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Assay of the activity of malonyl–coenzyme A decarboxylase by gas chromatography–mass spectrometry

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Abstract

We developed a gas chromatography–mass spectrometry (GC–MS) assay to measure the activity of malonyl–coenzyme A (CoA) decarboxylase (MCD) in crude tissue homogenates. Liver extracts are incubated with [U-$^{13}C_3$]malonyl–CoA to form [U-$^{13}C_2$]acetyl–CoA by the action of MCD. The reaction mixture contains 2 mM ADP to prevent the hydrolysis of [1,2-$^{13}C_2$]acetyl–CoA by acetyl–CoA hydrolase present in the extracts. Newly formed [U-$^{13}C_2$]acetyl–CoA and internal standard of [$^2H_3$,1-^{13}C]acetyl–CoA are analyzed as thiophenol derivatives by GC–MS. This assay was applied to a study of the kinetics of MCD in rat liver. Using the Lineweaver–Burke plot of MCD kinetics, K_m of 202 μM and V_{max} of 3.3 μmol min^{-1} (g liver)$^{-1}$ were calculated. The liver MCD activities (μmol min^{-1} g^{-1} ± SD) in three groups of rats with different nutritional statuses—fed, 1-day fasted, and 2-day fasted—were 1.80 ± 0.41, 2.59 ± 0.37 ($P < 0.05$), and 3.07 ± 0.70 ($P < 0.05$), respectively. We report a practical, nonradioactive, sensitive assay of MCD in crude tissue extract.

Keywords: Malonyl–CoA; Malonyl–CoA decarboxylase (MCD); MCD activity; GC–MS; Thiophenol; Acetylthiophenol

Malonyl–coenzyme A (CoA)[1] is an intermediate of fatty acid synthesis. In lipogenic and nonlipogenic organs, malonyl–CoA contributes to the regulation of mitochondrial fatty acid oxidation by inhibiting carnitine palmitoyltransferase I, the enzyme that controls the transfer of long-chain fatty acyl–CoAs from the cytosol to the mitochondrial matrix [1–3]. Malonyl–CoA is formed by cytosolic acetyl–CoA carboxylase (ACC) and by a side reaction of mitochondrial propionyl–CoA carboxylase [4]. The fates of malonyl–CoA are (i) fatty acid synthesis and (ii) decarboxylation via malonyl–CoA decarboxylase (MCD). Thus, ACC and MCD catalyze a substrate cycle that contributes to the regulation of fatty acid metabolism.

MCD occurs in a wide array of organisms, from prokaryotes to mammals [5]. In rat liver, MCD originally was identified only in peroxisomes and cytosol [6]. However, a recent study reported that MCD is present in rat liver mitochondria, peroxisomes, and cytosol, with the peroxisomal enzyme showing the highest specific activity [7]. MCD participates in the regulation of fatty acid oxidation in heart and skeletal muscle [8,9]. In muscle, MCD is activated by phosphorylation [9]. Also, conditions associated with elevated plasma-free fatty acid levels (high-fat feeding, starvation, and streptozotocin-induced diabetes) increase MCD messenger RNA (mRNA) in rat heart and skeletal muscle [8]. MCD expression is activated by the peroxisome proliferator-activated receptor α (PPARα) [10], a member of the nuclear hormone receptor superfamily and a fatty acid-activated transcription factor that regulates lipid metabolism [10]. Indeed, in the hearts of PPARα-deficient mice, the expression of MCD is decreased, malonyl–CoA

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[1] *Abbreviations used:* CoA, coenzyme A; ACC, acetyl–CoA carboxylase; MCD, malonyl–CoA decarboxylase; mRNA, messenger RNA; PPARα, peroxisome proliferator-activated receptor α; GC–MS, gas chromatography–mass spectrometry; MCDi, MCD inhibitor; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; PCI, positive chemical ionization; TMS, trimethylsilyl; LC, liquid chromatography.

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concentration is increased, and the rate of fatty acid oxidation is decreased [11].

There are several assays currently available to measure MCD activity. The spectrophotometric assay [12] involves (i) forming acetyl–CoA from the decarboxylation of malonyl–CoA by MCD, (ii) generating oxaloacetate from malate + NAD^+ + malate dehydrogenase, and (iii) forming citrate from the condensation of oxaloacetate with acetyl–CoA via citrate synthase. The assay is monitored by the production of NADH. This technique has low sensitivity and is not applicable to crude preparations. The first described radiochemical assay [13,14] is based on the same reactions except that [^{14}C]oxaloacetate is generated by transamination of [^{14}C]aspartate with glutamate. The assay is monitored by the accumulation of [^{14}C]citrate. A second, more direct radiochemical assay [15] measures the formation of $^{14}CO_2$ from [3-^{14}C]malonyl–CoA. A third radiochemical assay, developed by Kerner and Hoppel [16], involves (i) the production of [2-^{14}C]acetyl–CoA from [2-^{14}C]malonyl–CoA, (ii) the conversion of [2-^{14}C]acetyl–CoA to [2-^{14}C]acetylcarnitine in the presence of excess L-carnitine and carnitine acetyltransferase, and (iii) the isolation of [2-^{14}C]acetylcarnitine on ion exchange resin and the counting of its radioactivity. The above three radiochemical assays are sensitive and have been used extensively. To avoid using radioactive substrates, we developed a stable isotope-based assay of MCD activity. Our assay involves (i) incubating [U-$^{13}C_3$]malonyl–CoA with the tissue extract containing MCD to form [1,2-$^{13}C_2$]acetyl–CoA, (ii) spiking the reaction mixture with [$^2H_3$,1-^{13}C]acetyl–CoA internal standard, (iii) reacting the acetyl–CoA with thiophenol to form the corresponding labeled acetylthiophenols [17], and (iv) assaying the concentration of acetylthiophenol by gas chromatography–mass spectrometry (GC–MS).

Materials and methods

Materials

Chemicals, biochemicals, and enzymes were obtained from Sigma–Aldrich. An MCD inhibitor (MCDi), CBM-301940 (3-{[5-(2,2,2-trifluoro-1-hydroxy-1- trifluoromethylethyl)-4,5-dihydro-isoxazole-3-carbonyl]-amino}-butyric acid *tert*-butyl ester), was generously provided by Chugai Pharma USA [13,18]. *N*-Methyl-*N*-trimethylsilyl-trifluoroacetamide was supplied by Regis Technologies. [U-$^{13}C_3$]Malonic acid and [$^2H_3$,1-^{13}C]acetic anhydride (99%) were purchased from Isotec. [U-$^{13}C_3$]Malonyl–CoA, was prepared from [U-$^{13}C_3$]malonic acid by the method of Weaire and Kekwick [19]. [$^2H_3$,1-^{13}C]Acetyl–CoA internal standard was prepared by reacting [$^2H_6$,1,1-$^{13}C_2$]acetic anhydride with CoA [20]. Both [U-$^{13}C_3$]malonyl–CoA and [$^2H_3$,1-^{13}C]acetyl–CoA were purified by high-performance liquid chromatography (HPLC) on a Hewlett–Packard 1090 liquid chromatograph equipped with a diode array UV detector monitoring absorbance at 260 nm, an autosampler, and a 250-mm C18 semipreparative column (30 cm, 10 mm i.d., Phenomenex, C18(2) Luna) with a Security Guard column (50 × 10 mm, Phenomenex, C18(2) Luna). Solvent reservoir A contained acetonitrile, and reservoir B contained 75 mM KH_2PO_4. The gradient profile was as follows: from 0 to 15 min, increase linearly the acetonitrile concentration from 5.8 to 7.0% and the flow rate from 3.25 to 3.40 ml/min; from 15 to 20 min, wash the column with 60% acetonitrile. Malonyl–CoA and acetyl–CoA elute at 7.54 and 17.74 min, respectively.

Sample preparation

Rats were killed by skull crushing just before laparotomy and excision of a liver lobe, which was quick-frozen between aluminum blocks precooled in liquid N_2 The frozen liver samples were powdered under liquid N_2, and the powder was kept in plastic tubes immersed in liquid N_2 until analysis.

Liver powder was extracted with a Polytron homogenizer in ice-cold 130 mM KCl/20 mM Hepes buffer (pH 7.4). To the slurry were added 0.1% Triton X-100 and 0.1% (v/v) Sigma protease inhibitor cocktail. The extract was kept on ice for 10 min with occasional vortexing before being centrifuged at 12,000*g* for 20 min. The supernatant was frozen until analysis.

Assay of MCD activity

Tissue extract corresponding to 0.25 mg of rat liver (25 μl) was added to 450 μl of buffer (45 mM potassium phosphate (pH 7.7), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, and 0.2 mM KCN). Water was also added to bring the final volume to 600 μl. When testing the linearity of the assay, 0.1 to 1.0 mg of tissue equivalent was used. When the MCD inhibitor CBM-301940 was tested, it was added in dimethyl sulfoxide (DMSO), resulting in a final DMSO concentration of 1% [13]. The same DMSO concentration was added to control incubations. After equilibration at 37 °C, 0.4 mM [U-$^{13}C_3$]malonyl–CoA was added to start the reaction. After 0 to 5 min incubation at 37 °C, six samples of 100 μl of incubation medium were removed at 1-min intervals and transferred to a glass tube containing 200 μl of ice-cold methanol and 7.6 nmol of [$^2H_3$,1-^{13}C]acetyl–CoA internal standard. This was followed by adding 0.5 ml of 100 mM potassium phosphate buffer (pH 8.5) and 0.15 ml of 1 mM thiophenol solution in tetrahydrofurane (dried on sodium, freshly distilled, and tested for the absence of acetylthiophenol). The reaction between acetyl–CoA and thiophenol was allowed to proceed for 4 h at 60 °C under agitation. Excess thiophenol was precipitated with 0.1 ml of 5 mM silver nitrate. Acetylthiophenol was extracted with 3 × 4 ml of diethyl ether. The extract was dried with Na_2SO_4, and the solvent was evaporated down to approximately 0.1 ml to avoid