Pitfalls in the Use of 2-Octynoic Acid as an In Vivo Model of Medium-Chain Acyl-Coenzyme A Dehydrogenase Deficiency: Ketone Turnover and Metabolite Studies in the Rat

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2-Octynoic acid was administered by intraperitoneal injection to fasted Sprague-Dawley rats in an attempt to simulate medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency. The resultant urine organic acid profile showed a mild dicarboxylic aciduria but lacked the glycine conjugates characteristic of MCAD deficiency. Further studies with infused $^{13}C_4$-3-hydroxybutyrate and $^{13}C_2$-acetoacetate demonstrated reduced ketone production in treated rats compared with control animals. Although plasma ketone body concentrations were low in treated rats, plasma free fatty acids were also low, thereby providing diminished substrate for ketone production. This is the reverse of the finding in children with MCAD deficiency, who have low levels of plasma ketones despite elevated free fatty acids. These animal studies were therefore not helpful in improving our understanding of ketone body kinetics in children with MCAD deficiency.

MEDIUM-CHAIN acyl-coenzyme A dehydrogenase (MCAD) deficiency is the most common disorder of fatty acid oxidation currently recognized, although fewer MCAD-deficient homozygotes than predicted are actually diagnosed. The reasons for this are uncertain and presumably reflect modifying factors, one of which may be residual ketone production capacity, which may be critical during periods of metabolic stress such as fasting and infection. The difference between the predicted and observed incidence of MCAD deficiency may reflect a higher residual rate of ketone synthesis in some MCAD-deficient patients.

To investigate ketone production in MCAD deficiency, we first studied an animal model of this condition. 2-Octynoyl coenzyme A (coA) was demonstrated by Freund et al[1] in 1985 to inhibit pig kidney MCAD in vitro. Following this, Montgomery and Mamer[2] published a description of induction of medium-chain dicarboxylic aciduria following administration to the rat of 2-octynoic acid, a presumed suicide inhibitor of the MCAD enzyme. We extended these studies by examining the metabolic effects of administration of intraperitoneal 2-octynoic acid to fasted Sprague-Dawley rats and subsequently studied ketone turnover using stable isotopes of acetoacetate and 3-hydroxybutyrate.

MATERIALS AND METHODS

Materials

The sodium salt of 1,3-$^{13}C_2$-acetoacetate was prepared by hydrolysis of an aqueous solution of labeled ethyl acetoacetate with a 10% molar excess of 1-mol/L sodium hydroxide. The mixture was incubated at 40°C for 75 minutes. The pH was reduced to 7 by titration with 2 mol/L hydrochloric acid. The material was then extracted three times with 2 vol diethyl ether to remove any unreacted ethyl acetoacetate. The aqueous component was lyophilized and stored at −80°C. Pyrogen testing was performed on an aqueous aliquot of the material. Purity was confirmed by gas chromatographic–mass spectrometric analysis and enzymatic analysis. 1,3-$^{13}C_2$-sodium acetoacetate prepared in this way was also used to synthesize 1,3-$^{13}C_2$-hydroxybutyrate for use as a standard, using a modification of the method of Passingham and Barton.[3] All other chemicals were commercially available.

Experimental Animals

Approval for the animal studies was granted by the Animal Ethics Committee of Royal Children's Hospital. Sprague-Dawley female rats weighing 189 to 272 g were used for the studies. The rats were fasted for 24 hours before commencement of the experiment. Water was freely available at all times. Each rat was maintained in a metabolic cage during this time, which permitted close observation. Pooled urine was collected and the volume measured in four periods: during the fasting period and during the periods postinjection from 0 to 3h, 3 to 6, and 6 to 24 hours.

In treated rats, a neutralized solution of 2-octynoic acid was injected intraperitoneally at a dose of 0.56 mmol/kg. There was no injection in control rats. Anesthesia was produced using a mixture of halothane and oxygen applied through an open-face cone. The rats were maintained sufficiently anesthetized to inhibit tail movement in response to touch. In stable-isotope studies, a cannula was inserted in the foot vein for infusion of isotopes. The isotopes were dissolved in sterile saline and infused at a rate of 3 mL/h. 1,3-$^{13}C_2$-acetoacetate was infused at a rate of 0.425 μmol/kg/min and 1,2,3,4-$^{13}C_4$-3-hydroxybutyrate at 0.6 μmol/kg/min.

Blood was collected from the tail vein. Approximately 0.5 mL blood for free fatty acid, glucose, lactate, and 3-hydroxybutyrate analysis was collected into fluoride oxalate tubes at 0, 30, 60, 120, and 180 minutes in most cases and analyzed by previously described methods.[4] At the same times, 100 μL blood was collected into 100 μL iced perchloric acid for the stable-isotope studies.

During the experimental period in anesthetized rats, urine was collected by regular bladder expression. A syringe was used to collect the urine as it emerged from the urethra.

Stable-isotope infusion studies of ketone turnover were performed on rats fasted for 24 hours prior to the study. Complete results were available for four control rats and five experimental rats.

Effect of 2-Octynoic Acid on Free Fatty Acid Levels

2-Octynoic acid is a medium-chain unsaturated fatty acid. Mixing experiments showed that 2-octynoic acid did not interfere with the enzymatic assay for free fatty acids.

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Mass Spectrometry Methods

A Finnigan (San Jose, CA) Mat 1020 gas chromatograph/mass spectrometer was used for analysis of rat urine samples and for initial purity analysis of the synthesized ketones. Later analyses for purity, isotopic purity, and concentration of these compounds were performed on a Hewlett-Packard (Palo Alto, CA) MSD-5890 gas chromatograph/MSD-5971 mass spectrometer. All standards and rat plasma samples were analyzed on the Hewlett-Packard gas chromatograph/mass spectrometer.

A 0.5-mL aliquot of each urine sample was extracted, and trimethylsilyl derivatives were prepared for organic acid analysis according to standard pediatric techniques.[5]

Calculations

A modification of the two-accessible-pools model of Bougnères and Ferré[6] was used for calculations of ketone kinetic parameters. This is a steady-state model and is represented in diagrammatic form in Fig 1. A single-pool model cannot be used here because there are two interconverting pools. 3-Hydroxybutyrate and acetoacetate interact together too slowly to be considered together. The published model was modified to allow the use of matrix equations for calculation of the rates of synthesis, interconversion, and utilization of acetoacetate and 3-hydroxybutyrate.

The following system of linear equations can be set up at steady-state, representing the various mass balances:

$$P_1 + R_{a1s} + R_{a1i} = R_{o1} + R_{a2i} \quad \text{total acetoacetate balance}$$

$$P_2 + R_{a2s} + R_{a2i} = R_{a1I} + R_{o2} \quad \text{total 3-hydroxybutyrate balance}$$

$$P_1 + R_{a1i} \cdot B = R_{o1} \cdot A + R_{a2i} \cdot A \quad {}^{13}C_2\text{-acetoacetate balance}$$

$$P_2 + R_{a2i} \cdot C = R_{o2} \cdot D + R_{a1i} \cdot D \quad {}^{13}C_4\text{-3-hydroxybutyrate balance}$$

$$R_{a2i} \cdot A = R_{a1i} \cdot B + R_{o2} \cdot B \quad {}^{13}C_2\text{-3-hydroxybutyrate balance}$$

$$R_{a1i} \cdot D = R_{a2i} \cdot C + R_{o1} \cdot C \quad {}^{13}C_4\text{-acetoacetate balance.}$$

A, B, C, and D are the steady-state enrichment of $^{13}C_2$-acetoacetate, $^{13}C_2$-3-hydroxybutyrate, $^{13}C_4$-acetoacetate, and $^{13}C_4$-3-hydroxybutyrate, respectively, and P_1 and P_2 are the rates of infusion of $^{13}C_2$-acetoacetate and $^{13}C_4$-3-hydroxybutyrate, respectively. The other kinetic parameters are defined in Fig 1. These equations can be rearranged as

$$\begin{vmatrix} 1 & 0 & -1 & 1 & -1 & 0 \\ 0 & 1 & 1 & -1 & 0 & -1 \\ 0 & 0 & -A & B & -A & 0 \\ 0 & 0 & C & -D & 0 & -D \\ 0 & 0 & A & -B & 0 & -B \\ 0 & 0 & -C & D & -C & 0 \end{vmatrix} \cdot \begin{vmatrix} R_{a1s} \\ R_{a2s} \\ R_{a2i} \\ R_{a1i} \\ R_{o1} \\ R_{o2} \end{vmatrix} = \begin{vmatrix} -P_1 \\ -P_2, \\ -P_1 \\ -P_2 \\ 0 \\ 0 \end{vmatrix}$$

or in matrix notation, $E \cdot R = P$, where E, R, and P are matrices containing enrichment, rate, and infusion parameters respectively. Therefore, $R = E^{-1} \cdot P$.

R was calculated using a spreadsheet macroprogram to perform the matrix manipulations (Lotus 1-2-3, version 2.3; Cambridge, MA). The validity of the calculations was checked using the experimental data of Bougnères and Ferré,[6] wherein a model was used that assumed that the rates of isotope infusion were insignificant compared with the in vivo kinetic parameters, ie, $P_1 \ll R_{a1s}$, R_{a1i} and $P_2 \ll R_{a2s}$, R_{a2i}. This was used to simplify the calculations. Values for the kinetic parameters calculated using the matrix method were essentially identical to those obtained by Bougnères and Ferré.

RESULTS

Induction of dicarboxylic aciduria was confirmed in 24-hour fasted rats following 2-octynoic acid injection (Table 1). Quantitatively, the majority of dicarboxylic acids found in the urine were metabolites of the injected 2-octynoic acid, 4-hydroxyoctynoic and 8-hydroxyoctynoic acid, rather than the endogenous medium-chain dicarboxylic acids (adipic, suberic, and sebacic). However, substantial amounts of 3-hydroxybutyrate continued to be excreted in the urine following injection of 2-octynoic acid. This is in contrast to the usual findings in human MCAD deficiency, in which ketonuria is usually absent.

In the first 6 hours following injection, a small amount of hexanoyl glycine, a characteristic metabolite found in MCAD deficiency, was detected in the urine. Other specific glycine

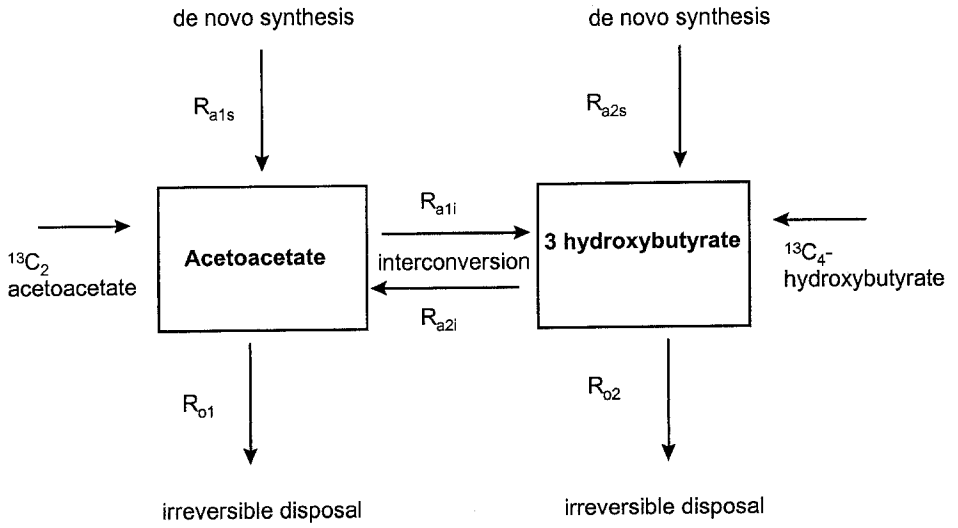

Fig 1. Two-accessible-pool model of steady-state kinetics of acetoacetate and 3-hydroxybutyrate turnover. Adapted from Bougnères and Ferré.[6]

Table 1. Major Urinary Organic Acids Before and After 2-Octynoic Acid Injection

Organic Acid	Pretreatment	Posttreatment 0-3 h	3-6 h	6-24 h
Lactate	++	+	++++	+++
3-Hydroxybutyrate	++	+	+++	++
2-Octynoic	−	+	+++	+
4-Hydroxyoctynoic	−	+	+	+
3-Ketooctanoic	−	TR	−	−
8-Hydroxyoctynoic	−	+	+	+
7-Hydroxy-2-octynoic	−	+	++	+
Pantothenic	+	TR	+	+
2-Octynedioic	−	+	−	−
Phenacetyl glycine	+++	+	+	−
Suberic	−	+	+	+
Sebacic	−	+	+	+
3-Hydroxysebacic	−	−	+	−
4-Hydroxyphenylacetic	++	+	−	−
Aconitic	++	+	−	+
Citric	+++	+	−	+

Abbreviations/symbols: −, not detected; TR, trace amount; +, detected; ++, mild elevation; +++, moderate elevation; ++++, gross elevation.

conjugates found in the human disorder, phenylpropionyl glycine and suberyl glycine, were not detected.

Experiments on rats fasted for 24 hours confirmed that this period of fasting is sufficient to stimulate fatty acid oxidation, as indicated by elevated levels of free fatty acids at commencement of the study (Fig 2A), which were not found in nonfasted rats. Both treated and control rats were ketotic after the 24-hour period of fasting, and free fatty acid levels decreased throughout the study period in 2-octynoic acid–treated rats but continued to increase in control rats (at 60 minutes, $P = .028$). Figure 2B shows the effect of 2-octynoic acid treatment on 3-hydroxybutyrate. These differences also reached statistical significance at 60 minutes post injection ($P = .029$).

Atom percent enrichment data confirm that steady state was reached with ^{13}C-labeled acetoacetate and hydroxybutyrate after 30 minutes of infusion.

Ketone kinetics were determined for treated and control animals and are represented in Fig 3. Interconversion of acetoacetate and hydroxybutyrate is shown by the appearance of the "reverse isotopes" $^{13}C_2$-hydroxybutyrate and $^{13}C_4$-acetoacetate.

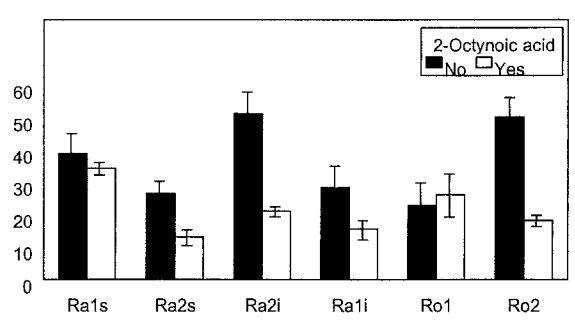

Fig 3. Mean rates of de novo synthesis (μmol/kg/min) of acetoacetate (R_{a1s}) and 3-hydroxybutyrate (R_{a2s}), interconversion of acetoacetate to 3-hydroxybutyrate (R_{a2i}) and 3-hydroxybutyrate to acetoacetate (R_{a1i}), and utilization of acetoacetate (R_{o1}) and 3-hydroxybutyrate (R_{o2}) in rats treated with 2-octynoic acid and control rats from stable-isotope studies. Bars represent the standard error of the mean.

The kinetics for ketone bodies determined from our stable-isotope studies show a general reduction in ketone production, interconversion, and utilization in rats treated with 2-octynoic acid compared with untreated rats. Treated rats have rates of production, interconversion, and utilization that are approximately two thirds of the values in control animals. The mean rate of total ketone body synthesis ($R_{a1s} + R_{a2s}$) and utilization ($R_{o1} + R_{o2}$) in control animals was 65.73 and 67.56 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively. The corresponding values for treated rats were 43.28 and 44.41 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively.

These results reflect reduced ketone production in 2-octynoic acid–treated rats, associated with mild suppression of free fatty acid release.

DISCUSSION

Most inborn errors of metabolism are individually rare. It is difficult in all but the largest referral centers to find sufficient patients in whom to study the consequences of abnormal metabolism. Animal models of inborn errors have much to offer in this setting by increasing our knowledge of the effect in vivo of a metabolic block. Gregersen et al[7] have investigated the metabolism of medium-chain fatty acids in the rat, and confirm that the rat is a valid animal model for studies of fatty acid oxidation defects.

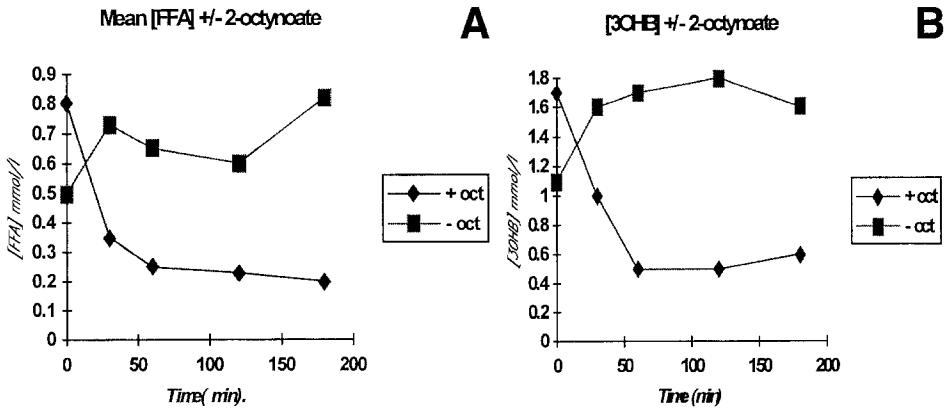

Fig 2. Mean plasma concentrations of (A) free fatty acids (FFA) and (B) 3-hydroxybutyrate (3OHB) for 5 treated (+oct) and 4 control (−oct) 24-hour fasted rats following injection of 2-octynoic acid.

Freund et al[1] used in vitro 2-octynoyl coA with kidney homogenates to demonstrate inhibition of the MCAD enzyme. Extrapolating from these results, Montgomery and Mamer[2] concluded that induced dicarboxylic aciduria following intraperitoneal 2-octynoic acid administration to the rat "closely, acutely and transiently resembles the human disorder" of MCAD deficiency and suggested that 2-octynoic acid is an inhibitor of medium-chain fatty acid oxidation in vivo. Although we confirmed the dicarboxylic aciduria, using ketone turnover studies, we have demonstrated reduced ketone production in treated compared with control fasted rats. We have shown that this is associated with reduced free fatty acid release (reduced substrate availability) with no evidence for specific enzyme inhibition, in contrast to MCAD deficiency in children, who have a low level of plasma ketone bodies despite an elevated level of circulating free fatty acids.

Under metabolic stress conditions, children with MCAD deficiency have a florid dicarboxylic aciduria and increased medium-chain glycine conjugates in the urine. Although showing a mild dicarboxylic aciduria, the rats we studied did not otherwise mimic human MCAD deficiency, with a comparative lack of glycine conjugates, particularly suberyl glycine, a key urinary metabolite in human MCAD deficiency, although this may simply reflect the different substrate specificity of rats and humans for glycine conjugation.[8]

We propose that the most likely explanation for the failure of 2-octynoic acid in vivo to mimic human MCAD deficiency is its failure to enter the mitochondria, the site of the MCAD enzyme, in appreciable amounts, in combination with its suppression of circulating free fatty acid levels. The appearance in the urine of ω and ω-1 metabolites of 2-octynoic acid is supportive evidence of absorption and metabolism, reflecting peroxisomal and microsomal oxidation. The mechanism of reduced free fatty acid release following 2-octynoic acid administration is uncertain, but may reflect inhibition of lipase.

The application of technology for the development of transgenic animals may provide a more satisfactory method for investigation of the metabolic consequences of defects in fatty acid oxidation than chemical inhibitors.

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