Changes in Kinetics of Carnitine Palmitoyltransferase in Liver and Skeletal Muscle of Dogs (Canis familiaris) throughout Growth and Development

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ABSTRACT This study was conducted to investigate developmental changes in the kinetics of carnitine palmitoyltransferase (CPT) within hepatic and skeletal muscle tissues of the canine species. Carnitine concentrations, CPT activity and the apparent K_m for carnitine were measured in tissue homogenates from dogs in six age categories: newborn; 24-h-old; 3-, 6- and 9-wk-old; and adult. Hepatic CPT activity was low at birth, increased by 100% during the suckling period (P < 0.05) and then declined after weaning to adult levels. In contrast, CPT activity in muscle continued to increase with age, reaching adult levels after 9 wk. Congruent with CPT activity, nearly identical concentration profiles of liver and muscle acylcarnitines were observed. The apparent K_m of hepatic CPT for carnitine also paralleled the increase in CPT activity during the suckling period; however, free and total liver carnitine concentrations declined by 50% during this time (P < 0.05). Beginning at 3 wk of age, the hepatic concentration of free carnitine was at or below the apparent K_m of CPT for carnitine. A similar relationship existed in muscle of young dogs, but in adults, the free carnitine concentration was markedly increased and exceeded the apparent K_m by 5-fold. Collectively, we infer that fatty acid oxidation capacity increases rapidly after birth in the canine, after ontogenic increases in CPT activity. Furthermore, based on the relatively low tissue carnitine concentrations when compared with the apparent carnitine K_m of CPT, we suggest that carnitine may have an important role in the regulation of fatty acid oxidation and that increased dietary carnitine may improve fatty acid oxidative capacity in developing dogs. J. Nutr. 133: 1113–1119, 2003.

KEY WORDS: • canine • carnitine • carnitine palmitoyltransferase • fatty acid oxidation • ontogeny

Long-chain fatty acid metabolism is tightly linked to the emergence of the carnitine palmitoyltransferase (CPT) system (2), in which three distinct enzymes have been identified: mitochondrial outer membrane CPT (CPT I), inner membrane CPT (CPT II) and carnitine-acylcarnitine translocase. Among these, CPT I catalyzes the rate-limiting step of long-chain acyl-CoA translocation into mitochondria for subsequent \( \beta \)-oxidation (1). Studies with food-deprived and diabetic adult animals (2,3) have shown that long-chain fatty acid oxidation is mainly controlled by changes in CPT I activity, malonyl-CoA concentration (a potent physiological inhibitor of CPT I) and/or the sensitivity of CPT-I to malonyl-CoA inhibition. This regulatory role of CPT I in long-chain fatty acid oxidation is observed not only in different physiological or pathological states but also in different stages of growth and development. It has been reported in rats (4,5), rabbits (6,7) and pigs (8,9) that CPT I activity was very low at birth but increased about 2-fold within 24 h of birth. These dramatic changes during the 1st d of life were paralleled by an increase in fatty acid oxidation. Therefore, the CPT system, especially CPT I, plays a very important role in controlling the rate of fatty acid oxidation in mitochondria.

In addition to mitochondria, CPT activity also is present in other subcellular locations such as peroxisomes and microsomes. The CPT in these subcellular compartments, as well as mitochondrial CPT, share a number of common kinetic and regulatory properties. Both malonyl-CoA–sensitive and –insensitive CPT (mitochondrial CPT I and CPT II, respectively) have been identified and characterized (10,11). Although the precise physiological role of the CPT system in the extramitochondrial compartments remains to be elucidated, it is clear that the enzymes work coordinately with mitochondrial CPT in fatty acid metabolism (12,13). The roles of these enzymes in lipid metabolism recently have been stressed and investigated extensively at the subcellular level [see McGarry and Brown (1) for review]. However, CPT as a whole—its activity and kinetic constants and their relationship with prevailing tissue carnitine concentrations during development—has not been carefully evaluated, especially in companion animal species.

L-Carnitine is an essential cofactor for the CPT enzyme system. Studies with mitochondria have shown that increasing the carnitine concentration in the mitochondrial matrix increases CPT activity, stimulates translocase activity and increases the flux of fatty acids through mitochondrial \( \beta \)-oxidation (14). As one of the substrates of CPT, carnitine's availability is very important for optimal CPT activity and fatty acid oxidation. Carnitine also participates in a variety of
other metabolic events, such as branched-chain amino acid metabolism, ketogenesis, lipolysis and de novo synthesis of fatty acids (15). All of these functions may vary with postnatal development and are especially important for the viability of newborns. Neonates cannot synthesize adequate amounts of carnitine de novo because of a low activity of butyrobetaine hydroxylase, and therefore carnitine status declines if exogenous carnitine is not supplied. On this basis, supplementation of carnitine to human neonates has been strongly advocated (16). Beneficial effects of adding carnitine to the diet also have been observed recently in growing farm animals (17–20). Depending on animal species, age and tissue, carnitine concentrations vary widely (15). The plasma carnitine level is commonly used to estimate carnitine status, but it does not necessarily reflect tissue carnitine levels (21). Thus, carnitine content in tissue is an important index to evaluate carnitine status, especially during early development (21).

In the present study, CPT activity and carnitine concentrations were examined in liver and skeletal muscle homogenates during postnatal development of dogs. The examination was specifically focused on changes in CPT activity and carnitine concentrations at birth, suckling and before and after weaning. The relationships between enzyme activity and carnitine requirement (evaluated by the carnitine $K_m$) and carnitine concentrations in the tissues are presented and discussed.

MATERIALS AND METHODS

Animals. Timed-pregnant Beagle bitches ($n = 15$) were housed in standard dog runs designed to satisfy PHS and AAALAC housing criteria. Details of dog feeding and management are reported elsewhere (22). Briefly, the bitches were given free access to commercially available complete food (Eukanuba Premium Performance, The Iams Co., Dayton, OH) that exceeded NRC 1985 nutrient recommendations (23). After whelping, puppies remained with their mothers and were allowed to suckle until 6 wk of age. At 2 wk before weaning (4 wk of age), puppies were fed only the dry puppy food that again was formulated commercially available complete food (Eukanuba Premium Performance, The Iams Co., Dayton, OH) mixed with water. After 6 wk, the puppies were fed only the dry puppy food that again was formulated to exceed established nutrient requirements (22). At designated ages, dogs were killed after consuming the morning meal as previously described (22) and tissues (liver and skeletal muscle) were separated by use of FC analysis directly, and the other was used for short-chain acylcarnitine (SC) analysis after alkaline hydrolysis with KOH at 60°C for 60 min. Long-chain acylcarnitines (LC) were analyzed after alkaline hydrolysis of the tissue pellet under the same alkaline conditions as for the SC. All samples were prepared according to the Michaelis–Menten equation, $V_i = V_{max}[s]/(K_m + [s])$, the apparent kinetic constants of CPT ($V_{max}$ and $K_m$ for carnitine) were calculated by use of the iterative NLIN procedure of SAS (28). The computed apparent $V_{max}$ is referred to as maximal activity throughout the manuscript. The calculated data (the kinetic constants) and all other analytical data were analyzed by one-way ANOVA appropriate for a completely random design by use of the GLM procedure of SAS (28), and means were separated by use of a protected LSD test (28). Differences were considered significant if $P < 0.05$.

RESULTS

Carnitine palmitoyltransferase activity. Hepatic CPT activity (Fig. 1) increased with age from birth to 6 wk of age and homogenate were determined by weight ($\sim 20\) g/100 g), and homogenate protein was analyzed by use of the Biuret method (25). Enzyme activity was expressed per g of wet tissue.

Carnitine analysis. Free carnitine (PC) and carnitine esters in tissues were measured by the enzymatic radiisotope method of McGarry and Foster (26), with a modification as described by Bhuiyan et al. (27). Frozen tissues (0.5 g) were homogenized in ice-cold HCICO$_4$ (1 mol/L) by use of a PowGen polytron (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 10,000 × g for 5 min and the supernatant was reserved in a 2-mL centrifuge tube. The pellet was washed with ice-cold HCICO$_4$ (0.1 mol/L) and recentrifuged. The supernatants from the two extractions were combined and neutralized with KOH (1 mol/L). After neutralization, the resultant precipitate was removed by centrifugation and the supernatant was divided into two parts. One part was used for FC analysis directly, and the other was used for short-chain acylcarnitine (SC) analysis after alkaline hydrolysis with KOH at 60°C for 60 min. Long-chain acylcarnitines (LC) were analyzed after alkaline hydrolysis of the tissue pellet under the same alkaline conditions as for the SC. All samples were prepared and analyzed in duplicate. Analytes were conducted in HEPES–EDTA buffer (pH 7.3) with 25.5 mmol [1-14C]Acetyl-CoA (37 kBq/μmol), 2 μmol N-ethylmaleimide and 1 μL carnitine acetyltransferase at 25°C for 30 min. Acetyl-carnitine was separated on a column packed with resin (AG 1×8, 100–200, chloride form; BioRad, Richmond, CA), and the radioactivity in the column effluent was measured by liquid scintillation.

Chloramphenicol was a gift from Lonza AG (4002, Basel, Switzerland). Carnitine acetyltransferase (EC 2.3.1.7), palmityl-CoA, acetyl-CoA and other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Statistics. According to the Michaelis–Menten equation, $V_i = V_{max}[s]/(K_m + [s])$, the apparent kinetic constants of CPT ($V_{max}$ and $K_m$ for carnitine) were calculated by use of the iterative NLIN procedure of SAS (28). The computed apparent $V_{max}$ is referred to as maximal activity throughout the manuscript. The calculated data (the kinetic constants) and all other analytical data were analyzed by one-way ANOVA appropriate for a completely random design by use of the GLM procedure of SAS (28), and means were separated by use of a protected LSD test (28). Differences were considered significant if $P < 0.05$.

RESULTS

Carnitine palmitoyltransferase activity. Hepatic CPT activity (Fig. 1) increased with age from birth to 6 wk of age and
Maximal activity of carnitine palmitoyltransferase in liver and skeletal muscle of developing dogs

<table>
<thead>
<tr>
<th>Age</th>
<th>Liver</th>
<th>Skeletal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/(h · g wet tissue)</td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>6.29 ± 1.15 (12)(^a)</td>
<td>2.77 ± 2.07 (11)(^a)</td>
</tr>
<tr>
<td>24 h</td>
<td>9.96 ± 0.94 (18)(^b)</td>
<td>5.53 ± 1.72 (16)(^a)</td>
</tr>
<tr>
<td>3 wk</td>
<td>10.71 ± 0.84 (18)(^b)</td>
<td>13.70 ± 1.72 (14)(^c)</td>
</tr>
<tr>
<td>6 wk</td>
<td>11.84 ± 0.92 (19)(^b)</td>
<td>9.38 ± 1.54 (18)(^b)</td>
</tr>
<tr>
<td>9 wk</td>
<td>10.84 ± 0.87 (21)(^b)</td>
<td>14.83 ± 1.67 (16)(^c)</td>
</tr>
<tr>
<td>Adult</td>
<td>7.63 ± 1.06 (14)(^a)</td>
<td>15.05 ± 1.84 (14)(^c)</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM (n = 11–21). Values in a column without a common letter differ, P < 0.05.
\(^2\) Maximal activity (under saturating carnitine concentration) was calculated using the NLIN procedure of SAS (19).

Effect of age on carnitine palmitoyltransferase activity. Fatty acid concentration in plasma increases dramatically after birth because of the mobilization of endogenous triglycerides and the hydrolysis of exogenous triglycerides from milk (32). To meet the energy needs of newborns, fatty acid oxidation must develop rapidly after birth in the liver and in extrahepatic tissues (32). Indeed, studies with rats have shown that fatty acid oxidation by isolated hepatocytes increased by 6- to 10-fold during the neonatal period (33). Moreover, the in-

with age from birth to 3 wk (Table 3). After 3 wk of age, SC decreased, whereas FC and LC remained relatively constant. Free carnitine and total carnitine (TC) were about 100% higher in neonatal dogs than in all other age groups (P < 0.05). SC concentrations in young dogs (from newborn to 3 wk) were 2.8-fold those in older dogs (6 and 9 wk of age), and nearly 10-fold the concentrations in adult dogs (P < 0.05).

Concentrations of FC and carnitine esters in muscle also varied with age (Table 3). As in liver, muscle FC and TC concentrations decreased rapidly with age from birth to 3 wk of age, but increased thereafter. Concentrations of FC and TC were extremely high in adult dogs compared to that in neonatal and young dogs (P < 0.01). Indeed, the concentrations measured in adult dogs were 6- to 20-fold those in neonatal and young dogs. Concentrations in newborn, 24-h and 9-wk-old dogs were 2.4-fold the concentrations in 3- and 6-wk-old dogs. The SC concentration in adult muscle was 4.2 times the concentration in neonatal and young dogs. There were no differences in FC and TC between neonatal and young dogs (P > 0.1). The concentration of LC esters was increased by about 100% in young dogs from 6 to 9 wk of age compared with neonates and 3-wk-old dogs, and was about 100% greater in adults compared with 6- and 9-wk-old dogs, indicating that the concentration of LC esters increased continuously with age from neonates to adults.

These variables did not differ between genders within age groups for either liver or muscle tissue (data not shown).
crease in fatty acid oxidation is paralleled by the development of the mitochondrial CPT enzymatic system. It was reported that hepatic mitochondrial CPT I activity increased 2- to 6-fold in the first 24 h, reaching the same level as that in adults (37,38). Although hepatic CPT activity (per g wet weight) fell after 6 wk, at which time the puppies were fully weaned onto solid food. Lonnerdal et al. (35) reported that the energy content of dog milk was 64.65 ± 15.68 nmol/g wet tissue.

### TABLE 3
Concentrations of free carnitine, short- and long-chain acylcarnitine and total carnitine in liver and skeletal muscle of developing dogs

<table>
<thead>
<tr>
<th>Age</th>
<th>Free carnitine (nmol/g wet tissue)</th>
<th>Short-chain acylcarnitine (nmol/g wet tissue)</th>
<th>Long-chain acylcarnitine (nmol/g wet tissue)</th>
<th>Total carnitine (nmol/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver, nmol/g wet tissue</td>
<td></td>
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</tr>
<tr>
<td>Newborn</td>
<td>851.15 ± 57.82a</td>
<td>178.82 ± 40.85a</td>
<td>81.12 ± 11.52a</td>
<td>945.10 ± 66.60a</td>
</tr>
<tr>
<td>24 h</td>
<td>246.05 ± 52.79b</td>
<td>138.65 ± 39.54b</td>
<td>54.65 ± 11.15b</td>
<td>853.82 ± 64.48b</td>
</tr>
<tr>
<td>3 wk</td>
<td>331.49 ± 54.32b</td>
<td>140.18 ± 39.54b</td>
<td>39.41 ± 11.15b</td>
<td>511.28 ± 62.56b</td>
</tr>
<tr>
<td>6 wk</td>
<td>305.51 ± 52.79b</td>
<td>61.30 ± 37.29b</td>
<td>40.90 ± 10.52b</td>
<td>407.71 ± 60.79b</td>
</tr>
<tr>
<td>9 wk</td>
<td>329.32 ± 50.08b</td>
<td>47.46 ± 35.38b</td>
<td>37.16 ± 9.98b</td>
<td>413.94 ± 57.67b</td>
</tr>
<tr>
<td>Adult</td>
<td>357.12 ± 64.65b</td>
<td>15.68 ± 45.67c</td>
<td>35.49 ± 12.88b</td>
<td>408.29 ± 74.46b</td>
</tr>
</tbody>
</table>

Skeletal muscle, nmol/g wet tissue

| Newborn | 576.80 ± 92.70b                   | 172.33 ± 91.56b                              | 58.84 ± 25.94c                              | 807.97 ± 133.42b                  |
| 24 h    | 480.18 ± 82.17b                   | 160.22 ± 81.16b                              | 93.62 ± 23.03c                              | 734.02 ± 118.26bc                 |
| 3 wk    | 173.40 ± 92.70c                   | 175.25 ± 91.56b                              | 122.92 ± 25.94c                             | 471.57 ± 133.42c                 |
| 6 wk    | 283.16 ± 97.22c                   | 199.46 ± 96.03c                              | 211.98 ± 27.21b                             | 694.60 ± 139.32bc                 |
| 9 wk    | 552.35 ± 79.38b                   | 166.46 ± 78.40c                              | 198.95 ± 22.22b                             | 917.77 ± 114.25b                  |
| Adult   | 3511.60 ± 97.22c                  | 759.00 ± 96.03c                              | 421.58 ± 27.21a                             | 4692.18 ± 139.92a                  |

1 Values are means ± SEM. Values in a column without a common letter differ, P < 0.05.
in neonates is very low because of a minimum activity of butyrobetaine hydroxylase (46,47). In addition, the ratio of acylcarnitine/carnitine in neonatal and younger dogs was 2–4.5 times that of adult dogs.

A similar profile was observed in serum from developing rats (48). Several explanations for the high acylcarnitine concentration in neonatal dogs are possible. First, the acylcarnitine may have originated from dog milk, given that it was reported that early human milk contains a higher ratio of acylcarnitine/carnitine than does mature milk (41). Second, the high acylcarnitine level correlates with a high milk lipid content and accelerated fatty acid oxidative capacity during the suckling period. Indeed, serum acylcarnitine concentration in rats is associated with dietary lipid content (49). When rats were weaned and the lipid contribution from milk was lost, the acylcarnitine concentration decreased as well. Corsi (50) suggested that the ratio of acylcarnitine to free carnitine was about 0.2 under normal conditions, but it could be affected by the composition of diet and availability of glucose (51). Third, acetate plays an important role in liver fatty acid metabolism of the canine, and we suggest that a high propensity to activate short-chain fatty acids (SCFA) for entry into metabolism (52) may result in the large proportion of acyl-soluble carnitine.

The carnitine concentrations in muscle at birth and during suckling were similar to those in liver, but generally increased after 3 wk of age. Concentrations were extremely low in neonatal and young dogs compared to adults. Tissue carnitine must be provided via plasma, where it originates from the diet or from carnitine synthesis in the liver, as it cannot be synthesized in cardiac or skeletal muscle (15). Thus, the tissue concentration depends on the dietary carnitine level, the rate of hepatic carnitine de novo synthesis, and the rate of carnitine uptake by the tissue. During suckling, when carnitine synthesis is low (46), carnitine in the milk seems to be the primary source. Thus, the decrease in muscle carnitine during the first 3 wk may be associated with declining milk carnitine content. After 3 wk, the concentration in muscle gradually increased. This increase could have been caused by the presence of more carnitine in the solid food compared to the milk from late lactation, but this is speculative because we did not measure milk carnitine concentrations. It also could be caused by an increase in carnitine uptake capacity of the tissue and/or by an increase in carnitine synthesis. It appeared that carnitine accumulation in muscle formed the largest reserve in the body. This was consistent with the report that in adult dogs, carnitine in cardiac and skeletal muscle constitutes 95–98% of the body pool (53).

Relationship between apparent carnitine $K_m$ and tissue carnitine concentration. Carnitine, as a substrate for CPT, plays a very important role in activating and controlling the carnitine-dependent fatty acid transport system. However, the carnitine concentration required for optimal CPT activity and fatty acid oxidation is unknown. Long et al. (54) in 1982 tested the relationship between carnitine and oleate oxidation in homogenates prepared from liver, heart, skeletal muscle and kidney of rats, and from canine and human skeletal muscle. He found that the carnitine requirement for long-chain fatty acid oxidation varied markedly, but was roughly proportional to the concentration of carnitine normally present in the tissue. However, the relationship between the carnitine requirement for CPT activity and tissue carnitine concentrations was not evaluated in their study. Apparent carnitine $K_m$ as one of the enzyme kinetic constants, could be a very useful index for evaluation of carnitine status in the tissue. In fact, many enzymes possess $K_m$ values that approximate the physiologic concentration of their substrate such that variation in sub-

 STRATE concentration will proportionally affect the rate of enzyme activity. Our study showed that the apparent carnitine $K_m$ in liver increased from birth to 3 wk of age, consistent with the postnatal increase in fatty acid oxidative capacity.

To ensure that fatty acid metabolism is favored toward oxidation, increased CPT activity is often accompanied by a reduced sensitivity to malonyl-CoA inhibition and a rise in carnitine $K_m$ because these parameters are inversely related (55,56). However, the carnitine concentrations decreased with age during the first 3 wk (Fig. 4). In the first 24 h after birth, carnitine concentrations in liver were 50% higher than the apparent carnitine $K_m$ of CPT and apparently meet the requirement of carnitine for a half-maximum velocity of CPT. This may be important for the newborn to aid in adaptation from the use of fetal carbohydrate fuel to the use of milk fat postnataally as a primary fuel. After 24 h, carnitine concentrations continued to decrease and at 3 wk of age, the apparent carnitine $K_m$ was significantly higher than the carnitine concentration in the tissue, suggesting that the initial velocity of CPT may be limited by the available carnitine. However, whether the potential limitation in velocity of CPT observed in the tissue from 3-wk-old dogs would result in a limitation in fatty acid oxidation in vivo is unknown. With respect to enzyme kinetics, the enzyme velocity depends on substrate concentration, especially when the substrate concentration is low; thus, supplementation of carnitine could be of benefit for the animal at this age. After 3 wk of age, the apparent carnitine $K_m$ decreased and remained similar in magnitude to the tissue carnitine concentration. This demonstrated that CPT, as a key enzyme, is affected by the substrate carnitine concentration, and suggests that carnitine, at least in liver, may play a regulatory role in fatty acid metabolism in vivo.

The $K_m$ and carnitine concentration relationship observed in skeletal muscle was considerably different from that in liver (Fig. 5). The free carnitine concentration in muscle was almost the same as the apparent carnitine $K_m$ for CPT in the first 24 h after birth, but was numerically lower than the $K_m$ at 3 wk of age. This is consistent with our finding in the liver tissue, supporting our speculation that developing dogs at 3 wk of age may not receive sufficient carnitine via the milk to maximize fatty acid oxidation. After 3 wk of age, carnitine
concentrations increased and were higher than the requirement for half-maximum velocity of the enzyme at 9 wk of age. Concentrations in adults were 10-fold the apparent carnitine Km. Because free fatty acids in plasma are a major energy source for muscle, especially during prolonged exercise or starvation, the high level of carnitine supports a high fatty acid oxidation capacity and thereby facilitates optimal muscle function. In addition, carnitine may play a physiological acetylstorage function as a buffer against excess formation of acetyl-CoA via the pyruvate dehydrogenase complex, during incremental (57) and high intensity exercises (58,59).

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LITERATURE CITED