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Impaired Cholesterol Esterification in Primary Brain Cultures of the Lysosomal Cholesterol Storage Disorder (LCSD) Mouse Mutant

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Esterification of cholesterol was investigated in primary neuroglial cultures obtained from newborn lysosomal cholesterol storage disorder (LCSD) mouse mutants. An impairment in ^{3}H-oleic acid incorporation into cholesteryl esters was demonstrated in cultures of homozygous LCSD brain. Primary cultures derived from other phenotypically normal pups of the carrier breeders esterified cholesterol at normal levels or at levels which were intermediary between normal and deficient indicating a phenotypic expression of the LCSD heterozygote genotype. These observations on LCSD mutant brain cells indicate that the defect in cholesterol esterification is closely related to the primary genetic defect and is expressed in neuroglial cells in culture.

The lysosomal cholesterol storage disorder (LCSD) mouse is an autosomal recessive murine mutant which displays neurovisceral lesions characterized by attenuated lysosomal sphingomyelinase and glucocerebrosidase activities, intralysosomal storage of free cholesterol, sphingomyelin, glucocerebroside and lactosylceramide

Abbreviations: ACAT, acyl-CoA: cholesterol acyltransferase; LCSD, lysosomal cholesterol storage disorder.

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(1-3) and dysmyelination within the central nervous system (4). Studies on cultured fibroblasts from the LCSD mutant indicate that the primary genetic lesion is closely linked to a rate limiting step in cholesterol esterification subsequent to delivery of cholesterol to lysosomes (3,5). A similar defect in cholesterol processing has been demonstrated in the human lysosomal storage disorder, Niemann-Pick disease, type C (6-8) for which the LCSD mouse is an animal model. In the present study we investigated cholesterol esterification in brain cells in primary culture and demonstrate a block in esterification of cholesterol in cells from LCSD brain.

Materials and Methods

Materials: [^{3}H]-oleic acid (specific activity 2.6 Ci/mmol) and cholesteryl oleate [oleate-1-^{14}C] (specific activity 56.6 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Oleic acid, triolein, and cholesteryl oleate were obtained from Sigma Chemical Company, St Louis, MO. Cholesterol was purchased from Supelco, Inc., Bellafonte, PA. Dulbecco's modified Eagle medium, gentamycin and trypsin were obtained from Gibco Laboratories, Grand Island, NY. Fetal bovine serum was purchased from Flow Laboratories, McLean, VA.

Mice: The affected animals were obtained from the Developmental and Metabolic Neurology Branch (DMNB) of the National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health (NINCDS, NIH), Bethesda, Maryland. LCSD homozygotes were obtained by mating proven carrier heterozygotes. The colony was propagated by brother-sister matings of phenotypically normal offspring born to known heterozygote carriers of the LCSD trait. Control mice were derived from a separate colony obtained from a BALB/c breeding stock.

Identification of affected mice: Affected (homozygote) pups in a litter from heterozygote parents were identified by their deficient hepatic lysosomal sphingomyelinase activity (1). The enzyme assay was carried out in liver homogenates using [^{14}C]-sphingomyelin as substrate by the method of Pentchev et. al. (9).

Cell culture: Primary cultures of mouse brain were obtained by the procedure adapted from Sensenbrenner (10). One day-old mice were decapitated, the brains removed and the cerebral hemispheres isolated. The hemispheres were stripped of meninges, minced in Dulbecco's modified Eagle medium and cells mechanically dissociated by pipetting through pasteur pipettes. The cells were resuspended in DMEM-gentamycin containing 10% fetal calf serum and plated on 6-well, 35mm tissue culture plates coated with poly D-lysine. Cells from each brain were plated at a density of two wells/brain. The cultures were grown at 37^{o}C in a humidified air and CO_2 incubator. The medium was initially changed after three days in culture. On day 6, the culture medium was replaced with

serum-free medium in order to deplete the cells of lipids. At the end of 24 hours, the cells were incubated without or with cholesterol dissolved in ethanol and added at a concentration of 50 ug cholesterol/ml medium.

[^{3}H]-oleic acid incorporation: The incorporation of [^{3}H]-oleic acid into cellular cholesteryl oleate was measured by incubating the cells with [^{3}H]-oleic acid (12.5 mM oleic acid in 12% bovine serum albumin and 0.9% NaCl; specific activity 200 dpm/pmol) (3,11). At the end of the specified time, the medium was removed and the cells washed three times with phosphate buffered saline (PBS). The cells were dissociated with 0.1% trypsin, 0.05% EDTA at 37^{o}C for 10 min. Proteolytic action was terminated by the addition of medium supplemented with 10% FCS. Cells pelleted by centrifigation were washed twice with PBS and the washed pellets disrupted by sonication. Carrier lipids and cholesteryl [^{14}C]-oleate as internal standard were added and lipids extracted with chloroform:methanol (2:1 v/v) by the method of Folch, et al.(12). After evaporation of chloroform under nitrogen, neutral lipids were separated by thin layer chromatography using the solvent system hexane:ether:glacial acetic acid (90:10:1). The individual lipid components were identified by exposure to iodine vapour and cholesteryl oleate and triolein spots scraped off and radioactivicy counted by scintillation counting.

Results and Discussion

Table 1 indicates the base line level of [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate and [^{3}H]-triolein in primary cultures of of newborn normal and affected mice brains.

TABLE 1

Cholesteryl (^{3}H)-oleate and (^{3}H)-triolein synthesis in normal and LCSD mutant brain cells in culture

Mice	Hepatic Sphingomyelinase (nmol/hr/mg protein)	Incorporation in brain cells in primary culture	
		(^{3}H)-oleic acid into cholesteryl oleate (pmol/hr/mg protein)	(^{3}H)-oleic acid into triolein (pmol/hr/mg protein)
Normal (3)	18.95 ± 0.595	234 ± 22.8	4,472 ± 559
Affected (1)	6.31	14.38	5,355

Primary brain cultures from one-day old LCSD homozygotes and normals were obtained as described in methods.On day 6, the medium was replaced with serum-free medium and the cells incubated for 24 hours followed by incubation with [^{3}H]-oleic acid (12.5 mM in 12% bovine serum albumin and 0.9% NaCl; specific activity 200 dpm/pmole) for 2 hours. The rate of [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate was determined as described in methods.

Results are expressed as mean ± S.E.M.

TABLE 2

Synthesis of cholesteryl (^{3}H)-oleate from (^{3}H)-oleic acid in the presence and absence of cholesterol

Mice	Hepatic sphingomyelinase (nmol/hr/mg protein)	(^{3}H)-oleic acid incorporation into cholesteryl oleate in brain primary cultures (pmol/hr/mg protein)	
		- Cholesterol	+ Cholesterol*
Normal (1)	18.72	225	944
Affected (2)	7.29 ± 0.56	58 ± 2	332 ± 33

Primary brain cultures from one-day old LCSD homozygotes and normals were obtained as described in methods. On day 6, the medium was replaced with serum-free medium and the cells incubated for 24 hours followed by incubation with or without cholesterol (50 ug/ml medium) added in 10 ul ethanol for a further 24 hours. [^{3}H]-oleic acid (12.5 mM in 12% bovine serum albumin and 0.9% NaCl; specific activity 200 dpm/pmole) was added and the incubation carried out for a further 2 hours. The rate of [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate was determined as described.

* 50 ug/ml cell culture medium.
Results are expressed as mean ± S.E.M.

Cells derived from affected brains showed markedly attenuated [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate. In contrast, [^{3}H]-oleic acid incorporation into [^{3}H]-triolein was comparable in affected and normal cells suggesting normal triglyceride synthesis and indicating that the murine lesion is specifically associated with cholesteryl ester synthesis. As previously reported (1), lysosomal sphingomyelinase activity was found to be deficient in the livers of homozygous mutants and assayed to be at 33% of normal levels. Table 2 shows the effect of exogenous non-lipoprotein cholesterol on [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate in affected and normal brain cells. Both the basal level and cholesterol-stimulated incorporation of [^{3}H]-oleic acid were found to be deficient in LCSD brain cells. The current finding of an impairment in cholesteryl ester synthesis in LCSD mutant brain cells supports

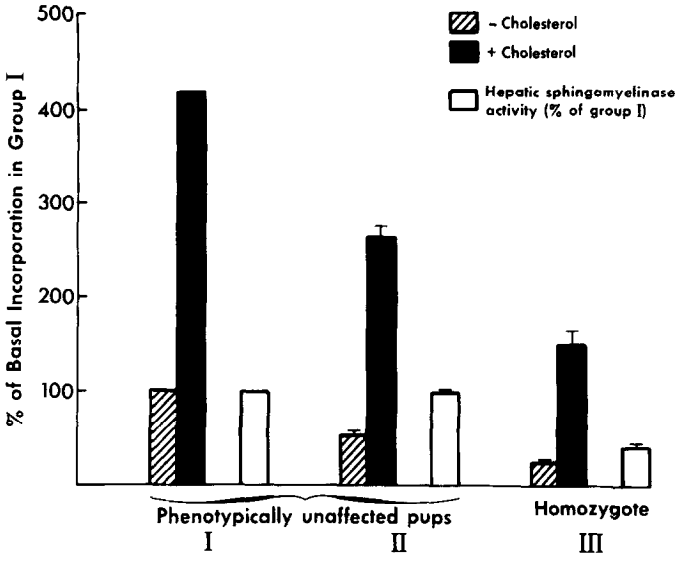

Fig. 1. Incorporation of [^{3}H]-oleic acid into cellular cholesteryl [^{3}H]-oleate in brain cells of newborn mice derived from a LCSD carrier breeder. Cells were incubated without or with 50 ug cholesterol/ml medium for 24 hours followed by incubation with [^{3}H]-oleic acid (12.5 mM in 12% bovine serum albumin and 0.9% NaCl; specific activity 200 dpm/pmol) for 2 hours and processed as described in methods. Values are represented as % of basal incorporation of [^{3}H]-oleic acid into cholesteryl [^{3}H]-oleate, expressed as pmol/hr/mg protein in group I. Sphingomyelinase activity is represented as % of activity in nmol/hr/mg protein in group I. Results are mean ± SEM; n: group I=1; group II=2; group III=2.

the earlier hepatic uptake studies (3) and cultured fibroblast investigations (3,5) and suggests that this mutation represents a broad and general lesion in the normal cellular processing of cholesterol.

Figure 1 depicts additional data on cholesterol esterification levels in primary brain cell cultures derived from 1 day-old animals from a litter from known heterozygous parents. This particular litter consisted of 5 animals. Assay of liver sphingomyelinase activity again indicated that the animals could be phenotypically differentiated into two groups. One group of three animals had normal enzyme activity and the other group of tw animals had 39% of control activity. The latter group with attenuated hepatic sphingomyelinase activity was identified as genotypically representing the LCSD/LCSD homozygous. On the basis

of [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate it appears possible to separate the sphingomyelinase-normal animals into two groups: group I having relatively high levels of cholesteryl ester synthesis (100% basal and 420% cholesterol-stimulated) and group II displaying intermediate levels of ester synthesis (52 ± 3% basal and 265 ± 10% cholesterol-stimulated). Homozygotes had distinctly low levels of cholesterol ester synthesis (26 ± 1% basal and 148 ± 15% cholesterol-stimulated). Cultured murine fibroblasts derived from obligate heterozygous carrier mice have been shown to express a partial deficiency in cholesteryl ester synthesis (5). Consequently, we interpret the results of the present study as possibly indicating that group I animals represent wildtype and that group II represents putative heterozygotes. Figure 2 shows [^{3}H]-oleic acid incorporation into cholesteryl [^{3}H]-oleate in brain cells in culture derived from a

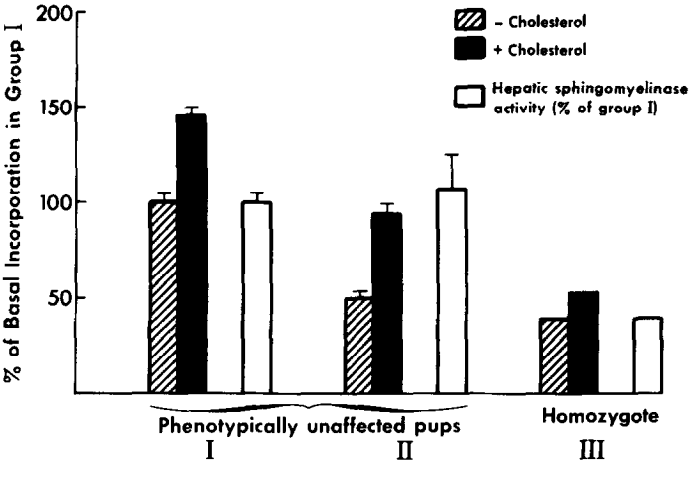

Fig. 2. Incorporation of [^{3}H]-oleic acid into cellular cholesteryl [^{3}H]-oleate in cultured brain cells of newborn mice derived from a separate LCSD carrier breeder. Cells were incubated without or with cholesterol (50 ug/ml medium) together with [^{3}H]-oleic acid (12.5 mM in 12% bovine serum albumin and 0.9% NaCl; specific activity 200 dpm/pmol) for 24 hours and processed as described. Values are represented as % of basal incorporation of [^{3}H]-oleic acid into cholesteryl [^{3}H]-oleate, expressed as pmol/hr/mg protein in group I. Sphingomyelinase activity is represented as % of activity in nmol/hr/mg protein in group I. Results are mean ± SEM; n: group I=6; group II=2; group III=1.

separate litter of newborn mice. This litter consisted of 9 animals, one of which could be identified as homozygous by its deficient liver sphingomyelinase activity. In this experiment, cells were continuously labelled with [^{3}H]-oleic acid in the absence or presence of cholesterol. The level of cholesteryl [^{3}H]-oleate formation could be differentiated into three groups. A group of six primary cultures expressed an activity which was higher than the remaining cultures (100 ± 4% basal and 145 ± 6% cholesterol-stimulated). One group of primary brain cultures derived from a hepatic sphingomyelinase-deficient pup (III) expressed a level of esterification which was 39% of the basal cholesteryl ester synthesis in group I and 50% of the level with cholesterol stimulation. Another group (II) of cultures derived from two pups displayed a level of esterification which was intermediary between that of group I and group III (50 ± 4% basal and 94 ± 6% cholesterol-stimulated). This differentiation according to levels of intracellular cholesterol ester synthesis is consistent with a genotypic classification of groups I, II and III as representing wildtype, heterozygous LCSD and homozygous LCSD. The earlier observations (3,5) support our present studies which indicate that cells of neural origin also express a metabolic defect in cholesterol esterification which is closely linked to the primary LCSD mutation.

The LCSD brain displays an array of neurochemical changes affecting gangliosides, neutral glycolipids and the pattern of long-chain fatty acids in cerebrosides (4). These changes are accompanied by hypomyelination in the absence of reactive gliosis. A possible explanation for the pleiotropic neurochemical alterations and the dysmyelination characteristic of the LCSD mouse is that a specific lesion in cholesterol homeostasis critically affects the normal synthesis and/or assembly of myelin components.

Studies on fibroblasts from the LCSD mutant have indicated that the defect in cholesterol esterifictaion could not be accounted for by attenuation in the *in vitro* activity of acyl-CoA: cholesterol acyltransferase (ACAT), the enzyme which catalyzes the formation cholesteryl esters from cholesterol and fatty acyl CoA. Investigations are in progress to determine whether the observed impairment of cholesterol esterification in primary brain cell cultures of the LCSD mutant can be traced to defective ACAT activity. However, other disturbances in the regulatory processes mediating cellular cholesterol homeostasis in brain must also be considered as plausible mechanisms associated with this genetic defect.

Acknowledgements

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References

1. Pentchev, P.G., Gal, A.E., Boothe, A.D., Omedeo-Sale,F., Fouks., Neumeyer, B.A., Quirk, J.M., Dawson, G. and Brady, R.O. (1980) Biochim. Biophys. Acta 619, 669-679.
2. Morris, M.D., Bhuvaneswaran, C., Shio, H. and Flower, S. (1982) Am. J. Pathol. 108, 140-149.
3. Pentchev, P.G., Boothe, A.D., Kruth, H.S., Weintraub, H., Stivers, J. and Brady, R.O. (1984) J. Biol. Chem. 259, 5784-5791.
4. Weintraub, H., Abramovici, A., Sandbank, U., Pentchev, P.G.,Brady, R. O., Sekine, M., Suzuki, A. and Sela, B. (1985) J. Neurochem. 45, 665-672.
5. Pentchev, P.G., Comly, M.E., Kruth, H.S., Patel, S.C., Proestel, M. and Weintroub, H. (1986) J. Biol. Chem. 261, 6:2772-2777.
6. Pentchev, P.G., Comly, M.E., Kruth, H.S., Vanier, M.T., Wenger, D.A., Patel S.C. and Brady, R.O. Proc. Natl Acad Sci, USA, 1985: 82, 8247-8251.
7. Kruth, H.S., Comley, M.E., Butler, J.D., Vanier, M.T., Fink, J.K., Wenger, D.A., Patel, S.C. and Pentchev, P.G. (1986) J. Biol. Chem:261,35:16769-16774.
8. Pentchev, P.G., Kruth, H.S., Comly, M.E., Butler, J.D., Vanier, M.T., Wenger, D.A. and Patel, S.C. (1986) J. Biol. Chem: 261,35:16775-16780.
9. Pentchev P., Brady R.O., Gal A.E. and Hibbert S.R. (1977) Biochim. Biophys. Acta 488, 312-321.
10. Sensenbrenner, M., Maderspach, K., Latzkovits, L. and Jaros, G.G. (1978). Develop. Neurosci. 1, 90-101.
11. Goldstein, J,.L., Basu, S.K. and Brown, M.S. In Methods in Enzymology eds. Fleischer, . and Fleischer, B. (1983) Academic Press, NY. 98:241-260.
12. Folch-Pi, J., Lees, M.B. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.