Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 440-446

MYELIN PROTEINS: DEGRADATION IN RAT BRAIN INITIATED BY METABOLITES CAUSATIVE OF MAPLE SYRUP URINE DISEASE

Diane Tribble and Raymond Shapira

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322

Received May 27, 1983

SUMMARY: Maple syrup urine disease (MSUD), an inborn error of metabolism in humans, is expressed as an inability to oxidatively decarboxylate the branched-chain α-keto acids derived from leucine, isoleucine and valine. Rats 14 days old were injected intracranially with a solution containing leucine, α-ketoisocaproate, and tracer amounts of ^{3}H-lysine. Myelin isolated from these rat brains at 28 days of age had a washed dry weight 85 per cent of controls. The protein content of the myelin prepared from treated and control rats was identical, as were the specific activities of the individual proteins separated by polyacrylamide gel electrophoresis. Myelin protein from treated rats was deficient in myelin high molecular weight proteins including glycoproteins, and degradation products of these proteins were observed in myelin of treated rats. MSUD associated metabolites in man may initiate a process leading to the proteolytic degradation of myelin proteins, thereby producing abnormal myelin sheaths.

Maple syrup urine disease (MSUD) affected children exhibit rigidity and seizures, and survivors are spastic and severely retarded. Brains of these children at autopsy are spongy, with marked changes in white matter. There is a decrease in myelin content and areas of focal astrocytosis are observed. (1) The relationship between the branched-chain α-keto acid accumulation in cerebrospinal fluid and the pathological changes in the brains of victims is unknown. (2) The myelinotoxic properties of the ketoacids has been demonstrated *in vitro* in myelinating cerebellar explants. (3) In this study, Silberberg showed that α-ketoisocaproate (ketoleucine) disrupted myelinating cultures at concentrations found in MSUD *in vivo*, suggesting that this was the major myelinotoxic metabolite. Liao *et al.*, reported that the α-ketoacids together with the amino acids elevated in MSUD had irreversible inhibitor effects on the cell cycle of a cloned glioma cell line with some properties of differentiating glia. (4).

Efforts to develop an animal model of MSUD have not been successful since normal animals readily metabolize injected branched-chain keto and amino acids in liver, thereby protecting the nervous system. By a similar mechanism, the MSUD fetus is protected in utero by the mother's ability to catabolize these metabolites. In an attempt to evaluate the effects of elevated MSUD metabolites on the nervous system, we have injected a mixture of L-leucine, α-ketoisocaproate and tracer amounts of ^{3}H-lysine directly into the brains of actively myelinating rats, and examined the myelin of these brains at near complete myelination. Our hypothesis is that a single specific insult to the developing nervous

0006-291X/83 $1.50

system at a crucial time might be sufficient to initiate the changes which lead to the pathology and symptomology of the disease. In this report, we document a significant loss of myelin proteins in the treated animals. This is the first animal experiment demonstrating significant alteration in myelin protein composition after exposure to MSUD metabolites. Myelin glycoproteins have been implicated in the synthesis of the myelin sheath and its compaction. (5) Degradation of myelin proteins could contribute to the pathogenesis of MSUD.

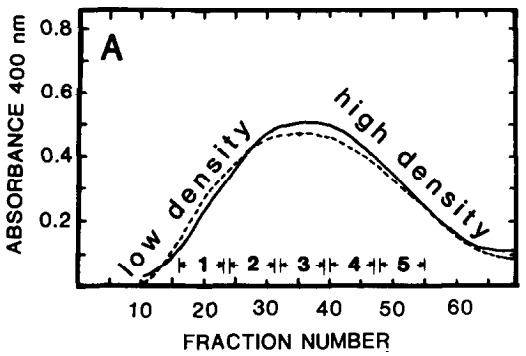
MATERIAL AND METHODS

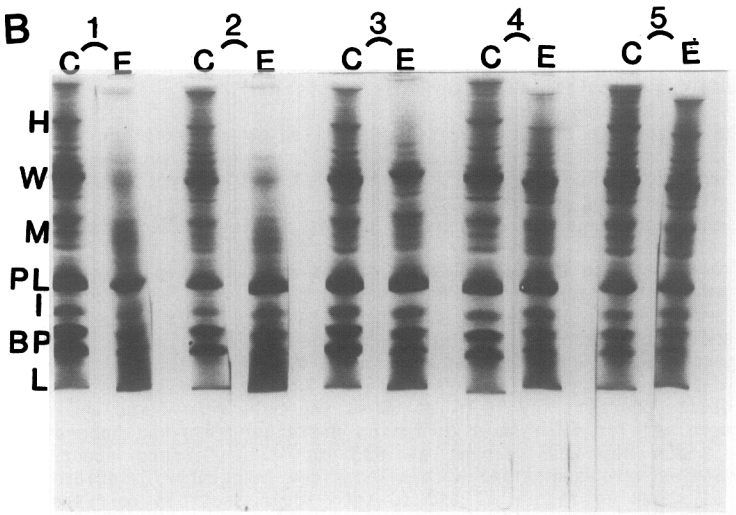
Fourteen days after birth, two Sprague-Dawley rat pups (Sprague-Dawley Inc., Indianapolis, Indiana) chosen at random from two litters each received a 30 ul intracerebral injection containing 0.5 mg L-leucine, 4.5 mg α-ketoisocaproic acid, sodium salt (Sigma Chemical Co., St. Louis, Missouri) and 1 mCi L-(4,5-H)-lysine (40 mCi/mmol, ICN Chemical and Radioisotope Division, Irvine, California) in freshly distilled water. Two weight-matched litter mates received 30 ul equimolar injection of sodium acetate (Mallinkrodt, Inc., St. Louis, Missouri) and 1 mCi of the labeled lysine. All injections were made midline between the ears to a depth of 3 mm with a 50 ul capacity syringe (Precision Sampling Corporation, Baton Rouge, Louisiana).

Two weeks after injection, the rats were killed by asphyxiation with carbon dioxide. Whole brains were immediately removed and washed in 5% (w/w) sucrose on ice. All subsequent steps were performed at 2-4°C; experimental and control brains were manipulated separately. Homogenation and separation of the myelin fractions by continuous sucrose density gradient centrifugation was carried out as previously described. (6) Fractions of 12 ml were collected and washed by centrifugation several times in distilled water. Individual fractions were combined into 5 groups of 8 fractions by taking 20 fractions from either side of the midpoint of the turbidity peak. (Fig. 1A) Each of the 5 fractions was lyophilized and delipidated with ether/ethanol (3:2v/v), and the proteins were solubilized and fractionated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as described by Greenfield et al (7) and Morell et al. (8) Approximately 100 g of the delipidated total myelin proteins in 100 l of protein solvent was applied to each gel. After electrophoresis for 10 h. at 60 v. the gels were fixed and stained with Fast Green, and destained for 60 h in a diffusion destainer (Bio-Rad Laboratories, Richmond, California). Each gel was scanned at 635 nm in a Gilford spectrophotometer, and individual proteins were quantified with a Technicon Integrator/Calculator. The gels were fractionated as shown in figure 1B and 1C into seven sections corresponding to (1) low molecular weight proteins (L), (2) basic proteins (BP), (3) intermediate proteins (I), (4) proteolipid protein (PL), (5) medium molecular weight proteins (M), (6) Wolfgram proteins (W), and (7) high molecular weight proteins (H). These sections were cut into 1 mm slices and solubilized as previously described. (9) The radioactivity was determined in a Beckman Model LS70000 liquid scintillation spectrometer. The specific activity of each sample was determined by dividing the area under the major densitometer peaks in the gel sections into the counts per minute for the corresponding section.

RESULTS

The myelin from the control rat brains (Fig. 1A, dashed line) showed a typical 28-day-old rat myelin pattern from sucrose density gradient centrifugation, (9) while the treated rat myelin (solid line) showed a decrease in light myelin and an increase in denser myelin. Myelin from the experimental animals constituted 85 per cent of the myelin from control animals on the basis of dry weight. After delipidation of the five myelin fractions from treated and control groups, 30-35 per cent of the dry weight was recovered. The delipidated myelin fractions were analyzed for protein content by digestion of aliquots in 6N HCl for 48 h. at 110° followed by amino acid analysis on a JEOL 6AH analyzer. The corresponding experimental and control fractions had identical amino acid





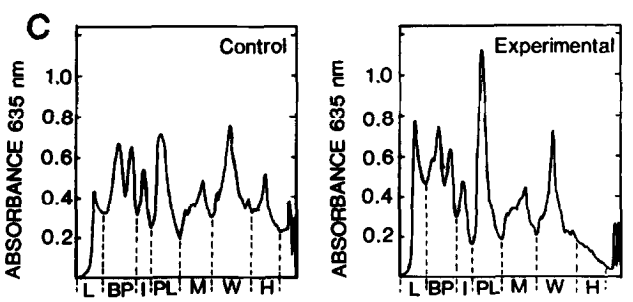


Figure 1. (A) Turbidity profiles of myelin fractions from sucrose density gradient centrifugation of homogenized rat brains, density fractions 1-5, low-high density, experimental treated rats (solid line), control rats (dashed line). (B) SDS-polyacylamide gels of proteins from myelin fractions 1-5 in A, experimental (E), control (C). (C) Densitometric scans of electrophoretic gels of myelin proteins from fraction 3, also showing where the gels were sliced for determination of radioactivity from incorporated ^{3}H-lysine. Low molecular weight proteins (L), basic proteins (BP), intermediate proteins (I), proteolipid protein (PL), medium molecular weight proteins (M), Wolfgram proteins (W), and high molecular weight proteins (H).

compositions. Equal weight of delipidated myelin were solubilized in SDS and separated by electrophoresis on 15% SDS polyacrylamide gels. (Fig. 1B and 1C) The major differences between all pairs of gels was a striking loss of high molecular weight proteins (H) in the myelin of the treated rats, and a corresponding increase in the low molecular weight proteins (L). In the treated animals' light myelin fractions (Fig. 1B, 1 and 2) less Wolfgram protein was seen. The peak density fractions densitometer tracings shown in figure 1C illustrates the major differences seen in all of the density fractions; the decrease in the major H bands and the increase in the L bands. Myelin glycoproteins were also visualized on SDS-polyacrylamide gel electrophoresis of the same fractions, and the periodic acid-Schiff-stained (10) tube gels confirmed the loss of glycoproteins.

Table 1 shows the counts per minute, Fast Green dye binding, and specific activity of each gel section. The dye binds PL and I proteins to a lower degree than the other

Table 1. Analysis of SDS-polyacrylamide electrophoresis gels from five density fractions of myelin from control rat brains (C1-C5) and experimental treated rat brains (E1-E5). Gels were stained with Fast Green and densitometry reported in Fast Green dye binding units (FGDB). Gels were sliced and radioactivity measured (CPM) as described in the test. Gel sections are described in Fig. 1C. Specific activity, (SA) CPM/FGDB, cannot be compared in absolute terms because the dye does not stain the myelin proteins equally

FRACTION	CPM	FGDB	SA	FRACTION	CPM	FGDB	SA
C1/L	380.4	21.4	17.78	E1/L	799.7	31.7	25.23
C1/BP	1235.2	57.5	21.48	E1/BP	1248.6	56.2	22.22
C1/I	419.8	11.2	37.48	E1/I	450.0	16.7	26.95
C1/PL	1384.8	39.3	35.24	E1/PL	1040.2	30.8	33.77
C1/M	660.2	40.1	16.46	E1/M	813.3	45.6	17.84
C1/W	1265.1	65.4	19.34	E1/W	621.0	23.5	26.43
C1/H	610.3	41.4	14.74	E1/H	262.3	19.7	13.31
C2/L	218.8	15.7	13.94	E2/L	990.4	34.9	28.38
C2/BP	986.0	53.5	18.43	E2/BP	1519.4	56.1	27.08
C2/I	260.7	13.3	19.60	E2/I	547.9	16.0	34.24
C2/PL	806.1	32.6	24.73	E2/PL	1662.0	41.6	39.95
C2/M	470.6	38.4	12.26	E2/M	857.0	38.1	22.49
C2/W	892.3	62.4	14.30	E2/W	619.6	20.7	29.93
C2/H	461.2	28.9	15.96	E2/H	221.9	7.1	31.25
C3/L	390.8	17.7	22.08	E3/L	658.3	33.0	19.95
C3/BP	1279.1	66.2	19.32	E3/BP	1161.1	60.9	19.07
C3/I	431.6	16.4	26.32	E3/I	447.6	19.7	22.72
C3/PL	1414.4	45.1	31.36	E3/PL	1725.7	50.8	33.97
C3/M	663.1	43.1	15.39	E3/M	925.8	37.9	24.43
C3/W	1248.9	70.1	17.82	E3/W	1097.2	45.8	23.96
C3/H	511.2	35.2	14.52	E3/H	364.8	12.2	29.90
C4/L	359.2	21.6	16.63	E4/L	527.1	30.4	17.34
C4/BP	871.1	65.0	13.40	E4/BP	978.3	60.2	16.25
C4/I	330.7	17.7	18.68	E4/I	510.8	22.0	23.22
C4/PL	1009.2	56.3	17.93	E4/PL	1382.5	57.7	23.96
C4/M	599.7	41.5	14.45	E4/M	940.8	51.9	18.13
C4/W	1210.2	78.6	15.40	E4/W	1435.6	73.4	19.56
C4/H	618.6	45.9	13.48	E4/H	521.4	27.4	19.03
C5/L	248.2	14.2	17.48	E5/L	393.1	18.3	21.48
C5/BP	541.3	42.0	12.89	E5/BP	609.2	45.8	13.30
C5/I	244.6	13.6	17.99	E5/I	307.6	14.5	21.21
C5/PL	598.4	33.7	17.76	E5/PL	691.6	36.2	19.10
C5/M	569.3	46.0	12.38	E5/M	769.8	43.6	17.66
C5/W	1096.1	84.4	12.99	E5/W	1416.7	70.7	20.04
C5/H	708.1	52.5	13.49	E5/H	900.2	35.8	25.15

proteins, thereby giving an apparently high specific activity. The denser fractions tend to have lower specific activities due to the greater synthesis of denser myelin during the two weeks following administration of label. (9) Similar changes in the density of myelin during early development have been reported. (11) These data show that ^{3}H-lysine was incorporated into control and experimental rat myelin proteins to a similar degree and indicate that no inhibition of protein synthesis occurred. Since the myelin glycoproteins and their degradation products were equally labelled, this degradation must have occurred after the lysine was incorporated into the myelin proteins. The myelin was prepared by procedures designed to remove non-myelin and nonmembrane proteins. Therefore, the H-proteins and their degradation products appear to be integral components of the myelin. This suggests that limited localized proteolysis affecting only susceptible proteins occurred in the myelin since most of the myelin protein appear to be intact.

The lighter density myelin fractions are synthesized earlier than the denser myelin. (9,11) Since the effect upon the high molecular weight proteins was more pronounced in the lighter fractions (Fig. 1B, 1 and 2), this proteolysis occurred at an early time in myelinogenesis. Consistent with this was the obsevation that synthesis of denser myelin appeared to be much less affected as judged by the electrophoretic profile of proteins asociated with the denser fractions (Fig. 1B, 4 and 5). These data predict the sequence of events in production of abnormal myelin in the treated rats as follows: (1) protein synthesis is not affected by the MSUD metabolites; proteins are synthesized and incorporated into myelin, (2) for some reason, the myelin H-proteins become susceptible to proteolysis, and (3) following proteolytic attack, the myelin is altered, a small amount is lost, and the rest is maintained by the synthesis of normal denser myelin.

Quarles (5) has speculated on the role of myelin-associated glycoprotein (MAG) in the nervous system. MAG is concentrated in the periaxonal regions (perinodal loops) of the CNS and PNS myelin sheaths where it may be involved in glia-axon interactions. The membranes in these regions are not fully compacted, and MAG may possibly play a role in controlling the compaction process. Without MAG, the proper attachment of myelin to axon would be lacking, and the treated myelin would be more susceptible to destruction.

Sato, Quarles and Brady recently reported that MAG was susceptible to a neutral protease associated with highly purified myelin from human and rat brain. (12). Incubation of highly purified myelin at 25°C and pH 8 in ammonium bicarbonate buffer for 18 h resulted in the degradation of about half of the rat MAG to a smaller derivative, while the human MAG was completely degraded. These authors state, "The very high susceptibility of human MAG to this enzyme indicates that the effect of neutral proteases on this glycoprotein should be considered in connection with demyelinating diseases." We

believe that this enzyme may also play a key role in the degradation of MAG and other susceptible proteins which we have observed in the rats treated with MSUD metabolites.

The experimental rats in this study may compare favorably to a partially affected MSUD child who is detected after some damage has occurred, and then given the proper low branch-chain amino acid diet. It is well documented that this child will never attain normal mental development. (13) This study predicts that the myelin and especially the lighter density myelin, will, in these children show a deficit in intact H-proteins and increased degradation products. Also, it predicts that normal nerve conduction through the axons with modified myelin would be unlikely because the important glial-axon attachments would not be present to the proper extent.

Wallerian degradation of rabbit optic nerve produced within two days a decrease in the concentration of myelin glycoproteins, while loss of other myelin proteins took weeks. (14) Thus, after axonal disintegration, the first change in myelin composition affects the glycoproteins at the paranodal regions without loss of myelin, followed by disintegration of the paranodal loops and eventually, the compact myelin. Glycoproteins in myelin may be dependent upon an intact axon for their existance. Therefore, it is possible that the α-keto and amino acids may affect the axons initially and the myelin only secondarily.

The events which occur in the brain between the injection of α-ketoisocaproate-leucine and the observed loss of proteins is not understood. A reasonable hypothesis is that these MSUD metabolites interfere with the processing of the proteins in the rough endoplasmic reticulum and golgi, such that the H-proteins are abnormal. These proteins which are terminally located primarily in the open loop regions of myelin would be more susceptible to degradation by cytoplasmic enzymes. Further characterization of these susceptible proteins and their degradation products may provide evidence for a complete pathogenic chain leading from the elevated levels of branched-chain keto and amino acids to the synthesis of abnormal myelin in brain development. Other amino acid excesses such as exists in phenylketonuria may cause similar effects on brain myelin.

References

1. Crome, L., Dutton, G., Ross, C.F. (1961). J. Path Bact. 81, 379.
2. Hershkowitz, N., in Inborn Errors of Metabolism in Humans, Cockburn, F. and Gitzelmann, R.,Eds. (1982) Alan R. Liss, Inc., New York. "To my knowledge, no complete pathogenic chain leading from the abnormal enzyme activity to an abnormal function has been shown for any inborn error of metabolism affecting brain development."
3. Silberberg, D. H. (1969) Neurochem., 16, 1141.
4. Liao, C. L., Herman, M.M., Bensch, K. G. (1978) Lab. Invest., 38, 122.
5. Quarles, R. H. (1982) Trans. Amer. Soc. Neurochem. 13, 79.
6. Shapira, R., Mobley, W. C., Thiele, S.B., Wilhelmi, M. R., Wallace, A, Kibler, R. F. (1978) J. Neurochem., 30, 735.
7. Greenfield, S., Norton, W. T., Morell, P. (1971) J. Neurochem., 18, 2119.

8. Morell, P., Wiggins, R. C., Gray, M. J. (1975) Anal. Biochem, 68, 148.
9. Shapira, R., Wilhelmi, M. R., Kibler, R. F. (1981) J. Neurochem. 36, 1427.
10. Quarles, R. H., Everly, J. L., Brady, R. O. (1973) J. Neurochem. 21, 1171.
11. Lane, J.D. and Fagg, G.E. (1980) J. Neurochem., 34, 163.
12. Sato, S., Quarles, R. H., Brady, R. O. (1982) J. Neurochem., 39, 97.
13. Tanaka, K. and Rosenberg, L. E., in the Metabolic Basis of Inherited Disease, Stanbury, J. B. et al., Eds. 5th edition (1983) p. 456, McGraw-Hill, New York.
14. Reigner, J., Matthieu, J.-M., Kraus-Ruppert, R., Lassmann, H., Poduslo, J. F., (1981) J. Neurochem 36, 1986.
15. D. T. was partially supported by the Human Genetics Section, Department of Pediatrics. We thank L. Elsas for constructive discussion; J. Kinkade and D. Groth for their critical review of this manuscript. This work was supported by N. I. H. Grant NS-08278 and N. S. F. Grant PCM-7916169.