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Developmental Maturation and Segmental Distribution of Rat Small Intestinal L-Carnitine Uptake

P. García-Miranda, J.M. Durán, M.J. Peral, A.A. Ilundáin
Depto. Fisiología y Zoología, Facultad de Farmacia, Universidad de Sevilla, Spain

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Abstract. Oral L-carnitine supplementation is commonly used in sports nutrition and in medicine; however, there is controversy regarding the mechanisms that mediate intestinal L-carnitine transport. We have previously reported that the Na^+/L-carnitine transporter OCTN2 is present in the small intestinal apical membrane. Herein we aimed to find out if this step of intestinal L-carnitine absorption is ontogenically regulated, and if so, to determine the molecular mechanism(s) involved. L-[^{3}H]-Carnitine uptake was measured in the jejunum and ileum of fetuses (E17 and E21), newborn (1 day-old), suckling (15 day-old), weaning (1 month-old) and adult (2 and 6 month-old) Wistar rats. Both, Na^+-dependent and Na^+-independent L-carnitine uptake rates, normalized to intestinal weight, significantly increased during the late gestation period, and then declined during the suckling period. After weaning, the rate of Na^+-dependent L-carnitine uptake is no longer measurable. In E21- fetuses and newborn rats, L-carnitine uptake was higher in the ileum than in the jejunum. The decline in Na^+-dependent L-carnitine uptake with maturation was mediated via a decrease in the V_{max} of the uptake process with no change in its apparent K_m. Semi-quantitative RT-PCR assays showed that OCTN2 mRNA levels were significantly higher in E21-fetuses and newborn rats compared to suckling rats, which were in turn significantly higher than that in adult rats. Neither retardation of weaning nor L-carnitine supplementation prevented the down-regulation of Na^+/L-carnitine transport activity. The results demonstrate for the first time that intestinal Na^+-dependent L-carnitine uptake activity is under genetic regulation at the transcriptional level.

Correspondence to: A.A. Ilundáin; email: ilundain@us.es

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Introduction

L-Carnitine (beta-hydroxy-gamma-trimethylaminobutyric acid) has indispensable roles in intermediary metabolism. It facilitates the transfer of activated long-chain fatty acids from the cytoplasm to the mitochondria, where they are processed by oxidation to produce ATP. It is involved in the transfer of the products of peroxisomal beta-oxidation to the mitochondria. It removes short-chain and medium-chain fatty acids from the mitochondria and therefore maintains coenzyme A levels in these organelles. L-carnitine also ensures elimination of xenobiotic substances (*see* Vaz and Wanders, 2002, for a recent review). Primary (SCD) and secondary L-carnitine deficiency syndromes led to a reduced usage of fatty acids in energy production. The SCD is an autosomal recessive disorder caused by mutations in the gene that codes for the Na^+/L-carnitine transporter OCTN2 (Tamai et al., 1998).

Oral L-carnitine supplementation is commonly used in sports nutrition and in the medical field to treat primary and secondary L-carnitine deficiency syndromes, and there is evidence indicating that L-carnitine has positive effects on liver, kidney, immune disorders, in diabetes, Alzheimer's disease, on male fertility, etc. However, there is controversy regarding the mechanisms that mediate intestinal L-carnitine absorption. Some reports have suggested that both, active and passive mechanisms are involved in intestinal L-carnitine transport (Shaw et al., 1983; Gudjonsson et al., 1985; Hamilton et al., 1986), while others concluded that the uptake process is a passive mechanism (Li et al., 1990; Gross, Henderson & Savaiano, 1986; Gross &

Henderson, 1984; Marciani et al., 1991). Gross and Savaiano (1993) reported that L-carnitine is actively transported only by enterocytes isolated from neonates but not by those from adult rodents. More recently, functional and molecular studies revealed that L-carnitine crosses the intestinal apical membrane by an active, Na^+-dependent and electrogenic transport system that resembles the OCTN2 transporter (Durán et al., 2002), whereas the OCTN3 transporter appears to mediate L-carnitine exit across the basolateral membrane (Durán et al., 2005). Explanations for these differences could be species variation, the intestinal preparation used and the age of the animals, among others.

The goal of the current study is to determine whether intestinal L-carnitine uptake across the apical membrane domain (i.e., OCTN2 transporter) is ontogenically and segmentally regulated, and if so, to determine the molecular mechanism(s) involved in such regulation. The rat was used as the experimental animal model because of the similarity of its intestinal transport physiology to that of humans. The age of the rats studied spanned five phases of intestinal development: gestation, neonatal, suckling, weaning and adulthood. The results showed that Na^+-dependent L-carnitine transport across the intestinal apical membrane is under ontogenic regulation, and that this regulation involves a transcriptional mechanism(s).

Materials and Methods

Materials

L-[^{3}H]-carnitine was purchased from Amersham Biosciences. Unless otherwise indicated, the other reagents used in the current study were obtained from Sigma Chemical, Madrid, Spain.

Animals and Diets

The experiments were performed in accordance with national/local ethical guidelines. Litters of 10–14 Wistar rats were born on day 22–23 of gestation. Foetuses (E17 and E21), newborn (1 day-old), suckling (15 day-old), weaning (1 month-old) and adult (2 and 6 month-old) rats were used. Rats were weaned on a rat chow diet (Panlab 04). For some experiments, 15-day-old pups were either left with the mothers or weaned onto either rat chow or commercial dry milk (ESBILAC-1, Lab. Dr. Esteve S.A.) resembling their mother's milk with or without 300 μM L-carnitine in the drinking water, and maintained under these conditions for either 7 or 14 days.

L-Carnitine Uptake Experiments

Rats were anaesthetized by 5–10 min ether inhalation. Except for the duodenum, the whole small intestine was removed, opened longitudinally, rinsed clean with ice-cold saline solution (0.9% NaCl) and divided into three similar parts. The first 2/3 of the small intestine (following the duodenum) were considered as jejunum and the last 1/3, ileum. 1- to 2-cm pieces of either jejunum or ileum were incubated at 37°C in a thermostatic bath, with continuous shaking, in Ringer's solution containing, in mM: 140 NaCl, 1.2 $CaCl_2$, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 10 $KHCO_3$, 1.2 $MgCl_2$, pH 7.4, and 4×10^{-6} L-[^{3}H]-carnitine, and it was continuously bubbled with 95%O_2/ 5%CO_2. When required, Na^+ was isosmotically substituted by N-methylglucamine (NMG^+). At the end of the incubation period the tissues were washed with gentle shaking in ice-cold Na^+-free Ringer's solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed wet and extracted with $Ba(OH)_2$ and $ZnSO_4$ (Barium/Zinc). Samples were taken from the bathing solution and from the extracts of the tissues for radioactivity counting.

To evaluate the apparent V_{max} and K_m of the Na^+-dependent L-carnitine transport system, 5 min L-carnitine uptake was measured at 0.5, 1, 5, 25, 50 and 100 μM L-carnitine in the presence and absence of Na^+. The data were analyzed using the ENZFITTER computer program.

Semi-Quantitative RT-PCR Analysis

Total RNA was isolated as described by Chomczynski and Sacchi (1987) from rat small intestine of different ages. 2 μg of total RNA were reverse-transcribed and amplified using specific primers for OCTN2 as before (Duran et al., 2002) and the β-actin cDNA sequences (antisense, 5′-ACCCACACTGTGCCCATCTA-3′ and sense, CGGAACCGCTCATTGCC). The cycling conditions were: 40 s at 94°C, 40 s at 58°C, and 40 s at 72°C, followed by an extension of 5 min at 72°C. The primers amplified a 651 bp segment from OCTN2 cDNA and a 290 bp segment from β-actin cDNA with 30 and 25 cycles, respectively. With these numbers of cycles the linear phase of reaction was maintained, β-Actin was used as internal control to assess relative levels of OCTN2 transcripts. The amount of DNA was quantified by measuring the optical density of the bands using the PCBAS 2.0 program (Raytest).

Statistical Analysis

Data are presented as mean ± SEM for n separate animals. In the figures, the vertical bars that represent the SEM are absent when they are smaller than the symbol height. Two-way ANOVA followed by Newman-Keul's test ($P < 0.05$) was used for multiple comparisons.

Results

Time Course of Intestinal L-Carnitine Uptake

L-carnitine uptake versus time was measured in jejunum and ileum of newborn and 1-month-old rats, and in the presence and absence of Na^+ in the incubation buffer (Fig. 1). L-Carnitine uptake follows an exponential time course and at the steady-state equilibrium uptake (60 min) the Na^+-dependent to Na^+-independent L-carnitine uptake ratio was approximately 4.3 for the ileum and 3.8 for the jejunum of newborn rats. In the intestine of 1-month-old rats, the ratio was 1.3 for the ileum and 1.6 for the jejunum.

Since Na^+-dependent L-carnitine uptake increased linearly up to 10 min, a 5 min incubation time period was adopted to measure initial rates of uptake.

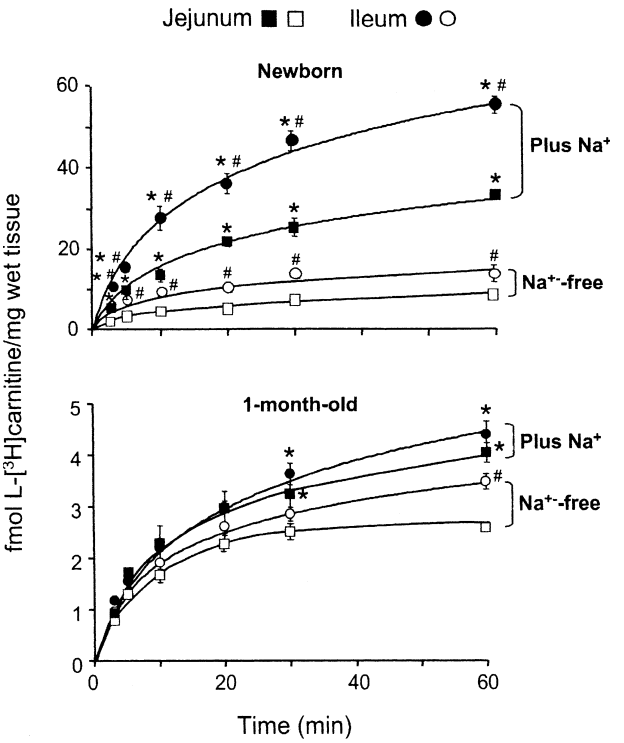

Fig. 1. Time course of L-carnitine uptake. L-[^{3}H]-carnitine uptake was measured in jejunum (*squares*) and ileum (*circles*) of newborns (1 day-old) and 1 month-old rats, in the presence (*filled symbols*) and absence (*empty symbols*) of extracellular Na^+. Na^+ was isosmotically replaced by NMG^+. Means ± SEM. The number of animals used per age was 5. $^*P < 0.001$ as compared with nominally Na^+-free conditions. $^{\#}P < 0.001$ ileum compared with jejunum.

Intestinal L-Carnitine Uptake *vs.* Age

The Initial rate of L-carnitine uptake was measured in the jejunum and ileum of fetuses (E17 and E21), newborn (1 day-old), suckling (16 day-old), weaning (1 month-old) and adult (2 and 6 month-old) rats, with and without Na^+ in the incubation buffer. Figure 2 shows that the Na^+-dependent L-carnitine uptake rates (total uptake minus that measured in the absence of Na^+), normalized to intestinal weight, increased during the late gestation period. This high level was maintained during the first days after birth and declined during the suckling period. After weaning, the rate of Na^+-dependent L-carnitine uptake is no longer measurable. In E21 fetuses and newborn rats, L-carnitine uptake was higher in the ileum than in the jejunum. In suckling, weaning and adult rats, however, no significant differences between jejunal and ileal L-carnitine uptake rates were observed. In nominally Na^+-free conditions, intestinal L-carnitine uptake increased during the late gestation and thereafter fell during the suckling period until it reached the adult values. In the early stages of life, Na^+-independent intestinal L-carnitine uptake was higher in ileum than in jejunum.

Both, jejunal and ileal Na^+-dependent L-carnitine uptake rates also declined with age when they were normalized to intestinal length (Fig. 2).

Kinetic Study of Intestinal L-Carnitine Uptake

The maximal initial uptake rate, V_{max}, and the Michaelis-Menten constant, K_m, were evaluated by measuring the initial rate of L-carnitine uptake *vs.* its extracellular concentration and in the presence and absence of Na^+ (Fig. 3*A* and 3*B*). Jejunum and ileum of newborn and suckling rats were used. In the presence of Na^+, L-carnitine uptake fit best ($r = 0.997$) a transport model describing a single saturable transport system plus a nonsaturable diffusion component:

$$V = (V_{max} S / K_m + S) + K_D S$$

where V is the initial rate of uptake; S, the external L-carnitine concentration and K_D, the apparent diffusion constant.

In nominally Na^+-free conditions, the relationship between L-carnitine uptake and its external concentration was linear (Fig. 3*A* and *B*) and the data were used to calculate the apparent diffusion constant, K_D (Table 1). The K_D was higher in newborn than in suckling rats and, in the former, it was higher in ileum than in jejunum. Total L-carnitine uptake minus that measured in the absence of Na^+ follows first-order kinetics (Fig. 3*C*). The Eadie-Hofstee plots of these data yield in all cases a linear relationship and reveal that the apparent V_{max} was greater in the ileum than in the jejunum, and that in both intestinal segments it decreases with age (Table 1). In contrast, no significant changes in the apparent K_m were observed with either maturation or intestinal segment. The apparent K_m values were similar to that previously reported for chicken enterocytes (Durán et al., 2002). The linearity of the Eadie-Hofstee plots, and that the same K_m value was found in jejunum and ileum, indicate the existence of a single L-carnitine transport system in the two intestinal segments.

Expression of Intestinal OCTN2 During Development

Since the OCTN2 is the carrier system involved in apical L-carnitine uptake in the gut (Duran et al., 2002), the effect of ontogeny on the level of OCTN2 mRNA was examined by semi-quantitative RT-PCR assay. The results (Fig. 4) showed that OCTN2 expression peaks in E21 fetuses and newborn rats and thereafter it decreases with age. OCTN2 expression in the ileum was higher than in jejunum.

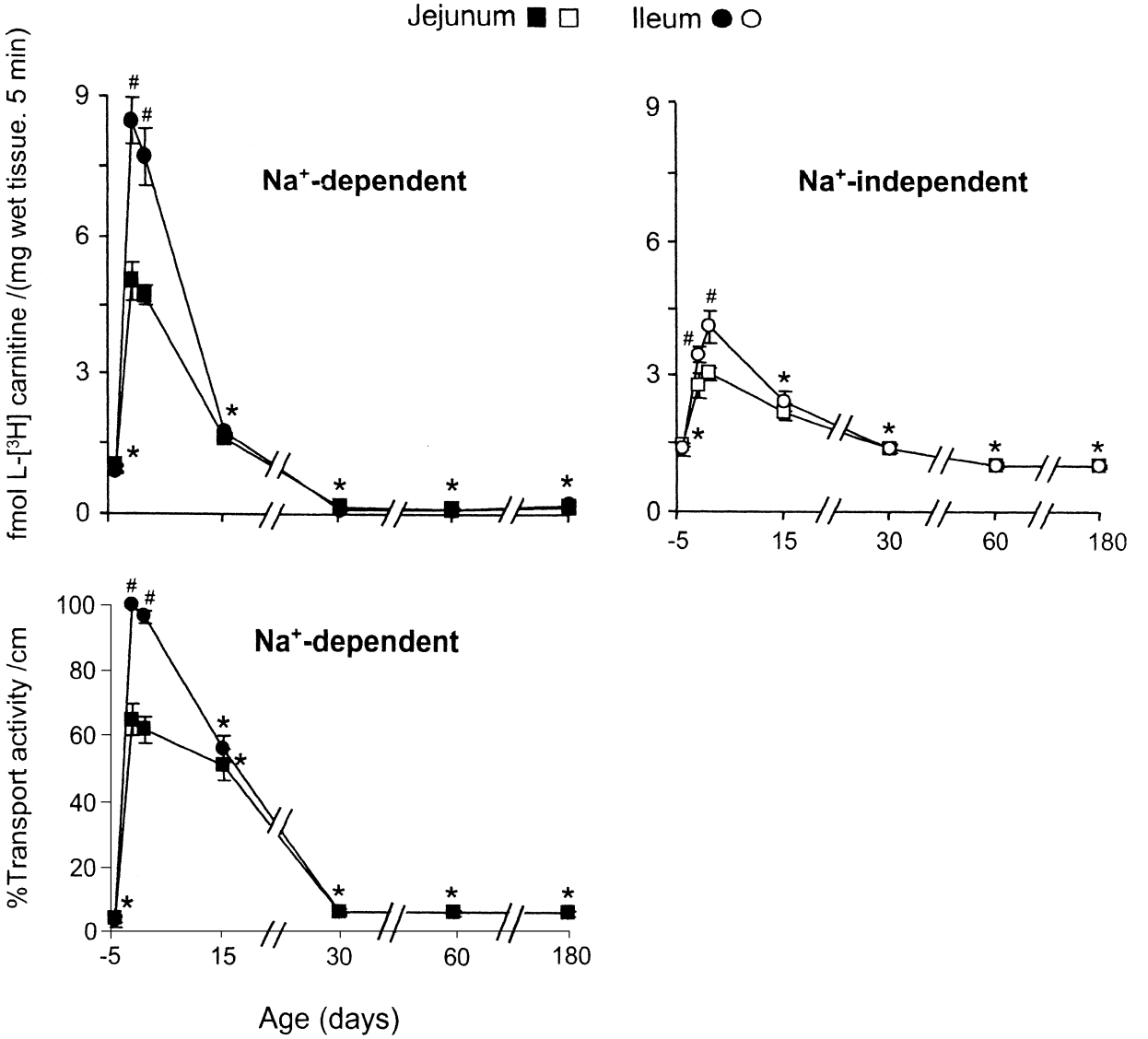

Fig. 2. Intestinal L-carnitine uptake rate *vs.* age. L-[^{3}H]-carnitine uptake rate was measured in jejunum (*squares*) and ileum (*circles*) isolated from fetuses (E17 and E21), newborn (1 day-old), suckling (15 day-old), weaning (1 month-old) and adult (2 and 6 month-old). 5 min uptake was measured in Na^+-containing (*filled symbols*) and (*empty symbols*) Na^+-free (NMG^+) buffers. Na^+-dependent uptake (total uptake minus that measured in Na^+-free conditions: Na^+-independent L-carnitine uptake). When L-carnitine uptake rate was normalized per cm intestinal length, 100%: uptake rate is that measured in the ileum of E21 -fetuses. Means ± SEM. The number of animals used per age was 8, except for 6 month-old animals it was 4. Two-way ANOVA showed an effect of maturation ($P < 0.001$) and segmental distribution ($P < 0.001$). Newman-Keul's test: *$P < 0.001$ as compared with E21 fetuses and newborn rats #$P < 0.001$, comparisons between jejunum and ileum.

Effect of the Diet on Intestinal L-Carnitine Uptake Rate

As the diet content of L-carnitine decreases at weaning (Gross & Savaino, 1993) it could be inferred that this could trigger the periweaning-induced changes in Na^+-dependent L-carnitine uptake activity shown in Fig. 2. However, they could also be genetically programmed. To distinguish between these two possibilities, 15-day-old pups were either left with the mothers or weaned onto either rat chow or commercial dry milk, with or without 300 μM L-carnitine in the drinking water, and maintained under these experimental conditions for either 7 or 14 days.

Figure 5 reveals that, as compared with pups either left with the mothers or weaned onto a rat chow, weaning onto a commercial milk diet slowed the decrease in Na^+/L-carnitine transport activity during the first week of treatment. After 2 weeks treatment Na^+/L-carnitine transport activity reached undetectable values regardless of the diet. Supplementation with L-carnitine in the drinking water had

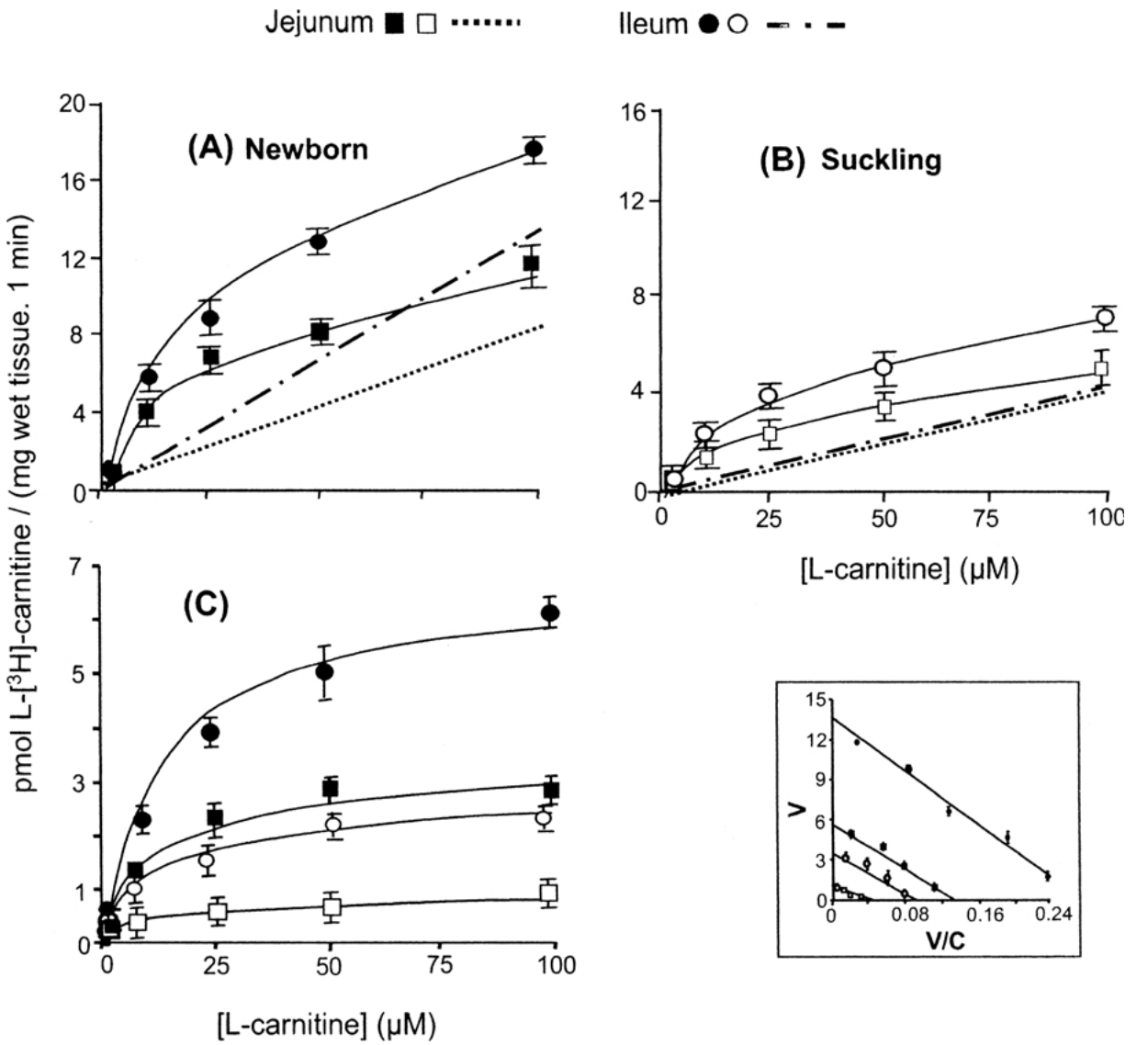

Fig. 3. L-Carnitine uptake *vs.* its concentration. Jejunal (*squares* and *dotted line*) and ileal (*circles* and *dashed line*) L-carnitine uptake, respectively, was measured in newborn (*A*) and suckling (*B*) rats in Na^+-containing (*filled symbols*, total uptake) and in nominally Na^+-free incubation buffers (*dashed lines*). (*C*) Saturable component: total uptake minus that measured in nominally Na^+-free buffers. Insert: Eadie-Hofstee plots of the saturable components. Means ± SEM. Three animals were used per age.

Table 1. Kinetic parameters of the Na^+-dependent L-carnitine uptake system measured in jejunum and ileum of rats of different ages

		V_{max}	K_m	K_D
Jejunum	Newborn	4.50 ± 0.22	24.23 ± 2.3	0.42 ± 0.03
	Suckling	0.84 ± 0.04*	16.05 ± 3.30	0.21 ± 0.01*
Illeum	Newborn	12.83 ± 0.22$^{\#}$	22.47 ± 2.20	0.66 ± 002$^{\#}$
	Suckling	3.23 ± 0.09*$^{\#}$	21.00 ± 2.63	0.22 + 0.01*

The apparent K_m (in μM), K_D (pmol/mg wet tissue/min μM) and V_{max} (pmol/mg wet tissue/min) values were calculated using non-linear regression analysis. Means ± SEM. The number of animals used per age group was 4. *$P < 0.001$ as compared with newborn values. $^{\#}P < 0.001$ as compared with jejunum.

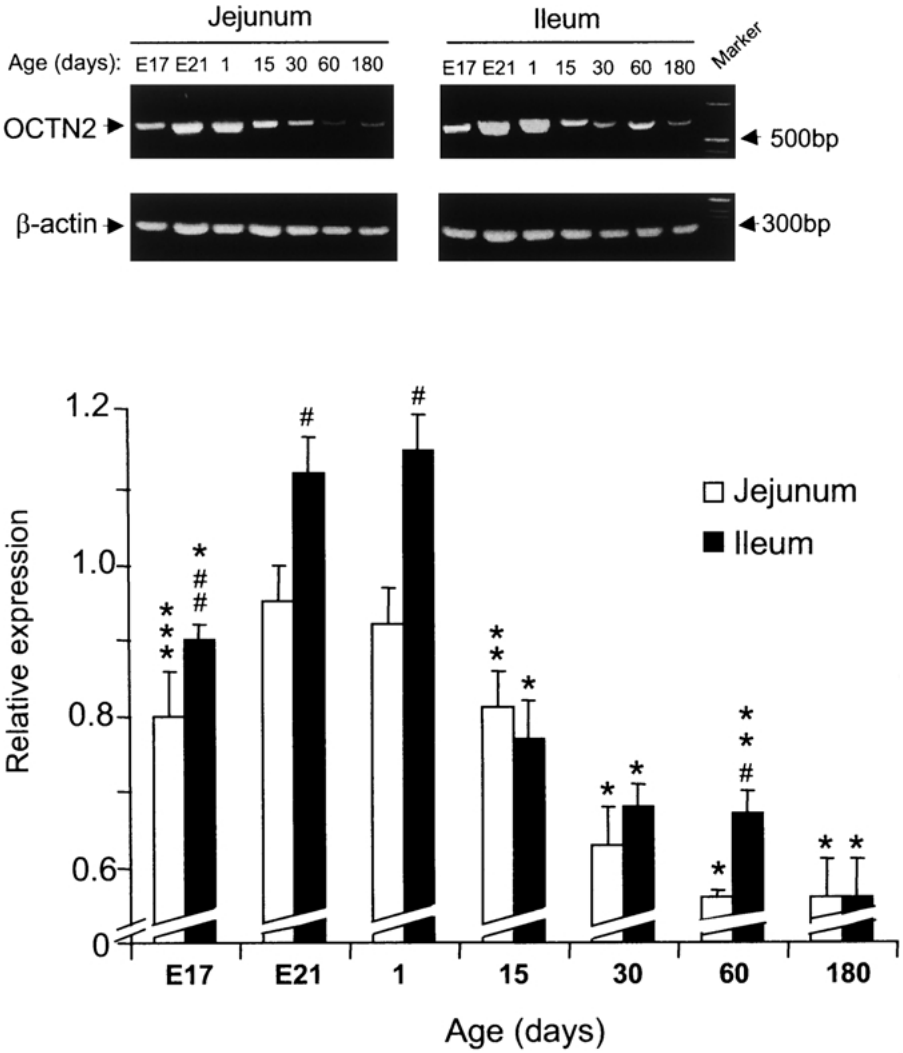

Fig. 4. Semi-quantitative RT-PCR analysis of OCTN2 mRNA. Total RNA was isolated from the jejunum and ileum of rats of different ages. The OCTN2 gene band displayed a motility corresponding to a size of ca. 650 bp. Histograms represent arbitrary units of OCTN2 expression. Two-way ANOVA showed an effect of maturation ($P < 0.001$) and segmental distribution ($P < 0.001$). Newman-Keul's test: "*", as compared with E21 fetuses and newborn rats. "#", comparisons between jejunum and ileum. $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$, $^{\#}P < 0.001$, $^{\#\#}P < 0.01$.

no effect on intestinal L-carnitine uptake activity. These observations indicate that the periweaning changes in intestinal L-carnitine uptake activity are not related to the L-carnitine content in the diet.

Discussion

Oral L-carnitine supplementation is currently used in sports nutrition and in medicine. However, information regarding intestinal L-carnitine transport is scarce and controversial. The present study reveals that rat small intestinal L-carnitine uptake is genetically regulated. Na^+-dependent L-carnitine uptake rate was significantly higher in both, pre-term fetuses and newborn rats than in suckling rats. After weaning, Na^+-dependent L-carnitine uptake was no longer measurable in either of the intestinal regions examined. In pre-term fetuses and newborn rats, Na^+-dependent L-carnitine uptake was significantly higher in the ileum than in the jejunum. The rate of Na^+-independent L-carnitine uptake also increased after birth, and thereafter it decreased during the suckling period until it reached adult values.

The high rates of Na^+-independent L-carnitine uptake observed in late gestation periods and newborn pups may be due to high rates in L-carnitine metabolism, since a similar pattern has been reported for the gene expression of enzymes involved in intestinal L-carnitine esterification (Asins et al., 1995; Miliar et al., 2001). However, the existence of a Na^+-independent L-carnitine transporter ontogenically regulated can no be ruled out.

The down-regulation in Na^+-dependent L-carnitine uptake with maturation was mediated via a decrease in the V_{max} of the uptake process with no change in its apparent K_m. These observations suggest that developmental maturation is associated with a decrease in the number (and/or activity) of the intestinal L-carnitine uptake carriers. The absence of changes in the apparent K_m excluded a maturation-related expression of different transporter isoforms. The RT-PCR assays support the view that the maturation-induced decrease in intes-

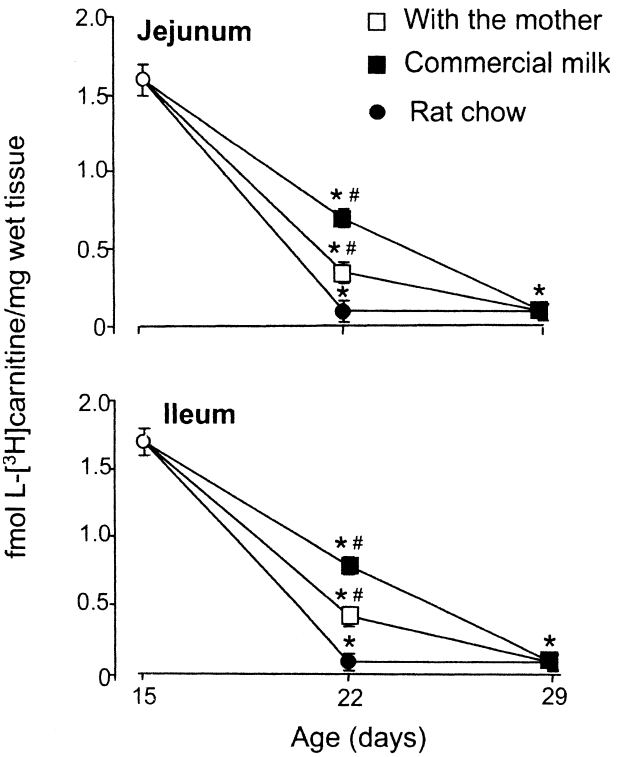

Fig. 5. Effect of L-carnitine supplementation on intestinal L-carnitine uptake. At day 15 post partum (○), 6 pups were left with the mothers (□), another 6 pups were weaned onto either rat chow (●) or commercial dry milk (■) with or without 300 μM L-carnitine in the drinking water, and maintained under these conditions for either 7 or 14 days. Means ± SEM of 4 treatments. Two-way ANOVA showed an effect of diet ($P < 0.001$) and maturation ($P < 0.001$). Newman-Keul's test: $*P < 0.001$ as compared with uptakes measured in 15-day-old rats. $\#P < 0.001$, as compared with rat chow.

tinal Na^+-dependent L-carnitine uptake is due to a decrease in the number of transporters involved. Thus, the pattern of OCTN2 expression is similar to that seen with Na^+/L-carnitine uptake activity: the higher levels of transport activity in the intestine of E21 fetuses and newborn rats compared to weaning and adult rats, were associated with a higher level of OCTN2 mRNA in the former compared to the latter age groups.

Since the suckling period is characterized by a rapid growth of the intestine and by changes in the composition and dynamics of the enterocyte population (Smith, 1988), decreases in rates of transport could arise from the dilution of transporting cells by non-transporting immature enterocytes. This will reduce transport rates of intestinal tissue normalized to mg, but would not affect overall uptake rates per unit length. However, intestinal L-carnitine uptake normalized to intestinal length decreases with age, indicating that dilution of transporting cells is unlikely to contribute to the maturation-induced decrease in intestinal Na^+-dependent L-carnitine uptake.

External (e.g., changes in dietary solute inputs associated with weaning) and internal (genetic) signals are involved in "instructing" the intestine to turn on/off transporters at the appropriate times (Dyer et al., 2003). The current results reveal that external signals had little effect on the ontogeny of intestinal L-carnitine transport. Thus, maintenance of the pups on a diet like their mother's milk reduced, but it did not prevent, the decrease in Na^+/L-carnitine transport activity observed after birth. Furthermore, L-carnitine supplementation had no effect on the maturation-induced changes in intestinal Na^+/L-carnitine transport activity. Gross and Savaino (1993) found that L-carnitine content in adult rat intestinal tissue is affected by dietary L-carnitine levels. These authors, however, did not measure initial rates of intestinal L-carnitine transport and do not distinguish between Na^+-dependent and Na^+-independent components of the L-carnitine uptake process.

Human and other mammals may obtain their L-carnitine requirement by dietary intake and by endogenous biosynthesis, but at least 75% of the L-carnitine requirement of well nourished adult humans come from the diet (*see*, Vaz & Wanders, 2002, for a recent review). This indicates that the adult intestine absorbs L-carnitine efficiently even though, as shown here, its intestinal Na^+-dependent L-carnitine uptake activity is not measurable. An increase in intestinal surface area with maturation might allow significant intestinal transport of L-carnitine and hence compensate the down-regulation of Na^+/L-carnitine cotransporter observed in adults.

L-carnitine deficiency is an infrequent problem in a healthy, well nourished adult population because a well balanced diet contains significant amounts of L-carnitine and of nutrients needed for its biosynthesis. However, it can be a conditionally essential nutrient during the abrupt perinatal transition from carbohydrate to fatty acids as the major substrate for energy production. L-carnitine is needed for fat metabolism and neonates depend upon external sources of L-carnitine to meet their metabolic needs because they have an immature L-carnitine biosynthetic pathway (Borum, 1985; Borum & Bennett, 1986; Rubaltelli et al., 1987). In rats, the liver is the site for L-carnitine biosynthesis, because it is the only tissue containing gamma-butyrobetaine hydroxylase (BBH), and the liver BBH activity (Hahn, 1981) and mRNA (Galland et al., 1999) increased from undetectable levels in fetuses and the perinatal period to maximal values at the adult stage.

Therefore, in rats, the regulation of body L-carnitine content involves at least the intestine and liver. In the early stage of life, when the demand for L-carnitine exceeds the endogenous capacity for its biosynthesis, rat pups receive most of their L-carnitine from the mother's milk (Robles-Valdes, McGarry & Foster, 1976) and the intestine presents

an active Na^+-dependent L-carnitine transporter to supply L-carnitine to the rest of the body. In the growing rat, however, the endogenous capacity for L-carnitine synthesis is sufficient, even with an L-carnitine-free diet (Borum, 1978), and its intestinal Na^+/L-carnitine transport activity is no longer measurable. Therefore, in the same individual, up-regulation of the liver L-carnitine biosynthesis is accompanied by the down-regulation of its intestinal Na^+/L-carnitine transport activity. However, during the late gestation period and early stage of life, both, the mother's liver L-carnitine biosynthesis (McGarry, Robles-Valdés & Foster 1975; Fernández-Ortega, 1989) and the fetuses' intestinal Na^+/L-carnitine transport system are up-regulated. How the capacity of intestine to transport L-carnitine and that of the liver to synthesize L-carnitine are interconnected remains unknown. Li et al. (1992) have suggested that in pigs glucagon and insulin may participate in the enterohepatic L-carnitine distribution after birth.

In humans the regulation of body L-carnitine pools may differ from that in rats, because BBH activity is present in liver, kidney and brain, and though liver BBH activity is ontogenically regulated, kidney BBH activity is already present at birth (Olson & Rebouche, 1987). In any case, as in rats, human neonates also require external sources of L-carnitine for their fat metabolism (Borum, 1985; Borum & Bennett, 1986; Rubaltelli et al., 1987; Feller & Rudman, 1988).

In conclusion, the rat intestinal Na^+/L-carnitine uptake system is ontogenically regulated. These maturation-related changes involve transcriptional mechanism(s) and do not appear to be affected by dietary L-carnitine.

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