Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 14-19

METABOLISM OF SPHINGOMYELIN BY INTACT CULTURED FIBROBLASTS: DIFFERENTIATION OF NIEMANN-PICK DISEASE TYPES A AND B

Arthur L. Beaudet and Aesta A. Manschreck

Departments of Pediatrics and Cell Biology,
Baylor College of Medicine, Houston, Texas 77030

Received January 18, 1982

When [choline-methyl-^{14}C]sphingomyelin was added to tissue culture medium containing human serum, there was efficient uptake of the phospholipid by cultured human fibroblasts. The great majority of the sphingomyelin was hydrolysed by normal cells, but not by cells from patients with Niemann-Pick disease type A suggesting that the phospholipid entered the lysosome and was hydrolysed by lysosomal sphingomyelinase. Studies comparing fibroblasts from Niemann-Pick disease types A and B demonstrated substantially greater hydrolysis by Niemann-Pick type B cells, indicating that the phenotypic differences in the two diseases are explained by residual sphingomyelinase activity in intact type B cells.

Niemann-Pick disease is a sphingomyelin lipidosis which has been separated into types A through F based on clinical and biochemical differences (1,2). Deficiency of lysosomal sphingomyelinase was demonstrated as the enzymic defect in Niemann-Pick type A (3), and was demonstrated subsequently in type B disease also (4). Niemann-Pick type A is a severe, fatal disorder with neurologic involvement while type B disease is a relatively benign disorder without neurologic involvement. Both diseases are associated with a severe reduction of sphingomyelinase activity in extracts of tissues and cultured cells. Some reports suggest detectable but very low levels of activity in Niemann-Pick type B cells as compared to virtual absence of activity in type A cells (4,5). Other reports detect no significant sphingomyelinase activity in cells from Niemann-Pick type B patients (6), and the biochemical basis of the different phenotypes has remained problematical. It was hypothesized that two forms of the enzyme might occur with one form being present in the brain in type B patients but not type A patients (7). A subsequent report suggested that one form of the enzyme represented an aggregate of the other (8). A recent analysis of sphingomyelinase activity in the brain of a fetus with type B disease demonstrated no detectable sphingomyelinase activity (6). This was strong evidence against a central nervous system form of enzyme which was spared in type B disease. We present evidence here that residual sphingomyelinase activity in intact cultured fibroblasts explains the phenotypic differences between Niemann-Pick disease types A and B.

0006-291X/82/050014-06$01.00/0

METHODS

Cell lines GM 112, GM 370, GM 406 and GM 3252 were obtained from the Genetic Mutant Cell Repository, Camden NJ. Control fibroblasts and those from Niemann-Pick B patient MT (sib of GM 3393) were established in this laboratory. The cell line JP was from a patient with typical type B disease and was provided by Dr. William Sly. Fibroblasts were grown in an atmosphere of 10% CO_2, in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. Cells were analysed regularly for mycoplasma contamination by fluorescent staining (9). Cells were plated in 60 mm dishes at low density, allowed to grow, and were studied 3 to 10 days after achieving confluence.

The [choline-methyl-^{14}C]sphingomyelin (specific activity 58 mCi/mmol) was either purchased or was prepared in our laboratory by Dr. Andrew Alpert according to Stoeffel (10) using [^{14}C]methyl iodide (specific activity 58mCi/mmol). Radioisotopes were obtained from New England Nuclear, Boston, MA. The sphingomyelin was delivered to medium without serum by adding 0.3 uCi in 5 ul of 95% ethanol per 1 ml of medium. A 10% volume of human fasting serum was then added to complete the incorporation medium. Similar results were obtained if the phospholipid was added in aqueous solution to the medium followed by sonication. Experiments were initiated by removal of tissue culture medium, washing once with phosphate buffered saline and addition of 1.1 ml of incorporation medium per 60mm plate. Experiments were terminated by removal of the radioactive medium, washing the plate 3 times with phosphate buffered saline and scraping the plate with 0.6 ml of water using a teflon policeman. The resultant extracts were sonicated for 10 seconds x 3 using a Branson sonifier set at 20 watts. After sonication, the content of [^{14}C]label was determined by scintillation counting and the proportion of counts taken up by the cells was designated as the percentage uptake. The proportion of counts which were TCA soluble (11) served as a rapid index of the percentage of hydrolysis of sphingomyelin by the cultured cells, but these data are not presented here. Aliquots of 25 ul were applied for thin layer chromatography on Polygram silica gel G sheets (Brinkmann Instrument, Inc., Westbury, N.Y.) with development in chloroform: methanol: water, 65:25:4. The chromatograms were exposed approximately 48 hours for autoradiography with Kodak SB-5 film and radioactive spots were cut out for scintillation counting. Treatments of radioactive products with phospholipases A_2 and C were performed as previously described (12).

RESULTS

When sphingomyelin was added to tissue culture medium containing human serum and incubated with cultured human fibroblasts, there was a substantial uptake of [^{14}C]sphingomyelin with up to 30% of radioactivity in the medium being taken up in a 24 hour period. The uptake of sphingomyelin was approximately linear over a 24 hour period. There was no consistent difference in uptake of radioactivity between fibroblasts from controls and from Niemann-Pick disease patients (Table I). The method used for determination of "uptake" by the cultured cells does not distinguish between internalization of the sphingomyelin, binding of the sphingomyelin to the surface (perhaps as part of a lipoprotein complex) or exchange with the plasma membrane. In other experiments we have evidence that the lipoprotein content of the medium markedly influences the fate of the sphingomyelin.* In experiments with whole human serum, evidence

*Alpert AJ and Beaudet AL, unpublished data.

TABLE I

UPTAKE AND HYDROLYSIS OF [^{14}C]SPHINGOMYELIN BY CULTURED FIBROBLASTS*

CELLS	UPTAKE (CPM x 10^{-3}/PLATE)			% SPHINGOMYELIN HYDROLYSED		
	3H	6H	24H	3H	6H	24H
CONTROL						
W.S.	14.1	52.7	130	51.5	74.7	92.8
C.C.	17.7	49.6	96.7	56.1	77.6	90.3
R.N.	13.6	34.0	73.4	30.5	60.2	79.4
NIEMANN-PICK A						
GM 406	8.71	29.6	136	1.7	3.3	7.5
GM 307	26.5	68.3	205	1.0	2.1	6.5
GM 112	15.4	36.6	141	0.0	0.0	1.8
NIEMANN-PICK B						
GM 3252	18.3	63.0	237	7.2	10.9	15.3
J.P.	13.9	32.1	115	6.6	18.9	36.8
M.T.(sib of GM3393)	16.2	42.2	112	5.1	19.6	49.6

* For each group, the first two cell lines were studied in a single experiment using [^{14}C]sphingomyelin from Amersham. The third cell line in each group was studied in a second experiment with [^{14}C]sphingomyelin synthesized in our laboratory.

is presented below that the bulk of the radioactivity designated as uptake in these experiments is internalized as evidenced by further lysosomal metabolism.

The fate of radioactive sphingomyelin taken up by cultured human fibroblasts was determined by recovery of the cells and fractionation of radioactive components by thin layer chromatography. Radioactive sphingomyelin was rapidly hydrolysed by control fibroblasts (Figure 1). The major product of hydrolysis after 3 hours of incubation migrated with an R_f identical to that of phosphorylcholine. Much lesser amounts of material with an R_f identical to that of choline were present routinely but phosphorylcholine and choline were cut out from the thin layer chromatograms as a single spot for scintillation counting. At 3 hours there was also a significant amount of material with an R_f identical to that of phosphatidylcholine. In order to confirm the identity of the putative phosphatidylcholine, the product was incubated with phospholipases A_2 and C to yield the expected products with R_f's compatible with lysophosphatidylcholine and phosphorylcholine respectively. With increased incubation time, there was a progressive accumulation of phosphatidylcholine and a progressive decrease in the percentage of counts in sphingomyelin. These data indicated a hydrolysis of sphingomyelin to yield primarily phosphorylcholine which was subsequently incorporated into phosphatidylcholine. Addition of nonradioactive choline to the tissue culture medium gave only a tiny decrease in the accumulation of radioactive phosphatidylcholine. These data suggested that the sphingomyelin was hydrolysed by sphingomyelinase. This was consistent with

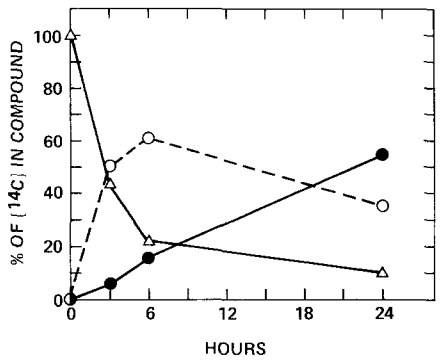

Figure 1. Time course of metabolism of [^{14}C]sphingomyelin by normal cultured fibroblasts. The uptake and thin layer chromatographic analysis were performed as described in Methods. The total uptake as [^{14}C]cpm per plate was 13,900 at 3 hours, 32,200 at 6 hours and 115,500 at 24 hours. Symbols are △, sphingomyelin; ○, phosphorylcholine (plus traces of choline); and ●, phosphatidylcholine.

the fact that sphingomyelin was not hydrolysed by fibroblasts which were deficient in lysosomal sphingomyelinase. All experiments were conducted with continuous incorporation so that the total counts at each time point were increasing, and the data presented in Figure 1 indicate what percentage of the counts taken up by the cells was present in various compounds.

The metabolism of radioactive sphingomyelin also was studied in fibroblasts from patients with Niemann-Pick types A and B. All of the cell lines utilized had less than 5% of control levels of sphingomyelinase activity by our determination, when assayed with a radioactive natural substrate and extracts of the cultured cells (11). Intact cells from patients with Niemann-Pick disease type A showed a marked deficiency of hydrolysis of [^{14}C]sphingomyelin. The difference between the Niemann-Pick cell lines and the control cell lines indicated that the bulk of the sphingomyelin taken up was hydrolysed by a pathway which was deficient in the Niemann-Pick A cells, presumably involving lysosomal sphingomyelinase. The same type of experiments showed a consistently greater level of sphingomyelin hydrolysis by intact cells from patients with Niemann-Pick disease type B compared to type A. These differences were consistent and substantial when a number of Niemann-Pick cell lines were compared using multiple preparations of [^{14}C]sphingomyelin. There was no difficulty in distinguishing Niemann-Pick disease fibroblasts of the A and B types whether one used data at 3 hours, 6 hours, or 24 hours. Thus, we observed very low or undetectable levels of sphingomyelinase activity in extracts of cultured fibroblasts from patients with Niemann-Pick A and B disease, but there was substantially greater hydrolysis of sphingomyelin by intact cultured fibroblasts from patients with Niemann-Pick disease type B.

DISCUSSION

The uptake and metabolism of sphingomyelin by cultured cells has been studied relatively little. Gatt and Bierman (13) examined the uptake of liposomes containing sphingomyelin and octadecylamine, and they observed that 97% of sphingomyelin taken up in this way was not hydrolysed. The intracellular localization of the sphingomyelin was not established in those experiments. The sphingomyelin might not have entered the lysosome, or the octadecylamine in the liposome might have impaired hydrolysis by intact cells. In contrast, we have observed extensive hydrolysis of sphingomyelin by intact cultured fibroblasts. The defective hydrolysis in cells from Niemann-Pick type A patients strongly suggests that sphingomyelin entered the lysosome where it was hydrolysed by sphingomyelinase. Ongoing studies using bovine serum albumin and various lipoprotein fractions suggest that specific lipoproteins are important in targeting the sphingomyelin to the lysosome. This experimental approach may be useful in defining a lipoprotein mediated pathway for intracellular metabolism of sphingomyelin similar to the LDL pathway for cholesteryl esters. The observation that lysosomal sphingomyelinase can be activated by apolipoprotein C-III-1 in vitro (14) may be relevant.

The fact that Niemann-Pick diseases A and B can be distinguished based on residual hydrolytic capacity of intact cells offers a more satisfactory explanation for the clinical differences in the disorders. The result is not surprising or unique among lysosomal storage diseases. There is considerable analogy to the biochemical basis for the difference between infantile and adult Gaucher disease, Hurler and Scheie mucopolysaccharidosis, and Wolman disease and cholesteryl ester storage disease. In these examples, dramatically different phenotypes are associated with the same enzyme deficiency. Significant residual enzyme activity can be easier, (e.g. Gaucher disease) or more difficult (e.g. cholesteryl ester storage disease) to demonstrate in vitro. The analogy to Wolman and cholesteryl ester storage disease is striking since residual acid lipase is difficult to demonstrate in either disease, but metabolism of cholesteryl esters by intact cultured cells is much greater for cholesteryl ester storage disease than for Wolman disease (15). Similarly, the severity of the phenotype in metachromatic leukodystrophy correlates well with the intracellular metabolism of cerebroside sulfate, while assays of cell extracts show very low activity for all phenotypes (16). We favor the explanation that the mutation in Niemann-Pick type B disease results in altered enzyme which has residual activity. This activity may be difficult to quantitate in vitro and may behave variably when exposed to different methods for extract preparation and enzyme assay. This explanation would assume that the brain is protected more easily than the viscera by low levels of residual

enzyme activity. Sphingomyelin content in the brain in Niemann-Pick type B disease has not been studied extensively. The content in brain from a type B fetus showed a minimal increase similar to that found in fetal brain from type A disease. Sphingomyelin accumulation is greater in liver than in brain in type A Niemann-Pick disease, and we suggest that the level of residual activity in type B disease is sufficient to prevent neurologic but not visceral symptoms.

We favor the view that Niemann-Pick disease types A and B are allelic, but this has not been tested directly. We have conducted preliminary studies with cells considered to represent type C or "atypical" Niemann-Pick disease. These studies have indicated normal hydrolysis and have not clarified the basis for the occurrence of Niemann-Pick disease with normal or substantial levels of sphingomyelinase activity. The experimental approach used here would allow testing of a rather speculative hypothesis that some forms of Niemann-Pick disease could be due to an alteration in an apolipoprotein which normally activates sphingomyelinase (14).

ACKNOWLEDGEMENT

We want to thank Dr. Andrew J. Alpert for helpful discussions. The work was supported by a grant from the USPHS, GM-25397.

References

1. Brady, R.O. (1978) in The Metabolic Basis of Inherited Disease, 4th ed., pp. 718-730 (Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S., Eds., McGraw-Hill, New York.
2. Schneider, E.L., Pentchev, P.G., Hibbert, S.R., Sawitsky, A., and Brady, R.O. (1978) J. Med. Genet. 15,370-374.
3. Brady, R.O., Kanfer, J.N., Mock, M.B., and Fredrickson, D.S. (1966) Proc. Natl. Acad. Sci. U.S.A. 55,366-369.
4. Schneider, P.B., and Kennedy, E.P. (1967) J. Lipid Res. 8,202-209.
5. Sloan, H.R., Uhlendorf, J.N., Kanfer, R.O., and Fredrickson, D.S. (1969) Biochem. Biophys. Res. Commun. 34,582-588.
6. Wenger, D.A., Kudoh, T., Sattler, M., Palmieri, M., and Yudkoff, M. (1981) Am. J. Hum. Genet. 33,337-344.
7. Yamaguchi, S., and Suzuki, K. (1977) Biochem. Biophys. Res. Commun. 77,999-1003.
8. Yamanaka, T., Hanada, E., and Suzuki, K. (1981) J. Biol. Chem. 256,3884-3889.
9. Del Giudice, R.A., and Hopps, H.E. (1978) in Mycoplasma Infection of Cell Cultures, pp. 57-69, (McGarrity, G.J., Murphy, D.G. and Nichols, W.W., Eds.) Plenum Press, New York.
10. Stoeffel, W. (1975) Methods Enzymol. 35,533-541.
11. Kanfer, J.N., Young, O.M., Shapiro, D., and Brady, R.O. (1966) J. Biol. Chem. 241,1081-1084.
12. Beaudet, A.L., Hampton, M.S., Patel, K., and Sparrow, J.T. (1980) Clin. Chim. Acta 108,403-414.
13. Gatt, S., and Bierman, E.L. (1980) J. Biol. Chem. 255,3371-3376.
14. Alpert, A.J., and Beaudet, A.L. (in press) J. Clin. Invest.
15. Goldstein, J.L., and Brown, M.S. (1977) Annu. Rev. Biochem. 46,897-930.
16. Porter, M.T., Fluharty, A.L., Trammell, J., and Kihara, H. (1971) Biochem. Biophys. Res. Commun. 44,660-666.