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Phosphoethanolamine Elevation in Plasma of Spinal Muscular Atrophy Type 1 Patients

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BACKGROUND: Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by degeneration or loss of lower motor neurons. The survival of motor neuron (*SMN*) 1 gene, which produces the SMN protein, has been identified as a responsible gene for the disease. SMN is ubiquitously expressed in any tissue and may play an important role on the metabolism in the human body. However, no appropriate biomarkers reflecting the alteration in the metabolism in SMA have been identified. METHODS: Low-molecular-weight metabolites were extracted from plasma of 20 human infants (9 SMA type 1 patients and 11 controls) and 9 infant mice (5 SMA-model mice, 4 control mice), and derivatized with N-methyl-N-trimethylsilyltrifluoroacetamide. Finally, the derivatized products were applied to Gas Chromatography/Mass Spectrometry apparatus. To confirm the metabolite abnormality in SMA type 1 patients, we performed *SMN*-silencing experiment using a hepatocyte-derived cell line (HepG2). RESULTS: We performed a comprehensive metabolomics analysis of plasma from the patients with SMA type 1 and controls, and found that phosphoethanolamine (PEA) was significantly higher in the patients than in the controls. HepG2 experiment also showed that *SMN*-silencing increased PEA levels. However, comprehensive metabolomics analysis of plasma from SMA-model mice and control mice showed different profile compared to human plasma; there was no increase of PEA even in the SMA-model mice plasma. CONCLUSION: Our data suggested that PEA was one of the possible biomarkers of human SMA reflecting metabolic abnormalities due to the SMN protein deficiency.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder caused by degeneration of α-motor neurons in the spinal cord, leading to muscle weakness and progressive loss of movement of the limbs and trunks. According to the age of onset and achievement of motor milestones, SMA is classified into four subtypes [1]: type 1 (severe form; age of onset 0–6 months, unable to sit unaided), type 2 (intermediate form; age of onset <18 months, unable to stand or walk unaided), type 3 (mild form; age of onset >18 months, able to stand or walk unaided), and type 4 (milder form; age of onset >21 years, able to stand or walk unaided).

The survival motor neuron (*SMN*) genes, which are considered as SMA-related genes, exist as two highly identical genes: *SMN1* (telomeric SMN) and *SMN2* (centromeric SMN) [2]. SMA patients show homozygous

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deletion or intragenic mutation of *SMN1* [2]. However, *SMN2* is never absent in SMA patients and increased *SMN2* copy number is associated with improved survival outcomes and maintenance of motor function, i,e., clinical subtypes [3-5].

SMN1 exclusively generates full-length (FL)-*SMN* transcript, leading to production of the functional SMN protein. Meanwhile, *SMN2* generates low levels of FL-*SMN* transcript, and high levels of exon 7-lacking (Δ7)-*SMN* transcript which is translated into an unstable truncated SMN protein [5]. A correlation between the levels of the FL-*SMN* protein and disease severity has been observed [6]. Taken together with genotype-phenotype relationship, low copy number (1 or 2) of *SMN2* may produce much less abundance of FL-SMN protein, leading to severe phenotype of SMA type 1 [3-6].

SMN protein, which is ubiquitously expressed in cells, is an important component of many cellular activities: anti-apoptotic function, anti-stress function, spliceosomal snRNP biogenesis, transcription, splicing, translation, etc. [7-15]. SMN may play an important role on the metabolism in the human cells, because its functions, involving transcription, splicing and translation, may influence the production of enzyme proteins. However, no appropriate biomarkers reflecting the alteration in the metabolism in SMA have been identified.

The advance of mass-spectrometry technique has allowed us to perform comprehensive profiling of metabolites using biofluid or cell lysate as samples. The metabolome is a unique chemical fingerprinting which specific “protein actions” leave behind, therefore the metabolome is closer to disease phenotype compared to genome and proteome studies. Thus, we aimed to apply metabolome analysis to the research of systemic and cellular metabolisms in SMA phenotypes.

In this study, to clarify the function of SMN protein in metabolisms, especially in non-neuronal tissues, we examined metabolomics in plasma from SMA type 1 patients and controls. SMA type 1 patients were selected because they represent the most severe phenotype in SMA. Here, elevation of phosphoethanolamine (PEA) was observed in SMA type 1 patients. Such PEA elevation was also observed in the *SMN*-silenced human hepatocytes (HepG2 cells). These findings suggest that SMN protein may have some roles involving metabolisms not only in neuronal tissues, but also in non-neuronal tissues.

MATERIALS AND METHODS

1. Experiments with plasma samples

1.1. Backgrounds of the subjects

[SMA patients and controls] A total of 20 individuals (n = 9: SMA type 1 patients, n = 11: controls, other patients without SMA) participated in this study. Patients and controls background characteristics were shown in Table IA. This study was approved by the research ethics committee of Kobe University Graduate School of Medicine (approval number: 1089) and informed consent was obtained from the parents of the patients.

Peripheral blood samples of patients and controls were collected as EDTA-plasma. Blood samples were then centrifuged at 5,000 x rpm in 4ºC for 5 minutes. Supernatant was separated as plasma and then stored in -80ºC until further use.

[SMA-model mice and control mice] SMA-model mice (mSmn-/-, SMN2+/+, SMNΔ7+/+) and control mice (mSmn+/+, SMN2+/+, SMNΔ7+/+) [16,17] were purchased from the Jackson laboratory (Bar Harbor, Maine, U.S.A.) and maintained in Department of Molecular Pharmacology, Gifu Pharmaceutical University. A total of 9 mice (n = 5: SMA-model mice, n = 4: control mice) were included in this study. Mice age and body weight were shown in Table IB. Blood samples were collected from mice heart with heparin preparation and then immediately centrifuged at 1,800 g in 4ºC for three minutes. Supernatants were collected as plasma and stored in -20°C until further use. This study was approved by the research ethics committee of Gifu Pharmaceutical University (approval number: 2017-267).

Table I. Backgrounds of the subjects analyzed in the study

A. Controls and SMA type 1 patients

	Number (F/M)	Age (months)	*SMN1* copy number	*SMN2* copy number
Controls	11 (6/5)	0 - 45	2	1 or 2
SMA type 1 patients	9 (2/7)	0 - 7	0	2

B. Control mice and SMA model mice

	Number	Age (weeks)	Weight (grams, mean ± SEM)
Control mice	4	0 - 1	2.70 ± 0.17
SMA model mice	5	0 -1	2.44 ± 0.28

1.2. Preparation for GC/MS analysis

Low-molecular-weight metabolites were extracted as follows: 50 µl of human plasma was mixed with 250 µl of methanol-water-chloroform solvent mixture (2.5:1:1, v/v/v) containing 10 µl of an aqueous solution of sinapinic acid (1.0 mg/ml in distilled water; Sigma Aldrich, Tokyo, Japan) as an internal standard. The mixture was shaken at 1,200 rpm for 30 min at 37°C, followed by centrifugation at 22,000 g for 3 min at 4°C. Next, 225 µl of the supernatant was transferred into a new tube, to which 200 µl of distilled water was added. Then, the mixture was centrifuged again at 22,000 g for 3 min at 4°C, and 250 µl of its supernatant was transferred into a new tube and lyophilized using freeze dryer. For oximation, 40 µl of methoxyamine hydrochloride (20 mg/ml in pyridine; Sigma-Aldrich) was mixed with the lyophilized samples and shaken at 1,200 rpm for 90 min at 30°C. Subsequently, 20 µl of N-methyl-N-trimethylsilyltrifluoroacetamide (GL Science, Tokyo, Japan) was added for derivatization and the mixture was incubated at 37°C for 30 min, then followed by centrifugation at 22,000 g at 4°C for 5 min. This supernatant was used for Gas Chromatography/Mass Spectrometry (GC/MS) analysis.

The methods for low-molecular-weight metabolite extraction from mouse plasma were exactly the same as the methods used in human plasma. However, due to the small amount of the mouse plasma, initial plasma volume used for the metabolite extraction was 5 µl. For oximation, the adjusted volume of methoxyamine hydrochloride (20mg/ml in pyridine; Sigma-Aldrich) and N-methyl-N-trimethylsilyl-trifluoroacetamide (GL Science) were 13 µl and 7 µl, respectively.

1.3. Condition of GC/MS analysis

GC/MS analysis was performed in the Integrated Center for Mass Spectrometry, Kobe University Graduate School of Medicine, using a GCMS-QP2010 Ultra (Shimadzu Co, Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30 m x 0.25 mm inner diameter, 0.25 µm film thickness; Agilent Co, Palo Alto, CA, USA). The front inlet temperature was 230°C and the helium gas flow rate through the column was 39.0 cm/s. The column was maintained at 80°C for 2 min isothermally, followed by a temperature increased to 330°C at the rate of 15°C/min and maintained for 6 min isothermally. The transfer line and ion-source temperatures were 250°C and 200°C, respectively. Twenty scans were recorded over the 85-500 m/z mass range using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co). MS data were exported in the net CDF format, and peak detection and alignment were performed using MetAlign software (Wageningen UR The Netherlands). The resulting data were exported in the CSV format file and analyzed using in-house analytical software (AI output) that enables peak identification using our in-house metabolite library. For quantification, the peak height of each ion was calculated and normalized using the peak height of sinapinic acid as the internal standard.

2. Experiments with HepG2 cells

2.1. Culture of HepG2 cells and *SMN*-silencing

A HepG2 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were maintained in a 5 % humidified CO_2 atmosphere at 37°C with Dulbecco's modified Eagle's medium (DMEM)-high glucose (Nacalai Tesque, Inc, Kyoto, Japan). The medium contains 10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B (Fungizone; Life Technologies, Carlsbad, CA, USA), and 10 % heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich).

For the *SMN*-silencing experiment, short-hairpins RNA (shRNA) constructs targeting human SMN in retroviral GFP vector TG301496 (Origene, Inc., Rockville, MD, USA) and scrambled shRNA cassette in pGFP-VRS vector TR30013 (Origene, Inc) were separately transfected into HepG2 using Neon™ Transfection System (Invitrogen, Carlsbad, CA, USA) to produce *SMN*-silencing and control cells, respectively. Transfection condition and procedures used were according to the company's manual.

2.2. Western blot analysis with anti-SMN antibody

The cells were cultured in a 6-well plate with a starting condition of 2 x 10^6 cells and 4 µg of sh-RNA plasmids transfected into each group (*SMN*-silenced group and "scramble" group). After 48 h incubation, protein samples were prepared by adding Radioimmunoprecipitation assay (RIPA) buffer and sonication. A total of 5 µg homogenized protein samples were electrophoresed by 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane in iBlot™ gel transfer stack using iBlot dry blotting system (Invitrogen, Carsbald, CA, USA). The mouse anti-β-actin (Sigma-aldrich, St. Louis, MO, USA) and mouse anti-SMN (BD Transduction Laboratories, Franklin Lakes, NJ, USA) primary antibodies were used to detect beta-actin and SMN, respectively. The anti-mouse IgG (Cell Signaling #7076 Anti-mouse IgG, HRP-linked) was used for secondary antibody. Chemiluminescent signals were produced by using Immobilon™ Western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA), and they were detected using a luminescent image analyzer Amersham Imager 600 (GE

Healthcare Life Sciences, Chicago, IL, USA). The signal intensity of the membrane was determined using ImageJ. Western blotting experiments and signal-intensity analyses were repeated at least three times. β-actin was an endogenous reference protein, and its amount was used as the relative denominator for SMN.

2.3 Metabolomic analysis using GC/MS

The cells were cultured in a 10-cm dish with a starting condition of 5 x 10^6 cells and 10 μg of sh-RNA plasmids for each group (*SMN*-silenced group and "scramble" group). After 48 h incubation, the cells were harvested using 1 mL of 80 % methanol. The harvested cells were then centrifuged 15,000 rpm in 4 °C for 15 minutes to separate the metabolites dissolved in supernatant and protein pellet. The supernatant combined with 10 μl of an aqueous solution of sinapinic acid (1.0 mg/ml in distilled water; Sigma Aldrich) as an internal standard. The mixture was lyophilized using freeze dryer. GC/MS analysis, as well as oximation and further sample preparation, followed the same procedures as in human plasma samples.

The protein pellet was dissolved into sodium hydroxide and its concentration was measured. The final protein amount was used as the relative denominator for the measured metabolites in GC/MS analysis.

3. Statistics

The software used for statistical analysis was Excel (Microsoft 2007) for calculation of descriptive statistics and t-tests. P-value of less than 0.05 was considered to indicate a significant difference.

RESULTS

1. Plasma metabolomic profiles of SMA patients and controls

We examined the comprehensive plasma metabolomic profiles from nine SMA patients and eleven age-matched controls using GC/MS analysis. The metabolomics identified 71 metabolites from the plasma, majority of which were amino acids and organic acids (Table II). Among them, four metabolites showed significant differences between controls and SMA patients' samples: aminoisobutyrate, phenylglycine, xylose, and PEA (Table II).

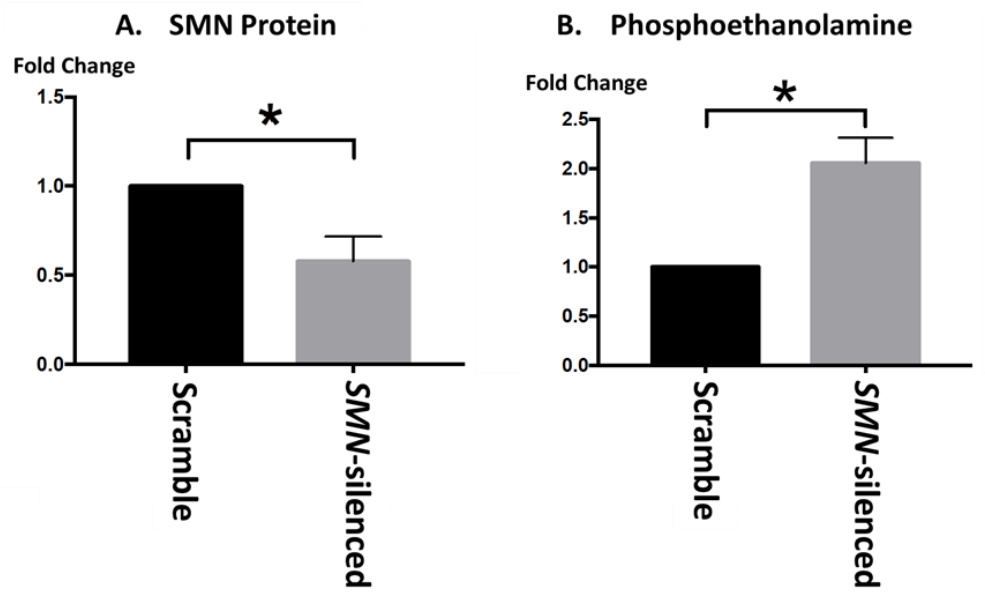

Figure 1. Phosphoethanolamine (PEA) elevation in *SMN*-silenced HepG2.
A. Western blotting of SMN in *SMN*-silenced HepG2. β-actin was used as endogenous reference protein. Relative quantification of western blot results from three independent experiments is presented in bar graphs.
B. GC/MS analysis of PEA in *SMN*-silenced HepG2 cells. Relative quantification of PEA from three independent experiments is presented in bar graphs. Here, data were presented in mean±SEM. * $p<0.05$

2. PEA increase in *SMN*-silenced HepG2

Having found that plasma PEA levels significantly elevated in SMA patients, we employed HepG2, which is derived from human hepatocyte, to investigate specific impact on metabolome by *SMN*-silencing. To examine the efficacy of *SMN*-silencing, we determined the SMN protein expression level by western blot in

SMN-silenced and control HepG2. In *SMN*-silenced HepG2, we observed approximately 50 % reduction of SMN protein expression compared to control (Figure 1).

After confirming the significant reduction of the SMN protein, we performed GC/MS based metabolomics using SMN-silenced and "scramble" HepG2 cells. "Scramble" HepG2 cells were transfected with scrambled shRNA cassette-containing pGFP-VRS vector, and considered as control cells. Of the 63 identified metabolites in HepG2, PEA was the only metabolite that showed significant elevation by SMN-silencing. PEA in SMN-silenced HepG2 showed approximately two-fold increase compared to the control cells (Figure 1B). Any other metabolites, including aminoisobutyrate, phenylglycine and xylose, did not changed by SMN-silencing. These results suggested that decreased SMN protein level increased PEA level in non-neuronal tissues, which may explain the higher PEA levels in the plasma of SMA patients.

Then, we expected that in SMA patients, PEA level might be elevated not only in liver, but also in kidney and in neuronal cells. Thus, we did metabolome analysis with SMN-silencing using HEK-293 (as a representative cell line with kidney cell characteristics) and SK-N-BE(2) (as a representative cell line with neuronal cell characteristics). However, these cells failed to show any correlation between SMN protein levels and PEA levels (data not shown).

Table II. Plasma metabolites examined in controls and SMA patients

	Control			SMA					
	Mean	SEM		Mean	SEM		Fold Induction	*P*	
Oxalate	0.1785	0.0417		0.2256	0.1000		1.26	0.195	
Sarcosine	0.0022	0.0019		0.0018	0.0008		0.82	0.576	
2-Aminoisobutyrate	0.0097	0.0023		0.0145	0.0057		1.50	0.024	#
Ketoisoleucine_1	0.0148	0.0055		0.0139	0.0078		0.94	0.770	
3-Hydroxyisovaleric acid	0.0025	0.0031		0.0040	0.0034		1.58	0.350	
Glyceraldehyde_2	0.0436	0.0402		0.0755	0.0460		1.73	0.134	
Oxamic acid	0.0044	0.0029		0.0043	0.0030		0.96	0.897	
Phosphate	10.4264	1.7061		12.2570	5.7574		1.18	0.354	
Threonine(2TMS)	0.0035	0.0051		0.0104	0.0087		2.98	0.050	
b-N-Methyl-amino-L-alanine_1	0.0013	0.0003		0.0025	0.0019		1.87	0.081	
Fumaric acid	0.0035	0.0013		0.0032	0.0021		0.93	0.773	
Nonanoic acid(C9)	0.0140	0.0056		0.0142	0.0088		1.01	0.972	
b-Alanine	0.0060	0.0041		0.0094	0.0116		1.56	0.407	
Malic acid	0.0095	0.0044		0.0113	0.0056		1.19	0.463	
Homoserine lactone	0.0290	0.0069		0.0371	0.0173		1.28	0.192	
α-Phenylglycine	0.0026	0.0013		0.0048	0.0013		1.88	0.002	#
Methionine	0.0029	0.0021		0.0043	0.0020		1.52	0.145	
trans-4-Hydroxy-L-proline	0.0360	0.0278		0.1391	0.1746		3.87	0.084	
Pyroglutamic acid	0.0601	0.0182		0.0767	0.0494		1.28	0.340	
Glutamic acid	0.4463	0.2792		1.3178	1.3908		2.95	0.070	
Phenylalanine	0.1153	0.0716		0.1738	0.0875		1.51	0.136	
Xylose_2	0.0027	0.0015		0.0045	0.0020		1.66	0.044	#
Lyxose_2	0.0016	0.0014		0.0036	0.0041		2.17	0.187	
Arabinose	0.0083	0.0022		0.0092	0.0046		1.11	0.602	
Lauric acid	0.0235	0.0173		0.0377	0.0261		1.61	0.182	
Asparagine	0.0180	0.0077		0.0185	0.0087		1.03	0.901	
Taurine	0.0075	0.0106		0.2596	0.6903		34.43	0.266	
Xylitol	0.0027	0.0009		0.0052	0.0042		1.92	0.087	
Rhamnose_1	0.0040	0.0007		0.0044	0.0016		1.11	0.458	

Arabitol	0.0229	0.0146		0.0275	0.0209		1.20	0.591	
2-Aminoadipic acid	0.0041	0.0019		0.0057	0.0037		1.39	0.253	
Ribitol	0.0028	0.0026		0.0033	0.0021		1.16	0.690	
Aconitate	0.0031	0.0021		0.0029	0.0015		0.93	0.817	
Glutamine	0.2973	0.1462		0.4054	0.3311		1.36	0.368	
O-Phosphoethanolamine	0.0111	0.0032		0.0174	0.0072		1.57	0.024	#
Citric acid + Isocitric acid	0.5435	0.1325		0.5707	0.2341		1.05	0.760	
Ornithine	0.6162	0.4321		1.1546	1.1545		1.87	0.192	
Hypoxanthine	0.0816	0.0949		0.1189	0.0910		1.46	0.409	
Tagatose_1	0.0057	0.0119		0.0026	0.0019		0.45	0.470	
Psicose_1	0.0041	0.0047		0.0030	0.0048		0.73	0.635	
1,5-Anhydro-D-glucitol	0.5998	0.4012		0.4996	0.4439		0.83	0.622	
α-Sorbopyranose_1(or Fructose_1)	0.0094	0.0045		0.0116	0.0078		1.24	0.444	
Lysine(3TMS)	0.0150	0.0066		0.0188	0.0241		1.26	0.635	
Fructose_1	0.1368	0.1104		0.1497	0.2543		1.09	0.887	
Glucose_1	12.2604	2.9329		15.2600	7.1964		1.24	0.247	
Allose_2	0.0346	0.0075		0.0409	0.0265		1.18	0.481	
Galactose_2	0.0219	0.0091		0.0299	0.0139		1.37	0.158	
Gulcono-1,4-lactone	0.0251	0.0308		0.0297	0.0425		1.18	0.796	
Lysine(4TMS)	0.6880	0.3028		1.0015	0.7289		1.46	0.235	
Histidine	0.0110	0.0100		0.0198	0.0145		1.80	0.145	
Glucuronate_1	0.0063	0.0037		0.0099	0.0070		1.57	0.178	
Glucosamine_2	0.0066	0.0058		0.0111	0.0084		1.68	0.198	
Tyrosine	0.7945	0.3486		0.8049	0.4832		1.01	0.959	
Galacturonic acid_2	0.0078	0.0019		0.0114	0.0062		1.47	0.099	
Pantothenate	0.0031	0.0016		0.0041	0.0031		1.35	0.359	
S-Benzyl-L-Cysteine_1	0.0018	0.0020		0.0037	0.0030		2.08	0.125	
Xanthine	0.0038	0.0022		0.0064	0.0060		1.69	0.220	
Palmitoleate	0.0030	0.0018		0.0031	0.0038		1.06	0.897	
N-Acetyl-D-Glucosamine_2	0.0019	0.0012		0.0031	0.0017		1.68	0.082	
Inositol	0.3478	0.2047		0.5405	0.4694		1.55	0.260	
Uric acid	0.5701	0.1517		0.6043	0.3441		1.06	0.782	
Tryptophan	0.3105	0.0942		0.4064	0.2207		1.31	0.232	
Elaidic acid	0.0021	0.0013		0.0018	0.0009		0.82	0.480	
3-Hydroxy-DL-Kynurenine	0.0020	0.0010		0.0016	0.0006		0.83	0.413	
5-Hydroxy-L-tryptophan_1	0.0250	0.0237		0.0472	0.0435		1.89	0.187	
2'-Deoxyuridine_2	0.0022	0.0012		0.0022	0.0008		1.02	0.916	
b-Lactose_2	0.0014	0.0004		0.0033	0.0044		2.39	0.180	
Lactitol	0.0020	0.0016		0.0019	0.0009		0.96	0.913	
Turanose	0.0015	0.0010		0.0023	0.0022		1.59	0.283	
Melibiose_2	0.0015	0.0005		0.0021	0.0011		1.47	0.087	
Maltotriose_1	0.0018	0.0007		0.0026	0.0009		1.44	0.051	

The plasma levels of each metabolite were normalized to the peak intensity of the internal standard, sinapinic acid. #, metabolites that showed significant change ($p < 0.05$).

3. Plasma metabolomic profile of SMA-model mice and control mice

The comprehensive plasma metabolomic profiles from five SMA-model mice and four control mice using GC/MS analysis were presented in Table III. The metabolomics identified 42 metabolites from the mice plasma. Among them, only kynurenate level showed significant reduction in SMA-model mice plasma compared to control mice plasma. When we compared the identified plasma metabolites in human and mice, some metabolites identified in human could not be found in mice, and vice versa, indicating plasma metabolome profile could have species-specificity.

From the results of metabolomics in both human plasma and HepG2 cell line, SMN reduction seemed to contribute to the elevated PEA levels. However, in control and SMA-model mice at zero to one week of age, there was no detectable amount of PEA in the comprehensive metabolome analysis.

Table III. Plasma metabolites examined in control mice and SMA-model mice

	Control			SMA-model				
	Mean	SEM		Mean	SEM		Fold induction	*P*
n-Propylamine	0.1208	0.0177		0.1326	0.0123		1.10	0.590
Lactic acid	5.1135	1.3422		3.7007	0.9843		0.72	0.413
Sarcosine	0.0682	0.0057		0.0582	0.0076		0.85	0.343
3-Hydroxy-Butyrate	0.3686	0.1327		0.4381	0.0973		1.19	0.678
Valine(2TMS)	0.0524	0.0096		0.0416	0.0155		0.79	0.595
Serine(2TMS)	0.0553	0.0121		0.0386	0.0060		0.70	0.226
Glycerol	1.2196	0.2659		0.8507	0.1244		0.70	0.218
Phosphate	14.3174	3.8947		8.7707	1.1846		0.61	0.175
Isoleucine	0.0220	0.0030		0.0164	0.0059		0.74	0.458
Glycine(3TMS)	2.1879	1.2954		0.8570	0.0731		0.39	0.282
Serine(3TMS)	0.1055	0.0321		0.1268	0.0653		1.20	0.796
2,3-Bisphospho-glycerate	0.1357	0.0268		0.1231	0.0186		0.91	0.701
Malic acid	0.0516	0.0159		0.0257	0.0057		0.50	0.137
meso-erythritol	0.0829	0.0212		0.0829	0.0170		1.00	0.998
Homoserine lactone	0.6743	0.2197		0.8361	0.0911		1.24	0.484
Aspartic acid	0.0417	0.0146		0.0237	0.0137		0.57	0.403
Pyroglutamic acid	0.1238	0.0515		0.0586	0.0075		0.47	0.200
Glutamic acid	0.0651	0.0255		0.0358	0.0104		0.55	0.285
Phenylalanine	0.0187	0.0073		0.0181	0.0068		0.97	0.950
Ribose	0.0482	0.0188		0.0642	0.0135		1.33	0.500
Citric acid + Isocitric acid	0.2210	0.0745		0.1492	0.0383		0.68	0.391
2-Aminopimelic acid	0.0458	0.0184		0.0363	0.0082		0.79	0.626
Ornithine	0.1883	0.0814		0.0347	0.0098		0.18	0.072
1,5-Anhydro-D-glucitol	0.0384	0.0110		0.0424	0.0054		1.10	0.734
Lysine(3TMS)	0.0393	0.0147		0.0273	0.0029		0.69	0.394
Mannose_1	0.1053	0.0316		0.0924	0.0202		0.88	0.732
2-Dehydro-D-gluconate_1	0.0289	0.0089		0.0311	0.0057		1.08	0.833
Glucose_1	13.9195	3.0809		13.2067	3.0673		0.95	0.876

Mannose_2	0.0512	0.0390		0.0141	0.0036		0.28	0.317	
2-MethylHippurate_2	0.5432	0.1650		0.4832	0.1222		0.89	0.773	
Glucose_2	3.2932	0.7858		3.1683	1.1291		0.96	0.934	
Gulcono-1,4-lactone	0.4512	0.1750		0.2087	0.0644		0.46	0.197	
Lysine(4TMS)	0.6944	0.2778		0.4047	0.1604		0.58	0.373	
Tyrosine	0.1966	0.0610		0.1260	0.0347		0.64	0.323	
Galacturonic acid_2	0.0436	0.0161		0.0301	0.0070		0.69	0.433	
Coniferyl aldehyde_1	0.0928	0.0825		0.0211	0.0065		0.23	0.357	
N-Acetyl-D-Glucosamine_1	0.0337	0.0194		0.0413	0.0260		1.22	0.830	
Inositol	1.1647	0.4617		1.4399	0.6707		1.24	0.759	
Kynurenate	0.0305	0.0071		0.0115	0.0026		0.38	0.028	#
Sakuranetin_2	0.0509	0.0205		0.0636	0.0267		1.25	0.728	
Myricetin	0.7323	0.2736		0.3761	0.1251		0.51	0.242	
Maltotriose_2	0.0242	0.0132		0.0630	0.0218		2.60	0.199	

The plasma levels of each metabolite were normalized to the peak intensity of the internal standard, sinapinic acid. #, metabolites that showed significant change ($p < 0.05$).

DISCUSSION

1. PEA elevation in the serum of patients with SMA type 1

In this study, we performed a comparative plasma metabolomics using SMA type 1 patients and control (Table II). Of note, both groups showed equivalent age and muscle weakness. We identified four significantly elevated metabolites in SMA type 1 patients' plasma compared to control group, and one of the metabolites, PEA, was reproducibly elevated in *SMN*-silenced hepatocytes. Interestingly, PEA has not been included in the list of biomarker candidates for SMA presented by Finkel [18]. We revealed the increased level of PEA in *SMN*-silenced hepatocyte with unbiased, comprehensive metabolome analysis. These findings might suggest that SMN reductions had significant impact on human PEA metabolism pathways.

2. Cellular activity related to PEA elevation in the SMA patients

PEA is a phosphomonoester metabolite of the phospholipid metabolism. PEA is a product of sphingolipid degradation from the sphingolipid metabolism pathway and also a precursor of glycerophospholipid biosynthesis from the glycerophospholipid metabolism pathway (Figure 2). These pathways are common in men and mice.

PEA can accumulate when cellular membrane remodeling or degradation increases [19]. Our result showed the increase of PEA levels in the plasma of SMA patients and in *SMN*-silenced hepatocytes, suggesting that the increased membrane remodeling occur in the liver cells or non-neuronal cells. As for the relationship between elevated PEA and bone metabolism, we will discuss it later in this section.

3. Absence of PEA elevation in SMA mice

Plasma from SMA-model mice and control mice showed different profile compared to SMA patients and control human plasma; there was no increase of PEA even in the SMA-model mice plasma.

According to our data, SMA-model mouse plasma showed different metabolomics profile compared to SMA patient plasma (Table 2 and Table 3). Of the four significant different metabolites between SMA patients and control subjects, none of them could be identified in SMA-model mouse plasma or control mouse plasma. Between SMA-model mice and control mice, no metabolites other than kynurenate differ significantly at the plasma concentration level. Kynurenate showed significant reduction in SMA-model mice compared to control mice (Table III).

A study reported that a specific abnormal splicing pattern was observed in the transcript of alanine-glyoxylate aminotransferase 2- like 1 (AGXT2L1) of the brain tissue of SMA-model mice [20]. AGXT2L1 has PEA-phospholyase activity to specifically degrade PEA into acetaldehyde, phosphate and ammonia [19, 21]. However, our study did not show any change of AGXT2L1 transcript level and its protein level in *SMN*-silenced HepG2 cells (data not shown).

We failed to identify the alteration of PEA level in our SMA-model mice in this study, as mentioned above. It suggested that SMN protein regulated the PEA metabolism differently between our patients and the SMA-model mice used in this study.

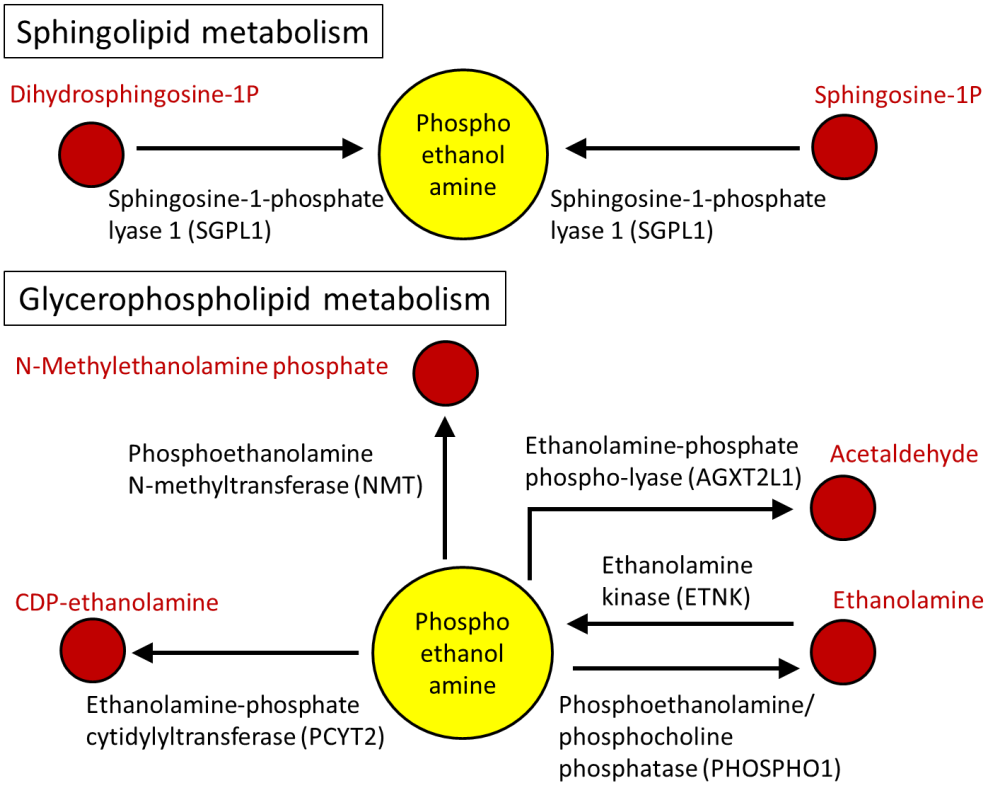

Figure 2. Schematic diagram of PEA metabolism in men and mice.
Arrows indicate direction of enzyme reaction. Enzyme names are shown near the arrows.

4. Clinical perspective: PEA as a possible biomarker of bone fragility in SMA

We clarified the relationship between decreased SMN protein and PEA elevation. However, we do not know what tissues are most responsible for PEA elevation in SMA patients.

Recently, bone fragility of SMA patients have often been reported [22, 23], which will lead to the idea that PEA elevation may be related to the bone metabolism. If it is the case, PEA can be a biomarker of bone metabolism in SMA. However, unfortunately, any clinical data or laboratory data of SMA patients were not available in this study.

It is well-known that PEA is elevated in hypophosphatasia (HPP). HPP is a metabolic disorder that features low serum alkaline phosphatase activity (ALP). Hypophosphatasemia is mainly caused by a loss-of-function mutation of the gene encoding the tissue-nonspecific isoenzyme of ALP (TNSALP). PEA is usually elevated in serum and urine in HPP, though less reliably for diagnosis [24]. PEA is a phosphoethanolamine/ phosphocholine phosphatase (PHOSPHO1) [25]. Additional deficit of the enzyme in osteoblasts and chondrocytes may increase PEA in HPP.

According to Morcos et al. [26], PHOSPHO1 is essential for normal bone fracture healing. It suggests that PHOSPHO1 should be the first target to be investigated in bone fragility of SMA patients. Though our experiments on bone metabolism are still on the way and have much to be done, our tentative hypothesis is as follows; (1) decrease of SMN protein may have some negative effect on the PHOSPHO1 activity in osteoblasts and chondrocytes, and, consequently, (2) PEA is accumulated in osteoblasts and chondrocytes, (3) accumulated PEA is released into the plasma, (4) inorganic phosphate (Pi) cannot be properly used in hydroxyapatite formation (Figure 3).

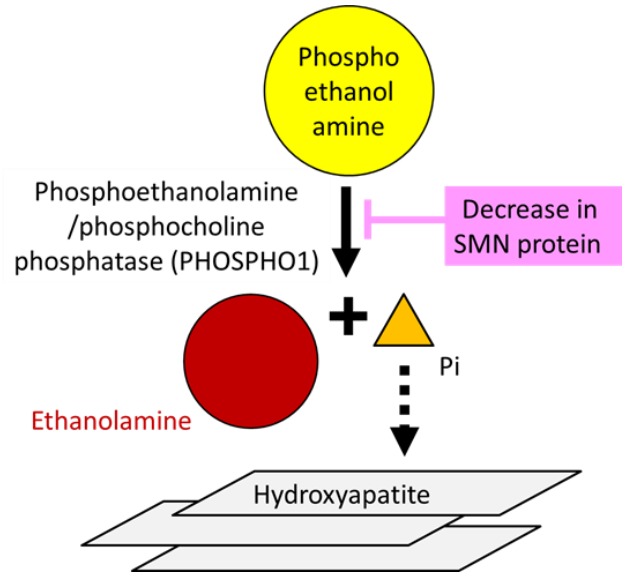

Figure 3. Hypothesis of PEA metabolism in osteoblasts and chondrocytes.
"Pi" denotes inorganic phosphate. Decease in SMN protein may have suppressive effect on PHOSPHO1 activity.

5. Conclusion

Our data suggested that PEA is one of the possible biomarkers of human SMA reflecting metabolic abnormalities due to SMN deficiency. The increased plasma PEA suggested the systemic impact of SMN deficiency on metabolism of SMA patients. However, SMA-model mice did not show PEA elevation in plasma. Further investigation is necessary to clarify the responsible organs and underlying mechanisms of PEA elevation in the plasma of SMA type 1 patients.

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