±1.70</td>
</tr>
<tr>
<td>+ simvastatin</td>
<td>1.71±0.12</td>
<td>0.26±0.07</td>
<td>2.46±1.60</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>2.13±0.22</td>
<td>0.82±0.22</td>
<td>2.70±0.05</td>
</tr>
<tr>
<td>Cholestyramine</td>
<td>2.30±0.09</td>
<td>0.74±0.07</td>
<td>2.30±0.19</td>
</tr>
<tr>
<td>Control</td>
<td>2.13±0.22</td>
<td>0.82±0.22</td>
<td>2.70±0.05</td>
</tr>
</tbody>
</table>

The results are expressed as the means±SEM.
*The animals were fed cholesterol for 42 days. There were six animals in each group.

incubation in 20 mM sodium deoxycholate for 30 minutes, which substantially lowered the background on the film. The membrane was then incubated with the ligand, 125I-labeled HDL3 (10 μg/ml in blocking buffer for 1 hour, at 20°C). Excess unbound ligand was removed by three 5-minute washes in phosphate-buffered saline (PBS, 145 mM NaCl, 7.5 mM Na2HPO4, and 2.5 mM NaH2PO4 *2H2O)+0.1% Tween (Sigma, St. Louis, MO), followed by three washes in PBS alone. After drying at room temperature, the membrane was applied to X-ray film (Hyperfilm-MP, Amersham, England) and was exposed for 64 hours at −80°C with Lightning Plus Intensifying screens. Preflashing the film did not alter the intensity of the bands. Regions of the nitrocellulose displaying binding activity were cut out and counted in a gamma counter (Minaxi autogamma 5000, Packard Instrument Co., Downers Grove, IL) for 30 minutes. Pieces of nitrocellulose of equivalent areas, which contained no HDL3-binding proteins, were also excised and counted to give estimates of background radiation. The net bound cpm of 125I-HDL3, which was proportional to the amount of protein on the filter, was compared for treated and control rats.

Results

Effect of Treatment on Serum and Liver Lipid Values

Cholestyramine treatment alone did not change serum triglyceride or cholesterol levels (Table 1). Simvastatin, when administered either alone or in combination with cholestyramine, produced a small decrease in serum cholesterol (about 25%) with large reductions in triglycerides of approximately 65% to 75%. Cholesterol feeding markedly increased both serum and liver cholesterol values, but the drug treatments did not alter liver cholesterol.

Lipoprotein Cholesterol Distribution

The effect of the various treatments on lipoprotein distribution was examined by size-exclusion chromatography of lipoproteins isolated from serum on an agarose gel column calibrated with the markers, human very low density lipoprotein (VLDL), LDL, and HDL (Figure 1). Cholestyramine plus simvastatin treatment produced no apparent changes in the ratios of VLDL and HDL (Figure 1A).

The profiles from cholesterol-fed rats suggested that the diet-induced hypercholesterolemia was predominantly due to a rise in the VLDL level and in lipoproteins of intermediate density remnants or LDL. HDL levels were
Assay of High Density Lipoprotein

treatment are presented (Figure 1B).

plasma membranes. After electrophoresis and electroblotting, tatin alone, cholestyramine alone, nor cholesterol feeding

ferred to nitrocellulose. Ligand blotting showed that bind-

of lipoprotein were subjected to electrophoresis on 4% to

changes in HDL particle size, which may be associated

HB1-

HB2-

Figure 2. Quantitation of radiolabeled high density lipoprotein (HDL3) binding to HB1 and HB2. A. Ligand blot shows duplicate lanes loaded with 50, 100, 200, 400, and 500 µg of rat liver plasma membranes. After electrophoresis and electroblotting, nitrocellulose sheets were incubated with radiolabeled HDL3 as described in the text. B. Plot of nanograms of radiolabeled HDL3 bound to HB1 and HB2 after excision and radioassay of the corresponding nitrocellulose pieces.

unaffected. These findings were consistent for all treatment periods, of which only the profiles after 6 weeks of treatment are presented (Figure 1B).

To determine whether the treatments produced changes in HDL particle size, which may be associated with changes in the expression of HB1 and HB2, aliquots of lipoprotein were subjected to electrophoresis on 4% to 30% acrylamide gradient gels (results not shown). Neither cholestyramine plus simvastatin treatment, simvas-
tain alone, cholestyramine alone, nor cholesterol feeding caused a change in the HDL particle size.

Assay of High Density Lipoprotein Binding Proteins

Preliminary experiments were carried out to validate the method for quantitative measurement of radiolabeled HDL3 bound to HB1 or HB2. Increasing amounts of plasma membrane from 50 to 500 µg of protein were applied in duplicate lanes loaded with 50, 100, 200, 400, and 500 µg of rat liver plasma membranes. After electrophoresis and electroblotting, nitrocellulose sheets were incubated with radiolabeled HDL3 as described in the text. B. Plot of nanograms of radiolabeled HDL3 bound to HB1 and HB2 after excision and radioassay of the corresponding nitrocellulose pieces.

When the above method was used to study the changes in HB1 and HB2 induced by diets or drugs, simvastatin in combination with cholestyramine significantly lowered the expression of both HDL binding pro-
teins (Table 2, Figure 3). That this effect was probably attributable to simvastatin was confirmed in studies where the two drugs were administered separately. In two experiments, HB2 was lowered significantly. The level of HB1 was also reduced in both experiments, reaching statistical significance in experiment 3 only (Table 2). Cholesterol feeding produced a 28% rise in the activity of HB2, although this increase (which was also seen after feeding cholesterol for 3 weeks) just failed to reach statistical significance.

Discussion

The liver is the major organ responsible for apolipoprotein E-free HDL catabolism in the rat; through a mechanism distinct from the LDL receptor. Two liver membrane proteins of 120 and 100 kDa molecular mass (HB1 and HB2, respectively) have been found to bind specifically to apoprotein E-free human HDL3 and it is suggested that these proteins, which appear to be distinct polypeptides, may play an important role in the metabolism of HDL by the liver.

Although the biochemical stimuli regulating these proteins are unknown, influence may be expected to occur through pathways that affect lipid metabolism and possibly through those factors that alter the concentration of the ligand, plasma HDL. Rats were therefore treated with various drugs or diets with the potential for directly or indirectly altering the metabolism of HDL or changing liver cholesterol concentrations. The expression of the HDL-binding proteins in treated and control rats was then compared by a ligand blotting assay similar to that used to quantify the LDL receptor. This technique, which currently provides the only means of examining these specific proteins, is sensitive to nanogram quantities of radiolabeled HDL3.

Of the several treatments tested, the most convincing changes to HB1 and HB2 occurred after cholestyramine plus simvastatin treatment or with simvastatin alone, both of which decreased the expression of HB1 and HB2 by approximately 50% (0.01 < p < 0.005). This effect on the binding proteins did not appear to correlate with liver cholesterol but was accompanied by a 25% decrease in serum cholesterol.22 This moderate decrease may reflect the potency of the simvastatin used, since other analogues appear to produce little change in serum cholesterol in the rat.22,23 Lipoprotein distribution, as evaluated by size-exclusion chromatography, showed no change with these treatments, although serum triglyceride content was lowered. No alterations in HDL particle size were detected by gradient gel electrophoresis.

Simvastatin treatment initially decreased the rate of cholesterol synthesis by competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key rate-controlling enzyme in the biosynthesis of cholesterol in rat liver. However, this lowered rate of cholesterol synthesis is compensated in part by the ability of the liver to increase HMG-CoA reductase mRNA24 and by synthesis of the enzyme.25 Cholestyramine, a bile acid sequestrant, potentiates this increased enzyme synthesis. Our data suggest that an additional mechanism for the maintenance of liver cholesterol content may be the down-regulation of HB1 and HB2 to decrease cholesterol efflux. This proposal is in
Table 2. Effect of Drugs or Cholesterol Feeding on Binding of High Density Lipoprotein to HB, or HB2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>HB1 (pmoles x 10^3)</th>
<th>% change</th>
<th>HB2 (pmoles x 10^3)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>6</td>
<td>11.8±0.22</td>
<td>-54 (p&lt;0.001)</td>
<td>6.9±0.16</td>
<td>-51 (p&lt;0.001)</td>
</tr>
<tr>
<td>Simvastatin + cholestyramine</td>
<td>6</td>
<td>5.4±0.13</td>
<td>3.4±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Control</td>
<td>5</td>
<td>17.8±2.7</td>
<td>-30 (NS)</td>
<td>20.9±3.8</td>
<td>-54 (p&lt;0.05)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>5</td>
<td>12.4±3.8</td>
<td>9.6±2.4</td>
<td>32.8±4.8</td>
<td>-62 (p&lt;0.01)</td>
</tr>
<tr>
<td>3. Control</td>
<td>6</td>
<td>28.0±4.8</td>
<td>12.5±2.4</td>
<td>29.5±2.5</td>
<td>-28 (NS)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>6</td>
<td>16.1±2.2</td>
<td>21.2±3.5</td>
<td>8.5±2.0</td>
<td>+28 (NS)</td>
</tr>
<tr>
<td>4. Control</td>
<td>6</td>
<td>45.0±7.8</td>
<td>21.2±3.5</td>
<td>10.9±2.0</td>
<td>+28 (NS)</td>
</tr>
<tr>
<td>Cholestyramine</td>
<td>6</td>
<td>37.8±6.0</td>
<td>21.2±3.5</td>
<td>8.5±2.0</td>
<td>+28 (NS)</td>
</tr>
<tr>
<td>5. Control</td>
<td>6</td>
<td>13.2±2.6</td>
<td>8.5±2.0</td>
<td>10.9±2.0</td>
<td>+28 (NS)</td>
</tr>
<tr>
<td>Cholesterol (6 weeks)</td>
<td>6</td>
<td>13.9±2.1</td>
<td>8.5±2.0</td>
<td>10.9±2.0</td>
<td>+28 (NS)</td>
</tr>
</tbody>
</table>

The data are given as the means±SEM; pmoles were calculated by assuming a molecular weight of 1.7 x 10^5 and 50% of protein content for HDL3.

Figure 3. Ligand blot of liver plasma membrane as described in Figure 2. The first six lanes show 125I-HDL3 binding to HB1 and HB2 from control animals, and the remainder are from simvastatin-treated rats.

Keeping with the reported up-regulation of HDL receptors in cholesterol-loaded cells, it is also consistent with an apparent, although weaker, up-regulation of HB2 activity that we observed after cholesterol loading of the liver when the sterol had been fed for 6 weeks. Although not reaching statistical significance, the 28% increase in liver HB2 activity may reflect an up-regulation of an HDL binding protein similar to that observed by Graham and Oram after cholesterol loading of various cell types in tissue culture. Thus, one mechanism controlling cholesterol efflux from the liver may be regulation through the HDL binding proteins, which in the case of cholesterol loading increases the input of sterol into plasma via HDL (after binding), a pathway possibly more important in the rat, in which HDL is the principal carrier of cholesterol. In the present study, the excessive cholesterol feeding could not be compensated for by the HDL acceptor route, which resulted in massive cholesterol loading of the liver. This does not, however, negate the role of HB1 and HB2 participation in HDL cholesterol homeostasis in rats fed normal diets.

Another possible explanation for a lowering of HB1 and HB2 content is that simvastatin or simvastatin together with cholestyramine may influence the intracellular content of a mediator, which has opposing effects on the expression of HB1, HB2, and HMG-CoA reductase. Such a mediator could be a putative nonsterol regulator of HMG-CoA reductase, which is suppressed after the administration of simvastatin. The demonstration of the HDL receptor suppression is consistent with other results recently obtained in our laboratory, which showed that lovastatin, when added to the medium of cultured rat adrenal-cortical cells, produced a marked lowering of HDL binding (unpublished observations). The demonstration that HB1 and HB2 are subject to regulation by simvastatin suggests that these proteins may function as part of a membrane complex that influences HDL metabolism.

Acknowledgments
The authors thank the National Heart Foundation of Australia for funds supporting this research and Merck, Sharp & Dohme for generous supplies of simvastatin.

References
1. Tozuka M, Fidge NH. Purification and characterization of two high density lipoprotein binding proteins from rat and human liver. Biochem J 1989;261:239-244


4. Chako GK. Human high density lipoprotein (HDL) binding to rat liver plasma membranes. Biochim Biophys Acta 1982;712:129–141

5. Glass CK, Pittman RC, Civen M, Steinberg D. \( 15 \)


15. Ha YC, Barter PJ. Rapid separation of plasma lipoproteins by gel permeation chromatography on agarose gel Superose 6B. J Chromatogr 1985;341:154–159


Index Terms: high density lipoprotein binding • liver • hypolipidemic drugs • cholesterol • rats
Regulation of hepatic high density lipoprotein binding proteins after administration of simvastatin and cholestyramine to rats.
D Mathai, N Fidge, M Tozuka and A Mitchell

doi: 10.1161/01.ATV.10.6.1045

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/10/6/1045

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/