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CHOLESTEROL ESTER HYDROLASE IN HUMAN RED BLOOD CELLS

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Summary

1. Cholesterol ester hydrolytic activity (sterol-ester hydrolase EC 3.1.1.13) was detected in human red blood cells. Enzyme activity appeared confined to the cell membrane and was most marked in washed preparations of red cell ghosts.

2. Hydrolytic activity was stimulated by the anti-oxidants D-α-tocopherol and butylated hydroxytoluene. Marked inhibition was produced by erythrocyte hemolysate, sodium taurocholate, and Triton X-100.

3. Optimal pH for the reaction was 5.4—5.7.

4. Because red cell cholesterol is all unesterified, it is speculated that the hydrolase serves to maintain the erythrocyte membrane free of esterified cholesterol.

Introduction

Cholesterol ester hydrolytic activity has been reported in a variety of tissues including liver, intestine, pancreas, adrenal, adipose tissue, brain, aorta, and placenta [1—6]. In the present study we document a cholesterol ester hydrolase in human red blood cell membranes and report some of its characteristics. The presence of such an enzyme is of particular interest because red cells contain cholesterol only in the unesterified form [7].

Materials and Methods

Chemicals

[7-^{3}H]Cholesterol (spec. act. 10 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., U.S.A.; cholesterol, oleoyl chloride and choles-

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terol oleate standard from Nu Chek Prep, Elysian, Minn., U.S.A.; silicic acid, Triton X-100, sodium taurocholate and cellulose phosphate from Sigma Chemical Co., St. Louis, Mo., U.S.A.; butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol) from J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.; D-α-tocopherol from Eastman Kodak Co., Rochester, N.Y., U.S.A.; Silicia gel G from E. Merck, Darmstadt, Germany; and Sephadex G-25 (medium) from Pharmacia, Uppsala, Sweden. All solvents were obtained from Fisher Chemical Co., Fairlawn, N.J., U.S.A., and were reagent grade. Benzene was redistilled before use.

Synthesis of [7-^{3}H] cholesterol oleate

Labelled ester was synthesized by the procedure of Deykin and Goodman [8]. The reaction mixture contained 250 μCi [7-^{3}H] cholesterol, 3 mg cholesterol and 20 μl oleoyl chloride in 0.3 ml dry pyridine. Incubation was carried out under nitrogen at 55°C for 4 h, then the mixture was extracted with petroleum ether and washed serially with 0.5 M HCl, 0.1 M NaOH in 50% ethanol, and water. The cholesterol ester was isolated by silicic acid column chromatography [9], pooled, taken to dryness in nitrogen and dissolved in benzene. It had a specific activity of 28—30 μCi/μmol and was stable for at least one month when stored as a benzene solution in the dark at 8°C. Co-chromatography with a mixture of standards on $AgNO_3$-impregnated Silica gel G plates [10] (solvent: benzene/hexane, 60 : 40, v/v) proved that over 97% of the total radioactivity was present as monounsaturated cholesterol ester.

Isolation of red blood cells

Venous blood from normal individuals fasted overnight was drawn into heparinized tubes and the red cells isolated by a modification of the column chromatographic method of Nakao et al. [11]. Sephadex G-25 (3 g) was allowed to swell overnight in the elution buffer (0.138 M NaCl, 0.005 M $MgCl_2$, 0.01 M potassium phosphate buffer, pH 7.5, and 10 units heparin/ml) and was then mixed with 0.5 g cellulose phosphate. The mixture was transferred to a 22 mm column and washed with 20 ml elution buffer. Fresh heparinised blood (3.0 ml) was mixed with an equal volume of elution buffer and transferred onto the column, followed by another 20 ml buffer to elute the red cells. The elution step took about 45 min at room temperature.

Eluate was collected in a nitrocellulose tube and centrifuged at 2000 × *g* for 10 min at 4°C. The supernatant was discarded and the packed erythrocytes washed twice with 20 ml isotonic potassium phosphate buffer (310 mOsm) [12], pH 7.4, then resuspended in fresh buffer to a volume of 3.0 ml. There was no visible hemolysis either in the initial suspension collected from the column or in the subsequent washes.

Red and white cell counts were determined by a Coulter counter, platelet counts by hemacytometer, and reticulocyte counts by examination of blood smears stained with new methylene blue [13].

Preparation of red cell suspension and whole hemolysate for assay

After the cell counts, 0.8 ml washed red cell suspension was centrifuged and the buffer discarded. Hemolysing buffer, 20 mOsm sodium phosphate buffer [12], pH 7.4, was added to the packed cells to make 2.0 ml total

volume. This was used as whole hemolysate in the appropriate studies. When intact erythrocyte suspension was required, the hemolysing buffer was replaced by isotonic buffer of the same pH.

Red cell ghost preparation

Red cell ghosts were prepared as described by Dodge et al. [12]. Washed erythrocyte suspension, 1.0 ml, was added to 14 ml hemolysing buffer. The mixture was stirred at room temperature for 2 min and centrifuged at 20 000 × *g* for 40 min at 4—8 °C, then the supernatant was carefully decanted and the ghost button resuspended in fresh hemolysing buffer to 15 ml. The centrifugation and decantation steps were repeated to obtain the first washed ghost button; second and third washes were carried out in the same manner. The required ghost button was resuspended in isotonic sodium phosphate buffer, pH 7.4, to 2.5 ml. Suspensions were stored at 4 °C and assayed for cholesterol ester hydrolytic activity within 12 h.

Assay procedure

The standard assay mixture contained 0.9 ml 0.1 M citrate/0.2 M sodium phosphate buffer, pH 5.4, and 0.1 ml enzyme source: either red cell suspension, whole hemolysate, initial ghost preparation, or first, second or third washed ghost preparation. Incubations contained about $1.5 \cdot 10^8$ erythrocytes or their equivalent of whole hemolysate or ghosts. The mixture was kept in ice and [7-^{3}H]cholesterol oleate, 0.20—0.23 μCi in 0.03 ml acetone, was added by means of a Hamilton syringe and the mixture immediately stirred by a vortex mixer. Incubations were carried out in a shaking water bath at 37 °C for one hour, then terminated by the addition of chloroform/methanol (2 : 1, v/v).

In experiments involving D-α-tocopherol or butylated hydroxytoluene, an appropriate amount was dissolved in the acetone solution of [^{3}H]cholesterol ester. Sodium taurocholate or Triton X-100 when required were added to the 0.9 ml citrate/phosphate buffer.

Analytical procedure

Lipids were extracted from incubation mixtures by the method of Folch et al. [14] fractionated by thin-layer chromatography on Silica gel G (solvent: petroleum ether/diethyl ether/acetic acid, 90 : 15 : 1.5, v/v), and visualized by iodine vapour. Fractions corresponding to cholesterol and cholesterol ester were scraped into scintillation vials containing 10 ml scintillation fluid (4 mg PPO and 50 μg POPOP per ml toluene). Counting was done in a Packard Tri Carb liquid scintillation spectrometer, Model 2425.

Results

Elution of heparinised blood through cellulose phosphate supported by a Sephadex G-25 matrix resulted in the quantitative removal of white cells and platelets. An average of 91% of the red cells were recovered, with essentially the same proportion of reticulocytes as in the initial whole blood (Table I). No platelets could be detected in the eluate and the column retained an average of 92% of the white blood cells. Thus contamination of the eluted red cell frac-

TABLE I

ISOLATION OF RED BLOOD CELLS BY COLUMN CHROMATOGRAPHY

Figures represent mean values ± S.D. of 9 experiments.

	Whole blood	After column
White cells/red cells	1/(767 ± 181)	1/(10088 ± 5674)
% white cells removed	—	92 ± 4
% red cells recovered	—	91 ± 5
% reticulocytes	1.1 ± 0.5	1.0 ± 0.3

tion ranged from 1 white cell per 6000 erythrocytes to as low as 1 per 21 800 erythrocytes.

As shown by a representative experiment in Table II, red cell ghosts exhibited cholesterol ester hydrolytic activity. Moreover under the standard assay conditions hydrolytic activity rose markedly as the ghost preparations were progressively washed free of contaminating hemolysate. Addition of D-α-tocopherol at a concentration of 5 μg/ml strikingly stimulated hydrolysis. Maximum stimulation was usually produced even with unwashed ghosts, and washing of the ghost preparations generally did not further enhance tocopherol-stimulated activity.

In contrast to erythrocyte ghosts, intact red cells showed only minimal hydrolytic activity under standard assay conditions (Table II). Nevertheless the addition of α-tocopherol strongly stimulated the reaction, though not to the same level as in ghosts. The experiments with intact cells, however, were complicated by the fact that visible hemolysis was noted in the incubations. Whole hemolysate itself contained insignificant hydrolytic activity and this was unaffected by the addition of α-tocopherol (Table II).

The possibility that contaminating white blood cells were responsible for these results was examined by assaying hydrolytic activity in buffy coat preparations freed of red cells by hypotonic lysis [15]. Under the standard assay

TABLE II

CHOLESTEROL ESTER HYDROLYTIC ACTIVITY IN INTACT RED BLOOD CELLS, WHOLE HEMOLYSATE, AND RED CELL GHOSTS

A representative experiment with standard assay conditions as described under Methods. Each incubation contained either $1.44 \cdot 10^8$ red cells, whole hemolysate from the same number of cells, or an equal number of erythrocyte ghosts. Values are the mean of triplicate determinations.

Enzyme source	% Cholesterol ester hydrolyzed	
	Standard	α-Tocopherol*
Intact red cells	1.4	14.2
Whole hemolysate	1.2	1.4
Unwashed ghosts	3.5	25.0
1st washed ghosts	8.8	27.2
2nd washed ghosts	14.6	26.1
3rd washed ghosts	16.4	26.7

* 5.0 μg D-α-tocopherol added to the 1.0 ml incubation mixture.

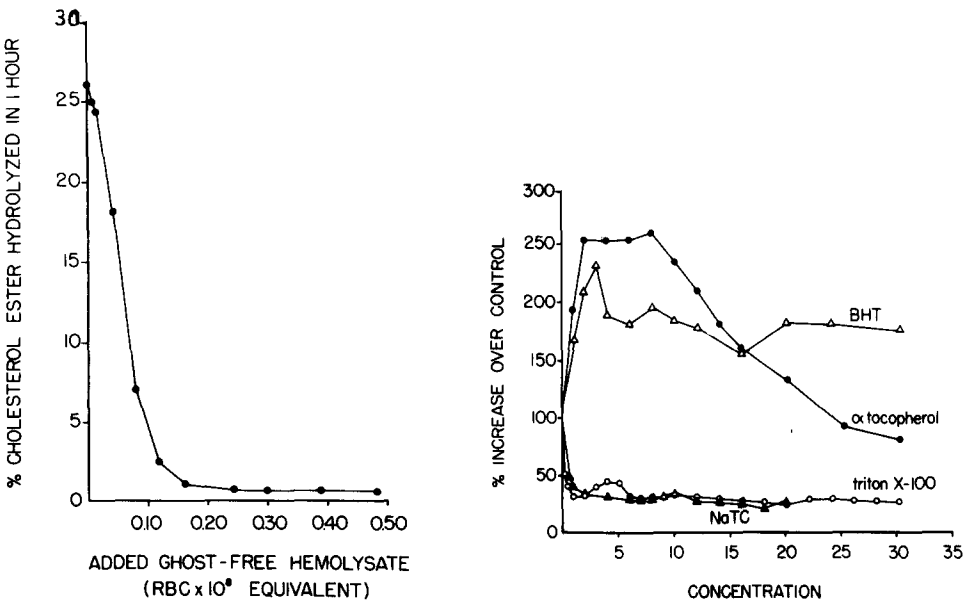

Fig. 1. Inhibition of red cell cholesterol ester hydrolytic activity by hemolysate. Second washed erythrocyte ghosts equivalent to $1.44 \cdot 10^8$ red cells were used as the source of enzyme in each incubation. Ghost-free hemolysate was obtained from the supernatant fraction during preparation of the initial ghost button. Standard assay conditions were used as described under Methods, with the addition of 5 μg D-α-tocopherol to each incubation.

Fig. 2. Effect of D-α-tocopherol, butylated hydroxytoluene (BHT), Triton X-100, and sodium taurocholate (NaTC) on red cell cholesterol ester hydrolytic activity. Standard assay conditions were used as described under Methods, with second washed red cell ghosts as the source of enzyme. Indicated concentrations on the horizontal axis: ●———●, α-tocopherol, μg/ml; △———△, BHT, μg/ml; ○———○, Triton X-100, % × 10^{-2}; and ▲———▲, NaTC, mM.

conditions described above, the number of white cells present in our erythrocyte preparations could hydrolyze only about 0.04% of the ^{3}H-labeled ester. Hydrolytic activity in platelets was not directly measured, but as noted above the red cell preparations contained virtually no platelets. Thus contamination by other formed elements of the blood could not account for the findings.

The above results suggested that hydrolytic activity in the red cell membrane was being inhibited by the products of hemolysis. To test this further, washed red cell ghosts were incubated with increasing amounts of ghost-free hemolysate. As shown in Fig. 1, ghost hydrolytic activity was indeed strikingly inhibited by small amounts of hemolysate; virtually total inhibition was produced by hemolysate derived from one-tenth the number of red cells as were present in the incubation mixture.

Fig. 2 shows the effect of several compounds on the hydrolysis of cholesterol oleate using second washed ghost preparations as the source of activity. The presence of α-tocopherol in the incubation mixture again stimulated hydrolysis of cholesterol ester. Optimal concentrations were 2—8 μg/ml, and at higher concentrations the stimulatory effect gradually diminished. Butylated hydroxytoluene was slightly less active than α-tocopherol at comparable concentrations. Maximum stimulation was found at 3 μg/ml but unlike D-α-

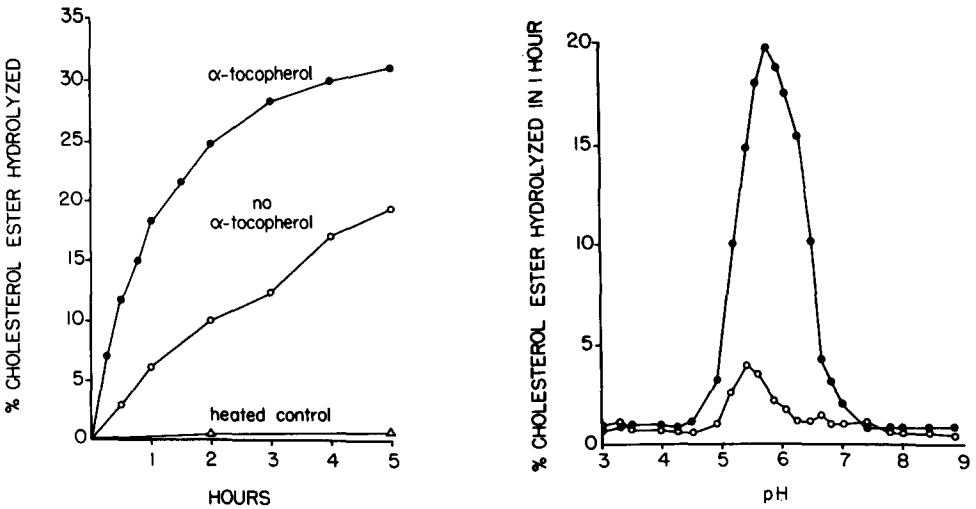

Fig. 3. Time course of cholesterol oleate hydrolysis by red cell ghosts. Standard assay conditions were used as described under Methods, with second washed ghosts equivalent to $1.80 \cdot 10^8$ erythrocytes in each incubation. ●———●, 5 μg D-α-tocopherol added to the incubation mixture; ○———○, no added α-tocopherol; △———△, 5 μg α-tocopherol added but ghost suspension preheated at 60 °C for 30 min.

Fig. 4. Effect of pH on red cell cholesterol ester hydrolytic activity. Second washed ghosts equivalent to $1.40 \cdot 10^8$ erythrocytes were used as the source of enzyme, with standard assay conditions as described under Methods. ●———●, 5 μg D-α-tocopherol added to the incubation mixture; ○———○, no α-tocopherol added.

tocopherol, the stimulatory effect was maintained at higher concentrations. Both Triton X-100 and sodium taurocholate were markedly inhibitory in small amounts. The inhibition was never complete, however, since even at high concentrations hydrolysis of ester remained at about 25% of the original level.

A comparison of the time course of [^{3}H]cholesterol oleate hydrolysis in the presence and absence of α-tocopherol (5 μg/ml) is shown in Fig. 3. Note that heating of the ghost preparation at 60 °C for 30 min completely abolished its hydrolytic activity.

Fig. 4 shows the effect of pH on the hydrolytic activity using second washed ghost preparations as the source of enzyme. Under standard assay conditions the optimal pH was 5.4, but in the presence of α-tocopherol it shifted to 5.7. A similar shift in optimal pH was observed in other experiments. When pH measurements were done before and after 1 h of incubation, however, it was observed that in the presence of α-tocopherol there was a decrease of 0.1 to 0.2 pH units. Thus the apparent shift in optimal pH may actually have reflected a slight pH change in mixtures containing tocopherol.

Discussion

These studies document the presence of cholesterol ester hydrolytic activity in human erythrocyte membranes. Only sparse information on this point was previously available. Guardamagna et al. [16] reported a cholesterol esterase in human erythrocytes, but their red cell preparations contained a high proportion of esterified cholesterol and therefore must have been contaminated

with plasma or other formed elements of the blood, since red cells lack esterified cholesterol [7]. In another study, Rotenberg and Misniakiewicz [17] claimed that pig red cell hemolysate was able to hydrolyze serum cholesterol ester, but neither intact red cells nor erythrocyte ghosts were studied.

In the present studies hydrolytic activity appeared confined to the red cell membrane, and there was considerable evidence for inhibition by products of hemolysis. Thus activity was highest in erythrocyte ghost preparations, and a progressive increase in activity occurred as the ghosts were washed free of contaminating hemolysate. Furthermore hydrolytic activity fell dramatically when small amounts of cell-free hemolysate were added to the incubation mixtures. For this reason the results with intact erythrocytes (Table II) should be interpreted with caution, as visible hemolysis was noted in incubations containing whole cells. Whether cholesterol ester hydrolytic activity in intact red cells equals, exceeds, or is less than that of isolated erythrocyte membranes therefore cannot validly be determined from these studies.

We assessed the influence of anti-oxidants on the reaction because hemoglobin and other hematin compounds are known to be powerful catalysts of lipid peroxidation [18], which impairs membrane function [19], and this effect can be prevented by the natural anti-oxidant D-α-tocopherol [20]. The results provide circumstantial evidence that hemolysate may exert its inhibitory action via a peroxidative mechanism. Thus in the presence of α-tocopherol, activity in unwashed ghost preparations increased and was not further enhanced by repeated washings. This effect was produced by concentrations of α-tocopherol (5 μg/ml) which are physiologic in normal adult plasma [21]. Activity in intact erythrocytes was also much higher in the presence of tocopherol. In addition, the synthetic anti-oxidant butylated hydroxytoluene also stimulated the reaction (Fig. 2). Bunyan et al. [20], in their study of the role of tocopherol in preventing red cell hemolysis, observed enhanced peroxide formation in dialuric acid-treated red cell stroma when hemolysate was present, and suggested a catalytic role for hemoglobin. We did not directly assess lipoperoxidation in our incubations, however, and the mechanisms by which hemolysate and anti-oxidants respectively impair and stimulate enzyme activity require further study.

Activity of the enzyme against different substrates also deserves future evaluation. We chose cholesterol oleate as substrate for these experiments because it is the principal monounsaturated ester of human plasma [1], but comparative assays with other important plasma cholesterol esters was not done. Whether the enzyme activity is specific for esters of cholesterol also requires study.

Hydrolytic activity occurred over a relatively narrow pH range (Fig. 4). The observed pH optimum of 5.4 under standard assay conditions shifted to 5.7 in the presence of α-tocopherol. Incubation mixtures containing tocopherol demonstrated a slight drop in pH during incubation, however, so the change in optimal pH may have been more apparent than real. This fall in pH was most likely due to the greater amount of free fatty acid released by the tocopherol-stimulated reaction, although this premise was not directly examined.

Finally, the physiologic significance of erythrocyte cholesterol ester hydrolase remains to be clarified. Free cholesterol and phospholipids are impor-

tant components of the red cell membrane, and there is extensive evidence that abnormalities of erythrocyte shape and physiologic behavior are produced by alterations in the membrane lipids [7,22]. Red cell cholesterol freely exchanges with cholesterol in plasma lipoproteins yet no cholesterol ester is present in erythrocyte membranes despite the fact that two-thirds of the plasma cholesterol is esterified [7]. Indeed the red cell is unique in that none of its cholesterol exists in ester form. Conceivably the hydrolase described herein could be responsible for maintaining the erythrocyte membrane free of esters from the surrounding plasma. If so, this newly documented enzyme would be of major physiologic significance in man.

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References

1 Goodman, D.S. (1965) Physiol. Rev. 45, 747—839
2 Stokke, K.T. (1972) Biochim. Biophys. Acta 270, 156—166
3 Arnaud, J. and Boyer, J. (1974) Biochim. Biophys. Acta 337, 165—168
4 Eto, Y. and Suzuki, K. (1973) J. Biol. Chem. 248, 1986—1991
5 Kothari, H.V., Miller, B.F. and Kritchevsky, D. (1973) Biochim. Biophys. Acta 296, 446—454
6 Chen, L. and Morin, R. (1971) Biochim. Biophys. Acta 231, 194—197
7 Cooper, R.A. (1970) Semin. Hematol. 7, 296—322
8 Deykin, D. and Goodman, D.S. (1962) J. Biol. Chem. 237, 3649—3656
9 Horning, M.G., Williams, E.A. and Horning, E.C. (1960) J. Lipid Res. 1, 482—485
10 Morris, L.J. (1963) J. Lipid Res. 4, 357—359
11 Nakao, M., Nakayama, T. and Kankura, T. (1973) Nature New Biol. 246, 94
12 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
13 Dacie, J.V. and Lewis, S.M. (1966) Practical Haematology, 3rd edn, J. and A. Churchill Ltd, London
14 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
15 Fallon, H.J., Frei, E., Davidson, J.D., Trier, J.S. and Burk, D. (1962) J. Lab. Clin. Med. 59, 779—791
16 Guardamagna, C., Massari, N. and Santambrogio, C. (1960) Giorn. Gerontol. 8, 161—166
17 Rotenberg, S. and Misniakiewicz, A. (1969) Acta Physiol. Polon. 20, 606—612
18 Tappel, A.L. (1953) Arch. Biochem. Biophys. 44, 378—395
19 Tappel, A.L. (1973) Fed. Proc. 32, 1870—1874
20 Bunyan, J., Green, J., Edwin, E.E. and Diplock, A.T. (1960) Biochem. J. 77, 47—51
21 Chieffi, M. and Kirk, J.E. (1951) J. Gerontol. 6, 17—19
22 Cooper, R.A., Diloy-Puray, M., Lando, P. and Greenberg, M.S. (1972) J. Clin. Invest. 51, 3182—3192