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Measurement of tissue acyl-CoAs using flow-injection tandem mass spectrometry: acyl-CoA profiles in short-chain fatty acid oxidation defects

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ABSTRACT

The primary accumulating metabolites in fatty acid oxidation defects are intramitochondrial acyl-CoAs. Typically, secondary metabolites such as acylcarnitines, acylglycines and dicarboxylic acids are measured to study these disorders. Methods have not been adapted for tissue acyl-CoA measurement in defects with primarily acyl-CoA accumulation. Our objective was to develop a method to measure fatty acyl-CoA species that are present in tissues of mice with fatty acid oxidation defects using flow-injection tandem mass spectrometry.

Following the addition of internal standards of [$^{13}C_2$] acetyl-CoA, [$^{13}C_8$] octanoyl-CoA, and [C_{17}] heptadecanoic CoA, acyl-CoA's are extracted from tissue samples and are injected directly into the mass spectrometer. Data is acquired using a 506.9 neutral loss scan and multiple reaction-monitoring (MRM).

This method can identify all long, medium and short-chain acyl-CoA species in wild type mouse liver including predicted 3-hydroxyacyl-CoA species. We validated the method using liver of the short-chain-acyl-CoA dehydrogenase (SCAD) knock-out mice. As expected, there is a significant increase in [C_4] butyryl-CoA species in the SCAD −/− mouse liver compared to wild type. We then tested the assay in liver from the short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) deficient mice to determine the profile of acyl-CoA accumulation in this less predictable model. There was more modest accumulation of medium chain species including 3-hydroxyacyl-CoA's consistent with the known chain-length specificity of the SCHAD enzyme.

1. Introduction

The oxidation of fatty acids in the mitochondria results in the production of energy to be used when there is an increased demand for energy including fasting, illness, and muscular exertion. The process of fatty acid oxidation (FAO) includes the carnitine cycle, the beta-oxidation cycle, the electron-transport chain, and, in liver, ketone synthesis. Free fatty acids are activated to their coenzyme A (CoA) esters in the cytosol at the outer mitochondrial membrane. Long-chain (C16 and C18) fatty acyl-CoAs enter the mitochondria as acylcarnitines via the carnitine transport system and are reconverted back to their respective acyl-CoA's at the inner mitochondrial membrane. With each step of FAO, fatty acyl-CoAs are chain-shortened by two carbons until completely converted to acetyl-CoA. Energy released during beta-oxidation is transferred to the electron transport chain resulting in the production of adenosine triphosphate (ATP). In the liver, most of the acetyl-CoA is used to synthesize ketone bodies (3-hydroxybutyrate and acetoacetate), which can be used as fuel by tissues that are unable to oxidize fatty acids, most notably, the brain. Tissues such as cardiac and skeletal muscle are capable of direct utilization of the fatty acids as a source of energy [1]. Disorders in FAO can result in various phenotypes including hypoglycemia, brain damage, liver disease, kidney dysfunction, and damage to cardiac and skeletal muscle [2,3]. Early detection of FAO disorders is critical to ensure that the proper evaluation and management are performed in a timely fashion [4]. The primary accumulating metabolites in fatty acid oxidation defects are intramitochondrial acyl-CoAs. Typically, secondary metabolites such as acylcarnitines, acylglycines and dicarboxylic acids are measured to study these defects. Methods have not been readily available for tissue acyl-CoA measurement. Most published methods involve HPLC separations and all previously published methods do not detect a full range of acyl-CoA species, mainly due to hydrophobicity differences between long-, medium- and short-chain acyl-CoA species [5–16]. We have developed an analytical approach to directly measure fatty acyl-CoA species of all chain lengths using flow-injection tandem mass spectrometry. The method was validated using liver from the short-chain-acyl-CoA dehydrogenase (SCAD) deficient mouse and tested using the medium- and short-chain-3-hydroxyacyl-CoA dehydrogenase (SCHAD) knock out mouse.

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2. Materials and methods

2.1. Materials

Acetyl-$^{13}C_2$ CoA, octanoyl-$^{13}C_4$ CoA, and heptadecanoyl CoA for internal standards and unlabeled acyl-CoA's were purchased from Sigma Aldrich (St. Louis, MO). Methanol and chloroform were HPLC or LC/MS grade from Fisher Scientific (Pittsburgh, PA). Strata X-AW 33 μ Polymeric Weak Anion 200 mg/3 ml Solid Phase Extraction (SPE) columns were from Phenomenex (Torrance, CA).

2.2. Sample preparation

Stable isotope labeled acetyl-$^{13}C_2$ CoA, octanoyl-$^{13}C_4$ CoA, and heptadecanoyl CoA solutions were mixed as internal standards before liver extraction. About 100 mg of frozen liver was weighed out in a 12×75 mm polypropylene tube. Then, 30 nmol of the acetyl-^{13}C2 CoA and 14.4 nmol of octanoyl-^{13}C4 CoA and heptadecanoyl CoA internal standard mixture was added to the tube. Acyl-CoAs were extracted with 3 ml of methanol-chloroform (2:1 by volume). A PowerGen 125 homogenizer (Fisher Scientific, PA) was used to homogenize the liver specimen twice whilst keeping the polypropylene tube on ice. Following each 30 second homogenization, the homogenate was centrifuged at 1300*g* for 15 minutes at 4 °C [17]. The supernatants were combined in a 15 ml polypropylene tube and were then phase separated by adding 1.5 ml of 10 mM ammonium formate and 1.5 ml of chloroform and vortexed for 10 seconds. The upper layer containing the acyl CoA fraction was collected after a 15 minute centrifugation at 1300*g* at 4 °C and further purified by elution through a weak anion reverse phase SPE column. The SPE protocol for acyl CoA clean up was to condition the SPE column with 3 ml methanol, then to equilibrate with 3 ml water, load the supernatant fraction, wash the column with 2.4 ml of 2% formic acid, followed by an additional wash with 2.4 ml of methanol. The first elution of the CoAs was with 2.4 ml of 2% ammonium hydroxide followed by a second elution with 2.4 ml of 5% ammonium hydroxide. The two eluted fractions were then combined in 13×100 mm glass tube and dried under a nitrogen stream at room temperature. Prior to injection into the mass spectrometer, the samples were reconstituted in 100 μl of 50% methanol.

2.3. Flow injection tandem mass spectrometry

Flow injection tandem mass spectrometry analysis was performed using a Waters Quattro Ultima electrospray tandem mass spectrometer fitted with a Waters 2795 high-performance liquid chromatography system (Waters Corporation, Milford, MA). The running buffer used was 5 mM ammonium formate in methanol. The Quattro Ultima was operated in positive ionization mode. The capillary voltage was set at 3.40 kV, cone voltage at 40 V, source temperature was 120 °C, desolvation temperature was 400 °C, and desolvation nitrogen flow was 800 l/h. Collision energy was 35 V, the dwell time 0.07 seconds and the flow injection rate was 0.15 ml/minute.

Samples were flow injected into the tandem mass spectrometer. A complete acyl-CoA profile was identified using the unique neutral loss of *m/z* 507 (Fig. 1). The MRM transitions for C2, C3, C4, C6, C8, C10, C12, C14, C16, C18, C18:1, and C18:2 acyl-CoA respectively were *m/z* 810→303, 824→317, 838→331, 866→359, 894→387, 922→415, 950→443, 978→471, 1006→499, 1034→527, 1032→555, and 1030→553. The acyl-CoA concentrations were calculated by measuring the ratio of the intensity of each acyl-CoA species to the intensity of the nearest chain-length internal standard.

3. Results

3.1. Selection of criteria for acyl-CoA identification and quantification

Fig. 1 shows the fragmentation pattern of acetyl-CoA identifying the neutral loss pattern and demonstrates the mass-specificity and the fragment used for quantitation.

3.2. Recovery and precision experiments

Intraassay imprecision studies were performed by analyzing 5 replicates of a single control tissue seeded with unlabeled acetyl-, octanoyl- and palmitoyl-CoAs at low and high concentrations and determining the mean, standard deviation and coefficient of variation of the calculated data. Interassay imprecision was determined by analysis of the same tissue extracted on 5 separate days and analyzed in 5 separate runs. Recoveries were determined by seeding in the same three acyl-CoAs prior to the extraction process at a predicted additional concentration of 0.5 μmol/l and repeating the extraction and analysis process 6 times.

Table 1 lists the recovery and imprecision data obtained using acetyl (C2) octanoyl (C8) and palmitoyl (C16) CoA species. In the initial phases of these experiments we detected residual carryover of the C16 species. Subsequent to this observation we added an additional flush cycle of 50% methanol to the analytical program which has now removed the contaminating carryover of long-chain species.

3.3. Measurement of Acyl-CoA Species in wild type mouse liver

Fig. 2 shows the full scan mass spectrometric profile of all acyl-CoA species that were detected in fasted wild-type mouse liver and demonstrates that this method can identify all long- medium- and short-chain acyl-CoA species in wild type mouse liver including putative 3-hydroxy and 3-ketoacyl-CoA species based on fragmentation patterns and mass determination.

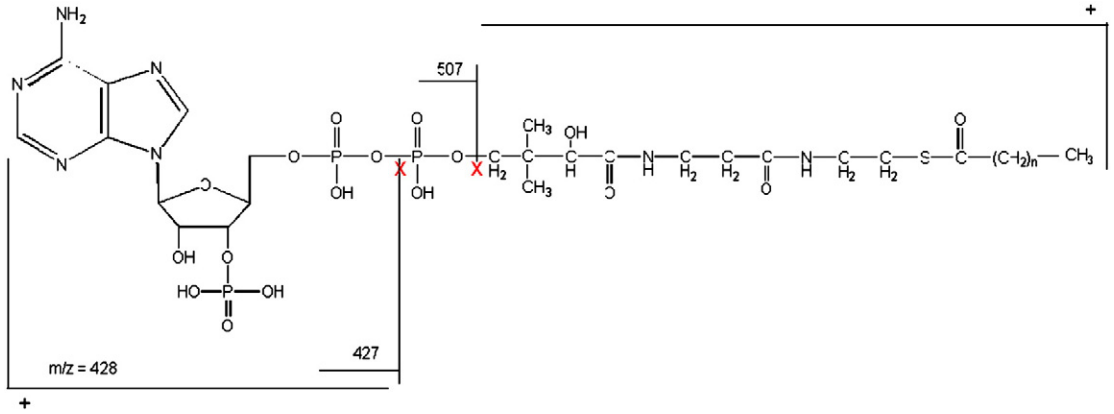

Fig. 1. The acyl-CoA fragmentation pattern showing the mass-specific fragment and the neutral loss of *m/z* 507.