Carnitine palmitoyltransferase I control of acetogenesis, the major pathway of fatty acid β-oxidation in liver of neonatal swine

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Lin X, Shim K, Odle J. Carnitine palmitoyltransferase I control of acetogenesis, the major pathway of fatty acid β-oxidation in liver of neonatal swine. Am J Physiol Regul Integr Comp Physiol 298: R1435–R1443, 2010. First published March 17, 2010; doi:10.1152/ajpregu.00634.2009.—To examine the regulation of hepatic acetogenesis in neonatal swine, carnitine palmitoyltransferase I (CPT I) activity was measured in the presence of varying palmitoyl-CoA (substrate) and malonyl-CoA (inhibitor) concentrations, and [1-14C]-palmitate oxidation was simultaneously measured. Accumulation rates of 14C-labeled acetate, ketone bodies, and citric acid cycle intermediates within the acid-soluble products were determined using radio-HPLC. Measurements were conducted in mitochondria isolated from newborn, 24-h (fed or fasted), and 5-mo-old pigs. Acetate rather than ketone bodies was the predominant radio-labeled product, and its production increased twofold with increasing fatty acid oxidation during the first 24h suckling period. The rate of acetogenesis was directly proportional to CPT I activity. The high activity of CPT I in 24-h-suckling piglets was not attributable to an increase in CPT I gene expression, but rather to a large decrease in the sensitivity of CPT I to malonyl-CoA inhibition, which offset a developmental decrease in affinity of CPT I for palmitoyl-CoA. Specifically, the IC50 for malonyl-CoA inhibition and m value for palmitoyl-CoA measured in 24-h-suckling pigs was 1.8- and 2.7-fold higher than measured in newborn pigs. The addition of anaplerotic carbon from malate (10 mM) significantly reduced 14C accumulation in acetate (P < 0.003); moreover, the reduction was much greater in newborn (80%) than in 24-h-fed (72%) and 5-mo-old pigs (55%). The results demonstrate that acetate is the primary product of hepatic mitochondrial β-oxidation in Sus scrofa and that regulation during early development is mediated primarily via kinetic modulation of CPT I.

Acetate; anaplerosis; carnitine palmitoyltransferase I activity; ketone bodies; mitochondria; Sus scrofa

It is well established that hepatic long-chain fatty acid oxidation is acutely controlled by a system in which carnitine palmitoyltransferase I (CPT I) is regulated allosterically by a change in malonyl-CoA concentration and/or the sensitivity to malonyl-CoA inhibition. This regulatory mechanism dictates fatty acid flux into mitochondria and controls the rates of β-oxidation and ketogenesis based on the animal’s physiological status. The neonatal period represents a physiological state characterized by marked enhancement of fatty acid oxidation and ketogenesis. Both humans and rats present a significant hyperketonemia during the suckling period (40). Evidence confirms that the physiological hyperketonemia is a result of the high hepatic ketogenic rate in neonates consuming a diet (i.e., milk) that is high in fat and low in carbohydrate. In fact, more than 90% of the non-CO2 carbon derived from fatty acid oxidation in liver homogenates is ketone bodies (21). While this is true for species possessing significant ketogenic capacity, it is not the case for the animals that have a low ketogenic capability, such as neonatal piglets. The blood concentration of ketone bodies in neonatal piglets is extremely low compared with other mammalian neonates during the suckling period. On the basis of our previous research with isolated piglet hepatocytes, 60–70% of the radiolabel identified in acid-soluble products (ASP) was found in acetate rather than ketone bodies (1, 19), suggesting that the regulation and fate of fatty acid oxidation in newborn piglets differ from other species.

Most recent studies indicate that the key regulatory enzyme—hepatic CPT I—has an atypical molecular structure with a limited expression in pigs compared with other mammalian species (28, 29). The porcine CPT I protein is a natural chimera of the more typical mammalian liver (L) and muscle (M)-CPT I isotypes, containing the L-CPT I binding site for acetyl-CoA and the M-CPT I binding sites for carnitine and malonyl-CoA. Besides the unique structure of porcine CPT I, the activity of mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase (mHMGCS), the key enzyme regulating ketogenesis, is extremely low in suckling piglets, which results in the reduced ketogenesis (8). The attenuated activity of mHMGCS appears not to be associated with a low mRNA abundance but rather a low rate of translation (5). The physiological significance of the unique structure of hepatic CPT I is not known, but it was suggested that fatty acid oxidation in mitochondria may be limited in neonatal pigs due to the diminished ketogenic pathway (8). This prompted us to investigate the oxidation occurring in peroxisomes (which is usually considered an ancillary oxidative pathway to mitochondria oxidation), because acetate is reported to be the primary product of peroxisomal β-oxidation (18) due to the high level of short-chain acyl-CoA thioesterase (39). We observed that peroxisomal β-oxidation develops rapidly in piglets after birth and can account for up to 40% of total oxidation (31, 43). However, a question has arisen as to whether the origin of acetate is from peroxisomal β-oxidation because it is not clear whether the acetyl-CoA produced in peroxisomes is hydrolyzed by an enzyme within the peroxisome or in the cytosol by an extra-peroxisomal enzyme (37). Furthermore, we noticed that radiolabel accumulation in acetate (from 1-[14C]-palmitate) was decreased by 40–60% by the addition of antimycin/rotenone, inhibitors of the electron-transport chain and mitochondrial β-oxidation. In addition, the production of acetate was increased by approximately twofold when malonate, an inhibitor of succinate dehydrogenase, was present in the incubation of liver homogenates (1). These results suggest that the primary origin of acetate is from mitochondria rather than from peroxisomes. Although our hypothesis was supported when acetate was found to be the primary product of hexanoate oxidation in liver homogenates, it is not the case for the animals that have a low ketogenic capability, such as neonatal piglets.

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oxidation, given that hexanoate is primarily a mitochondrial substrate (2), it has never been proven directly. Moreover, whether the acetate from β-oxidation is formed in the cytosol or mitochondria itself is not clear from our previous studies. In the work presented herein, we further investigated the mitochondrial origin of acetate production and the role of CPT I in its regulation. The relationship between CPT I and acetoogenesis and the effect of increasing citrate synthesis on aceto genesis also were evaluated. A better understanding of aceto genesis and its regulation as an alternate pathway of fatty acid carbon flux could have important implications for understanding metabolic derangements that produce either hypo-ketonic or hyper-ketonic states.

MATERIALS AND METHODS

Animals and mitochondria preparation. All animal procedures were approved by the Institutional Animal Care and Use Committee. Twelve crossbred neonatal pigs were obtained from a commercial swine farm either immediately as new borns (body weight, 1.43 ± 0.38 kg; n = 4), after 24 h of fasting (body weight, 1.13 ± 0.27 kg; n = 4), or after 24 h of feeding/suckling (body weight 1.34 ± 0.26 kg; n = 4). Piglets were anesthetized with pentobarbital sodium (20 mg/kg body wt), and livers were removed and chilled in ice-cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM EGTA, and 1% of fatty acid-free BSA, and the reaction was conducted in a buffer containing 50 mM KCl, 10 mM HEPES, 0.2 mM EGTA, and 1% of fatty acid-free BSA, and the reaction was con ducted at 30°C in a final volume of 1 ml. For CPT I kinetic measurements, mitochondria (1 mg protein) were incubated with nine concentrations of malonyl-CoA (0–150 μM). For the measurements of CPT I inhibition by malonyl-CoA, mitochondria were preincubated for 3 min with 80 μM palmitoyl-CoA in the presence of seven concentrations (0–2.5 μM) of malonyl-CoA. All reactions were initiated by adding 1 μmol of (i-methyl-3H)-carnitine (18.5 MBq/μmol) and stopped after 6 min by the addition of 2 ml of 6% of HClO4 (vol/vol). The labeled palmitoyl-carnitine generated from the reactions was extracted with scintillation spectrometry (Beckman LS 6000IC).

Palmitic acid oxidation. Mitochondria (~5 mg of protein) were incubated in reverse Krebs-Henseleit bicarbonate medium (8), pH 7.4, at 30°C under an atmosphere of O2/CO2 (19:1) in a final volume of 3.0 ml. All incubations were conducted in duplicate with 0.5 μCi of [1-14C]-palmitate (0.5 mM) complexed to BSA (1:3.5) in the presence of seven concentrations (0–2.5 μM) of the inhibitor malonyl-CoA (identical concentrations with CPT I analysis) and malate (10 mM). Peroxisomal contamination of mitochondrial preparations was evaluated by incubation with rotenone plus antymycin (10+50 μM) or in the absence of carnitine. The incubations were terminated after 15 min by adding 0.5 ml of HClO4 (35%, vol/vol). Accumulation of radio labeled CO2 and acid-soluble products (ASP) was determined as described previously (30).

Identification of organic acids in ASP. Organic acids and ketone bodies in ASP samples were separated by HPLC-radio chromatography using an Aminex HPX-87 H cation exchange column (300 × 7.8 mm), protected by a micro guard column (Bio-Rad, Richmond, CA). The chromatographic conditions and flow rates were used as described by Odle et al. (30). Peaks were presumptively identified by their retention times compared with authentic standards. The radioactivity accumulated in each peak was collected using a fraction collector (ISCO Retriever II; Lincoln, NE) and quantified by liquid scintillation spectrometry.

Mitochondrial acetyl-CoA hydrolase activity. Acetyl-CoA hydrolase activity was analyzed in mitochondria isolated from newborn, 24-h-fasted and adult animals using the spectrophotometric method as described by Alexson and Nedergaard (3). The reaction was conducted in a buffer containing 50 mM KCl, 10 mM HEPES, 0.3 mM DTNB, 0.025% Triton X-100 (pH 7.4), and 0.35 mg of mitochondrial protein. Absorbance was measured at 412 nm after the addition of 100 μM acetyl-CoA, and the enzyme activity was calculated using an extinction coefficient of 13,600 M·cm.

Quantitative RT-PCR. On the basis of results from fatty acid oxidation, liver samples collected from newborn and 24-h-fed piglets also were used for mRNA analysis. RNA was extracted using guanidine isothiocynate and phenol (TRIZol Reagent, Sigma Chemical, St. Louis, MO). The extracted RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of the RNA was confirmed using gel electrophoresis with SYBR Safe TM DNA gel stain from Invitrogen Life Technologies (Carlsbad, CA). The RNA (10 μg/50 μl) then was treated with TurboDNase (Ambion, Austin, TX), according to the manufacturer’s instructions for removal of genomic DNA and transcribed using the iScriptTM Select cDNA Synthesis Kit provided with oligo (dT) primer mix. (Bio-Rad Laboratories, Hercules, CA). The mRNA abundances of CPT I (L isoform), mHMGCS, 3-ketoacyl-CoA thiolase, and citrate synthase were measured using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories). Primers (Table 1) were designed from pig-specific sequences available via GenBank, purchased from Sigma Genosys, and resultant amplicons

### Table 1. Sequence of forward (F) and reverse (R) primers utilized in quantitative RTPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primers: Sequence (5’-3’)</th>
<th>Amplicon Length, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: GTC TGG AGA AAC CTA CCA AA</td>
<td>228</td>
<td>AF017079</td>
</tr>
<tr>
<td></td>
<td>R: CCC TGT TGG TGT AGC CAA AT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-CPT-1</td>
<td>F: TCA CAA GCG AAT TGG AGT GC</td>
<td>242</td>
<td>AF288789</td>
</tr>
<tr>
<td></td>
<td>R: AAA TCC AGA CCG CAG TGG CT</td>
<td>173</td>
<td>AF028007</td>
</tr>
<tr>
<td>3-Ketoacyl-CoA thiolase</td>
<td>F: CAG TTT GAT GTT GTG TGG GC</td>
<td>151</td>
<td>U90884</td>
</tr>
<tr>
<td></td>
<td>R: CGA GGG AGC TCA GGT AGT AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mHMGCS</td>
<td>F: AAA ACC AAG CCC TGC TCT TA</td>
<td>145</td>
<td>NM_214276</td>
</tr>
<tr>
<td></td>
<td>R: CCA AGC CAG ACAT AAG AG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>F: TCT CAG CTC AGT GCA GCC ATT ACA</td>
<td>145</td>
<td>NM_214276</td>
</tr>
<tr>
<td></td>
<td>R: CTA CAG CAA AGG GTA GCT TGC GGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

L-CPT, liver-carnitine palmitoyltransferase I; mHMGCS, mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase.
were sequence verified. Samples were analyzed in triplicate after amplification efficiencies were verified to be similar for the endogenous control GAPDH and the measured genes. Each reaction contained cDNA and 0.4 μM each of reverse and forward primers. All templates were amplified for 40 cycles under the following conditions: denaturation for 30 s at 95°C, primer annealing and extension for 30 s at 60°C. Fluorescence readings were collected at the end of each elongation phase. At the end of the amplification cycles, melt curves were examined to ensure the absence of nonspecific products and primer dimers. The relative change in gene expression was calculated from the real-time RT-PCR data using the 2−ΔΔCT method, where ΔΔCT = (Ct_Target − Ct_GAPDH) age x − (Ct_Target − Ct_GAPDH) age 0 (20).

Chemicals. The L-[methyl-3H]-carnitine hydrochloride (2.30 TBq/ mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), and the sodium salt of [1-14C]-palmitic acid (217 MBq/ mmol) was obtained from MP Biomedicals (Irvine, CA). The unlabeled sodium palmitic acid, CoA derivatives (malonyl-CoA, palmitoyl-CoA), and inhibitors (rotenone and antimycin) were purchased from Sigma Chemical. Lonza AG (CH-4002, Basel, Switzerland) provided the L-carnitine. All other chemicals were obtained from Fisher Scientific (Iusca, IL).

Statistical analysis. The apparent kinetic constants of CPT I (Vmax and Km for palmitoyl-CoA) were calculated according to the Michaelis-Menten equation, $V_i = V_{max,i}/(K_m + [s])$, using the iterative NLIN procedure of SAS (Carey, NC). The IC50 (concentration yielding 50% inhibition) for CPT I inhibition by malonyl-CoA also was calculated according to a model, $P_{act} = B0 + B1 \times Exp^{Km-CoA}$, which was fit using the SAS NLIN procedure. The Vmax and Km for palmitoyl-CoA and IC50 for malonyl-CoA were subsequently analyzed by the GLM procedure of SAS according to a completely randomized design, and the data from [1-14C]-palmitate oxidation and organic acid analyses were analyzed by ANOVA, according to a split plot design (SAS, Carey, NC). The relationship between CPT I activities and products of palmitate β-oxidation was evaluated using a simple linear regression model. Results are presented as least square means ± SE and considered significantly different when $P < 0.05$.

RESULTS

Effect of palmitoyl-CoA concentration on CPT I activity. The activities of CPT I in liver mitochondria isolated from newborn, 24-h-fed, 24-h-fasted piglets, and 5-mo-old pigs are shown in Fig. 1. The activity of CPT I increased as palmitoyl-CoA concentration increased, but the rate and extent of increase were different among ages ($P < 0.01$).

Table 2. Kinetic parameters of hepatic carnitine palmitoyltransferase I from swine of various ages

<table>
<thead>
<tr>
<th>Age</th>
<th>$V_{max}$, nmol/(h·mg protein)</th>
<th>$K_m$, μM</th>
<th>IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>54.8 ± 55.5^a</td>
<td>79.3 ± 79.2^a</td>
<td>0.19 ± 0.076^a</td>
</tr>
<tr>
<td>24 h-fed</td>
<td>144.8 ± 64.2^b</td>
<td>142.7 ± 91.5^b</td>
<td>0.26 ± 0.076^b</td>
</tr>
<tr>
<td>Adult</td>
<td>342.0 ± 64.2^b</td>
<td>461.1 ± 91.5^b</td>
<td>0.21 ± 0.088^a</td>
</tr>
</tbody>
</table>

Values are means ± SE of $n = 4$ observations. $V_{max}$ is maximal velocity, $K_m$ is the Michaelis constant for palmitoyl-CoA, and IC50 is the concentration of malonyl-CoA required to produce 50% inhibition. Letters that differ within a column denote significant difference, $P < 0.05$.

Effect of malonyl-CoA concentration on CPT I activity. The activities of CPT I were inhibited potently by malonyl-CoA, but the percentage of inhibition was greater in newborn pigs than in 24-h and adult pigs at a malonyl-CoA concentration of 1 μM (Fig. 2). The sensitivity to malonyl-CoA inhibition estimated by calculating malonyl-CoA concentration required for 50% inhibition of CPT I (IC50) activity from newborns was similar to 24-h-fasted piglets and adults. However, the IC50 from 24-h-fed pigs was 2.3-fold higher than newborn and 24-h-fasted pigs, as well as adult pigs, indicating a significant decrease in the sensitivity of CPT I to malonyl-CoA inhibition in 24-h-fed piglets (Table 2).

Effect of postpartum age and malonyl-CoA on palmitate β-oxidation. Postpartum age and physiological state (fed vs. fasted) had a significant influence on the palmitate oxidative flux to CO2 and ASP (Fig. 3C). Oxidation rates increased progressively from newborn to 24-h-fasted to 24-h-fed piglets. The radioactivity accumulation (nmol/(h·mg protein)) in CO2 and ASP was 78% higher from 24-h-fed pigs than newborn pigs ($P < 0.01$), and 70% higher than adult pigs ($P < 0.0004$). There was no difference in the accumulation of CO2 and ASP...
among newborn, fasted piglets, and adult pigs. The addition of 2.5 μM malonyl-CoA decreased radiolabel accumulation in CO2 and ASP by 5.5-, 2.8, and 1.8-fold from newborn, 24-h fasted, and fed piglets, respectively, but there was no decrease in adults. The addition of malate increased radioactivity accumulation in ASP by 28% (Fig. 3B) and reciprocally decreased accumulation in CO2 by 89% in 24-h-fed piglets. No effect was detected in newborns, 24-h-fasted, and adult pigs.

The total radiolabel accumulation rate (CO2/ASP) was not affected by addition of malate, but the metabolic distribution between CO2 and ASP was changed significantly. The 14C accumulation decreased 96% in CO2 in the presence of rotenone and antimycin and 100% in ASP in the absence of carnitine, suggesting that potential contamination from cytoplasm and peroxisomes was minimal (data not shown).

Identification of radioactivity in acid-soluble products. Radiolabel accumulation in acetate was 90% higher from 24-h-fed pigs than from newborn and 24 h-fasted pigs, and 3.4 times higher than adults. There was no difference between newborn and 24 h-fasted piglets (Fig. 4A). The addition of 2.5 μM malonyl-CoA decreased the radioactivity accumulation in acetate by 70% in newborns, 75% in 24-h-fasted pigs, and 42% in 24-h-fed pigs, but there was no decrease in adults (Fig. 4A). Similarly, the radioactivity in acetate was reduced by 80% in newborns, 90% in 24-h-fasted pigs, and 73% in fed piglets by the addition of malate (P < 0.001), but no effect was detected in adults. Radiolabel accumulation in ketone bodies from adults was 7.4-fold higher than newborns, 13.8-fold higher than in 24-h-fasted, and 4.6-fold higher than in 24-h-fed piglets (Fig. 4B). There were no differences among newborn and 24-h-old piglets. The addition of malonyl-CoA had no effect on radiolabel accumulation in ketone bodies for pigs of any age. However, the addition of malate reduced the radiolabel accumulation by 47% in adults but had no significant effects in newborns or 24-h-old piglets. Radiolabel accumulations in citric acid cycle intermediates (Fig. 4C) were similar for animals of all ages with or without addition of malonyl-CoA, but the accumulations were significantly increased by the addition of malate (P < 0.001). Also, the increase was significantly higher in 24-h-fed piglets and adult piglets than newborn pigs and 24-h-fasted piglets.

The relationship between palmitate β-oxidation rate and CPT I activity. The rates of fatty acid β-oxidation and acetogenesis measured at different CPT I activities modulated by malonyl-CoA are illustrated in Figs. 5 and 6. Both fatty acid oxidation and acetate production were strongly correlated with

![Fig. 3. Effects of malonyl-CoA and malate on hepatic mitochondrial β-oxidation from pigs of different postnatal ages and physiological states (fed vs. fasted) in CO2 (A), acid-soluble products (ASP; B), and CO2 and ASP (C). The values are means ± SE (n = 4). abcdLetters that differ in each panel denote significant difference (P < 0.05).](image)

![Fig. 4. Effects of malonyl-CoA and malate on 14C-accumulation rates in acetate (A), ketone bodies (B), and citric acid cycle intermediates (citrate, fumarate, and succinate) (C) from pigs at different postnatal ages and physiological states during incubation of liver mitochondria with 1-14C-palmitate. The values are expressed as means ± SE; n = 4. abcdLetters that differ in each panel denote significant difference (P < 0.05).](image)
CPT I activity, and linear relationships were observed under all physiological states examined in this study. Physiological condition had no significant influence on the slope of each equation obtained from fatty acid oxidation (Fig. 5), but the slope obtained from acetate production in newborns was 29% greater than in 24 h-old fed piglets (Fig. 6).

The mitochondrial activity of acetyl-CoA hydrolase. There was no significant increase in acetyl-CoA hydrolase activity during 24 h after birth, but the activity measured in adult pigs [894.4 μmol/(h·mg protein)] was on average 5.8 times higher than that in neonatal pigs [155.2 μmol/(h·mg protein); P < 0.05].

Effects on gene transcript abundance. The gene transcript amounts of hepatic CPT I and citrate synthase were 5- and 2.4-fold higher in newborn than 24 h-fed pigs (P < 0.05), and CPT I message was 2.75 times higher in adult compared with newborn liver. The transcript amount of 3-ketoacyl-CoA thiolase also tended to be higher in newborn than in 24 h-fed and adult pigs (P < 0.07). No change in the transcript amount of mHMGCS was detected.

**DISCUSSION**

Acetate is the primary product of mitochondrial fatty acid β-oxidation in swine. Findings from this study convincingly demonstrate that acetate is the primary product of mitochondrial fatty acid β-oxidation in domestic swine. About 88% of ASP or 75% of the total oxidative products (CO₂ + ASP) derived from mitochondrial palmitic acid oxidation were identified as acetate (Fig. 4), while ketone bodies accounted only for 7–10% of the total oxidation or 1/10th of the acetate level. But, could this extensive production of acetate from hepatic mitochondrial fatty acid oxidation be reflected in the circulatory system? We reported previously that plasma acetate concentrations in newborn, fasted, or suckled piglets (230–343 μM) were at least 10-fold higher than ketone bodies (2), which is consistent with the proportion of acetate to ketone bodies observed in the present work; however, we were unable to increase plasma acetate appearance when hexanoate was infused in vivo (2). Acetate can be produced in the gut of adult pigs by cecal fermentation of dietary fiber, but for neonatal pigs this is not very likely given the extent of ketone body production compared to newborn piglets.
piglets, the diet (i.e., sow milk) contains limited fiber and should have limited influence on blood acetate concentration. Acetate also can be produced by peroxisomal β-oxidation, but more recent studies showed that the acetate produced by the hydrolysis of acetyl-CoA formed in peroxisomes is used for local malonyl-CoA synthesis (13), suggesting that peroxisomal acetate production may contribute little to the plasma acetate pool. Thus, plasma acetate could be largely derived metabolically from hepatic mitochondrial fatty acid oxidation and/or from pyruvate dehydrogenase and distributed to other tissues for utilization. Acetate is preferentially metabolized (over ketone bodies) in pig liver, brain, and colonocytes (7, 36), suggesting it is an important physiological fuel. In addition, the production and distribution of acetate may be important for lipogenesis, which occurs primarily in adipose tissue in swine due to the extremely low hepatic lipogenesis (26). Furthermore, we found that the acetate produced in hepatic mitochondria of the mature pigs was significantly reduced (38% of the ASP) compared with neonatal piglets, but it was still 90% greater than ketone production. By contrast, the acetate production in rats increased along with ketone bodies in the fatty-acid-perfused liver (34), but ketone body production was 2.2–3-fold higher than acetate production when palmitic acid was perfused (41). Collectively, the acetate formed from hepatic fatty acid oxidation is significant and may play an important role in energy metabolism in this nonketogenic species.

Free acetate production is catalyzed by acetyl-CoA hydro-lase, which has been well studied in rats (35). The enzyme is highly regulated by ATP (stimulator) and ADP (inhibitor) and is considered as a mediator of cytosolic acetyl-CoA and free CoA concentrations (32), while the acetate production and its regulation in the mitochondria have not been fully investigated since the enzyme activity was confirmed in rat liver mitochondria (9, 35). Our study was conducted with isolated mitochondria, indicating that the enzyme catalyzing the conversion of acetyl-CoA to acetate is present in their mitochondria. Therefore, we measured mitochondrial acetyl-CoA hydrolyase activity and found the specific activity from neonatal and adult pigs [155–894 μmol/(h·mg protein)] was 129–745 times higher than that [1.2 μmol/(h·mg protein)] observed in adult rat mitochondrial fraction (42) and 237–1,367 times higher than that [0.65 μmol/(h·mg protein)] observed in the total purified enzymes from rat mitochondria (37), suggesting that the high production of acetate was associated with the remarkably high acetyl-CoA hydrolyzing activity in mitochondria. However, acetyl-CoA hydrolyase has never been recovered and characterized completely from mitochondria. Svensson et al. (37) in 1996 isolated an acetyl-CoA-hydrolyzing enzyme from the mitochondrial matrix with a molecular mass about 48 kDa, but immunoprecipitation confirmed that the purified enzyme only corresponded to a minor portion of the total mitochondrial acetyl-CoA hydrolyase activity. Interestingly, an enzyme recently purified from rat mitochondria displayed both acetyl-CoA hydrolase and 3-ketoacyl-CoA (acetoacetyl-CoA) thiolase activities (42). We found that the activity was highly expressed at birth compared with other mitochondrial fatty acid oxidation-related enzymes such as CPT I, mHMGCS, and citrate synthase. However, the high production of acetate in 24-h-old pigs might not be associated with an increase in gene expression of this enzyme. The results indicated that acetyl-CoA-hydrolyzing activity of 3-ketoacy-CoA thiolase might be regulated primarily by its substrate level, as suggested by Yamashita et al. (42), if it plays a primary role in the production of acetate in pigs. As the activity of mHMGCS in neonatal pig is limited due to an extremely low amount of the enzyme protein (5), the accumulation of acetyl-CoA may activate the enzyme toward acetyl-CoA hydrolase activity for acetate production. To confirm this, the enzyme(s) responsible for mitochondrial acetate production needs to be characterized, and the role of the enzyme(s) during fatty acid β-oxidation needs to be investigated further in this nonketogenic species.

Role of CPT I in mitochondrial acetate production. Hepatic mitochondrial fatty acid oxidation increases dramatically in piglets after birth (43). The oxidative products (CO₂ + ASP) on average were 60% higher from 24-h-old piglets than from newborns. However, the increase is 3 times greater in 24-h-fed piglets than in 24-h-fasted piglets, emphasizing the importance of food intake in the development of the mitochondrial oxidative system during the transition to postnatal life, which was observed also in our previous study with liver homogenates (44) and hepatocytes (30). Consistent with the enhancement of β-oxidation, acetate production from fed piglets was increased by twofold compared with newborn and 24-h-fasted piglets (Fig. 4A), but there was no difference in ketone body production, either between newborn and 24-h-old piglets or between fed and fasted piglets (Fig. 4B). The transcript abundance of mHMGCS did not differ among newborn, 24 h-old, and adult pigs, which was consistent with the ketone body production, although it was transcriptionally active. These results clearly illustrate that acetogenesis rather than ketogenesis is the primary pathway of acetyl-CoA flux during β-oxidation in liver mitochondria of neonatal piglets. Moreover, the induction of acetogenesis 24 h after birth accompanied with the marked increase in fatty acid β-oxidation was associated with a twofold increase in CPT I activity. This was supported strongly by the linear relationships between the enzyme activity and fatty acid oxidation, and enzyme activity and acetate production, as well as between fatty acid oxidation and acetate production obtained from this study (Figs. 5 and 6). However, the increase in CPT I activity after 24 h is not likely due to an increase in gene expression (22), it but could be associated with modifications in the mitochondrial environment (such as membrane fluidity; 15) or/and in the enzyme (such as phosphorylation; 14). The kinetic assay for substrate (palmitoyl-CoA) yielded Km estimates (Table 2) that were relatively high (80–300 μM), attributable in part to the nonlinear computational method and the fixed BSA concentration employed in our analysis. Notably, the increase in CPT I activity in 24-h-fed pigs was accompanied with an increase in Km for palmitoyl-CoA, illustrating that the increase in activity is related to a decrease in affinity of the enzyme for substrate, which was observed also in digitonin-permeabilized rat hepatocytes (12). The decrease would allow the enzyme to deal with the high fatty acid concentrations, as typically observed in ketotic states (11, 24). This stresses the importance of CPT I’s regulatory role in substrate availability and acetogenesis for newborn pigs to acquire enough energy after birth. Furthermore, malonyl-CoA, as the physiological inhibitor of CPT I, reduced both mitochondrial β-oxidation and acetogenesis significantly, but the
reductions were much greater in newborn (85% and 70%) and 24-h-fasted pigs (75% and 74%) than in 24-h-fed pigs (55% and 40%). These data suggest that the sensitivity of CPT I to malonyl-CoA inhibition may play an important role in regulating the increased fatty acid oxidation and acetogenesis. Indeed, the induction of β-oxidation and acetogenesis was congruent with a decrease in sensitivity of CPT I to malonyl-CoA inhibition because the concentration of malonyl-CoA required for 50% inhibition (IC50 values) was 2.4 times higher in 24-h-fed than newborn pigs (Fig. 2). The molecular basis of changes in malonyl-CoA sensitivity under changing physiological states remains unclear. Changes may be related to modification of the malonyl-CoA domain of CPT I (16, 27), changes in the local membrane architecture/fluidity (15, 23), or even phosphorylation state (14). In contrast, the pig IC50 value was much lower than that determined in newborn rabbits and fasted rats (23, 33). The difference may be due to the atypical chimeric structure of the porcine liver enzyme, containing a muscle-CPT I binding site for carnitine and malonyl-CoA (28). In ketogenic animals, regulation of fatty acid oxidation and ketogenesis may be mediated by both changes in the amount of malonyl-CoA and/or activity of CPT I. Considering that the changes in amount of malonyl-CoA may be minimal due to the extremely low lipogenesis in piglets, the significant decrease in the sensitivity of CPT I to malonyl-CoA inhibition might be the primary contribution to the increase in fatty acid oxidation and acetogenesis during the suckling period. Collectively, these results demonstrate that CPT I provides a main regulatory role in fatty acid oxidation and acetogenesis by controlling fatty acid entrance into mitochondria in pigs.

Effect of citric acid cycle activity on acetate production: the role of hepatic acetogenesis. The main pathways for acetyl-CoA disposal in liver mitochondria are the citric acid cycle and the synthesis of ketone bodies (in species with ketogenic capacity). In general, when the rate of acetyl-CoA production exceeds the capacity of the citric acid cycle (or the activity of citric acid cycle is limited), ketogenesis is engaged because free CoA must be regenerated to sustain thiolase activity of fatty acid β-oxidation (17, 38). Thus, the activity of the citric acid cycle influences the flux of acetyl-CoA into the ketogenesis pathway. Because neonatal pigs fail to produce ketone bodies because of the limited mHMGCS activity, the role of the citric acid cycle in intramitochondrial β-oxidation and its effect on the acetogenesis become important. Therefore, we tested the effect of citric acid cycle activity on the flux of acetyl-CoA channeled into acetogenesis by the addition of anaplerotic carbon from malate, the immediate precursor of oxaloacetate. We found that isotope accumulations were increased greatly in citric acid intermediates when malate was added to the medium. With the increase of radiolabel accumulation in these intermediates, the accumulation in acetate production was reduced significantly, indicating that the acetyl-CoA formed from β-oxidation flowed into the citric acid for further oxidation. By contrast, we showed previously (1) that acetate production was increased significantly from palmitate oxidation when the citric acid cycle was blocked with malonate (an inhibitor of succinate dehydrogenase), indicating that acetyl-CoA diverted to acetate production when the citric acid cycle is diminished. Combing these results together, we suggest that acetate production may be an overflow of the citric acid cycle to maintain the thiolase activity of fatty acid β-oxidation by releasing CoA. The data further underscore the importance of CPT I in the control of fatty acid oxidative flux. However, the distributions of 14C accumulation in acetate, ketone bodies, and citric acid cycle intermediates changed with age (Fig. 7). These results suggest that the distribution of acetyl-CoA metabolism among the citric acid cycle, ketogenesis, and acetogenesis might be regulated during development of the pig.

Perspectives and Significance

Our results demonstrate that acetate but not ketone bodies is produced predominantly in mitochondria during β-oxidation in pigs and that acetate production varies with physiological state. The extensive production of acetate in the first 24 h of suckling appeared to be associated with a high acetyl-CoA hydrolyzing activity and to be governed by CPT I activity that was altered via a large decrease in sensitivity (of CPT I) to malonyl-CoA inhibition and a decreased affinity for palmitoyl-CoA substrate but was not associated with changes in mRNA abundance of 3-ketoacyl-CoA thiolase or changes in abundance of mHMGCS mRNA. Increasing citric cycle activity by the addition of anaplerotic carbon from malate significantly reduced 14C-acetate production, but the degree of reduction was greater in newborn than in 24-h-fed piglets. These results suggest that CPT I is the primary site for controlling hepatic fatty acid β-oxidation flux, but acetogenesis might be regulated also by the activity of the citric acid cycle. The molecular regulatory mechanisms of porcine acetogenesis merit further investigation.

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DISCLOSURES

No conflicts of interest are declared by the authors.
REFERENCES


