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ABNORMAL EXCRETION OF URINARY PHOSPHOLIPIDS AND SULFATIDE IN PATIENTS WITH MITOCHONDRIAL ENCEPHALOMYOPATHIES

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SUMMARY: We found that patients with mitochondrial encephalomyopathies excreted urinary phosphatidylethanolamine, cardiolipin, and phosphatidylserine most likely derived from mitochondria and sulfatide which is specific to myelin or the kidney. It is of interest that four patients with myoclonus epilepsy with ragged-red fibers and one patient with chronic progressive external ophthalmoplegia all showed qualitatively similar abnormal excretion of such urinary lipids. It is conceivable that the urinary acidic phospholipids reflect abnormalities in the mitochondrial phospholipids, which are very important for mitochondrial enzymatic activities.

Mitochondrial encephalomyopathies, clinically characterized by lactic acidosis and ragged-red fibers in the muscle, are caused by mitochondrial DNA mutations(1) and classified into the following subgroups: myoclonus epilepsy associated with ragged-red fibers(MERRF); chronic progressive external ophthalmoplegia(CPEO) including Kearns-Sayer syndrome; and mitochondrial myopathy, encephalopathy, lactic acidosis,

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Abbreviations: MERRF, myoclonus epilepsy with ragged-red fibers; CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns-Sayer syndrome; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. C/M, chloroform/methanol; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; PE, phosphatidylethanolamine; CA, cardiolipin; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; CMH, cerebroside; CS, sulfatide.

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and stroke-like episodes(MELAS) (2). Although the features of these disorders have been recently elucidated from the viewpoints of clinicopathology, biochemistry, and molecular genetics, the pathogenesis remains unclear. We found that patients with mitochondrial encephalomyopathies showed abnormal urinary excretion of both mitochondria-specific phospholipids and myelin- or kidney- specific glycolipids. These findings may be important for the clarification of this pathogenesis and lead to effective treatment or prevention of these diseases.

MATERIALS AND METHODS

Materials: The 48-hr urine samples were collected from five female patients with mitochondrial encephalomyopathies, three other patients and two normal subjects as a control. Each sample was then filtered to isolate the lipids, which were retained on the filter paper (No. 51A; Toyo Roshi Co. Ltd., Tokyo).

Case reports: We examined four patients with MERRF (Patient 1 through Patient 4) and one patient with CPEO (Patient 5). Patient 1 (age 32 y), the proband of family Y, developed seizure, myoclonus, and cerebellar ataxia since at age 13 y. Patient 2 (age 30 y) was the sister and Patient 3 (age 56 y) was the mother of Patient 1. Patient 4 (age 47 y) was unrelated to the family Y. Patient 5 (age 26 y) was sporadic. All patients showed lactic acidosis and ragged-red fibers in the muscle. According to previously described methods(3-5), the $tRNA^{Lys}$(8344) mutation in the patients of family Y and the common deletion of mtDNA(deletion position: 8483-13460) in Patient 5 were detected by Southern blot analysis and the polymerase chain reaction. We also examined one patient with myotonic dystrophy (C1, age 49 y, female), one patient with Becker muscular dystrophy (C2, age 17 y, male), one patient with familial hypoceruloplasminemia (C3, age 27 y, male), and two normal males (C4, age 36 y; C5, age 25 y). Routine renal function studies indicated all patients were normal except for Patient 3: serum BUN 28.1 (normal, 7-20) mg/dl, serum creatinine 1.4 (normal, 0.6-1.2) mg/dl, and serum β2-microglobulin 2.88 (normal, 0.85-1.65) mg/dl.

Methods: Lipids were extracted from the filter paper containing urinary sediment with 300 ml of chloroform/methanol(2:1, v/v) and again with 300 ml of C/M(1:1). The extracts were combined together and dried in vacuo by a rotary evaporator. The residue was dissolved with 40 ml of C/M(2:1) and partitioned into the upper and lower phases with 10 ml of H_2O according to Folch et al.(6), and the lower phase was again partitioned with 20 ml of the theoretical upper phase. The lower phase was evaporated, and the residue was weighed and dissolved with a small volume of C/M/H_2O(30:60:8). The solution was applied to a DEAE-Sephadex A-25(Pharmacia Fine Chemical, Uppsala) column prepared as follows: the ion-exchange resin was equilibrated with C/M/H_2O(30:60:8) and loaded to a column(1 cm in diameter and 25-cm-high). Neutral lipids were eluted with 200 ml and again with 100 ml of the same solvent mixture, whereas acidic lipids were eluted with 200 ml of C/M/0.8M sodium acetate (30:60:8). The neutral and acidic lipid fractions were applied to TLC plates(Silica Gel 60, Merck) for analysis of phospholipids and glycolipids, respectively. The following solvent systems were used: C/M/H_2O

(65:25:4 or 60:35:8) for one-dimensional TLC and C/M/2.5N aqueous ammonium hydroxide(60:35:6) and C/M/acetone/acetic acid/H_2O(70:15:30:15:7.5) for two-dimensional TLC. Lipid bands were visualized with cupric phosphoric acid, ninhydrin, and ammonium molybdate reagents, and then measured densitometrically by a Dual-Wavelength Flying-Spot Scanner(CS-9000, Shimazu). Individual phospholipids were isolated by preparative TLC and their phosphorus contents determined by the method of Bartlett (7). Some phospholipids were purified by silicic acid column chromatography. Sulfatide was isolated by silicic acid column chromatography from the acidic fraction treated with alkali to cleave the ester linkage. After methanolysis of the isolated phospholipids and sulfatide, the fatty acid methylesters thus obtained were analyzed by GLC, while the methyl-glycosides were converted to TMS-derivatives, which were also analyzed by GLC as reported by us (8,9).

RESULTS

Urinary neutral lipid fraction: The neutral lipid fraction was applied to TLC. As shown in Fig.1, all bands of the neutral lipids were visualized by cupric phosphoric acid charring reagent. However, three of them were phosphorus-positive and measured densitometrically by a scanner, as shown in Fig. 2. A major phosphorus-positive band was also ninhydrin-positive and thus identified as phosphatidylethenolamine judging from its Rf-value. A second one was also ninhydrin-positive and thus thought to be lysophosphatidylethanolamine, although it may have possibly been overlapped with phosphatidylcholine. A third doublet was ninhydrin-negative and determined to be sphingomyelin. The neutral phospholipid contents and compositions were determined as described in the Materials and Methods. These results are described in Table 1. Although patients with MERRF and CPEO differed in the contents of their urinary neutral phospholipids, they and the patient(C1) with myotonic dystrophy showed qualitatively similar abnormal excretion of a major phosphatidylethanolamine, in contrast with other control subjects.

Since other phosphorus-negative lipid bands showing a higher Rf-value than cerebroside were common in the five patients and the control subjects, they were neglected in this experiment.

Urinary acidic lipid fraction: The acidic lipid fraction was analyzed by TLC. As shown in Fig. 1, all bands of the acidic lipids were visualized by the charring reagent. However, among them, two major and two

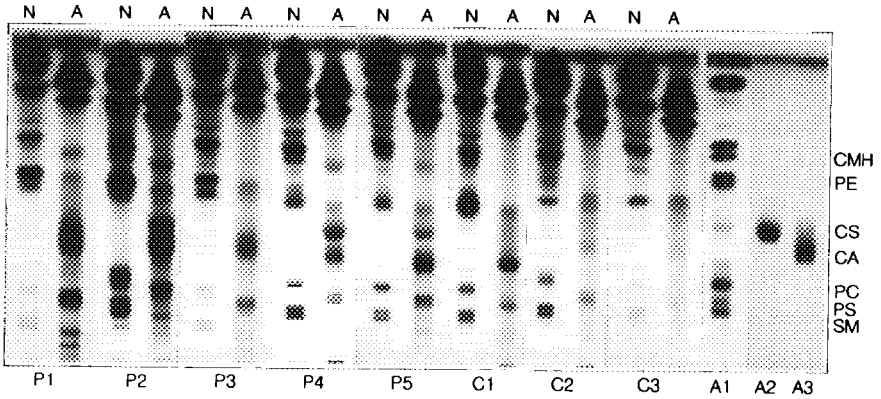

Fig. 1. One-dimensional TLC patterns of urinary neutral(N) and acidic(A) lipid fractions which were separated by DEAE-Sephadex A-25 column chromatography. P1, P2, P3, and P4: patients 1, 2, 3, and 4 with MERRF; P5: patient 5 with CPEO as described in Materials and Methods. C1: patient with myotonic dystrophy; C2: patient with Becker muscular dystrophy; C3: patient with familial hypoceruloplasminemia. A1: CMH (cerebroside), PE(phosphatidylethanolamine), PC(phosphatidylcholine), PS(phosphatidylserine), and SM(sphingomyelin) as authentic samples; A2: CS(sulfatide) as an authentic sample; A3: CA(cardiolipin) as an authentic sample. Development solvent: C/M/H_2O(65:25:4). Bands were visualized by cupric phosphoric acid reagent.

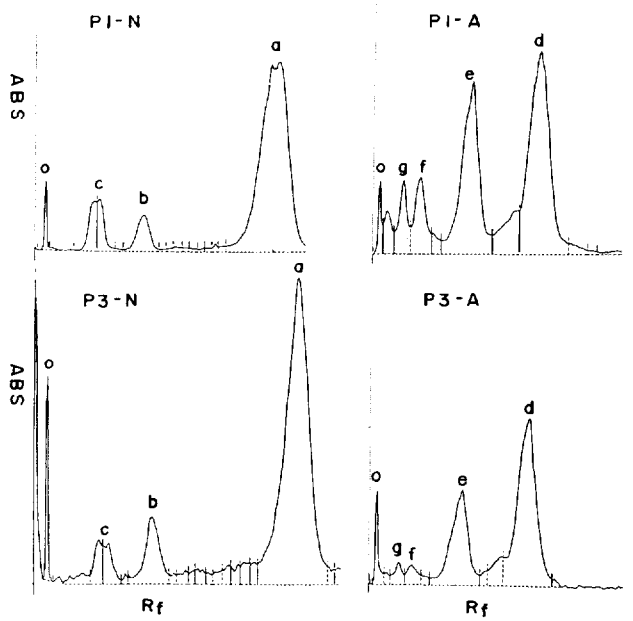

Fig. 2. Densitometrical patterns of TLC of urinary neutral(N) and acidic(A) lipid fractions of P1(Patient 1) and P3(Patient 3) as described in Materials and Methods. a: phosphatidylethanolamine; b: lysophosphatidylethanolamine + phosphatidylcholine; c: sphingomyelin; d: cardiolipin; e: phosphatidylserine; f: lysocardiolipin; g: lysophosphatidylserine; o: origin.
ABS: absorbance at 530 nm.

TABLE 1. URINARY PHOSPHOLIPID AND SULFATIDE ANALYSIS

Patient Numbers	1	2	3	4	5	C1	C2	C3	C4	C5
Content (μmol/24-hr urine)										
Neutral PL[a]	1.61	0.93	2.08	1.82	1.61	1.20	0.22	0.40	0.18	0.21
Acidic PL	2.79	0.88	1.39	0.75	0.66	0.26	-	-	-	-
Content (nmol/24-hr urine)										
Sulfatide	90.0	25.0	179.1	60.4	78.1	-	-	-	-	-
Phospholipid composition (%)										
PE[b]	32.9	34.8	46.2	41.8	44.4	57.6	82.2	37.6	35.3	43.9
Lyso-PE+PC[c]	3.1	9.5	6.2	12.4	11.6	8.4	2.2	16.7	-	-
SM[d]	4.8	20.0	4.4	20.2	18.4	23.8	15.6	45.5	64.7	56.1
CA[e]	28.5	24.3	25.8	17.7	15.4	12.9	-	0.2	-	-
PS[f]	20.5	9.9	14.0	7.0	10.0	4.0	-	-	-	-
Lyso-CA	5.9	1.3	1.8	0.8	0.2	0.9	-	-	-	-
Lyso-PS	4.3	0.2	1.6	0.1	-	-	-	-	-	-

a, Phospholipids; b, phosphatidylethanolamine; c, phosphatidylcholine; d, sphingomyelin; e, cardiolipin; f, phosphatidylserine.

minor phosphorus-positive bands were also measured densitometrically by a scanner, as shown in Fig. 2. The major band showing the lower Rf-value was ninhydrin-positive and identified as phosphatidylserine. After the other ninhydrin-negative band was isolated by preparative TLC, it was identified as cardiolipin by two-dimensional TLC (data not shown). After methanolysis, GLC-analysis showed that it contained more polyunsaturated fatty acid than did the other phospholipids (data not shown). The minor band showing the lower Rf-value was ninhydrin-positive and thus thought to be lysophosphatidylserine. The other one was ninhydrin-negative and thus considered to be lysocardiolipin, according to its Rf-value. The acidic phospholipid content and composition were determined, and the data are shown in Table 1. Patients with MERRF and CPEO and the patient with myotonic dystrophy alone showed qualitatively similar abnormal urinary excretion of cardiolipin and phosphatidylserine, which are very specific to the mitochondria (10,11).

Since other phosphorus-negative lipids showing a higher Rf-value than cerebroside were common in both the patients and the control subjects, they were also neglected.

Identification of sulfatide in the acidic lipid fraction: As shown in Fig. 1, the neutral lipid fraction gave doublet bands corresponding to glucosylceramide or cerebroside not only in patients with MERRF and CPEO, but also in the control subjects. Thus, such neutral glycolipids were neglected in this experiment. On the other hand, a band corresponding to sulfatide was found in the acidic lipid fraction of MERRF and CPEO patients, but not in that of the control subjects or the patient with myotonic dystrophy. GLC-analysis confirmed it as sulfatide by finding galactose, non-hydroxy and hydroxy fatty acids, and sphingosine base (data not shown). The urinary sulfatide contents of the five patients are shown in Table 1.

DISCUSSION

It is remarkable that qualitatively similar abnormal excretions of urinary phospholipids and sulfatide were found for the first time in four patients with MERRF and one with CPEO, whose mitochondrial gene mutations have been clarified to some extent. In particular, it was noted that the urinary phospholipids were mainly composed of phosphatidylethanolamine, cardiolipin, and phosphatidylserine, which are specific to the mitochondria, rather than phosphatidylcholine, which is the most common lipid substance in the cell membrane and blood plasma. The abnormal excretion of these phospholipids in the urinary sediment containing the desquamated glomerular and tubular epithelial cells and casts of patients with mitochondrial encephalomyopathies seemed to reflect the abnormalities of the mitochondrial phospholipids which are very important for mitochondrial enzymatic activities. According to Kagawa (12), reconstitution of mitochondrial function revealed at least two roles for phospholipids in the mitochondria: (i) to activate enzymes bound to the membrane structure and (ii) to form membrane vesicles.

Thus, the loss of these roles may be considered a cause of these diseases. The findings by Fleischer et al.(10) that cardiolipin was much more effective than phosphatidylcholine in reactivation of succinate:cytochrome c oxidoreductase also supported our findings. Although the evidence of renal involvement in patients with CPEO is not known, we suggest that mitochondrial abnormalities may be present in them as well as in patients with MERRF or MELAS.

Biochemical analysis using TLC of the urine sediment has been utilized for screening the patients with sphingolipidoses or disorders of glycoprotein degradation. In these diseases of inborn error of metabolism, the pattern of abnormal urinary excretion of sulfatide has been known only in metachromatic leukodystrophy. To our knowledge, this is the first description of this pattern in diseases of the mitochondrial DNA. Although the sulfatide is specific to myelin or the kidney, we presume that the sulfatide in the urine sediment may be derived from the kidney. Even with maximal variation, glycosphingolipids from a normal 24-hr urinary sediment are not easily visualized on TLC (14). In addition, the patients with Fabry disease, usually affecting the kidney, show large amount of specific triaosylceramide and digalactosylceramide in urine, but the abnoraml urinary excretion of sulfatide is not shown (14). Furthermore, routine renal function studies in our patients were normal except for Patient 3. Therefore, we suggest that there may be no correlation between the amount of uriary excretion of sulfatide and the degree of renal dysfunction in patients with mitochondrial encephalomyopathies.

It was noted that among the control subjects, the myotonic dystrophy patient excreted abnormal urinary phospholipids but no sulfatide. Although this disease differs from MERRF and CPEO, the abnormal excretion of urinary phospholipids was recognized in terms of the incidence of ragged-red fibers in the muscle of patients with myotonic dystrophy as well as patients with mitochondrial myopathies (15). It was also

noted that the patient with Becker muscular dystrophy and the patient with familial hypoceruloplasminemia with suspected mitochondrial abnormality as well as normal subjects did not show any abnormal excretion of urinary lipids. In conclusion, it was found that chemical analyses of urinary neutral and acidic lipids are useful for the diagnosis of mitochondrial encephalomyopathies.

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