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Hydrolysis of Cholesterol Ester in Artificial Lipid Mixtures by Cholesterol Esterase Released from Particulate Fractions of Rat Arterial Wall

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SHIRAI, K., SHINOMIYA, M., MATSUOKA, N., SAITO, Y. and YOSHIDA, S. *Hydrolysis of Cholesterol Ester in Artificial Lipid Mixtures by Cholesterol Esterase Released from Particulate Fractions of Rat Arterial Wall.* Tohoku J. exp. Med., 1984, **143**(2), 171–176 —— Acid and neutral cholesterol esterase activities in rat arterial wall were released from the lysosomal fraction and microsomal fraction respectively into the 105,000 ×g supernatant fraction by treatment with Triton X-100, heparin and dextran sulfate. The percentage releases of acid cholesterol esterase by Triton X-100 (0.1%), heparin (50 μg/ml) and dextran sulfate (1 mg/ml) were 21%, 18% and 4%, respectively, while those of neutral cholesterol esterase were 66%, 56% and 39%, respectively. The cholesterol esterase released by dextran sulfate, especially that from the microsomal fraction, hydrolyzed cholesterol ester in artificial lipid mixtures with similar lipid compositions to those of the deposits in fatty streaks and fibrous plaques of atheromatous lesions. ———— arterial wall; acid cholesterol esterase; neutral cholesterol esterase; artificial fibrous plaque; atheroma

A major lipid accumulated in atheromatous lesions is cholesterol ester, which forms round crystalline deposits containing other lipids such as triglyceride, free cholesterol and phospholipids (Hata et al. 1974). The contents of these components differ in different stages of atherosclerosis, such as fatty streaks and fibrous plaques (Hata 1975). For reduction of deposited cholesterol ester, the cholesterol ester has to be hydrolyzed and carried away by high density lipoproteins (Tall and Small 1980), and the possibility that this actually occurs is supported by the existence of cholesterol ester hydrolases in the arterial wall (Brecher et al. 1977; Shinomiya et al. 1979). In the rat arterial wall, cholesterol esterase hydrolyzing activity has two pH optima, pH 4.5 (acid CEase) and pH 7.5 (neutral CEase) (Shinomiya et al. 1979). Acid CEase and neutral CEase are presumably located in lysosomes and microsomes, respectively (Shinomiya et al. 1979). For hydrolysis of cholesterol ester in deposited particles, these enzyme-bound organellae may

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fuse with the deposited lipid, or the enzyme may be released from the organellae to interact with the deposited lipid inclusion. In this work, we studied the latter possibility by measuring the releases of acid CEase and neutral CEase from the particulate fractions, and released enzyme activities were assayed by measuring the hydrolysis of cholesterol ester in lipid mixtures with the same lipid compositions as that of lipid deposits in atheroma.

Materials and Methods

Chemicals

Cholesterol [1-^{14}C]oleate (specific activity = 54 mCi/mmol) and tri[1-^{14}C]oleoyl glycerol (specific activity = 45 mCi/mmol) were purchased from New England Nuclear. Triolein, cholesterol, pig liver phosphatidylcholine, pig liver phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine were obtained from Serdary Research Laboratories. Dextran sulfate was a gift from Kowa Pharmaceutical Co., Tokyo. Heparin (pig intestine, 144 ISU/mg) was purchased from Sigma Co., St. Louis, Mo, USA.

Preparation of enzyme solutions

Enzyme solution was prepared from the arterial wall of male Wistar-King albino rats as reported previously (Shinomiya et al. 1979). The aortas were washed with ice-cold 0.9% NaCl solution and the adventitia was carefully removed. The tissue was cut up with scissors and homogenized with 4 volumes of 5 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose in a Hiscotron (Nichion Co., Tokyo). The homogenate was centrifuged at 800× g for 5 min and the resultant supernatant was used as enzyme source.

Assay of cholesterol esterase activity and lipase activity

For assay of cholesterol esterase, the reaction mixture consisted of 500 μM choletserol oleate, 0.05 μCi of cholesterol [1-^{14}C]oleate, 1 mM dilinoleoyl phosphatidylcholine and 50 mM acetate buffer, pH 4.5, or 50 mM Tris-HCl buffer, pH 7.5 in a final volume of 200 μl. Details of the method have been reported (Shinomiya et al. 1979).

For assay of lipase activity, substrate mixture consisting of 2 mM trioleoyl glycerol, 0.04% Triton X-100 and 1.25 μCi/ml of tri[1-^{14}C] oleoylglycerol was sonicated for 3 min with an ultrasonic vibrator. The reaction mixture consisted of 50 μl of sonicated substrate mixture, and 50 μl of 0.4 M acetate buffer, pH 5.0, or 0.4 M potassium phosphate buffer, pH 7.0 and 100 μl of enzyme solution. The reaction mixture was incubated at 37°C for 60 min and then [^{14}C]oleic acid released was measured by the method of Belfrage and Vaughan (1969).

Preparation of artificial lipid mixtures

Artificial lipid mixtures were prepared as described previously (Shinomiya et al. 1981). The compositions of mixture corresponding to that of the fatty streak were cholesterol ester 82.1%, free cholesterol 4.9%, triglyceride 4.3% and phospholipids 8.7% (phosphatidylcholine 4.6%, phosphatidylethanolamine 1.2%, sphingomyelin 2.2%, lysophosphatidylcholine 0.7%). Mixture corresponding to that of fibrous plaques consisted of cholesterol ester 60.8 %, free cholesterol 13.0%, triglyceride 10.4%, phospholipids 15.8% (phosphatidylcholine 5.2%, phosphatidylethanolamine 1.3%, sphingomyelin 3.8%, lysophosphatidylcholine 1.5%) as reported by Hata (1975).

Hydrolysis of cholesterol ester in artificial lipid inclusions

The reaction mixture consisted of 50 μl of artificial lipid mixture containing 500 μM cholesterol oleate, 0.05 μC of cholesterol [1-^{14}C]oleate, equivalent amounts of the other lipid

components to those described above, 100 μl of acetate buffer (pH 4.5) or Tris-HCl buffer (7.5) and 50 μl of enzyme solution in a final volume of 200 μl. Incubations were performed at 37°C for 120 min and the reaction was stopped by adding extraction mixture as described for CEase assay.

Subcellular fractionation

Subcellular fractionation was performed by ultracentrifugation as described previously (Shinomiya et al. 1979) The supernatant of a homogenate of arterial wall obtained at 500 $\times$g was centrifuged at 5,500 $\times$g for 30 min to precipitate the mitochondria. Then the resultant supernatant fraction was cetrifuged at 14,000 $\times$g for 60 min to precipitate lysosomes and at 105,000$\times$g for 60 min to precipitate microsomes. Each fraction was washed by suspending it in an appropriate volume of the same solution and recentrifugating it as before.

Release of lipase and cholesterol esterase from particulate fractions

For measurement of release of enzymes from the lysosomal and microsomal fractions, 2 ml of lysosomal or microsomal fraction (protein content 2 mg/ml) in 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4, was incubated at 37°C for 10 min in the presence of various amounts of dextran sulfate, heparin, and Triton X-100. After incubation, the mixtures were centrifuged at 105,000 $\times$g for 60 min. The resultant supernatant and the pellet fraction, which was resuspended with standard buffer containing the same amount of each solubilizer as in the supernate, were used for enzyme assay.

Other methods

Acid phosphatase activity was measured by the method of Kind and King (1954), and protein by the method of Lowry et al. (1951).

Results

Solubilization of CEase from the lysosomal and microsomal fractions by heparin and Triton X-100

When the particulate fraction was incubated with various concentrations of heparin or Triton X-100, less acid CEase than neutral CEase was released into the 105,000 $\times$g supernatant fraction, as shown in Table 1.

Table 1. *Effects of heparin and Triton X-100 on the release of cholesterol esterase activity from particulate fractions*

Treatment		Released cholesterol esterase activity (percent activity in 105, 000$\times$g supernatant)	
		pH 4.5	pH 7.5
Heparin	10 μg/ml	16	45.5
	50 μg/ml	18	56.0
Triton X-100	0.01%	12	10
	0.05%	17	21
	0.10%	21	66

Values are means for three experiments.
The standard deviation was less than 5% of the mean.

Solubilization of CEase and lipase from the lysosomal and microsomal fractions by dextran sulfate

When the lysosomal fraction was incubated with dextran sulfate at 37°C for 15 min, acid CEase was released into the 105,000 ×g supernatant fraction, but even with 1,000 μg/ml of dextran sulfate, only 4% was released, while the releases of acid lipase and acid phosphatase were 7% and 11%, respectively, as shown in Fig. 1.

Fig. 2 shows the release of enzyme activities from the microsomal fraction. When the microsomal fraction was incubated with dextran sulfate at 37°C for 15 min, release of neutral CEase into the supernatant increased up to 40% with increase in dextran sulfate 1,000 μg/ml. Scarcely any neutral lipase was released by dextran sulfate.

Hydrolysis of cholesterol ester in mixtures simulating fatty streak lipid and fibrous plaque lipid by various enzymes

As shown in Fig. 3A, the activities of acid CEase in the 800 ×g supernatant on cholesterol ester in mixtures simulating fatty streak and fibrous plaque lipids were 16 and 19 nmoles/mg protein per 2 hr, respectively. The enzyme solubilized from the lysosomal fraction by treatment with dextran sulfate scarcely hydrolyzed cholesterol ester in these artificial lipid mixtures. As shown in Fig. 3B, the activities of neutral CEase in the 800 ×g supernatant on cholesterol esters in mixtures simulating fatty streak and fibrous plaque lipids were 3 and 7 nmoles/mg protein per 2 hr, respectively, whereas the enzyme solubilized from the microsomal fraction with dextran sulfate hydrolyzed 38 and 36 nmoles/mg protein per 2 hr, respectively.

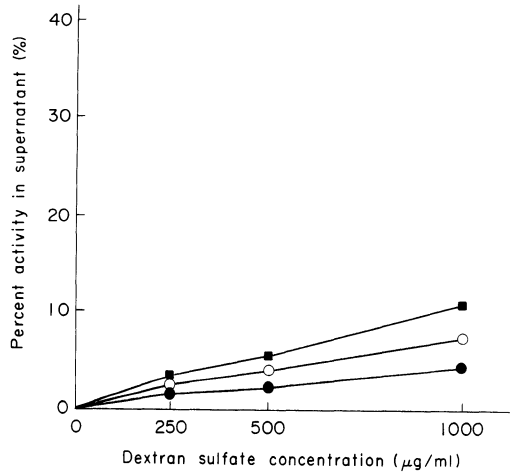

Fig. 1. Effect of dextran sulfate on the release of acid lipase, acid cholesterol esterase and acid phosphatase from the lysosomal fraction. ○——○, acid lipase; ●——●, acid CEase; ■——■, acid phosphatase.

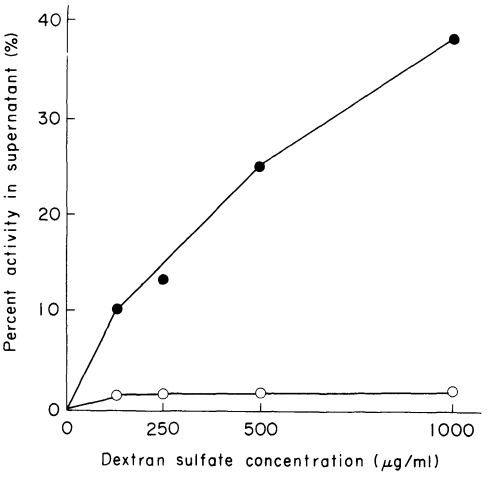

Fig. 2. Effect of dextran sulfate on the release of neutral lipase and neutral cholesterol esterase from the microsomal fraction.
○——○, neutral lipase; ●——●, neutral CEase.

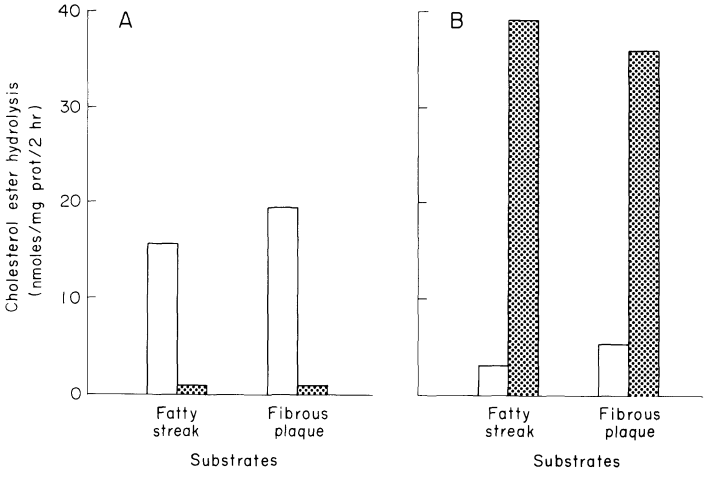

Fig. 3. Hydrolysis of cholesterol ester in mixtures simulating fatty streak lipid and fibrous plaque lipid by various enzyme sources.
A: pH 4.5. B: pH 7.5. □, 800×g supernatant fraction; ▨, 105,000×g supernatant fraction after dextran sulfate treatment.

Discussion

The results that less acid CEase than neutral CEase was released from particulate fractions on treatment of heparin, Triton X-100 or dextran sulfate, suggesting that neutral CEase is easily detouched from microsomes. The specific activities of the enzymes could not be compared exactly because the enzymes were not pure, but it is noteworthy that on solubilization the specific activity of neutral

CEase for cholesterol ester in artificial lipid mixtures increased, whereas that of acid CEase decreased. The poor release of CEase from lysosomes, and the inability of the solubilized fraction to hydrolyze cholesterol ester support the idea that acid CEase is tightly bound to the lysosome membrane and acts on LDL cholesterol ester when it is incorporated into lysosomes through LDL pathway (Goldstein and Brown 1977), but its exact action is still to be investigated. Neutral CEase was readily released from the microsomal fraction, and showed activity on cholesterol ester in artificial lipid mixtures with similar lipid compositions as those of fatty streak and fibrous plaque in atheromatous lesions. These results indicate that neutral CEase may play an important role to hydrolyze deposited cholesterol ester in atheromatous lesion. In this work, dextran sulfate and other detergents were used to solubilize these enzymes. Enzyme releases from the particulate fraction in pathological conditions such as in atheromatous lesions and their properties require further investigation.

The extents of release of CEase and lipase from the lysosomal fraction and microsomal fraction were different. Patients with Wollman disease were reported to lack both CEase and lipase activity, suggesting that the two activities are due to the same enzyme (Fredrickson and Ferrans 1978). Further study is required on the identities of these two enzymes.

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