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Biochimica et Biophysica Acta 1282 (1996) 274–282

BBA Biochimica et Biophysica Acta

Sodium-dependent high-affinity binding of carnitine to human placental brush border membranes

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Received 23 January 1996; revised 29 March 1996; accepted 1 April 1996

Abstract

The interaction of carnitine with human placental brush-border membrane vesicles was investigated. Carnitine was found to associate with the membrane vesicles in a Na^+-dependent manner. The time course of this association did not exhibit an overshoot, which is typical of a Na^+ gradient-driven transport process. The absolute requirement for Na^+ was noticeable whether the association of carnitine with the vesicles was measured with a short time incubation or under equilibrium conditions, indicating Na^+-dependent binding of carnitine to the human placental brush-border membranes. The binding was saturable and was of a high-affinity type with a dissociation constant of 1.37 ± 0.03 μM. Anions had little or no influence on the binding process. The binding process was specific for carnitine and its acyl derivatives. Betaine also competed for the binding process, but other structurally related compounds did not. Kinetic analyses revealed that Na^+ increased the affinity of the binding process for carnitine and the Na^+/carnitine coupling ratio for the binding process was 1. The dissociation constant for the interaction of Na^+ with the binding of carnitine was 24 ± 4 mM. This constitutes the first report on the identification of Na^+-dependent high-affinity carnitine binding in the plasma membrane of a mammalian cell. Studies with purified rat renal brush-border membrane vesicles demonstrated the presence of Na^+ gradient-driven carnitine transport but no Na^+-dependent carnitine binding in these membrane vesicles. In contrast, purified intestinal brush-border membrane vesicles possess neither Na^+ gradient-driven carnitine transport nor Na^+-dependent carnitine binding.

Keywords: Carnitine; High-affinity binding; Sodium ion dependence; Brush-border membrane; (Human placenta)

1. Introduction

Carnitine (β-hydroxy-γ-*N*-trimethylaminobutyrate) is an essential cofactor in the oxidation of long-chain fatty acids because it facilitates their transport from the cytoplasm into the mitochondria across the inner mitochondrial membrane [1]. The ability to oxidize fatty acids is extremely important for fetal adaptation to extrauterine life because during the immediate postnatal period, there is an abrupt transition from reliance upon glucose as the main energy fuel to fatty acids as the major substrate for energy production. Thus, there is a need for the developing fetus to build up the tissue reserves of carnitine in preparation for this critical metabolic transition at birth. In adults, carnitine is synthesized endogenously using the carbon skeleton of lysine and the methyl group of *S*-adenosyl methionine [2]. Due to this endogenous biosynthetic capacity, there is no dietary requirement for carnitine in adult mammals [3]. However, available evidence suggests that developing fetuses and neonates may not be capable of synthesizing adequate amounts of carnitine despite an adequate supply of the necessary precursor amino acids lysine and methionine due to immature development of the carnitine biosynthetic pathway in the liver and kidney [4–7]. And yet, carnitine levels in neonatal and cord blood have been found to be significantly higher when compared to maternal blood [8,9]. The apparent inability of the fetus to synthesize carnitine and the higher levels of carnitine in the fetal circulation than in the maternal circulation suggest that the placenta is capable of active transfer of carnitine from mother to fetus.

To date, there has been only a single reported study on the transport of carnitine across the human placenta [10]. This study, utilizing an in vitro placental perfusion tech-

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PII S0005-2736(96)00068-5

nique, could not demonstrate concentrative transport of carnitine across the placenta from the maternal side to the fetal side. This study, however, did show that carnitine is taken up by the placental tissue in a stereospecific manner, indicating participation of a mediated process. There is no information available at present on the identity and nature of this mediated process. The present investigation was undertaken to delineate the mechanism of carnitine transport in the placenta at the membrane level rather than at the whole organ level. The syncytiotrophoblast, which is the primary functional unit of the placenta, is a polarized cell with its brush-border membrane in direct contact with maternal blood and its basal membrane facing fetal circulation. Thus, the maternal-facing brush-border membrane forms the first barrier between the maternal and fetal circulations. It is known that this membrane possesses active transport mechanisms for various nutrients which are transported in a concentrative manner across the placenta from mother to fetus [11]. Therefore, we studied the interaction of carnitine with purified human placental brush-border membrane vesicles in an attempt to understand the mechanism responsible for active maternal-to-fetal transfer of carnitine across the placenta. For comparison, we also studied the interaction of carnitine with brush-border membrane vesicles isolated from two other transport organs, namely the kidney and the intestine.

2. Materials and methods

2.1. Materials

L-[*methyl*-^{3}H]Carnitine (specific radioactivity, 77 Ci/mmol) was purchased from Amersham. 5-[1,2-^{3}H]Hydroxytryptamine (serotonin) (specific radioactivity, 30.4 Ci/mmol) was purchased from Du Pont-New England Nuclear. L-Carnitine, D-carnitine, acetyl-DL-carnitine, stearoyl-DL-carnitine, palmitoyl-DL-carnitine, betaine, γ-aminobutyrate, β-hydroxybutyrate, 6-*N*-trimethyllysine, choline, and creatinine were obtained from Sigma. Propionyl-DL-carnitine was kindly provided by Dr. A.L. Carter (Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA). All other chemicals were of analytical grade.

2.2. Preparation of brush-border membrane vesicles

The Mg^{2+}-aggregation method [12] was used to isolate the maternal-facing brush-border membrane vesicles from normal human term placentas obtained within 1 h of delivery. Brush-border membrane vesicles from rat renal cortical tissue and from rabbit intestinal mucosal tissue were prepared as described previously [13–15] by using a similar Mg^{2+}-aggregation method. The membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5), containing 75 mM potassium gluconate and 150 mM mannitol. Protein concentration in final membrane preparations was adjusted to either 5 mg/ml (carnitine transport/binding studies) or 1.5 mg/ml (serotonin transport studies). The membrane vesicles were stored in small aliquots in liquid nitrogen and used within 2–3 weeks of preparation.

2.3. Measurement of transport / binding in membrane vesicles

Transport/binding was initiated by mixing 40 μl of membrane vesicles with 160 μl of transport/binding medium containing radiolabeled substrate [16]. The composition of the medium varied depending upon the individual experiment. In most cases, the medium was 10 mM Hepes-Tris buffer (pH 7.5), containing 150 mM NaCl. The mixture was then incubated at room temperature for the desired time. At the end of the incubation, transport/binding was terminated by the addition of 3 ml of ice-cold 5 mM Hepes-Tris buffer (pH 7.5) containing 160 mM KCl, followed by filtration under vacuum on a Millipore filter (DAWP type, 0.65 μm pore size). By this procedure, the brush-border membrane vesicles were retained on the filter. The filter was washed four times with 5 ml of the same KCl-buffer, following which the radioactivity associated with the filter was determined by liquid scintillation spectrometry.

2.4. Data analysis

Measurements were made in duplicate or triplicate and each experiment was done with 1–3 different membrane preparations. Results are given as means ± S.E. Kinetic analyses were carried out using a commercially available computer software called *Fig. P.6.0* (Biosoft, Cambridge, UK).

3. Results

3.1. Influence of Na^+ on the transport / binding of carnitine in human placental brush-border membrane vesicles

Fig. 1 describes the time course of carnitine transport/binding in human placental brush-border membrane vesicles in the presence and absence of an inwardly directed Na^+ gradient. The membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 150 mM mannitol and 75 mM potassium gluconate and the transport/binding of carnitine was determined in the presence of KCl or NaCl in the extravesicular medium. In the absence of Na^+, the amount of carnitine associated with the membrane vesicles was very small even though it increased with time. The presence of Na^+ in the extravesicular medium markedly increased the amount of carnitine that was associated with the membrane vesicles. This

Na^+-dependent increase was evident at all time periods (0.5–240 min). With a 240 min incubation, the transport/binding of carnitine was almost 10-fold greater in the presence of Na^+ than in its absence.

When placental brush-border membrane vesicles preloaded with a Na^+-free buffer are mixed with a Na^+-containing buffer, there exists initially an inwardly directed Na^+ gradient which disappears gradually with time. At equilibrium, there is no concentration gradient for Na^+ across the membrane (i.e., $[Na^+]_{inside} = [Na^+]_{outside}$). Under these experimental conditions, Na^+ gradient-dependent transport systems exhibit an 'overshoot' phenomenon in which the transport is much greater at shorter periods of incubation than at equilibrium. Previous studies from our laboratory have demonstrated such an overshoot phenomenon in human placental brush-border membrane vesicles for several Na^+ gradient-dependent transport systems. Examples of these transport systems include those which transport taurine [17], succinate [18], serotonin [19], and catecholamines [20,21]. Therefore, it was interesting that even though the association of carnitine with the placental brush-border membrane vesicles was Na^+-dependent, the process did not exhibit an overshoot. To provide evidence that an inwardly directed Na^+ gradient did exist at the initial stages of incubation which dissipated with time, we studied serotonin transport in these membrane vesicles under the same experimental conditions in which carnitine transport/binding was measured. As shown in Fig. 2, the transport of serotonin in the presence of Na^+ was many-fold greater at 2.5 min incubation than at equilibrium (240 min incubation), demonstrating the overshoot. Furthermore, the transport was Na^+-dependent at 2.5 min, but this Na^+-dependence was not seen at 240 min. These results clearly indicate that there was a complete dissipation of the

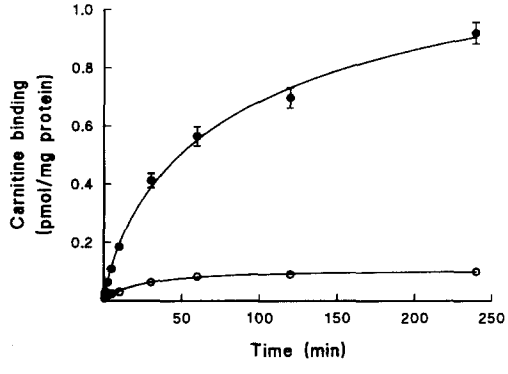

Fig. 1. Time course of carnitine binding to human placental brush-border membrane vesicles. Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5), containing 75 mM potassium gluconate and 150 mM mannitol. Binding of carnitine was initiated by mixing 40 μl of membrane suspension (200 μg membrane protein) with 160 μl of 10 mM Hepes-Tris buffer (pH 7.5), containing [^{3}H]carnitine and either 150 mM NaCl (●) or 150 mM KCl (○). The mixture was incubated for varying time periods and bound carnitine was measured by rapid filtration assay. Final concentration of carnitine in the incubation mixture was 10 nM. Values represent means ± S.E. ($n = 4$).

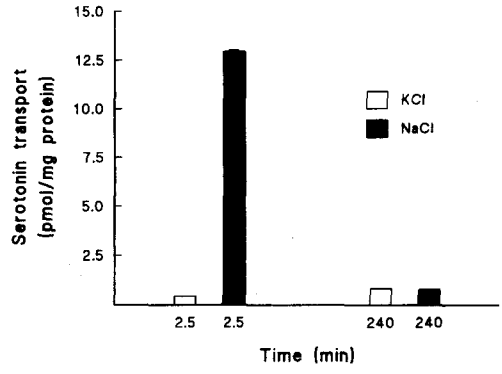

Fig. 2. Influence of Na^+ on serotonin transport in placental brush-border membrane vesicles. Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 75 mM potassium gluconate and 150 mM mannitol. Transport of serotonin was initiated by mixing 40 μl of membrane suspension (200 μg membrane protein) with 160 μl of 10 mM Hepes-Tris buffer (pH 7.5), containing [^{3}H]serotonin and either 150 mM NaCl or 150 mM KCl. The mixture was incubated for either 2.5 min or 4 h and the amount of serotonin transported into the vesicles was determined by rapid filtration assay. Final concentration of serotonin in the incubation mixture was 20 nM. Values represent means ± S.E. ($n = 3$).

Na^+ gradient within 240 min incubation. Since the association of carnitine with the membrane vesicles shows an obligatory dependence on Na^+ even under these Na^+-equilibration conditions, this means that the process is dependent on Na^+ rather than the transmembrane Na^+ gradient.

The intravesicular volume in human placental brush-border membrane vesicles is in the range of 1–2 μl/mg of protein [16,22,23]. If carnitine is sequestered in the intravesicular space in free form in these membrane vesicles, the maximal amount of carnitine that can be expected to be associated with the vesicles at equilibrium and at a concentration of 10 nM is about 0.02 pmol/mg of protein. Interestingly, the amount of carnitine associated with the vesicles under these conditions is about 1 pmol/mg of protein, a value which is 50-times greater than the expected value. This indicates that carnitine binds to the placental brush-border membrane vesicles and that the binding process is Na^+-dependent. In order to differentiate the binding from the transport, we studied the influence of increasing osmolarity in the extravesicular medium on the equilibrium association (4 h incubation) of carnitine with the membrane vesicles in the presence of Na^+. It was found that osmolarity of the extravesicular medium had negligible effect on the amount of carnitine associated with the membrane vesicles (Fig. 3). These results strongly suggest that carnitine binds to the human placental brush-border membranes.

3.2. Ion specificity of carnitine binding

We studied the influence of various monovalent cations and anions on the binding of carnitine to the placental brush-border membrane vesicles. In this experiment, the vesicles were preloaded with a buffer containing 150 mM

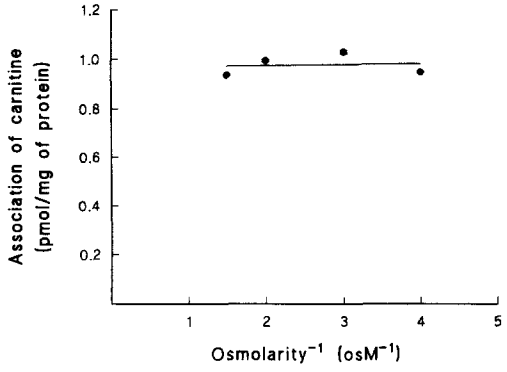

Fig. 3. Influence of medium osmolarity on the association of carnitine with human placental brush-border membrane vesicles. Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5), containing 75 mM potassium gluconate and 150 mM mannitol. Equilibrium association of [^{3}H]carnitine with the vesicles was measured with a 4 h incubation in the media consisting of 10 mM Hepes-Tris (pH 7.5), 60 mM NaCl, and varying concentrations of mannitol. Final concentration of carnitine in the incubation mixture was 10 nM. The osmolority values of the final incubation media were used in the analysis.

mannitol and 75 mM potassium gluconate and the binding was measured in buffers containing 120 mM of different inorganic salts. The binding was measured with two different incubation periods, 10 min (initial binding) and 4 h (equilibrium binding). As is evident in Table 1, the initial as well as equilibrium binding was maximal when Na^+ was present, and substitution of Na^+ with other monovalent cations such as K^+ and Li^+ reduced the binding considerably. This shows that neither of these two cations can effectively substitute for Na^+ in the binding process. It is also evident from the data that the initial rates of Na^+-dependent carnitine binding (10 min incubation) was only slightly affected by various anions. However, when the incubation time was increased to 4 h to measure the Na^+-dependent carnitine binding at equilibrium, the binding was significantly affected by the anions SCN^- and gluconate. When compared to the binding measured in the presence of Cl^-, the binding in the presence of SCN^- was significantly lower whereas the binding in the presence of gluconate was significantly higher. Considering the relatively long incubation time, it is likely that the observed effects of SCN^- and gluconate are not specific. These data show that the binding of carnitine to the placental brush-border membrane vesicles exhibits an obligatory requirement for Na^+ with no or little dependence on anions.

Table 1
Influence of monovalent cations and anions on carnitine binding to human placental brush border membranes

Salt	Carnitine binding			
	10 min		4 h	
	pmol/mg	%	pmol/mg	%
NaCl	0.17±0.01	100	0.70±0.01	100
NaF	0.15±0.01	93	0.68±0.01	97
$NaNO_3$	0.13±0.00	81	0.59±0.02	84
Na SCN	0.14±0.01	86	0.48±0.01	69
Na gluconate	0.16±0.01	99	0.92±0.01	131
LiCl	0.06±0.00	38	0.18±0.00	25
KCl	0.02±0.00	13	0.07±0.00	11

Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5), containing 75 mM potassium gluconate and 150 mM mannitol. Binding of carnitine (10 nM) was initiated by mixing 40 μl of membrane suspension with 160 μl of 10 mM Hepes-Tris buffer (pH 7.5), containing indicated inorganic salts (150 mM). The mixture was incubated for 10 min or 4 h and bound carnitine was determined by rapid filtration assay. Values are means ±S.E. ($n = 3$).

Table 2
Substrate specifity of carnitine binding sites in human placental brush border membranes

Substrate analog	[^{3}H]Carnitine binding	
	pmol/mg of protein	%
None	0.61±0.01	100
L-Carnitine	0.05±0.00	9
D-Carnitine	0.06±0.00	9
Acetyl-DL-carnitine	0.06±0.00	9
Propionyl-DL-carnitine	0.04±0.00	7
Palmitoyl-DL-carnitine	0.07±0.00	12
Stearoyl-DL-carnitine	0.15±0.01	26
Betaine	0.06±0.00	9
6-*N*-Trimethyllysine	0.55±0.00	91
β-Hydroxybutyrate	0.65±0.01	106
γ-Aminobutyrate	0.65±0.00	106
Choline	0.53±0.01	87
Creatinine	0.53±0.01	87

Binding of [^{3}H]carnitine (10 nM) to placental brush-border membranes was measured with a 4 h incubation in the presence of Na^+. Final concentration of unlabeled substrate analogs was 100 μM. Values are means ±S.E. ($n = 3$).

3.3. Substrate specificity of the carnitine binding process

To determine the substrate specificity of the binding process in the placental brush-border membrane vesicles, we performed competition experiments in which the influence of various unlabeled substrate analogs on the binding of labeled carnitine was assessed (Table 2). The binding was measured with a 4 h incubation in the presence of Na^+. At a concentration of 100 μM, L-carnitine, D-carnitine, acetyl-DL-carnitine, propionyl-DL-carnitine, palmitoyl-DL-carnitine, and betaine inhibited the binding of 10 nM labeled carnitine by 90%. Stearoyl-DL-carnitine was slightly less effective, causing 75% inhibition. On the other hand, 6-*N*-trimethyllysine, β-hydroxybutyrate, γ-aminobutyrate, choline, and creatinine showed very little effect on carnitine binding. These results show that the binding is specific for carnitine and its acyl derivatives. The short-chain as well as long-chain acyl derivatives are effective substrates. Among the compounds studied, betaine was the only non-carnitine compound which effec-

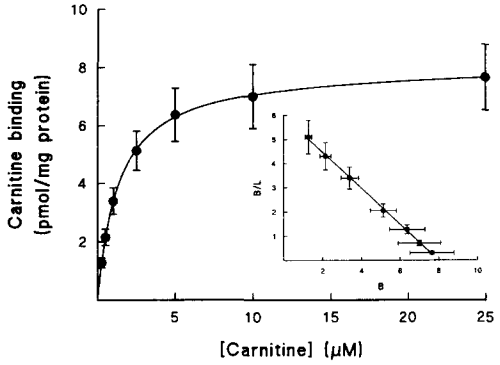

Fig. 4. Scatchard analysis of carnitine binding. Human placental brush-border membranes were incubated with [^{3}H]carnitine in the presence of NaCl for 4 h and bound carnitine was measured by rapid filtration assay. The concentration of [^{3}H]carnitine was kept constant at 10 nM in the experiment and concentration of carnitine was varied (range, 0.25–25 μM) as desired by the addition of unlabeled carnitine. Nonspecific binding was determined in the presence of excess amount (1 mM) of unlabeled carnitine. Data for specific binding are given as carnitine concentration versus binding or as Scatchard plot, i.e., carnitine binding (B) versus carnitine binding/carnitine concentration (B/L) (inset). The amount of carnitine bound to the membranes was less than 1% of total carnitine present in the incubation mixture at all carnitine concentrations. Therefore, concentrations of total carnitine present during incubation were used directly in Scatchard analysis. Values are means ± S.E. ($n = 6$).

tively competed with carnitine for the binding. 6-*N*-Trimethyllysine, which is the precursor for carnitine biosynthesis, and β-hydroxybutyrate and γ-aminobutyrate, both of which bear significant structural resemblance to carnitine, are not recognized by the binding process. Similarly, the quaternary ammonium compound choline and the guanidino compound creatinine are also not substrates for the binding process.

3.4. Saturation kinetics of carnitine binding

Fig. 4 describes the influence of carnitine concentration on the binding, measured with a 4 h incubation (equilibrium binding) in the presence of Na^+. The physiologically occurring L-stereoisomer of carnitine was used in these studies. The binding was measured over a carnitine concentration range of 0.25–25 μM. Since the amount of carnitine bound to the membrane vesicles was less than 1% of total carnitine present in the assay medium, the concentrations of total carnitine (instead of free carnitine) in the medium were used directly in the kinetic analysis. Nonspecific binding was determined in the presence of excess amount (1 mM) of unlabeled carnitine. The relationship between specific binding and carnitine concentration was hyperbolic, demonstrating saturability of the binding process. Scatchard analysis of the data provided evidence for the presence of a single binding site. The dissociation constant (K_d) for the binding process was 1.37 ± 0.03 μM and the maximal binding capacity (B_{max}) was 8.0 ± 0.1 pmol/mg of membrane protein.

3.5. Dose–response relationship for the inhibition of [^{3}H]carnitine binding by L-carnitine, D-carnitine, acetyl-DL-carnitine, and propionyl-DL-carnitine

The data in Table 2 show that both L- and D-forms of carnitine are potent inhibitors of [^{3}H]carnitine binding. Similarly, the racemic mixtures of the acetyl and propionyl esters of carnitine are also very effective in inhibiting [^{3}H]carnitine binding. To determine the relative affinities of these four compounds for the carnitine binding process, we assessed the ability of increasing concentrations (range, 0.25–25 μM) of these compounds to inhibit the binding of [^{3}H]carnitine (10 nM). From the dose–response curves, given in Fig. 5, it was calculated that the IC_{50} values (concentration of the compound necessary to cause 50% inhibition of the specific binding of radiolabeled carnitine) for L-carnitine, D-carnitine, acetyl-DL-carnitine, and propionyl-DL-carnitine were 0.97 ± 0.08 μM, 1.99 ± 0.06 μM, 2.63 ± 0.28 μM and 1.26 ± 0.16 μM, respectively. Since the concentration of L-[^{3}H]carnitine used in these experiments was very small compared to the K_d value (0.01 μM versus 1.37 μM), these IC_{50} values closely approximate to inhibition constants (K_i). The K_i value calculated for L-carnitine (0.97 μM) is very close to the K_d value calculated for L-carnitine (1.37 μM) by Scatchard analysis. It is evident from the K_i values that the binding process exhibits about two-fold less affinity for D-carnitine than for L-carnitine. If this is also true for the stereoisomers of acetylcarnitine and propionylcarnitine, the actual K_i values for the L-isomers of these carnitine esters are likely to be lower than the K_i values determined in this study for

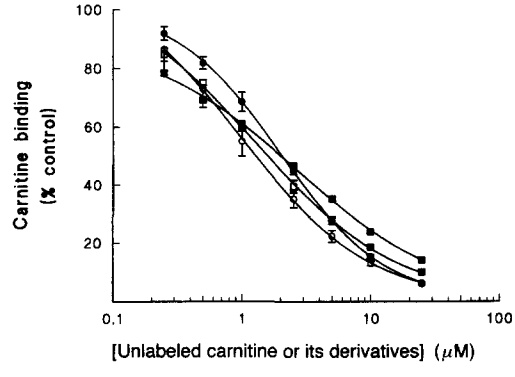

Fig. 5. Dose–response relationship for the inhibition of [^{3}H]carnitine binding to human placental brush-border membranes by unlabeled D-carnitine, L-carnitine, acetyl-DL-carnitine, and propionyl-DL-carnitine. Binding of [^{3}H]carnitine (final concentration, 10 nM) to placental membranes was measured with a 4 h incubation in the presence of NaCl. Concentration of unlabeled carnitine or its derivatives was varied between 0.25 and 25 μM. Binding of [^{3}H]carnitine measured in the absence of inhibitors was 0.86 ± 0.07 pmol/mg of protein and this value was taken as 100% (control). Values represent means ± S.E. ($n = 4$). Key: ●, D-carnitine; ○, L-carnitine; ■, acetyl-DL-carnitine; □, propionyl-DL-carnitine. The S.E. bars for the 10 and 25 μM values are within the symbol and are not shown for clarity.

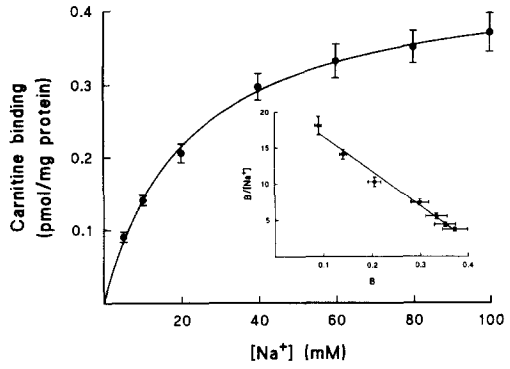

Fig. 6. Influence of Na^+ concentration on carnitine binding. Human placental brush-border membranes were incubated with 10 nM [^{3}H]carnitine for 4 h in the presence of various concentrations of Na^+ (as NaCl) (range, 5–100 mM). Appropriate concentrations of KCl were used to maintain osmolality. Na^+-dependent binding was calculated by subtracting the binding measured in the absence of Na^+ from the binding measured in the presence of Na^+. Values (means ± S.E., $n = 4$) represent Na^+-dependent binding. Data are presented as [Na^+] versus carnitine binding or as a Hill-type plot, i.e., carnitine binding (B) versus carnitine binding/[Na^+] (inset).

the respective racemic mixtures. The L-isomers of these two carnitine esters are currently in therapeutic use [24,25].

3.6. Dependence of carnitine binding on Na^+ concentration

To analyze kinetically the dependence of carnitine binding on the concentration of Na^+, equilibrium binding of carnitine was measured in the presence of increasing concentrations of Na^+ in the binding buffer (range, 0–100 mM). The relationship between Na^+-dependent carnitine binding (i.e., binding in the presence of Na^+ minus binding in the absence of Na^+) and Na^+ concentration was found to be hyperbolic (Fig. 6), suggesting a Na^+/carnitine coupling ratio of 1. The data were analyzed using the Hill-type equation

$$B = \frac{B_M[Na^+]^n}{K_{0.5}^n + [Na^+]^n}$$

where B is Na^+-dependent carnitine binding, B_M is carnitine binding at infinite concentration of Na^+, $K_{0.5}$ is the concentration of Na^+ necessary to cause carnitine binding equal to 50% of B_M, and n is the number of Na^+ ions involved in the binding of one molecule of carnitine. This analysis gave an n value of 0.95 ± 0.08 and $K_{0.5}$ value of 24 ± 4 mM. The n value is very close to 1 and provides supporting evidence for the Na^+/carnitine coupling ratio of 1 in the binding process.

3.7. Influence of Na^+ on the kinetics of carnitine binding

Since the binding of carnitine is a Na^+-dependent process, it was of interest to determine the influence of Na^+ on the kinetic parameters of carnitine binding. The binding of carnitine was kinetically analyzed to calculate the K_d and B_{max} at two different Na^+ concentrations, 150 mM and 20 mM (Fig. 7). At a Na^+ concentration of 150 mM, the K_d for carnitine binding was 1.36 ± 0.03 μM and B_{max} was 9.9 ± 0.1 pmol/mg of membrane protein. When the concentration of Na^+ was lowered to 20 mM, the K_d value for carnitine increased 2-fold to 2.73 ± 0.11 μM. There was no significant change in B_{max} (10.7 ± 0.3 pmol/mg of membrane protein). These results show that Na^+ activates carnitine binding by enhancing the affinity of the binding site for carnitine without altering the maximal number of binding sites.

3.8. Carnitine transport/binding in renal and intestinal brush-border membrane vesicles

We compared the handling of carnitine by the human placental brush-border membrane vesicles with the handling of carnitine by brush-border membrane vesicles from two other transporting epithelia, namely, the renal and intestinal absorptive cells. The time course of carnitine association with the renal brush-border membrane vesicles in the presence and absence of an inwardly directed Na^+ gradient is described in Fig. 8A. The amount of carnitine associated with the vesicles in the absence of Na^+ was small, increased with time, and reached equilibrium within 60 min. The presence of an inwardly directed Na^+ gradient markedly stimulated the initial rates of carnitine association and the time course under these conditions exhibited the 'overshoot' phenomenon that is typical of a Na^+ gradient-dependent transport process. The Na^+-dependence of the process was evident only with short incubation periods. At 60 min incubation, the amount of carnitine associated with the membrane vesicles was the same in the presence as well as in the absence of Na^+. These data provide evidence for the presence of a Na^+ gradient-driven uphill transport of carnitine in renal brush-border mem-

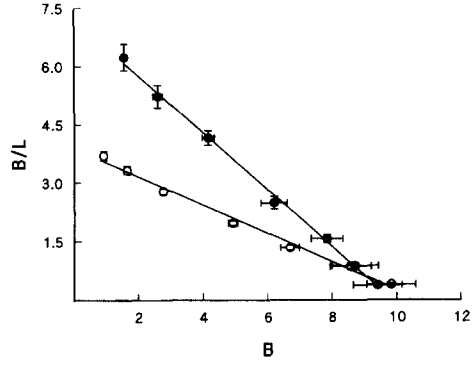

Fig. 7. Influence of Na^+ on the kinetics of carnitine binding. Human placental brush-border membranes were incubated with varying concentrations (range, 0.25–25 μM) of carnitine in the presence of either 20 mM NaCl (○) or 150 mM NaCl (●). KCl was used to maintain osmolality. Values (means ± S.E., $n = 4$) represent specific binding. Data are presented as Scatchard plots, i.e., carnitine binding (B) versus carnitine binding/carnitine concentration (B/L).

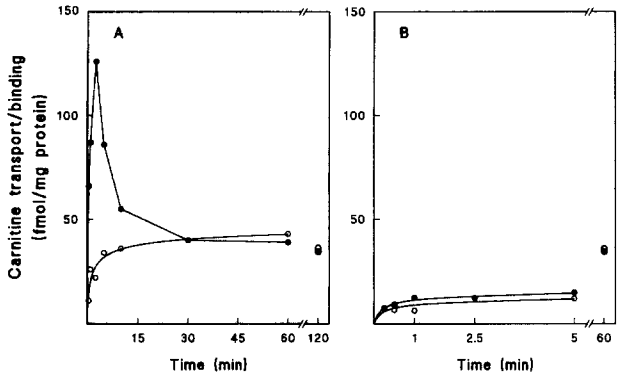

Fig. 8. Binding/transport of carnitine in rat renal (A) and rabbit intestinal (B) brush-border membrane vesicles. Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5), containing 75 mM potassium gluconate and 150 mM mannitol. Binding/transport of carnitine was initiated by mixing 40 μl of membrane suspension (200 μg of membrane protein) with 160 μl of 10 mM Hepes-Tris buffer (pH 7.5), containing [^{3}H]carnitine and either 150 mM NaCl (●) or 150 mM KCl (○). The mixture was incubated for varying time periods and binding/transport was determined by rapid filtration assay. Final concentration of carnitine in the incubation mixture was 10 nM in the case of renal membrane vesicles and 20 nM in the case of intestinal membrane vesicles. Values are averages of duplicate determinations.

brane vesicles. At equilibrium with the transmembrane Na^+ gradient no longer in existence, there is no net transport of carnitine into the vesicles. Accordingly, there is no evidence for Na^+-dependence of carnitine transport under these equilibrium conditions. Thus, the transport of carnitine in response to a transmembrane Na^+ gradient is clearly demonstrable in renal brush-border membrane vesicles. These characteristics of carnitine transport in renal brush-border membrane vesicles differ markedly from those of carnitine transport in placental brush-border membrane vesicles.

Fig. 8B describes the time course of the association of carnitine with intestinal brush-border membrane vesicles in the presence and in the absence of an inwardly directed Na^+ gradient. In contrast to the renal and placental brush-border membrane vesicles, there was no Na^+-dependent association of carnitine with the intestinal brush-border membrane vesicles. This shows that there is neither Na^+-dependent carnitine transport nor Na^+-dependent carnitine binding in the intestinal brush-border membrane vesicles.

4. Discussion

In this paper we describe the characteristics of the interaction of carnitine with purified human placental brush-border membrane vesicles. This study was undertaken to delineate the mechanism involved in the maternal-to-fetal transfer of carnitine across the placenta. There is unequivocal evidence for the transfer of carnitine from mother to fetus, but the mechanism underlying this transfer process has not been identified. Transfer of carnitine across the placental syncytiotrophoblast has to involve at least two steps – transfer across the maternal-facing brush-border membrane, followed by transfer across the fetal-facing basal membrane. The purpose of the study reported in this paper was to elucidate the mechanism of carnitine transport in purified brush-border membrane vesicles. The results of the study indicate that the human placental brush-border membranes bind carnitine in a Na^+-dependent manner. The binding process is substrate-specific, exhibiting high-affinity only for carnitine and its acyl (short-chain as well as long-chain) derivatives. The requirement of the binding process for Na^+ is absolute. The presence of Na^+ increases the affinity for carnitine. The Na^+/carnitine coupling ratio appears to be 1. These findings are unique because the presence of Na^+-dependent high-affinity binding of carnitine has not been described thus far in any mammalian tissue.

There have been earlier reports on the handling of carnitine by isolated renal [26,27] and intestinal [28] brush-border membrane vesicles. Mammalian kidney is known to reabsorb carnitine actively from the tubular filtrate [29,30] and accordingly the renal brush-border membrane vesicles possess the ability to take up carnitine in a Na^+ gradient-dependent manner [26,27]. The characteristics of interaction of carnitine with renal brush-border membrane vesicles are as expected for a typical Na^+ gradient-driven transport process. There is no evidence for Na^+-dependent binding of carnitine in these membranes. Intestinal brush-border membrane vesicles were used to study carnitine transport [28], prompted by the reports of successful treatment of carnitine deficiency with large oral doses of carnitine [31,32] and by the findings which suggested the presence of a Na^+-dependent transport mechanism for carnitine in intact intestinal tissue preparations [33–35]. Surprisingly, it was concluded from the study with purified intestinal brush-border membrane vesicles that these membranes do not possess a Na^+-dependent carnitine transport mechanism. There is also no evidence for Na^+-dependent carnitine binding in these membranes.

The aforementioned results obtained by other investigators with purified renal and intestinal brush-border membrane vesicles have been confirmed in the present study, ruling out any possible contribution of methodological differences to the interpretation of the data obtained with placental brush-border membrane vesicles. Thus, the present study provides evidence for the first time for Na^+-dependent high-affinity binding of carnitine to human placental brush-border membranes. Such binding in the plasma membrane has not been described in any other tissue. Recently, Vesci et al. [36] reported on the characterization of carnitine binding sites in rat brain. These binding sites are localized to synaptic vesicles. There are important differences between these neuronal carnitine binding sites and the placental carnitine binding process. The neuronal binding sites appear to be Na^+-independent because these sites show saturable high-affinity ($K_d = 0.28$ μM) carnitine binding in the total absence of Na^+. Furthermore, the

neuronal binding sites are very specific for carnitine. The affinity of these sites for acetylcarnitine is 500-fold less than that for carnitine. These characteristics are clearly different from those of the binding process in the placental brush-border membrane. Therefore, the high-affinity carnitine binding described in the present study in human placental brush-border membranes are not identical with the neuronal carnitine binding.

It is likely that the Na^+-dependent, high-affinity binding process specific for carnitine and its acyl derivatives in the brush-border membrane of the human placenta described in this paper are involved in the transfer of carnitine across the brush-border membrane in the placenta. However, the renal brush-border membrane vesicles which transport carnitine into the intravesicular space in a Na^+ gradient-dependent manner do not exhibit measurable Na^+-dependent binding of carnitine. In most cases, this is the normal characteristic of a typical ion gradient-driven solute transporter. Apparently, in placental brush-border membrane vesicles, carnitine binds to some component on the internal surface of the membrane following Na^+-dependent transport across the membrane.

Even though available evidence for active transport of carnitine from mother to fetus across the human placenta is unequivocal, there is a lot that needs to be understood regarding the role of carnitine in fetal development. There is very little contribution of fatty acid oxidation to the energy needs of the fetus and therefore, building up the tissue carnitine reserves to facilitate fatty acid oxidation in the immediate postnatal period seems to be an obvious function of the placental transfer of carnitine. However, it seems unlikely that carnitine has no active role in the developing fetus prior to birth. Animal studies have shown that untreated carnitine deficiency in the mother during pregnancy is associated with severe metabolic derangement in the newborns that is detectable within 5 days of birth [37–39]. These findings were from a genetically mutant mouse, called juvenile visceral steatosis (*jvs*) mouse, which exhibits systemic carnitine deficiency most likely due to a genetic defect in the renal carnitine transport system [40]. Facilitation of fatty acid oxidation within the mitochondria does not appear to be the only function of carnitine in animal tissues. Evidence has been accumulating in recent years suggesting involvement of carnitine in other functions such as in the regulation of immune response, apoptosis, and oxidative stress [41]. Therefore, it is very likely that placental transfer of maternal carnitine to the fetus is obligatory for normal growth and development of the fetus in utero, since the developing fetus has no or little capacity for endogenous biosynthesis of this compound.

Acknowledgements

This work was supported in part by National Institutes of Health Grant HD 24451 (V.G.) and Wyeth-Lederle Neonatology Research Grant (A.S.R.). The authors thank Sarah Taylor for expert secretarial assistance.

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