Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 328-333

CHOLESTERYL ESTER HYDROLASE IN GALLBLADDER MUCOSA: EVIDENCE FOR THE PRESENCE OF AN INHIBITOR PROTEIN IN THE CYTOSOL

Dewey H. Neiderhiser

Medical Research Service, Cleveland VA Medical Center and the Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received December 24, 1981

SUMMARY: The presence of a cholesteryl ester hydrolase (EC 3.1.1.13) with optimal activity at pH 4.5 in the guinea pig gallbladder is described. The activity was enriched in the mitochondrial-lysosomal subcellular fraction. A protein present in the cytosol was found to competitively inhibit the activity of this enzyme.

INTRODUCTION: Cholesterosis of the gallbladder is characterized by the intramural accumulation of lipid, principally cholesteryl ester, in the gallbladder wall (1,2). One source of this cholesteryl ester is the bile within the gallbladder (3). In previous studies we reported that the guinea pig gallbladder mucosa absorbs cholesterol from bile (3) and then forms cholesteryl ester through a mechanism which utilizes the enzyme acylCoA: cholesterol acyltransferase (EC 2.3.1.26) (4). However, it is not clear what causes cholesteryl ester to accumulate in the gallbladder wall to result in cholesterosis. In the course of these experiments we looked for the presence of a cholesteryl ester hydrolase which would in turn degrade cholesteryl ester. No hydrolytic activity was observed with the neutral pH conditions used to assay for acyl CoA:cholesterol acyltransferase. But in keeping with reports that an acid cholesteryl ester hydrolase (EC 3.1.1.13) is present in tissues such as aorta (5) and intestinal mucosa (6), we here report evidence for the presence of this activity in the guinea pig gallbladder mucosa; in addition, we report the presence in the cytosol of a protein which competitively inhibits the activity of gallbladder acid cholesteryl ester hydrolase.

MATERIALS AND METHODS: Materials. Male guinea pigs (300-500g) were obtained from Wm Ky Cavies (Fern Creek, KY). The cholesteryl [1-^{14}C] oleate (50 to 60 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA), carrier cholesteryl oleate from Applied Science Labs (State College, PA) and sodium taurocholate from Calbiochem (La Jolla, CA). The purity of these compounds was confirmed by TLC using previously described methods (3). Chromatographically pure

0006-291X/82/050328-06$01.00/0

egg phosphatidylcholine was prepared as previously described (2). Porcine cholesterol esterase and snake venom phospholipase A_2 (Naja, naja) were obtained from Calbiochem.

Analytical Procedures. Previously described procedures were used for assay of protein (8), phospholipid phosphorus (9) and bile acids (3). Radioactivity was measured in a Beckman LS 8000 Spectrometer and the data are expressed as or calculated from disintegrations per min (9). Cholesterol was assayed by a modified Sperry-Webb micro-procedure (3). Cholesteryl ester and fatty acid were separated on silica gel G TLC plates with a solvent system containing petroleum ether (35-60°C)/diethyl ether/acetic acid (80:20:1, v/v/v) (3).

Preparation of Gallbladder Homogenate and Subcellular Fractions. Gallbladder was washed thoroughly in 0.25 M sucrose and the mucosa was removed by scraping with a microscope slide. Mucosa was homogenized in 0.25 M sucrose (1 ml per gallbladder). In a typical experiment, the mucosae from 4 gallbladders were pooled and 3 ml of the resulting homogenate were used for subcellular fractionation and the remaining 1 ml for enzyme assay of homogenate (8). The 970 g pellet (nuclei, cells and debris), 5,200 g pellet (mitochondrial-lysosomal fraction) 12,500 g pellet (light-mitochondrial fraction), 100,000 g pellet (microsomal fraction) and 100,000 g supernatant (cytosol fraction) were isolated as previously described (8). The 5,200 g pellet used as a source of cholesteryl ester hydrolase was washed once with 2 ml of 0.25 M sucrose using mild sonication to suspend the pellet. In most experiments, the fractions were assayed immediately, however, in some the 100,000 g supernatant and the 5,200 g pellet were frozen and stored at -20°C. We found that these fractions could be stored for up to 2 months without appreciable loss of activity. Freeze-thawing once did not change the enzyme activity. No measurable amount of bile acid could be detected in the homogenate or the subcellular fractions obtained from the homogenate.

Substrate Preparation. The substrates for cholesteryl ester hydrolase were prepared according to modification of the procedures of Brecker et al. (10) and Lundberg et al. (11). Appropriate amounts of egg phosphatidylcholine, cholesteryl [1-^{14}C]oleate and carrier cholesteryl oleate in ethanol were aliquoted and the ethanol evaporated with warming under nitrogen. The lipids were suspended in 12 ml 5 mM Tris, pH 7.39 containing 50 mM NaCl and the appropriate amount of sodium taurocholate. The suspension was first sonicated for 10 min at 45 to 51°C using a Branson B 75 Sonifier and then centrifuged for 10 min at 20,000 g to remove titanium fragments from the disruptor horn (5). All lipids were recovered in the supernatant fraction and no degradation as a result of sonication was observed.

Enzyme Assays. All assays used 0.5 ml of the appropriate substrate preparation, 0.1 ml of 2 M buffer (acetate, pH 4.5 or Tris, pH 7.0), 0.1 ml of the enzyme source and sufficient 0.25 M sucrose to make a final volume of 1.0 ml. When inhibitor materials were tested, they were added in the 0.25 M sucrose solution. The final incubation mixture contained cholesteryl [1-^{14}C]oleate (0.02 μCi, 2 to 40 μM), egg phosphatidyl-choline (5.6 μM) sodium taurocholate (500 μM) and sodium acetate (0.2 M, pH 4.5). For studies on neutral cholesteryl ester hydrolase, the mixture was identical except Tris (0.2 M, pH 7.0), phosphatidylcholine (5.6 mM) and sodium taurocholate (8.3 mM) were used to obtain optimal activity. All incubations were carried out at 37°C with shaking for 0 to 60 min. Incubations were terminated by addition of 5 ml chloroform/methanol (1:4, v/v) and then 1 μmole of carrier oleic acid and cholesteryl oleate were added. The lipids were extracted and radioassayed as previously described (8). The proportion of radioactivity in the oleic acid fraction was converted to pmol on the basis of the known amount of cholesteryl [1-^{14}C]oleate added to the incubation. The activity of the marker enzymes was determined by standard procedures (8). Phospholipase A_2 activity was assayed as previously described (12).

RESULTS: Table 1 presents the activity of acid cholesteryl ester hydrolase in the guinea pig gallbladder mucosa and in the subcellular fractions obtained from the homogenate. The activity of the enzyme was enriched in the 5,200 g pellet and the total recovery of the activity in this fraction was as much as 5 fold greater than that

Table 1. The Subcellular Distribution and Specific Activity of Cholesteryl Ester Hydrolase in Guinea Pig Gallbladder Mucosa.[a]

Fraction	Protein	Cholesteryl Ester Hydrolase	
	(%)[b]	Specific Activity (pmol/min/mg)	(%)[c]
Homogenate	100	25.8 ± 6.4	100
970 g pellet	29.6 ± 2.9	51.5 ± 16.0	39.2 ± 5.0
5,200 g pellet	6.8 ± 0.2	849.8 ± 149.7	268.7 ± 66.7
12,500 g pellet	8.9 ± 1.1	105.5 ± 34.4	29.3 ± 7.1
100,000 g pellet	9.7 ± 0.7	50.3 ± 21.2	15.2 ± 7.4
Supernatant	46.5 ± 2.2	8.5 ± 5.9	7.0 ± 4.1
Total Recovery	93.4 ± 6.4		359.3 ± 58.4

[a]Values Represent means ± SEM, n=4.

[b]Percent of protein recovered in subcellular fractions is based on 100% in the homogenate.

[c]Percent of enzyme activity in subcellular fractions is based on 100% activity in the homogenate.

observed in the homogenate. Whereas, 44 and 33% respectively of our mitochondrial (succinate dehydrogenase) and lysosomal (acid phosphatase) marker enzymes were found in this fraction, we recovered 164 to 464% of the acid cholesteryl ester hydrolase (Table 1). Some activity was found in the 970 g and 12,500 g pellets while minimal activity was associated with the 100,000 g pellet and the cytosol fraction.

Hydrolysis of cholesteryl oleate by the 5,200 g precipitate was linear with time through 60 min ($r = 0.992$) and with protein ($r = 0.997$) (Fig. 1). Studies on varying the concentration of phosphatidylcholine and bile salt showed that the conditions used by this assay were optimal for the activity in the 5,200 g pellet and further that the activity in the homogenate was not increased or decreased by addition of bile salts (up to 5 mM) or phosphatidylcholine (up to 1 mM). The activity of this enzyme was optimal at pH 4.5 and minimal at pH 3.5 & 7.5.

Experiments were performed to determine the reason for the latent activity in the homogenate. Addition of the cytosol fraction to the 5,200 g pellet resulted in a competitive inhibition of the enzyme activity (Fig. 2). The K_m for cholesteryl ester hydrolase alone was 6.7 μM. Addition of 30 μg cytosol protein gave a K_i of 17.7 μM.

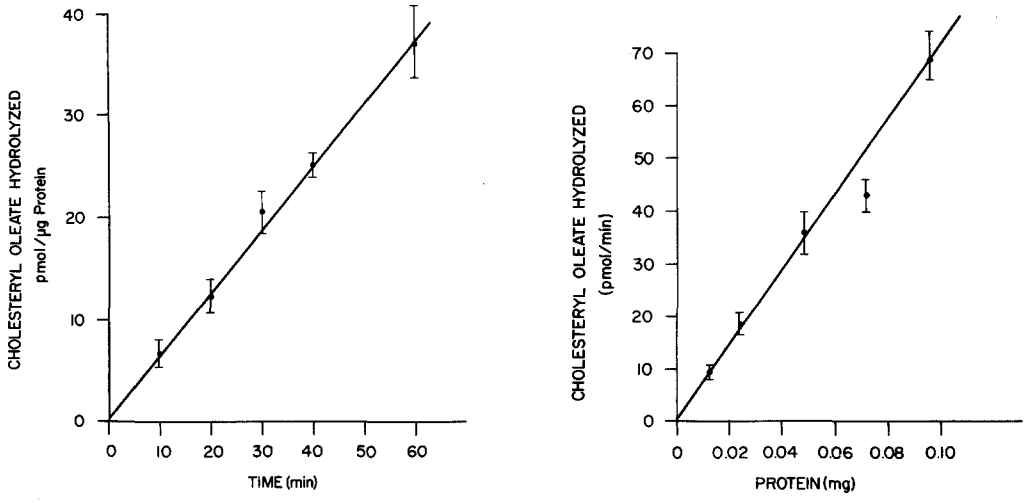

Figure 1. Properties of cholesteryl ester hydrolase in gallbladder mucosa. The time course of the reaction is shown on the left; each incubation mixture contained 34 µg of mitochondrial-lysosomal protein. Dependence on protein is shown on the right; incubations were performed for 20 min. In both cases, the incubation mixture contained cholesteryl [$1^{-14}C$] oleate (0.02 µCi, 20 µM), egg phosphatidylcholine (5.6 µM), sodium taurocholate (500 µM) and sodium acetate (0.2 M, pH 4.5), in a total volume of 1.0 ml and incubations were carried out at 37°C.

Addition of (up to 100 µg) microsomal protein from the gallbladder or fatty acid free bovine serum albumin, bile salts (up to 5 mM), phosphatidylcholine (up to 1 mM) or EDTA (20 mM) did not inhibit activity in the 5,200 g pellet. The inhibition was not removed from the cytosol by dialysis or by heating for 10 min at 60°C. Heating at 100°C for 10 min at pH 7.2 resulted in precipitation of 32% of the protein in the cytosol

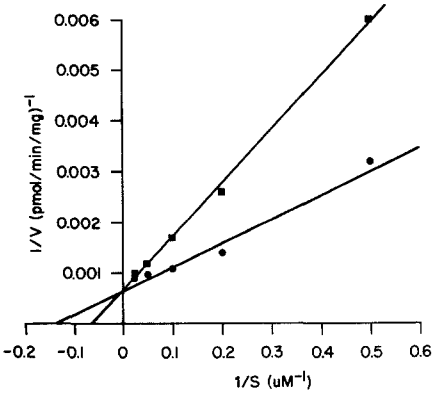

Figure 2. Lineweaver-Burk plot of cholesteryl ester hydrolase in the presence of cytosol fraction. —●—No addition, —■—In presence of cytosol fraction, 30 µg protein.

and a 32% loss in the inhibition. Heating at 100°C for 10 min at pH 5.0 precipitated most of the protein and destroyed the inhibition by the cytosol.

We tested the ability of the cytosol protein from the guinea pig gallbladder to inhibit the activity of pancreatic neutral cholesteryl ester hydrolase and snake venom phospholipase A_2. Using the identical substrate concentrations as in the above experiments, no inhibition of neutral cholesteryl ester hydrolase was observed. Similar results were found with snake venom phospholipase A_2.

We have isolated and partially purified a protein from the cytosol of the guinea pig gallbladder which inhibits the activity of acid cholesteryl ester hydrolase. After chromatography on Sephadex G-75, the inhibition was found to be associated with a protein fraction of molecular weight between 20,000 and 50,000. The inhibition appears to be associated with a single protein band in this fraction which was isolated by polyacrylamide gel electrophoresis (6.5%, pH 8.9) and eluted from the gel with 1.0 M NaCl in 0.1 M Tris, pH 7.0.

DISCUSSION: Although a cholesteryl ester hydrolase with optimal activity in acid pH has been reported to be in various tissues, this is the first report to our knowledge on the presence of this activity in the gallbladder mucosa. We found that the activity of this enzyme was low in the homogenate but was enriched in the 5,200 g subcellular fraction. Since this enzyme activity has been associated with the lysosomal fraction of other tissues, and since the optimal activity was at pH 4.5, these data suggest that this activity is associated with the lysosomes of gallbladder mucosa. We also found that the activity of cholesteryl ester hydrolase was competitively inhibited by the cytosol and that this inhibition was associated with a protein in this fraction.

The inhibitory protein has properties which are similar to a protein recently reported by Kondo et al. (13) which inhibits the activity of δ-aminolevulinate dehydratase in bone marrow cells and one reported by Kirate et al. (14) which suppressed the activity of phospholipase A_2 in rabbit neutrophils. However, the first authors reported that a noncompetitive inhibition of their enzyme occurred with their inhibitory protein whereas the protein from the gallbladder appears to act competitively. Our protein appears to be specific for acid cholesteryl ester hydrolase and does not inhibit

the activity of phospholipase A_2 from snake venom or the neutral cholesteryl ester hydrolase in porcine pancreas.

Feeding guinea pigs a diet high in cholesterol results in a marked elevation in the level of cholesterol, both esterified and unesterified, in tissues and blood (15). Cholesterol is toxic to the guinea pig and leads to hemolytic anemia and eventually death (16). The mechanism of this toxicity in the guinea pig is not known; however, the increased infiltration of cholesterol and cholesteryl ester in liver and other tissues probably contributes. The results from our study on an inhibitory protein present an intriguing possibility that an inhibition of the hydrolysis of cholesteryl ester to cholesterol, an important and obligatory step in conversion of cholesteryl ester to its metabolites, could possibly play a role in cholesteryl ester accumulation as occurs in cholesterosis of the gallbladder.

ACKNOWLEDGEMENTS: The author acknowledges the technical assistance of Mr. J. Stager. This research was supported by funds from the Medical Research Service of the Veterans Administration.

REFERENCES:

1. Salmenkivi, K. (1964) Acta Chir. Scand. Supp. 324, 1-93.
2. Holtzbach, R.T., Marsh, M., and Tang, P. (1977) Exp. Mol. Path. 27, 324-338.
3. Neiderhiser, M.D., Harmon, C.K., and Roth, H.P. (1976) J. Lipid Res. 17, 117-124.
4. Neiderhiser, D.H. (1981) Lipids in press.
5. Haley, N.J., Fowler, S., and deDuve, C. (1980) J. Lipid Res. 21, 961-969.
6. Murthy, S.K., and Ganguly, J. (1962) Biochem. J. 83, 460-469.
7. Neiderhiser, D.H., Roth, H.P., and Webster, L.T. (1966) J. Lab. Clin. Med. 68, 90-102.
8. Harmon, C.K., and Neiderhiser, D.H. (1978) Biochim. Biophys. Acta 530, 217-226.
9. Neiderhiser, D.H., Morningstar, W.A., and Roth, H.P. (1973) J. Lab. Clin. Med. 82, 891-897.
10. Brecker, P., Pyun, H.Y., and Chobanian, A.V. (1977) J. Lipid Res. 18, 154-163.
11. Lundberg, B., Klemets, R., and Lovgren, T. (1979) Biochim. Biophys. Acta 572, 492-501.
12. Neiderhiser, D.H., and Roth, H.P. (1970) Gastroenteology 58, 26-31.
13. Kondo, M., Kajimoto, M., Kimura, H., Suzuki, T., Sasaki, A., Niwa, M., and Urata, G. (1981) Arch. Biochem. Biophys. 208, 189-194.
14. Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1980) Proc. Natl. Acad. Sci. 77, 2533-2536.
15. Beher, W.T., Baker, G.D., and Penney, D.G. (1963) J. Nut. 79, 523-530.
16. Hansma, H., and Ostwald, R. (1974) Lipids 9, 731-737.