EFFECT OF LIPOPROTEIN ON 3-HYDROXY-3-METHYLGLUTARYL COENZYME A (HMG CoA) REDUCTASE ACTIVITY IN RAT LIVER CELL CULTURE: SPECIAL SUPPRESSANT EFFECT OF A LIPOPROTEIN ISOLATED FROM HYPERCHOLESTEROLEMIC RAT PLASMA

Jan L. Breslow*†, Duane R. Spaulding*, David A. Lothrop* and Alexander W. Clowes**
Department of Pediatrics* and Pathology,** Harvard Medical School, Boston, Massachusetts
**Department of Surgery, Case Western Reserve University School of Medicine, Cleveland, Ohio

Received September 3, 1975

SUMMARY

A significant advance in cell culture methodology has permitted an analysis of the regulation of liver cell cholesterol metabolism by lipoproteins. The effect of rat lipoproteins on the activity of the rate limiting enzyme in cholesterol biosynthesis, HMG CoA reductase, was studied in monolayer cultures of adult rat hepatic parenchymal cells. Lipoproteins isolated from normocholesterolemic rat plasma, including low density lipoprotein, did not suppress HMG CoA reductase activity. However, enzyme activity was profoundly suppressed by a cholesterol rich $d < 1.063$ lipoprotein(s) isolated from hyperlipemic rat plasma. This lipoprotein may regulate the suppression of endogenous hepatic cholesterol biosynthesis which occurs after cholesterol feeding.

INTRODUCTION

The rate of cholesterol biosynthesis in rat liver is determined by the activity of the enzyme HMG CoA reductase (1). Diverse stimuli affect the level of this enzyme *in vivo*. For example, enzymatic activity is profoundly depressed by cholesterol feeding (2). The exact role of lipoproteins in these regulatory events is unknown. In this study, lipoproteins were isolated from normocholesterolemic and hypercholesterolemic rat plasma and their effects on HMG CoA reductase activity in monolayer cultures of adult rat hepatic parenchymal cells were examined.

MATERIALS AND METHODS

Lipoprotein Preparation

Rat lipoproteins were obtained from two groups of Sprague Dawley rats weighing about 300g. One group was maintained on standard Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and the other group on rat chow with added cholesterol (4%), cholic acid (2%), and propylthiouracil (0.5%), (Sigma Chemical Co., St. Louis, Mo.) for 3 months to induce hypercholesterolemia (3). After an overnight fast the rats were bled by cardiac puncture under diethyl ether anaesthesia. Blood from each set of rats was pooled, kept on ice, and anticoagulated with EDTA (1 mg/ml). Plasma obtained from blood centrifuged at 900g

†Address: Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Massachusetts 02115.

for 30 minutes was recentrifuged in a Beckman preparative ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.) at varying densities. Density adjustments were made with solid KBr according to standard techniques in order to float various lipoproteins (4). Plasma from rats fed on standard chow was spun sequentially at d 1.006, d 1.040, d 1.063, d 1.21. The supernatants were refloated through salt solutions of their own density, isolated and dialyzed extensively against buffer A (0.15 M NaCL and 0.3 mM EDTA (ph 7.4)). Lipoproteins of d < 1.006, d 1.006-1.040, d 1.063-1.21 were designated very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), respectively (5). The turbid hyperlipidemic plasma obtained from rats fed the cholesterol rich diet was spun at d 1.063. Under these conditions the turbid material floated and the isolated supernatant was refloated through a salt solution, d 1.063, reisolated, and dialyzed extensively against buffer A. This material was designated CLp. All lipoprotein fractions were passed through a Millipore filter (0.45 μ) and stored at 4°C.

LIVER CELL CULTURE

Nonproliferating monolayer cultures of adult rat liver parenchymal cells were generated by the Bissell technique from Sprague Dawley rats maintained on standard rat chow(6). 5 x 10^6 cells were plated in 60 mm plastic Petri dishes in 2.5 ml of L-15 medium (Microbiological Associates, Bethesda, Maryland), which contained 1 mM succinate. The cells were incubated at 37°C in air and a complete medium change was carried out after the first 24 hours of incubation. In the ensuing experiments this point is referred to as 0 time.

LIVER CELL HMG CoA REDUCTASE ACTIVITY

Liver cells were incubated either in 2.5 ml of medium or in 2.3 ml of medium plus lipoproteins in 0.2 ml of Buffer A for the times and at the lipoprotein protein concentrations indicated in the figure legends. Cell extracts were then prepared and analyzed for HMG CoA reductase activity as described for human fibroblasts by Brown (7). Twenty to 100 μg of cell extract protein was used for each assay. Preliminary experiments revealed that for these liver cell extracts the rate of conversion of substrate to product was proportional to protein concentration in this range. In addition, the conversion of substrate to product was linear for the 2 hours of the assay.

LIPID ANALYSES, LIPOPROTEIN ELECTROPHORESIS, AND PROTEIN ANALYSIS

Total cholesterol and triglyceride determinations were made on rat plasma and isolated lipoproteins using the Technicon AutoAnalyzer II method (Technicon Instruments Corporation, Tarrytown, New York) (8). Aliquots of whole serum and isolated lipoproteins were subjected to agarose electrophoresis using the Millipore Panagel Electrophoresis System (Millipore Biomedica, Acton, Mass.). Electrophoresis was performed at PH 8.6. The lipoproteins were stained with Sudan Black B (9). Protein analysis was by the method of Lowry (10).

RESULTS

HMG CoA REDUCTASE ACTIVITY IN LIVER CELL CULTURES

The level of liver cell HMG CoA reductase activity in the presence of medium was evaluated at 0 time and after 24, 48, 72 hours of incubation. HMG CoA reductase activity increased 4 fold by 48 hours and was then relatively stable through 72 hours of incubation.

CHARACTERIZATION OF LIPOPROTEINS

The plasma pool derived from rats fed standard rat chow had a cholesterol level of 78 mg% and a triglyceride level of 62 mg%. The lipoproteins isolated from this pool were designated VLDL, LDL, and HDL and on lipoprotein electro-

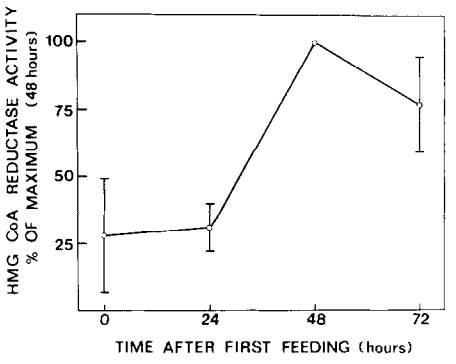

FIGURE 1

At 0 time (See Methods) the liver cells were incubated in 2.5 ml of medium for 0°, 24°, 48°, and 72°. Cell extracts were then prepared and analyzed for HMG CoA reductase activity. The data shown are a composite of 3 experiments each done in duplicate. In each experiment the maximum HMG CoA reductase activity was at 48 hours and in order to directly compare these 3 experiments the levels of enzyme activity at 0°, 24°, and 72° was expressed as a percent of this activity. The percent HMG CoA reductase activity at 48 is represented in the figure in the ordinate and the incubation time on the abscissa (hrs). The standard deviations are shown.

phoresis had pre-beta, beta and alpha mobility respectively with cholesterol/triglyceride (C/T) ratios of 0.06, 0.47, and 30, respectively. The plasma pool from rats fed a cholesterol-rich diet had a cholesterol level of 1140 mg% and a triglyceride level of 40 mg%. The isolated lipoprotein subclass $d < 1.063$ designated CLp migrated in the pre-beta region and had a C/TG ratio of at least 60 (Figure 2).

THE EFFECT OF LIPOPROTEINS ON RAT LIVER CELL HMG CoA REDUCTASE

Lipoproteins derived from either normo- or hypercholesterolemic rats were incubated with rat liver cultures for 24 hours and the HMG CoA reductase levels then measured. Lipoproteins derived from normocholesterolemic rats, including LDL, did not suppress rat liver cell HMG CoA activity. The VLDL and HDL from these same animals was actually able to stimulate enzyme activity (Fig. 3A).

On the other hand, the lipoprotein subclass $d < 1.063$, isolated from hypercholesterolemic rats suppressed HMG CoA reductase levels to 5% of control. (Fig. 3B). This effect was not due to a general suppression of hepatocyte metabolic activity since this lipoprotein subclass caused no decrease in cellular protein

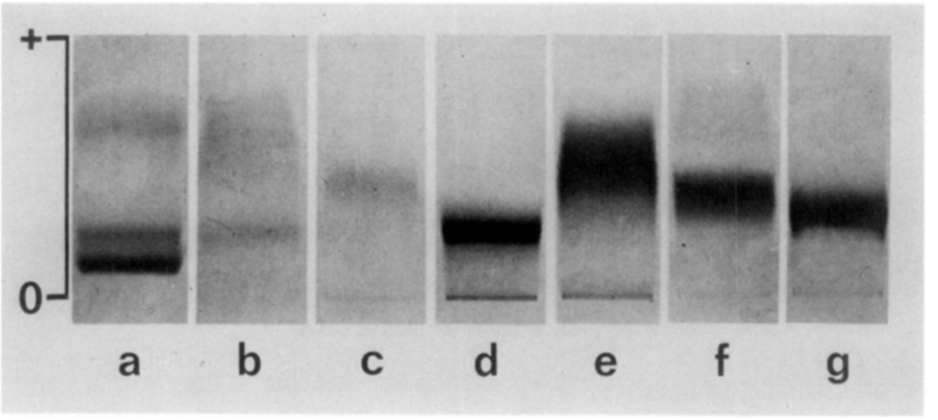

FIGURE 2

The lipoprotein electrophoresis of aliquots of whole serum and isolated lipoproteins on agarose are shown: a. normolipemic human plasma; normolipemic rat: b. plasma c. VLDL d. LDL e. HDL; hypercholesterolemic rat: f. plasma g. lipoprotein fraction d < 1.063.

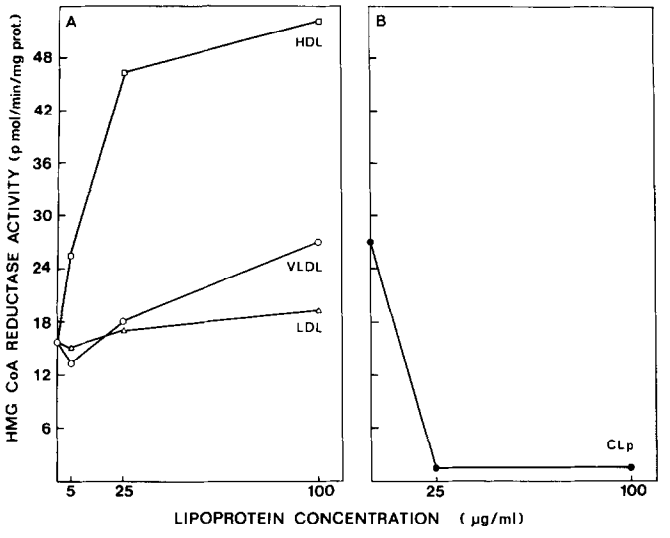

FIGURE 3

At 0 time (See Methods) the liver cells were incubated in 2.3 ml of medium for 48 hours. Then.2 ml of Buffer A containing the indicated concentration of lipoprotein protein were added for 24 hours. Cell extracts were then prepared and analyzed for HMG CoA reductase activity. The ordinate represents HMG CoA reductase activity (pmol/min/mg prot) and the concentration of lipoprotein protein is on the abscissa (µg/ml). Each experiment was done in duplicate and the mean result is shown. Duplicates were within 10% of each other. Fig. 3a. The cells were incubated with the lipoproteins derived from normolipemic rat plasma. Figure 3b. The cells were incubated with the d < 1.063 lipoprotein subclass isolated from hypercholesterolemic rat plasma. (CLp).

synthesis (as measured by incorporation of radiolabeled amino acids into TCA insoluble material) and actually stimulated cholesterol esterification (unpublished results).

DISCUSSION

Although the liver is considered to be the major site of cholesterol biosynthesis in the whole organism (11), most of the previous cell culture studies concerned with the regulation of cholesterol biosynthesis have used fibroblasts. In part, this was because no reliable system for maintaining hepatocytes in culture was yet available (12). Using a new liver cell culture technique developed by Bissell (6), this report shows that the regulation of cholesterol biosynthesis in rat hepatocytes differs from that seen previously in fibroblasts

Brown and Goldstein have shown that lipoproteins isolated from normocholesterolemic human plasma, LDL and VLDL but not HDL, could inhibit human fibroblast HMG CoA reductase activity (7). In addition, Bates and Rothblatt have found that human LDL depresses L cell fibroblast cholesterol biosynthesis (13). In the hepatocyte system lipoproteins isolated from normocholesterolemic rat plasma, including LDL, were unable to suppress HMG CoA reductase activity. However, the lipoprotein subclass $d < 1.063$ isolated from the plasma of rats fed a high cholesterol diet did suppress HMG CoA reductase activity. These differences in cholesterol biosynthesis regulation may reflect true differences between liver cells and fibroblasts. It is also possible that the differences seen were due to interspecies variation.

Brown and Goldstein have also found that in human fibroblasts LDL was bound by a high affinity receptor site and degraded by lysosomal enzymes (14). They also found that LDL stimulated fibroblast cholesterol esterification (15) and increased cellular cholesterol ester content (16). In preliminary experiments in the hepatocyte system referred to in this report, the cholesterol-rich particle produced by feeding rats a high cholesterol diet stimulated cholesterol esterification. It is possible that the cholesterol-rich particle acts on liver cells in a manner analogous to the action of LDL on fibroblasts.

In this report HDL stimulated hepatocyte HMG CoA reductase activity. Bates and Rothblatt have also observed a stimulation of cholesterol biosynthesis by HDL in L cell fibroblasts. In their system HDL promoted cholesterol efflux from the cell and this may have been involved in the mechanism whereby cellular cholesterol biosynthesis was increased (13).

The regulation of hepatocyte cholesterol biosynthesis by lipoproteins has been examined in systems other than the monolayer cell culture model. Edwards has studied single cell suspensions of rat hepatocytes in short term culture (17). He found that HMG CoA reductase levels were stimulated by normal or hypercholesterolemic human serum, normal or cholesterol fed rat serum, rat VLDL, LDL, and HDL. This nonspecific stimulation by all sera and lipoprotein fractions tested may have resulted because the cells were studied within 4 hours of preparation and were in a very unsteady state with regard to cholesterol biosynthesis. In the present study the liver cells were used at least 24 hours after preparation and probably had sufficiently recovered from the culture manipulations to better represent the *in vivo* situation.

In yet another system Nervi *et. al.* reported that chylomicrons isolated from the thoracic duct of cholesterol fed rats and then infused into other rats inhibited liver cholesterol biosynthesis and increased liver cholesteryl ester content (18). Even though the chylomicrons are triglyceride-rich these actions are similar to those of the cholesterol-rich particles studied here. The two sets of observations may be related. Chylomicrons after they leave the thoracic duct may be acted upon by lipases and converted to cholesterol-rich remnants (19). This indeed may be the true origin of the particles described in this paper. It is not clear from Nervi's *in vivo* work whether the chylomicrons themselves or remnants are the particles which ultimately interact with the liver cells. This current study provides evidence that a cholesterol-rich particle can interact directly with the liver cell to lower endogenous cholesterol biosynthesis.

The nature of the cholesterol-rich particle which inhibits liver cholest-

erol biosynthesis must be further defined. In preliminary experiments a high cholesterol ester to cholesterol ratio has been found. This particle may be analogous to the cholesterol ester-rich, arginine-rich lipoproteins described in the cholesterol fed rabbit (20), dog (21), and miniature swine (22) as well as in human Type III hyperlipoproteinemia (23).

ACKNOWLEDGEMENTS:

The authors are indebted to Doctors Samuel Lux, Harvey Colten, John Watkins, and James Epstein for their helpful suggestions in preparing the manuscript and Ms. Helen Hourihan for typing the manuscript. Miss Elizabeth Tuttle performed the cholesterol, triglyceride, and lipoprotein electrophoresis determinations. This work was supported by NIH Grant 1R0 1HL 15895 and the PHS Training Grant GM 01588.

REFERENCES:

1. Siperstein, M.D. (1970) Curr. Top. Cell. Regul, 2, 65-100.
2. Siperstein, M.D., and Fagan, V.M. (1966) J. Biol. Chem, 241, 602-609.
3. Page, I.H., and Brown, H.B. (1952) Circ, 6, 681-687.
4. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest, 34, 1345-1353.
5. Koga, S., Horwitz, D.L., and Scanu, A.M. (1969) J. Lipid Res, 10, 577-588.
6. Bissell, D.M., Hammaker, L.E., and Meyer, U.A. (1973) J. Cell Biol, 59, 722-734.
7. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1974) J. Biol. Chem, 249, 789-796.
8. Technicon AutoAnalyzer II, (1972), Simultaneous Cholesterol and Triglyceride Clinical Method No. 24. Technicon Instruments Corporation, Tarrytown, N.Y.
9. Procedures Manual for PANAGEL Electrophoresis System, (1975), Millipore Biomedica Corporation, Acton, Massachusetts.
10. Lowry, O.H., Rosebrough, N.J., Fark, A.L., and Randall, R.J. (1951) J. Biol. Chem, 193, 265-275.
11. Dietschy, J., and Wilson, J.D. (1970) N. Engl. J. Med, 282, 1128-1138, 1179-1183, 1241-1249.
12. Potter, V.R., (1972) Cancer Res. 32, 1998-2000.
13. Bates, S.R., and Rothblatt, G.H. (1974) Biochim. Biophys. Acta, 360, 38-55.
14. Goldstein, J.L., and Brown, M.S. (1974) J. Biol. Chem, 249, 5153-5162.
15. Goldstein, J.L., Dana, S.E., and Brown, M.S. (1974) Proc. Nat. Acad. Sci. USA, 4288-4292.
16. Brown, M.S., Faust, J.R., and Goldstein, J.L. (1975) J. Clin. Invest, 55, 783-793.
17. Edwards, P.A. (1974) Fed. Proc, 33, 1346.
18. Nervi, F.D., Weis, H.J., and Dietschy, J.M. (1975) J. Biol. Chem, 250, 4145-4151.
19. Redgrave, T.G. (1970) J. Clin. Invest, 49, 465-471.
20. Shore, V.G., Shore, B., and Hart, R.G. (1974) Biochem, 13, 1579-1585.
21. Mahley, R.W., Weisgraber, K.H., and Innerarity, T. (1974) Circ. Res, 35, 722-733.
22. Mahley, R.W., Weisgraber, K.H., Innerarity, T., Brewer, B., and Assmann, G. (1975) Biochem, 14, 2817-2823.
23. Havel, R.J., and Kane, J.P. (1973) Proc. Natl. Acad. Sci. USA, 70, 2015-2019.