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Uptake and Utilization of Double-Labeled High-Density Lipoprotein Sphingomyelin in Isolated Brain Capillaries of Adult Rats

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Abstract: Isolated rat brain capillaries were incubated in the presence of high-density lipoprotein (HDL) containing [*stearic acid*-^{14}C, (*methyl*-^{3}H)*choline*]sphingomyelin. This double-labeled sphingomyelin was taken up in a concentration-dependent manner. Cerebral capillary-associated sphingomyelin had a $^{3}H/^{14}C$ ratio close to that of the incubation medium, a result indicating uptake of sphingomyelin without prior hydrolysis. TLC of lipid extracted from capillaries showed that part of the sphingomyelin (up to 40%) was hydrolyzed in the brain capillaries to ceramide and free fatty acids. The hydrolysis was proportional to the amount of incorporated sphingomyelin and reached a plateau when the HDL sphingomyelin concentration in the medium was 237 nmol/ml. The results of "pulse-chase" experiments showed that the choline moiety of sphingomyelin was recovered in the incubation medium after the chase period and that there was no redistribution of liberated choline in phosphatidylcholine of capillaries. **Key Words:** High-density lipoprotein—Sphingomyelin—Brain capillaries. **Homayoun P. et al.** Uptake and utilization of double-labeled high-density lipoprotein sphingomyelin in isolated brain capillaries of adult rats. *J. Neurochem.* **53,** 1031–1035 (1989).

The microvessels of the cerebral cortex are characterized by a continuous layer of endothelial cells joined together by tight junctions that restrict intercellular diffusion (Rapoport, 1976). Carrier-mediated transport systems are considered to be important mechanisms for the entrance of hexoses, amino acids, purine compounds, and monocarboxylic acids from blood to the brain (Pardridge and Oldendorf, 1977). However, little information is available concerning the interaction of plasma lipids with brain microvessels. In vivo studies have shown that albumin-bound fatty acids (Dhopeshwarkar and Mead, 1973) and lysolecithin (Illingworth and Portman, 1972) given intravenously are taken up by brain and metabolized. However, Pardridge and Mietus (1980) have reported that a limited amount of albumin-bound palmitate was transported through the blood–brain barrier.

Few data concerning brain uptake of complex lipids from lipoproteins are available. Koelz et al. (1982) did not find any uptake of iodine-labeled lipoproteins by brain. Moreover, lipoprotein-bound cholesterol does not seem to be taken up by brain in a substantial amount (Andersen and Dietschy, 1977; Turley et al., 1981). However, Bentejac et al. (1988) demonstrated that small quantities of sphingomyelin were recovered in the brain of rats 24 h after injection of double-labeled high-density lipoprotein (HDL) sphingomyelin. Although sphingomyelin could be taken up at nonbarrier regions of the brain by vesicular transport, the possibility of sphingomyelin uptake through endothelial cells of brain microvessels cannot be ruled out. The present investigation was performed to study the uptake and utilization of double-labeled HDL sphingomyelin by brain capillaries.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (weighing 225–250 g) were purchased from Iffa-Credo (France). Chemicals and materials were obtained as follows: [^{3}H]methyl iodide (10 Ci/mmol) and [1-^{14}C]stearic acid (60 mCi/mmol) from the Radiochemical Centre (Amersham, U.K.); bovine brain sphingomyelin and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO, U.S.A.); dextran from Pharmacia (Upp-

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Abbreviation used: HDL, high-density lipoprotein.

sala, Sweden); nylon mesh (pore size, 118 μm) from Desjobert (Paris, France); glass beads from B. Braun (Mensungen, F.R.G.); and TLC plates of silica gel (60 F 254 and G) and solvents from Merck (Darmstadt, F.R.G.).

Preparation of double-labeled sphingomyelin

[(*methyl*-^{3}H)*choline*]Sphingomyelin was prepared from bovine brain sphingomyelin and CH_3I according to the method of Stoffel (1975). Tritiated sphingomyelin was deacylated using a slight modification of the method of Kaller (1961): 1–10 mg of [^{3}H]sphingomyelin and 0.1 ml of a mixture of water/12 *M* HCl/*n*-butanol (1:1:2 by volume) was heated at 100°C for 20 min. [^{3}H]Sphingosylphosphorylcholine was reacylated by [^{14}C]stearic acid in the anhydride (Masson et al., 1981) or free (Cohen et al., 1984) form. Double-labeled sphingomyelin was purified by TLC on silica gel G using chloroform/methanol/NH_4OH (16% in water) (60:35:8 by volume) as the developing solvent. Sphingomyelin was extracted from the powder using chloroform/methanol/water (50:50:4 by volume), subjected to mild alkaline hydrolysis (Stoffel and Melzner, 1980), and purified by HPLC using a column (4.5 mm o.d. × 40 cm) of 5-μm Si-60 (Merck) and acetonitrile/methanol/water (72:18:10 by volume) as the eluting solvent (2 ml/min) under isocratic conditions. The specific radioactivities of the final product were 16–47 and 122–137 mCi/mmol for ^{14}C and ^{3}H, respectively.

Transfer of labeled sphingomyelin to HDL

[*stearic acid*-^{14}C, (*methyl*-^{3}H)*choline*]Sphingomyelin was diluted with unlabeled bovine brain sphingomyelin (containing 44.8% of $C_{18:0}$). Preparation of labeled sphingomyelin liposomes, their transfer to HDL, and then separation and purification of the labeled HDL were performed according to the method previously described (Bentejac et al., 1988). In brief, to obtain HDL containing various amounts of labeled sphingomyelin, various quantities of liposomes of labeled sphingomyelin were incubated with 1 ml of rat serum at 37°C for 1 h. HDL was separated by ultracentrifugation using a discontinuous gradient of KBr and was purified by gel filtration on a Sepharose column. HDL was concentrated on Amicon concentrators, and its purity was checked by electrophoresis on a polyacrylamide gradient gel.

Brain capillary preparation

Brain capillaries were isolated from cerebral cortices of 2-month-old rats according to the method of Goldstein et al. (1975) with slight modifications (Homayoun et al., 1985). The quality of each preparation was monitored by phase-contrast microscopy and by measuring the γ-glutamyl transferase activity.

Incubations

Isolated capillaries (~0.6 mg of protein) were suspended in 0.5 ml of incubation medium (Krebs buffer at pH 7.4) containing various concentrations (164–298 nmol/ml) of double-labeled sphingomyelin incorporated into HDL (0.71 ± 0.24 mg of protein/ml). The preparations were incubated at 37°C for 2 h (4°C for controls). The incubations were terminated by adding ice-cold buffer. The capillaries were washed twice with ice-cold buffer to remove free labeled HDL sphingomyelin. Lipids were extracted by adding chloroform/methanol (2:1 vol/vol) followed by sonication (Folch et al., 1957; Pollet et al., 1978), and aliquots were measured for their radioactivity.

Total lipids were separated into phospholipid classes by TLC using chloroform/methanol/water (65:25:4 by volume) as the developing solvent. In some cases, the ceramide fraction was separated from the free fatty acid fraction by TLC using hexane/diethyl ether/acetic acid (90:30:1 by volume) as the developing system. Lipids were visualized by short exposure to iodine vapor. Distribution of radioactivity was determined by scraping lipid bands from chromatographic plates into counting vials to which 10 ml of Beckman (HP) scintillation solution was added. Radioactivity was also measured in aliquots of the incubation plus washing medium.

In "pulse–chase" experiments, capillaries were similarly incubated in the presence of an appropriate concentration of labeled substrate, for 2 h. They were washed twice with ice-cold buffer and then reincubated in the presence of the same concentration of nonlabeled substrate for an additional 2 h. The incubations were stopped by addition of ice-cold buffer, and the capillaries were washed again with ice-cold buffer. Radioactivity was measured in the incubation plus washing medium after reincubation and in the lipid classes separated by TLC from capillary lipid extracts. Blank incubations were performed at 4°C under the same conditions.

The protein content of the capillaries was determined according to the procedure of Lowry et al. (1951) and that of HDL according to the technique of Peterson (1977).

In vivo experiments

Rats were fasted overnight and lightly anesthetized with ether before injection. Saline (0.4 ml) containing 100 nmol of [(*methyl*-^{3}H)*choline*]sphingomyelin incorporated into HDL (0.25 mg of protein) was injected into the penis vein. The animals were killed 24 h after injection, and cerebral capillaries were isolated. Lipids were extracted from brain and capillaries and separated as described above.

RESULTS

When the capillaries were incubated in the presence of substrate at 4°C, part of the radioactivity was associated (whether by capillarity, adsorption, or binding) with capillaries. The proportion of the medium radioactivity recovered in the capillaries rose from 2 up to 5% along with an increasing concentration of substrate but less rapidly than when capillaries were incubated at 37°C. The data reported subsequently are the result of subtraction of the values obtained at 4°C from those obtained at 37°C.

Figure 1 shows that the uptake of sphingomyelin by isolated brain capillaries rose when the concentration of substrate in the medium (up to 298 nmol of sphingomyelin incorporated into 0.71 mg of HDL/ml of incubation medium) increased. Below 150 nmol of sphingomyelin/ml, the statistical variations were too high to discriminate between the values determined at 4 and 37°C, but that does not exclude a probable uptake at low concentrations. The sphingomyelin uptake tended to plateau at ~298 nmol of sphingomyelin/ml. However, because the incorporation of >298 nmol of sphingomyelin into HDL equivalent to 0.71 mg of protein was impossible, we could not measure a possible uptake at higher sphingomyelin concentrations. The ^{3}H/^{14}C ratio of the cell sphingomyelin was similar to that of the exogenous sphingomyelin added to the

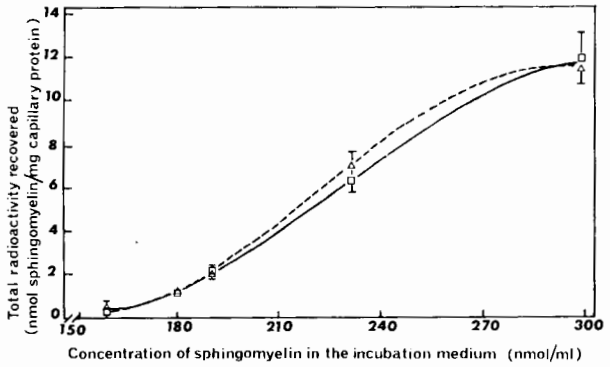

FIG. 1. Incorporation of [*stearic acid*-^{14}C, (*methyl*-^{3}H)*choline*]-sphingomyelin into isolated brain capillaries: ^{3}H (□) and ^{14}C (△). Labeled sphingomyelin (164–298 nmol) incorporated into HDL (0.71 ± 0.24 mg of protein)/ml of incubation medium (Krebs buffer, pH 7.4) was incubated with capillaries (0.6 mg of protein) at 37 or 4°C (control) for 2 h. Total lipids extracted from the capillaries by the method of Folch et al. (1957) were found to contain all the radioactivity. The values obtained at 4°C were subtracted from those obtained at 37°C. Results are expressed as nmol of sphingomyelin/mg of capillary protein. The values were fitted using a polynomial regression program. Data are mean ± SE (bars) values from at least three experiments.

medium. That indicates that labeled sphingomyelin was taken up by capillaries without prior hydrolysis.

Figure 2 shows that the sphingomyelin incorporated was degraded into ceramide plus free fatty acid. This degradation rose in parallel with the increasing amount of sphingomyelin taken up as a result of the increase in concentration of substrate in the incubation medium. However, the hydrolysis of sphingomyelin peaked at ~237 nmol of sphingomyelin/ml. At this concentration, the sphingomyelinase seemed to be saturated by the substrate. When a chase period was added to experiments, the hydrolysis products reached 40% of the ^{14}C remaining in capillaries (Table 1), and free fatty acids represented 16–19% of the total cell ^{14}C.

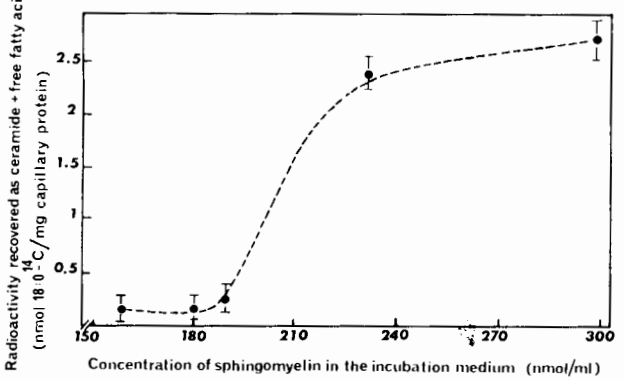

FIG. 2. Production of ceramide plus free fatty acid from incorporated sphingomyelin in isolated brain capillaries. Results are expressed as nmol of stearic acid-^{14}C/mg of capillary protein. Data are mean ± SE (bars) values from at least three experiments. The experimental conditions were identical to those in Fig. 1.

The production of free fatty acids suggests the presence of ceramidase in the brain capillaries.

Table 1 first shows the radioactivity distribution among the lipid classes after in vitro incubation of isolated capillaries with double-labeled substrate for 2 h and with unlabeled substrate for an additional 2 h. More ^{14}C than ^{3}H was recovered in cell lipids, mainly sphingomyelin (54%) and ceramide plus free fatty acid (40%). Cell ^{3}H was almost exclusively recovered in sphingomyelin (98%). This indicates that phosphocholine was not retained in the cells. Results from in vivo experiments are also shown in Table 1; 24 h after injection of ^{3}H-labeled bovine brain sphingomyelin incorporated into HDL, 20% of the radioactivity recovered in the lipid extracts from isolated capillaries was still associated with the sphingomyelin fraction. This suggests that sphingomyelin was taken up through the luminal surface of capillary endothelial cells and that all the sphingomyelin incorporated was not hydrolyzed in the endothelial cells. However, Table 1 shows that a high percentage of the radioactivity was recovered in phosphatidylcholine, in contrast to the in vitro experiments.

Figure 3 shows the results from "pulse–chase" experiments. Part of the radioactivity previously associated with capillaries was recovered in the medium after the chase period. More ^{3}H than ^{14}C was recovered in the medium. In the capillaries, almost all the radioactivity was found in the lipid extracts. The ^{3}H value was fairly constant, whereas the ^{14}C content increased in parallel with the rising concentration of substrate in the medium. The excess of ^{3}H in the medium com-

TABLE 1. *Radioactivity distribution in lipid fractions of isolated brain capillaries after in vitro incubation with double-labeled HDL sphingomyelin (Sph) or 24 h after injection of single-labeled HDL Sph into rats*

Lipid class	In vitro incubation			In vivo injection
	% ^{3}H	% ^{14}C	^{3}H/^{14}C	(% ^{3}H)
Or	0.3 ± 0.05	0.1 ± 0.00	—	0.9
Sph	98.1 ± 0.04	52.1 ± 2.45	1.14 ± 0.02	20.3
PC	1.6 ± 0.40	4.1 ± 0.60	0.24 ± 0.10	76.9
Other PL	—	2.7 ± 0.20	—	1.9
Ce + FFA	—	40.8 ± 1.75	—	—

Results are percentages of isotope recovery in tissue lipids. Data are mean ± SE values (n = 3). Isolated capillaries (0.6 mg of protein) were incubated with 237 nmol of [*stearic acid*-^{14}C, (*methyl*-^{3}H)*choline*]Sph incorporated into HDL (0.71 ± 0.24 mg of protein)/ml of Krebs buffer (pH 7.4) at 37 or 4°C for 2 h. Capillaries were washed and incubated in the presence of the same quantity of unlabeled substrate at 37 or 4°C for 2 h. Values obtained at 4°C were subtracted from those obtained at 37°C. The ^{3}H/^{14}C ratio of original Sph was taken as equal to 1. In in vivo experiments, 100 nmol of bovine brain [(*methyl*-^{3}H)*choline*]Sph incorporated into HDL (0.25 mg of protein) was injected into rats. The recovery (50 nCi) in the capillaries was 0.004% of the injected dose. Values are the means of four pooled experiments. Or, original; PC, phosphatidylcholine; PL, phospholipids; Ce, ceramide; FFA, free fatty acids.

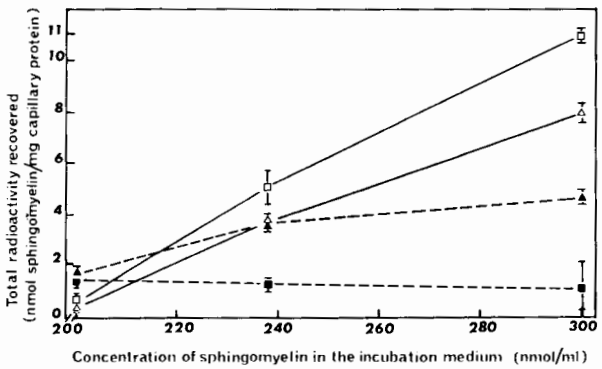

FIG. 3. Isotope recovery as ^{3}H (squares) and ^{14}C (triangles) in incubation medium (open symbols) and capillaries (solid symbols) after the chase period. Isolated capillaries (0.6 mg of protein) were incubated with 200–298 nmol of labeled sphingomyelin incorporated into HDL (0.71 ± 0.24 mg of protein)/ml of Krebs buffer (pH 7.4) at 37 or 4°C (control) for 2 h. Capillaries were washed twice with ice-cold buffer and reincubated in the presence of the same quantity of unlabeled substrate at 37 or 4°C (control) for 2 h. Values obtained at 4°C were subtracted from those obtained at 37°C. Radioactivity in the capillaries and medium after reincubation is expressed as nmol of sphingomyelin/mg of capillary protein. Data are mean ± SE (bars) values from at least three experiments.

pared with ^{14}C was recovered in the aqueous phase after lipid extraction by the method of Folch et al. (1957). This suggests that intracellular hydrolysis of sphingomyelin and release of tritiated phosphocholine or choline into the medium were responsible for this excess of ^{3}H. The separation by TLC of the medium lipids was carried out only at the 237 nmol/ml concentration of substrate. These results (data not shown) indicated that ^{3}H was located in sphingomyelin and that ^{14}C was mainly located in sphingomyelin and to a small extent (10–18%) in the ceramide plus free fatty acid fraction. These data suggest a displacement or exchange of intact sphingomyelin and a release of some of the hydrolysis products (preferentially the phosphocholine moiety) into the medium.

DISCUSSION

This is the first study to document the interaction of lipoprotein with brain capillaries. The present data show that isolated brain capillaries are able to take up intact sphingomyelin and metabolize it. Uptake of sphingomyelin by other cells has been reported (Spence et al., 1983; Levade and Gatt, 1987; Bentejac et al., 1988). We previously reported (Carré et al., 1985) that cerebral capillaries possess both neutral and acid sphingomyelinases and that neutral sphingomyelinase (membrane bound) has higher affinity for sphingomyelin. However, the present results show that in brain capillary endothelial cells, unlike in other isolated cell types, such as fibroblasts (Spence et al., 1983; Vanier et al., 1985; Levade and Gatt, 1987), or in the brain in vivo (Bentejac et al., 1988), [^{3}H]phosphocholine (or [^{3}H]choline) released after hydrolysis is not reutilized to form phosphatidylcholine, although the existence of phosphocholine transferase in the plasma membrane has been established (Voelker and Kennedy, 1982; Kudoh et al., 1983; Marggraf and Kanfer, 1984).

Several explanations are possible: (a) This enzyme is not active in isolated capillaries. (b) The rate of phosphatidylcholine biosynthesis is low in isolated capillaries. (c) The main hydrolysis site is located elsewhere than in the plasma membrane, i.e., in lysosomes. (d) Sphingomyelin hydrolysis is located on the external cell side, so the phosphocholine moiety would be released in the medium and could not be reutilized. In our in vivo experiments, 24 h after injection of HDL [(*methyl*-^{3}H)*choline*]sphingomyelin, 77% of the capillary radioactivity was recovered in phosphatidylcholine. This figure could bear out the second hypothesis and rule out the first one. However, it could be the result of an uptake of lyso- or phosphatidylcholine from the plasma after redistribution of radioactivity by the liver, as has been observed in the whole brain (Bentejac et al., 1989). In this case, the first hypothesis could be accepted.

Because most of the ^{14}C hydrolysis products were recovered in the capillary lipids and because existence of an active and saturable choline transport in the blood–brain barrier has been reported (Cornford et al., 1978; Pardridge et al., 1979; Braun et al., 1980), the fourth hypothesis could be discarded. However, from an anatomic point of view, it can also be presumed that the fate of the sphingomyelin hydrolysis products is dependent on the uptake site: luminal or abluminal. When uptake is luminal, sphingomyelin is first hydrolyzed by neutral sphingomyelinase, and the phosphocholine moiety is reutilized to form phosphatidylcholine, whereas abluminal uptake of sphingomyelin (predominant in in vitro experiments) could lead to a loss of hydrolysis products from capillary endothelial cells.

Whatever the true explanation, the capacity of brain capillaries to hydrolyze sphingomyelin could be of some importance in the understanding of Niemann-Pick and Farber's diseases. Under physiological conditions, brain capillaries could control the flux of sphingomyelin through the blood–brain barrier. Further experiments are needed to verify these points.

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