Postnatal Changes in Neonatal Acylcarnitine Profile

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ABSTRACT

Electrospray-tandem mass spectrometry represents a powerful method for detection of inborn errors of fatty acid metabolism. In the present study, it was used to examine neonatal carnitine metabolism, which reflects fatty acid metabolism. In 70 healthy neonates, blood samples were taken from the umbilical cord and by heel-stick puncture in full-term neonates on postnatal d 5. Cord blood specimens were also obtained from 15 preterm and 10 small-for-gestational-age infants. Acylcarnitine concentrations were measured in dried blood spots by electrospray tandem mass spectrometry. Compared with cord blood, the levels of nearly all acylcarnitine species were significantly higher on the postnatal d 5, whereas free carnitine remained unchanged. Total acylcarnitine/free carnitine-ratio increased, whereas the free carnitine/total carnitine-ratio (0.54 ± 0.05; $p < 0.01$) further decreased. A reduced availability of free carnitine in the early neonatal period may affect fatty acid oxidation and thus be of potential pathophysiological relevance under conditions with higher energy demands, *e.g.* in sepsis. Cord blood concentrations of free carnitine, total carnitine, and total acylcarnitines were strongly related to birth weight ($p < 0.01$). Lower umbilical artery pH, *i.e.* mild hypoxia, caused accumulation of mainly long-chain acylcarnitines. This implicates that long-chain acylcarnitines could serve as a parameter of perinatal asphyxia. (***Pediatr Res* 49: 125–129, 2001**)

Abbreviations:
FC, free carnitine
m/z, mass to charge ratio of ions
tAC, total acylcarnitines
TC, total carnitine

In utero, the main substrates for fetal metabolism are amino acids and glucose. The human placenta is also permeable to FFA. However, they are mainly stored as triglycerides in adipose tissues and liver because of the constant supply of glucose and amino acids and a low capacity of fetal tissues for FFA oxidation. The cutoff of the nutrient supply in the presuckling period after birth leads to a transient period of starvation. Thus, the newborn infant is entirely dependent on the mobilization of glycogen and fat stores. With the initiation of milk feeding, neonatal metabolism switches to a high-fat diet. Therefore, it is obvious that fatty acid oxidation is very important in the early postnatal period (1).

Carnitine plays an important role in oxidation of especially long-chain fatty acids. Carnitine palmitoyltransferases I and II mediate the transport of fatty acid-carnitine esters (acylcarnitines) across the mitochondrial membranes. Carnitine acyltransferases can also be found in peroxisomes (carnitine octanoyltransferase) and endoplasmatic reticulum (microsomal carnitine acyltransferase), although their function is not yet fully understood. In the mitochondrion, carnitine removes excess acyl groups and thus regulates the concentration of free CoA in the mitochondrial matrix (2). Several studies have shown that the carnitine pool in the neonate is limited (1, 3, 4). Tandem mass spectrometry allows quantitative determination of free carnitine and various carnitine esters like acylcarnitines (5, 6).

In the present study, we used this technique to investigate the changes in neonatal carnitine and fatty acid metabolism during the first 5 d after birth. Special attention was given to perinatal factors of potential pathophysiological relevance, such as umbilical artery pH, Apgar score, gestational age, and birth weight.

METHODS

Solvents, reagents, and internal standards. High-purity grade methanol was obtained from Merck (Darmstadt, Germany). Butanolic HCl (3 M) was prepared from high-purity butanol (Merck) and HCl-gas. Stable isotopes used as internal standards were obtained from Neo Gen Screening (Pittsburgh, PA, U.S.A.).

Patients and blood specimen collection. We studied a total of 70 healthy appropriate-for-gestational-age (AGA) neonates,

32 girls and 38 boys. Birth weight was 3422 ± 509 g (mean ± SD) and gestational age was 39.4 ± 1.3 wk. Thirteen infants were delivered by cesarean section. None of the neonates had a 1-min Apgar score lower than 6, 10-min Apgar scores were 9 or 10 in all subjects. To test for a possible influence of birth weight or gestational age, 10 small-for-gestational-age (SGA) infants (birth weight below third centile: 2502 ± 507 g, gestational age 39.4 ± 0.7 wk) and 15 preterm AGA infants (birthweight 1166 ± 329 g, gestational age 28.8 ± 2.2 wk) were also included in the study. Blood was taken immediately after birth by puncture of the umbilical artery. Two drops of blood were spotted on a Guthrie card. A second blood sample was obtained from the term AGA infants on the postnatal d 5 by heel-stick puncture during routine neonatal metabolic screening. On d 5, breast-feeding was well established in these children with a mean daily intake of 120 mL/kg body weight (mean fat intake 5.4 g/kg, mean carnitine supply 0.9 μmol/kg) (7). Heel-stick puncture was performed 30 min after feeding. Informed consent was obtained from all mothers. The study was approved by the Ethical Committee of the University Children's Hospital, Department of Neonatology.

Sample preparation. All dried blood spots were investigated by means of electrospray-tandem mass spectrometry by the method previously described in detail (5, 6). Briefly, one 3-mm-diameter dot per sample was punched out from a 10-mm-diameter dried blood spot into a vial of a 96-well microtiter plate. A methanol stock solution of internal standards was prepared, containing 0.76 μM [^{2}H$_9$]carnitine (m/z 227,3), 0.19 μM [^{2}H$_3$]acetylcarnitine (m/z 263,4), 0.04 μM each of [^{2}H$_3$]propionylcarnitine (m/z 277,4), [^{2}H$_3$]butyrylcarnitine (m/z 291,4), [^{2}H$_9$]isovalerylcarnitine (m/z 311,4), [^{2}H$_3$]octanoylcarnitine (m/z 347,5), [^{2}H$_9$]myristoylcarnitine (m/z 437,6), and 0.08 μM [^{2}H$_3$]palmitoylcarnitine (m/z 459,6). The samples were allowed to elute in 100 μL of the stock solution for 20 min before the blood dots were removed. They were evaporated to dryness in a freeze dryer and then 60 μL of 3 n HCl in butanol were added. The microtiter plates were sealed and incubated at 65°C in a forced air oven for 15 min. After removal of the seal, excess HCl-butanol was evaporated to dryness in a freeze dryer. The derived samples were reconstituted with 100 μL acetonitril/water (1:1), containing 0.025% formic acid. The internal standard was used for quantification of acylcarnitines by forming the ratio of the signals. When the specific stable isotope was not available, the following ratios were used for calculation: C2/C3-d3, C5:1/C5-d9, C6/C5-d9, C8:1/C8-d3, C10/C8-d3, C10:1/C8-d3, C12/C14-d9, C14:1/C14-d9, C14OH/C14-d9, C16:1/C16-d3, C16OH/C16-d3, C16:1OH/C16-d3, C18/C16-d3, C18:1/C16-d3, C18:1OH/C16-d3

Mass spectrometry. A PE Sciex API 365 triple quadrupole tandem mass spectrometer (PE Sciex, Concord, Ontario, Canada) was used with an ion spray source. Twenty-five microliters of the samples were introduced by using a PE 200 HPLC-pump (40 μL/min acetonitril/water (1:1 vol/vol) containing 0.025% formic acid) and a PE 200 autosampler. The first analyzer was set to scan all ions between m/z 200 and 500. Acylcarnitines were detected by looking for the precursor ions of m/z = 85. The acylcarnitine butylester passes analyzer 1,

collides with nitrogen (fragmentation), and one of the typical fragments (m/z = 85) passes analyzer 2, which is fixed to m/z = 85. It is registered by the detector while the m/z that has passed analyzer 1 is recorded.

To enable comparisons to reported values, acylcarnitine concentrations were summarized to obtain levels for short- (acetyl-, propionyl-, butyryl-, and isovalerylcarnitine), medium- (hexanoyl-, octanoyl-, octenoyl-, decanoyl-, decenoyl-, and lauroylcarnitine) and long-chain acylcarnitines (myristoyl-, myristoleyl-, hydroxymyristoyl-, palmitoyl-, hydroxy-palmitoyl-, palmitoleyl-, hydroxypalmitoleyl-, stearoyl-, hydroxystearoyl-, oleyl-, and hydroxyoleylcarnitine). Short-, medium- and long-chain acylcarnitines were again summarized to yield total acylcarnitine concentrations. Cord blood and postnatally drawn blood samples were compared with the *t* test for paired samples. Cord blood samples of the AGA, SGA, and preterm infants were tested for differences with the *t* test for unpaired samples. Correlations with perinatal factors were calculated using the Pearson correlation. Differences with $p < 0.05$ were regarded statistically significant.

RESULTS

The levels of FC and acylcarnitines in cord blood and in the postnatal blood samples are summarized in Table 1. No differences in the levels of FC were noted between cord and 5-d blood. The concentrations of carnitine esters with saturated acyl chains, however, were found to be considerably higher on the postnatal d 5. Similar results were obtained for acylcarnitines with monounsaturated acyl chains. Hydroxylated long-chain acylcarnitines remained unchanged during the first 5 d. The ratio of tAC to FC increased significantly on d 5. On the other hand, the ratio of FC to TC decreased.

In the cord blood samples, concentrations of TC, FC, tAC, short-chain (all $p < 0.01$), and long-chain ($p < 0.05$) acylcarnitines increased with increasing birth weight (Fig. 1). Significant positive correlations were noted for acetyl- ($p = 0.01$), propionyl- ($p < 0.001$), butyryl- ($p < 0.05$), isovaleryl- ($p < 0.05$), oleyl- ($p < 0.05$), and hydroxyoleylcarnitine ($p < 0.05$).

In cord blood, concentrations of acetyl- ($p < 0.01$), propionyl- ($p < 0.05$), myristoyl- ($p < 0.01$), myristoleyl- ($p < 0.01$), palmitoyl- ($p < 0.05$), stearoyl- ($p < 0.05$), and oleylcarnitine ($p < 0.05$) increased with decreasing umbilical arterial pH (Fig. 2) and 5-min Apgar score.

The influence of birth weight on the concentrations of FC and short-chain acylcarnitines was still present in the postnatal blood samples drawn on d 5. Correlations were found for FC ($p < 0.01$), acetyl- ($p < 0.01$) and propionylcarnitine ($p < 0.01$). On the postnatal d 5, FC and acylcarnitine levels were not related to gestational age or umbilical artery pH. Carnitine levels did not correlate with gestational age, neither at birth nor on d 5. No differences were noted regarding the concentrations of FC and acylcarnitines between girls and boys or between normal labor and delivery by cesarean section.

Cord blood levels of free carnitine, total carnitine, and total, short-, medium-, and long-chain acylcarnitine were lower in preterm compared with term infants (Fig. 3). Significantly lower values were also found for propionyl- ($p = 0.001$),

Table 1. *Concentrations of FC and acylcarnitines in cord blood and postnatally drawn blood samples (mean ± SD)*

	Cord blood [μmol/L]*	Day 5 after birth [μmol/L]*	*p*-Value
TC	51.5 ± 14.4	55.7 ± 16.0	<0.01
FC	31.6 ± 10.2	30.6 ± 10.6	NS
tAC	19.9 ± 4.8	25.1 ± 6.4	<0.001
Short-chain acylcarnitines	13.1 ± 3.5	15.5 ± 4.6	<0.001
Medium-chain acylcarnitines	0.8 ± 0.2	0.9 ± 0.2	<0.001
Long-chain acylcarnitines	6.0 ± 1.3	8.6 ± 2.1	<0.001
tAC/FC	0.65 ± 0.12	0.86 ± 0.17	<0.01
FC/TC	0.61 ± 0.04	0.54 ± 0.05	<0.01
Short-chain acylcarnitines			
Acetylcarnitine (C2)	10.3 ± 3.1	12.6 ± 4.1	<0.001
Propionylcarnitine (C3)	2.0 ± 0.6	2.2 ± 0.7	<0.05
Butyrylcarnitine (C4)	0.41 ± 0.13	0.47 ± 0.13	NS
Isovalerylcarnitine (C5)	0.24 ± 0.09	0.27 ± 0.08	<0.05
Isovalerylcarnitine (C5:1)	0.13 ± 0.05	0.13 ± 0.05	NS
Medium-chain acylcarnitines			
Hexanoylcarnitine (C6)	0.21 ± 0.06	0.23 ± 0.06	NS
Octanoylcarnitine (C8)	0.12 ± 0.06	0.14 ± 0.06	<0.05
Octenoylcarnitine (C8:1)	0.13 ± 0.06	0.17 ± 0.08	<0.01
Decanoylcarnitine (C10)	0.10 ± 0.05	0.12 ± 0.05	<0.05
Cecenoylcarnitine (C10:1)	0.10 ± 0.04	0.11 ± 0.04	NS
Lauroylcarnitine (C12)	0.15 ± 0.06	0.18 ± 0.09	<0.05
Long-chain acylcarnitines			
Myristoylcarnitine (C14)	0.23 ± 0.09	0.30 ± 0.11	<0.001
Myristoleylcarnitine (C14:1)	0.12 ± 0.05	0.13 ± 0.06	NS
Palmitoylcarnitine (C16)	2.6 ± 0.8	3.8 ± 1.3	<0.001
Palmitoleylcarnitine (C16:1)	0.17 ± 0.06	0.29 ± 0.11	<0.001
Stearoylcarnitine (C18)	1.3 ± 0.4	1.6 ± 0.5	<0.001
Oleylcarnitine (C18:1)	1.1 ± 0.4	1.9 ± 0.6	<0.001
Hydroxymyristoylcarnitine (C14OH)	0.13 ± 0.06	0.13 ± 0.05	NS
Hydroxypalmitoylcarnitine (C16OH)	0.14 ± 0.07	0.14 ± 0.06	NS
Hydroxypalmitoleylcarnitine (C16:1OH)	0.14 ± 0.07	0.16 ± 0.08	NS
Hydroxyoleylcarnitine (C18:1OH)	0.07 ± 0.04	0.08 ± 0.04	NS

* Except tAC/FC and FC/TC ratios.

Figure 1. Correlation of FC, TC, and tAC with birth weight in cord blood of AGA infants.

Figure 2. Correlation of tAC and short- and long-chain acylcarnitine with umbilical artery pH in cord blood of AGA infants.

lauroyl- (*p* = 0.002), myristoleyl- (*p* = 0.001), hydroxymyristoyl- (*p* < 0.05), palmitoyl- (*p* < 0.001), palmitoleyl- (*p* < 0.05), hydroxypalmitoyl- (*p* < 0.05), stearoyl- (*p* < 0.01), and oleylcarnitine (*p* < 0.001). Carnitine levels in the cord blood of SGA infants were lower than in term AGA children (Fig. 3). However, the differences were not statistically significant. Compared with the SGA infants, the cord blood levels of the preterm infants tended to be lower.

DISCUSSION

Maternal plasma carnitine concentrations are significantly lower from wk 12 of gestation, compared with nonpregnant women. These differences become more pronounced toward the end of the pregnancy. Levels of FC decrease to a greater extent than acylcarnitine concentrations (8). Maternal plasma carnitine concentrations have been reported to be lower than in umbilical cord blood, implicating active carnitine transport across the placenta (9, 10). However, no differences between maternal and cord blood have been detected in another study (11).

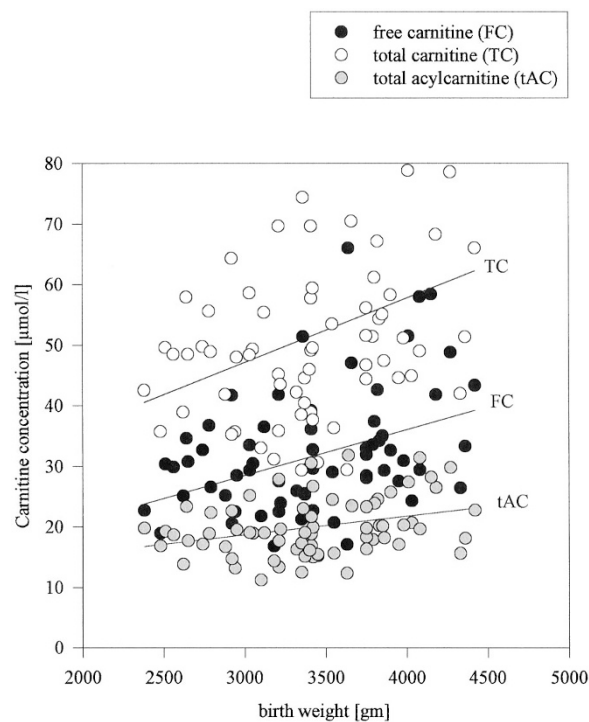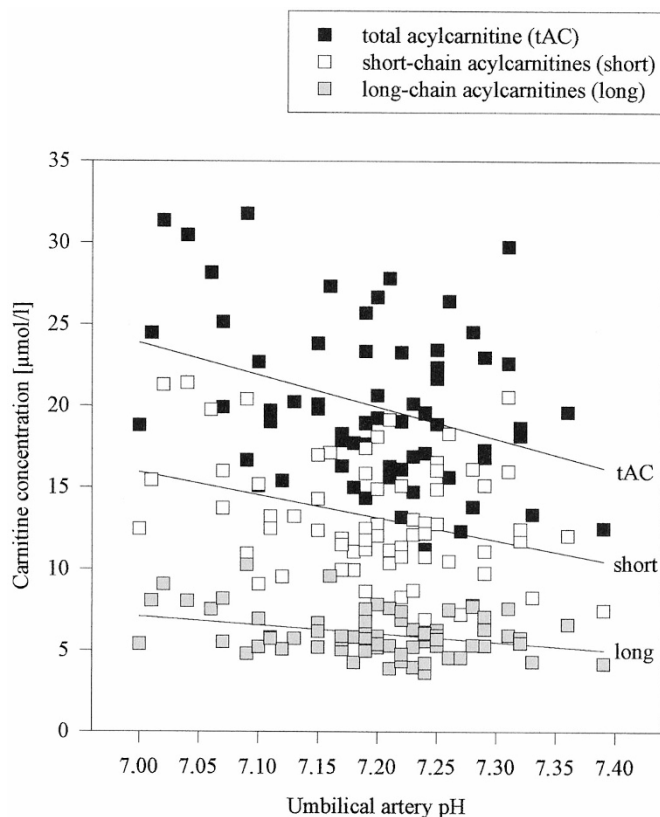Only a few studies on carnitine status in cord blood have been carried out before (4, 12, 13). In all these studies, plasma carnitine levels were measured. Because the intraerythrocytic carnitine pool can comprise to 73% of whole blood carnitine content (4), investigations on whole blood by tandem mass
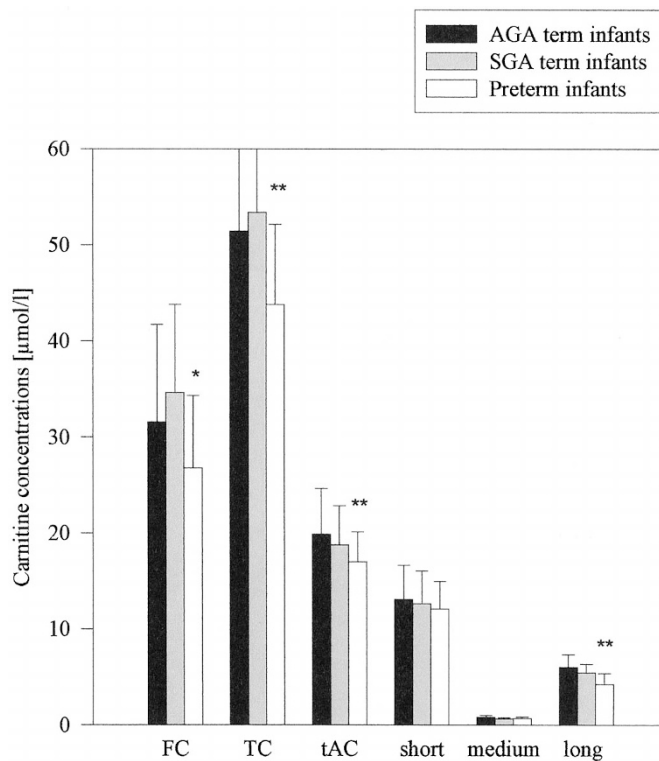
Figure 3. Cord blood levels of FC, TC, tAC, and short-, medium-, and long-chain acylcarnitines in AGA, SGA, and preterm infants (*$p < 0.05$; **$p < 0.01$ preterm compared with AGA term infants).

spectrometry may be more appropriate. In whole blood samples of 14 full-term infants, we found markedly higher levels of total (47.8 ± 15.1 *versus* 37.8 ± 9.7 μmol/L) and long-chain acylcarnitines (7.1 ± 2.3 *versus* 1.8 ± 0.5 μmol/L) compared with plasma. Because of these differences, carnitine levels obtained by tandem mass spectrometry cannot be easily compared with previous data.

The main function of carnitine is the transport of fatty acids across the mitochondrial membranes. In the liver carnitine is only needed for the transport of long-chain fatty acids, whereas in other tissue like skeletal muscle or myocardium, carnitine is also important for the transport of short- and medium-chain fatty acids (2). The intramitochondrial relationship between acyl-CoA and free CoA is reflected by the extramitochondrial tAC to FC ratio. Thus, high plasma levels of acylcarnitines reflect reduced bioavailability of acyl-CoA in the mitochondrion. The tAC/FC has therefore been called a marker of "carnitine insufficiency" (14, 15). On the other hand, the ratio of FC to TC is a useful parameter to assess the availability of FC. Because higher acylcarnitine levels in whole blood are compensated for by a tendency to overestimate FC concentrations using tandem mass spectrometry (16), the ratios obtained by different techniques should be comparable. In fact, our tandem mass spectrometry data corresponds well to published data for cord blood (12, 13) and neonates (17).

Longitudinal observations of carnitine metabolism within the neonatal period and early infancy are rare. Plasma levels of TC, FC, and tAC increase after 1–4 wk after birth (9, 17) and are significantly higher in older children when compared with

neonates (18). During infancy, tAC/FC decreases to 0.4 whereas FC/TC increases to 0.7 (15). The highest tAC/FC and lowest FC/TC can thus be found within the neonatal period. Our results support this observation in detail. Within the first 5 postnatal d, levels of all acylcarnitines increased, whereas the concentration of FC remained unchanged (Table 1). Decreased availability of free carnitine within the first postnatal days may impair fatty acid oxidation. For the newborn, this may be potentially harmful in conditions with higher energy demand, *e.g.* during septicemia.

Levels of TC, FC, and tAC were significantly related to birth weight. Lower values were found in the SGA group compared with the AGA infants. Rising carnitine levels with higher birth weights may reflect increasing tissue carnitine stores, which are known to be strongly related to body weight and muscle mass (3, 19). In our study on neonates with 37–42 wk of gestation, no significant influence of gestational age could be detected. Nevertheless, cord blood levels of preterm children were significantly lower than those of full-term infants. Reported cord blood plasma levels of TC, FC, and tAC are higher in preterm compared with term infants (4, 12, 13, 20).

Acylcarnitine concentrations were inversely correlated with umbilical artery pH and 5-min Apgar score. This suggests that mild acidemia and hypoxia leads to accumulation of short- and long-chain acylcarnitines. Accumulation of long-chain acylcarnitines is known as one of the main causes of arrhythmias and cellular damage in ischemic myocardium (21, 22). This has also been shown for neonatal rat myocytes (23). In six infants with severe perinatal asphyxia (pH < 7.0, base excess < -20) we found significantly higher levels of total (26.7 ± 4.9 *versus* 19.9 ± 4.8 μmol/L) and long-chain acylcarnitines (7.8 ± 1.5 *versus* 5.9 ± 1.4 μmol/L) immediately after birth compared with cord blood of healthy term infants. We therefore speculate that levels of long-chain acylcarnitines could serve as a useful parameter for perinatal asphyxia.

In summary, we present representative levels of carnitine and its esters as determined by electrospray tandem mass spectrometry in whole blood specimen within the early neonatal period. Acylcarnitines increase after birth while free carnitine remains unchanged. In full-term infants, carnitine concentrations depend strongly on birth weight, and further studies on the influence of gestational age are needed. Mild acidosis and hypoxia lead to accumulation of long-chain acylcarnitines. The availability of free carnitine, given by the tAC/FC and FC/TC ratios, is lower compared with cord blood and older children. This restriction of fatty acid oxidation capacity could be of potential pathophysiological relevance during the first days of life, especially under conditions with higher energy demands.

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