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THE ROLE OF CHOLESTERYL ESTER HYDROLYSIS AND SYNTHESIS IN CHOLESTEROL TRANSPORT ACROSS RAT INTESTINAL MUCOSAL MEMBRANE A NEW CONCEPT

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SUMMARY: The rate of accumulation of cholesteryl ester in rat intestinal sacs is enhanced up to 3-fold by the addition of porcine cholesterol esterase to the uptake medium. For this the enzyme must be catalytically active and does not exert its effect via its activity in the medium. A mechanism for this rate enhancement is proposed in which the action of cholesterol esterases on both sides of a bilayer membrane can catalyze the net transmembrane movement of cholesterol.

The absorption of dietary cholesterol in the intestine has been extensively studied with regard to the delivery of free cholesterol to the mucosal membrane and its reesterification within the mucosal cell (1,2). Two roles for pancreatic cholesterol esterase in this process have been proposed. It catalyzes the hydrolysis of dietary cholesteryl ester and may catalyze cholesterol reesterification after being taken up by the villus cell (1). Our recent studies of the properties of porcine pancreatic cholesterol esterase (3,4) showed an affinity of the enzyme for free fatty acids, a product of lipolysis. This observation, together with the effects of fatty acid on cholesterol absorption (1) and the requirement of cholesterol esterase for cholesterol absorption *in vivo*, suggested that the enzyme may also function on the luminal surface of the villus membrane. That a protein is involved in the transbilayer movement of cholesterol in the intestine has been suggested by the work of Chow and Hollander who showed that uptake had a free

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energy of activation energy of 20 Kcal/mole (5). This argues against diffusion of cholesterol to the villus membrane as the sole rate determining step (1). In this communication, we examine the role of cholesterol esterase(s) in the transport of cholesterol.

EXPERIMENTAL PROCEDURES

Materials: Cholesterol, oleic acid and monooleoylglycerol were obtained from Nu-Chek Prep, Elysian, MN. [^{14}C]Cholesterol and [^{3}H]inulin were obtained from Research Products International Company, Mount Prospect, IL, and Amersham, respectively. Sodium taurocholate and diisopropylfluorophosphate were obtained from Calbiochem and p-nitrophenylacetate from Sigma. Cholesterol esterase from porcine pancreas was purified and its activity was measured as previously described (6). Diisopropylfluorophosphate (DFP) inactivated enzyme was prepared by treating the enzyme with 0.1 mM DFP at 4^{o}C. After complete loss of activity the DFP was removed by dialysis.

Methods: The proximal half of rat small intestine was removed from ether anesthetized, male Sprague-Dawley rats weighing 180-250 g, rinsed with cold saline and immediately everted over a glass rod. From this 5 cm sacs were prepared, avoiding Peyer's patches, and filled with Krebs-Ringer carbonate buffer (Ca^{++}, Mg^{++} omitted). Prior to the experiments, the everted sacs were washed at 37^{o}C for 15 min with 20 ml of buffer containing 10 mM sodium taurocholate and 5 mg/ml of glucose.

Clear, micellar lipid solutions for absorption studies containing [^{14}C]cholesterol (as marker for cholesterol absorption) and [^{3}H]inulin (as marker to measure the volume of the adherent medium) were prepared as reported by Rampone and Machida (7). The final concentrations of the components were free cholesterol (^{14}C, 0.04 Ci/ml), 0.75 mM; oleic acid, 3.0 mM; monooleoylglycerol, 1.5 mM; sodium taurocholate, 24 mM; glucose, 5 mg/ml; and ^{3}H-inulin, 0.15 Ci/ml. During cholesterol uptake experiments, the medium was vigorously stirred at 3,000 rpm with a round stirring bar, and a slow bubbling of 5% CO_2 in oxygen was provided. Following incubation, sacs were removed and rinsed thoroughly with ice cold saline. After opening along the mesenteric line, the flat segment of intestine was mounted on dissecting wax, mucosal side facing upwards. Two circular segments of tissues, 0.38 cm^{2} each, were quickly removed using a sharp edged cork borer. One sample was placed in a vial with 1 ml of Soluene-350 (Packard Instrument Company) and was subsequently incubated at 50^{o}C until the tissue completely dissolved (1.5 - 2.0 h). After cooling to room temperature, 10 ml of scintillation cocktail (Dimilume-30, Packard) was added to the vial and both [^{3}H] and [^{14}C] were measured. Net cellular uptake of [^{14}C]cholesterol was calculated as outlined by Rampone and Machida (7). The contribution of non-absorbed [^{14}C]cholesterol to the total was generally less than 20%. The second tissue punch was placed in 2 ml of 0.5 M Tris-HCl, pH 7.4, containing 6 mM sodium taurocholate. Subsequently, this was

homogenized using a Polytron tissue homogenizer for 30 s, centrifuged at 10,000 x g for 15 min, and assayed for cholesterol esterase activity using either p-nitrophenylacetate or [^{14}C]cholesteryl oleate. The remaining tissue was homogenized in 3 ml of 0.5% trichloroacetic acid to stop metabolic activity. The total lipid was then extracted with chloroform/methanol (2:1) (8). Carrier lipid was added and free and esterified cholesterol were separated by thin layer chromatography on a silica gel plate using a solvent system of petroleum ether/ethyl ether/acetic acid (90:10:1). The carrier was localized by brief exposure to iodine vapor, the spots were scraped off and radioactivity was determined by liquid scintillation counting. In independent experiments it was shown that the percentage of cholesterol in the medium which was esterified via the action of added or endogenous cholesterol esterase was less than 0.2%. This data, together with the measurements of [^{14}C]cholesterol distribution between the tissue and adherent medium, was used to calculate the quantities of free and esterified cholesterol associated with the tissue.

RESULTS AND DISCUSSION

To minimize diffusion barriers and interference from endogenous rat cholesterol esterase, gut sacs were incubated before use with stirring at 37^{o} in buffer containing 10 mM sodium taurocholate. A 15 min wash removed 30-40% of the total cholesterol esterase activity which could be removed by several hours of washing. No ultrastructural damage was discernible by electron microscopy with up to one hour of washing. The accumulation of free and esterified cholesterol by the tissue was approximately linear for up to 20 min (Fig. 1). The presence of porcine pancreatic cholesterol esterase in the medium increased the rates of both free and esterified cholesterol accumulation. Although the accumulation of cholesteryl ester is small relative to free cholesterol, the presence of enzyme increased its rate 2- to 3-fold. A similar enhancement has been observed with rat enterocytes and rat pancreatic cholesterol esterase (9). The lack of a demonstrable lag in this enhancement argues against the internalization of the enzyme as a prerequisite for this effect. Also, the porcine enzyme, MW 80,000 (6), was chosen because it is immunologically distinct from rat pancreatic, MW 65,000 (10), and intestinal cholesterol esterase (10) and, hence, would not be

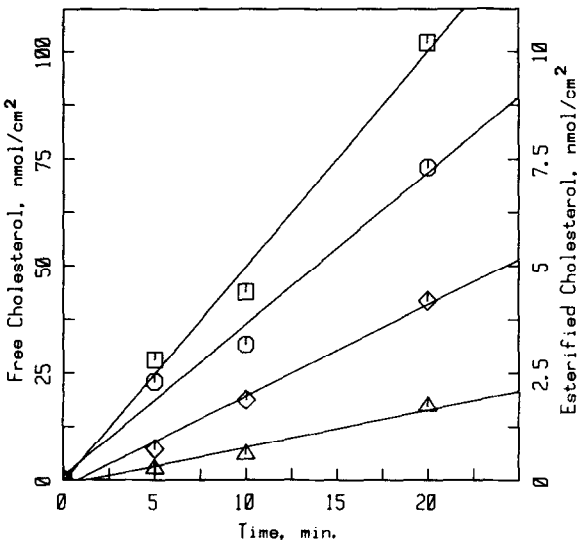

Fig. 1. Time course of cholesterol and cholesteryl ester uptake by rat intestinal everted sacs in the presence and absence of porcine pancreatic cholesterol esterase. Concentration (mM) of components of the absorption medium in Krebs-Ringer bicarbonate buffer were: cholesterol, 0.75; monooleoylglycerol, 1.5; oleic acid, 3.0; sodium taurocholate, 24.0, and enzyme, 0.78 M. Free cholesterol with (□) and without (○) enzyme; esterified cholesterol with (◇) and without (△) enzyme.

expected to be taken up by rat intestine. Figure 2 shows that the rate of enhancement approaches saturation at an enzyme

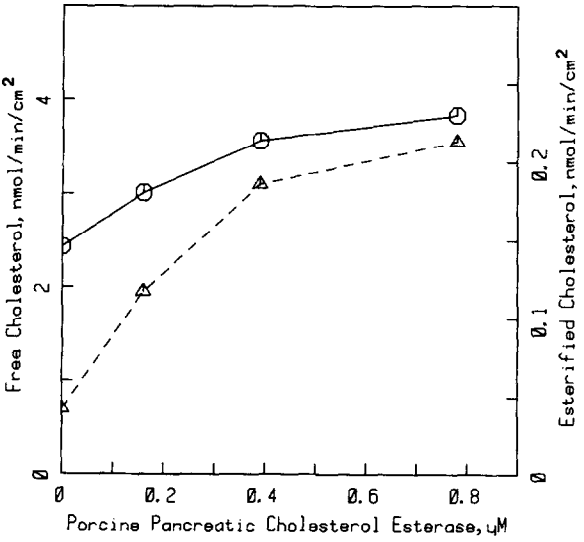

Fig. 2. Effect of initial concentration of porcine pancreatic cholesterol esterase in the absorption medium on the uptake of cholesterol and cholesteryl ester by rat intestinal everted sacs. Cholesterol (○——○) cholesteryl ester (△---△).

TABLE I

EFFECT OF PORCINE PANCREATIC CHOLESTEROL ESTERASE ON UPTAKE OF CHOLESTEROL AND CHOLESTERYL ESTER BY EVERTED RAT INTESTINAL SACS

	Cholesterol (nmols/min/cm^2)	
Addition	Free Cholesterol	Esterified
None	2.76	0.06
Porcine pancreatic cholesterol esterase (0.78 μM)	3.65	0.22
Diisopropylfluorophosphate treated enzyme (0.8 μM)	2.40	0.07

Absorption medium was prepared in Krebs-Ringer bicarbonate buffer consisting of the following components: cholesterol, 0.75 mM; monooleoylglycerol, 1.5 mM; oleic acid, 3.0 mM; sodium taurocholate, 24 mM; and enzyme.

concentration of 10^{-6} M. That the enzyme must be catalytically active to effect this is shown in Table I; its prior inactivation with DFP blocks the enhancement.

A possible mechanism for the enhancement is that in the medium the enzyme catalyzes the formation of cholesteryl ester which is taken up intact by the tissue. To test this we used unlabelled cholesterol in the medium to which was added 0.2 mol%, the equilibrium level, of [^{14}C]cholesteryl oleate. The rate of cholesteryl ester absorption via this route was less than 0.003 nmole/cm^2/min as compared with the expected level of 0.22 nmol/cm^2/min (Table I). This shows that direct uptake of intact cholesteryl ester from the medium cannot account for the observed accumulation when ^{14}C-cholesterol was used. Thus, our data suggest that exogenous cholesterol esterase enhances the accumulation of both free and esterified cholesterol by acting catalytically at the luminal surface of the villus membrane.

A plausible mechanism by which this occurs is shown in Figure 3. Once adsorbed to the luminal surface of the membrane,

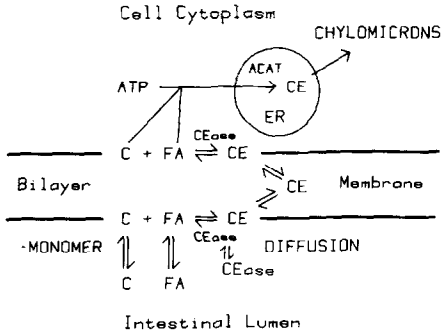

Fig. 3. Mechanism of synthesis/hydrolysis induced transport of cholesterol across membranes. Abbreviations: C, cholesterol; CE, cholesteryl ester; CEase, cholesterol esterase; FA, fatty acid; ATP, adenosine triphosphate; ACAT, acyl CoA:cholesterol acyltransferase; ER, endoplasmic reticulum.

the enzyme catalyzes the esterification of a few percent of the cholesterol in the membrane (3). Being highly apolar, this ester readily partitions to the hydrocarbon region of the bilayer from where it can return or partition to the inner surface of the membrane. On the inner surface the intestinal cholesterol esterase, perhaps of pancreatic origin (1), again catalyzes the free/esterified cholesterol equilibrium which favors hydrolysis. Thus, cholesterol esterases on each surface of the membrane can catalyze the "flip-flop" of cholesterol (via the ester) from one leaflet of the membrane to the other. Free cholesterol is now available inside the cell for transport to and reesterification in the endoplasmic reticulum via the energy driven pathway (11).

The proposed pathway is consistent with the known properties of cholesterol absorption, such as its specificity and the requirements for a source of fatty acid such as triglyceride and for cholesterol esterase (1). This pathway should not be unique for intestine and could operate in virtually all cells. For example, it could explain how cholesterol exits the lysosome, via the acid cholesterol esterase inside the lysosome (12) and the cytoplasmic enzyme outside (13). Thus, our results suggest a general mechanism for the transbilayer movement or "flip-flop" of

cholesterol under conditions where the uncatalyzed pathway is very slow (14).

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