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Oxidized phosphatidylcholines suggest oxidative stress in patients with medium-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

Inborn errors of metabolism encompass a large group of diseases caused by enzyme deficiencies and are therefore amenable to metabolomics investigations. Medium chain acyl-CoA dehydrogenase deficiency (MCADD) is a defect in β-oxidation of fatty acids, and is one of the most well understood disorders. We report here the use of liquid chromatography–mass spectrometry (LC–MS) based untargeted metabolomics and targeted flow injection analysis–tandem mass spectrometry (FIA–TMS) that lead to discovery of novel compounds of oxidative stress. Dry blood spots of controls ($n=25$) and patient samples ($n=25$) were extracted by methanol/water (1/1, v/v) and these supernatants were analyzed by LC–MS method with detection by an Orbitrap Elite MS. Data were processed by XCMS and CAMERA followed by dimension reduction methods. Patients were clearly distinguished from controls in PCA. S-plot derived from OPLS-DA indicated that medium-chain acylcarnitines (octanoyl, decenoyl and decanoyl carnitines) as well as three phosphatidylcholines (PC(16:0,9:0(COOH))), PC(18:0,5:0(COOH)) and PC(16:0,8:0(COOH)) were important metabolites for differentiation between patients and healthy controls. In order to biologically validate these discriminatory molecules as indicators for oxidative stress, a second cohort of individuals were analyzed, including MCADD ($n=25$) and control ($n=250$) samples. These were measured by a modified newborn screening method using FIA–TMS (API 4000) in MRM mode. Calculated *p*-values for PC(16:0,9:0(COOH)), PC(18:0,5:0(COOH)) and PC(16:0,8:0(COOH)) were 1.927×10^{-14}, 2.391×10^{-15} and 3.354×10^{-15} respectively. These elevated oxidized phospholipids indeed show an increased presence of oxidative stress in MCADD patients as one of the pathophysiological mechanisms of the disease.

1. Introduction

Fatty acid oxidation disorders (FAODs) are metabolic diseases with a cumulative incidence of approximately 1:9300 (measured in 2010) based on newborn screening (NBS) in Australia, Germany and USA [1]. At least 15 different disorders of fatty acid metabolism are known today [2]. Medium chain acyl-CoA dehydrogenase deficiency (MCADD OMIM #201450) is one of the most

Abbreviations: MCADD, Medium chain acyl-CoA dehydrogenase deficiency; LC–MS, liquid chromatography–mass spectrometry; FIA–TMS, flow injection analysis–tandem mass spectrometry; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; MRM, multi-raction-monitoring mode; FAODs, fatty acid oxidation disorders; NBS, newborn screening; MCAD, medium chain acyl-CoA dehydrogenase enzyme; C8, octanoylcarnitine; C6, hexanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C2, acetylcarnitine; C12, dodecanoylcarnitine; PGPC, 1-O-hexadecanoyl-2-O-(9-carboxybutanoyl)-sn-glyceryl-3-phosphocholine; PAzPC, 1-O-hexadecanoyl-2-O-(9-carboxyoctanoyl)-sn-glyceryl-3-phosphocholine; FWHM, full width at half maximum; QC, quality control; CID, collision-induced dissociation; HCD, higher-energy collisional dissociation; FTMS, fourier transform mass spectrometry; LOESS, local regression; clr, centered logratio transformation; MSI, Metabolite Standards Initiative; ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; PoxnoPC, 1-O-hexadecanoyl-2-O-(9-oxononanoyl)-sn-glyceryl-3-phosphocholine; PAPC, 1-O-hexadecanoyl-2-O-(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoyl-sn-glyceryl-3-phosphocholine

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common fatty oxidation defects and it is inherited as an autosomal recessive trait. The medium chain acyl-CoA dehydrogenase (MCAD) enzyme (EC 1.3.99.3) metabolizes medium long chain fatty acids (carbon moiety length from 4 to 12) in a β-oxidation pathway which is localized in mitochondria and starts the mitochondrial β-oxidation pathway by introducing a double bond into a β-position of acyl-Coenzyme A (acyl-CoA). *ACADM* gene is located at 1p31.1 and one prevalent mutation c.985A > G accounts for up to 90% of mutant alleles identified so far. Up to now 36 mutations have been identified by newborn screening [2] and the frequency of mutation varies substantially between different populations with a particularly high incidence in north-central Europe: Denmark 1:8954 [3], England 1:10,000 [4], Netherlands 1:30,000 [5], Germany 1:133,000 [6], Greece 1:16,000 [7], and an overall incidence in USA 1:15,000 [8]. By contrast, with the exception of Greece, low incidence was generally found in southern Europe (1:300,000) [9] and no cases have been reported in Japan.

The clinical picture of the disease is highly variable ranging from severe-life threatening forms to asymptomatic patients identified retrospectively in families with positive newborn screening. The symptoms can appear at any age from newborn to adult, but the most common age of presentation is between 3 and 15 months. Symptoms in untreated patients cover hypoglycemic, acute encephalopathy initiated by common infectious diseases or fasting [10]. Up to 50% of the patients die during the first episode. Those, who survive, suffer from development delay, hypotonia and (cardio-)myopathy. The conditions can be effectively managed by preventing hypoglycemia. This is achieved by increasing feeding frequency with food containing a high starch content and a limited supply of medium chain triglyceride oils. Acute patients exhibit hyperamonemia and characteristic profile of elevated organic acids in urine – adipic, suberic, sebacic acids, hexanoic, octanoic acids [11] – and several other glycine conjugates – hexanoylglycine, 3-phenylpropionylglycine and suberylglycine [12]. This biochemical picture depends on the clinical status and can disappear during the period of normalcy.

The disease is screened in the majority of developed countries in the world. Nowadays, the common technique used in NBS is tandem mass spectrometry. This enables the rapid (typically 1 min/sample) measurement of dozens of diagnostically important metabolites with very high sensitivity and specificity [13]. The main diagnostic marker for MCADD is octanoylcarnitine [C8] and secondary markers include hexanoylcarnitine [C6], decanoylcarnitine [C10] and decenoylcarnitine (C10:1), respectively [14]. The screening of MCADD is based on determination of elevated levels of several acylcarnitines and their ratios with other acylcarnitines (e.g. acetylcarnitine (C2), dodecanoylcarnitine (C12) – C8/C2; C8/C12; C8/C6) in dried blood spots usually taken 1–3 days after birth [14].

In the area of inherited metabolic disorders untargeted metabolomics was firstly utilized by Siuzdak's group [15]. They used capillary reverse phase liquid chromatography with nonlinear alignment software (XCMS) data processing on samples from patients suffering from methylmalonic and propionic acidemia. Recently, this approach was already successfully applied on various defects in amino acid metabolism, organic acidurias and mitochondrial defects (including MCADD) [16]. The aim of this work was to investigate patho-physiological/-biochemical changes associated with MCADD using untargeted MS-based metabolomics.

2. Materials and methods

2.1. Chemicals

All solvents (acetonitrile, methanol and water) were of LC–MS quality purchased from Sigma-Aldrich (St. Louis, USA). Acetic acid and ammonium hydroxide were also purchased from Sigma-Aldrich. Standards of PGPC (1-O-hexadecanoyl-2-O-(9-carboxybutanoyl)-*sn*-glyceryl-3-phosphocholine) and PAzPC (1-O-hexadecanoyl-2-O-(9-carboxyoctanoyl)-*sn*-glyceryl-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

2.2. Samples

Dry blood spots from healthy newborns (control group, $n=25$) and patients suffering from MCADD (8 patients, $n=25$; from subsequent sampling) were chosen for the experiments. Four patients were compound heterozygotes and four were homozygous with mutation c.985A > G. In order to confirm the results and validate the chemical species (metabolites) identified in untargeted metabolomics experiment a second set of samples from 250 newborns was used for targeted metabolite analysis. All samples were collected in laboratory for inherited metabolic disorders (University Hospital Olomouc, CZ) within the pilot project of Czech newborn screening program. Written informed consent was obtained for all samples that were used in the analyses.

2.3. Untargeted metabolomics method

Two disks (3.2 mm) were dissected from dry blood spot and extracted in pure methanol (100 μL). After shaking (30 min, 25 °C) the sample was centrifuged (24,400*g*, 15 min, 4 °C). Supernatant (50 μL) was mixed with water (50 μL) and analyzed by the LC–MS untargeted metabolomics method adopted from Bajad et al. [17].

The stationary phase employed an aqueous normal phase separation system using amino-propyl stationary Luna NH_2 3 μm 100 Å, 150 × 2 mm^2 (Phenomenex, Torrance, USA). An Ultimate 3000 RS (Thermo Fisher Scientific, MA, USA) was used for liquid chromatography and binary gradient elution consisted of 20 mm ammonium acetate in water, pH 9.45 (mobile phase A) and acetonitrile (mobile phase B). The gradient elution with flow rate of 0.3 mL/min was as follows: $t=0.0$, 95% B; $t=15.0$, 30% B; $t=17.0$, 5% B; $t=23.0$, 5% B; $t=23.1$, 95% B; $t=28.0$ min 95% B. The injection volume was 10 μL.

An Orbitrap Elite (Thermo Fisher Scientific, MA, USA) operating in positive full scan mode (120,000 FWHM) within range of 70–1200 *m/z* was used for untargeted metabolomics experiments. Settings of the electrospray ionization were: heater temperature of 300 °C, sheath gas of 35 arb. units, auxiliary gas of 10 arb. units, capillary temperature of 350 °C and source voltage was +3.0 kV. A Thermo Tune Plus 2.7.0.1103 SP1 was used as instrument control software and data were acquired in profile mode using Thermo Excalibur 2.2 SP1.48 software (Thermo Fisher Scientific, MA, USA).

Quality control (QC) samples were prepared by pooling of all patient and control samples (10 μL). Blank sample was prepared by the same procedure without disks from dry blood spots. The order of healthy controls and MCADD patient's samples was randomized in the experiment batch. QC samples were analyzed and used as previously published [18]. Fragmentation spectra MS^n were acquired on an Orbitrap Elite using CID and HCD fragmentation method with detection via FTMS (resolution 60,000 FWHM) in both positive and negative mode as required. Settings for MS^2 and MS^3 experiments were as follows: act.Q. of 0.25, act. time of 10 ms (for MS^3 20 ms) and normalized collision energy of 35%. HCD fragmentation settings were as follows: act.Q. of 0.10 and normalized collision energy of 40%.