BIOLOGICAL STABILITY OF [^{3}H]CHOLESTERYL OLEYL ETHER IN CULTURED FIBROBLASTS AND INTACT RAT

Y. STEIN, G. HALPERIN and O. STEIN

Lipid Research Laboratory, Department of Medicine B, Hadassah University Hospital, and *Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel*

Received 12 December 1979

1. Introduction

The synthesis of the majority of cholesterol esters in human plasma is catalyzed by lecithin cholesterol acyl transferase (LCAT) [1] and while the reaction takes place on the HDL particles, the cholesterol esters are transferred to other plasma lipoproteins by a protein carrier [2]. On the basis of measurement of the LCAT reaction in vivo and in vitro [3] it appears that the production of cholesterol esters exceeds considerably the amount cleared from the circulation in the form of LDL and HDL particles [4]. Thus plasma cholesterol esters need to be removed by some additional pathways. Since cholesterol esters are hydrolyzed quite rapidly following uptake by the liver [5] and by cultured cells [6,7] a cholesterol ester analogue, which is not rapidly degraded might prove useful in the elucidation of the role of various tissues in the uptake of lipoprotein cholesterol esters. [^{14}C]Sucrose covalently bound to low density lipoproteins (LDL) was used in [8] as a non-degradable marker to follow the protein portion of the LDL particle into the lysosome. To follow the fate of the cholesterol ester moiety we have synthesized cholesterol alkyl ethers [9] and incorporated them into serum lipoproteins. This report deals with the determination of the non-degradability of these cholesterol ethers and their potential suitability to serve as analogs for lipoprotein cholesterol esters.

2. Materials and methods

Human LDL and high-density lipoprotein (HDL) were isolated according to [10], rat HDL was isolated at d = 1.085–1.21 g/ml. The labeled cholesteryl alkyl ethers and cholesteryl linoleate were introduced into partially delipidated LDL using unlabeled cholesteryl linoleate as carrier [6]. HDL (6 mg protein/tube) were delipidated [6] and relipidation was carried out with 6 mg cholesteryl linoleate containing trace amounts of either [^{3}H]cholesteryl alkyl ethers or [^{3}H]cholesteryl linoleate.

Human skin fibroblasts were cultured as in [7]. The labeled lipoproteins were added to culture medium containing $d > 1.25$ fraction of human serum, 2.5 mg protein/ml. After 2–4 days incubation, the cell layer was washed with 0.2% albumin in phosphate-buffered saline [7], the cells were released with trypsin [11] and the cell suspension washed by repeated centrifugation and resuspension in excess buffered saline [11]. The labeled lipids were extracted from the pellet and trypsinate according to [12] and were analyzed by thin-layer chromatography (3% ethyl ether in light petroleum, b.p. 30–60°C) using cholesterol linoleate and free cholesterol as reference standards. The relipidated rat HDL was injected into rats 180–200 g body wt, which were killed by exsanguination under ether anesthesia 2–48 h thereafter. The radioactivity in organs was determined in the chloroform phase after extraction of lipids according to [12], with a β liquid scintillation spectrometer, Packard, Model 2650. [^{3}H]Cholesteryl linoleate was prepared as in [7] and >98% was recovered in esterified form. Synthesis of cholesteryl alkyl ethers was carried out by a modification [9] of the method in [13]. [7-α-^{3}H]Cholesterol was reacted with p-tosyl-chloride in dry pyridine at 38°C for 20 h. The cholesterol tosylate extracted with hexane was heated at 110°C in the presence of an excess of fatty acid alcohol. Following extraction with hexane in the presence of excess $NaHCO_3$ the cholesterol alkyl ether was eluted from a silicic acid

 Elsevier/North-Holland Biomedical Press

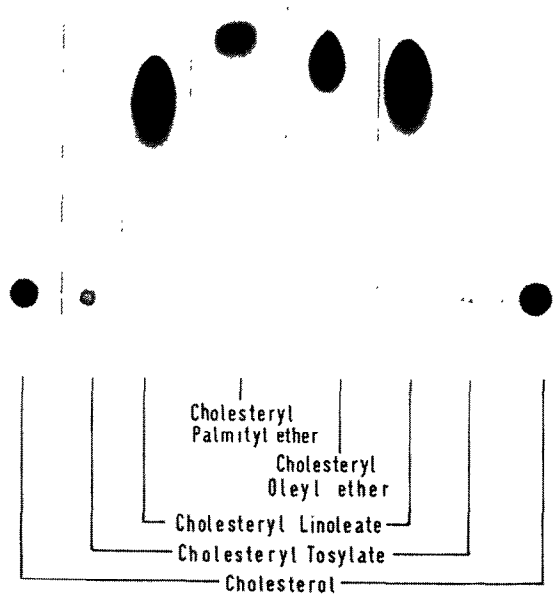

Fig.1. Thin-layer chromatograph of labeled cholesteryl alkyl ethers.

column in 15% benzene in hexane [9]. The R_f of the cholesteryl alkyl ethers was similar to that of cholesteryl linoleate (fig.1). More than 98% of the ^3H label was recovered as cholesteryl ether. Protein was determined as in [14]. [7-α-^{3}H]Cholesterol was obtained from The Radiochemical Centre, Amersham and the fatty acid alcohols from Nu Chek, Elysian, MN.

3. Results

Cultured fibroblasts were incubated for 4 days with LDL labeled with [^{3}H]cholesteryl oleyl ether and ~10% of the added label were recovered in the cells after removal of surface-bound radioactivity by trypsinization (table 1). About 92–94% of the cellular [^{3}H] lipid was recovered as unhydrolyzed cholesteryl ether. In cells exposed for 4 days to LDL-labeled with [^{3}H]cholesteryl linoleate 98% of the labeled cellular lipid was found as free cholesterol. LDL labeled with [^{3}H]cholesteryl cetyl or linoleyl ether was also taken up and the intracellular labeled lipid was recovered in unhydrolyzed form (94–96%). To verify the uptake of 'reconstituted' LDL by the cultured cells, the lipoprotein was delipidated as above and relipidated with non-labeled cholesterol linoleate and cholesteryl oleyl ether, and iodinated with ^{125}I [11]. The uptake and degradation [11] of the protein moiety of the 'reconstituted' and native LDL were compared after 6 h incubation with human skin fibroblasts. The uptake and degradation of native ^{125}I LDL were 10.9 ± 0.3 and 12.0 ± 0.4 ng LDL protein/dish, while the uptake and degradation of the 'reconstituted' LDL ranged between 32–40 and 29–45 μg LDL protein/dish. Next the labeled compounds were injected i.v. into rats in the form of relipidated rat HDL. 2 h after injection of HDL labeled with [^{3}H]cholesteryl oleyl ether 86% of the injected label was recovered in plasma and liver (table 2) and 95% of the labeled lipid was in unhydrolyzed form. When rats had been injected with

Table 1
Retention of [^{3}H]cholesteryl oleyl ether in cultured human skin fibroblasts

Labeled lipid in LDL	Incubation (days)	[^{3}H]Lipid in		
		Trypsin (dpm)	Cells	
			(dpm)	% Non-hydrolyzed
Cholesteryl oleyl ether	2	930	6520	94
Cholesteryl oleyl ether	4	1280	10 200	92
Cholesteryl linoleate	4	600	3700	2

Conditions: LDL relipidated with [^{3}H]cholesteryl oleyl ether or with [^{3}H]cholesteryl linoleate, 60 μg protein/ml and 5 × 10^4 dpm/ml were added to 2 ml culture medium containing $d > 1.25$ fraction of human serum, 2.5 mg protein/ml. At the end of incubation, the cell layer was washed with 0.2% albumin in buffered saline and the cells were released by trypsinization. Values are means of duplicate dishes

Table 2
Retention of [^{3}H]cholesteryl oleyl ether in rat liver in vivo

Labeled lipid in HDL	Time after injection (h)	Liver		Plasma	
		% injected dose	% hydrolyzed	% injected dose	% hydrolyzed
Cholesteryl oleyl ether	2	50.3	4.8	35.7	5.3
	48	65.7	6.1	7.8	79.0
Cholesterol linoleate	2	32.6	96.6	23.5	91.0
	48	7.0	–	5.3	–

Rats were injected with 0.7–1.0 mg relipidated HDL protein which contained 2×10^5 dpm of either [^{3}H]cholesteryl oleyl ether or [^{3}H]cholesteryl linoleate. Values are means of duplicate determinations using 3 rats for each time

HDL labeled with [^{3}H]cholesteryl linoleate >90% of the labeled lipid was found in free cholesterol. 48 h after injection of [^{3}H]cholesteryl oleyl ether, the label in the liver increased to 65% injected dose of which 94% were in non-hydrolyzed form.

4. Discussion

This study contains the first description of the metabolic fate of unsaturated cholesteryl alkyl ethers complexed to plasma lipoproteins. Since in the experiments with cultured human skin fibroblasts the cellular label had been determined after trypsinization it provides evidence that these modified lipoproteins had been taken up by the cells. However, while the labeled cholesteryl ester of the reconstituted LDL had undergone complete hydrolysis, the labeled cholesteryl ether remained almost intact. The reconstituted LDL was internalized and its protein moiety (^{125}I-labeled) was degraded more avidly than the native LDL, probably owing to a change in particle size and shape as seen in negatively stained preparations. This different behavior proved beneficial for the purpose of the experiments here, the aim of which was to study the intracellular degradability of the cholesteryl alkyl ethers. The reconstituted HDL injected in vivo was also cleared more rapidly from the circulation than the native ^{125}I-labeled HDL [15] and was probably removed in part by the scavenger pathway. This permitted to determine whether the cholesteryl ether will be stable also when ingested by tissue macrophages. Even after 48 h, when ~66% of the injected dose was recovered in the liver, 94% of the labeled cholesteryl oleyl ether had not been hydrolyzed. This finding has to be contrasted with the fate of cholesterol ester which had been complexed with HDL, and following injection into rats had been hydrolyzed in <2 h.

Studies are in progress to introduce the labeled cholesterol ethers into plasma lipoproteins by a more physiological procedure, and preliminary findings indicate that indeed these compounds may serve as reliable markers to trace the extravascular fate of the cholesterol ester of plasma lipoproteins.

References

[1] Glomset, J. A. (1979) Lipoprot. Metab. 15, 41–66.
[2] Chajek, T. and Fielding, C. J. (1978) Proc. Natl. Acad. Sci. USA 75, 3445–3449.
[3] Barter, P. J. (1974) J. Lipid Res. 15, 234–242.
[4] Stein, Y. and Stein, O. (1980) in: Atherosclerosis V, Springer Verlag, in press.
[5] Stein, Y., Ebin, V., Bar-On, H. and Stein, O. (1977) Biochim. Biophys. Acta 486, 286–297.
[6] Krieger, M., Brown, M. S., Faust, J. R. and Goldstein, J. L. (1978) J. Biol. Chem. 253, 4093–4101.
[7] Stein, Y., Halperin, G. and Stein, O. (1978) Biochim. Biophys. Acta 530, 420–428.
[8] Pittman, R. C. and Steinberg, D. (1978) Biochem. Biophys. Res. Commun. 81, 1254–1259.
[9] Halperin, G. and Gatt, S. (1980) Steroids, in press.
[10] Havel, R. J., Eder, H. A. and Bragdon, J. A. (1955) J. Clin. Invest. 34, 1345–1353.
[11] Stein, O. and Stein, Y. (1975) Biochim. Biophys. Acta 398, 377–384.
[12] Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509.
[13] Stoll, W. (1932) Z. Physiol. Chem. 207, 147–151.
[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Roheim, P. S., Rachmilewitz, D., Stein, O. and Stein, Y. (1971) Biochim. Biophys. Acta 248, 315–329.